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THE OPTIMISATION OF ACCELERATED YOGURT PRODUCTION

by

ESTI-ANDRINE SMITH

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University of the Free State

SUPERVISOR : DR J. MYBURGH

November 2010

DECLARATION

I declare that the dissertation hereby submitted for the qualification Master of Agricultural Science in Food Science Degree at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at another university or faculty. I furthermore concede copyright of the dissertation to the University of the Free State.

Esti-Andrine Smith

November 2010

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In this dissertation each chapter is an individual entity and some repetition between chapters has therefore been unavoidable.

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
α-LA	α-lactalbumin
β-LG	β-lactoglobulin
ACH	acid casein hydrolysate
AR	analytical grade
BSA	blood serum albumin
CDM	chemically defined medium
CMP	casein macropeptide
EMP	Embden-Meyerhof-Parnas
EPS	extracellular polysaccharides
FBP	folate-binding protein
FGM	fat globule membrane
g	acceleration due to gravity
GRAS	generally regarded as safe
µg	microgram
HCl	hydrochloric acid
HTST	high-temperature-short-time
kPa	kilopascal
kg	kilogram
kt	kiloton
l	litre
L. bulgaricus	*Lactobacillus delbruekii* spp. *bulgaricus*
LPL	lipoprotein lipase
min	minutes
ml	milliliter
NaOH	sodium hydroxide
nm	nanometer
N	normal
OD	optical density
PEP	phosphoenolpyruvate
PMF	proton motive force
RDA	recommended dietary allowance
rpm	revolutions per minute
s	seconds
S. thermophilus	*Streptococcus salivarius* spp. *thermophilus*
UHT	ultra high temperature
WPC	whey protein concentrate

DEFINITIONS OF TERMINOLOGY USED IN DISSERTATION

TERMINOLOGY	DESCRIPTION
Setting pH	The pH at which yogurt coagulates
Yogurt milk	Milk used for yogurt fermentation
Control	Standard yogurt fermentation process with no variables

CHAPTER 1

INTRODUCTION

The yogurt making process is an ancient craft which dates back thousands of years. It can possibly even be traced back to the domestication of the cow, sheep or goat, but it is safe to assume that prior to the nineteenth century the various stages involved in the production of yogurt were little understood. The continued existence of the process through the ages can therefore be attributed to the fact that the scale of manufacture was small, and the craft was handed down from parents to children (Tamime and Robinson, 1996). The first fermented dairy products were produced by a fortuitous combination of events. The main contributor was the ability of lactic acid bacteria to grow in milk and to produce just enough acid to reduce the pH of milk to the iso-electric point of the caseins, at which these proteins coagulate. Neither the lactic acid bacteria nor the caseins were however designed for this function. The caseins were 'designed' to be enzymatically coagulated in the stomach of neonatal mammals at pH 6, which is much higher than the iso-electric point of the casein proteins. The ability of lactic acid bacteria to ferment lactose was acquired relatively recently in the evolution of these bacteria. Their natural habitats are vegetation and intestines, from which they presumably colonised the teats of mammals which was contaminated with milk containing lactose. Through evolutionary pressure, these bacteria subsequently acquired the ability to ferment lactose. While the use of rennets to coagulate milk for cheese manufacture was intentional, the coagulation of milk by the *in-situ* production of lactic acid which resulted in yogurt was presumably accidental (Fox, 2004).

Yogurt is a popular fermented milk product widely accepted and consumed worldwide. More than 73 % of the yogurt that was manufactured in 2001 was produced in Europe alone (Sodini et al., 2005). A steady increase in total sales of yogurt occured from 779 kt in 1999 to 1083 kt in 2003 in the United States of America (Boeneke and Aryana, 2008).

Micro-organisms, i.e. the yogurt starter cultures, play an important role during the production of yogurt. A traditional method and an improved method for yogurt manufacture exist (Tamime and Robinson, 1996). These methods are depicted in Figure 1.1 and Figure 1.2.

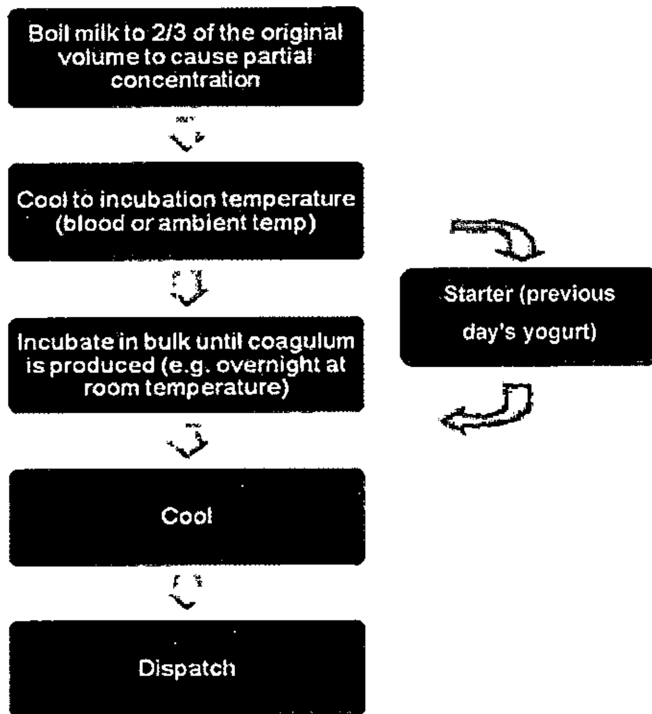

Figure 1.1 The traditional process of yogurt making (Tamime and Robinson, 1996).

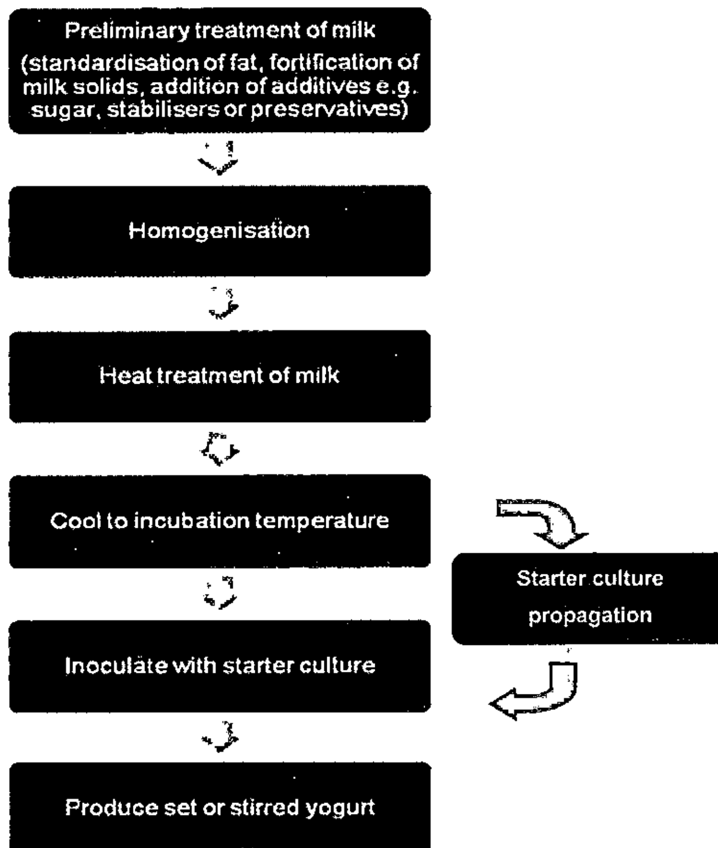

Figure 1.2 The improved process of yogurt making (Tamime and Robinson, 1996).

Tamime and Robinson (1996) observed that the traditional method has several drawbacks, such as:

- Consecutive inoculations of the starter culture tend to alter the ratio between *Lactobacillus delbruekii* spp. *bulgaricus* and *Streptococcus salivarius* spp. *thermophilus*, or may lead to mutation beyond the 15-20th subculturing;
- In comparison to the optimum conditions of 40-45 °C for 2½-3 hours, the acidification of milk is slow (18 hours or more) due to the low incubation temperature, i.e. ambient;
- Undesirable side effects may be promoted due to the slow rate of acid development, e.g. whey syneresis, which adversely affects the yogurt quality;
- The level of lactic acid production during the fermentation cannot be controlled during the traditional process.

Even though the improved process is a superior process to the traditional method, there is a possibility that it can be optimised even further. Research in this study was focused on the following areas:

- Optimisation of starter culture which involves precise determination of inoculum loads as well as addition of pre-inoculum activators;
- The influence of physical conditions (temperature, electricity- and pressure pretreatment of milk) on the fermentation time of yogurt;
- Modification of physical properties of the casein micelle in milk by addition of certain enzymes;
- Addition of possible accelerants to yogurt milk. This includes vitamins, minerals and certain extracts and hydrolysates.

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CHAPTER 2

LITERATURE REVIEW

2.1 YOGURT MANUFACTURE

Yogurt has developed into one of the most well-accepted and consumed fermented dairy products over the last few years. A mildly acidic taste, good digestibility, variations in taste and a high dietetic value have significantly contributed to its increased marketplace acceptance (Vandewegh et al., 2002).

High quality yogurts must have particular attributes. The pH must be between 4.2 and 4.3 at the time of consumption and the texture must be such that it contains no grittiness or effervescence. It must also have a distinctive taste and aroma and be sufficiently viscous to be eaten with a spoon (Weinbrenner et al., 1997).

In general, the overall properties of yogurt, such as acidity level, free fatty acid content, production of aroma compounds (diacetyl, acetaldehyde, acetoin) as well as the sensory profile and nutritional value, are important traits of the product. These aspects are influenced by numerous factors such as the chemical composition of the milk base, processing conditions and the activity of starter culture during the incubation period (Mahdian and Tehrani, 2007). Yogurt contains at least 8.25 % nonfat solids and has a titrable acidity of not less than 0.9 % expressed as lactic acid (Weinbrenner et al., 1997). The acidification process during yogurt fermentation can be monitored by pH measurement (de Brabandere and de Baerdemaeker, 1999).

Two popular yogurt products are set yogurt and stirred yogurt. Set yogurt is manufactured by incubating inoculated milk in the container in which the end product is sold. This leads to a product with high viscosity. Stirred yogurt is prepared by incubating milk in a tank where fermentation takes place whereafter the product is stirred and packaged. Stirring disrupts the protein network in yogurt, decreasing the viscosity and resulting in a product known as stirred yogurt (Klaver et al., 1994; Rawson and Marshall, 1997; Renan et al., 2009). Basic yogurt manufacturing processes generally use a dairy medium such as milk or a milk component as starting material. The dairy medium is typically chosen from, but is not limited to, pasteurised or unpasteurised milk, cream, non-fat dried milk or concentrated milk and water. Other ingredients such as stabilisers (e.g. hydrocolloids such as starches or gelatins), whey protein and milk powder concentrates can be added to stabilise the viscosity and consistency of the end product. Once the dairy medium has been chosen and the desired ingredients have been added, the mixture is heated to induce pasteurisation. This is generally accomplished by heating the mixture to between 82 °C and 93 °C for about 6 to 10 minutes which also leads to denaturing of the whey protein. After this heating phase the mixture is allowed to cool to 40-46 °C and placed into a fermentation tank wherein a constant temperature is maintained. When 42 °C is reached, starter cultures are added (de Brabandere and de Baerdemaeker, 1999; Klaver et al., 1994; Vandewegh et al., 2002). These yogurt-producing starter cultures generally consist of two organisms, namely *Lactobacillus delbruekii* spp. *bulgaricus* and *Streptococcus salivarius* spp. *thermophilus*. Selected *Bifidobacteria* species are usually added as an additional probiotic (Klaver et

al., 1994). Starter bacteria are used in yogurt production due to the fact that they produce lactic acid at the temperatures used in conventional yogurt manufacturing (42 °C) (Vandewegh et al., 2002). Starter culture inoculation loads vary from 0.025 to 5 % (Klaver et al., 1994). Fermentation proceeds until the milk mixture reaches a pH value below 4.6, which indicates appropriate levels of acidity. Below a pH of approximately 4.6 the final product is considered a high acid food and the product will not support growth of pathogenic bacteria (Vandewegh et al., 2002).

Acidification causes iso-electric coagulation of the proteins that are responsible for the typical yogurt texture, while yogurt flavour also develops during the acidification process. Fruit pulp, flavourings or colourants can optionally be added in varying concentrations of up to 10 % to the yogurt after fermentation to produce the final commercial product (Vandewegh et al., 2002).

2.2 ENZYMES PRESENT IN MILK

Enzymes are present in milk for two main reasons. Firstly, innate enzymes are enzymes which are inherent to milk and originate from several different sources including the apical membrane and cytoplasm of secretory cells, somatic cells and blood plasma of animals. Esterases, proteinases, phosphatase, amylase, peroxidase, catalase and lactase are only a few of the enzymes inherent to milk (Fox and Kelly, 2006). The second source of enzymes in milk is enzymes produced by micro-organisms such as psychrotrophic bacteria present in the milk tank during storage at 2-5 °C (Cousin, 1982).

Innate milk enzymes have little or no beneficial effect on the organoleptic or nutritional qualities of milk. The destruction of these enzymes is done by many dairy processes through heat application. The same applies to microbial enzymes like lipases and proteases which are denatured during pasteurisation conditions of 90 °C for 6-10 minutes (Fox and Kelly, 2006).

2.2.1 Indigenous enzymes

2.2.1.1 Lipoprotein lipase

Lipoprotein lipase (LPL) is the main indigenous milk enzyme and is the main, if not only, lipase in cow's milk. This enzyme is mainly found in the plasma in association with the casein micelles and is able to attack the lipoproteins of the fat globule membrane (FGM). Lipolysis may be caused in this way, and the lipolytic activity subsequently causes rancidity. Its level of activity is usually low due to the difficult access of the enzyme to its substrates in milk. LPL is a relatively unstable enzyme, and is deactivated by ultraviolet light, heat, acid, oxidising agents and prolonged freezing (Chavarri et al., 1998; Deeth and Fitz-Gerald, 2006).

2.2.1.2 Protease

Two ubiquitous protease systems, which are both derived from blood, are present in milk. All innate proteases present in milk originate from blood and enter milk either via the lysosomes of somatic cells or in a soluble form for example plasmin. Plasmin is the main indigenous protease in milk and is responsible for dissolving blood clots, whereas lysosomal proteases of somatic cells are effective against invasion by micro-organisms (Fox and Kelly, 2006; Kelly et al., 2006). The κ-caseins in milk are resistant to proteolysis by plasmin (Silanikove et al., 2006). The enzyme activity in the milk and the length of exposure to the enzymes are unquestionably correlated to the degree of casein hydrolysis. Low temperature storage however leads to plasmin autolysis which reduces its catalytic activity (Fox and Kelly, 2006; Kelly et al., 2006).

Concerning starter organisms, *L. bulgaricus* possesses a more effective proteolytic enzyme system than *S. thermophilus.* This enzyme system consists of proteases which degrade milk proteins, mainly caseins, to peptides and subsequently amino acids (Kang et al., 1997; Tari et al., 2009).

2.2.1.3 Lactoperoxidase

The lactoperoxidase system (lactoperoxidase/thiocyanate/hydrogen peroxide) is a natural antimicrobial system present in milks from many species. The enzyme lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide, the antimicrobial effect being due to intermediate reaction products. Thiocyanate is widely distributed in animal tissues, with levels in bovine milk ranging between 1 and 10 ppm. Hydrogen peroxide could be generated in milk by leucocytes and by lactic acid bacteria (Chavarri et al., 1998).

2.2.2 Microbial enzymes: (Exogenous enzymes)

2.2.2.1 Proteases and lipases

Psychrotrophs are micro-organisms which are common contaminants of milk and are ubiquitous in nature (Cousin, 1982). Most of the psychrotrophic bacteria that are found in dairy products are Gram-negative, non-sporulating, oxidase-positive small rods. The three genera that these organisms are commonly placed in include *Pseudomonas, Flavobacterium* and *Alcaligenes* (Nelson, 1981). Common areas which contribute to the psychrotrophic population include poorly sanitised valves in pipes and milk tanks, post-pasteurisation contamination of product or equipment, improperly sanitised containers as well as airborne contamination. During the refrigerated storage of milk these organisms increase in number and synthesise protease enzymes which biochemically alter the milk composition. This in turn eventually leads to spoilage. A large number of psychrotrophic bacteria is not necessarily needed to produce significant amounts of protease enzyme (Cousin, 1982; Gassem and Frank, 1991). Yogurt is often made from milk that at some point has been at risk of proteolytic degradation (Gassem and Frank, 1991).

The major microbial lipases are also produced by psychrotrophic bacteria. Extracellular lipases produced by psychrotrophic bacteria have considerable potential for causing hydrolytic rancidity in milk and milk products (Deeth and Fitz-Gerald, 2006).

In studies done by Gassem and Frank (1991), yogurt made from milk pretreated with microbial protease had higher syneresis, apparent viscosity and firmness than the product used as control. Although fermentation was more rapid in the treated milks, no consistent effects on yogurt culture levels due to the proteolysis of milk were noted (Gassem and Frank, 1991).

Most raw milk which is used for yogurt manufacturing contains either heat-stable proteases or bacteria which are able to produce proteases. These proteases can lead to bitter flavour developments and coagulation of the milk by attacking the whey and casein proteins present in the milk (Cousin, 1982). During studies performed by Cousin and Marth (1977) milk precultured with psychrotrophic bacteria was used to produce yogurt. The end product showed an increase in firmness of the coagulum together with a decrease in fermentation time (Cousin and Marth, 1977). Other studies that were done with milk containing previous psychrotrophic growth resulted in yogurt with unacceptable flavour scores (Cousin, 1982).

Although the presence of psychrotrophic bacteria is generally seen as a drawback and great care is taken to ensure its absence in milk, these organisms can possibly lead to acceleration of yogurt fermentation under controlled conditions (Cousin, 1982). Gassem and Frank (1991) reported in their studies that milk that was precultured with psychrotrophic spoilage bacteria took 25-30 minutes less to reach pH 4.25 when compared to the control that was not treated with protease.

Adams et al. (1975) and Liu and Guo (2008) also reported that the addition of psychrotrophic bacteria accelerates yogurt fermentation time. The reason for this is possibly due to the fact that the protease enzyme originating from the psychrotrophic bacteria only selectively destabilises some of the κ-casein on the casein micelle, where the casein hydrolysate totally destabilises and digests the entire casein micelle. Where the κ-casein is only partly destabilised, the residual κ-casein is hydrolysed during acid development due to charge upliftment. This results in a less aggressive alteration of the casein micelle which in turn leads to accelerated fermentation (Adams et al., 1975; Liu and Guo, 2008).

From the above information it can be deduced that recombinant enzymes, for example proteases, could possibly assist in food processing applications, especially fermentations. Chymosin, or rennet as it is also known, is proteolytic and specifically cleaves the milk protein κ-casein on the Phe(105)–Met(106) position. This enzyme is used in fermentations and leads to curd formation in cheese processing (Ross et al., 2000).

2.2.2.2 β-Galactosidase

β-Galactosidase, also known as lactase, is an enzyme produced by lactic acid bacteria in the gastro-intestinal tract of lactose tolerant individuals which hydrolises the disaccharide D-lactose in mammalian milk to D-glucose and D-galactose. Approximately 50 % of the world's human population is unable to utilise lactose due to the fact that they lack this enzyme. This condition is known as lactose intolerance. Due to the presence of β-galactosidase in yogurt, lactose intolerance sufferers who cannot drink milk are able to consume yogurt without adverse effects (Gorbach, 1990; Rings et al., 1994; Burton and Tannock, 1997; Stanton et al., 2001; Vasiljevic and Jelen, 2001; Ouwehand et al., 2002; Haider and Husain, 2009; Tari et al., 2009).

A study was done by Vasiljevic and Jelen (2001) to determine the optimal conditions for the maximum production of β-galactosidase. It was found that *L. bulgaricus* cultivated in skim milk yielded the highest enzyme activity compared to cultivation in dried whey powder and whey powder supplemented with yeast extract or De Man-Rogosa-Sharpe (MRS) broth. The production of lactase was found by Vasiljevic and Jelen (2001) to increase steadily during yogurt fermentation and reach a maximum level after four hours of incubation.

β-Galactosidase synthesis is enhanced in the presence of an inducer (lactose) and suppressed by a repressor (glucose) (Vasiljevic and Jelen, 2001). Studies done by Hickey et al. (1986) on several *L. bulgaricus* strains indicated that the addition of small amounts of glucose to a growing culture resulted in a significant reduction in β-galactosidase activity. Glucose could therefore lead to partial repression of the *lac* operon (constituted by the transcription rate of the *lac* gene) which results in decreased activity of β-galactosidase in the starter culture. The enhancement of β-galactosidase activity is however achieved by addition of a suitable neutraliser to control the pH of the buffered medium (Vasiljevic and Jelen, 2001).

2.3 CASEIN MICELLES

Bovine milk consists of approximately 2.8 % protein. Milk proteins can be divided into two classes, based on their solubility at pH 4.6: the soluble whey proteins, which represent ±20 % of total milk protein; and the insoluble casein proteins, which represent ±80 % of total milk protein. The whey proteins are very heterogenous; the principle proteins are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), with lesser amounts of blood serum albumin (BSA) and immunoglobulins. BSA and immunoglobulins begin to denature at 65 °C, whereas β-LG and α-LA are more heat stable and significant denaturation occurs only at temperatures above 70-75 °C (Fox and Kelly, 2004; Phadungath, 2005; Huppertz et al., 2006; Considine et al., 2007; Ceballos et al., 2009). On heating, the denatured whey proteins can interact with each other and with the casein micelles (Considine et al., 2007).

Casein proteins are hydrophobic, have a relatively high charge and can be fractionated into four distinct proteins namely α_{s1}-, α_{s2}-, β- and κ-caseins. The protein fraction in cow's milk is displayed in Table 2.1 (Horne, 2002; Singh and Bennett, 2002; Fox and Kelly, 2004; Phadungath, 2005; Huppertz et al., 2006). The α_{s1}- and α_{s2}-caseins are situated in the core region of the micelle whereas κ-casein is located on the surface of the micelle where it exercises a stabilising effect upon the native micelles and prevent them from coagulating. The stabilising effect is due to the two distinct regions of the κ-casein, namely the hydrophobic para-κ-casein (residues 1-105) and the hydrophilic macropeptide (residues 106-169). In its natural position on the surface of the micelles, the κ-casein is linked to the remainder of the micelle via the hydrophobic para-κ-casein moiety of the molecule, allowing the casein macropeptide (CMP) to protrude from the surface into the surrounding solution. This hydrophilic moiety interacts with the solvent to stabilise the micelles. The β-casein is situated in the core of the native micelle in a milk environment but can move to the surface when the micelle is in a non-native environment, e.g. <10 °C. The phosphate groups of the casein micelles bind large amounts of calcium. The so-called colloidal calcium phosphate is an important stabilising factor and is responsible for holding the network together by crosslinking the protein, which is important in regard to the structure of the micelles (Singh and Bennett, 2002; Dalgleish, 2004). Removal of the colloidal calcium phosphate results in the casein micelles dissociating into smaller particles which in turn aggregates and forms a coagulum. These particles are referred to as subunits or submicelles (Shaker et al., 2000; Considine et al., 2007). A pictorial representation of the effect on proteins when milk is heated can be seen in Figure 2.1.

Yogurt is pasteurised at a higher temperature than fresh milk in order to prevent whey syneresis. Figure 2.2 represents changes of casein micelle surface during pasteurisation, observed with an electron microscope. When the temperature of the milk reaches 85 °C, the κ-caseins which are situated at the surface of the casein micelles, react with the whey protein β-LG. This interaction produces minute 'bumps' on the casein micelle surfaces (Figure 2.2A). The β-LG-κ-casein complex prevents other casein micelles from attaching at these sites when yogurt bacteria metabolise lactose and produce lactic acid, which subsequently leads to coagulation of the milk. Due to the surfaces of the heated casein micelles being partially blocked, only a few micelles can interact. This leads to the formation of short branched micellar chains. When the coagulation is complete, the milk has changed into a gel i.e. yogurt. Milk that has not been heated consists of casein micelles with smooth surfaces (Figure 2.2B) (Kalab, 1997).

The mechanisms of yogurt production rely mainly on the casein micelle, which is very stable in milk. It is homogeneously distributed in milk and is responsible for its characteristic white colour. The negative charge on the κ-casein outside of the micelle stabilises the caseins in the milk. These negative charges repel the casein micelles from each other and prevent the micelles from settling to the bottom and separating into whey and casein in fresh milk (Goff, 1999).

When lactic acid bacteria produce lactic acid from lactose hydrolysis in milk, positively charged hydrogen ions are introduced into milk. The positive charges uplift the negative charges on the casein micelles and the charges on the micelles are neutralised (Goff, 1999). The destabilised micelles then form a three dimensional network in which whey is trapped. Destabilisation of casein micelles occurs at pH 5.2-5.3 (de Brabandere and de Baerdemaeker, 1999; Singh and Bennett, 2002). As the pH lowers during yogurt fermentation and the iso-electrical point (pH 4.6) is reached, the casein micelles completely precipitate and form a coagulum (Rawson and Marshall, 1997; de Brabandere and de Baerdemaeker, 1999; Shaker et al., 2000).

Variations in pH also have a strong influence on casein hydrolysis. This effect is more evident in streptococci than in lactobacilli. It is therefore advisable to select the bacterial population of a starter culture on the basis of its proteolytic activity and its rate of lactic acid production. This should be done over the entire range of temperatures and pH values occurring during the manufacturing process to avoid the development of off-flavours and bitterness in cheese as well as yogurt (de Giori et al., 1985).

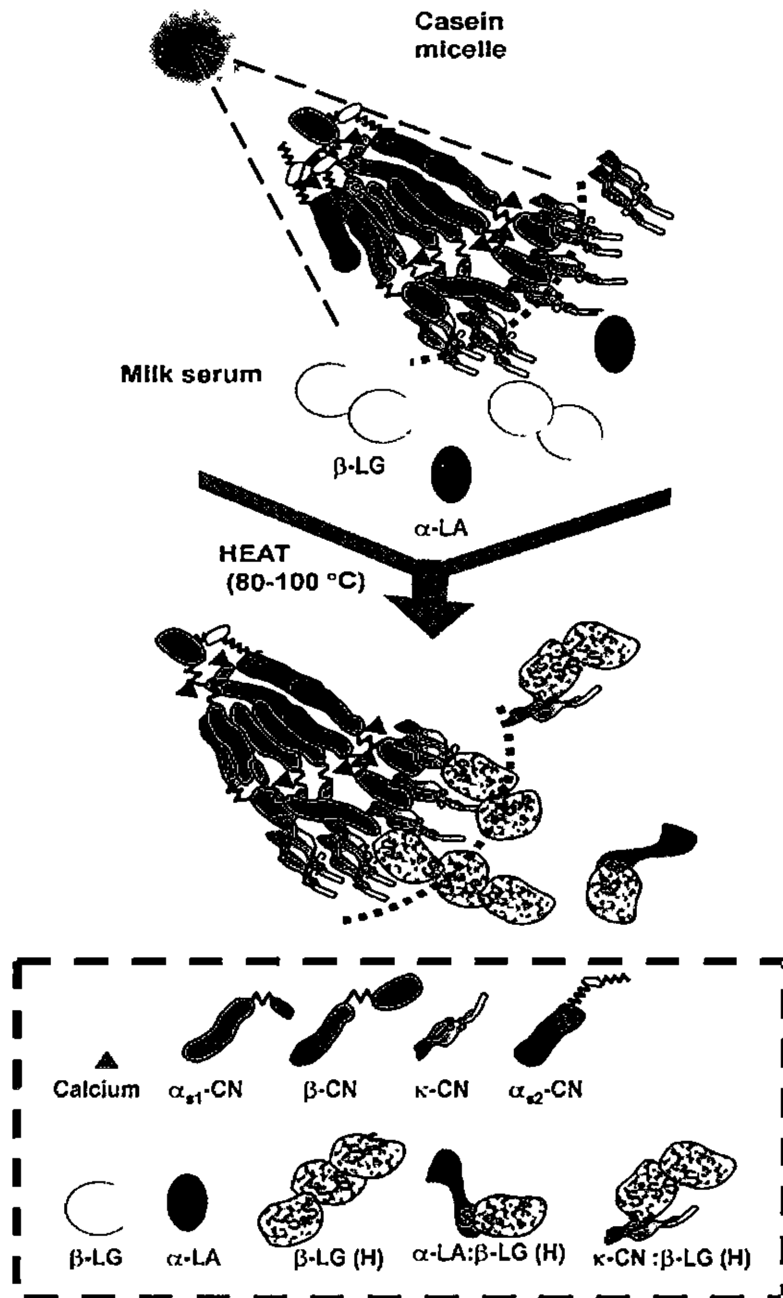

Figure 2.1 The effect of heating milk to 90 °C on the casein micelle. The native β-LG dimer dissociates and the monomer undergoes internal disulfide-bond interchange to give reactive monomers that react with κ-casein (κ-CN) at the surface (outer region) of the casein micelle. Native β-LG monomers can also form an adduct with α-LA, which then give rise to α-LA dimers and α-LA:β-LG dimers. In the severely heat-treated samples, α_{s2}-CN also forms disulfide bonds with other proteins. The (H) indicates heat treated proteins (Considine et al., 2007).

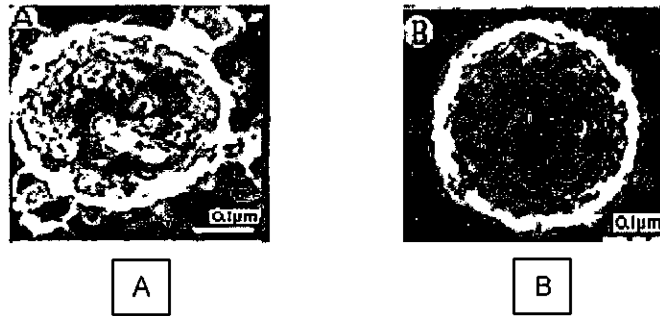

Figure 2.2 Changes evident in the casein micelle structure during pasteurisation of milk. Figure 2.2A represents the casein micelle after pasteurisation, and Figure 2.2B before pasteurisation (Kalab, 1997).

Table 2.1 Protein fraction (g/100 g protein) of cow's milk (n = 30) (Fox and Kelly, 2004; Considine et al., 2007; Ceballos et al., 2009).

Protein	%
Casein (Cn)	82.65
α_{S1}-Cn	30.80
α_{S2}-Cn	7.50
β + $_{\kappa}$-Cn	44.35
Whey proteins	17.35

2.4 STARTER ORGANISMS

2.4.1 Background

Starter bacteria play a vital role in the manufacturing of fermented products. These organisms produce lactic acid which influences the texture, taste and moisture content of the end product. Modern yogurt starter cultures have been developed by retaining small quantities of yogurt from previous completed batches and using it as the inoculums for the succeeding days' production. This process is widely known as "back-slopping" (Mullan, 2001). Starter bacteria growth depends on many factors including milk composition, inoculum size and time and temperature of incubation (Mahdian and Tehrani, 2007).

The major contributions that starter cultures have on yogurt production are the initial hydrolysis of casein micelles and the degradation of polypeptides to peptides and free amino acids. These products of hydrolysis are used by the starter cultures for lactic acid production (Simov et al., 2006). This will be covered in greater detail in Section 2.4.4.

2.4.2 Microbiological aspects of starter cultures

S. thermophilus is a thermophilic Gram-positive bacterium that is a natural inhabitant of raw milk. It requires certain amino acids and B-vitamins for optimal growth and displays limited proteolytic ability (Rajagopal and Sandine, 1989; Mullan, 2001; Robinson et al., 2004; Tamime, 2004). The growth and metabolism of *S. thermophilus* are optimal at a neutral pH of about 6.8 and are normally inhibited at a lactic acid content of above 10 g of lactic acid per kg of yogurt. This usually occurs at a pH of 4.3-4.5, which is indicative of an acid-sensitive bacterium (Robinson et al., 2004; Tari et al., 2009).

