FRACTIONATION AND CHARACTERISATION OF A COMMERCIAL YEAST EXTRACT TO FACILITATE ACCELERATION OF YOGURT FERMENTATION

by

ESTI-ANDRINE SMITH

Submitted in accordance with the requirements for the degree of

PHILOSOPHIAE DOCTOR (FOOD SCIENCE)

in the

Faculty of Natural and Agricultural Sciences
Department of Microbial, Biochemical and Food Biotechnology
University of the Free State

Supervisor: Dr J. Myburgh
Co-supervisors: Prof G. Osthoff
Dr M. de Wit

November 2013

DECLARATION

I declare that the thesis hereby submitted for the qualification Philosophiae Doctor in Food Science 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 thesis to the University of the Free State.

Esti-Andrine Smith

November 2013

ACKNOWLEDGEMENTS

I wish to thank the following:

Dr. J. Myburgh for his support, advice, guidance, patience and friendship;

Prof. G. Osthoff for his valuable advice, support and expertise;

Dr. M. de Wit for her contribution;

Proffs. E. van Heerden and J.C. du Preez for valuable advice and troubleshooting;

The staff and students at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, for their support and the use of infrastructure;

The staff at the Division of Food Science, University of the Free State, for their help and advice;

My parents, André and Cathy Smith, for their continuous support and encouragement.
I could not have wished for better parents;

The Creator for giving me the perseverance, strength and gifts for completing this study.

TERMINOLOGY USED IN THIS STUDY

The terms “positive control” and “yeast extract supplemented milk” are used interchangeably in this study.

“Negative control” refers to yogurt containing no supplementations.

All instances where reference is made to the current study refer to this specific research study.

During all experiments discussed in this study a positive control and a negative control were used.

American Psychological Association (APA) 6th Edition Reference style was used throughout this document.

OUTCOMES OF THIS STUDY

Patents:

Myburgh, J., & Smith, E. (2012). Production of a fermented foodstuff. Application number 2012/01139. Johannesburg, RSA: DM Kisch Inc.

Publications submitted to International Journals:

Smith, E., & Myburgh, J. (2013). The optimisation of accelerated yogurt production. *Journal of Microbiology, Biotechnology and Food Sciences*.

Smith, E., Myburgh, J., Osthoff, G. & de Wit, M. (2013). Characterisation of the active component in yeast extract which decreases yogurt fermentation time. *Journal of Dairy Research.*

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

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

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

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, Montella, & Tong, 2005). A steady increase in total sales of yogurt occurred from 779 000 ton in 1999 to 1 083 000 ton in 2003 in the United States of America (Boeneke & Aryana, 2008). Yogurt is a milk product widely consumed as functional food due to its good sensory and nutritional properties, and beneficial effects on human health.

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 & Robinson, 1999). The first fermented dairy products were produced by a fortuitous combination of events. The main contributor was the ability of lactic acid bacteria (LAB) 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 LAB 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 LAB 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).

In many modern societies, yogurt constitutes a substantial proportion of total daily food consumption primarily because of its long history of proven health benefits and taste. Studies encompassing scientific parameters of yogurt have proved it as the best source of natural probiotics (Khan, Hussain, Wajid, & Rasool, 2008).

The name yogurt should be used when the milk is only fermented by *Streptococcus thermophilus* and *Lactobacillus delbruekii* spp. *bulgaricus* (Tabasco, Paarup, Janer, Peláez, & Requena, 2007). A mildly acidic taste, good digestibility, variations in taste and a high

dietetic value have significantly contributed to its increased marketplace acceptance (U.S. Patent No. 6,399,122 B2, 2002). Both historically and commercially, yogurt is the most popular product made with thermophilic cultures (Tamime & Robinson, 1999).

Inoculation, pasteurisation and flavour addition are the steps of yogurt manufacture that take the least amount of time and can not be significantly shortened. Due to the fact that the fermentation process takes the most amount of time, it is the most viable step to focus on with the aim of decreasing yogurt production time (Figure 1).

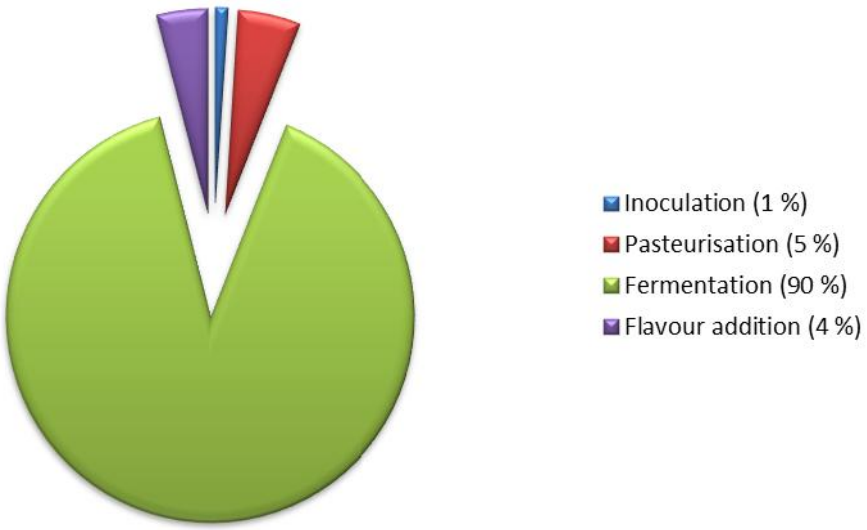


Figure 1 Time profile of yogurt production

In my M.Sc study it was hypothesised that the yogurt fermentation process can be optimised. A variety of supplements, such as vitamins, amino acids, proteins, sugars and extracts were evaluated. Only yeast extract (YE) was observed to accelerate the yogurt fermentation process in comparison to the control. The active component responsible for the shortened fermentation time, however, had to be identified, as the yogurt had an unacceptable yeast-like flavour. The aims of in the present study therefore focused on the following areas:

- Identification and characterisation of the accelerant present in YE
- Determining whether yeast YE enhances starter bacterial growth or rate of lactic acid production

1.2 MILK AND MILK CONSTITUENTS

1.2.1 PROTEINS IN MILK

Bovine milk consists of approximately 2.8 % protein (Considine, Patel, Anema, Singh, & Creamer, 2007). 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.

1.2.1.1 WHEY PROTEINS

The whey proteins are very heterogeneous; the principal 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 (Ceballos *et al*., 2009; Considine *et al*., 2007; Fox & Kelly, 2004; Huppertz, Fox, de Kruif, & Kelly, 2006; Phadungath, 2005a).

In contrast to the caseins, the whey proteins possess high levels of secondary, tertiary and, in most cases, quaternary structures. They are typical globular proteins and are denatured on heating to 90°C for 10 min. On heating, the denatured whey proteins can interact with each other and with the casein micelles. Whey proteins are not phosphorylated and are insensitive to Ca^{2+}. All whey proteins contain intra-molecular disulphide bonds that stabilise their structure (Considine *et al*., 2007; Shihata, 2004).

It has recently been shown that whey proteins and caseins contain cryptic peptides, which display various biological activities when released during digestion, for example. In addition to supplying amino acids, whey proteins have a definite biological role: the lactoglobulin fraction contains immunoglobulins G, A and M, which are present at very high levels in colostrum and play important protective roles during the first few days postnatal (Fox & Brodkorb, 2008).

1.2.1.2. CASEIN PROTEINS

Casein, the major milk protein, is not truly soluble in water. In the milk system it is dispersed in small units called casein micelles which do not settle under normal gravitational conditions. This gives milk its whiteness, since the casein micelles, containing several hundred casein molecules, are large enough to scatter light. The only known function of caseins is nutritional, i.e. to supply amino acids, calcium and phosphate. The majority of the

casein protein present in milk occurs with an internal disulfide bond between cysteine residues 36 and 40 forming a small loop in the structure (Farrell, Brown, Hoagland, & Malin, 2003; Farrell *et al*., 2004). The conformation of caseins is much like that of denatured globular proteins. The high number of proline residues in caseins causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures. The lack of tertiary structure accounts for the stability of caseins against heat denaturation because there is very little structure to unfold. Without a tertiary structure there is considerable exposure of hydrophobic residues. This results in strong association reactions of the caseins and renders them insoluble in water (Fox & Brodkorb, 2008; Shihata, 2004).

The mechanisms of yogurt production rely mainly on the casein micelle, which is very stable in milk. 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).

In fresh milk, the caseins are present in the form of essentially spherical particles containing many protein molecules and amorphous calcium phosphate. These particles average 150 nm in diameter with sizes ranging from 15 to 1000 nm in diameter (De Kruif & Holt, 2003; Farrell *et al*., 2003; Farrell *et al*., 2004). 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 1 (Fox & Kelly, 2004; Horne, 2002; Huppertz *et al*., 2006; Phadungath, 2005a; Singh & Bennett, 2002). The α_{s1}- and α_{s2}-caseins are situated in the core region of the micelle. The α_{s1}-casein, which is the least compact molecule of all the casein molecules, is a major component of the total caseins and has the largest net negative charge in neutral pH buffer with only monovalent cations present. The α_{s2}-casein, in contrast, is a minor component and is the least hydrophobic casein component that is the most highly and variably phosphorylated of the caseins. β-casein, a major casein component, is the most hydrophobic of the intact caseins and has the largest regions of high hydrophobicity. It 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 (De Kruif & Holt, 2003; Farrell *et al*., 2003; Singh & Bennett, 2002).