As in the case of *S. thermophilus*, *L. bulgaricus* is also Gram-positive and both are facultative anaerobic bacteria. The amount of lactic acid produced by *L. bulgaricus*, the more acid-tolerant of the two bacteria, ranges up to 18 g per kg of yogurt, which indicates that *L. bulgaricus* has an increased acid producing ability in comparison to *S. thermophilus* (Cais-Sokolinska et al., 2004; Tari et al., 2009).

The synergism that exists between the two starter bacteria, termed protocooperation, is evident in their growth in yogurt milk. The exchange in growth factors between the two species leads to an increase in acidifying activity and product yield (Bouzar et al., 1996; Sodini et al., 2000; Robinson et al., 2004; Tari et al., 2009).

Streptococcus genera *Streptococcus pneumonia, -pyogenes, -salivarius, -agalactiae and -vestibularis* are all pathogenic, whereas *Streptococcus salivarius* spp. *thermophilus* is not virulent.

S. thermophilus has lost its virulence through the years due to the inactivation of the genes in the bacteria coding for the protein responsible for virulence. This genus therefore has Generally Regarded As Safe (GRAS) status (Stiles and Holzapfel, 1997; Bolotin et al., 2004; Delorme, 2008). The growth of *Streptococcus* and *Lactobacillus* is so intimate that through the years they have exchanged genes which enabled *S. thermophilus* to hydrolise lactose as is the case for *L. bulgaricus* (Bolotin et al., 2004).

2.4.3 Role of starter cultures in acid production

The most important factor concerning the role of lactic acid bacteria is the acidifying ability of the organisms (Quiberoni et al., 2003). The optimal growth temperature of *S. thermophilus* ranges from 35-42 °C and that of *L. bulgaricus* is between 43 and 46 °C. The incubation temperature of 42 °C is a compromise between the optimum temperatures of the two organisms (Radke-Mitchell and Sandine, 1986; Gueguim-Kana et al., 2007). These growth temperatures are 2-8 °C below that of the optimum temperature where the organisms produce acid. This is proven by the fact that coagulation occurs sooner at temperatures above that at which the greatest amount of cell growth occurs. The temperature where maximum acid production occurs is at 40-48 °C for *S. thermophilus* species. An increase in incubation temperature from 37-45 °C leads to an increase in proteolytic activity of *L. bulgaricus* (Radke-Mitchell and Sandine, 1986).

2.4.4 Impact of starter bacteria on one another

Unlike *S. thermophilus,* which has limited proteolytic ability, *L. bulgaricus* possesses a proteinase which hydrolises casein to release peptides and polypeptides which stimulate *S. thermophilus* growth. Peptidase activity in turn is limited in *L. bulgaricus*, and due to the presence of *S. thermophilus* which can easily hydrolise peptides to free amino acids, the free amino acids are available for *L. bulgaricus* to utilise (Kang et al., 1997; Robinson et al., 2004). The growth of *L. bulgaricus,* which provides flavour compounds, is further enhanced by formic acid which is produced by *S. thermophilus* (Fung, 1996; Stiles and Holzapfel, 1997; Tari et al., 2009).

The growth association that exists between *S. thermophilus* and *L. delbruekii* spp. *bulgaricus* enhances acid production in comparison to the two organisms used individually (Rajagopal and Sandine, 1989; Tamime, 2004; Tari et al., 2009). The optimum ratio that must be maintained between the two starter organisms is *L. bulgaricus:S. thermophilus* as 1:1 (Penna et al., 1997; Cais-Sokolinska et al., 2004; Gueguim-Kana et al., 2007). *L. bulgaricus* does not preserve well because of its sensitivity to freezing and drying. These cells must therefore be harvested in the early stages of the stationary phase, when they are less sensitive (Tamime, 2004).

2.5 LACTOSE UTILISATION

2.5.1 Fermentation of lactose

The first step in the fermentation of yogurt is the active transport of lactose across the cell membranes of the starter bacteria (Robinson et al., 2004). This occurs through the mediation of a membrane-located enzyme, galactoside permease. This transport requires energy due to lactose transport taking place against a concentration gradient. The required energy is obtained by *S. thermophilus* and *L. bulgaricus* by the use of a proton motive force (PMF). Once inside the cell, the enzyme β-galactosidase hydrolises lactose to glucose and galactose. This enzyme has a maximal activity at pH 7.0 and is virtually inactive at pH ≤ 4.0. The glucose is metabolised via the Embden-Meyerhof-Parnas (EMP) Pathway to the end product, namely lactic acid. The lactic acid is released extra cellularly into the cultivation medium. The formation of organic acids (citric, acetic, pyruvic and lactic acid) during fermentation acts as natural preservatives. This results in a pH decrease to around 4.3 where further fermentation is inhibited due to the inhibition of β-galactosidase activity (Fernandez-Garcia, 1994; Kotz et al., 1994; Cogan and Hill, 2004; Robinson et al., 2004).

Although galactose and lactic acid are transported out of the cell and accumulate in the medium, some strains of *S. thermophilus* and *L. bulgaricus* possess a galactokinase enzyme. This enzyme converts the galactose to galactose-1-P which in turn is further converted to either glucose-1-P which enters the EMP Pathway, or to galactose-6-P which is metabolised by the D-Tagatose 6-phosphate pathway. Hereafter it enters the EMP Pathway and is metabolised to lactic acid as the final product (Hickey et al., 1986; Robinson et al., 2004). This pathway is illustrated in Figure 2.3.

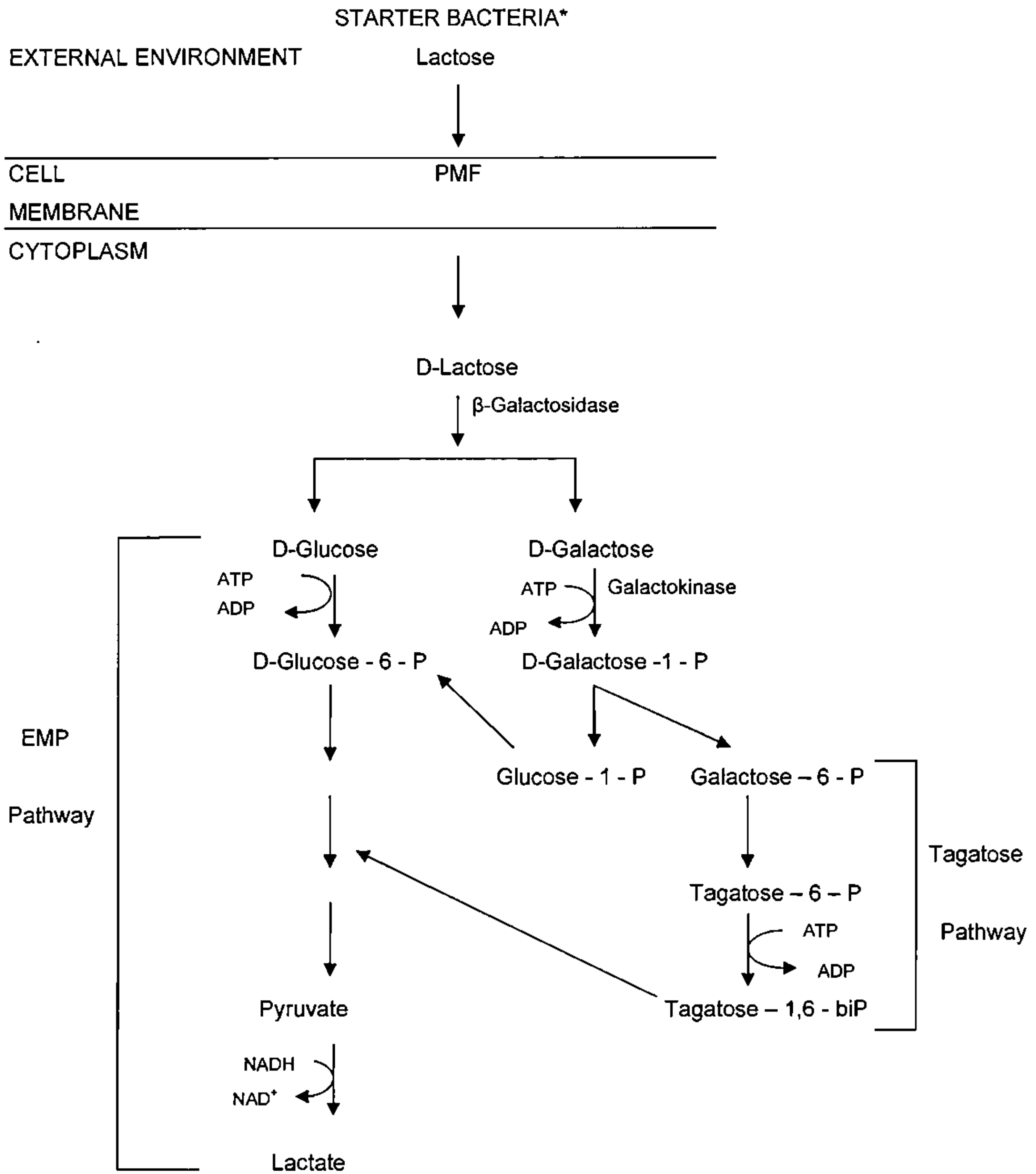

Figure 2.3 Sugar metabolism by starter cultures via the Embden-Meyerhof-Parnas (EMP) pathway. Adapted from Cogan and Hill (2004). * *Streptococcus salivarius* spp. *thermophilus, Lactobacillus delbruekii* spp. *bulgaricus and Bifidobacterium lactis.*

2.5.2 Lactose transport

Lactic acid bacteria transport lactose into the cell by means of two systems: a phosphoenolpyruvate (PEP)-lactose phosphotransferase system and a lactose permease system (Hickey et al., 1986).

L. bulgaricus uses a permease system rather than a PEP-dependent phosphotransferase for the transport of lactose and galactose into the cell. The permease system only transfers the sugars from the medium into the cell, whereas the phosphotransferase system starts hydrolising the sugars while they are being transported into the cell. Most *S. thermophilus* strains possess a β-galactosidase permease and PEP-sugar phosphotransferase system. The former is responsible for lactose transport into the cell whereas the latter facilitates transport of lactose, glucose and galactose into the cell. *S. thermophilus* only ferments the glucose moiety of lactose and releases unmetabolised galactose into the medium (Hickey et al., 1986; Farkye and Vedamuthu, 2002).

In studies done by Hickey et al. (1986) it was found that *L. bulgaricus* grown in the presence of excess lactose does not metabolise the galactose originating from lactose hydrolysis. The activity of β-galactosidase was found to be much higher for cells grown on lactose in comparison to using glucose as substrate. β-Galactosidase activity was immediately repressed by adding glucose and galactose to bacterial cells grown on lactose as substrate. The utilisation of galactose and lactose present in the growth medium of the bacterial cells are greatly reduced by the presence of glucose due to an exclusion effect exerted by glucose (Hickey et al., 1986).

2.6 POST ACIDIFICATION

During refrigerated storage, the lactobacilli in yogurt may continue to produce acid and lower the pH to 3.5. This affects the viability of probiotic bacteria and moreover results in an excessively sour product. This occurrence is known in the industry as post acidification (Weinbrenner et al., 1997; Dave and Shah, 1998). Three common methods for preventing excessive acid formation by yogurt cultures have been identified. The first is pasteurisation of the finished yogurt in order to eliminate the viable starter bacteria. This eliminates the beneficial probiotic cultures in yogurt. The other two methods include increasing the proportion of streptococci to lactobacilli in the yogurt culture, and rapidly cooling the finished product to reduce starter culture activity. These two methods delay, but do not prevent acid formation (Weinbrenner et al., 1997).

2.7 FLAVOUR AND TEXTURE COMPONENTS IN YOGURT

2.7.1 Acetaldehyde

Acetaldehyde is the most important flavour compound which is formed during yogurt fermentation and is also considered the main indicator of flavour. The two starter organisms normally associated with yogurt production, *L. bulgaricus* and *S. thermophilus*, use lactose, amino acids and nucleic acids as the main sources for acetaldehyde production. Increased incubation time enables the yogurt micro-organisms to produce more acetaldehyde which enhances the flavour of the product (Gardini et al., 1999; Ozer and Atasoy, 2002). In mixed cultures *L. bulgaricus* is the most prominent in synthesising acetaldehyde. Although *S. thermophilus* also produces this flavour component, it is to a much lesser extent (Robinson et al., 2004). When *L. bulgaricus* dominates, or when excessive amounts of starter bacteria are used for yogurt fermentation, a harsh acid flavour will be present in the end product. This is an indication of overproduction of acetaldehyde (Collado and Hernandez, 2007). The final concentrations of acetaldehyde which contribute to yogurt's characteristic flavour range between 2.4-41 µg/g (Robinson et al., 2004).

2.7.2 Extracellular polysaccharides

The extracellular polysaccharide (EPS) is a gum-like material which forms filamentous links between cell-surfaces of the protein matrix and bacteria. The exopolysaccharide filaments interfere with the casein network and partly inhibit protein-protein interaction as well as protein strand formation. This leads to reduction in rigidity of the yogurt gel. EPS, which is a ropy exopolysaccharide substance, has a slimy consistency and is formed by a so-called "slimy" culture (Robinson et al., 2004; Guzel-Seydim et al., 2005; Sodini et al., 2005). The polysaccharide substances produced by lactic acid bacteria mainly consist of glucose and galactose. A few strains however also produce rhamnose (Petry et al., 2000; Guzel-Seydim et al., 2005). These slime producing cultures can be different strains of either *S. thermophilus* or *L. bulgaricus*. These strains secrete "slimy/ropy" polysaccharides, which either migrate into the surrounding medium or form a capsular envelope around the cell. The gel of the capsular polysaccharides is less prone to damage due to stress and gives a thicker texture to the final yogurt product. Viscosity, which is lost during packaging, is recovered by the encapsulated gels which "clump" together while binding the casein micelles. Syneresis is retarded and viscosity may be further enhanced by additional polysaccharide synthesis provided that yogurt is held at room temperature before it is chilled (Fajardo-Lira et al., 1997; Robinson et al., 2004; Robitaille et al., 2009). Using ropy (EPS-producing) starter strains reduce syneresis of yogurt to a larger extent than non-ropy strains of starter bacteria (Robitaille et al., 2009).

Petry et al. (2000) confirmed in their studies that in order for *L. bulgaricus* to achieve optimal EPS yields, maximum bacterial growth is needed. For certain strains investigated by Petry et al. (2000) it was evident that the proportions of EPS produced in milk varied as a function of the growth phase but

not of the carbon source. Application of exopolysaccharide producing cultures provide better texture in low fat yogurts than additives for example fat replacers (Guzel-Seydim et al., 2005).

2.8 HEALTH ASPECTS OF YOGURT

2.8.1 Probiotics

Functional foods is a term used to describe foods fortified with ingredients promoting health. Yogurt which contains probiotics is one of the products which falls in this category (Stanton et al., 2001). A probiotic is defined by McKinley (2005) as living micro-organisms (single or mixed cultures), which upon ingestion in required quantities exert health benefits beyond inherent general nutrition. Such an organism can exert its effects during passage through the gastrointestinal tract and does not need to colonise the tract to do so. Lactobacilli are generally used as probiotics. The most common way however of administrating probiotics is by using fermented dairy products (Stanton et al., 2001; Ouwehand et al., 2002; Collado and Hernandez, 2007). Organic acids, bacteriocins and other metabolic products of probiotic bacteria may control undesired micro-organisms and also lead to an increased shelf life of the product (Ekinci and Gurel, 2008).

The beneficial effects exerted by probiotics are usually associated with products that are fermented with *L. bulgaricus* and *S. thermophilus* due to their ability to degrade lactose to its respective monomers (Ouwehand et al., 2002). *Bifidobacterium*, which is an anaerobic genus that is usually present in yogurt products, is also classified as a probiotic (Dave and Shah, 1996; Stanton et al., 2001; Collado and Hernandez, 2007). Due to the lack of proteolytic activity by probiotic bacteria, the efficacy of this organism in a probiotic mixture is improved by the presence of *S. thermophilus* and *L. bulgaricus* (Dave and Shah, 1998; Oliveira et al., 2001; Delorme, 2008).

Yogurt contains various organic acids, peptones, peptides, other trace activators and lactic acid bacteria. It also has an intestine-cleaning function to promote the proliferation of intestinal lactic acid bacteria and probiotics (Park and Oh, 2007). The contribution of bifidobacteria to good health has been recognised for quite some time. This has lead to the widespread utilisation of bifidobacteria as probiotics for maintaining or improving human health (Ouwehand et al., 2002). The increase in commercial interest in the proposed health benefits of probiotics has contributed to the rapid growth in this sector of the market (Stanton et al., 2001).

2.9 POSSIBLE ENHANCEMENTS DURING YOGURT PREPARATION AND FERMENTATION

2.9.1 Changes in physical conditions of yogurt fermentation

2.9.1.1 Temperature

The overall quality of yogurt is affected by the incubation temperature. Two methods of yogurt manufacture exist i.e. low temperature of 30-37 °C for 7-8 hours, or incubation at 42 °C for 3 hours. Although the low temperature incubation method allows more production of flavour substances such as acetaldehyde, the high temperature method is more economical for dairy plants due to the reduced fermentation time (Guzel-Seydim et al., 2005).

In studies done by de Brabandere and de Baerdemaeker (1999), it was found that the decrease in pH was significantly lower when fermentation was done at temperatures below that of optimum acid production. The pH decrease had the shortest lag time in milk that had been sterilised (133 °C for 8 seconds) before the starter culture was added. It also yielded the fastest pH decrease measured against time. Sterilisation of milk therefore enhances pH development of yogurt during fermentation (de Brabandere and de Baerdemaeker, 1999). Considine et al. (2007) found that severe heat treatment of milk (130 °C for several minutes) causes substantial increases in the size of the particles in milk. These changes were found to be due to the aggregation of the casein micelles.

Whereas β-LG denatures and adheres to κ-caseins at ±80 °C, heat treatment of α-LA has very little effect on aggregation. Evidence shows that α-LA is readily heat denatured, but has a great tendency to renature rather than form aggregates (Considine et al., 2007).

Incubation temperature also has an influence on the production of EPS. Low temperatures cause increased ropy polysaccharide production (Guzel-Seydim et al., 2005).

2.9.1.2 Pressure pretreatment

De Ancos et al. (2000) found that pressure application (100-400 MPa) to yogurt did not have any significant effect on the pH profile during yogurt manufacturing. The only effect that was noted in comparison to untreated yogurt was an increase in viscosity and amino acid content. In addition to loss in solubility of both β-LG and α-LA at pH 4.6, high pressure treatment at pressures exceeding 100 MPa also denatures these two whey proteins. β-LG is one of the most pressure-sensitive proteins whereas α-LA, due to its more rigid molecular structure, is one of the most pressure resistant proteins. Although some of the denatured β-LG remains non-sedimentable (either in the form of whey aggregates or associated with casein particles too small to be sedimented), the majority of denatured β-LG associates with the casein micelles. On release of pressure, unfolded α-LA and β-LG molecules

that have not interacted with another protein may refold to a state closely related to that of native β-LG. The reason for the increase in viscosity of yogurt that underwent prior pressure treatment, is due to the fact that the pressure disintegrated the casein micelle into smaller subunits. This resulted in a product with increased aggregating properties. Pressures higher than 200 MPa lead to a reduction in activity of starter culture and thus reduction in the acidifying ability (de Ancos et al., 2000; Huppertz et al., 2006; Considine et al., 2007).

2.9.1.3 Oxygen limited conditions

L. bulgaricus and *S. thermophilus* are both facultative anaerobes that can grow in aerobic conditions, but prefer anaerobic environments (Beshkova et al., 2002; Horiuchi et al., 2009).

An improved, short-time process for fermentation of comestible products is described by Fung (1996). This process enhances the growth of operative micro-organisms in a fermentation system and consequently reduces the required incubation time. The process involves inoculating yogurt milk with an amount of an oxygen-reactive enzyme such as OXYRASE™ (a trademark of Oxyrase, Inc. of Akron, Ohio) which is not naturally generated during the fermentation process. Oxyrase is known to be an effective oxygen-reducing enzyme used to create anaerobic conditions. Reduced time fermentation can be achieved in a wide variety of systems, such as in the production of fermented liquid, semi-solid and solid dairy products, fermented meat products, fermented cereal-based products, yeast-raised baked and fried products and alcoholic beverages. Fermentation is improved by the addition of an oxygen-reactive enzyme into the fermentation system to accelerate the activity of fermentative micro-organism(s) present therein (Fung, 1996).

The amount of Oxyrase to be used depends on the amount, activity and growth characteristics of the micro-organisms present in the fermentation system. When using this enzyme, a larger population of starter culture caused higher acidity and this in turn caused a more developed acidic flavour (Fung, 1996).

2.9.2 Supplementations added to yogurt milk

There are three main strategies that have been considered for the acceleration of cheese ripening (Martinez-Cuesta et al., 2001) which can be modified and adapted during optimised yogurt fermentation studies.

- Use of optimised starter organisms
- Higher ripening temperature
- Addition of exogenous enzymes

The levels of essential minerals, amino acids, trace elements and vitamins that are present in milk and dairy products are influenced by various factors. These include environmental conditions, lactation stage and also technological handling of the product (Gambelli et al., 1999). Growth of starter bacteria depends on adequate supplies of suitable sources of nitrogen and carbon. The carbon source is not limiting if the starter organisms possess an enzyme which hydrolises lactose, e.g. β-galactosidase. This however is not the case for nitrogen, due to the fact that free amino acids and peptides are present only to a limited degree in milk (Rajagopal and Sandine, 1989). Lactic acid production is evidently influenced by the type as well as the initial concentration of the nitrogen source which is present in the medium (Hujanen and Linko, 1996). The manner of preparing seed cultures seems to be especially significant regarding lactose fermentation (Amrane and Prigent, 1994).

2.9.2.1 Different products used as supplements

An invention by Vandewegh et al. (2002) relates to a process for decreasing the time required for the production of yogurt without compromising the product quality. Fermentation is done at 40 °C to about 46 °C followed by direct acidification by using any acid appropriate for addition to foodstuffs. The yogurt composition is directly acidified when the pH of the composition reaches a pH of about 4.8 to 5.2. The composition can be acidified while the temperature is at 40 °C to about 46 °C, or the composition can be acidified during or after cooling. Previously used yogurt production processes rely on the production of lactic acid by the yogurt culture to lower the pH below 4.6. Depending on the bacterial culture added and the method of addition of the culture (i.e., bulk or direct), the fermentation time is typically 2 to 5 hours. Using the yogurt cultures combined with the present acidification step, fermentation times have been dramatically shortened, usually by about 50 %. This process can be used in the production of any fermented dairy product such as yogurt (Vandewegh et al., 2002).

Extracts

Yeast extract

Yeast extracts, also known as yeast autolysates, are concentrates of the soluble components of yeast cells which are mainly derived from *Saccharomyces cerevisiae* and to a lesser extent from *Kluyveromyces fragilis* or *Candida utilis*. These extracts are mainly produced by autolysis where the

yeasts are degraded by their own endogenous enzymes without the addition of other enzymes. This is done by applying controlled temperature or osmotic shock (Sommer, 1996).

Yeast extract is of great importance in the preculture medium because of the purine and pyrimidine bases and the B-vitamins which are present. In studies done by Hujanen and Linko (1996), it was clearly established that addition of yeast extract had a significant effect on lactic acid production during the lag phase of bacterial growth. An increase in yeast extract level lead to a linear increase in lactic acid concentration.

Cogan et al. (1968) found yeast extract to be a good stimulant for *L. delbrueckii* growth. Studies done by Elli et al. (1999) coincides with this by stating that bacterial growth can be improved by the addition of substances such as yeast extract or peptones (of various origins) to the growth medium. The stimulatory role of substances such as yeast extract and peptones on bacterial growth in milk has been related to their nucleotide content (Elli et al., 1999).

Elli et al. (1999) had successful results when adding yeast extract and peptones to milk which had been inoculated with *L. johnsonii*. All investigated *L. johnsonii* strains were unable to grow in skim milk as well as in whole fat UHT milk samples without the addition of chemically undefined substances like yeast extract or peptones. The supplementation of yeast extract or peptones resulted in a two log increase of viable cell numbers. Unfortunately, changes in flavour and colour are usually observed in fermented dairy products supplemented with yeast extract. The ability of these complex materials to stimulate the growth of lactobacilli was previously linked to the presence of nucleotides and amino acids. Further studies by Elli et al. (1999) confirmed the importance of four key amino acids (L-cysteine, L-alanine, L-serine and L-isoleucine). Although the strain *L. johnsonii* could grow in the absence of L-alanine, L-serine or L-isoleucine, these amino acids strongly stimulated its growth when exogenously supplied. The most important amino acid is L-cysteine, confirming that an absence of this sulphydrilic compound in milk negatively affects bacterial growth. In contrast to the stimulatory effect associated with the addition of four key amino acids (L-cysteine, DL-alanine, DL-serine and DL-isoleucine), a significant negative impact was observed on the remaining amino acids (Elli et al., 1999).

Although experiments done by Hujanen and Linko (1996) showed the addition of grass extract and malt sprouts resulting in the same end percentage of lactic acid, the fermentation time was greatly decreased by addition of yeast extract during yogurt fermentation. Concentrations of 0.4 % and 2.2 % yeast extract respectively showed good potential as growth factors and nitrogen sources for lactic acid production.

Casein hydrolysate

Acid casein hydrolysate (ACH) is obtained by using hydrochloric acid to digest casein. This results in an end product in which a proportion of the vitamins, amino acids and other growth-promoting substances are retained (Lamprecht and Haberhauer, 2001). The incorporation of peptides and amino acids which are present in casein hydrolysate may reduce the fermentation time of yogurt where only *S. thermophilus* is used as pure culture (Oliveira et al., 2001). Casein hydrolysate addition to a pure culture of *Lactobacillus casei* however does not have a significant effect on the fermentation time of yogurt (Hujanen and Linko, 1996).

In research done by Dave and Shah (1998) it was determined that the addition of acid casein hydrolysate and tryptone to yogurt, affected the acidifying rate significantly in comparison to the control. Due to the increase in available micronutrients, the time needed to reach pH 4.5 subsequently decreased by 25 %. Yogurt supplemented with 500 mg/l of ACH or tryptone yielded results that were similar to those of yogurt supplemented with 250 mg/l of ACH or tryptone, respectively.

In similar research, Oliveira et al. (2001) found that the acidifying ability of the starter organisms used in yogurt fermentation was greatly increased by the supplementation of casein hydrolysate. A 55 % reduction in fermentation time was noted by casein hydrolysate supplementation in comparison to the addition of whey powder and milk protein supplementation. A conclusion was reached that the addition of casein hydrolysate and the use of mixed starter cultures as an alternative to pure cultures, results in increased acidification. Textural properties also showed improvement in the end product (Oliveira et al., 2001; Mahdian and Tehrani, 2007). Studies done by Lucas et al. (2004) produced similar results stating that the acidifying activity of *S. thermophilus* is positively influenced by supplementation of hydrolysates. The addition of a small amount of casein hydrolysate also improved the growth of *L. acidophilus*, a probiotic frequently used in yogurt fermentation (Masuda et al., 2003).

Proteose peptone

Proteose peptone is produced by the enzymatic digestion of animal tissues. It is commonly used in the preparation of culture media for the production of toxins, and in the fermentation industry for starter cultures. It is a highly nutritious medium for the growth of a wide range of micro-organisms (Condalab, 2008). Innocente et al. (1998) described the proteose peptone fraction of bovine milk as the heat-stable and acid-soluble protein fraction that has important functional properties.

Bacterial growth can be improved in milk by the addition of substances of undefined composition to the growth medium, such as yeast extract or peptones of various origins. The off-flavours associated with these compounds, as well as the cost and variability in stimulating bacterial growth, however, render these substances less suitable for industrial production of commercial dairy products (Elli et al., 1999).

Whey

Yogurt quality is affected by numerous factors. These factors include chemical composition of the milk and methods which increase the total non-fat solids content by fortification (Guzman-Gonzalez et al., 1999). Creaminess and smooth consistency are important qualities of yogurt. One of the ways to obtain these features is by the addition of milk solids in the form of milk powder or whey powder. Whey has a high concentration of minerals that can change yogurt's ionic strength (Penna et al., 1997). It is used more readily than millk powder due to its cost effectiveness (Sodini et al., 2005). Penna et al. (1997) however found in their studies (which compared milk powder addition to whey powder addition) that the use of milk powder resulted in the shortest fermentation time. When equal parts of skim milk powder and demineralised whey powder were used, it was necessary to increase the amount of starter culture that was used in order to maintain the same fermentation time (Penna et al., 1997). Addition of whey powder also resulted in a product with softer gel texture than a product where skim milk powder was added (Gonzalez-Martinez et al., 2002). Although the addition of whey protein concentrates to yogurt during manufacture increased the water-holding ability of yogurt by higher cross-linkage of the network, no acceleration in fermentation was noted (Sodini et al., 2005). Studies done by de Brabandere and de Baerdemaeker (1999) established that pH development was not affected by fortification with dry matter.

Utilising whey or ultra filtrated (UF) whey permeate as a medium for *L. bulgaricus* or *S. thermophilus* fermentation is a cheap and readily available source. In spite of this fact both media require supplementation of yeast extract or another complex additive in order for lactic acid bacteria to proliferate. The addition of 1 or 2 % of whey protein concentrate (WPC) to whey or UF whey permeate broths, can lead to significantly higher bacterial counts and an increase in lactic acid development by the starter bacteria. During ultra filtration some growth factors are removed, which presents whey broth as a better growth medium for acid production and bacterial growth than UF whey permeate broth (Bury et al., 1998).

The acidifying rate of yogurt fermentation was significantly affected over 24 hours of fermentation by the supplementation of whey powder, whey powder concentrate, acid casein hydrolysates and cysteine. The control, in comparison, containing 2 % skim milk powder, did not show a notable change in the acidifying rate (Dave and Shah, 1998).

Teas

The many health benefits that have been ascribed to tea due to its strong antioxidant activity make it the second most popular consumed beverage worldwide (Jaziri et al., 2009). Studies done by Jaziri et al. (2009) showed that although black and green tea possess antioxidant and antimicrobial properties, their addition to yogurt have no influence on the final pH or lactic acid levels of the yogurt. The survival of the yogurt micro-organisms and the fermentation time were also not affected (Jaziri et al., 2009).

Vitamins

Vitamin B: vitamins B_2, B_6 and B_{12}

Vitamin B_2, also known as riboflavin, is stable against heat application while it is easily destroyed by light in both alkaline and acid media. Due to the fact that riboflavin is photosensitive, the most significant losses of this vitamin occur once milk is exposed to light during processing. Vitamin B_6, also known as pyridoxal, is sensitive to heat as well as light, and the sensitivity is to some extent dependent on the pH of the medium (Biesalski and Back, 2002).

With the exception of vitamin B_{12} and folic acid, milk is an excellent source of B-vitamins (Rao et al., 1984). Arkbage (2003) disagrees with this statement and claims that milk and dairy products are good sources of vitamin B_{12} as well as folate.

The average vitamin B_{12} content in milk was recorded as 6.6 µg/l by Collins et al. (1950). In studies by Arkbage (2003) the heat treatment of milk (96 °C for 5 minutes) caused no loss in vitamin B_{12}, whereas the vitamin B_{12} content decreased by 9 % after starter culture had been added to the milk. At the point of product packaging, vitamin B_{12} content further decreased by an additional 16 %. Arkbage (2003) found that the fermentation of milk followed by storage at 4 °C for 14 days decreased vitamin B_{12} concentrations in fermented milk by 40-60 %. This was most likely attributed to the fact that vitamin B_{12} was utilised by starter cultures. Although this study group reported the decrease in vitamin B_{12} content during the fermentation of milk, they stated that it is however possible to find certain strains and conditions where vitamin B_{12} is produced. Studies done by Rao et al. (1984) showed no increase in vitamin B_{12} content, regardless of the culture used during fermentation. Rao et al. (1984) further reported that *S. thermophilus* is not effective in synthesising vitamin B_{12} and that *L. bulgaricus* utilises more B_{12} than other lactic cultures.

Folic acid

Folic acid is a water soluble B-vitamin. The average folic acid content in milk varies from 1 to 4 µg/l. Two different forms of folic acid exist; a natural form known as folate, and a synthetic form found in multivitamins and fortified foods (Prieto et al., 2006). Folic acid mostly exibits substantially greater stability than folates. Folates rapidly becomes unstable in food over a period of days, while folic acid remains stable for months or even years (Forssen et al., 2000; WHO and FAO, 2002; Boeneke and Aryana, 2008). This stability is pH-dependent, with folates being most stable above pH 8 and below pH 2. Dairy products are not good sources of folic acid, and cow's milk contains only 5-10 µg of folic acid per 100 g of milk, while the RDA is 400 µg (Forssen et al., 2000; Boeneke and Aryana, 2008; Cueva and Aryana, 2008).

Gorbach (1990) reported that fermentation increases the riboflavin and folic acid content in yogurt. Studies done by Boeneke and Aryana (2008) established that the average folic acid content in yogurt was higher when folic acid was added after pasteurisation in comparison to addition prior to

pasteurisation. Ristow et al. (1982) found folic acid to be stable at processing temperatures of 120 °C for 20 minutes. In similar studies Wigertz et al. (1996) examined folate present in milk and yogurt and reported significantly reduced levels of total folate in pasteurised milk and yogurt exposed to 90 °C for 10 minutes. In contrast, Forssen et al. (2000) found that pasteurisation only had minor effects on the folate content of milk, causing losses of less than 10 %. These differences in opinions are possibly due to the influence of different starter cultures and strains on the folate content (Boeneke and Aryana, 2008).

Rao and Shahani (1987) and Forssen et al. (2000) found that the total folate levels in milk fermented by *L. bulgaricus*, decreased from 9.8 µg to 1.6 µg/100 g during an incubation time of 36 hours. Folates already present in the milk were utilised, whereas in the case where *S. thermophilus* was used, the total folate levels increased substantially. In the case where both *L. bulgaricus* and *S. thermophilus* were used, the former might have consumed the folates produced by the latter. This explains the minor effect on total folate content in the final product (Rao et al., 1984; Forssen et al., 2000; Boeneke and Aryana, 2008). Rogosa et al. (1961) reported that the addition of folic acid at a concentration of 10 µg/l led to inhibition in the growth of *L. bulgaricus*. In order for normal bacterial growth to continue, it was necessary to decrease the folic acid content to below 1 µg/l (Rogosa et al., 1961).

Almost all naturally occurring folate in milk is bound to a folate-binding protein (FBP) (Arkbage, 2003). Wigertz et al. (1996) and Arkbage (2003) found that all folates in unprocessed milk and pasteurised milk were protein bound, while folates in UHT-processed milk and yogurt occurred freely. A significantly lower concentration of FBP was found in pasteurised milk in comparison to unprocessed milk. Only low levels of FBP concentrations were found in UHT milk and plain yogurt (Forssen et al., 2000). Since an acidic pH releases folates bound to FBP, fermentation may affect protein binding. Folates bound to FBP were found to be absorbed in a different and more efficient manner than free folates. Another possible effect of this protein binding is that milk folates are sequestered and thereby prevented from being consumed by intestinal micro-organisms (Wigertz et al., 1996). Conflicting data on the reduction of the folate-binding capacity after pasteurisation has been reported, ranging from 10 % (Ford, 1974; Wigertz et al., 1996; Arkbage, 2003) to more than 90 % (Areekrul et al., 1978). Ford (1974), Wigertz et al. (1996), Forssen et al. (2000) and Arkbage (2003) all found a decrease in folate-binding properties of 10 % after low-temperature, long-term pasteurisation. Areekul et al. (1978) on the other hand noted that a reduction of more than 90 % in folate-binding capacity in milk pasteurised for 15 s at 75 °C (HTST). These inconsistent results may be due to the pasteurisation conditions being very close to those at which FBP denaturation takes place (Wigertz et al., 1996). An increase of more than 200 % in folate concentration was noted by Rao et al. (1984) during fermentation of skim milk by *S. thermophilus* and *L. acidophilus*, whereas *L. bulgaricus* reduced folic acid to negligible within hours.

Contradictory reports of biosynthesis of folic acid and vitamin B_{12} have been numerously reported. Different strains, species and incubation times might be reasons for the variations in folic acid synthesis and folate content determined by several research groups (Rao et al., 1984; Forssen et al., 2000).

The many complexities involved in measurement of the folic acid content in food have been emphasised by the disagreement between methods used and between laboratories (Indyk, 2010).

Ascorbic acid

Contrary to popular belief, the ascorbic acid content in milk is not entirely destroyed during processing. During UHT treatment only up to 25 % is destroyed. This is due to the destruction of the dehydroascorbic acid formed by the influence of dissolved oxygen on ascorbic acid. The instability of ascorbic acid i.e. vitamin C, is due to this antioxidant's sensitivity to heat, light and oxygen. In food it can be partly or completely destroyed by lengthy storage or overcooking. Milk, whether pasteurised, UHT-treated or sterilised, loses significant amounts of riboflavin and vitamin C when exposed to direct sunlight, diffused daylight or fluorescent light. Losses of vitamin C are also markedly influenced by the residual oxygen level in milk (Rolls and Porter, 1973; DSM, 2008).

When ascorbic acid (commonly used as a food additive), is used to fortify yogurt, it can act as an oxygen scavenger and can prove useful to maintain low oxidation-reduction potential which is necessary for the viability of probiotic bacteria. The incorporation of ascorbic acid in yogurts, however, can reduce the amount of oxygen required for the activities of the facultative anaerobic *S. thermophilus*, a vital micro-organism in the manufacture of yogurt. This can subsequently have detrimental effects on the textural and nutritional qualities of yogurt. For the above reasons the use of ascorbic acid in yogurts may not be practical (Talwalkar and Kailasapathy, 2004).

Minerals

Calcium

Nutritionists and consumers alike recognise dairy products as good sources of calcium. An unfortified 170 g serving of fruit-flavoured low fat yogurt provides only approximately 200 to 250 mg of calcium, while the current recommended dietary allowance (RDA) is 1000 mg. Calcium fortification is usually done with consumer interest in mind (Park, 1988; Fleury et al., 1998). There is therefore great public interest in the use of food products that will supply the RDA of calcium.

Calcium fortification is usually done after the fermentation process has been completed and the fruit pulp and flavouring have been added. In milk, 60-70 % of the calcium exists as insoluble colloidal calcium phosphate associated with the casein micelles. Added acid-soluble calcium salts tend to settle at the bottom making uniform dispersions difficult during the manufacturing process. Sterile calcium can be added after the fruit pulp, as the acidity of the fruit assists solvation of the calcium.

This procedure however is cumbersome and uneconomical (Hansen and Fligner, 1993; Fleury et al., 1998).

Tricalcium phosphate (TCP) can be added prior to fermentation, but results in an unacceptably gritty end product. Calcium gluconate is eliminated for pre-pasteurisation fortification as it is not heat stable (Hansen and Fligner, 1993).

In isolated cases where calcium was added prior to fermentation, calcium-fortified soy milk required an inoculation load double the amount needed for unfortified soy milk. This resulted in higher titratable acidity and increased syneresis (Yazici et al., 1997).

Iron

Despite its rich nutrient composition, milk displays a strong functional iron-deficiency due to the fact that iron is complexed as lactoferrin, therefore being unavailable for the metabolism of lactobacilli. The absence of the available iron can negatively affect the cell-division of certain *Lactobacillus* species e.g. *L. acidophilus* (Elli et al., 1999). In a study by Simova et al. (2008) however, iron fortification in yogurt with concentrations ranging from 8 to 27 mg/kg showed no significant influence on yogurt fermentation.

Sugars

Masuda et al. (2003) established that the addition of glucose to milk did not promote the growth of *L. acidophilus*. Donkor et al. (2007) however found that a 1 % glucose supplementation (in combination with 1 % raffinose) to soy milk increased the bacterial population (*L. bulgaricus, S. thermophilus* and *L. acidophilus*) and also the amount of lactic acid.

The observations reported by Masuda et al. (2003) suggesting that glucose transport is inefficient in *L. acidophilus*, stands in contrast to the general idea that glucose is the most energetic or hierarchically preferred sugar for bacterial growth. Chervaux et al. (2000) ascribed this to bacterial adaptation to a given growth medium.

L. bulgaricus and *L. acidophilus* are usually found and extensively cultured in milk in which lactose is present at a high concentration (±5 %). The addition of glucose in concentrations ranging from 0.1 % to 2 % by Chervaux et al. (2000) did not affect the growth rate of these organisms on lactose. Moreover, no diauxic growth curve could be observed, suggesting that both sugars were more likely utilised simultaneously by *L. bulgaricus* (Chervaux et al., 2000).

It is interesting to note that, although it is the glucose portion of lactose that is preferentially metabolised, when adequate free glucose is available for fermentation, utilisation of lactose by *S. thermophilus* does not subside (O'Leary and Woychik, 1976).

L. bulgaricus ferments lactose, glucose, fructose, and mannose and does not grow on galactose. Galactose has been reported to accumulate in the medium after fermentation of the glucose moiety of lactose (Chervaux et al., 2000). O'Leary and Woychik (1976) confirmed this statement by declaring that *S. thermophilus* preferentially ferments the disaccharides lactose and sucrose.

Lactose is the major carbohydrate present in milk and constitutes 4.8 % of milk composition. The solubility of lactose is less than that of other sugars and results in about 17.8 g/100 g solubility at 25 °C. High processing temperatures lead to the Maillard reaction which in turn leads to browning and off-flavour development. The reaction is dependent on water activity, pH of the medium, temperature and duration of exposure. At temperatures exceeding 100 °C, lactose is converted to acids, mainly formic acid. The presence of these acids lowers the pH of the milk (Singh and Bennett, 2002) and reduces the availability of lactose to starter organisms. Sterilisation of milk (133 °C for 8 seconds) however enhanced pH development of yogurt during fermentation, which implies that the starter organisms were not affected by the possible reduction in available lactose (de Brabandere and de Baerdemaeker, 1999). Bury et al. (1998) found that the addition of extra lactose within WPC did not increase growth or acid development of *L. bulgaricus* when added to whey or whey ultra filtrated permeate broths.

During pasteurisation and the subsequent fermentation of yogurt, one third of lactose is hydrolised (Richmond et al., 1987). Mouillet et al. (1977) confirmed this by stating that during their studies, only 33 % of the original lactose present in milk was hydrolised during yogurt fermentation. Glucose remained at trace concentrations while galactose accumulated in the medium during fermentation (Richmond et al., 1987). An interesting observation noted by Chervaux et al. (2000) was that lactose, rather than glucose, markedly increased the growth rate of *L. bulgaricus*.

Amino Acids, organic acids, purines and pyrimidines

Amino acids

Milk is generally low in free amino acid and peptide content (Rajagopal and Sandine, 1989; Elli et al., 1999). Yogurt, however, contains higher levels of free amino acids as compared to milk due to proteolysis by the yogurt starter cultures (Gorbach, 1990).

Studies performed by Grobben et al. (1998) established that the omission of aspartic acid, glutamic acid, and glycine respectively affected *L. bulgaricus* growth only slightly. The omission of glutamine, asparagine and threonine in respective tests, resulted in a stronger reduction in growth. All other amino acids were essential. Multiple omissions of amino acids caused an almost complete loss of *L. bulgaricus* growth (Grobben et al., 1998). Corresponding studies done by Robinson et al. (2004) reported that amino acids such as histidine, valine, methionine, cysteine and glutamic acid are not present in milk at levels high enough to support the essential growth of *S. thermophilus*.

In contrary to the above, Vasiljevic and Jelen (2001) stated that even though supplementation with peptides and amino acids may increase the viability of probiotic organisms in milk, the casein fraction of the milk contains all the amino acids that are necessary for the growth of lactic acid bacteria in milk. As a matter of fact, according to Vasiljevic and Jelen (2001), less than 1 % of the available casein is needed for optimal starter bacteria growth.

Dave and Shah (1998) determined that the addition of cysteine as supplement during yogurt fermentation affected the acidifying rate significantly in comparison to the control which received no cysteine supplementation. The respective additions of 250 and 500 mg/l of cysteine to yogurt, increased the time to reach pH 4.6 by 50 %. Samples with added cysteine at a concentration of 50 mg/l showed a drop in pH during fermentation that was similar to that of the control yogurt. An increase in the concentration of cysteine above 50 mg/l adversely affected the rate of acid production. The addition of cysteine at concentrations above 50 mg/l caused damage to the cell wall and the cell membrane of *S. thermophilus*. The viability of the organism was affected and therefore subsequently increased the time to reach pH 4.5. Dave and Shah (1998) and Talwalkar and Kailasapathy (2004) deduced that L-cysteine concentrations of 250 and 500 mg/l adversely affected the growth of *S. thermophilus* and *L. bulgaricus*, whereas 500 mg/l ACH or tryptone supported the growth of *S. thermophilus*. Multiplication of *S. thermophilus* was also found to be faster in yogurts supplemented with the above ingredients, which could have been the reason for the shorter incubation time needed to reach a pH of 4.6.

Organic acids

Organic acids are of significant importance in fermented dairy products as they act as natural preservatives and contribute to sensory characteristics of the final product. Certain organic acids act as growth factors for *L. bulgaricus* and *S. thermophilus* and can therefore be beneficial during yogurt fermentation (Fernandez-Garcia, 1994).

Purines and pyrimidines

Nucleic acid derivatives have been shown to stimulate several lactobacilli. Milk contains only traces (5 µmoles/l) of the purine adenine and its derivatives (Cogan et al., 1968). The poor bacterial growth in milk due to the low content of adenine and guanine nucleotides could be overcome by choosing *Lactobacillus* species which possess a 'de-novo' synthesis of DNA and RNA precursors (Elli et al., 1999).

L. bulgaricus cells cultivated with *S. thermophilus* cells can utilise exogenously formed formic acid. The addition of purines accordingly stimulates *L. bulgaricus* growth. Stimulating effects of guanine however are less than those of adenine. This is due to the fact that *L. bulgaricus* is unable to convert exogenously supplied guanine into adenine nucleotides (Suzuki et al., 1985).

Adenine supplementation was found to be responsible for slight stimulation of *L. bulgaricus* growth in tomato juice at 45 °C at all concentrations tested (0, 1, 5, 12.5, 25, 50 and 100 µg/ml) but was inhibitory at 37 °C at concentrations greater than 12.5 µg/ml (Cogan et al., 1968).

Petry et al. (2000) reported that the addition of 20 mg/l adenine and 40 mg/l xanthine, a product in the pathway of purine degradation (Cysewski and Jeziorek, 1998), stimulated *L. bulgaricus* growth. Suzuki et al. (1985) found that concentrations of 135 mg/l adenine stimulated the growth of most *L. bulgaricus* strains whereas xanthine inhibited its growth.

According to Voegtlin and Sherwin (1918) 1 litre of milk contains at least 5 mg adenine and 10 mg of guanine. One litre chemically defined medium (CDM) prepared by Petry et al. (2000) contained 20 mg of adenine, 40 mg of xanthine and no guanine. CDM prepared by Grobben et al. (1998) contained 10 mg adenine, 10 mg xanthine and 10mg guanine and that of Rogosa et al. (1961) contained 5 mg of each. The increased amount of adenine and xanthine present in the CDM prepared by Petry et al. (2000) stimulated growth of of *L. bulgaricus* in milk. This indicates that the quantities in which these purines are present in milk, are not high enough for optimal starter bacteria growth.

2.10 CONCLUSIONS

There are many factors which influence yogurt quality. The formulation and quality of the milk base as well as the processing conditions play important roles. The starter culture, supplementations and modifications made to the milk substrate all contribute to yogurt fermentation time as well as final product quality.

Limited information in the field of optimisation of the yogurt process is available in scientific publications, and that which can be found is scattered within articles on related subjects. This demonstrates that a limited amount of focussed research has seemingly been done in this field.

Due to the constant improvement of process technology and starter cultures concerned with yogurt fermentation, great potential lies in the improvement of the yogurt manufacture process. Faster yogurt fermentation processes are necessary. It will lead to increased production plant capacity and will result in cost savings during the manufacturing process. It would therefore be valuable if studies concerning optimisation, and accordingly the acceleration of the yogurt fermentation process, be continued and expanded.

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CHAPTER 3

STANDARDISATION OF YOGURT STARTER CULTURE

A PROVISIONAL PATENT WAS FILED ON THIS CHAPTER DURING 2009 WITH PATENT NUMBER P41266ZAPO. THE FILING OF THE FINAL PATENT WILL BE PERFORMED IN NOVEMBER 2010.

3.1 INTRODUCTION

Fermented milk products are manufactured by using single or mixed cultures of lactic acid bacteria. The exchange of growth factors between the strains leads to an increase in acidifying ability, as in the case where *Streptococcus salivarius* spp. *thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* are used as mixed cultures in yogurt production (Sodini et al., 2000). These two bacteria are predominantly used during yogurt fermentation due to the fact that both flourish and produce lactic acid at the temperatures used in yogurt manufacture (Vandewegh et al., 2002). The growth association that exists between *S. thermophilus* and *L. bulgaricus* enhances acid production in comparison to the two organisms used individually (Rajagopal and Sandine, 1989; Sodini et al., 2000; Tamime, 2002; Tari et al., 2009). The starter culture used in our studies was selected due to the fact that the two most common organisms involved in yogurt fermentation i.e. *S. thermophilus* and *L. bulgaricus,* (Bouzar et al., 1996; Weinbrenner et al., 1997; Grobben et al., 1998; de Brabandere and de Baerdemaeker, 1999; Sodini et al., 2000; Collado and Hernandez, 2007; Jaziri et al., 2009) are present in this freeze-dried culture.

The variation in starter culture loads used in independent experiments influences the fermentation process significantly. To be able to make a comparison between the fermentation profiles of controls in different experiments, the need arose to standardise the starter culture load which is used for yogurt inoculation.

In order to achieve the above, a reliable method was needed to monitor bacterial growth in milk. After extensive literature study, it was concluded that no standard methods are available. To overcome this problem, a suitable technique had to be developed.

Milk composition of domestic cows varies due to breed-, cow to cow-, herd to herd-, seasonal- and geographical variations as well as the feeding regime (Goff, 1999). All of these factors play a crucial role in the yogurt fermentation process, as variation in milk composition affects the time to reach pH 4.6.

The opaque colour of milk can be attributed to the presence of the casein proteins. Therefore, the removal of the casein from milk would result in a more transparent, yellowish serum which could sustain bacterial growth and subsequently allow spectrophotometrical growth monitoring. The yellowish colour of the milk serum is due to the presence of riboflavin, or vitamin B_2 as it is more commonly known (Walstra, 1990).

Milk serum is defined in the 'Consumer's Dictionary of Food Additives' as the part of skim milk remaining after the coagulation and separation of the casein proteins. This product is regarded as safe by the U.S. Department of Agriculture's Meat Inspection Department and is used in imitation sausage, soups, baked goods and ice cream due to the lactose, whey protein, water soluble vitamins and minerals present (Winter, 1978).

By using milk serum as bacterial growth medium, the problem of monitoring growth in milk specrophotometrically will be addressed in this study.

3.2 MATERIALS AND METHODS

3.2.1 Rehydration of skim milk powder

Skim milk powder (Nestlé, South Africa) was reconstituted with tap water (10 % w/v). The rehydration process was performed at 50 °C for 60 minutes.

3.2.2 Development of cultivation medium for growth monitoring

Six possible variations of serum preparation were evaluated in order to develop the clearest and least turbid milk serum.

3.2.2.1 Trial 1. After rehydration was completed (described in Section 3.2.1), the milk was heated to 90 °C for 40 minutes. The skim milk was left to reach room temperature, whereafter the pH was adjusted to pH 4.6 with 36 % HCl (Merck, analytical grade (AR)). Directly after the HCl addition, the pH of the milk was adjusted to pH 6.5, using 10 N NaOH (Saarchem, AR). The destabilised milk was then heated to 42 °C for 1 hour after which it was centrifuged (Beckman Model J2-21 centrifuge) at 4 °C at 9820 g for 10 minutes using a JA-20 rotor. A white precipitate and an opaque yellow supernatant (milk serum) were obtained. The casein-free milk was from this point on referred to as serum.

3.2.2.2 Trial 2. After skim milk preparation (Section 3.2.1), the milk was heated to 90 °C for 40 minutes. The skim milk was left to cool down to room temperature, whereafter the pH was adjusted to pH 4.6 with 36 % HCl (Merck, AR). Directly after this acidification step, the pH of the milk was adjusted to pH 6.5, using 10 N NaOH (Saarchem, AR). The milk was centrifuged for 5 minutes at 9820 g. This was followed by heating to 42 °C for 30 minutes, and another centrifugation at 9820 g for 5 minutes. The serum was finally reheated to 42 °C for 15 minutes and centrifuged at 9820 g for 10 minutes. The supernatant represented the milk serum.

3.2.2.3 Trial 3. The milk (prepared as described in Section 3.2.1) was heated to 90 °C for 40 minutes and left to cool to room temperature. Concentrated HCl (36 %) was used to adjust the pH to 4.6 (Merck, analytical grade). The milk was centrifuged for 5 minutes at 9820 g. The pH of the serum was adjusted using 10 N NaOH to pH 6.5 (Saarchem, AR). The serum was then heated to 42 °C for 45 minutes, which was followed by 10 minutes of centrifugation at 9820 g. The supernatant represented the milk serum.

3.2.2.4 Trial 4. The remaining fat present in the skim milk powder was removed by adding 225 ml diethyl ether ($(C_2H_5)_2O$) (Merck) to 45 g of skim milk powder in order to eliminate the influence of fat on serum clarity. This mixture was transferred to a glass funnel lined with filter paper (Whatman 2)

and washed with 150 ml diethyl ether. The retentate dried overnight at room temperature (25 °C). The fat free milk powder was rehydrated and was used to prepare milk serum as described in Trial 3.

3.2.2.5 Trial 5. Skim milk (as prepared in Section 3.2.1) was heated to 90 °C for 40 minutes, whereafter it was allowed to cool to room temperature. The pH was adjusted to pH 4.6, using 36 % HCl (Merck, AR). The milk was centrifuged for 5 minutes at 9820 g, after which the pH of the serum was adjusted to pH 6.5 using 10 N NaOH (Saarchem, AR). The serum was reheated to 42 °C for 30 minutes, and submitted to a second centrifugation for 5 minutes at 9820 g. Finally, the serum was reheated to 42 °C for 15 minutes, and centrifuged yet again for 10 minutes at 9820 g. The supernatant represented the milk serum.

3.2.2.6 Trial 6. The skim milk (as prepared in Section 3.2.1) was left to reach room temperature after which the pH was adjusted to pH 4.6 using 36 % HCl (Merck, AR). The milk was heated to 90 °C for 40 minutes after which it was cooled to room temperature and centrifuged for 5 minutes at 9820 g. The pH of the serum was adjusted to pH 6.5 using 10 N NaOH (Saarchem, analytic grade). The serum was then reheated to 42 °C for 30 minutes, followed by 5 minutes of centrifugation at 9820 g. The serum was once again heated to 42 °C for 15 minutes and centrifuged at 9820 g for 10 minutes. The supernatant represented the milk serum.

3.2.3 Starter cultures

A thermophilic yoghurt culture, FD-DVS YF-L812 Yo-Flex, supplied by Chr. Hansen, was used throughout all experiments. The starter culture was received as freeze-dried granules and grinded with a mortar and pestle to reduce particle size. This simplified the weighing process in comparison to weighing the whole granules, and resulted in better distribution of the starter bacteria.

3.2.4 Pre-inoculum preparation

The prepared serum (300 ml) (prepared as described in Section 3.2.2.6) was heated to 42 °C whereafter it was inoculated with 0.1 g of ground starter culture. The inoculated serum was incubated in a shake machine (New Brunswick Scientific, Edison, USA) at 42 °C and 100 rpm. Growth was monitored spectrophotometrically at OD_{640} at hourly intervals for 8 hours. The OD was monitored up until the onset of the stationary growth phase of the starter culture.

The average of data obtained from 7 individual growth curves was used to set up the growth profile of the starter organisms (Figure 3.6). Serum aliquots that were taken at one hour intervals were respectively used as inocula for individual yogurt fermentation runs.

3.2.5 Spectrophotometry

A Genesys 10 vis/UV ThermoSpectronic (Rochester, USA) spectrophotometer was used in all absorbance determinations. Quartz cuvettes with a volume of 1 ml were used in all experiments.

3.2.6 Determination of cell counts

A Neubauer haemocytometer was used for the determination of direct cell counts (http://people.oregonstate.edu/weisv/protocols/symbiodinium/cellcounts.pdf). A pre-inoculum with an OD_{640} of 0.35-0.4 was prepared whereafter a 10 fold dilution was made using distilled water. An aliquot of 25 µl was applied and the cells were directly counted using a phase contrast microscope at 400 times magnification (Phase Contrast-2, Nikon).

3.2.7 Standardisation of inoculation load

The pre-inoculum was prepared as described in Section 3.2.4 and 1 ml aliquots were hourly removed and subsequently used as inocula for individual yogurt fermentations in 2 litre batches of reconstituted skim milk. These individual milk batches were incubated at 42 °C (Tamime and Robinson, 1996; Gueguim-Kana et al., 2007) and the pH was monitored at 15 minute intervals. From these results the optimum yogurt fermentation profiles were selected.

3.2.8 pH Measurements

The pH was measured with a portable pH Spear (Eutech Instrument Co., Oakton, USA), specifically designed for dairy products. The pH meter was calibrated with pH 4 and pH 7 buffer solutions (Hanna instruments, USA) prior to use. The pH decrease was an indication of the rate of lactic acid production.

3.2.9 Statistics

Calculations on average growth between triplicates and standard deviation were performed using the computer program Excel 2007 by Microsoft Corporation.

3.2.10 Yogurt manufacture

Skim milk powder was rehydrated as described in Section 3.2.1. A concentration of 2.5 % sucrose (Huletts, South Africa) was added after rehydration was completed. The milk was heated to 90 °C for 8-10 minutes to pasteurise and was subsequently cooled to 42 °C. Similar heating conditions were also used by Tamime and Robinson (1996), Vandewegh et al. (2002) and Arkbage (2003). A batch of 2 litres of milk was then inoculated with 1 ml milk serum that was prepared as described in Section 3.2.4. Yogurt fermentation was stopped at pH 4.6, the iso-electric point of casein proteins. Stainless steel yogurt fermentation tubes (2 litres) with lid and stirrer were used during all yogurt fermentations.

No stabilisers were added during any experiments. Yogurt prepared in this way served as control in all yogurt experiments.

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3.3 RESULTS AND DISCUSSION

3.3.1 Comparability of controls between milk batches

3.3.1.1 Variation in fresh milk controls vs. skim milk powder controls

The use of fresh milk for yogurt fermentation may not pose a problem for industrial use. When used on laboratory scale, however, the variation in the composition of fresh milk influences yogurt fermentation time.

The effect of the variation in fresh milk composition (from different batches) on fermentation time of yogurt can clearly be seen in Figure 3.1. The fermentation time varied from 195-345 minutes when using the same inoculation load, milk and incubation conditions. There was also a variation in initial pH of fresh milk which ranged between pH 6.3 and pH 6.6. This variation indicated that fresh milk is not a reliable or trustworthy source to be used for yogurt fermentation experiments. Vercet et al. (2002) encountered a similar problem and observed fluctuation in fermentation times in each of the control yogurt batches in their studies. They reported that within a specific experiment that was performed in triplicate, the time to reach pH 4.6 varied between 210 and 310 minutes. They also stated that this occurs frequently even when no variation in the fermentation process is introduced.

De Brabandere and de Baerdemaeker (1999) overcame this problem by preparing base milk batchwise at the beginning of each experimental run and storing it in a deepfreezer until later use. In this research group's studies it was possible to deepfreeze milk batchwise, however this was not the case in our studies, due to the fact that results obtained from individual experiments needed to be compared across batches. In order to address the above mentioned problem, fresh milk was substituted with skim milk powder.

The use of skim milk powder eliminated all variables associated with the use of different fresh milk batches, and the results of the standardisation can be seen in Figure 3.2. The initial pH of the controls varied between pH 6.4 and 6.6., and all the controls in Figure 3.2 reached pH 4.6 within a time span of 285-315 minutes. The use of skim milk powder resulted in a 30 minute variation in fermentation time and therefore eliminated the problem experienced when using fresh milk in which a 150 minute variation was observed in time to reach pH 4.6 (Figure 3.1).

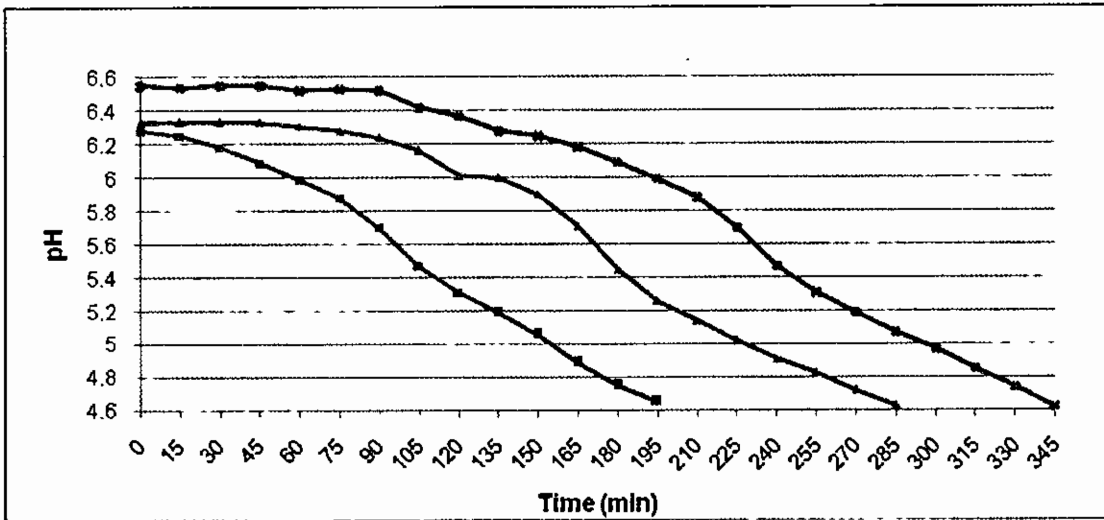

Figure 3.1 Internal controls of yogurt produced from three individual fresh milk batches: Batch 1 (—■—), Batch 2 (—▲—) and Batch 3 (—●—).

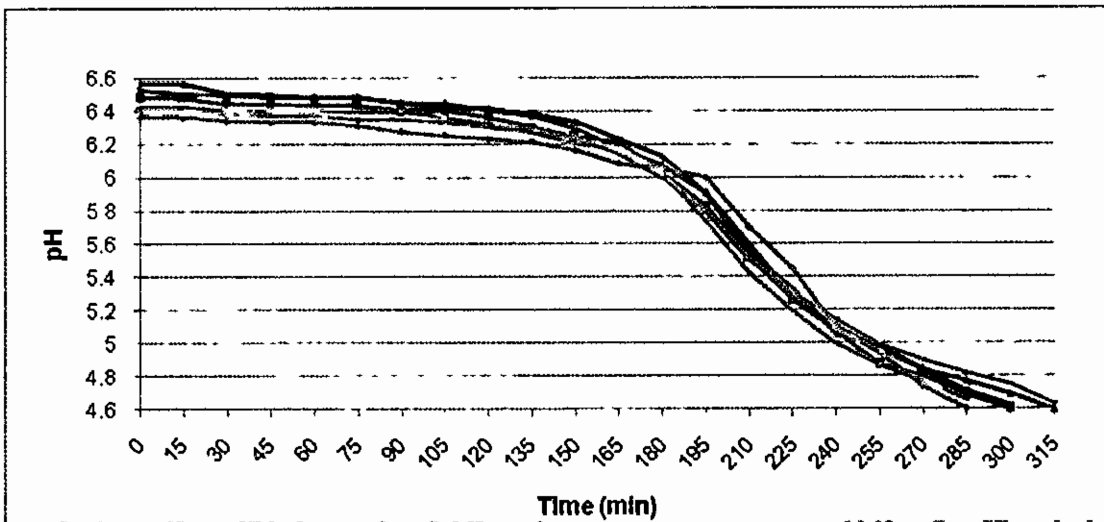

Figure 3.2 Internal controls of yogurt produced from six individual skim milk powder batches: Batch 1 (—■—), Batch 2 (—▲—), Batch 3 (—■—), Batch 4 (——), Batch 5 (——) and Batch 6 (—▼—).