κ-casein constitute 10-12 % of the entire casein and 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 distinctly different regions of the κ-casein,

the N-terminal and the C-terminal. These two regions can be separated by cleavage of a bond that is hydrolysed by an acid protease at a neutral pH (De Kruif & Holt, 2003; Farrell *et al*., 2003).

The N-terminal carries a net positive charge, is very hydrophobic and interacts strongly with the other casein molecules. The C-terminal carries a net negative charge and is highly hydrophilic. The bond between the two regions is a peptide bond that carries a net positive charge. It is well conserved in most species and is well recognised by chymosin that readily and specifically cleaves the Phe 105-Ser 106 bond resulting in two peptides: a largely positively charged hydrophobic peptide referred to as para-κ-casein (residues 1-105) and a smaller hydrophilic casein macropeptide referred to as CMP (residues 106-169) (Dalgleish, 2004; De Kruif & Holt, 2003; Farrell *et al*., 2003; Singh & Bennett, 2002).

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 CMP to protrude from the surface into the surrounding solution and exists as a flexible hair-like structure. This hydrophilic moiety interacts with the solvent to stabilise the micelles. The phosphate groups of the casein micelles bind large amounts of calcium. The so-called colloidal calcium phosphate (CCP) is an important stabilising factor and is responsible for holding the network together by crosslinking the caseins, which is important in regard to the structure of the micelles.

Variations in pH also have a strong influence on casein hydrolysis. 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, de Valdez, Holgado, & Oliver, 1985).

Table 1 Protein fraction (g/100 g protein) of cow's milk (n = 30) (Ceballos *et al*., 2009; Considine *et al*., 2007; Fox & Kelly, 2004; Shihata, 2004).

Protein	% of total protein
Casein (Cn)	**82.65**
α_{S1}-Cn	30.80
α_{S2}-Cn	7.50
β-Cn	33.25
κ-Cn	11.1
Whey proteins	**17.35**
α-lactalbumin	3.47
β-lactoglobulin	10.41
Other minor proteins	3.47

1.2.2 ENZYMES 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 & 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 85- 95 °C for 6-10 min (Fox & Kelly, 2006).

1.2.2.1 INDIGENOUS ENZYMES

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, Santisteban, Virto, & de Renobales,

1998; Deeth & Fitz-Gerald, 2006). This enzyme is therefore deactivated during the pasteurisation process.

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 & Kelly, 2006). The κ-caseins in milk are resistant to proteolysis by plasmin (Silanikove, Merin, & Leitner, 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 & Kelly, 2006).

Lactoperoxidase

The lactoperoxidase system is a natural antimicrobial system present in milks from many species. The enzyme lactoperoxidase catalyses 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 µg/g. Hydrogen peroxide could be generated in milk by leucocytes and by LAB (Chavarri *et al*., 1998; Nakada, Dosako, Hirano, Oooka, & Nakajima, 1996). In studies performed by Barrett, Grandison and Lewis (1999) it was found that the keeping quality of milk pasteurised at 72 °C for 15 seconds was found to be better than that of milk heated at 80 °C for 15 seconds. This can be ascribed to the fact that lactoperoxidase is completely deactivated at the higher temperature, whereas residual lactoperoxidase activity was found to be 70 % in milk pasteurised at 72 °C for 15 seconds. Higher levels of hypothiocyanite (the major antimicrobial agent produced by the lactoperoxidase system) were also detected in milk processed at 72 °C than at 80 °C, which supports the theory that the lactoperoxidase system has a role in the keeping quality of pasteurised milk (Barrett *et al*., 1999). Milk used for yogurt fermentation which is subjected to pasteurisation temperatures of 90-95 °C for 5-10 min therefore contains no lactoperoxidase.

1.2.2.2 Microbial enzymes (Exogenous enzymes)

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. Yogurt is often made from milk that, at some point, had been at risk of proteolytic degradation (Cousin, 1982; Gassem & 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 & 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 & 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 & 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 that milk that was precultured with psychrotrophic spoilage bacteria took 25-30 min less to reach pH 4.25 when compared to the control that was not treated with protease.

Adams, Barach and Speck (1976) 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, while 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*., 1976; Liu & Guo, 2008).

Beta-Galactosidase

β-Galactosidase, also known as lactase, is an enzyme that 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 (Haider & Husain, 2009; Ouwehand, Salminen, & Isolauri, 2002; Tari, Ustok, & Harsa, 2009). During personal communication on November 19, 2013, Doctor Jacobus Myburgh stated that during the setting process of yogurt, a gel matrix is formed wherein the various components of yogurt are entrapped, resulting in the constraining of lactose movement. This shielding effect consequently limits lactose absorption in the body, preventing the onset of lactose intolerance symptoms.

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 *Lactobacillus bulgaricus* cultivated in skim milk yielded the highest enzyme activity compared to cultivation in dried whey powder and whey powder supplemented with YE 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 & Jelen, 2001). Studies done by Hickey, Hillier and Jago (1986) on several *Lactobacillus 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 maintain the pH of the buffered medium at pH 5.6±0.1 (Vasiljevic & Jelen, 2001).

1.3 STARTER BACTERIA USED FOR YOGURT MANUFACTURE

1.3.1 BACKGROUND

The microorganisms employed in milk fermentation are single strain or multiple strain cultures of lactic acid bacteria, producing different types of fermented milk products. Usually, one or two strains dominate the milk environment. In the case of yogurt fermentation, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* are the two main organisms used. Both these organisms are thermophilic starter cultures (Surono & Hosono, 2011). Starter cultures are so called because they 'start' the production of lactic acid from lactose, which occurs in the early phase of manufacture of fermented milks and cheeses (Tamime, 2005). 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 & Tehrani, 2007).

The two most obvious beneficial roles of lactobacilli are to produce acid rapidly and as probiotics (Tamime, 2005). 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, Simova, & Beshkova, 2006). This will be covered in greater detail in Section 1.3.6.

Streptococcus thermophilus shares many phenotypic and genetic properties with the other lactic acid bacteria, although it does not fit conveniently into any systematic grouping. It is, for example, fairly closely related by DNA hybridisation to *Streptococcus salivarius*, a common oral bacterium. This finding led to the reclassification of the organism as a new subspecies, *Streptococcus salivarius* ssp. *thermophilus*. It has recently been returned to the species level again following a more detailed DNA hybridisation study (Tagg, Wescombe, & Burton, 2011).

1.3.2 CHARACTERISTICS OF STARTER CULTURES

Streptococcus thermophilus is a thermophilic Gram-positive bacterium that is a natural inhabitant of raw milk. It occurs in long chains of 10-20 cells, and ferments lactose homofermentatively to give L(+) lactic acid as the principle product. Glucose, fructose and mannose can also be metabolised, but the fermentation of galactose, mannose and sucrose is strain specific (Robinson, Tamime, & Wszolek, 2004).

The growth and metabolism of *Streptococcus 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 *Streptococcus thermophilus*, *Lactobacillus bulgaricus* is also Gram-positive and both are facultative anaerobic bacteria. *Lactobacillus bulgaricus* occur in milk as chains of 3-4 short rods with rounded ends. Its basic metabolism is also homofermentative, although the end product is D(-) lactic acid. Lactobacilli ferment hexoses almost entirely to lactic acid, while pentoses or gluconate is not fermented. This form of lactic acid is less readily metabolised by humans than the L(+) isomer. In addition to lactose, fructose, glucose and, in some strains, galactose, can all be utilised by *Lactobacillus bulgaricus*. The tolerance of *Lactobacillus bulgaricus* to acidity also contrasts dramatically with that of *Streptococcus thermophilus*, and the amount of lactic acid produced by *Lactobacillus bulgaricus*, the more acid-tolerant of the two bacteria, can range up to 18 g per kg of yogurt (Cais-Sokolinska, Michalski, & Pikul, 2004; Tamime, 2005; Tari *et al*., 2009) which indicates that *Lactobacillus bulgaricus* has an increased acid producing ability in comparison to *Streptococcus thermophilus.*

Acid tolerance is an important property of lactic acid bacteria (LAB) in a food environment because the sugar concentration in milk is sufficient to decrease the pH below 4.5. In several LAB species, unlike most other cells, the intracellular pH decreases concurrently with the decrease of extracellular pH. The decrease in acid sensitive bacteria like *Streptococcus thermophilus* is slower than that in acid tolerant lactobacilli for example *Lactobacillus bulgaricus* (Adamberg, 2003).

Streptococcus genera *Streptococcus pneumonia, -pyogenes, -salivarius, -agalactiae and -vestibularis* are all pathogenic, whereas *Streptococcus thermophilus* is not virulent. It 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 species therefore has Generally Regarded as Safe (GRAS) status. The interrelationship of the growth of the *Streptococcus* and *Lactobacillus* species is so intimate that through the years they have exchanged genes which enabled *Streptococcus thermophilus* to hydrolise lactose as is the case for *Lactobacillus bulgaricus* (Bolotin *et al*., 2004; Delorme, 2007; Iyer, Tomar, Maheswari, & Singh, 2010; Stiles & Holzapfel, 1997).