3.3.1.2 Starter culture load fluctuation

The irregular distribution of the starter culture in granular form does not pose a major problem when used on industrial scale (1 sachet of 20g per 5000 litres of milk). In laboratory scale experiments (0.01 g per 2 litres of milk) however, the irregular distribution of starter culture granules during the weighing process causes vast differences in fermentation time of yogurt. These differences are even evident in yogurt manufactured with the same batch of milk, which prevents obtaining repeatable results (Figure 3.3). The unrepeatability can be attributed to the fact that the two bacteria used as starter culture are freeze dried with milk powder which acts as a buffer. In the sachet, the two starter cultures are represented as two different coloured granules with irregular sizes (Figure 3.4). Due to this fact it is impossible to get both organisms equally represented during the weighing process. The ratio of the two starter cultures, as well as the proportion of starter cultures to milk powder therefore deviated significantly, consequently influencing yogurt fermentation time.

The above mentioned problem was solved by grinding the starter culture granules in order to ensure equal distribution of the two starter organisms and thus consistent inoculation loads (Figure 3.4A).

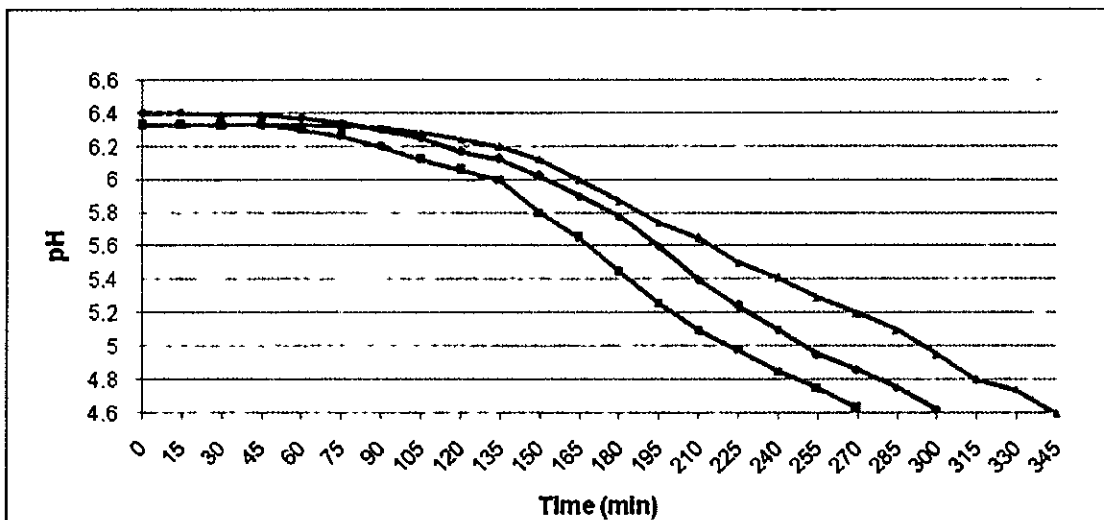

Figure 3.3 The effect of irregular starter culture inoculation loads on yogurt fermentation time: Batch 1 (—■—), Batch 2 (—▲—) and Batch 3 (—◆—).

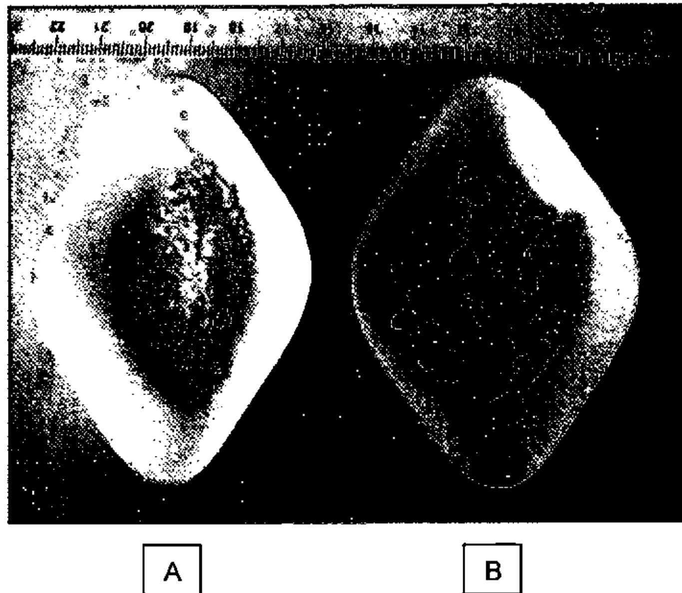

Figure 3.4 Ground starter culture can be observed in Figure 3.4A. The irregularity of the starter culture direct from the sachet can clearly be seen in Figure 3.4B.

3.3.2 Development of cultivation medium for growth monitoring

The fact that milk is an opaque liquid complicates spectophotometrical readings of bacterial growth in milk. Radke-Mitchell and Sandine (1986) attempted to address this problem by means of alkalising the milk to above pH 12 with NaOH. This alkalisation process solubilised the casein micelles in the milk, leaving turbidity in the milk due only to bacterial cells present. During trial experiments performed in our laboratories it was found that this method did not deliver repeatable results (data not shown). This technique did not meet with our requirements and therefore the need arose to develop a more consistent and reliable technique to monitor bacterial growth in a milk analogue.

Due to the fact that the casein present in milk is responsible for the opaque colour of milk, it was decided to prepare a milk serum in which the casein was removed by acid precipitation. In trials using this serum it was observed that the serum became turbid after heating to the optimal growth temperature of starter bacteria (42 °C).

For this reason six different serum trial runs were prepared and subsequently evaluated in order to develop the clearest and least turbid milk serum to be used during incubation.

3.3.2.1 Milk serum development

In all experiments performed in this section the initial step entailed the heating of the milk to 90 °C in order to pasteurise the skim milk and also to partially denature the globular whey proteins. During the subsequent casein destabilisation steps, the denatured whey proteins adhered to the precipitated casein proteins (Kalab, 1997).

All the trials evaluated during the development of a clear milk serum can be divided into two parts; destabilisation and precipitation, and heat treatment and clarification.

The serum obtained from Trial 1 was completely opaque (See Figure 3.5). The serum from Trial 2 was also completely opaque even though it was clarified 3 times by centrifugation. Although Trial 2 had three clarification (centrifugation) steps and Trial 1 only had one, both methods delivered unclear milk serum. Neither of these two trial runs can therefore be used to deliver a medium which will enable bacterial growth monitoring spectrophotometrically. Based on a hypothesis that the NaOH addition directly after the HCl may solubilise some of the components precipitated by the acid, it was decided to add the NaOH after the centrifugation step, and not directly after the addition of HCl. This resulted in a clearer serum, as can be observed in the milk serum obtained by Trial 3 (Figure 3.5).

During the preparation of Trial 3, it was observed that the last heating and centrifugation steps (as described for Trial 2) did not contribute to any further clearing effect. The last 2 heating steps (30 minutes and 15 minutes respectively at 42 °C) of Trial 2 could therefore successfully be condensed into only 1 prolonged heating step (45 minutes at 42 °C) in Trial 3.

In Trial 4 the effect of the 0.5 % fat present in the skim milk powder was evaluated to establish whether the presence of fat had an influence on the clarity of milk serum. The serum that was obtained in Trial 4 was not clear and displayed signs of turbidity (OD_{640nm} of 0.257) in comparison to the serum obtained during Trial 3 (OD_{640nm} of 0.024), where the fat was still present. The fat was therefore not removed in the following trials.

Due to the significant difference in turbidity between the serums obtained by Trial 2 and Trial 3, it was decided to modify Trial 2 by altering only the destabilisation step. The addition of NaOH after the serum had been centrifuged, and not before as in Trial 2, resulted in a serum that was clear and translucent in Trial 5 (OD_{640nm} 0.010).

In the case of Trial 6, the initial acidification process was preformed prior to pasteurisation, and not after pasteurisation as in the cases of Trials 1-5. The last two centrifugation steps were performed as described in Section 3.2.2.6, although no difference in absorbance was evident after the last centrifugation step. The last heating and centrifugation step in this trial run can therefore be omitted. The serum obtained by this technique delivered a clear end product (OD_{640nm} 0.00).

From the above results obtained it was evident that the preparation of both Trial 5 and Trial 6 produced the clearest serum. Due to fewer steps involved in the preparation of Trial 6, this trial run was subsequently implemented in all experiments.

The OD of the various trial runs was spectrophotometrically measured at 640 nm using distilled water as blank (See Table 3.1). The final serums of the above mentioned 6 preparations can be seen in Figure 3.5.

Table 3.1 The optical density (OD) of the six procedures obtained during development of milk serum.

	$OD_{640\ nm}$
Trial 1	2.434
Trial 2	0.927
Trial 3	0.024
Trial 4	0.257
Trial 5	0.010
Trial 6	0.000

All values mentioned above are the average of three measurements

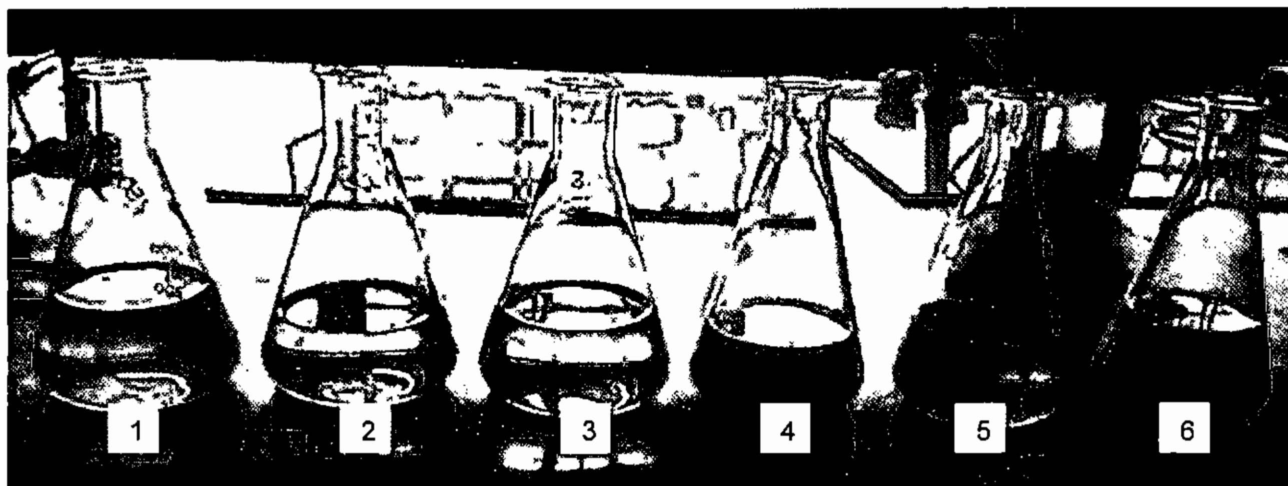

Figure 3.5 The milk serums obtained by the six evaluated trial runs. Flask 1 represents Trial 3; flask 2-Trial 5; flask 3-Trial 6; flask 4-Trial 2; flask 5-Trial 4 and flask 6-Trial 1.

3.3.3 Pre-inoculum preparation

The above developed milk serum, which acted as the pre-inoculum, was used as growth medium during the determination of the growth profile of starter bacteria. The mean of 7 independent growth profiles are depicted in Figure 3.6. From this linear growth profile it could clearly be seen that the standard deviation increased from 1 hour up to 6 hours after incubation. It was evident that growth of 1-4 hours exibited small standard deviation values and therefore the highest repeatability to serve as standardised inocula for yogurt fermentation. Growth of 5-8 hours exhibited higher standard deviation values and could possibly compromise repeatability. After 7 and 8 hours respectively, the standard deviation notably decreased. The fact that the standard deviation values after 1-4 hours of incubation were very small renders the experiment repeatable and therefore reliable. The OD_{640} after 1 hour of incubation was the lowest, therefore growth after 2-4 hours was more adequate to be used as inoculations for yogurt fermentations due to their higher cell load.

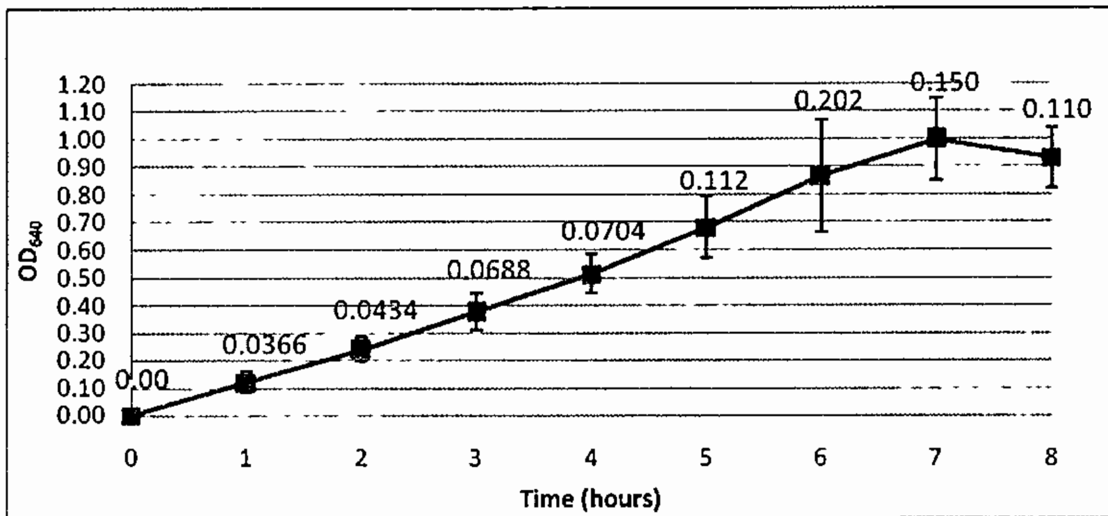

Figure 3.6 Average and standard deviation of 7 individual growth curves obtained from starter culture cultivated in milk serum.

3.3.3.1 Standardisation of pre-inoculation load

Aliquots of the pre-inoculum (kept at 42 °C) were taken hourly for 8 hours in succession and acted as inocula for independent yogurt experiments in order to determine the individual yogurt fermentation times (Figure 3.7). All the individual inoculations followed the conventional yogurt fermentation profile. It was observed that the pre-inoculum in the early growth stage (0-1 hour) took extensively longer to reach pH 4.6 than the pre-inoculum incubated for 2-4 hours. This indicated that the use of a pre-inoculum enables the starter bacteria to be in a higher concentration and a more active growth state in comparison to starter bacteria directly added to milk without pre-inoculum preparation. A decrease in yogurt fermentation time of 17 % was observed for the pre-inoculum incubated for 3 and 4 hours, respectively, in comparison to 0 and 1 hour. These two time intervals (0 and 1 hour) were eliminated

due to their prolonged fermentation times respectively. It was therefore apparent that as the OD_{640} of the pre-inoculum increased after 1-4 hours of incubation, the yogurt fermentation time consequently decreased. Even though the OD of the pre-inoculum increased from 5-8 hours, it resulted in an increase in yogurt fermentation time which makes it unsuitable to act as a pre-inoculum. It is evident that the shortest yogurt fermentation time was obtained from a pre-inoculum that had been incubated for 3-4 hours at 42 °C (Figure 3.7). Due to no significant difference in results obtained between 3 hours and 4 hours and to limit the total run time of experiments, serum incubated for 3 hours at 42 °C was selected as inoculum. The OD_{640} for this selected pre-inoculum was found to be approximately 0.45 with a direct cell count of approximately 1.03×10^7 cells per ml. These values were used as standards in all successive experiments.

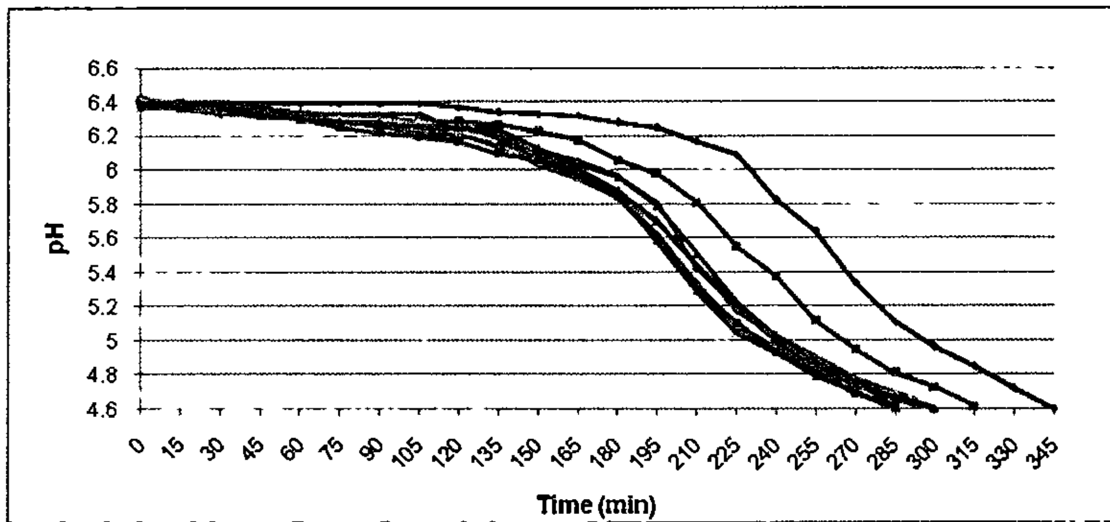

Figure 3.7 Independent yogurt runs inoculated with starter culture pre-incubated in milk serum for time intervals 0 (—◆—), 1 hour (—■—), 2 hours (—▲—), 3 hours (—×—), 4 hours (—✱—), 5 hours (—●—), 6 hours (—+—), 7 hours (—+—) and 8 hours (—■—).

3.4 CONCLUSIONS

Starter culture plays a key role in yogurt fermentation. For this reason the optimisation of starter culture by inoculating yogurt milk while the bacteria is in its optimal growth stage can play an important part in accelerating yogurt fermentation. This was confirmed in the results obtained in this chapter.

During the freeze drying process of yogurt starter cultures, skim milk is used as a buffer to prevent extensive damage to the cultures. No clear indication was evident that the ratio between starter culture and skim milk used as yogurt inoculum was constant and repeatable. The variation in starter culture load resulted in the variation in yogurt fermentation time between independent experiments. It was therefore essential to standardise the starter culture (load and growth stage) used for yogurt production by developing a new method which would enable the monitoring of bacterial growth in milk.

After the suitable medium had been developed, hourly aliquots of the pre-inoculum were used as inocula for individual yogurt fermentations in order to determine the optimal growth stage to be used as inoculum for yogurt fermentation. It was concluded that inoculations taken from different growth phases influenced the fermentation time of yogurt. Starter cultures in their mid growth phase used as inocula in independent yogurt runs exhibited the fastest lactic acid production and accordingly shortest yogurt fermentation time. It was found that starter culture incubated for 3 hours at 42 °C was the most suitable for use as inoculum for yogurt fermentation as it delivered the most repeatable results.

Although the acceleration of yogurt fermentation was obtained during the above mentioned experiments, the main aim of the work done was to develop a method which standardised yogurt fermentation time. By using the developed milk serum, it was possible to standardise the inoculum so that every completed yogurt run reached pH 4.6 within the same time frame. This procedure ensured that the starter culture used as inoculum was uniform and in the same growth phase for each experiment. It meant that any changes observed in yogurt fermentation would be due to the external factors under investigation and not as a result of experimental and starter culture variations.

Even though casein did not form part of the nutrient source available to the bacteria in the milk serum, this does not pose a problem. If the casein fraction was still present in the serum, no significant difference would have been imminent, and the casein could even possibly have acted as a sterical hindrance in nutrient diffusion.

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CHAPTER 4

EVALUATION OF THE EFFECTS OF VARIOUS SUPPLEMENTS ON THE YOGURT FERMENTATION PROCESS

4.1 INTRODUCTION

Fortification of soft drinks and dairy products, especially yogurt, is a growing trend (Prieto et al., 2006). Due to the high consumer demand, possible optimisation of yogurt fermentation by fortification can be a viable, inexpensive and wide-ranging market opportunity. This can be done by means of adding vitamins and various extracts (Cogan et al., 2007).

Food fermentation occurs as a result of microbial activity on suitable substrates under either controlled or uncontrolled conditions. This results in the production of more stable, palatable and nutritious food products (Fung, 1996). By adjusting the conventional yogurt fermentation process by adding supplements which could decrease production time, fermented products can be improved even further. Cogan et al. (1968) supports this assumption by stating that several extracts of plant and animal origin have been shown to stimulate the growth of lactic acid bacteria.

Among lactic acid bacteria, members of the *Lactobacillus* genus present complex nutrient requirements that can only be satisfied by a cultivation medium containing energy sources, precursors for cell growth and division and growth stimulatory substances. Yeast extract or peptones are commonly used as milk supplements in industrial fermentations to induce the growth of bacterial strains which are unable to grow in pure milk (Elli et al., 1999).

Based on the above reports, it was our view that by increasing the concentration of the previously mentioned energy sources and growth factors in the cultivation medium (milk) may therefore enhance bacterial growth which in turn may lead to a decrease in yogurt fermentation time.

After the optimisation of starter culture described in Chapter 3 had been completed it was possible to perform a screening process. This was done using an increased starter culture load and various supplements in order to determine the effect on the yogurt fermentation profile. It included a range of vitamins, amino acids, proteins, sugars and extracts.

4.1.1 Increased starter culture inoculum load

The apparent solution of accelerating yogurt fermentation by means of increasing the starter culture inoculum load was investigated by Dave and Shah (1997) and Olsen and Aryana (2008). When an excessively high inoculation load of *L. acidophilus* was added to a starter culture consisting of *L. bulgaricus* and *S. thermophilus*, it prolonged the yogurt fermentation time and resulted in inferior yogurt quality (Olson and Aryana, 2008). In similar research done by Dave and Shah (1997), varying concentrations of *S. thermophilus*, *L. bulgaricus*, *L. acidophilus* and bifidobacteria were used as constitutive microflora to study the effect of inoculum load on the viability of yoghurt bacteria. It was observed that the organisms had higher growth rates in the yogurt with the lower levels of inoculum than with the higher inoculum levels. The decrease in pH during fermentation was also more

significant in samples prepared with a lower inoculum load. This is due to *S. thermophilus* and *L. bulgaricus* possibly being biologically more active in the lower inoculum samples (Dave and Shah, 1997). Due to the above results, no further investigation was carried out in this study concerning the starter culture inoculation load during fermentation.

4.1.2 Supplements

Vitamins

According to studies done by Suzuki et al. (1986) and Fernandez-Garcia (1994), orotic acid, an intermediate of purine biosynthesis, is a growth factor for *L. bulgaricus*. This organism could not grow in milk from which orotic acid was removed. Bovine milk contains relatively high concentrations (80 mg/l) of orotic acid (Suzuki et al., 1986; Fernandez-Garcia, 1994). Due to the reportedly high orotic acid content present in bovine milk, the supplementation of orotic acid to yogurt was not further investigated.

In studies done by Rogosa et al. (1961) pyridoxal (vitamin B_6) was found to be essential for the growth of *L. jugurti* and *L. helveticus* but not for *L. bulgaricus*. Due to fact that *L. bulgaricus* is one of the key bacteria present in the starter culture used in this study, the supplementation of pyridoxal to milk used for yogurt fermentation did not warrant further investigation.

In studies done by Rogosa et al. (1961) and Grobben et al. (1998) the importance of nicotinic acid and riboflavin for optimal *L. bulgaricus* growth was established. Rogosa et al. (1961) further reported that the addition of folic acid inhibits the growth of *L. bulgaricus*. Due to all the discrepancies concerning folic acid, Indyk (2010) confirmed that recently the many complexities involved in measurement of the folic acid content in food have been emphasised by the disagreement between methods and between laboratories. Great controversy exists in terms of folic acid stability during milk pasteurisation and other heat treatments, therefore the impact of folic acid on yogurt fermentation therefore warrants further investigation.

Milk and dairy products are rich in vitamin B_{12}, which is bound to the milk proteins. Arkbage (2003) and Meydani and Ha (2000) confirmed that vitamin B_{12} is heat stable at pasteurisation temperatures (96 °C for 5 minutes) and although this vitamin is used during fermentation, yogurt consumed at "use by date" still contains 40-60 % of the vitamin B_{12} originally present in the milk. This indicates that not all the vitamin B_{12} is consumed by the starter organisms during the fermentation process, therefore supplementation with additional vitamin B_{12} would be futile.

Ascorbic acid, a common food additive, when used as fortification in yogurts, can act as an oxygen scavenger and can prove useful to maintain low oxidation-reduction potential necessary to the viability of probiotic bacteria (Talwalkar and Kailasapathy, 2004). This research group further stated that the incorporation of ascorbic acid in yogurts can reduce the amount of oxygen required for the activities of the facultatively anaerobic *S. thermophilus*, a vital micro-organism in the manufacture of yogurt.

Although the above can subsequently result in detrimental effects on the textural and nutritional qualities of yogurt which renders it impractical as a yogurt supplement (Talwalkar and Kailasapathy, 2004), it may contribute positively to *S. thermophilus* growth in yogurt. Due to the fact that vitamin C is utilised during fermentation (Meydani and Ha, 2000) and the possibility of increased bacterial growth, the supplementation of ascorbic acid to yogurt was evaluated.

Amino Acids

Milk is generally low in free amino acid content (Elli et al., 1999). The concentrations of certain essential amino acids present in milk, specifically glutamic acid and methionine, are far below the requirements of *S. thermophilus* and *L. bulgaricus* (Rajagopal and Sandine, 1989; Farkye and Vedamuthu, 2002; Letort et al., 2002; Robinson et al., 2004). Studies performed by Grobben et al. (1998) established that the omission of aspartic acid, glutamic acid, or glycine affected *L. bulgaricus* growth only slightly, and the omission of glutamine, asparagine, or threonine resulted in a stronger reduction of the growth. All other amino acids were essential and subsequently multiple omissions of amino acids caused an almost complete loss of growth (Grobben et al., 1998). Robinson et al. (2004) established that some amino acids such as glutamic acid, methionine and cysteine are not present in milk at levels sufficient to support the essential growth of *S. thermophilus*. The increase in cell numbers of *S. thermophilus* necessary to complete the yogurt fermentation process therefore depends on the absorption of short-chain peptides released by *L. bulgaricus* and the subsequent hydrolysis of these peptides to the constituent amino acids.

During studies on the supplementation of yogurt with cysteine, promising results were obtained by Dave and Shah (1998) and Talwalkar and Kailasapathy (2004). Due to the above information, the use of the amino acids glutamic acid, methionine and cysteine as supplements to milk used for yogurt fermentation therefore justified further research in this study.

Minerals

Due to the high public interest of calcium-fortified yogurt, it was decided to investigate the influence of B-Cal and B-Cal-DM (commericial sources of calcium supplements) on yogurt fermentation by using it as a supplement prior to fermentation.

Despite its rich nutrient composition, milk displays a strong functional iron-deficiency because iron is complexed as lactoferrin, therefore being unavailable to the metabolism of lactobacilli. The absence of the available iron can negatively affect the cell-division of certain *Lactobacillus* species e.g. *L. acidophilus* (Elli et al., 1999). In a study by Simova et al. (2008) however, iron fortification in concentrations ranging from 8 to 27 mg/kg in yogurt showed no significant influence on yogurt fermentation. Due to these findings, no additional research on iron fortification was done in our studies.

Undefined media

Proteose peptone is commonly used in the fermentation industry for starter cultures. It is a highly nutritious source for the growth of a wide range of micro-organisms (Innocente et al., 1998) which makes it suitable for our yogurt fermentation studies. Studies done by Hujanen and Linko (1996) and Elli et al. (1999) agree by stating that bacterial growth can be improved by the addition of substances of undefined composition such as yeast extract or peptones (of various origins) to the growth medium. Due to the above reasons yeast extract and proteose peptone were evaluated in our studies.

Oliveira et al. (2001) supplemented commercial pasteurised milk with 2 % casein hydrolysate (CH). The addition of CH combined with the use of mixed cultures resulted in better acidification in comparison to yogurt produced without the addition of CH and use of pure cultures. Due to the similar results obtained by acid casein hydrolysate (ACH) and tryptone supplementation by Dave and Shah (1998), only the effect of ACH on the fermentation process was evaluated in our studies.

Sugars

Richmond et al. (1987) found that heat treatment of milk by pasteurisation leads to a 7 % decrease in lactose content and subsequent fermentation leads to a further decrease of 27 % in lactose content. Mouillet et al. (1977) confirmed this by stating that during their studies, only 33 % of the original lactose present in milk was hydrolysed during yogurt fermentation. The supplementation of additional lactose would therefore most probably have no effect on the fermentation process and was thus not evaluated.

L. bulgaricus ferments lactose and glucose and does not grow on galactose. Glucose remains at trace concentrations while galactose accumulates in the medium during fermentation (Richmond et al., 1987; Chervaux et al., 2000). O'Leary and Woychik (1976) confirmed this statement by declaring that *S. thermophilus* preferentially ferments the disaccharides lactose and sucrose.

Purines

Nucleic acid derivatives have been shown to stimulate the growth of several lactobacilli. Milk contains only traces of the purine adenine and its derivatives which lead to poor bacterial growth in milk (Elli et al., 1999). Adenine supplementation was found to be responsible for slight stimulation of *L. bulgaricus* growth in tomato juice at 45 °C at concentrations ranging from 0 to 100 µg/ml but was inhibitory at 37 °C at concentrations greater than 12.5 µg/ml (Cogan et al., 1968). Suzuki et al. (1986) also found that adenine stimulated the growth of most *L. bulgaricus* strains whereas xanthine inhibited its growth. The increased amount of adenine and xanthine present in the chemically defined medium (CDM) prepared by Petry et al. (2000) stimulated growth of *L. bulgaricus* in milk. It was further reported that the growth of *L. bulgaricus* was stimulated by adenine, adenosine and adenine nucleotides, whereas guanine, xanthine and guanosine inhibited its growth (Suzuki et al., 1986; Fernandez-Garcia, 1994).

All of the above statements indicate that the quantities in which the purines adenine and xanthine are present in milk and their effects on bacterial growth are controversial, and that additional research on the effect of purines on the yogurt fermentation process need to be investigated.

4.2 MATERIALS AND METHODS

4.2.1 Rehydration of skim milk powder

Skim milk powder (Nestlé, South Africa) was reconstituted with tap water (10 % w/v). The rehydration process was performed at 50 °C for 60 minutes.

4.2.2 Milk serum preparation

The skim milk (as prepared in Section 4.2.1) was left to reach room temperature after which the pH was adjusted to pH 4.6 using 36 % HCl (Merck, AR). The milk was heated to 90 °C for 40 minutes after which it was cooled to room temperature and centrifuged for 5 minutes at 9820 g using a Beckman Model J2-21 centrifuge with a JA-20 rotor. The pH of the serum was adjusted to pH 6.5 using 10 N NaOH (Saarchem, AR). The serum was then reheated to 42 °C for 30 minutes, followed by 5 minutes of centrifugation at 9820 g. The serum was once again heated to 42 °C for 15 minutes and centrifuged at 9820 g for 10 minutes. The supernatant represented the milk serum.

4.2.3 Starter cultures

A thermophilic yoghurt culture, FD-DVS YF-L812 Yo-Flex, supplied by Chr. Hansen, was used throughout all experiments. The starter culture was received as freeze-dried granules and grinded with a mortar and pestle to reduce particle size. This simplified the weighing process in comparison to weighing the whole granules, and resulted in better distribution of the starter bacteria.

4.2.4 Pre-inoculum preparation

Serum (300 ml) prepared as described in Section 4.2.2 was heated to 42 °C whereafter it was inoculated with 0.1 g of ground starter culture. The inoculated serum was incubated in a shake machine (New Brunswick Scientific, Edison, USA) at 42 °C for 100 rpm. Growth was monitored spectrophotometrically at 640 nm until an OD of approximately 0.45 was reached with a coulter chamber count of approximately 1.03 x 10^7 cells per ml. An inoculation load of 1 ml of this pre-inoculum was then used for individual yogurt fermentation runs.

4.2.5 Spectrophotometry

A Genesys 10 vis/UV ThermoSpectronic (Rochester, USA) spectrophotometer was used in all absorbance determinations. Quartz cuvettes with a volume of 1 ml was used in all experiments.

4.2.6 pH Measurements

The pH values were measured with a portable pH Spear (Eutech Instruments Co., Oakton, USA) during incubation at 42 °C at 15 minute intervals. The pH meter was calibrated using pH 4 and pH 7

buffer solutions (Hanna Instruments, USA) prior to use. The pH decrease was an indication of the rate of lactic acid production.

4.2.7 Statistics

Calculations on average growth between triplicates were performed using the computer program Excel 2007 by Microsoft Corporation. Additional statistical analysis was not performed due to a too small variation in data.

4.2.8 Yogurt manufacture

Skim milk powder was rehydrated as described in Section 4.2.1. A concentration of 2.5 % sucrose (Huletts, South Africa) was added after rehydration was completed. The milk was heated to 90 °C for 8-10 minutes to pasteurise and was subsequently cooled to 42 °C. Similar heating conditions were also used by Tamime and Robinson (1996), Vandewegh et al. (2002) and Arkbage (2003). A batch of 2 litres of milk was then inoculated with 1 ml milk serum that was prepared as described in Section 4.2.4. Yogurt fermentation was stopped at pH 4.6, the iso-electric point of casein proteins. Stainless steel yogurt fermentation tubes (2 litres) with lid and stirrer were used during all yogurt fermentations. No stabilisers were added during any experiments. Yogurt prepared in this way served as control in all yogurt experiments.

4.2.9 Inoculation loads of supplements

The following supplements were evaluated in the stipulated concentrations in order to determine their influence on the yogurt fermentation process. The suppliers of the products are specified in brackets. All supplements were added after pasteurisation of the milk used for yogurt fermentation. All experiments were performed in triplicate.

Multivitamins

Various multivitamins were evaluated including B-Cal-DM, B-Cal, Elimni Rad, Pregnavit M, Folic Acid Forte and StaminoGro of which the components are listed in Tables 4.1-4.6.

The six multivitamins evaluated were each used in 2 concentrations: 0.9 g/l and 3.6 g/l of B-Cal-DM, 1 g/l and 4 g/l of B-Cal, 0.46 g/l and 1.8 g/l of Elimni Rad, 1.8 g/l and 3.5 g/l of Pregnavit M, 0.27 g/l and 1.06 g/l of Folic Acid Forte, 0.63 g/l and 1.26 g/l of StaminoGro (each concentration represents 1 or 2 tablets respectively).

Table 4.1 The composition of B-Cal-DM as provided by supplier.

Each capsule contains:	Per capsule
Elemental Calcium	500 mg
Vitamin D	250 iu
Elemental Magnesium	125 mg

Capsule weight: 1.79 g

Table 4.2 The composition of B-Cal as provided by supplier.

Each capsule contains:	Per capsule
Elemental Calcium	500 mg

Capsule weight: 2.02 g

Table 4.3 The composition of Elimni Rad as provided by supplier.

Each capsule contains:	Per capsule
L-Methionine	50 mg
Magnesium	100 mg
Vitamin B_6	20 mg
Vitamin C	300 mg

Capsule weight: 0.91 g

Table 4.4 The composition of Pregnavit M as provided by supplier.

Each capsule contains:	Per capsule
Vitamin A	2666 iu
Vitamin B_1	3 mg
Vitamin B_2	2 mg
Vitamin B_6	1 mg
Vitamin B_{12}	2 µg
Vitamin C	50 mg
Vitamin D	400 iu
Calcium	230 mg
Folic acid	795 µg
Iron	20 mg
Magnesium	0.5 mg
Niacin	10 mg

Capsule weight: 0.88 g

Table 4.5 The composition of Folic Acid Forte as provided by supplier.

Each capsule contains:	Per capsule
Folic acid	799 µg
Vitamin B_1	3 mg
Vitamin B_2	5 mg
Vitamin B_3	18 mg
Vitamin B_5	15 mg
Vitamin B_6	24 mg
Vitamin B_{12}	24 µg
Vitamin C	200 mg

Capsule weight: 0.53 g

Table 4.6 The composition of StaminoGro as provided by supplier.

Each capsule contains:	Per capsule
L-Glutamine	150 mg
L-Lysine	50 mg
Vitamin C	75 mg
Folic acid	198 µg
Vitamin B_1	0.75 mg
Vitamin B_2	1.25 mg
Vitamin B_3	6 mg
Vitamin B_5	6 mg
Vitamin B_6	6 mg
Vitamin B_{12}	6 µg
Elemental Calcium	100 mg
Vitamin D	75 iu
Magnesium	60 mg

Capsule weight: 1.26 g

All these individual tablets were grinded into a powder and added after pasteurisation together with starter culture inoculation.

All capsules were supplied by Georen Pharmaceuticals (Pty) Ltd.

Vitamins

The following vitamins were evaluated: 0.05 mg/l Folic acid (Sigma, AR), 1 g/l of nicotinic acid (Sigma, AR), 0.2 g/l of ascorbic acid (Merck, AR) and 1 mg/l of riboflavin (Sigma, AR).

Amino Acids and proteins

The concentrations of amino acids and proteins evaluated as supplements to individual yogurt fermentation runs prior to fermentation were as follows: 50 mg/l of cysteine (Sigma, AR), 0.2 g/l and 2.6 g/l respectively of glutamic acid (Sigma, AR), 0.2 g/l of L-methionine (Sigma, AR), 0.2 g/l of L-tyrosine (Sigma, AR), 0.25 g/l of purified casein (Difco, AR) and 2.5 g/l of casein (Difco, AR).

Undefined media (Extracts)

Various extracts were evaluated, including 3 g/l meat extract (Oxoid), 0.25 g/l and 0.5 g/l respectively of casein hydrolysate, 0.2 g/l of proteose peptone (Difco) and 10 ml/l soy milk (Good Hope). 3 g/l of yeast extract (Oxoid) was added at two different temperatures; at 80 °C after pasteurisation and at 42 °C directly before starter culture inoculation.

Sugars

The two sugars evaluated included 0.2 g/l glucose monohydrate (Saarchem, AR) and 0.2 g/l galactose (Merck, AR).

Purines and Pyrimidines

Adenine (Sigma, AR) supplementation was evaluated by using different concentrations which consisted of 20 mg/l, 0.1 g/l and 0.2 g/l. Xanthine (Sigma, AR) was added in concentrations of 40 mg/l and 0.2 g/l. Thymidine (Sigma, AR) was evaluated at a concentration of 1 mg/l.

4.3 RESULTS AND DISCUSSION

4.3.1 Supplements

4.3.1.1 Multivitamins and calcium

Due to complex metabolism of the two bacteria used as starter culture namely *S. thermophilus* and *L. bulgaricus* (Cogan et al., 2007), it could not be said with certainty which supplements will affect the growth profile of these organisms. In order for a preliminary screening process to be performed, the effect of 6 different multivitamin and mineral supplements on the yogurt fermentation process was evaluated before proceeding to evaluation of the individual supplements.

The effect of four multivitamins, Folic Acid Forte, StaminoGro, Pregnavit M and Elimni Rad and two calcium supplements, B-Cal-DM and B-Cal, on yogurt fermentation time were evaluated in our studies (See Figure 4.1 – Figure 4.5). The main objective of the multivitamin and calcium supplementations was to determine whether any of these 6 supplements, when added to milk prior to fermentation, would decrease yogurt fermentation time. In the cases where promising results were obtained, the vitamins and minerals present in the respective products were evaluated individually.

Vitamin B_6 was found to be non essential for *L. bulgaricus* growth (Rogosa et al., 1961) whereas it was found that more than half of the vitamin B_{12} present in milk was still present in yogurt at the "use-by-date" (Arkbage, 2003), therefore further supplementation would prove futile. Neither vitamin B_6 nor vitamin B_{12} was therefore evaluated as supplements in our studies. Results obtained by Simova et al. (2008) stated that the fortification of yogurt with iron showed no significant effect on yogurt fermentation. Iron supplementation was therefore not further investigated.

Calcium fortification (B-Cal-DM and B-Cal)

Yogurt, like milk, is a rich source of protein, riboflavin, folic acid, and calcium, although compositional changes occur as milk is converted into yogurt. Some minerals, for example calcium, are more bioavailable from yogurt than from milk (Meydani and Ha, 2000). Two calcium supplements were evaluated in our studies; B-Cal-DM and B-Cal (Figure 4.1). The effect of B-Cal-DM was first evaluated due to its high calcium and magnesium content. Results obtained during the evaluation of 0.9 g/l B-Cal-DM indicated that an increase of 5 % in fermentation time was noted when compared to the control. Where the concentration was increased to 3.6 g/l however (Figure 4.2), no change in the fermentation time was noted in comparison to that of the control. It was therefore decided to evaluate the effect of calcium supplementation on the fermentation process. This was done by using B-Cal, which contains only calcium. Results indicated a similar tendency in the fermentation time as obtained with B-Cal-DM supplementation (Figure 4.1). An increased concentration of 4 g/l of B-Cal was subsequently evaluated (Figure 4.4), which resulted in an increase of 5 % in fermentation time. It could therefore be concluded that neither calcium, magnesium nor vitamin D supplementation had a positive influence on yogurt fermentation time.

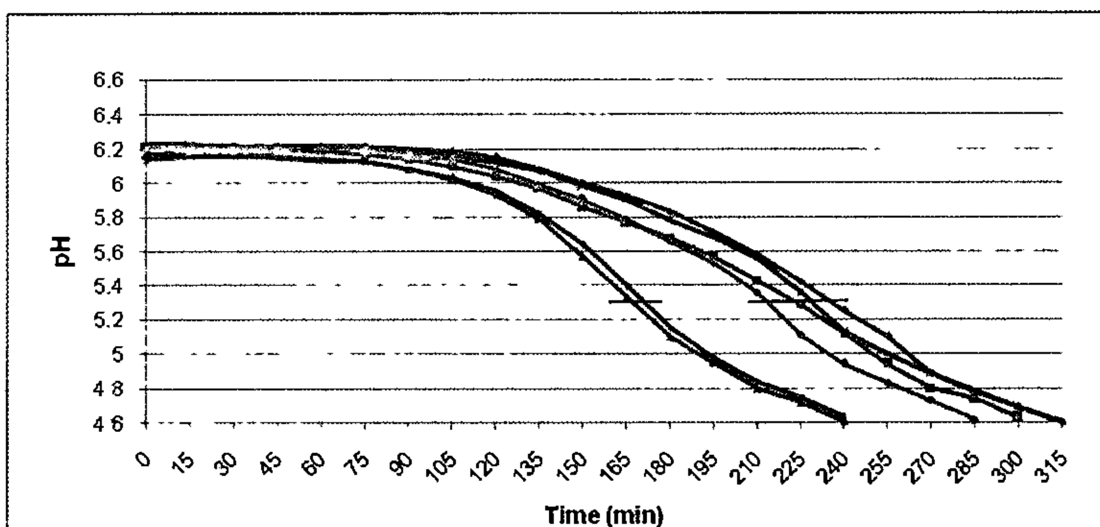

Figure 4.1 The effect of Elimni Rad (0.45 g/l) (—•—), B-Cal-DM (0.9 g/l) (—•—), B-Cal (1 g/l) (—•—), yeast extract added at 42 °C (3 g/l) (—*—) and yeast extract added at 80 °C (3 g/l) (—•—) supplementation to yogurt in comparison to the control (—•—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

Elimni Rad

Elimni Rad is a multivitamin that contains mainly magnesium, vitamin C and L-methionine, but no lactose, sucrose or gluten.

During evaluation of this multivitamin it was found that Elimni Rad decreased the time to reach pH 4.6 by 5 % (Figure 4.1), in comparison to the control. Due to this decrease in fermentation time, it was decided to evaluate the effect of an increased concentration (Figure 4.2), which resulted in no deviation from the control.

Due to the slight decrease in fermentation time achieved during supplementation with the lower concentration (0.45 g/l) of Elimni Rad, it was decided to further evaluate its main components magnesium, vitamin C and L-methionine individually. The ineffectiveness of magnesium supplementation has already been observed and discussed under Calcium Fortification. The influence of only vitamin C and L-methionine was therefore further investigated (Discussed in Sections 4.3.1.2 and 4.3.1.3).

Owing to the fact that these multivitamins showed little promise in accelerating yogurt fermentation, no further research was done using Elimni Rad, B-Cal or B-Cal-DM.

It is important to note that the effectiveness of the standardisation step of starter culture inoculum load, as developed in Chapter 3, is clearly noticeable. It is demonstrated in Figure 4.2 where all three graphs are virtually superimposable, which was not the case before standardisation.

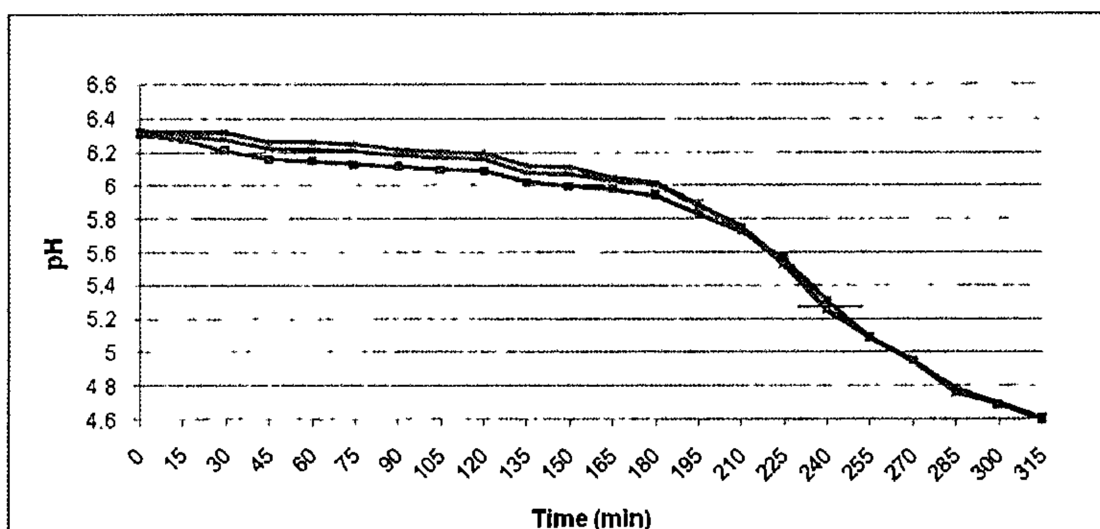

Figure 4.2 The effect of B-Cal-DM (3.6 g/l) (—) and Elimni Rad (1.8 g/l) (—) supplementation on yogurt fermentation time in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

Pregnavit M

The vitamin and mineral capsules Pregnavit M contain 795 µg folic acid per capsule, which contributes to 398 % of the recommended dietary allowance (RDA). This multivitamin also has high concentrations of other vitamins and minerals (See Table 4.4). In preliminary research performed using a concentration of 3.5 g/l Pregnavit M in a batch of 2 litres of milk, an increase of 42 % was noted in fermentation time to reach pH 4.6, in comparison to the control (Figure 4.3). Studies done by Rogosa et al. (1961) support these results and reported that 10 µg/l folic acid completely inhibited the growth of many *L. bulgaricus* strains. In the case where the inoculation load was decreased to 1.8 g/l as shown in Figure 4.4, no increase in fermentation time was observed, although no decrease was noted. Due to Pregnavit M having no significant effect on the fermentation process when evaluated in such a low concentration, it was not evaluated in different concentrations, as was the case for B-Cal-DM and Elimni Rad in Figure 4.2, and B-Cal in Figure 4.4.

In order to confirm that the initial increase in fermentation time was not due to only the folic acid content in Pregnavit M, several of its constituents were investigated. This included vitamin B_2, vitamin C, vitamin D, calcium, magnesium, folic acid and iron. As previously discussed, iron supplementation was not evaluated. Calcium, magnesium and vitamin D, which are all constituents of B-Cal-DM, showed no influence on the fermentation process as discussed in Section 4.3.1.1 under Calcium Fortification. For this reason only the effect of vitamin B_2, vitamin C and folic acid were evaluated (See Section 4.3.1.2).

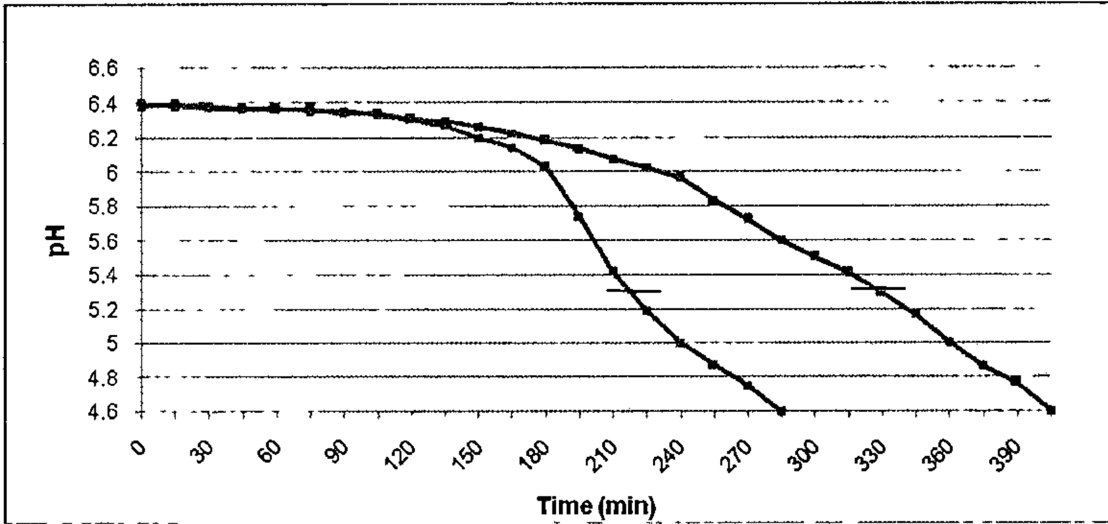

Figure 4.3 The effect of Pregnavit M (3.5 g/l) (—■—) supplementation on yogurt fermentation in comparison to the control (—□—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

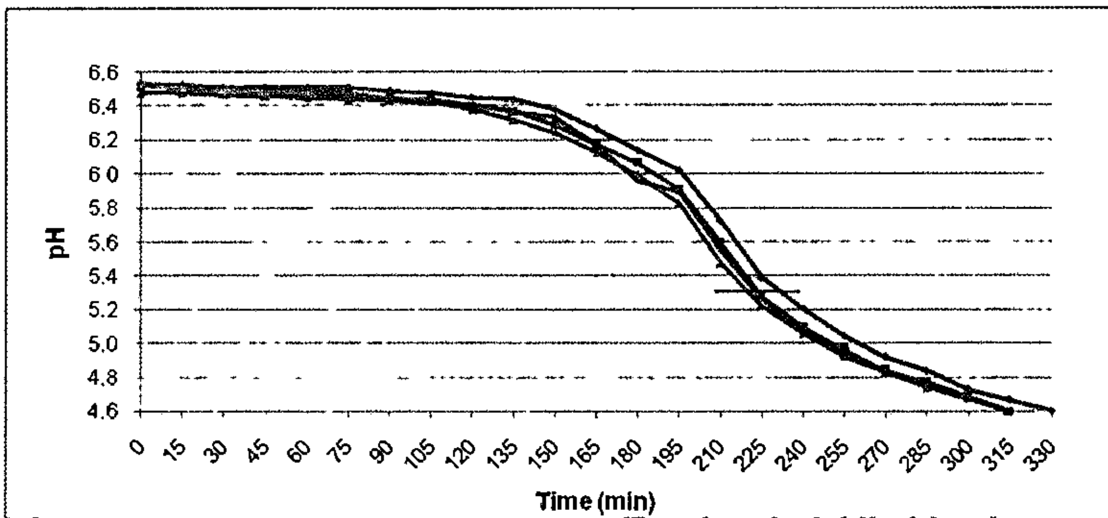

Figure 4.4 The supplementation of yogurt with B-Cal (4 g/l) (——), Pregna-Vit (1.8 g/l) (—✱—) and glucose monohydrate (0.2 g/l) (——) in comparison to the control (—✱—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

Folic acid Forte and StaminoGro

In trial experiments the effect of 1.06 g/l Folic Acid Forte tablets (containing 1590 µg of folic acid) and 1.26 g/l StaminoGro tablets (containing 198 µg of folic acid) were evaluated respectively. The supplementation of these two multivitamins resulted in a significant increase in fermentation time. In subsequent experiments the level of both supplements was lowered to 0.27 g/l of Folic Acid Forte and 0.63 g/l of StaminoGro (Figure 4.5) which resulted in an increase in fermentation time of 19 % and 14 % respectively. No further research was therefore performed using Folic Acid Forte and StaminoGro. Due to the fact that the combination of vitamins and minerals present in the multivitamins Folic Acid Forte and StaminoGro could possibly negatively influence one other, it was decided to identify the main components. These constituents were identified and subsequently individually evaluated based on their potential to accelerate the yogurt fermentation process. The main vitamins identified included folic acid, vitamin C, vitamin B_2, vitamin B_3 and vitamin B_6. Vitamin B_6 was excluded due to Rogosa et al. (1961) reporting that this vitamin was not essential for starter bacterial growth. Only the influence of folic acid, vitamin C, vitamin B_2 and vitamin B_3 on the fermentation process were therefore evaluated (See Section 4.3.1.2).

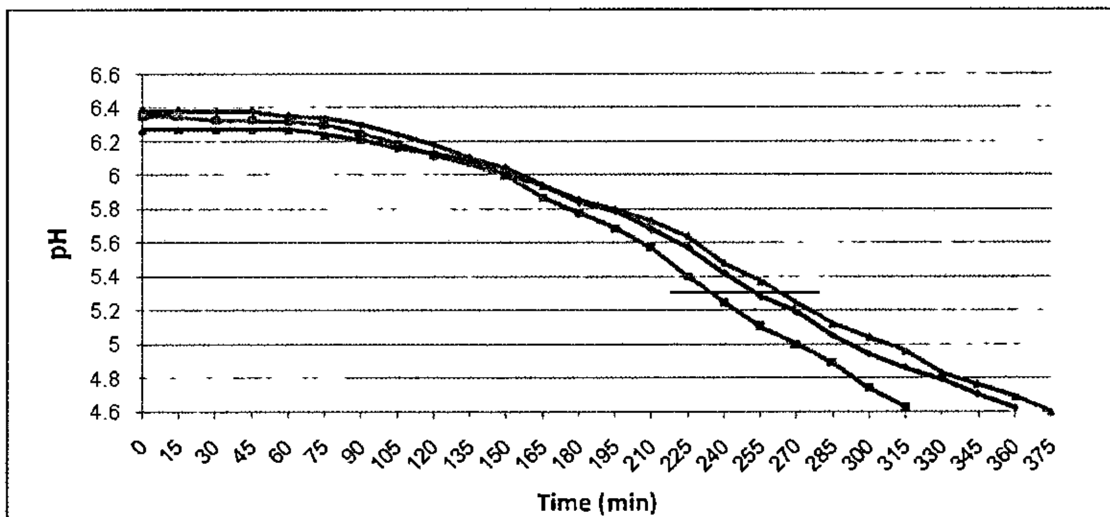

Figure 4.5 The effects of yogurt supplementation using the multivitamins StaminoGro (0.63 g/l) (—+—) and Folic Acid Forte (0.27 g/l) (—▲—) in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

4.3.1.2 Vitamins

Riboflavin (Vitamin B_2) and Thymidine

Concentrations of 1 mg/l of the vitamin riboflavin and the pyrimidine thymidine respectively were evaluated and the results are depicted in Figure 4.6. Thymidine had no effect on the fermentation time, whereas riboflavin increased the fermentation time by 5 %. In the case where the riboflavin load was increased to 2 mg/l, a more pronounced increase of 14 % in fermentation time was noted.

From the above results it is evident that the *L. bulgaricus* and *S. thermophilus* present in the yogurt starter culture does not require either thymidine or riboflavin supplementation for optimal growth. These results are in contrast to those obtained by Rogosa et al. (1961) who stated that whereas *L. delbruekii* does not require folic acid or vitamin B_{12} for optimal bacterial growth, it does however require riboflavin and thymidine. It is important to note that an increase in bacterial growth rate does not necessarily result in an increase in rate of lactic acid production.

Nicotinic acid (Vitamin B_3)

The addition of 1 g/l nicotinic acid to milk resulted in the immediate reduction of the initial pH 6.4 to pH 6 (Figure 4.8) due to the natural acidity of nicotinic acid. Despite this initial decrease in pH the fermentation time still decreased by 16 %. This increase in fermentation time, in combination with the reduction of the initial pH, renders nicotinic acid unsuitable for use as supplement in yogurt manufacture. These results are in contrast to the results reported by Rogosa et al. (1961) and Grobben et al. (1998) who stated that nicotinic acid is essential for the growth of *L. bulgaricus*. In our studies however, *L. bulgaricus* was used in association with *S thermophilus*. Lactic acid production was monitored in our studies whereas the above research groups monitored bacterial growth. This yet again confirms that growth rate cannot necessarily be linked to the rate of lactic acid production.

Folic acid (Vitamin B_9)

An increase in fermentation time during folic acid supplemention was evident in Figure 4.12. This coincides with data reported by Rogosa et al. (1961) who observed that the addition of folic acid to milk inhibited bacterial growth.

To establish whether the high folic acid content in the multivitamin supplements, Pregnavit M, Folic Acid Forte and StaminoGro was responsible for the fact that no decrease in fermention time was noted, the influence of pure folic acid on the fermentation process was evaluated. In Figure 4.12 pure folic acid supplementation increased the time to reach pH 4.6 by 30 minutes (11 %). This indicated that folic acid supplementation undoubtably increased the yogurt fermentation time. Due to this conclusion, the other vitamins and minerals present in Pregnavit M , Folic Acid Forte and StaminoGro were tested individually.

Ascorbic acid (Vitamin C)

The supplementation of yogurt with ascorbic acid had no effect on the yogurt fermentation process (Figure 4.9). It is therefore not practical to use this vitamin as supplement to decrease yogurt fermentation time. Talwalkar and Kailasapathy (2004) also conducted studies on ascorbic acid and found that ascorbic acid supplementation resulted in a detrimental effect on the texture of yogurt.

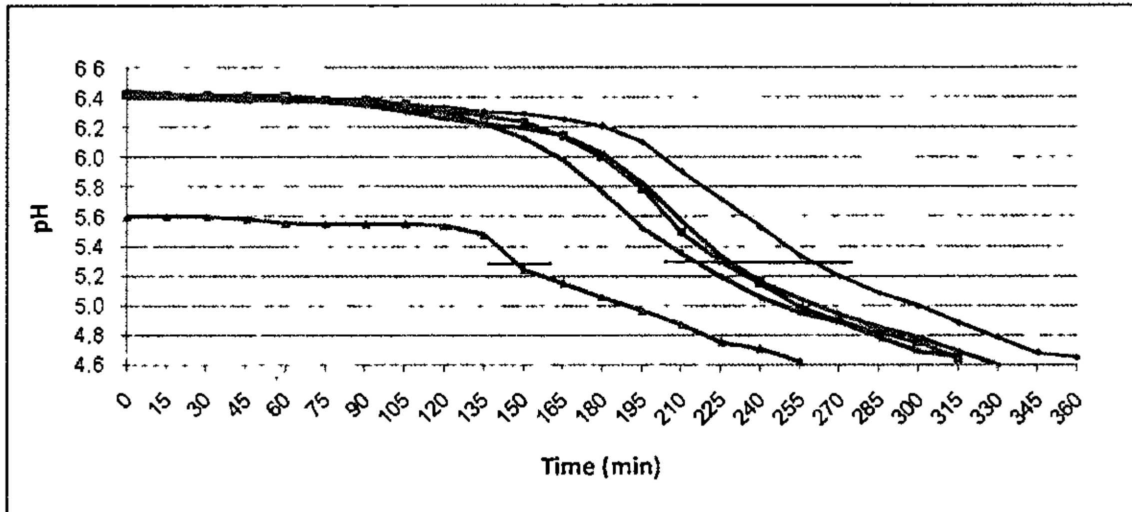

Figure 4.6 The supplementation of yogurt with riboflavin (1 mg/l) (—), riboflavin (2 mg/l) (—), thymidine (1 mg/l) (—) and glutamic acid (2.6 g/l) (—) in comparison to the control (—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

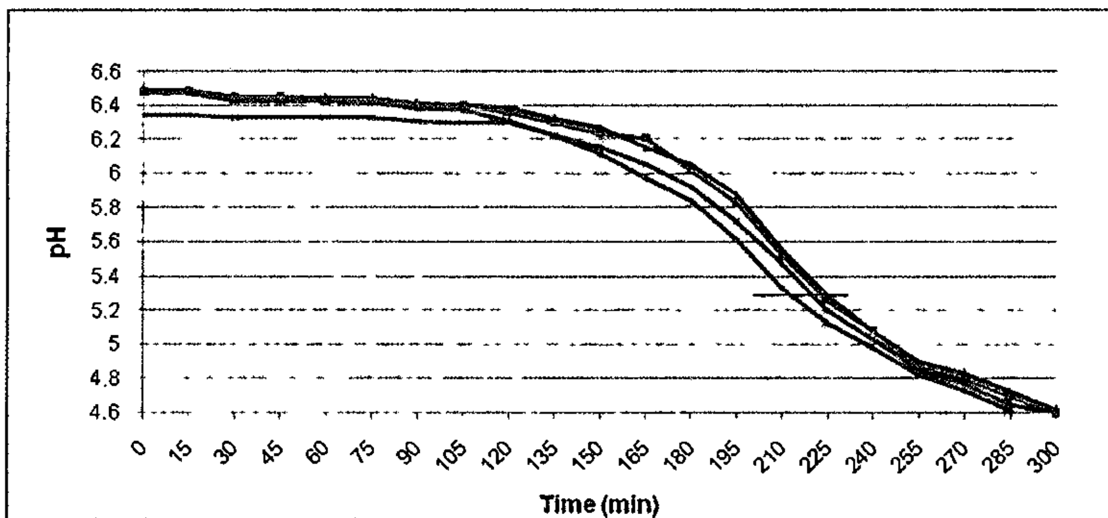

Figure 4.7 The supplementation of yogurt with glutamic acid (0.2 g/l) (—), galactose (0.2 g/l) (—) and proteose peptone (0.2 g/l) (—) in comparison to the control (—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

4.3.1.3 Amino Acids

Cysteine

The supplementation of yogurt with 50 mg/l cysteine resulted in an increase of almost 60 % in time to reach the setting point of pH 5.3 (Figure 4.8), which is considerably higher than pH 4.6 where the fermentation process is normally stopped. The fermentation time will therefore increase even more when the process is continued to pH 4.6. This compares well with data obtained by Dave and Shah (1998) who found a 25 % increase in fermentation time with cysteine supplementation. This research group reported that the increase in fermentation time was due to the cysteine causing damage to the cell wall of *S. thermophilus*, which resulted in changes in the viable bacteria count. When keeping in mind that not only *S. thermophilus*, but also *L. bulgaricus* was present in the starter culture used in our studies, the cell damage to both these bacteria could be a possible explanation for the significant increase in fermentation time depicted in Figure 4.8.