1.3.3 ROLE OF STARTER CULTURES IN ACID PRODUCTION

The most important factor concerning the role of LAB is the acidifying ability of the organisms (Quiberoni *et al*., 2003). The optimal growth temperature of *Streptococcus thermophilus* ranges from 35-42 °C and that of *Lactobacillus bulgaricus* is between 43 and 46 °C. The incubation temperature of 42 °C is a compromise between the optimum temperatures of the two organisms (Gueguim-Kana, Oloke, Lateef, & Zebaze-Kana, 2007; Radke-Mitchell & Sandine, 1986). 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 *Streptococcus thermophilus* species. An increase in incubation temperature from 37-45 °C leads to an increase in proteolytic activity of *Lactobacillus bulgaricus* (Radke-Mitchell & Sandine, 1986).

1.3.4 STARTER BACTERIA PROTO-COOPERATION AND PROTEOLYTIC ABILITY

The synergism that exists between the two starter bacteria, termed proto-cooperation, is evident in their growth in yogurt milk. The symbiotic relationship between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* has long been used in the manufacture of fermented milks and various Swiss and Italian cheeses. Compatible strains have been consistently observed to result in an increase in acidifying activity, product yield and flavour production (Bouzar, Cerning, & Desmazeaud, 1997; Radke-Mitchell & Sandine, 1984; Robinson *et al*., 2004; Sodini, Latrille, & Corrieu, 2000; Tari *et al*., 2009).

The growth association that exists between *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, specifically, enhances lactic acid production in comparison to the two organisms used individually (Rajagopal & Sandine, 1990; Robinson *et al*., 2004; Tamime, 2002; Tari *et al*., 2009). While the combined culture may generate an acidity of more than 10 g/l in 4 hours, the values in the individual cultures could be around 4 g/l for *Streptococcus thermophilus* and 2 g/l for *Lactobacillus bulgaricus*. Initially *Streptococcus thermophilus*

grows more rapidly in milk than *Lactobacillus bulgaricus* and releases more lactic acid. Most *Streptococcus thermophilus* strains produce CO_2 from milk urea by ureases. In this way, it can stimulate aspartate biosynthesis by lactobacilli, since the quantity of CO_2 dissolved in milk decreases after heat treatment and so remaining CO_2 is too low to meet the requirements of *Lactobacillus.* This developing acidity provides an environment that is conducive to the growth and metabolism of *Lactobacillus bulgaricus* so that, at the end of the 4 hours, *Lactobacillus bulgaricus* will be releasing more lactic acid than *Streptococcus thermophilus* (Robinson *et al*., 2004; Shihata, 2004). The optimum ratio that must be maintained between the two starter organisms is *Lactobacillus bulgaricus:Streptococcus thermophilus* as 1:1 (Cais-Sokolinska *et al*., 2004; Gueguim-Kana *et al*., 2007; Penna, Baruffaldi, & Oliveira, 1997). *Lactobacillus bulgaricus* is not easily preserved 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, 2002).

LAB possess proteolytic enzymes that are found in the cell wall, the cell membrane, and the cytoplasm, but only about 2 % of casein is broken down during milk fermentation. The proteinases are present outside the cell, whereas most, if not all, peptidases are found in the cytoplasm (Shihata, 2004).

The proteolytic system of *Streptococcus thermophilus* comprises of more than 20 proteolytic enzymes, and is composed of i) an extracellular cell-anchored protease capable of casein hydrolysis, ii) a set of amino acid and peptide transport systems required for the import of amino acids and iii) a set of intracellular peptidases involved in the hydrolysis of casein-derived peptides for various house-keeping processes. The cell wall-anchored proteases are reported to be present only in a minority of *Streptococcus thermophilus* strains studied to date, so the amino acid requirement of *Streptococcus thermophilus* is satisfied by the cooperation with other bacterial species growing in association in the dairy environment, such as *Lactobacillus bulgaricus.* The main components of the proteolytic system of lactobacilli are cell envelope-associated proteinases, amino acid and peptide transport systems, and a range of intracellular peptidases (Tamime, 2005). Studies done by Rajagopal and Sandine (1990) indicated that lactobacilli are highly proteolytic (61.0 to 144.6 µg of tyrosine/ml of milk) and *Streptococcus thermophilus* are less proteolytic (2.4 to 14.8 µg of tyrosine/ml of milk).

LAB used during yogurt fermentation are characterised by their high demand for essential growth factors such as peptides and amino acids. However, milk does not contain sufficient free amino acids and peptides to allow growth of LAB. Therefore, these LAB possess a

complex system of proteinases and peptidases, which enable them to use milk casein as a source of amino acids and nitrogen (Shihata, 2004). Optimal growth of *Streptococcus thermophilus* in milk requires hydrolysis of caseins, internalisation and hydrolysis of the resulting peptides; de novo amino acid synthesis, or depends on the utilisation of exogenous amino acids (Abraham, Antoni, & Anon, 1993; Iyer *et al*., 2010; Juille, Le Bars, & Juillard, 2005). Despite its protein-rich environment, *Streptococcus thermophilus* displays limited proteolytic ability. Its source of nitrogen, at least initially, is therefore limited to the free amino acids present in milk or that have been released during pasteurisation (Rajagopal & Sandine, 1990). The amino acids required by *Streptococcus thermophilus* include glutamic acid, histidine, methionine, cysteine, valine, leucine, isoleucine, tryptophan, arginine and tyrosine (Shihata, 2004). Studies done by Robinson (2001) indicated that some amino acids such as glutamic acid, histidine, cysteine, methionine, valine or leucine, are not present in milk at levels sufficient to support the essential growth of *Streptococcus thermophilus*. Some *Streptococcus thermophilus* strains appear to be auxotrophic for at least two amino acids, cysteine and histidine (Pastink *et al*., 2009). Consequently, the increase in cell numbers depends on the absorption of short-chain peptides released during protein hydrolysis by *Lactobacilllus bulgaricus* or by heating milk for yogurt manufacture suffiently to hydrolyse whey proteins (Mullan, 2001; Tamime, 2002; Tamime & Robinson, 1999).

Unlike *Streptococcus thermophilus,* which has limited proteolytic ability, *Lactobacilllus bulgaricus* possesses a wall-bound proteinase which is regulated by temperature and growth phase. This cell-bound proteinase has an optimum activity between 45 and 50 °C and pH values ranging from 5.2 to 5.8. The proteinase of *Lactobacilllus bulgaricus* is more active on caseins, specifically β-casein, than on whey proteins and degrades the proteins to peptides and subsequently amino acids (Kang, Vezinz, Laberge, & Simard, 1998; Tari *et al*., 2009). Studies by several researchers revealed a preferential but partial hydrolysis of β- and α_{s1}-caseins. It was also observed that after growth in milk, caseinolytic activity was 3 times higher than that measured after growth in a complex medium rich in short peptides (Shihata, 2004).

The function of cell wall-bound peptidases is to hydrolyse larger peptides into units no larger than four amino acids which can be transported through the cell membrane. Cytoplasmic peptidases subsequently hydrolyse the transported peptides (Shihata, 2004).

The proteinase of *Lactobacilllus bulgaricus* hydrolises casein to release peptides and polypeptides which then stimulate the growth of *Streptococcus thermophilus.* Peptidase activity in turn is limited in *Lactobacilllus bulgaricus*, and due to the presence of

Streptococcus thermophilus which can easily hydrolise peptides to free amino acids, the free amino acids are available for *Lactobacilllus bulgaricus* to utilise (Abraham *et al*., 1993; Kang *et al*., 1998; Robinson *et al*., 2004; Shihata, 2004). The growth of *Lactobacilllus bulgaricus,* which provides flavour compounds, is further enhanced by formic acid which is produced by *Streptococcus thermophilus* (Figure 2) (Stiles & Holzapfel, 1997; Tari *et al*., 2009).