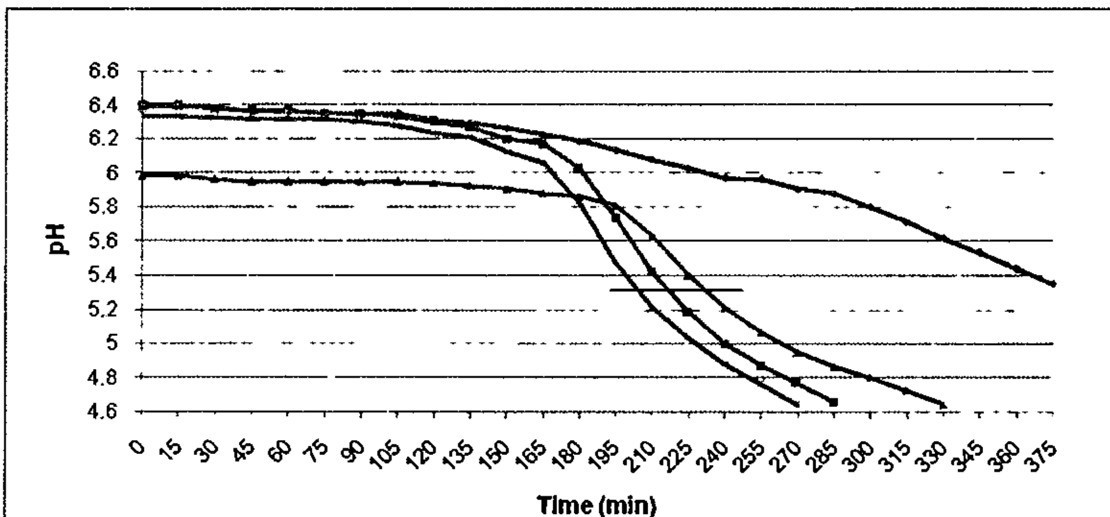

Figure 4.8 The effects of supplementation of yogurt with casein (2.5 g/l) (—•—), nicotinic acid (1 g/l) (—▲—) and cysteine (50 mg/l) (—+—) in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

Glutamic acid

The addition of 2.6 g/l glutamic acid decreased the fermentation time by 1 hour (Figure 4.6), although it resulted in the reduction of the initial pH from pH 6.4 to 5.6. This significant decrease was most probably due to the acidity of glutamic acid which resulted in an artificial decrease. Although the 20 % acceleration in fermentation time depicted in Figure 4.6 is highly noteworthy, it was necessary that the effect of a smaller concentration be evaluated in order to eliminate the effect of the acidity of glutamic acid on the initial pH. In the case where the glutamic acid load was reduced to 0.2 g/l (Figure 4.7), no effect was evident on the fermentation time, however, the effect of the acidity of this amino acid on the initial pH could still clearly be observed. Although the difference of the initial pH in Figure 4.7 was not as evident as in Figure 4.6, the pH at inoculation was nevertheless still lower than that of the control.

In a study done by Letort et al. (2002) it was established that an insufficient amount of glutamic acid is present in milk in order to support *S. thermophilus* growth. Our results confirm this statement in the case where the higher concentration (2.6 g/l) of glutamic acid was evaluated, although this was not the case for the lower concentration. Results obtained in Figure 4.6 depict that glutamic acid can stimulate lactic acid production by the starter bacteria, although it has not yet been established whether this acceleration in fermentation time is of physiological or chemical nature.

L-Methionine

From results depicted in Figure 4.9, the supplementation of milk with 0.2 g/l L-methionine did not have any effect on the yogurt fementation process. These results do not coincide with those obtained by Letort et al. (2002) who reported that the growth of *S. thermophilus* was clearly modified when milk was supplemented with 1 g/l L-methionine. Although the increased concentration reportedly stimulated *S. thermophilus* growth, this concentration is too high to be economically feasible. What is important to observe is that Letort et al. (2002) found increased bacterial growth, and not increased lactic acid production, on which our studies focussed. As previously stated, it is important to note that the two are not necessarily linked. A possible explanation for the contrast in results may be due to the fact that a mixed culture was used in our studies, whereas Letort et al. (2002) used a pure culture of *S. thermophilus*.

L-Tyrosine

The addition of 0.2 g/l L-tyrosine was also investigated, and no deviation from the control run was observed (Figure 4.9). After the evaluation of cysteine, glutamic acid, L-methionine and L-tyrosine, the effect of ACH, which contains all these amino acids, was investigated (Section 4.3.1.4).

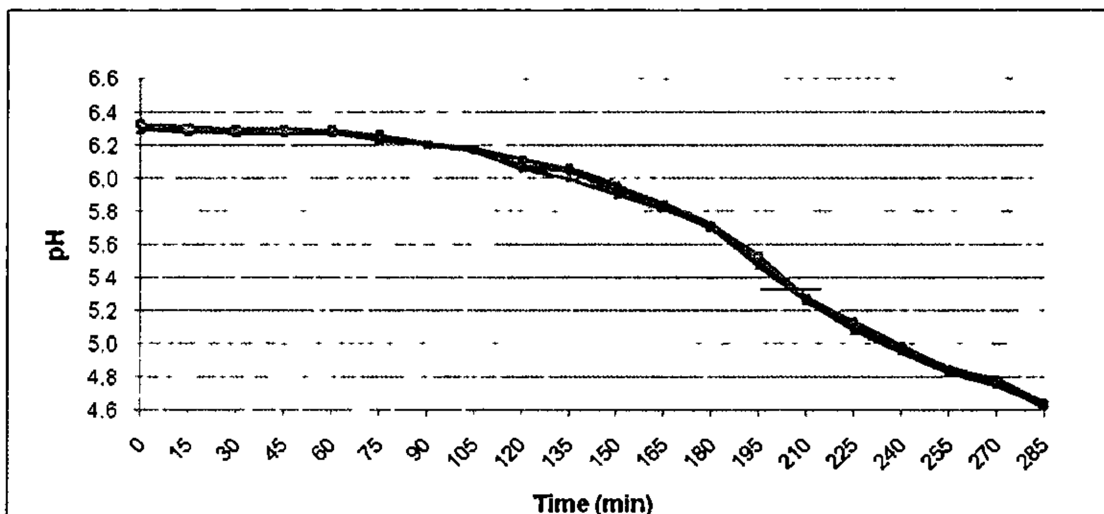

Figure 4.9 The evaluation of vitamin C (0.2 g/l) (—•—), L-methionine (0.2 g/l) (—•—) and L-tyrosine (0.2 g/l) (—•—) supplementation to yogurt in comparison to the control (—•—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

4.3.1.4 Undefined media (Extracts)

Yeast extract

During this study yeast extract was added in two different stages to evaluate the stability of yeast extract at different temperatures. In the first batch 3 g/l yeast extract was added shortly after pasteurisation when the milk had cooled to 80 °C, whereas in the second batch, 3 g/l yeast extract was added together with the starter culture inoculation at incubation temperature of 42 °C. No difference in fermentation time was noted between the two temperatures at which the extract was added (Figure 4.1). The yeast extract that was added at both temperatures during our experiments accelerated the fermentation process by 20 %. A product of poor quality was however delivered, which will not be acceptable to the consumer (Figure 4.10). In future studies yeast extract will be analysed and the compound responsible for the acceleration in fermentation time will be identified.

In similar studies performed by Tamime and Robinson (1996), guar and locust bean gum were added to milk to destabilise the casein micelle, but resulted in strong syneresis. The final product had a coarse coagulum with an open texture. This problem was minimised by blending the milk with carrageenan or alginates. A similar solution can possibly be applied in solving the problem of the coarse coagulum produced during yeast extract supplementation in our studies.

Figure 4.10 Yogurt produced by the supplementation of yeast extract.

Soy milk and Meat extract

The addition of 10 ml/l soy milk delivered unsatisfactory results in previous studies concerning the rheological properties of yogurt (data not shown). Yogurt was not smooth and formed whey pockets throughout the final product (syneresis). Supplementation of yogurt with 3 g/l meat extract was also evaluated, but due to the undesirable flavour and texture of the final product further research using this extract was not done. As a result of the undesirable products delivered by the above supplements, further investigation into the effects of these supplements on the yogurt fermentation process were immediately discontinued.

Elli et al. (1999) encountered similar problems to the above and concluded that the cost and variability in stimulating bacterial growth and the off-flavours which are associated with the addition of components such as yeast extract and soy render these substances less suitable for industrial production of commercial dairy products.

Casein hydrolysate

Dave and Shah (1998) ascertained that ACH, amongst others, contains 13 % glutamic acid, 1.3 % methionine, 2.14 % tyrosine and 0.16 % cysteine. It was therefore decided to evaluate the influence of these amino acids on the fermentation time individually. Thereafter, the effect of ACH was assessed.

The supplementation of yogurt with L-methionine and L-tyrosine did not affect the fermentation process (Figure 4.9), whereas glutamic acid supplementation accelerated the fermentation process (Figure 4.6) (Discussed in Section 4.3.1.3). Cysteine supplementation to yogurt significantly increased the fermentation time (Figure 4.8). Although the amount of cysteine present in ACH is very low, this significant increase in fermentation time can overall affect the influence of ACH on yogurt fermentation. Due to the varying results obtained during the evaluation of some individual components of ACH, the influence of all of them combined in ACH was evaluated. Supplementation of yogurt with casein hydrolysate was evaluated in our studies due to the positive results obtained by Dave and Shah (1998) with *S. thermophilus* and *L. acidophilus*, Oliveira et al. (2001) with *S. thermophilus*, *L. acidophilus* and *L. rhamnosus*, and Masuda et al. (2003) with a pure culture of *L. acidophilus*. None of these research groups, however, worked on the starter bacteria focussed on in this study, so for this reason we repeated this experiment with *S. thermophilus* and *L. bulgaricus*. From the results depicted in Figure 4.11 the addition of 0.25 g/l and 0.5 g/l casein hydrolysate respectively did not have an influence on yogurt fermentation. This could possibly be due to the slight decrease of fermentation time exerted by glutamic acid in combination with the increase by cysteine addition. Due to the limited effect of casein hydrolysate and 4 of its individual amino acids on the yogurt fermentation process, none of its other components were investigated.

In the case of purified casein supplementation, no difference was noted in comparison to the results obtained by casein hydrolysate supplementation (Figure 4.11).

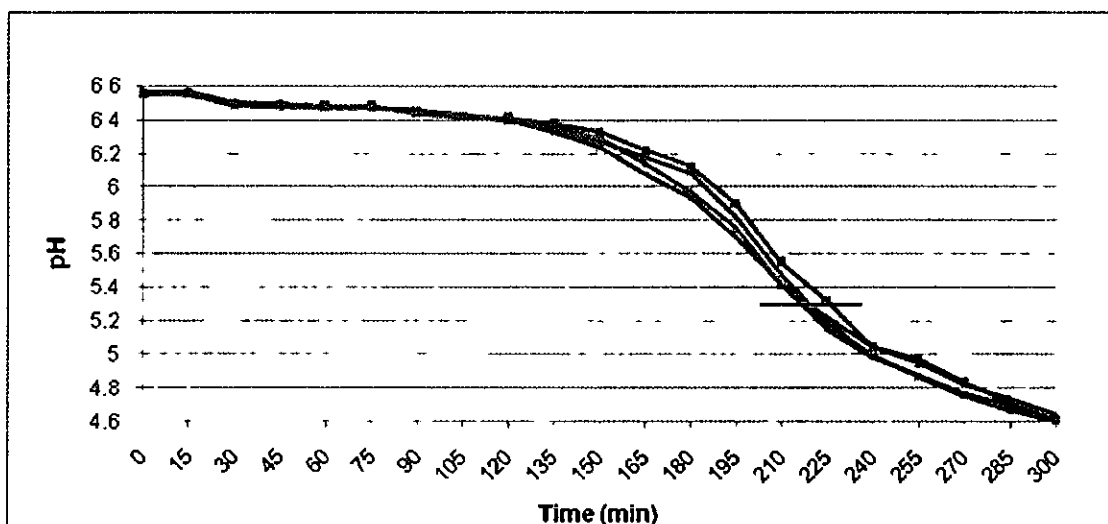

Figure 4.11 The effects of supplementing yogurt with casein hydrolysate (0.25 g/l) (—▲—), casein hydrolysate (0.5 g/l) (——) and purified casein (0.25 g/l) (—▲—) in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

Proteose peptone

The proteose peptone fraction of bovine milk represents about 10 % of total whey protein (Innocente et al., 1998) and due to this fact the effect of an increased concentration on yogurt fermentation was evaluated. Only a slight decrease of 5 % in fermentation time was noted in Figure 4.7 during supplementation of 0.2 g/l proteose peptone to milk used for yogurt fermentation, in comparison to the control. The slight decrease in fermentation time coincide with results obtained by Elli et al. (1999) and Hujanen and Linko (1996) who found improved bacterial growth during the addition of peptones of various origins to the growth medium. The concentration of 0.2 g/l proteose peptone used in our studies resulted in yogurt with an uneven texture and in which whey pockets formed throughout the product. Although a decrease in fermentation time was noted during peptone supplementation, higher concentrations were not evaluated due to the rheological undesirable product that was produced.

4.3.1.5 Total Solids

In Figure 4.8 the effect of 2.5 g/l casein on yogurt fermentation was evaluated. This concentration is 10 times larger than the supplementation of yogurt with casein hydrolysate and purified casein (Figure 4.11), and even in the presence of this significantly increased concentration, the fermentation time was not significantly affected. Similar results were obtained by de Brabandere and de Baerdemaeker (1999) who investigated the effect of certain protein supplements on the pH profile of the yogurt fermentation process.

4.3.1.6 Sugars

During studies on 0.2 g/l glucose monohydrate supplementation to skim milk, no significant difference was evident when compared to the control (Figure 4.4). This corresponds to studies done by Masuda

et al. (2003) on *L. acidophilus* and Chervaux et al. (2000) who confirmed that lactose rather than glucose, as previously considered, markedly increases the growth rate of *L. bulgaricus*.

No change in yogurt fermentation time was noted after supplementation of milk with 0.2 g/l galactose (Figure 4.7). This finding is similar to the findings of O'Leary and Woychik (1976) who established that the starter culture utilised almost none of the free galactose present in milk.

In milk pretreated with a lactase enzyme and supplemented with glucose, galactose, and lactose, only the glucose and lactose were fermented. Fermentation of lactose in the control milk, which contained no enzyme or supplements, was accompanied by the release of free galactose, with the result that carbohydrate utilisation was less efficient than in treated milk. Carbohydrate utilisation by a mixed yogurt culture was more rapid when the lactose in milk was partially prehydrolyzed (O'Leary and Woychik, 1976). The results obtained by O'Leary and Woychik (1976) suggest that the more rapid acid development that took place when a mixed yogurt starter culture was grown in milk containing prehydrolyzed lactose was the result of a more rapid and efficient utilisation of carbohydrates by *S. thermophilus* when free glucose, in addition to lactose, was available for fermentation.

O'Leary and Woychik (1976) further observed that glucose was utilised throughout the incubation period, whereas lactose utilisation took place only up to 4 hours, with the most rapid period of utilisation occurring between 2 and 4 hours of their process that continued up to 5 hours. Free galactose was not utilised. As lactose was consumed during the first 4 hours of incubation, galactose accumulated in the medium.

In experiments performed by this research group results indicated that virtually all the galactose produced from lactose through intracellular hydrolysis was released into the medium. In order to evaluate the above statements, galactose supplementation was evaluated to determine whether it has an influence on yogurt fermentation time. No change in the yogurt fermentation profile was noted during supplementation of yogurt with 0.2 g/l galactose (Figure 4.7).

4.3.1.7 Purines

As depicted in Figure 4.12, the supplementation of milk with 20 mg/l and 0.1 g/l adenine, respectively, and 40 mg/l xanthine prior to fermentation resulted in no decrease in fermentation time. While using the same concentrations of these two purines as Petry et al. (2000), who found that an increased concentration of adenine and xanthine stimulated growth, different results were obtained during our studies (Figure 4.12). To evaluate whether a further increase in the purine concentrations would possibly accelerate fermentation, the concentrations of both purines were increased to 0.2 g/l. As can be seen in Figure 4.13, the acceleration of 5 % obtained during our studies was not noteworthy, and therefore these supplements were not further investigated. Although *L. bulgaricus* growth was slightly stimulated during adenine supplementation by Cogan et al. (1968), when evaluating this purine in the

presence of *L. bulgaricus* and *S. thermophilus* in yogurt fermentation in our studies, no significant change was evident.

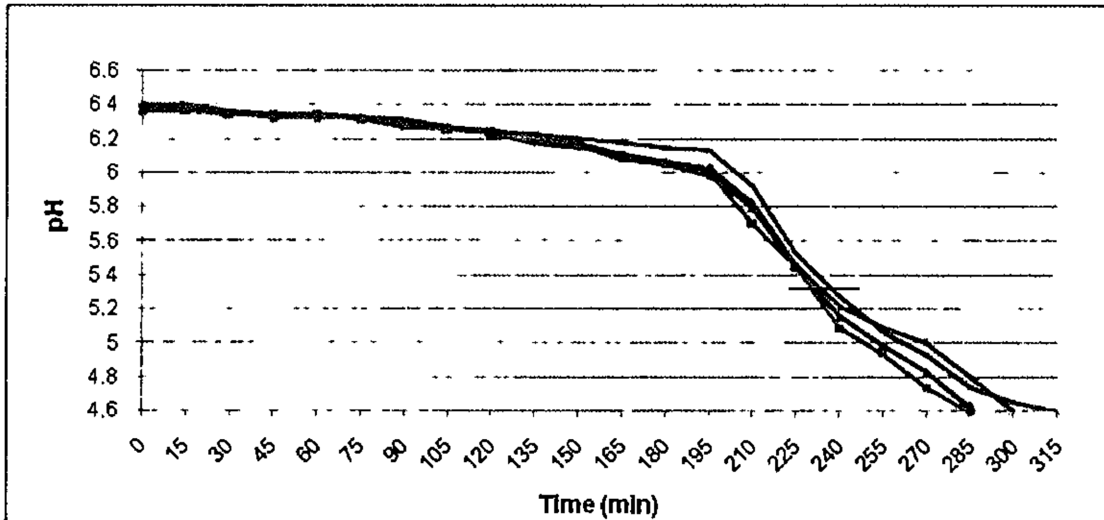

Figure 4.12 The evaluation of adenine (20 mg/l) (—▲—), adenine (0.1 g/l) (—+—), xanthine (40 mg/l) (—▲—) and folic acid (0.05 mg/l) (—•—) supplementation to yogurt in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

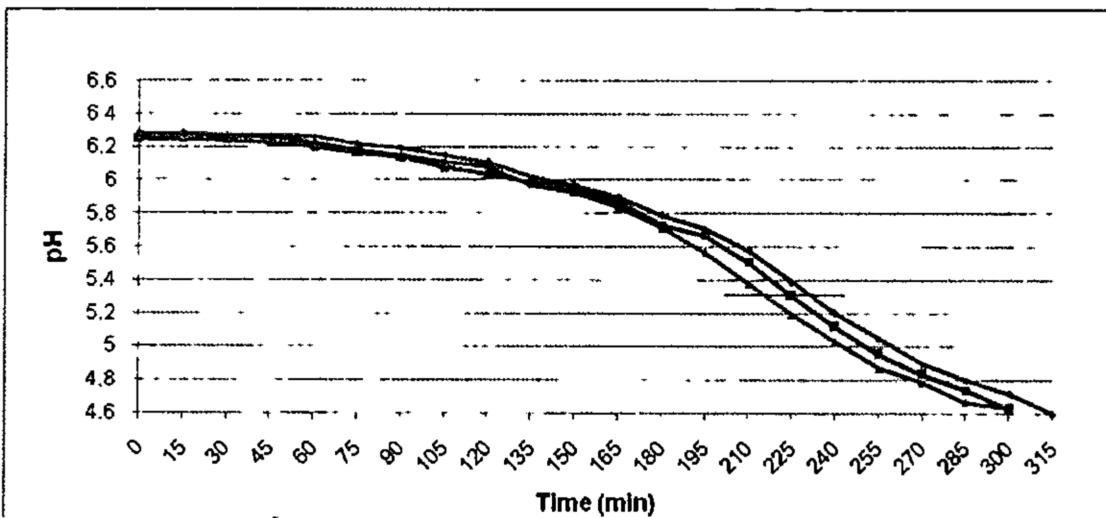

Figure 4.13 Adenine (0.2 g/l) (—■—) and xanthine (0.2 g/l) (—▲—) supplementation to yogurt in comparison to the control (—■—). The black horisontal line (——) depicts the pH at which the yogurt coagulated.

4.4 CONCLUSIONS

The supplementation of yogurt, a naturally healthy product, with selected vitamins, minerals and amino acids is a viable and practical way of further fortifying yogurt. By researching a wide range of supplements, it has been established that this will not only benefit the consumer, but it could possibly also have an accelerating effect on the yogurt fermentation process itself.

During the evaluation of the influence of supplements investigated in this study, it became evident that milk in itself is a complete growth medium for the two starter bacteria involved in yogurt fermentation. For this reason the evaluated supplements did not accelerate fermentation, which indicates that the starter organisms did not have a deficiency for any of these supplements for optimal lactic acid production.

None of the multivitamins that were evaluated in this study made any positive contributions to the yogurt fermentation process. B-Cal, B-Cal-DM, Elimni Rad and Pregnavit M had no effect, whereas Folic acid Forte and StaminoGro even increased the fermentation time.

In the case of the single vitamins investigated in this study, nicotinic acid increased the time to reach pH 4.6 considerably. This increase was evident even though this vitamin by itself decreased the initial pH of 6.4 to below 6. Both these factors eliminate the use of nicotinic acid as an accelerant for yogurt fermentation.

During studies done on the supplementation of yogurt with the amino acid cysteine, it was found that it increased the time to reach the yogurt setting point (pH 5.3) by 60 %. This increase does not even take into account the additional time needed to reach pH 4.6. The addition of glutamic acid to yogurt decreased the fermentation time by 20 %, although it also reduced the initial pH of the milk from pH 6.4 to 5.6. In the case where the concentration was lowered in order to eliminate the effect of the acidity of glutamic acid on the initial pH, no effect was evident on the fermentation time. The effect of the acidity of this amino acid on the initial pH could however still clearly be observed. The supplementation of yogurt with L-methionine and L-tyrosine did not have any effect on the yogurt fementation process in comparison to the control. The effect of all four these amino acids were evaluated separately before evaluating acid casein hydrolysate, which contains all these amino acids. Due to the variation in results obtained during yogurt supplementation by cysteine, glutamic acid, L-methionine and L-tyrosine on the fermentation time, it was decided to continue with the evaluation of casein hydrolysate.

Casein hydrolysate supplementation did not have an influence on the yogurt fermentation time. This could possibly be due to the fact that the positive effect of glutamic acid supplementation may be neutralised by the negative effect of cysteine supplementation. Due to the limited effect of casein hydrolysate on the fermentation process, none of its other components were investigated. In the case

of purified casein supplementation, no difference in fermentation time was noted in comparison to that of casein hydrolysate fortification as well as to the control.

In studies done on yeast extract at two different inoculation temperatures (42 °C and 80 °C) no difference was noted in fermentation time. It was therefore established that the composition of this extract is not affected by exposing it to temperatures as high as 80 °C. Although the yeast extract added at both temperatures decreased fermentation time by 20 % in comparison to the control, a product of unsatisfactory quality was delivered. It is considered important that the component responsible for the acceleration in fermentation time must be identified in future studies.

During investigations on the effect of sugar supplementations to yogurt fermentation, glucose monohydrate, galactose and lactose showed no significant difference in yogurt fermentation time in comparison to the control. This is not surprising due to the fact that literature reports that a significant fraction of lactose is still present in yogurt (66 %) after fermentation has been completed, which indicates an abundance of lactose and subsequently glucose and galactose.

What is evident throughout the results obtained in this study is the fact that in the isolated cases where a significant decrease in fermentation time was observed, an uneven, unacceptable yogurt with undesirable flavour was produced. It is therefore important to keep in mind that yogurt quality must not be compromised by accelerating the yogurt fermentation process. This problem can only be addressed if further research is performed in order to determine which of the various components in the specific supplements are responsible for the decrease in fermentation time. It will enable the evaluation of that specific component, which will most likely not have an adverse effect on the final product if used on its own. This new data will lead to a better understanding of the complex nutritional and growth requirements of the two bacteria used as starter culture for yogurt fermentation.

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CHAPTER 5

EVALUATION OF THE EFFECTS OF PHYSICAL CONDITIONS ON THE YOGURT FERMENTATION PROCESS

5.1 INTRODUCTION

Milk primarily consists of ±20 % whey and ±80 % casein proteins (Fox and Kelly, 2004; Considine et al., 2007). Micellar calcium phosphate, which consists mainly of calcium and phosphate as well as some magnesium and citrate, plays a crucial role in maintaining casein micelle integrity (Shaker et al., 2000; Huppertz et al., 2006). This colloidal calcium phosphate is an important stabilising factor responsible for holding the network together by crosslinking the protein, which is important with regard to the structure of the micelles (Singh and Bennett, 2004). The stability of calcium phosphate is influenced by the rate of pH decrease. Due to the stabilising function of the calcium phosphate, the disruption or dissolving of this micellar calcium phosphate will result in the destabilisation and disintegration of the casein micelle. As a result the micelle will coagulate and subsequently influence the pH at which yogurt coagulates (Shaker et al., 2000; Huppertz et al., 2006). For this reason milk was subjected to several conditions during which the stability of calcium phosphate and the casein micelle were investigated in this study.

Casein proteins can be fractionated into four distinct proteins namely α_{s1}-, α_{s2}-, β- and κ-caseins. The surface of the casein micelle consists primarily of κ-casein (Fox and Kelly, 2004; Phadungath, 2005; Huppertz et al., 2006). The negatively charged casein macro peptide (CMP) portion of the κ-casein provides electrostatic and steric repulsion, thereby preventing inter-micellar aggregation. Enzymatic hydrolysis or acid-induced neutralisation of the κ-casein proteins allows the conversion of milk into cheese curd or yogurt, respectively (Huppertz et al., 2006). By combining these two treatments, it was possible to decrease the time in which yogurt coagulates, therefore increasing the pH at which coagulation occurs.

The proteins present in milk can be modified and destabilised by either one of two independent mechanisms; rennet, which is a proteolytic enzyme originating from animal stomachs, and lactic acid, which is a product of the metabolism of lactic acid bacteria. The use of either of the above processes results in the conversion of milk to curd. During cheese manufacture, rennet is usually added to milk to proteolytically destabilise the casein micelle (Considine et al., 2007). The rennet is responsible for the removal of κ-caseins from the casein micelle, which results in the coagulation of the casein micelles. In contrast to this, lactic acid production during yogurt fermentation neutralises the negative charge on the casein micelle, which then results in coagulation and precipitation (Fox, 2004).

The κ-caseins in milk are resistant to proteolysis by plasmin (Silanikove et al., 2006). For this reason another protease, capable of hydrolysing κ-casein, had to be selected to determine its effect on the casein micelle and subsequently the pH at which yogurt coagulates. Neutrase is a bacterial protease produced by a selected strain of *Bacillus amyloliquefaciens* which hydrolises proteins to peptides (Apar and Ozbek, 2007; NCBE, 2008). This enzyme was suitable for our research purposes due to the fact that its optimum pH of 5.5-7.5, falls in the range of the pH of milk. The optimum temperature of the protease is 45-55 °C, and is inactivated when exposed to conditions of 85 °C for at least two

minutes which is reached during pasteurisation of milk used for yogurt manufacture (90 °C for 10 minutes) (NCBE, 2008).

Initially it was considered that by adding a suitable proteolytic enzyme to milk prior to yogurt fermentation, it could possibly have the same hydrolytic effect on the casein proteins as during cheese manufacture. Due to the partial hydrolysis of the κ-caseins from the casein micelle, it was anticipated that the amount of lactic acid needed to neutralise the κ-caseins would be lowered, which would result in an increase in pH at which the yogurt coagulates. This hypothesis was further investigated in this study.

Cousin (1982) stated that psychrotrophic bacteria can possibly lead to acceleration of yogurt fermentation under controlled conditions. Gassem and Frank (1991) supported this statement by reporting that milk that was precultured with psychrotrophic spoilage bacteria accelerated yogurt fermentation by decreasing the time to reach pH 4.3 by 25-30 minutes in comparison to the control which was not pretreated with psychrotrophic bacteria. From the above statements it was necessary to evaluate the effect of a protease enzyme on yogurt fermentation time and setting pH.

The application of an electrical current to milk was investigated due to the fact that the electrical current causes milk coagulation (Myburgh, 2010, personal communication). A possible explanation for this coagulation might be the solubilisation of the calcium phosphate in the casein micelle. The removal of the colloidal calcium phosphate results in the casein micelles dissociating into smaller particles which in turn aggregate and form a coagulum (Shaker et al., 2000; Considine et al., 2007). This could possibly influence the pH at which yogurt coagulates. By applying this principle, the effect of an electrical current on the casein micelle in milk was investigated. Theoretically, the electrical current would influence the calcium phosphate bond, which would then subsequently lead to accelerated yogurt coagulation. This theory was evaluated in our studies.

No reports are available in literature on the effects of the application of an electrical current on milk used for yogurt manufacture. The only report related to this field was on electro-heating where electricity was applied for a fraction of a second to concentrated, high viscosity products that are otherwise difficult to sterilise by means of heat. David Reznik, president and chief scientist of Raztek Corporation, claimed in 2008 that passing an electrical current through the product in order to pasteurise or sterilise it, eliminates the possiblity of off-odours and flavours forming. This is due to the fact that the heat is created from within, rather than being transferred by exchange.

Homogenisation is a process that reduces the size of fat globules in milk and creams (from 1–8 μm in raw milk to 0.3–0.8 μm in homogenised milk), preventing creaming during long shelf-storage. In classical homogenisation processes, milk heated to 60–70 °C is forced under moderate pressure (10-20 MPa) and high velocity through a narrow opening (Thiebaud et al., 2003).

Results obtained by various researchers (Parnell-Clunies et al., 1988; Sedlmeyer et al., 2004) show that the subjection of milk to pressures greatly exceeding the pressures at which homogenisation takes place, considerably increases the hydration of casein micelles. This is most probably due to the association of the casein micelles with denatured β-LG, which enhances micellar solvation as a result of an increase in the net-negative charge on the casein micelles (de Ancos et al., 2000; Huppertz et al., 2006; Ramasubramanian et al., 2008). Pressures lower than 100 MPa, however, have no significant effect on the denaturation of either β-LG or α-LA (Lopez-Fandino, 2006). From the above it is evident that pressure application to milk influences its solvation, which in turn might have an impact on casein destabilisation and therefore required further investigation.

Generally, the manufacture of yogurt is done by using lactic acid bacteria as starter culture. Lactic acid bacteria are facultative anaerobes that can grow in oxygenated environments, although they have a preference for anaerobic conditions (Beshkova et al., 2002; Horiuchi et al., 2009). In order for *S. thermophilus* (one of the bacteria used as starter culture in our studies) to function optimally, it requires an abundant supply of dissolved oxygen in the yogurt medium. This is made possible by the mixing and agitation involved in the steps of homogenisation, cooling (after pasteurisation), and inoculation with starter cultures during commercial yogurt manufacture (Talwalkar and Kailasapathy, 2004). Dave and Shah (1998) and Beshkova et al. (2002) confirmed this by stating that *S. thermophilus* prefers an aerobic environment to an anaerobic environment. In contrast, the growth of *L. bulgaricus* is inhibited by oxygen and this bacteria function optimally in anaerobic conditions (Beshkova et al., 2002).

In studies done by Beshkova et al. (2002), it was found that in comparison to anaerobically cultivated yogurt which reached pH 4.6 after 3.5 hours, yogurt incubated at levels of 10 % and 20 % oxygen subsequently decreased fermentation time by 43 %. The decrease in fermentation time was most probably related to the co-dependance of the starter culture, i.e. to the stimulating effect (due to the oxy-tolerance) of *S. thermophilus* on the growth of *L. bulgaricus*, which subsequently increased its proteolytic activity. An elevated oxygen concentration of 30 % however increased fermentation time by approximately 25 %. This indicated that above a concentration of 30 % oxygen, the oxygen tolerance of *S. thermophilus* became impaired. Yogurt bacteria viability was at its highest in the mixed culture at the time when 20 % dissolved oxygen was maintained in the milk. On the other hand, according to Horiuchi et al. (2009), starter bacteria only begin to produce acid actively after the dissolved oxygen concentration in the milk has been reduced to 0 mg/kg, suggesting that the dissolved oxygen retards acid production. Although Horiuchi et al. (2009) found that a dissolved oxygen concentration of 0 mg/kg decreased fermentation by 23 % in comparison to normal air conditions, this did not apply to all starter cultures of *L. bulgaricus* and *S. thermophilus* that were tested. Vercet et al. (2002) confirmed that the expulsion of oxygen had stimulatory effects on yogurt starter cultures. Fung (1996) also substantiated this by accelerating the yogurt fermentation process by adding an oxygen-reactive enzyme which removed all oxygen in the medium.

Talwalkar and Kailasapathy (2004) further noted that at present, research on the interaction of oxygen with probiotic bacteria is largely inadequate. Consequently, the problem of oxygen toxicity in these bacteria remains unclear. In order to evaluate all of the above findings, the effect of de-aerated milk on the growth of starter bacteria and subsequent yogurt fermentation time was investigated.

Different heat treatments can have complex effects on fermentation time. These effects can either be stimulatory or inhibitory on the fermentation process, depending on incubation time and temperature conditions (de Brabandere and de Baerdemaeker, 1999; Vercet et al., 2002). The industrial fermentation process of yogurt is carried out at a constant temperature of 42 °C. This temperature is a compromise between the optimal growth temperatures of 39 °C and 44 °C for *S. thermophilus* and *L. bulgaricus*, respectively (Gueguim-Kana et al., 2007). These three temperatures were evaluated in order to determine the effects of different incubation temperatures on yogurt fermentation time as well as on the pH at which yogurt coagulates.

5.2 MATERIALS AND METHODS

5.2.1 Rehydration of skim milk powder

Skim milk powder (Nestlé, South Africa) was reconstituted with tap water (10 % w/v). The rehydration process was performed at 50 °C for 60 minutes.

5.2.2 Milk serum preparation

The skim milk (as prepared in Section 5.2.1) was left to reach room temperature after which the pH was adjusted to pH 4.6 using 36 % HCl (Merck, AR). The milk was heated to 90 °C for 40 minutes after which it was cooled to room temperature and centrifuged for 5 minutes at 4 °C at 9820 g using a Beckman Model J2-21 centrifuge with a JA-20 rotor. The pH of the serum was adjusted to pH 6.5 using 10 N NaOH (Saarchem, AR). The serum was then reheated to 42 °C for 30 minutes, followed by 5 minutes of centrifugation at 9820 g. The serum was once again heated to 42 °C for 15 minutes and centrifuged at 9820 g for 10 minutes. The supernatant represented the milk serum.

5.2.3 Starter cultures

A thermophilic yoghurt culture, FD-DVS YF-L812 Yo-Flex, supplied by Chr. Hansen, was used throughout all experiments. The starter culture was received as freeze-dried granules and grinded with a mortar and pestle to reduce particle size. This simplified the weighing process in comparison to weighing the whole granules and resulted in better distribution of the starter bacteria.

5.2.4 Pre-inoculum preparation

Serum (300 ml) prepared as described in Section 5.2.2 was heated to 42 °C whereafter it was inoculated with 0.1 g of ground starter culture. The inoculated serum was incubated in a mechanical shaker (New Brunswick Scientific, Edison, USA) at 42 °C and 100 rpm. Growth was monitored spectrophotometrically until an OD_{640} of approximately 0.45 was reached with a coulter chamber count of approximately 1.03 x 10^7 cells per ml. An inoculation load of 1 ml of this pre-inoculum was then used for individual yogurt fermentation runs.

5.2.5 Spectrophotometry

A Genesys 10 vis/UV Thermo Spectronic (Rochester, USA) spectrophotometer was used in all absorbance determinations. Quartz cuvettes with a volume of 1 ml was used in all experiments.

5.2.6 pH Measurements

The pH values were measured with a portable pH Spear (Eutech Instrument Co., Oakton, USA) during incubation at 42 °C at 15 minute intervals. The pH meter was calibrated using pH 4 and pH 7

buffer solutions (Hanna instruments, USA) prior to use. The pH decrease was an indication of the rate of lactic acid production.

5.2.7 Statistics

Calculations on average growth between triplicates were performed using the computer program Excel 2007 by Microsoft Corporation. Additional statistical analysis was not performed due to a too small variation in data.

5.2.8 Yogurt manufacture

Skim milk powder was rehydrated as described in Section 5.2.1. A concentration of 2.5 % sucrose (Huletts, South Africa) was added after rehydration was completed. The milk was heated to 90 °C for 8-10 minutes to pasteurise and was subsequently cooled to 42 °C. Similar heating conditions were also used by Tamime and Robinson (1996), Vandewegh et al. (2002) and Arkbage (2003). A batch of 2 litres of milk was then inoculated with 1 ml milk serum that was prepared as described in Section 5.2.4. Yogurt fermentation was stopped at pH 4.6, the iso-electric point of casein proteins. Stainless steel yogurt fermentation tubes (2 litres) with lid and stirrer were used during all yogurt fermentations. No stabilisers were added during any experiments. Yogurt prepared in this way served as control in all yogurt experiments.

5.2.9 pH versus setting point

In Chapter 4 the fermentation time of yogurt, specifically the time to reach pH 4.6, was investigated. In this chapter however, closer attention was paid to the pH at which the yogurt coagulated when milk was subjected to different conditions prior to fermentation.

5.2.10 Protease treated milk

Yogurt preparation

Yogurt was prepared as described in Section 5.2.8. In this case the pasteurisation of the milk served a dual purpose; not only to eliminate pathogenic organisms, but also to inactivate the added proteolytic enzyme. The milk was cooled and no coagulation was present in any of the milk batches that were used for yogurt fermentation.

Protease preparation

A stock solution of protease was prepared by adding 0.3 g of Neutrase enzyme 1.5 MG (Novozymes, Denmark) to 200 ml of phosphate buffer (Sigma) with a final pH of 7.4.

Preliminary trial experiments performed on the supplier's recommended load of Neutrase indicated that 3.75 µg/ml was the highest load which could be used without directly leading to milk coagulation. No Neutrase activity remained after pasteurisation. The influence of various loads of the Neutrase

enzyme on the yogurt fermentation process was evaluated. This included 0.1875 µg/ml, 0.375 µg/ml, 1.875 µg/ml and 3.75 µg/ml, respectively. These concentrations were all added at 10 °C at the onset of pasteurisation.

5.2.11 Milk treated with an electrical current

Milk was treated with an electric current of 500 mA for 30 minutes. A LKB 2197 power supply and a Hoefer CHEF (Contour-clamped homogeneous electric field) separation unit were used in all electrical current studies. This specific current was found to be the highest that did not directly result in the alteration of the composition of the milk.

Calcium determination

After subjecting milk to an electrical current, 1.5 ml was precipitated with 1 ml TCA (Merck, 24 %). The milk that served as control was not exposed to an electrical current treatment. The free calcium in the respective supernatants was evaluated using the technique of Hesse (1971).

5.2.12 Pressure applications to milk

Fresh milk was supplied by Dairy Corporation (Bloemfontein) and the pressure treatments of milk took place on their premises. A Gaulin homogeniser was used for all pressure treatments.

Individual fresh milk batches were subjected to various pressures including 2.5 MPa, 5 MPa, 7.5 MPa, 11 MPa and 13 MPa. After the pressure treatments had been completed, individual yogurt runs were performed using the different pressure treated milk batches. Milk not subjected to pressure treatment served as the control.

5.2.13 De-aeration of milk

Reconstituted skim milk (prepared as described in Section 5.2.1) was heated to 90 °C for 10 minutes. The milk was then cooled to 42 °C, whereafter it was placed under vacuum. This was done in 500 ml increments in an airtight container (desiccator). The milk was gradually exposed to 625 mm of mercury vacuum (83 kPa) until the milk stopped bubbling (± 5 minutes). The de-aerated milk was then carefully transferred into stainless steel yogurt fermentation tubes. Fermentation proceeded as normal. Skim milk not exposed to vacuum application served as control.

5.2.14 Pasteurised and unpasteurised milk

Milk was pasteurised at 90 °C for 8-10 minutes and then cooled to 42 °C. The unpasteurised milk was not subjected to any heat treatment prior to fermentation.

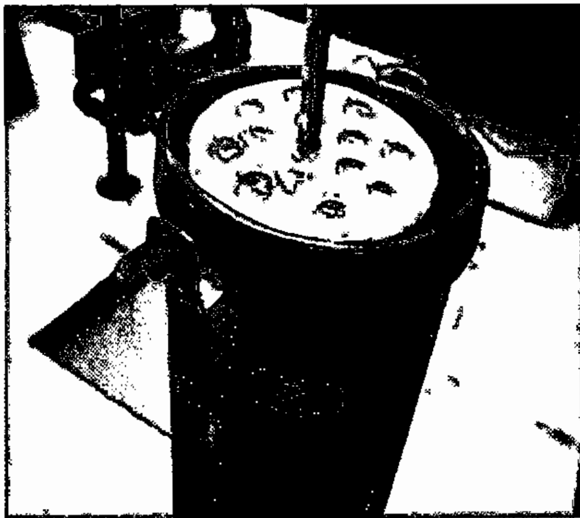
5.2.15 Various incubation temperatures

Three individual yogurt fermentations were performed at 39 °C, 42 °C and 45 °C respectively.

5.3 RESULTS AND DISCUSSION

5.3.1 Protease treated milk

The commercial bacterial protease, Neutrase, is inactivated when exposed to 85 °C for at least two minutes. Neutrase was therefore totally inactivated during the pasteurisation phase which was performed at 90 °C for 10 minutes. Producing yogurt using milk pre-treated with 3.75 µg/ml of Neutrase (supplier's highest recommended load) resulted in a smooth yogurt with acceptable consistency (Figure 5.1A). When higher than recommended Neutrase loads were added, it resulted in a cheesy yogurt product with a gritty texture which also exhibited strong syneresis (Figure 5.1B).



A

B

Figure 5.1 Yogurt produced by Neutrase loads of 3.75 µg/ml (Figure 5.1A) and 7.5 µg/ml (Figure 5.1B) respectively.

The milk used for yogurt manufacture that had been pre-treated with 0.375 µg/ml protease exhibited no difference in fermentation time in comparison to the control (Figure 5.3). Due to results obtained by Cousin (1982) and Gassem and Frank (1991) who found that milk treated with psychrotrophic bacteria (which produce protease enzymes) accelerated yogurt fermentation, it was decided to evaluate additional Neutrase loads. In the case where different loads of 0.1875 µg/ml, 1.875 µg/ml and 3.75 µg/ml, respectively, were added to milk prior to pasteurisation, no change in fermentation time was once again noted when compared to the control (Figure 5.4). The pH at which the yogurt coagulated however was increased from pH 5.2 to 5.4 when using a 0.375 µg/ml protease load, which resulted in a slightly sweeter yogurt. No significant difference in yogurt setting pH was noted where the other enzyme loads were evaluated.

Work was done by Adams et al. (1975) relating to proteolysis by proteases produced by psychrotrophic bacteria, and not by a commercial protease as used in this study. The results reported

by this research group coincide with findings in this study which indicated that as the protease activity increased, the κ-casein hydrolysis consequently also increased, which resulted in the destabilisation of the casein. This research group also found that raw milk which had undergone extensive κ-casein hydrolysis, coagulated during ultra high temperature (UHT) treatment. While the milk used in our experiments was pasteurised and did not receive UHT treatment, similar results were obtained when using concentrations higher than 3.75 µg/ml of Neutrase. In experiments done by Gassem and Frank (1991), yogurts made from milk treated with bacterial protease preparations were firmer and had greater apparent viscosity than that of the control. Similar results were observed during our experiments when using the Neutrase enzyme.

The variation in results concerning the acceleration of yogurt fermentation using a proteolytic enzyme, can be attributed to the fact that commercial Neutrase (used in our studies) is destroyed at yogurt pasteurisation temperatures (90 °C for 10 minutes). Natural protease produced by psychrotrophic bacteria as used by Cousin (1982) and Gassem and Frank (1991) was however capable of withstanding UHT treatments (135 °C for 5 s).

A hypothesis for the insignificant difference in yogurt setting pH between the milk that was subjected to protease activity, and that of the control, could be attributed to the CMP present in the medium. Although the proteolytic activity of the enzyme hydrolised the CMP portion of the κ-caseins to some extent, these peptides are still present in the medium, even though not as part of the casein micelle (Figure 5.2C). As lactic acid is produced, the negative charge on both the intact as well as the free CMP proteins needs to be neutralised. Due to the fact that the hydrolised κ-caseins are still present in the medium and not removed, it can therefore potentially still take part in the lactic acid-κ-casein-neutralisation action. The same amount of lactic acid is therefore needed to neutralise and thus destabilise the casein micelles as for the control.

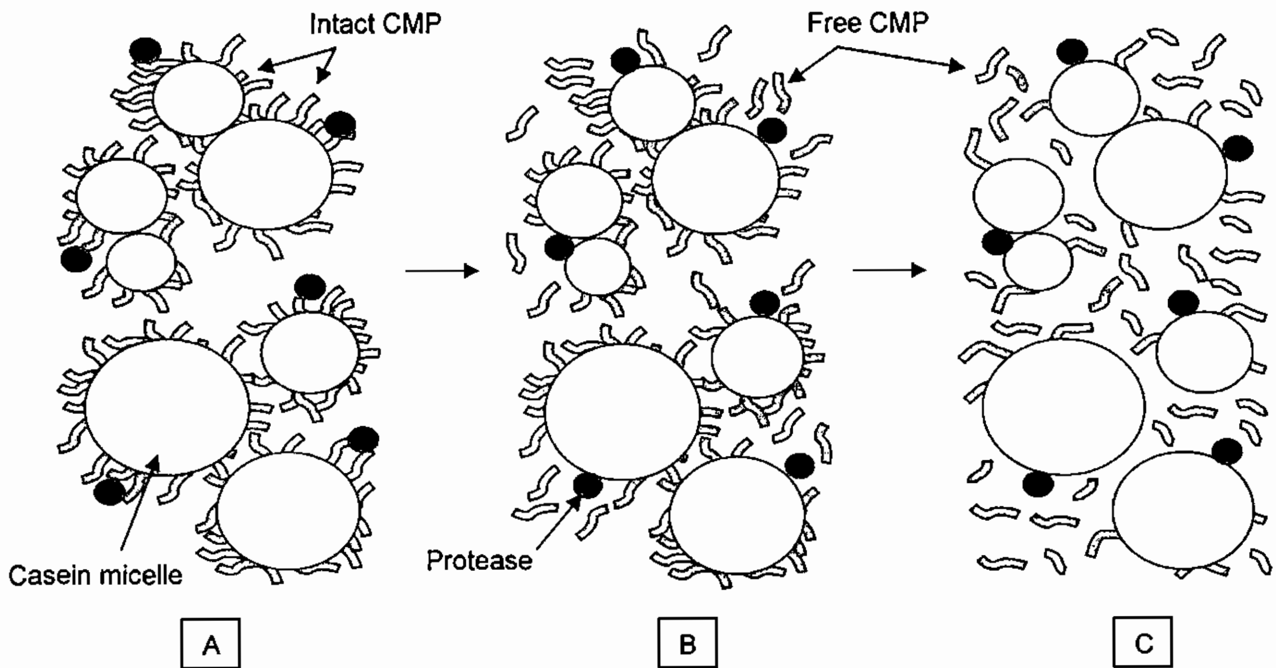


Figure 5.2 The effect of a proteolytic enzyme on the casein micelle. As the micelle is exposed to the proteolytic action for extended periods (Figures 5.2B and C), the CMP is hydrolised which increases the amount of free CMP. The amount of lactic acid needed to neutralise the micelle in Figure 5.2C is therefore much less than that needed for Figure 5.2A where all the CMP are still intact. Adapted from Dalgleish (2004).

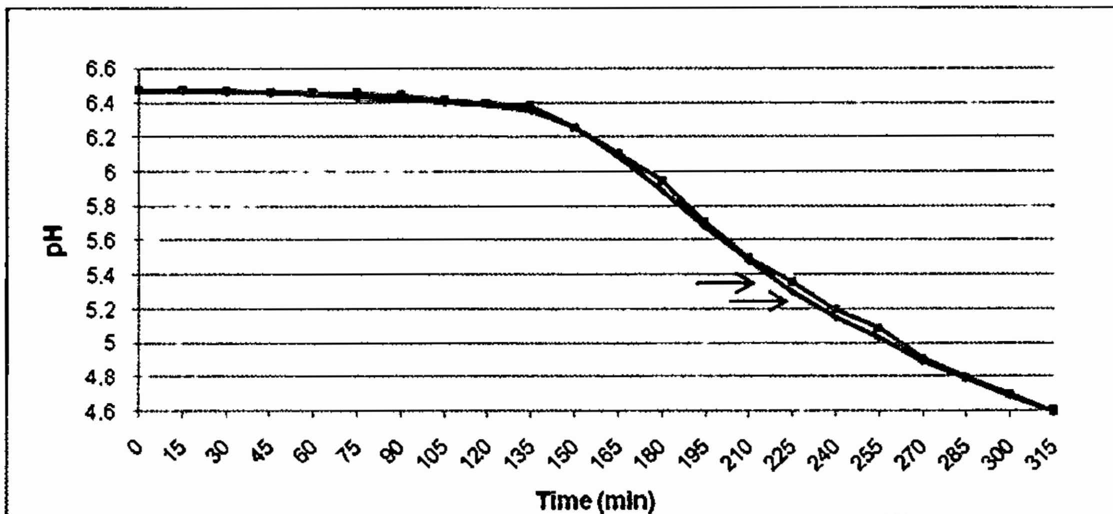


Figure 5.3 The yogurt fermentation profile of control milk (—■—) and milk pre-treated with 0.375 µg/ml Neutrase (—♦—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

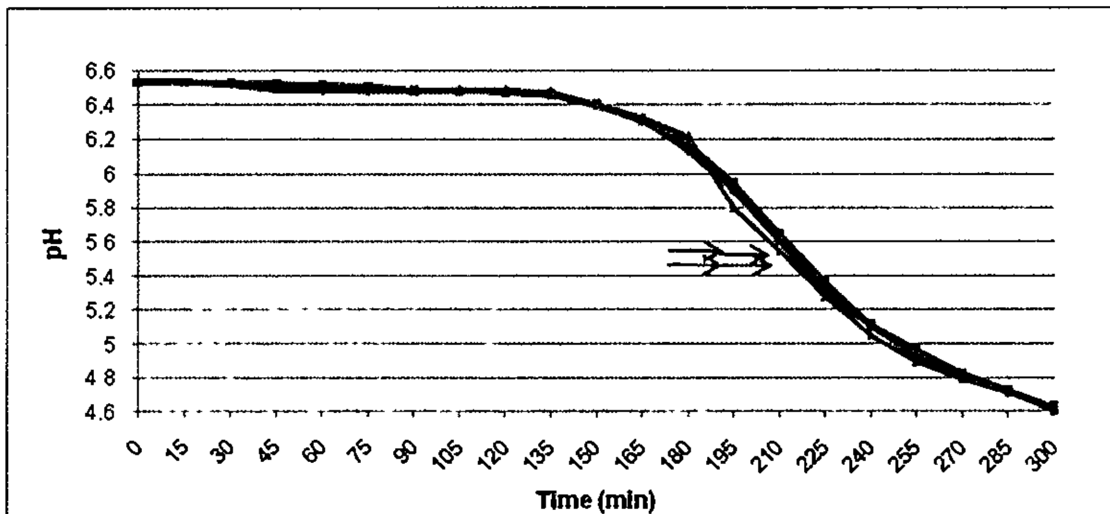


Figure 5.4 The effect of 0.1875 µg/ml (—•—), 1.875 µg/ml (—▲—) and 3.75 µg/ml (—*—) of Neutrase on yogurt fermentation in comparison to the control (—■—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

5.3.2 Milk treated with an electrical current

Milk which had been subjected to an electrical current, as well as untreated milk which served as control, were precipitated in order to determine the amount of free calcium present in the supernatant. This indicated the amount of free calcium present in the milk which was no longer bound in the casein micelle. When compared to the control milk, the pre-treated milk showed no significant difference in calcium content. The control milk contained 628 mg/l of calcium whereas the milk treated with an electrical current contained 654 mg/l of calcium. This indicated that subjecting milk to an electrical current of 500 mA prior to fermentation, did not solubilise the calcium phosphate aggressively enough to affect the structure of the casein micelle.

After the milk had been subjected to the electrical current, it was evident that the application of 500 mA to milk prior to fermentation decreased the time to reach pH 4.6 by 5 %, and increased the coagulation pH of 5.2 to pH 5.35. These results correspond to a certain extent with the data reported by Shaker et al. (2000), who noted that the removal of the calcium phosphate bond destabilised the casein micelle which resulted in coagulation. The effect on fermentation time and the difference in the pH at which the yogurt coagulated (Figure 5.5) however, was marginal. Due to fact that 500 mA was found to be the largest electrical current that could be applied without directly altering the milk structure, further studies on electrical current application to milk was not performed.

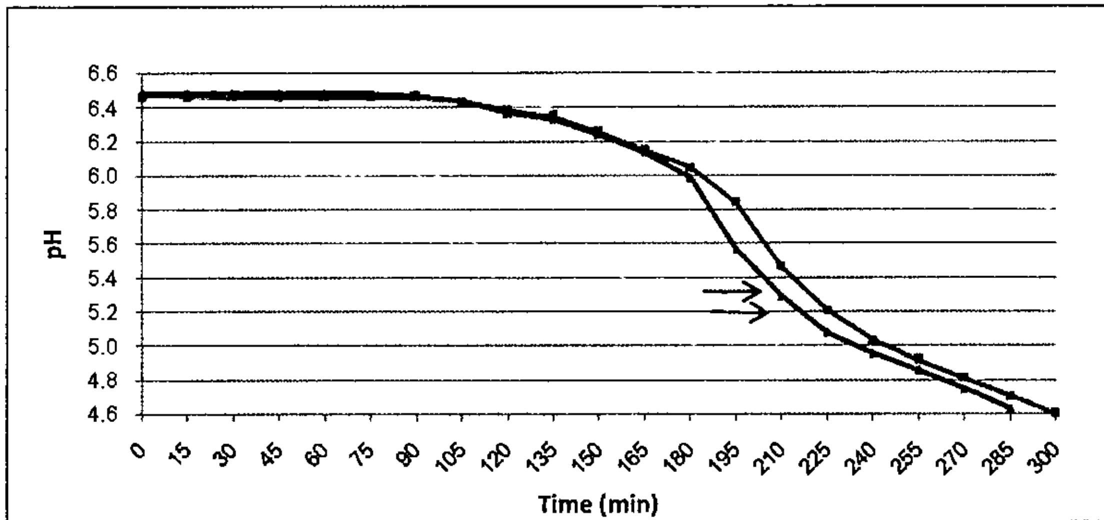


Figure 5.5 The effect of 500 mA (—+—) electrical current application to milk prior to fermentation in comparison to the control (—■—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

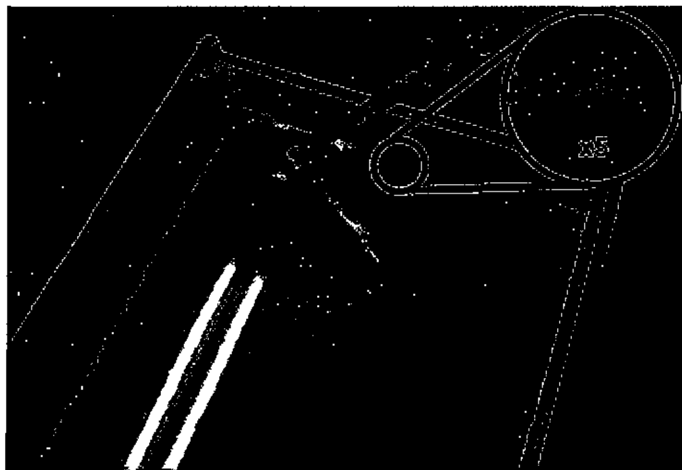


Figure 5.6 The application of an electrical current to milk. The effect of the electrical current on the milk can clearly be seen in the form of slight coagulation occuring at the negative electrode in the 5 times enlarged view.

5.3.3 Pressure applications to milk

During yogurt fermentations performed using pressure treated milk, it was evident that the rate and extent of casein micelle destabilisation increased with applied pressure. This subsequently influenced the setting pH of yogurt (Figure 5.7). This finding is confirmed by Huppertz et al. (2006) and Lopez-Fandino (2006), who observed that when high pressure-treated milk was acidified, the onset of gelation or coagulation point occured at a higher pH than in the case of unpressurised milk. This coincides with results obtained in our studies where significantly lower pressures (10 times lower) were evaluated.

The major difference between the two investigated milk batches during yogurt fermentation was the fact that the yogurt produced with the homogenised milk, coagulated at a significantly higher pH of 6.07 in comparison to the control which coagulated at pH 5.2 (Figure 5.7). Due to the difference in the coagulation pH between the homogenised and unhomogenised milk, the effect of a range of pressures necessitated further investigation.

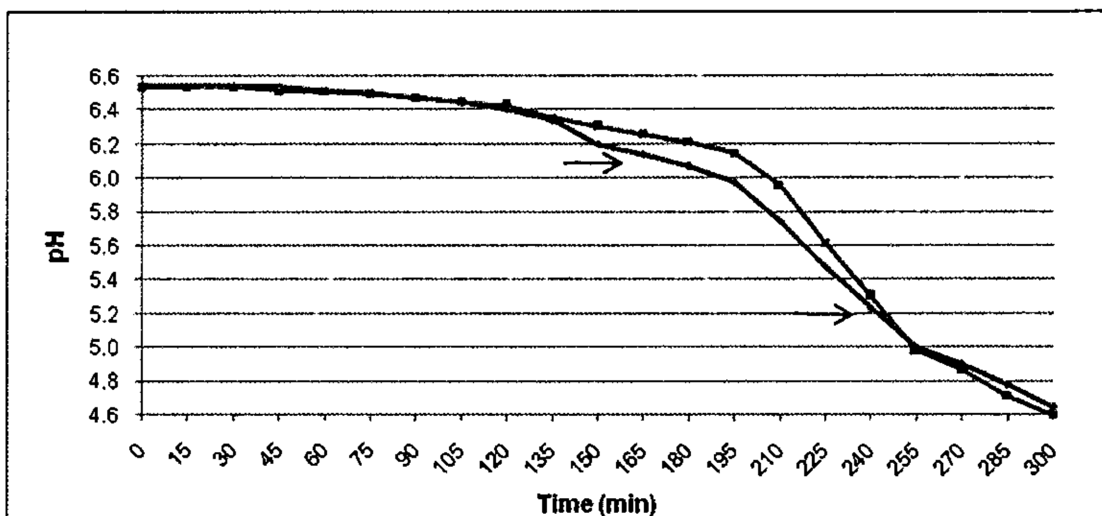


Figure 5.7 The fermentation profile of homogenised milk (—•—) in comparison to unhomogenised milk (—▪—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

Two clear groupings of the pH at which the yogurt coagulated were evident when milk was subjected to pressures ranging from 2.5 MPa, 5 MPa, 7.5 MPa, 11 MPa to 13 MPa prior to fermentation (Figure 5.8). Milk subjected to 2.5 MPa and 13 MPa, as well as the control, all coagulated in the normal setting pH range of between pH 5.2 and pH 5.3, whereas milk subjected to 5 MPa, 7.5 MPa and 11 MPa all coagulated between pH 6.0 and pH 6.2. It is clear that as the pressure increased, the setting pH of the milk subsequently increased. For reasons not yet known, the milk subjected to 13 MPa totally deviated from this tendency. Furthermore, no logical explanation can be given for the large difference in setting pH between pH 5.3 and pH 6.0 (Figure 5.8).

De Ancos et al. (2000) found that pressure application to yogurt milk did not have any significant effect on the fermentation time during yogurt manufacture. The only effect that was however reported

by de Ancos et al. (2000) was that the yogurt produced using the pressure treated milk had an increase in viscosity and amino acid content in comparison to the control. They also concluded that the higher viscosity was due to the fact that pressures exceeding 100 MPa disintegrated the casein micelle into smaller subunits, resulting in a product with increased aggregating properties. In milk subjected to pressures higher than 200 MPa prior to fermentation a reduction in growth activity of starter culture was observed (de Ancos et al., 2000). In literature no reports were found on the pressure range investigated in our studies.

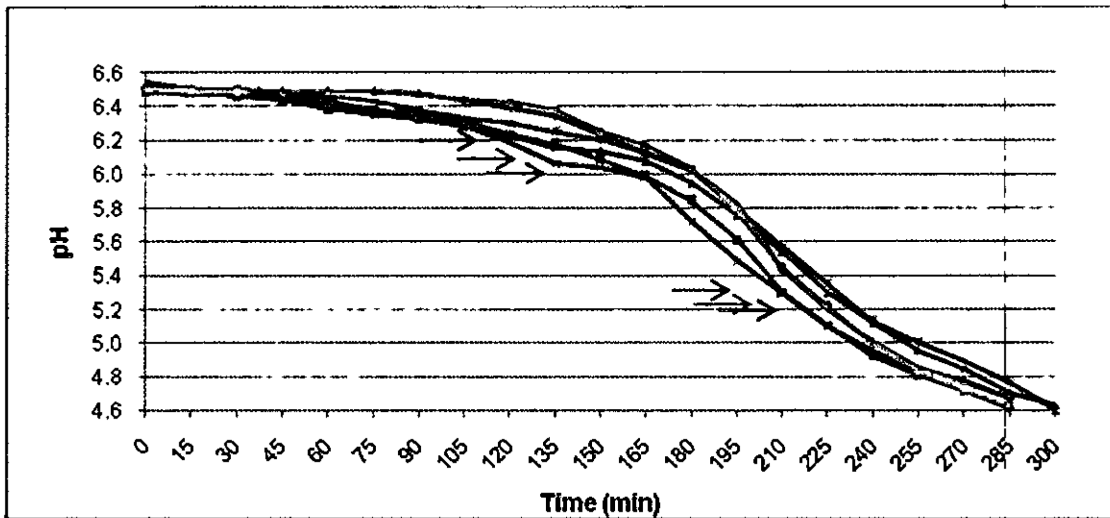


Figure 5.8 The effects of 2.5 MPa (—•—), 5 MPa (—•—), 7.5 MPa (—•—), 11 MPa (—•—) and 13 MPa (—•—) on the setting pH of yogurt in comparison to the control (—▪—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

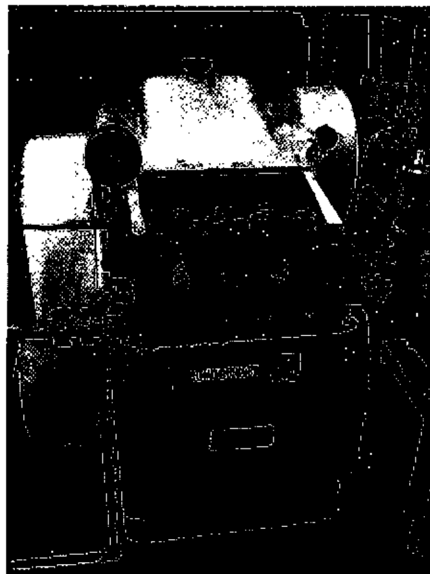


Figure 5.9 The homogeniser used in pressure studies.

5.3.4 De-aeration of milk

Due to positive results acquired by several research groups concerning the acceleration of yogurt fermentation time by de-aerating milk, we attempted to remove as much air (oxygen) as possible in a laboratory set-up using a vacuum pump. The investigation on the influence of oxygen on the yogurt fermentation process was nevertheless difficult. This was due to the milk being subjected to agitation during the preparation of stirred yogurt, which unavoidably incorporated small amounts oxygen. The results obtained (Figure 5.10) indicated no difference in fermentation time between the de-aerated and control milk fermentations. There was also no significant variation in the setting pH of the yogurt which ranged between pH 5.2 and 5.3. These results are contrary to those obtained by Fung (1996), Beshkova et al. (2002), Vercet et al. (2002) and Horiuchi et al. (2009).

What must be kept in mind however, is that the abovementioned studies were all performed on set type yogurt, where no agitation and consequently oxygen incorporation, was present. Stirred yogurt, which formed the basis of our studies, is however subjected to agitation.