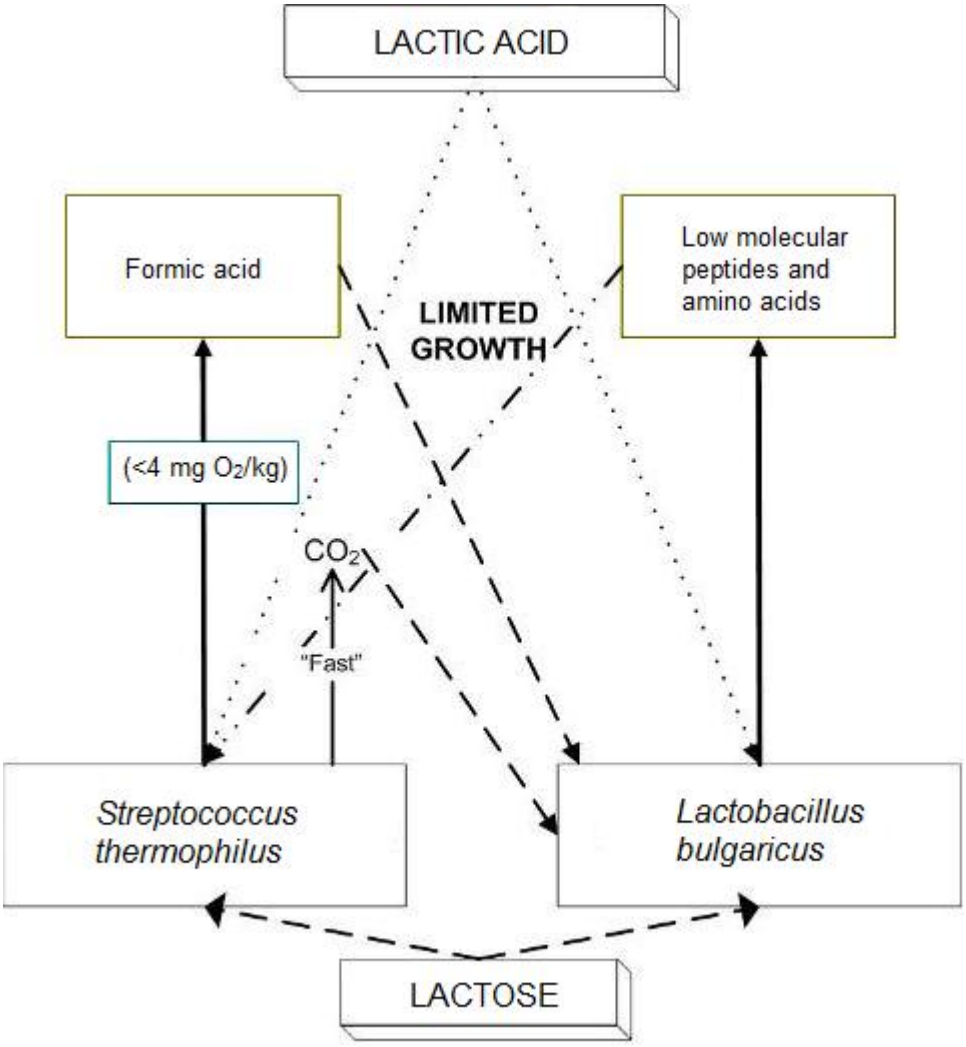


Figure 2 Scheme for proto-cooperation of yogurt starter culture (Angelov, Kostov, Simova, Beshkova, & Koprinkova-Hristova, 2009).

1.3.5 FOLATE PRODUCTION

Milk is a well-known source of folate. Reviews on microbiological assays report folate levels in cows' milk in the range of 5-7 µg/100 g (Crittenden, Martinez, & Playne, 2003; Iyer *et al*., 2010). Fermented milk products are reported to contain even higher amounts of folate due to the production of additional folate by bacteria during fermentation. *Streptococcus*

thermophilus has a strain-specific ability for folate production and has been reported to produce higher quantities compared with other lactic acid bacteria. The majority of this folate is excreted into milk. *Streptococcus thermophilus* was therefore found to be responsible for about a six-fold increase in the folate content of milk during fermentation. Great differences have however been observed in the folate production ability of different strains. The reason for this may be that some strains retain all the folate intracellularly, while with other strains almost complete excretion or leakage of the folate is observed. Folate production by bacteria is therefore highly dependent on the composition of the medium and the conditions in which the strains grow, i.e. temperature, oxygen, pH and incubation time (Crittenden, Martinez, & Playne, 2003; Iyer *et al*., 2010; Iyer, Tomar, Singh, & Sharma, 2009).

1.3.6 LACTOSE UTILISATION

1.3.6.2 LACTOSE TRANSPORT

LAB 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).

Lactobacilllus 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 phosphotransferasesystem starts hydrolising the sugars while they are being transported into the cell. Most *Streptococcus 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. *Streptococcus thermophilus* only ferments the glucose moiety of lactose and releases unmetabolised galactose into the medium (Farkye & Vedamuthu, 2002; Hickey *et al*., 1986).

In studies done by Hickey *et al*. (1986) it was found that *Lactobacilllus 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).

1.3.6.1 FERMENTATION OF LACTOSE

Lactose, a reducing disaccharide, is composed of galactose and glucose linked by a β 1-4 glycosidic bond. It is unique to milk and is the principal sugar in milk of most mammals. The first step in the fermentation of yogurt is the active transport of lactose across the cell membranes of the starter bacteria. 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 *Streptococcus thermophilus* and *Lactobacilllus bulgaricus* by the use of a proton motive force (PMF). Once inside the cell, the lactose is hydrolysed to glucose and galactose by the enzyme β-galactosidase. This enzyme has a maximal activity at pH 7.0 and is virtually inactive at pH ≤ 4.0. Under conditions of excess glucose and limited oxygen, homolactic LAB catabolise one mole of glucose via the Embden-Meyerhof-Parnas (EMP) Pathway to yield two moles of pyruvate. Lactic dehydrogenase converts the pyruvate to the end product, namely lactic acid. The lactic acid is released extra cellularly into the cultivation medium. Overall, 1 mol of lactose is fermented to 2 mol of lactic acid plus 1 mol of galactose. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles of ATP per mole of glucose consumed (Cogan & Hill, 2004; Fernandez-Garcia, 1994; Fox & Brodkorb, 2008; Kotz, Furne, Savaiano, & Levitt, 1994; Matalon & Sandine, 1986; Robinson *et al*., 2004).

Lactobacilllus bulgaricus does not use galactose but catabolises the glucose moiety of lactose. Enzymes responsible for glucose fermentation are thus present in *Lactobacilllus bulgaricus* (Chervaux, Ehrlich, & Maguin, 2000). Some *Streptococcus thermophilus* strains will use a small percentage of the excreted galactose, while others will not. Although galactose and lactic acid are transported out of the cell and accumulate in the medium, some strains of *Streptococcus thermophilus* and *Lactobacilllus 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 further metabolised to lactic acid as the final product (Hickey *et al*., 1986; Robinson *et al*., 2004). This pathway is illustrated in Figure 3.

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 (Cogan & Hill, 2004; Fernandez-Garcia, 1994; Robinson *et al*., 2004).

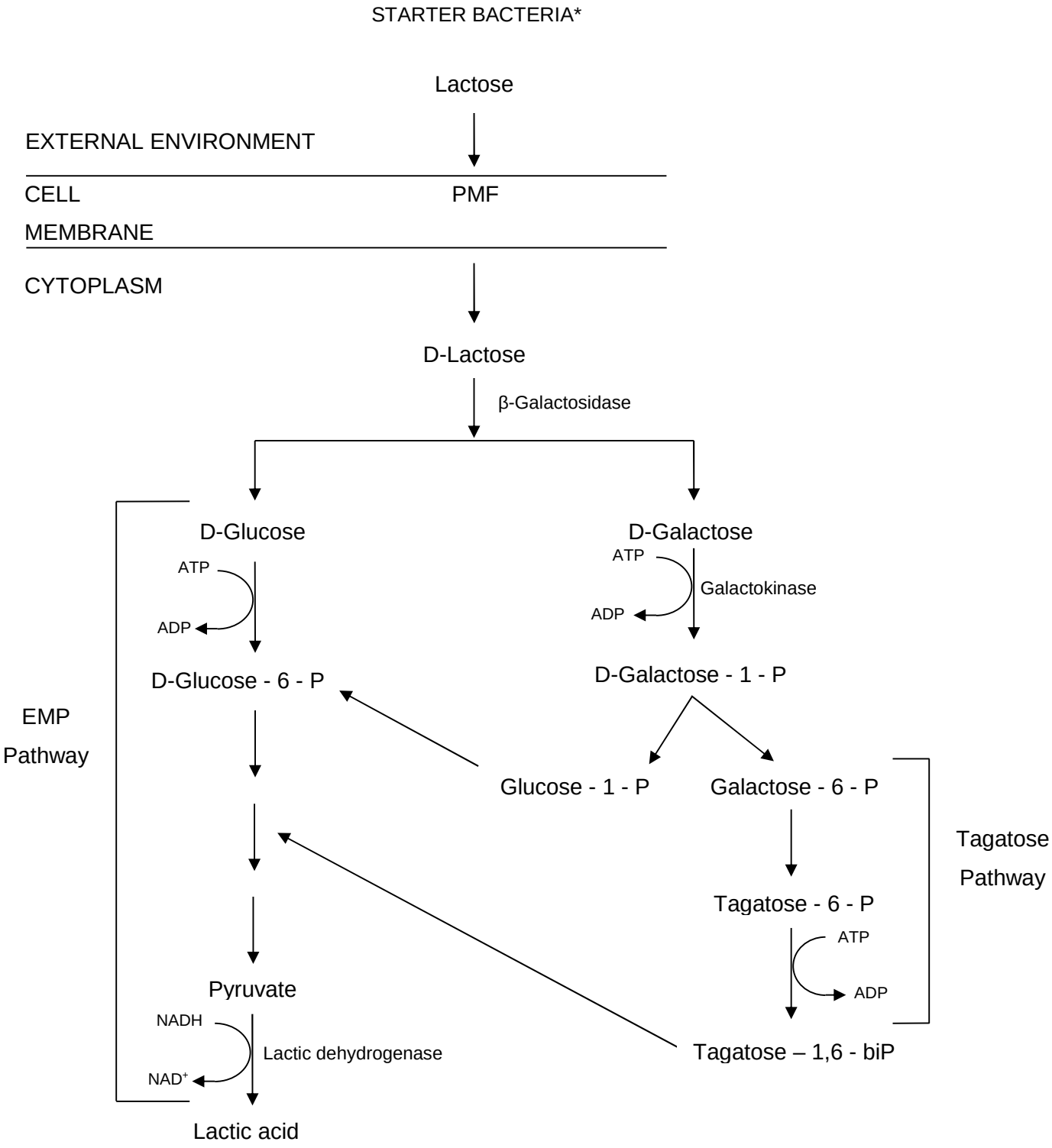


Figure 3 Sugar metabolism by starter cultures via the Embden-Meyerhof-Parnas (EMP) pathway. Adapted from Cogan and Hill (2004) and Robinson *et al*. (2004). * *Streptococcus thermophilus, Lactobacilllus bulgaricus* and *Bifidobacterium lactis.*

1.4 YOGURT MANUFACTURING PROCESS

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 standardised, the milk is homogenised. Homogenisation of the milk base is an important processing step for yogurts containing fat. When milk is homogenised, caseins and whey proteins form the new surface layer of fat globules, which increases the number of possible structure-building components in yogurt made from homogenised milk (Lee & Lucey, 2010).

1.4.1 TRADITIONAL AND IMPROVED YOGURT PROCESSES

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 & Robinson, 1999). These methods are depicted in Figures 4 and 5.

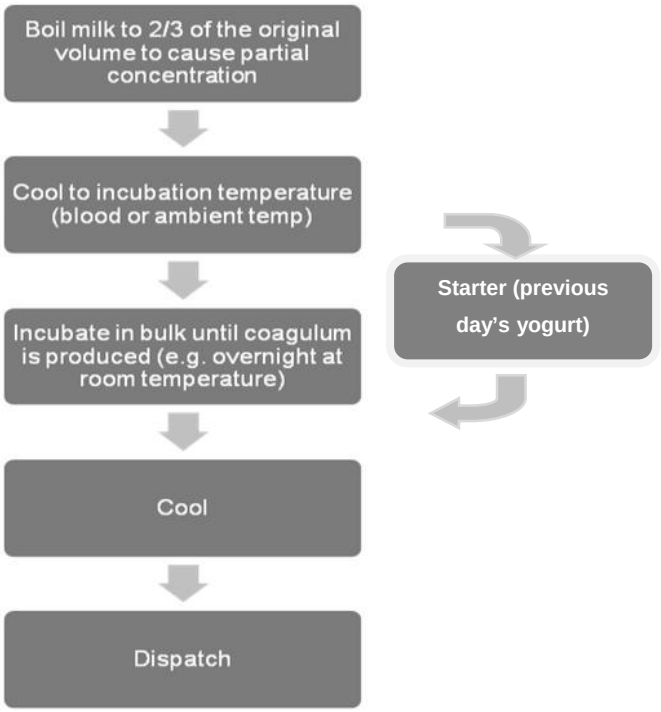


Figure 4 The traditional process of yogurt making (Tamime & Robinson, 1999).

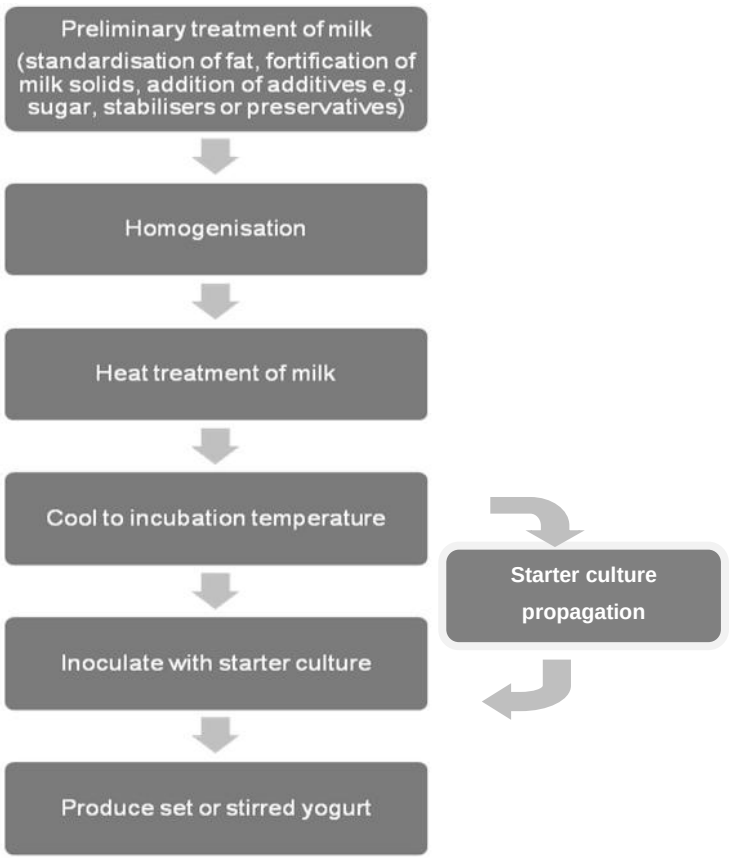


Figure 5 The improved process of yogurt making (Tamime & Robinson, 1999).

Tamime and Robinson (1999) 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 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

High quality yogurts must have particular attributes. The pH must be between 4.2 and 4.3 at 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, Barefoot, & Grinstead, 1997). Regarding viability, the

norm specifies that the sum of microorganisms constituting the starter culture should be at least 10^7 cfu/g, and the minimum counts of other labelled microorganisms should be 10^6 cfu/g (Tabasco *et al*., 2007).

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 & Tehrani, 2007). Yogurt contains at least 8.25 % non-fat solids and has a titratable acidity of not less than 0.9 % expressed as lactic acid (Lee & Lucey, 2010; Weinbrenner *et al*., 1997). The acidification process during yogurt fermentation can be monitored by pH measurement (de Brabandere & de Baerdemaeker, 1999). Fermentation proceeds until 20-30% of the lactose in the milk has been converted to lactic acid and a pH value below 4.6 is reached, 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 (Shihata, 2004; U.S. Patent No. 6,399,122 B2, 2002).

Codex regulations for yogurt indicate that the minimum milk protein content is 2.7% (except for concentrated yogurt where the minimum protein content is 5.6% after concentration) and the maximum fat content is 15 % (Codex standard for fermented milk, 2008). The total solids content of milk can be increased by concentration processes such as evaporation under vacuum and membrane processing (i.e. reverse osmosis and ultrafiltration). Stabilisers such as pectin or gelatine are often added to the milk base to enhance or maintain the appropriate yogurt properties including texture, mouthfeel, appearance, viscosity as well as to prevent whey separation. The use of stabilisers may help in providing a more uniform consistency and minimise batch to batch variation. However, there can be textural defects related to the use of stabilisers, including over-stabilisation and under-stabilisation. Over-stabilisation results in a “jelly-like” springy body of yogurt while a weak “runny” body or whey separation can be produced due to under-stabilisation (Lee & Lucey, 2010).

Gel formation is the most important functional property of fermented milk products. The physical and textural characteristics of this composite gel are governed by milk composition, dry matter content, type and quantity of the starter culture that is used to inoculate the milk, fermentation temperature, and the storage conditions of the final product. In addition, the consistency and water-holding capacity of acidified milk gels are strongly related to the quality of fermented milk products (Malbaša, Vitas, Lončar, & Milanović, 2012).

1.4.1.1 SET AND STIRRED YOGURT

Two popular yogurt products are set yogurt and stirred yogurt (Figure 6). Set yogurt, which includes fruit-on-the-bottom, 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 after which 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 (Lee & Lucey, 2010; Rawson & Marshall, 1997; Renan *et al*., 2009).