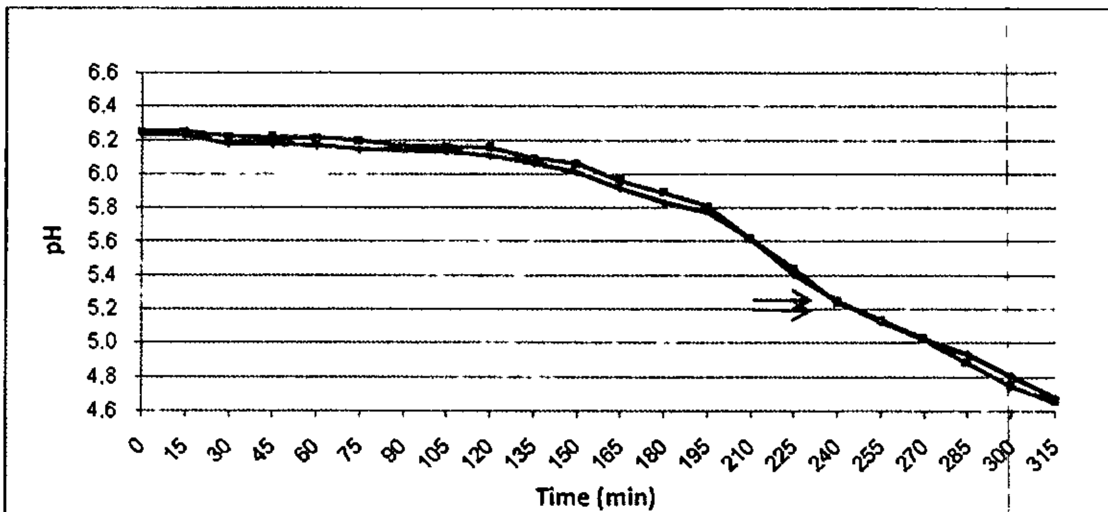


Figure 5.10 The effect of de-aerated milk (—+—) on the yogurt fermentation process in comparison to the control (—■—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

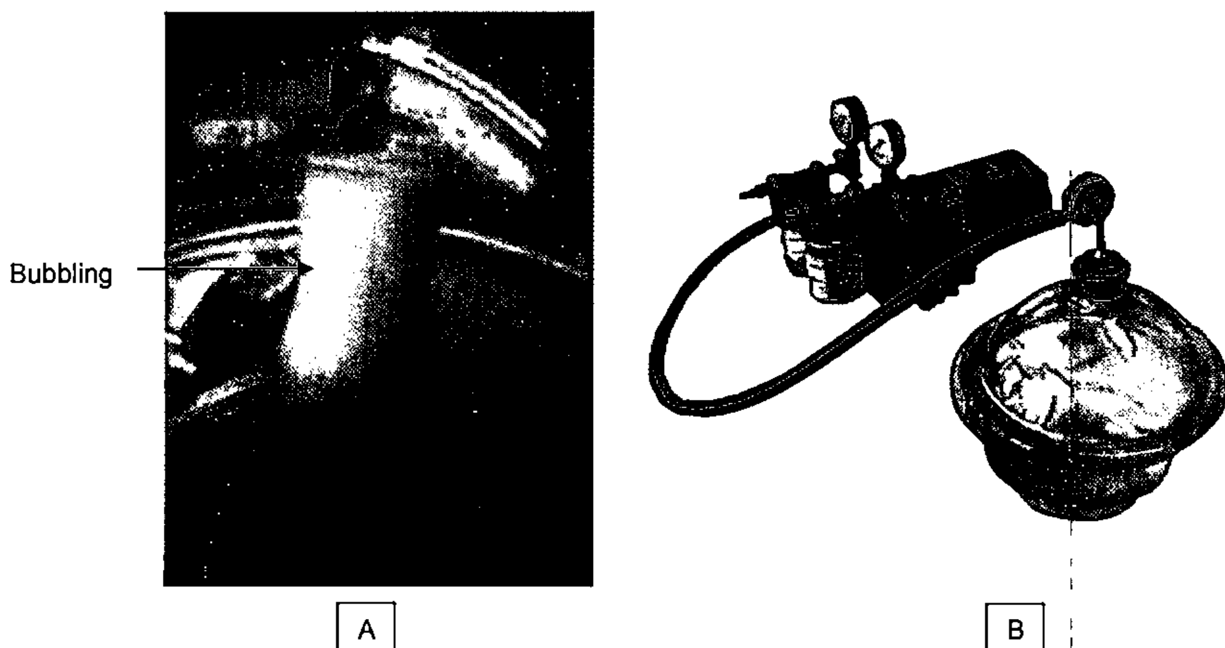


Figure 5.11 The effect of vacuum application on the milk can be seen in Figure 5.11A by the bubbling of the milk in the container. Figure 5.11B represents the equipment used to place to milk under vacuum.

5.3.5 Pasteurised and unpasteurised milk

The effect of pasteurised as well as unpasteurised milk on yogurt fermentation time and setting pH were evaluated. From results obtained (Figure 5.12) no significant difference in the pH at which the two different batches of yogurt coagulated was observed. Interestingly, pasteurised milk reached a pH of 4.6, 15 minutes before that of unpasteurised milk. These results coincide with results obtained by Parnell-Clunies et al. (1988). They reported that milk subjected to UHT treatment (140 °C for 6 seconds) or vat-pasteurisation (85 °C for 10 minutes) respectively reached pH 4.6 significantly faster than milk that was subjected to either high-temperature-short-time (HTST) (98 °C for 112 seconds) or the control milk, which received no additional treatment other than homogenisation. These individual temperature treatments not only affected the fermentation time, but also influenced the viscosity of the end product. Milk that was vat-pasteurised (which is also the method used in all our experiments) delivered yogurt with the highest viscosity, when compared to HTST, UHT and control milk batches (Parnell-Clunies et al., 1988).

During studies done by de Brabandere and de Baerdemaeker (1999), the application of heat treatment to the base milk also significantly affected yogurt fermentation time. Ultra high temperature treatment (142 °C for 10 s) resulted in a decrease in the lag phase for pH decrease.

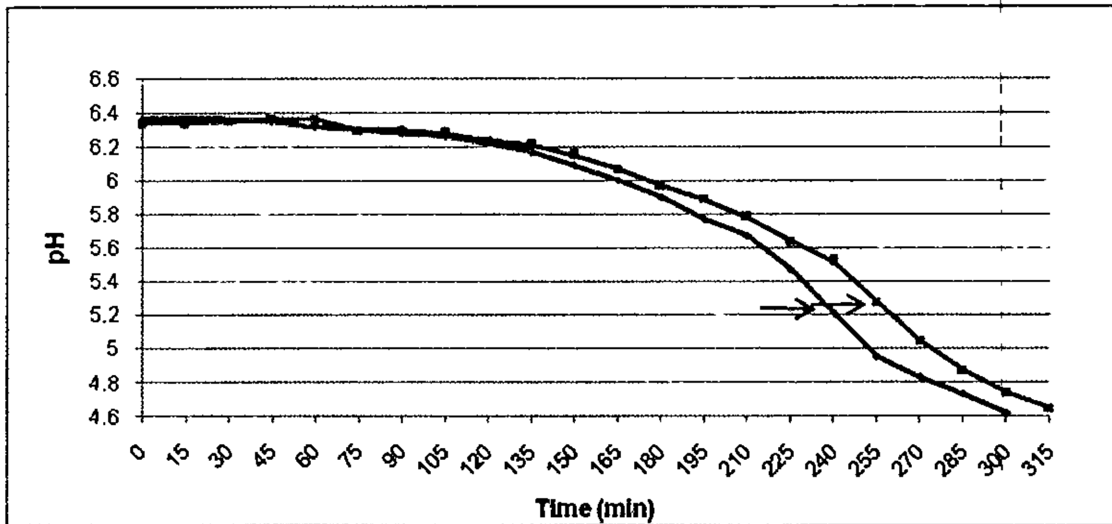


Figure 5.12 The fermentation profile of pasteurised (—◆—) and unpasteurised milk (—■—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

5.3.6 Various incubation temperatures

Yogurt fermentations were performed at 39 °C, 42 °C and 45 °C respectively (Figure 5.13). After the fermentation process had been completed, the texture of the end product was evaluated. The consistency of the yogurt fermented at 39 °C was smoother and slimier (containing more polysaccharides) than that obtained from fermentation at 42 °C and 45 °C. Cerning (1995) noted that the quantities of EPS produced in milk by different species and strains vary considerably. The amount of EPS range from 50 to 350 mg/l for *S. thermophilus* and 60 to 150 mg/l for *L. bulgaricus* (Cerning, 1995). The increased slime production observed in the yogurt fermented at 39 °C was therefore due to the increased amount of EPS produced by *S. thermophilus* at this temperature (its optimal growth temperature). Horiuchi et al. (2009) stated that the starter culture might produce more polysaccarides during low-temperature fermentation (37 °C), which results in a final product with a smoother texture. Cerning (1995) demonstrated an increased EPS production from *S. thermophilus* and *L. bulgaricus* at a fermentation temperature of 39 °C in comparison to 42 °C. Studies performed by Vercet et al. (2002) observed that longer fermentation times (as was evident with the milk incubated at 39 °C in our studies) increased the firmness of yogurt. Results obtained during our studies correlated well with the findings of all of the above research groups. No significant difference in setting pH between the three different incubation temperatures were observed.

The yogurt fermented at 45 °C decreased fermentation time by 14 % in comparison to fermentation performed at 42 °C. The increased incubation temperature however produced a product containing curd which compacted at the bottom of the flask and displayed strong syneresis. The yogurt exhibited very low slime production due to the dominance of *L. bulgaricus*, in which case less slime and more lactic acid was produced in a shorter time span (Cerning, 1995). Rajagopal and Sandine (1989) investigated the pH profiles of single strains of *L. bulgaricus* and *S. thermophilus* cultivated in pasteurised reconstituted non-fat dry milk. *L. bulgaricus* displayed increased acid production in comparison to *S. thermophilus*. In contrary, the yogurt fermented at 39 °C (the optimum growth temperature of *S. thermophilus*) contained excessive amounts of slime with a significant decrease in lactic acid production (Figure 5.13). Although fermentation performed at 39 °C resulted in a 23 % increase in fermentation time in comparison to incubation performed at 42 °C, this yogurt exhibited no syneresis. Similar results were obtained by de Brabandere and de Baerdemaeker (1999). The optimal ratio established between slime and lactic acid production was evident in the yogurt fermented at 42 °C, which proved why this temperature is used as fermentation temperature in the industrial yogurt fermentation process.

A comparable study was done by Gueguim-Kana et al. (2007), which concluded that the shortest yogurt fermentation time was achieved by initially heating milk to 45 °C for 1 hour. After this heating phase the milk was cooled down during fermentation in two steps. Firstly the temperature was decreased to 43 °C for 1 hour, then lowered to 38 °C for 1 hour. The temperature was then elevated to 41 °C until pH 4.5 was reached and fermentation was completed. Due to the complexity of this fermentation temperature profile and the possible adverse effect on the development of flavour

compounds, it is more suitable to maintain incubation temperature at a constant 42 °C throughout fermentation. This conclusion coincides with the results obtained during experiments performed in our studies.

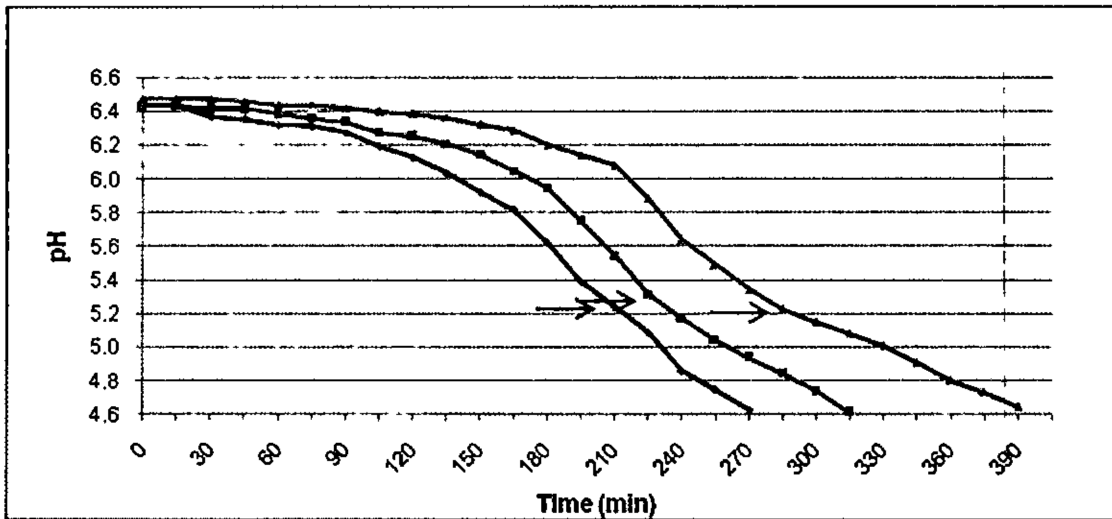


Figure 5.13 The effect of various incubation temperatures on yogurt fermentation time: 39 °C (—▲—), 42 °C (—■—) and 45 °C (—♦—). The coloured arrows represent the individual points at which the yogurt batches coagulated.

5.4 CONCLUSIONS

The solubilisation of the micellar calcium phosphate present in the casein micelle results in the destabilisation of the micelle, subsequently resulting in the coagulation of yogurt. The influences of several conditions on the yogurt fermentation process, and more specifically the stability of the casein micelle, were therefore investigated in order to attempt to increase the pH at which yogurt coagulates.

Milk treated with a proteolytic enzyme caused hydrolysis of the CMP from the casein micelle and consequently influenced the coagulation time of milk used for yogurt production. Neutrase treatment of milk resulted in a sweeter yogurt, and the yogurt coagulated at a higher pH in comparison to the control which received no enzyme treatment. A clear distinction must however be made between yogurt which coagulates at a higher pH due to enzymatic hydrolysis (which results in less lactic acid needed for coagulation) and coagulation which is directly caused by a high concentration of protease. In future studies the κ-casein hydrolised by the protease will have to be removed from the medium in order to result in yogurt coagulating at a higher pH. This will lead to a lower lactic acid requirement by the κ-casein still present on the casein micelle, which will then increase yogurt setting pH.

Due to the fact that the calcium concentration of milk pretreated with an elecrical current did not differ significantly from that of the control, it could be concluded that treating milk with 500 mA prior to yogurt fermentation did not result in calcium phosphate dissolution in the casein micelle. The insignificant impact on fermentation time as well as pH at which the yogurt coagulated can also be ascribed to this finding.

In studies done on different pressure treatments to milk prior to fermentation, the effect of pressure on the pH at which yogurt coagulated became evident. As the pressure increased from 2 MPa to 11 MPa, the setting pH of yogurt accordingly increased.

Although a homogenisation pressure of approximately 11 MPa is used in the dairy industry, it does not normally impact the yogurt setting pH, as was observed in this study. This can possibly be attributed to the introduction of a final cream separation step performed in the industry after homogenisation. The milk used in this study did not undergo cream separation. The presence of the fat globules in the milk with recombined fat globule membranes formed during homogenisation (as was the case in this study) could significantly affect the stability of the yogurt. This could result in the yogurt coagulating at a higher pH in comparison to yogurt prepared from milk from which the cream has been removed. It must however be kept in mind that the yogurt with the increased setting pH might be more susceptible to contamination than normal yogurt.

The increase in yogurt setting pH will result in a sweeter yogurt, due to the fact that less lactic acid is needed to destabilise the casein, which in turn means that more sugar remains in the medium. Less sugar therefore have to be added to the yogurt during the flavouring step, which results in an end

product with reduced calorie content. Due to consumers becoming more health conscious, such a product will be favourably accepted.

The influence of de-aerated milk on yogurt fermentation was investigated due to the controversy around the oxygenic growth requirements of the starter bacteria. Although most of the air (oxygen) present in milk was removed, results indicated no significant difference in the yogurt fermentation time nor the pH at which the yogurt coagulated when compared to the control.

Pasteurised milk decreased the yogurt fermentation time in comparison to unpasteurised milk. The fact that the South African Health Act No. 63 Regulation R185 (1977) states that raw milk and products prepared using raw milk is dangerous and should be avoided by consumers, makes the use of unpasteurised milk during yogurt fermentation impractical.

It was observed that the temperature at which yogurt fermentation takes place significantly influenced the fermentation time as well as the rheological characteristics of the end product. The incubation temperature affected the starter organisms differently, resulting in each organism delivering a unique product in accordance to their respective characteristics and optimal growth temperatures.

Submitting yogurt milk or yogurt to several physical conditions showed that changes in physical conditions influenced the pH at which yogurt coagulated. Further studies into these conditions would clearly be of great value.

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CHAPTER 6

FINAL CONCLUSIONS

The ease with which milk can be converted into a wide range of products is a very important feature from an industrial point of view.

Due to their unique properties, milk proteins are probably the most important constituents of milk, and are fundamental to the production and characteristics of yogurt (Huppertz et al., 2006).

The development of an optimised, accelerated yogurt fermentation process would enable the industry to produce higher volumes with existing infrastructure, although continuing to provide consumers with trustworthy, high quality products. As observed by Tamime (2002), while the quality of the end product can be exceptionally high, the time required to manufacture a standard industrial batch (5000 litres) of yogurt can be as long as 12 hours.

In order to accelerate yogurt fermentation the key aspect of fermentation, namely the starter culture, first had to be examined.

Two main problems concerning yogurt fermentation were identified and addressed in the first part of this study. Firstly, the variation in composition of fresh milk influenced the repeatability of yogurt fermentation experiments. This problem was overcome by substituting fresh milk with skim milk powder, which ensured repeatability.

The second problem that was encountered was the fact that commercial starter culture did not deliver consistent results when used on a laboratory scale. This was attributed to the fact that the starter culture was freeze dried with milk powder which acted as a buffer. When the starter culture was weighed, the ratio of starter culture to milk powder varied, consequently influencing yogurt fermentation time. A need therefore arose to standardise the starter culture in terms of inoculation load and growth stage. A medium which enables bacterial growth monitoring by spectrometry first had to be developed. After the suitable method was developed, this milk serum was used to monitor growth and subsequently optimise the starter culture inoculation load. Further studies determined that by preparing a pre-inoculum in which the starter bacteria can reach a specific cell load, individual yogurt batches could be inoculated with consistent inoculation loads. Not only did this deliver repeatable results, the yogurt fermentation time was also decreased by up to 17 % in comparison with starter cultures not incubated in milk serum prior to inoculation. Using this standardised starter culture meant that the effect of different supplements and different physical conditions could be evaluated without concerns of variations in starter cultures influencing results.

The development of standardised and optimised starter culture promises to have such a big influence on the dairy industry that a provisional patent was filed during 2009 (P41266ZAPO). The filing of the final patent will be performed in November 2010.

Supplementing yogurt with a wide array of vitamins, minerals, sugars, purines and amino acids were investigated in order to determine their effect on the yogurt fermentation process.

After the evaluation of the addition of several multivitamins, vitamins and sugars, it was determined that none of these supplements resulted in a decrease in fermentation time of yogurt. The only amino acid that had a positive influence on yogurt fermentation (concerning fermentation time) was glutamic acid. It could however not be determined whether the acceleration was due to the effects of the glutamic acid on the fermentation process or due to the acidity of the amino acid itself.

The effect of the main amino acids present in casein hydrolysate was evaluated individually; whereafter the influence of casein hydrolysate supplementation to yogurt fermentation was investigated. The insignificant influence of the casein hydrolisate can possibly be attributed to the fact that only isolated amino acids (glutamic acid and cysteine) affected the fermentation process when individually evaluated, whereas none of the other amino acids present in this medium had any effect.

Studies done on yogurt supplementation with yeast extract confirmed that temperatures as high as 80 °C do not influence yeast extract composition. Although the addition of yeast extract prior to the fermentation process significantly decreased fermentation time, a product with unacceptable texture and flavour was produced. This renders it unsuitable as a supplement for yogurt fermentation studies.

The fact that very few of the supplements that were evaluated resulted in the acceleration of the fermentation process indicates that milk already contains most of the components needed for optimal bacterial growth and acid production. It was clear that the starter bacteria do not need any of the evaluated supplements for sufficient growth during yogurt fermentation.

During the evaluation of the effects of several physical conditions on the yogurt fermentation process, pressure application, proteolytic treatment, de-aeration, different incubation temperatures and electrical current application to milk were investigated.

The treatment of milk with a commercial proteolytic enzyme prior to fermentation caused hydrolysis of the CMP from the casein micelle and subsequently influenced the coagulation pH of yogurt. The resulting product was sweeter and the yogurt coagulated at a higher pH in comparison to the control which received no enzymatic treatment.

Treating milk with an electrical current prior to yogurt fermentation did not significantly affect the calcium content when compared to untreated milk. The insignificant difference in fermentation time and the pH at which the yogurt coagulated could therefore be attributed to the fact that the treatment of milk prior to yogurt fermentation did not result in calcium phosphate dissolution in the casein micelle.

Individual milk batches subjected to incremental pressure treatments did not significantly influence the yogurt fermentation time, although it did influence the pH at which the individual yogurt batches coagulated. As the pressure treatments increased, the coagulation pH consequently increased. The yogurt that was produced with the milk subjected to the higher pressures was a sweeter yogurt due to less lactic acid that was produced.

This can be valuable to the industry due to the fact that less sugar needs to be added to obtain a product with the same degree of sweetness. The consumer can also benefit from this product as a result of lower calorie content. From the above trend it is clear that the effect of higher pressure treatments on the yogurt setting pH need to be evaluated.

The influence of de-aerated milk on yogurt fermentation was investigated due to the controversy that exists around the oxygenic growth requirements of the starter bacteria. Results indicated no significant difference in the yogurt fermentation time nor the pH at which the yogurt coagulated when compared to the control however.

In the case where the effect of pasteurisation on yogurt fermentation time was evaluated, it was observed that pasteurised milk decreased the yogurt fermentation time in comparison with unpasteurised milk. The South African Health Act No. 63 Regulation R185 (1977) states that in order to decrease health risks, all dairy products produced for human comsumption should however be pasteurised. This indicates that even if unpasteurised milk decreased the fermentation time, it could not be implemented in the yogurt industry except if the milk is sterilised by using methods other than heat application.

During incubation temperature studies it became evident that the incubation temperature during yogurt manufacture influences the rate of lactic acid production as well as the rheological properties of the end product. The optimal growth temperatures of the respective starter bacteria could also be clearly observed. The yogurt that was incubated at 39 °C was slimier and smoother than that obtained by 42 °C incubation, whereas incubation at 45 °C resulted in accelerated yogurt production, but it displayed syneresis and limited slime production.

6.1 FUTURE RESEARCH

The composition of the milk serum developed in this study could be determined. This will determine which components present in milk have been removed during the milk serum preparation. Due to the fact that the two starter bacteria concerned in our studies did not have difficulty growing in the serum, it can possibly be concluded that the specific components removed during preparation is not essential for starter bacteria growth.

In future studies the milk protein peptide bond (CMP) present on the κ-casein, which is hydrolised by the protease at positions Phe(105)-Met(106), will have to be removed from the medium in order to lead to yogurt coagulating at a higher pH. This will result in less acid needed by the κ-casein still present on the casein micelle, which will in turn increase the coagulation pH of yogurt.

Subjecting milk to an electrical current prior to yogurt fermentation can also be studied further. The basic technique applied in our studies can be further expanded by subjecting milk to an electrical current in a container in which the milk is constantly agitated. By applying this principle it would be possible to increase the electrical current without damaging the structure of the milk.

Studies done by Vercet et al. (2002) indicated that the application of ultrasound to milk decreased fermentation time, whereas the simultaneous application of heat and high-energy ultrasonic waves under moderate pressure increased the time to reach pH 4.6. Reduction in fermentation time by applying ultrasound prior to starter inoculation has also been observed by Mason et al. (1996). These findings can be evaluated and possibly optimised in future studies when access to applicable equipment is possible.

What is evident throughout the results obtained in this study is the fact that in the isolated cases where a significant decrease in fermentation time was observed, an uneven, unacceptable yogurt with undesirable flavour was produced. It is important to keep in mind that yogurt quality must not be compromised when accelerating the yogurt fermentation process. This problem can only be addressed if further research is performed in order to determine which of the various components in the specific supplements are responsible for the decrease in fermentation time. The influence of these individual components on the microbiological stability, flavour and texture of the yogurt, as well as the setting pH, should be included in such a study. This new data will lead to a better understanding of the complex nutritional and growth requirements of the two bacteria used as starter culture for yogurt fermentation.

Pressures exceeding those used in this study need to be evaluated. Although higher pressures will possibly increase the setting pH even more, the microbial stability (shelf life) must consequently be evaluated.

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CHAPTER 7

SUMMARY

Yogurt is one of the most popular of the dairy product range and a reduction in price would increase its popularity. If ways to accelerate the fermentation process could be found, making it possible to produce larger quantities with existing infrastructure, the affect would be reduced unit costs. This in turn should lead to a reduced shelf price.

It was therefore decided to undertake a study into identifying a way or ways to accelerate the process without affecting the quality of the final product.

Initially two main problems with the current yogurt fermentation process were identified. Firstly, the variation in the composition of fresh milk influences the repeatability of independent yogurt fermentations. This problem was eliminated by substituting fresh milk with milk powder.

Secondly, the need arose to standardise the starter culture's inoculation load and growth stage. In order to do this, a suitable growth medium which enables spectrophotometrical growth monitoring was developed. This medium is referred to as milk serum in this study. The standardisation of the pre-inoculum load enabled repeatability and resulted in a 17 % decrease in fermentation time. Thus, any variation observed in fermentation time can be attributed to the specific supplement or condition evaluated.

The development of the milk serum was an important breakthrough because it enabled the evaluation of the impact of several supplements and physical conditions on the yogurt fermentation process.

Various supplements were evaluated. They included a wide array of vitamins, minerals, sugars and amino acids. It was found that only a small number were capable of decreasing the fermentation time, and in those instances the final product was unsuitable for human consumption. This is an indication that the starter bacteria do not have a deficiency for any of those supplements when milk was used as growth medium. It could therefore be concluded that milk is an ideal and complete growth medium for the starter organisms.

After the evaluation of supplements had been completed, the effect of different conditions on the fermentation process was investigated. This included proteolytic treatment, electrical current application, pressure applications, de-aeration and different incubation temperatures.

The treatment of milk with a commercial proteolytic enzyme prior to fermentation increased the pH at which the yogurt coagulated marginally and also resulted in a sweeter product.

Milk pre-treated with an electrical current did not significantly affect the free calcium content, which indicates that the structure of the casein micelle was still intact.

Various pressure treatments of yogurt milk did not significantly influence the yogurt fermentation time. It did however influence the yogurt setting pH. As the pressure treatments increased, the setting pH subsequently also increased, which resulted in a sweeter yogurt.

The de-aeration of milk did not significantly influence the yogurt fermentation time or the setting pH.

Pasteurisation of milk resulted in the decrease of yogurt fermentation time in comparison to unpasteurised milk.

During studies done on various incubation temperatures, the optimal growth temperatures of the respective starter bacteria was clearly noticeable/visible. The individual yogurt batches obtained by incubations at 39 °C, 42 °C and 45 °C exhibited properties unique to each of the two starter bacteria. At the optimal growth temperature of *L. bulgaricus*, 45 °C, the yogurt exhibited higher lactic acid content (pH reduction), whereas the yogurt produced at 39 °C, the optimum growth temperature of *S. thermophilus*, had increased slime production. It was evident during incubation temperature studies that the incubation temperature during yogurt manufacture influences the rate of lactic acid production, as well as the rheological properties of the final product.

The results obtained in this study indicate that further research, whether it be to decrease yogurt fermentation time or to improve the product, would be of great value.

Keywords: fermentation, growth medium, pH, physical conditions, process optimisation, serum, supplements, yogurt.

OPSOMMING

Jogurt is 'n baie gewilde suiwelproduk en 'n prysverlaging sal die gewildheid verder laat toeneem. Dit kan geskied deur maniere te vind om die fermentasieproses te versnel en sodoende dit vir jogurt vervaardigers moontlik te maak om groter hoeveelhede te produseer sonder enige infrastruktuur uitbreidings. Die voordeel kan dan deurwerk tot 'n verlaagde verbruikersprys.

Daar is gevolglik besluit om 'n studie te onderneem om te bepaal of daar maniere gevind kan word om die proses te versnel maar steeds hoë kwaliteitsvlakke te handhaaf.

Aanvanklik is twee hoof probleme met die huidige jogurtfermentasieproses geïdentifiseer. Eerstens, die variasie in varsmelksamestelling beïnvloed die herhaalbaarheid van onafhanklike fermentasies. Hierdie probleem is geïllimineer deur vars melk te vervang met melkpoeier.

Tweedens het die behoefte ontstaan om die suurselkultuur se inokulasielading en groeifase te standaardiseer. 'n Gepaste groeimedium waarin groei spektrofotometries gemonitor kan word, moes egter eers ontwikkel word. Die medium word in hierdie studie beskryf as melkserum. Die standaardisering van die pre-inokulumlading het herhaalbaarheid bewerkstellig en het gelei tot 'n 17 % verbetering in fermentasietyd. Enige variasie in fermentasietyd sal dus toegeskryf word aan die spesifieke aanvulling of kondisie wat geëvalueer is.

Die ontwikkeling van die melkserum was 'n belangrike deurbraak, omdat dit die evaluering van die impak van verskillende aanvullings en fisiese kondisies op die jogurtfermentasieproses moontlik gemaak het.

Verskillende aanvullings is geëvalueer. Dit sluit in 'n wye reeks vitamiene, minerale, suikers en aminosure. Daar is gevind dat net 'n klein hoeveelheid van bogenoemde aanvullings die fermentasietyd verkort het maar in daardie gevalle was die finale produk nie geskik vir menslike gebruik nie. Hierdie is 'n bewys dat die suurselkultuur nie 'n tekort aan enige van die getoetste aanvullings het wanneer melk as groeimedium gebruik word nie. Daar kan dus aanvaar word dat melk 'n ideale en volmaakte groeimedium vir die suurselorganismes is.

Nadat die evaluering van die aanvullings voltooi is, is die effek van verskillende kondisies op die fermentasieproses getoets. Dit het proteolitiese behandeling, elektriese stroom blootstelling, druktoepassings, ontlugting en verskillende inkubasietemperature ingesluit.

Die behandeling van melk voor fermentasie met 'n kommersiële proteolitiese ensiem, het die stollings pH van die jogurt effens verhoog en het tot 'n soeter produk gelei.

Melk wat vooraf behandel is met 'n elektriese stroom se kalsiuminhoud is nie betekenisvol beïnvloed nie, wat aandui dat die struktuur van die kaseïnmisel onbeskadig is.

Verskeie drukbehandelings op melk voor fermentasie het nie die jogurtfermentasietyd betekenisvol beïnvloed nie. Die pH waarby die jogurt gestol het, is egter beïnvloed. Soos die drukbehandelings verhoog het, het die pH waarby die jogurt gestol het ook verhoog, wat tot 'n soeter produk gelei het.

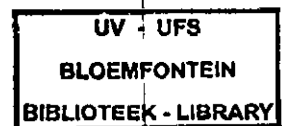
Die ontlugting van melk het geen betekenisvolle invloed op jogurtfermentasietyd of pH van stolling gehad nie.

Die gebruik van gepasteuriseerde melk het gelei tot korter fermentasietyd in vergelyking met ongepasteuriseerde melk.

Gedurende studies op verskeie inkubasietemperature was die optimale groeitemperature van die onderskeie suurselbakterië duidelik sigbaar. Die onderskeie jogurt lopies wat geproduseer is by onderskeidelik 39 °C, 42 °C en 45 °C het die unieke eienskappe van die twee onderskeie suurselbakterië vertoon. By die optimale groeitemperatuur van *L. bulgaricus*, naamlik 45 °C, het die jogurt verhoogde vlakke van melksuurproduksie gehad. Dit was in teenstelling met die jogurt wat geproduseer is by 'n fermentasietemperatuur van 39 °C, die optimale groei temperatuur van *S. thermophilus*, wat verhoogde slym produksie getoon het. Dit was duidelik tydens inkubasietemperatuurstudies dat die inkubasietemperatuur tydens jogurtfermentasie die tempo van melksuurproduksie, asook die reologiese eienskappe van die final produk, beïnvloed.

Die resultate van hierdie studie dui aan dat verdere navorsing van groot waarde sal wees, hetsy vir versnelde fermentasie of 'n verbeterde produk as einddoel.

Sleutelwoorde: aanvullings, fermentasie, fisiese kondisies, groeimedium, jogurt, optimisering, pH, proses serum.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