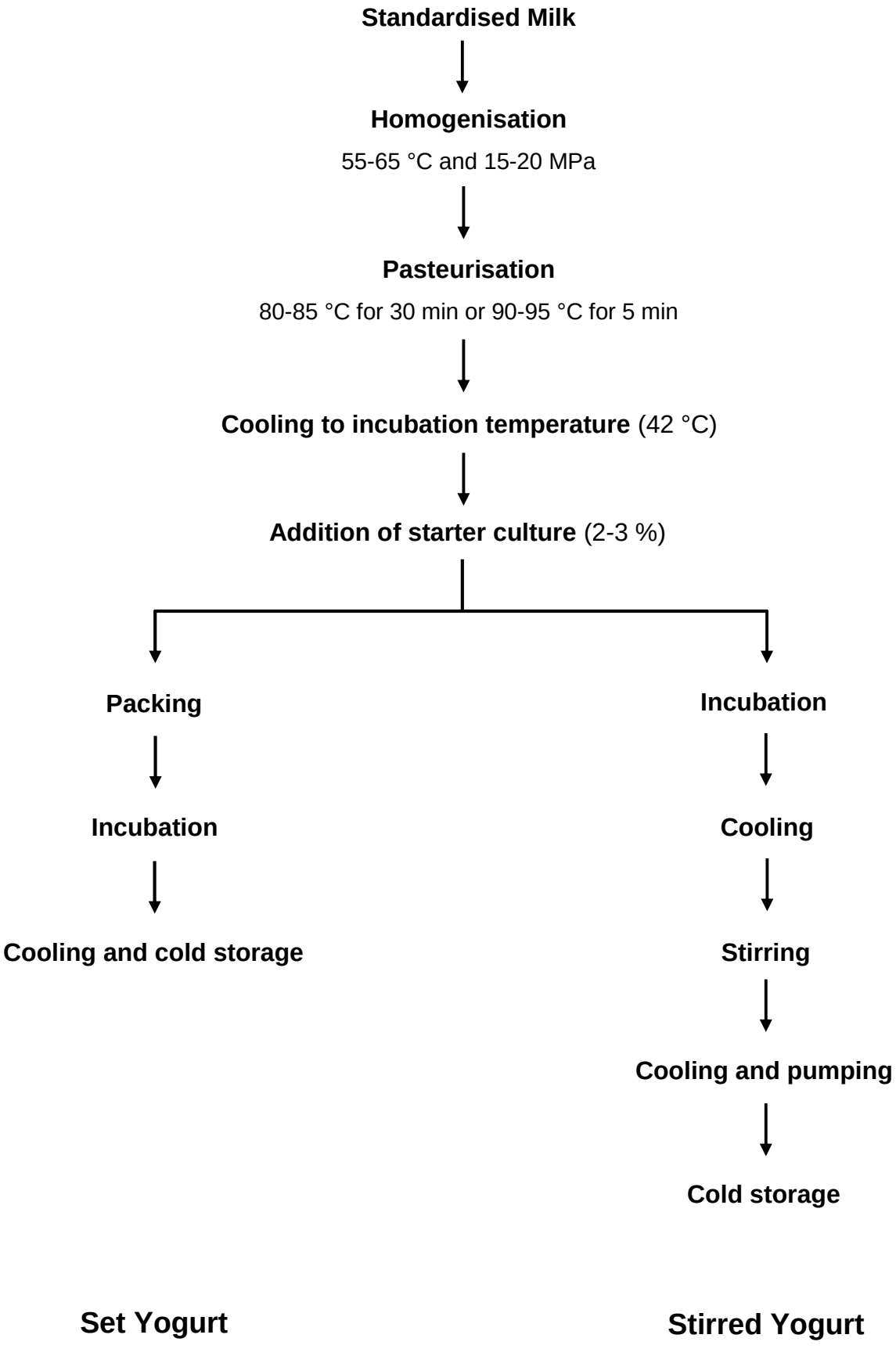


Figure 6 Main processing steps in the manufacture of set and stirred yogurt (Lee & Lucey, 2010).

1.4.2 Flavour and texture components in yogurt

1.4.2.1 Acetaldehyde

Acetaldehyde is the most important flavour compound which is formed during yogurt fermentation and is also considered the main indicator of flavour. Increased incubation time enables the yogurt micro-organisms to produce more acetaldehyde which enhances the flavour of the product (Gardini, Lanciotti, Guerzoni, & Torriani, 1999; Ozer & Atasoy, 2002; Robinson *et al*., 2004). In mixed cultures *Lactobacilllus bulgaricus* is the most prominent in synthesising acetaldehyde. Although *Streptococcus thermophilus* also produces this flavour component, it is to a much lesser extent. Acetaldehyde at levels up to 40 mg/kg is the major component of the flavour profile, and the major pathway for production by *Lactobacilllus bulgaricus*, and to a lesser extent *Streptococcus thermophilus*, is the conversion of threonine to glycine by threonine aldolase (Iyer *et al*., 2010; Robinson *et al*., 2004).

Acetaldehyde can also be produced directly from lactose metabolism as a result of decarboxylation of pyruvate (Iyer *et al*., 2010).

In some lactic acid bacteria, such as *Lactobacilllus acidophilus*, the enzyme alcohol dehydrogenase reduces the acetaldehyde to alcohol, but as neither *Streptococcus thermophilus* nor *Lactobacilllus bulgaricus* possess this enzyme, the acetaldehyde accumulates to a level dependent upon the strain involved. The activity of threonine aldolase produced by *Streptococcus thermophilus* decreases significantly as the temperature of incubation is raised above 30 °C, while the comparable enzyme in *Lactobacilllus bulgaricus* is unaffected. *Lactobacilllus bulgaricus* is therefore likely to be the main source of acetaldehyde in commercial yogurt (Robinson *et al*., 2004; Tamime, 2002).

When *Lactobacilllus 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 & Hernandez, 2007).

1.4.2.2 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 (Guzel-Seydim, Sezgin, & Seydim, 2005; Robinson *et al*., 2004; Sodini *et al*., 2005). Production of EPS by *Streptococcus thermophilus* is both strain-dependent and growth rate associated. Production is also significantly influenced by media and growth conditions such as temperature, pH, carbon source and carbon:nitrogen ratio (Iyer *et al*., 2010). EPS is a general term that refers to two types of secreted polysaccharides. The first is attached to the cell wall as a capsule, while the other is produced as loose unattached material (slime). Some microorganisms produce both types of EPS, whereas others only produce one type (Hassan, Frank, & Shalabi, 2001). The polysaccharides produced in yogurt mainly consist of galactose and glucose in a ratio 2:1. Minor quantities of fructose, mannose, arabinose, rhamnose and xylose are also present, individually or in combination (Guzel-Seydim *et al*., 2005; Petry, Furlan, Crepeau, Cerning, & Desmazeaud, 2000; Shihata, 2004; Surono & Hosono, 2011). These slime producing cultures can be different strains of either *Streptococcus thermophilus* or *Lactobacilllus bulgaricus.* The gel of the capsular polysaccharides is less prone to damage due to stress and gives a thicker texture to the final yogurt product. The bacterial cells are partly covered by polysaccharide and the filaments link the cells and the proteins in milk. The texture of yogurt results from a complex interaction between milk proteins, lactic acid and EPS produced. This influences important physical properties such as firmness, smoothness, viscosity and gel stability or susceptibility to syneresis. 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. The capsule also slows diffusion of lactic acid away from the cell, causing the cells to stop acid production earlier. This prevents the over acidification of yogurt (Fajardo-Lira, Garcia-Garibay, Wacher-Rodarte, Farres, & Marshall, 1997; Robitaille *et al*., 2009; Surono & Hosono, 2011). 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 *Lactobacilllus 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).

Recent studies consider EPS as having prebiotic properties since they may contribute to human health either as a nondigestible food fraction or because of their claimed antitumoral activity (Surono & Hosono, 2011).

1.4.3 PROBLEMS ENCOUNTERED WITH FINAL PRODUCT

1.4.3.1 WHEY SEPARATION

Whey separation (wheying-off) is defined as the expulsion of whey from the network which then becomes visible as surface whey. Wheying-off negatively affects consumer perception of yogurt as consumers think there is something microbiologically wrong with the product. Yogurt manufacturers use stabilisers, such as pectin, gelatine and starch, to try to prevent wheying-off. Another approach is to increase the total solids content of yogurt milk, especially the protein content, to reduce wheying-off. Spontaneous syneresis, which is contraction of gel without the application of any external force (e.g. centrifugation), is the usual cause of whey separation (Lee & Lucey, 2010).

1.4.3.2 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 (Dave & Shah, 1998; Weinbrenner *et al*., 1997). 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).

Excessive acidity, as a result of continued starter activity during prolonged storage above 5 °C, can also be a problem, due to the acid-tolerant *Lactobacillus bulgaricus* having the ability to generate lactic acid levels of 1.7 % or above (Tamime & Robinson, 1999).

1.4.4 HEALTH ASPECTS OF YOGURT

1.4.4.1 PROBIOTICS

Functional foods is a term used to describe foods fortified with ingredients promoting health. Yogurt which contains probiotics is one of the products that 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. Fermented milk products are the most popular means of delivering probiotic bacteria in food. Among them, strains *Lactobacilllus acidophilus*, *Lactobacilllus casei* and *Bifidobacterium lactis* predominate in commercial probiotic products. In yogurt, lactobacilli are generally used as probiotics (Collado & Hernandez, 2007; Ouwehand *et al*., 2002; Stanton *et al*., 2001; Tabasco *et al*., 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 & Gurel, 2008).

It has been suggested by Salminen, Ouwehand, Benno and Lee (1999) that the metabolic activity of the gut microbiota is potentially greater than that of the liver. The gut microbiota therefore plays an important role in the maintenance of health by aiding digestion, protecting the host from invading bacteria and viruses and modulating the activity of the immune system. The applications of probiotics have been shown to be one of the methods that can positively affect the composition and activity of the gut microbiota (Salminen, Bouley, & Boutron-Rualt, 1998; Salminen *et al*., 1999).

The beneficial effects exerted by probiotics are usually associated with products that are fermented with *Lactobacilllus bulgaricus* and *Streptococcus 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. Except for *Bifidobacterium bifidum,* other bacteria such as *Lactobacilllus acidophilus*, *Lactobacilllus casei* and *Bifidobacterium longum* have also been used as probiotics in humans (Collado & Hernandez, 2007; Dave & Shah, 1996; Stanton *et al*., 2001).

Lactobacilllus delbrueckii ssp. *bulgaricus* and *Streptococcus thermophilus* are not bile resistant and do not survive the passage through the intestinal tract. However, *Lactobacilllus acidophilus* and *Bifidobacterium bifidum* incorporated into the yogurt starter culture have the

ability to establish themselves among the gut flora (Shihata, 2004). 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 *Streptococcus thermophilus* and *Lactobacilllus bulgaricus* (Dave & Shah, 1998; Delorme, 2007; Oliveira, Sodini, Remeuf, & Corrieu, 2001). Although Salminen, Kenifel and Ouwehand (2011) stated that *Streptococcus thermopilus* and *Lactobacilllus bulgaricus* has no effect on rotavirus diarrhea and no immune-enhancing effect during rotavirus diarrhea, it is effective in alleviation of lactose intolerance symptoms.

Yogurt contains various organic acids, peptones, peptides and LAB. It also has an intestine-cleaning function to promote the proliferation of intestinal LAB and probiotics (Park & 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).

1.4.5 CHANGES IN PROTEIN STRUCTURE DURING YOGURT PROCESSING

CCP plays a major role in the colloidal stability, heat stability, rennet coagulability and alcohol stability of the casein micelle (Dalgleish, 2004; Fox & Brodkorb, 2008; Singh & Bennett, 2002). Removal of the CCP 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 (Figure 7) (Considine *et al*., 2007; Shaker, Jumah, & Abu-Jdayil, 2000).

After homogenisation the mixture is heated to induce pasteurisation. Yogurt milk is pasteurised at a higher temperature than fresh milk in order to prevent whey syneresis. Figure 9 represents changes of casein micelle surface during pasteurisation, observed with an electron microscope. When the temperature of the milk reaches 85 °C, the major whey proteins, such as β-lactoglobulin, are denatured and react with the κ-caseins which are situated at the surface of the casein micelles (Figure 8). This interaction produces minute ‘bumps’ on the casein micelle surfaces and results in increased gel firmness and viscosity of yogurt (Figure 9A). 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 9B) (Kalab, 1997; Lee & Lucey, 2010).

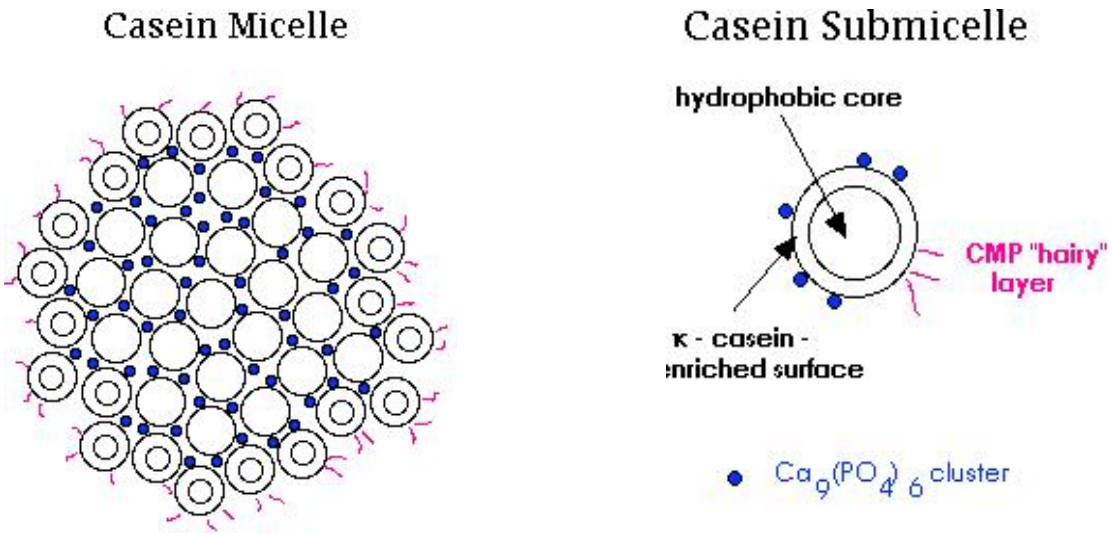


Figure 7 Casein micelle and submicelle model (Shihata, 2004).

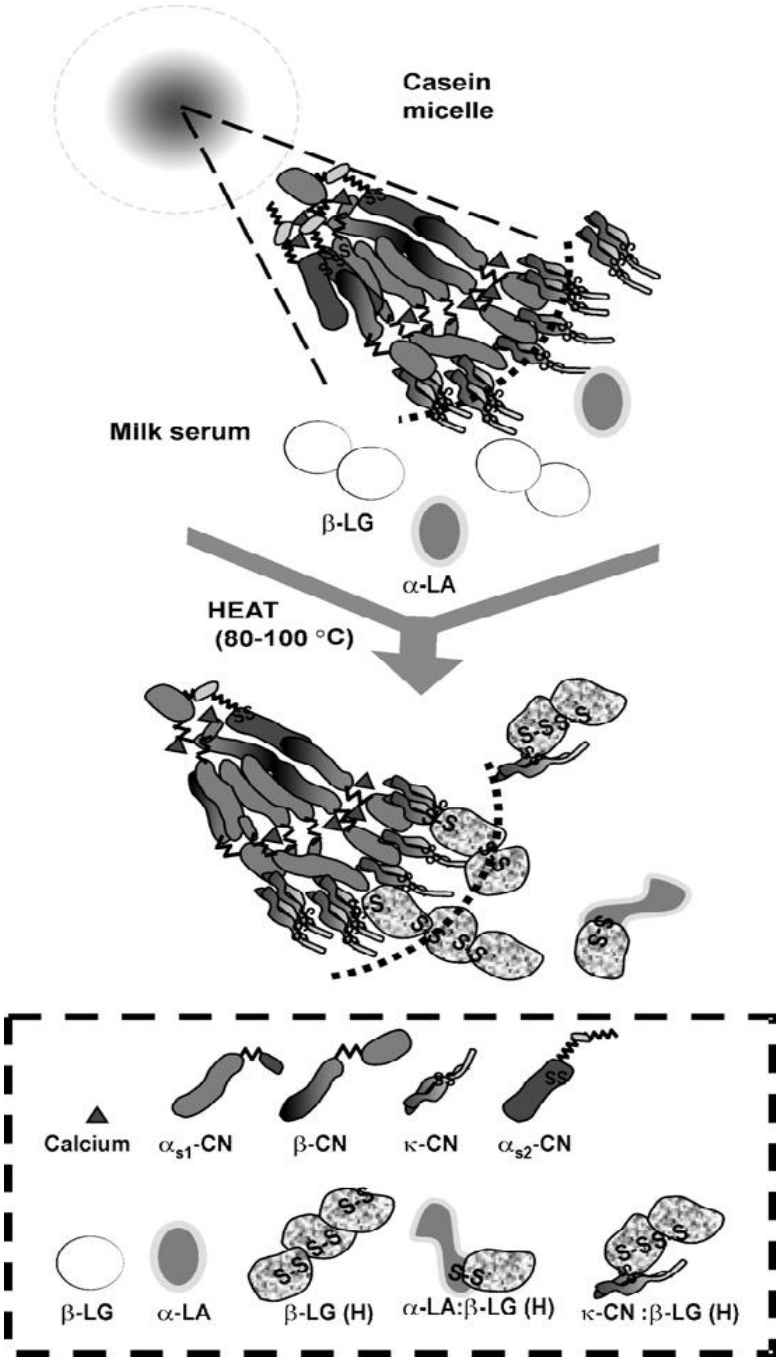


Figure 8 The effect of heating milk to 90 °C on the casein micelle. The native β-LG dimer dissociates and the monomer undergoes internal disulfide-bond interchange to give reactive monomers that react with κ-casein (κ-CN) at the surface (outer region) of the casein micelle. Native β-LG monomers can also 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).

A B

Figure 9 Changes evident in the casein micelle structure during pasteurisation of milk. Figure 9A represents the casein micelle after pasteurisation, and Figure 9B before pasteurisation (Kalab, 1997).

Milk used for yogurt fermentation is pasteurised between 85 °C and 95 °C for 5-10 min for the following reasons:

- To result in a yogurt with the desired textural properties. Thus, the heating step denatures the whey proteins, so that the β-lactoglobulin, in particular, may become attached to the casein micelles. This improves the texture of set yogurt and the viscosity of stirred yogurt
- To denature the whey proteins which release free amino acids that stimulates the activity of the starter culture
- To expel oxygen from the milk which is to the advantage of the facultative anaerobic starter culture
- To kill non-spore forming pathogens that may be present

In order to achieve these aims, the critical feature is the holding time, because high temperatures alone do not result in organoleptically attractive yogurts (Tamime & Robinson, 1999). After this heating phase the mixture is allowed to cool to 40-45 °C and placed into a fermentation tank where a constant temperature is maintained. When 42 °C is reached, starter cultures are added (de Brabandere & de Baerdemaeker, 1999; U.S. Patent No. 6,399,122 B2, 2002). The yogurt-producing starter cultures generally consist of *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* Selected *Bifidobacteria* species are usually added as an additional probiotic. 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) in inoculation loads varying from 0.025 to 5 % (Horiuchi & Sasaki, 2012; Lee & Lucey, 2010). The major disadvantage of using freeze-dried concentrate cultures is the longer lag phase in the vat, adding an additional 30-60 min to the time required to ferment milk (Surono & Hosono, 2011). When the starter 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. The destabilised micelles then form a three dimensional network in which whey is trapped (Goff, 1999; Shihata, 2004; Singh & Bennett, 2002; de Brabandere & de Baerdemaeker, 1999). Coagulation occurs at pH 5.2 to 5.4 for milk that was given a high heat treatment (Lee & Lucey, 2010). As the pH drops during yogurt fermentation and the iso-electrical point (pH 4.6) is reached, the casein micelles completely precipitate and form a coagulum (Rawson & Marshall, 1997; Shaker *et al*., 2000; de Brabandere & de Baerdemaeker, 1999). The characteristic 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 (U.S. Patent No. 6,399,122 B2, 2002).

1.4.6 PHYSICO-CHEMICAL MECHANISMS INVOLVED IN THE FORMATION OF YOGURT GELS

During acidification of milk, the pH decreases from 6.7 to ≤4.6. Acidification of milk leads to the disruption of the internal structural properties of casein micelles due to the solubilisation of CCP. As caseins approach their iso-electric point (pH 4.6), the net negative charge on casein is reduced, which decreases electrostatic repulsion between charged groups, including the phosphoserine residues that are exposed when the CCP is solubilised. Electrostatic attraction between proteins increases, but protein-protein attraction is also increased through enhanced hydrophobic interactions (Phadungath, 2005b). According to Lee and Lucey (2010), physico-chemical mechanisms for the formation of acid milk gels can be discussed for three pH regions.

pH 6.7 to 6.0

When the pH of milk decreases from 6.7 to 6.0, the net negative charge on the casein micelles decreases, which results in a decrease in electrostatic repulsion. Since only a small amount of CCP is solubilised at pH >6.0, the size of the casein micelles is largely unchanged.

pH 6.0 to 5.0

As the pH of milk decreases further from pH 6.0 to 5.0, the net negative charge on casein micelles greatly decreases. This results in a decrease in electrostatic repulsion and steric stabilisation, which are both responsible for the stability of casein micelles in the original milk. At pH ≤6.0 the rate of solubilisation of CCP increases, which weakens the internal structure of casein micelles and increases electrostatic repulsion between the exposed phosphoserine residues. In milk, CCP is completely solubilised in casein micelles at

pH ~5.0. The amounts and proportions of caseins dissociated from the micelles are both temperature- and pH-dependent. The pH at which maximum dissociation occurs is between pH 5.6 and ~5.1, which may be attributed to a partial loosening of bonds within and between caseins due to loss of CCP.

pH ≤5.0

When the pH of milk becomes close to the iso-electric point of casein (pH 4.6), there is a decrease in the net negative charge on casein, which leads to a decrease in electrostatic repulsion between casein molecules. On the other hand, casein-casein attractions increase due to increased hydrophobic and electrostatic charge interactions. The acidification process results in the formation of a three-dimensional network consisting of clusters and chains of caseins (Lee & Lucey, 2010).

1.5 YEAST EXTRACT

1.5.1 GENERAL INTRODUCTION

Although final biomass and activity of the yogurt starter cultures are important, short fermentation times are industrially advantageous (Barrette, Champagne, & Goulet, 2001).

Two main practical problems have to be considered in lactic acid fermentation: media supplementation, owing to the fastidious growth requirements of LAB (Torriani, Vescovo, & Scolari, 1994) and inhibition by accumulated metabolic product. Inhibition by undissociated lactic acid is undoubtedly proved. At acidic pH or in the absence of any pH control, inhibition is observed. At the usual working pH (±pH 6.0) however, the optimum value for lactic acid production, the free lactic acid concentration (approximately 0.3 g/l), is below the inhibitory level (Amrane & Prigent, 1998; Venkatesh, Okos, & Wankat, 1993). Cessation of growth may therefore be attributed to nutritional starvation due to either a deficiency in peptide sources or in growth factors (Amrane & Prigent, 1998; Mozzi, de Giori, Oliver, & de Valdez, 1994). From work done by Amrane and Prigent (1998) it appears that the starvation due to a deficiency of peptide sources is more important.

Bacterial growth can be improved in milk by the addition of substances of undefined composition to the growth medium, such as YE 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, Zink, Reniero, & Morelli, 1999). Studies have indicated that certain extracts and hydrolysates can accelerate lactic acid production and/or LAB growth. These include, amongst others, whey permeate (Fitzpatrick & O'Keeffe, 2001), tryptic digests of proteins, pancreas extract, liver extract (Speck, McAnelly, & Wilbur, 1958), peptones (Vázquez & Murado, 2008) and YE (Rojan, Anisha, Nampoothiri, & Ashok, 2009). Other studies confirmed that lactic acid productivity of specifically lactobacilli is significantly improved by the addition of YE, amino acids, protein concentrates, hydrolysates, vitamins and inorganic compounds such as $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ (Amrane & Prigent, 1998; Cheng, Mueller, Jaeger, Bajpai, & Lannotti, 1991; Demirci, Pouetto, Lee, & Hinz, 1998). The growth of lactic acid streptococci in milk has also been shown to be stimulated by the addition of various crude extracts of plant and animal tissues, and by enzymatic hydrolysates of proteins (Speck *et al*., 1958).

Ikawa and O'Barr (1956) also noted that when *Lactobacilllus delbruekii* is grown in the presence of pyridoxamine phosphate and thymidine, the time required for maximum growth

to occur is reduced substantially by the addition of an enzymatic hydrolysate of casein or a variety of natural extracts. This effect is especially noticeable when a short incubation period and a very dilute inoculum load are employed (Ikawa & O'Barr, 1956).

Nutrient supplements such as YE, corn steep liquor, and casein hydrolysate can improve the nutritional quality of the medium, because in addition to organic nitrogen and carbonic compounds, they contain growth promoting compounds. However, the use of these nutrient supplements in large quantities is very expensive and can reach as high as 32 % of the total lactic acid production cost (de Lima, Coelho, & Contiero, 2010). There is, therefore, a need to develop an industrially attractive process that considers productivity, residual lactose and economic levels of nutrient supplements (Ghaly, Tango, & Adams, 2003).

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. YE 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).

Although it is evident from the above that many extracts and hydrolysates have been found to improve LAB growth and lactic acid production, in the current study, only YE supplementation was investigated. This was due to Aeschlimann and Von Stockar (1990) and Smith, Hillier and Lees (1975) confirming that the beneficial effect of YE on the growth of LAB is well established.

1.5.2 BACKGROUND ON YEAST EXTRACT

YE from dried brewer's yeast cells can be used in a wide variety of foods as flavours, flavour enhancers, or flavor potentiators. Yeasts are extensively used in food products and "cheesy", "meaty" or "savoury" flavour notes may be imparted by yeast autolysates. Apart from being reliable sources of peptides, amino acids, trace minerals and B-vitamins, they are also nutritional additives (Chae, Joo, & In, 2001; Gaudreau, Champagne, Goulet, & Conway, 1997).

The Food Chemical Codex and the European Association for Speciality Yeast Products define YE as the following: "Yeast Extract comprises the water soluble components of the yeast cell, the composition of which is primarily amino acids, peptides, carbohydrates and

salts. YE is produced through the hydrolysis of peptide bonds by the naturally occurring enzymes present in edible yeast or by the addition of food-grade enzymes".

Yeast autolysates are known under the name of "yeast extracts" and are mainly used in the fermentation industry as substrates and in the food industry as flavour enhancers. The flavour improving properties come from the interaction of various amino acids (the most important being glutamic acid) in combination with 5 prime nucleotides and reaction products (Sommer, Haider, Cabaj, Heidenreich, & Kundi, 1996). Good yields can be expected when used in fermentation applications due to the high nutrient content of the extract. YE is commonly added to media as a source of vitamins and growth factors (Adamberg, 2003; Van der Walt, 1971).

The major raw material for YE is primarily grown high protein yeasts (strains of *Saccharomyces cerevisiae*) such as baker's or brewer's yeast, which is grown on molasses based media. The YE from *Saccharomyces* yeast have a specific yeast flavor. Other raw materials in use are yeasts such as *Kluyveromyces fragilis* which is fermented on whey, or *Candida utilis*, which is grown on high carbohydrate waste-products of the timber industry or on ethanol. The YE from genus *Candida* have almost no yeast flavor which is specific to YE that originated from yeast belonging to the genus *Saccharomyces* (US Patent No. 6,344,231 B1, 2002).

The concentrations of amino acids in milk are below the nutritional requirements for the growth of auxotrophic lactobacilli, and therefore, their complex proteolytic system degrades mainly caseins into small peptides and amino acids, which fulfill their nutritional needs (Tamime, 2005). This could be a possible explanation for the acceleration in yogurt fermentation observed in YE supplemented yogurt.

1.5.3 PRODUCTION PROCESS OF YEAST EXTRACT

YE used as growth medium is typically prepared by growing bakers' yeast, *Saccharomyces* spp., in a carbohydrate-rich plant medium (Acumedia, 2004). Brewers' yeast can also be used.

Bakers' YE have higher total nitrogen content than brewers' YE, but there is not always a correlation between the nitrogen content and growth (Champagne, Gaudreau, & Conway, 2003). A study had shown that lactic cultures preferred bakers' YE over brewers' YE (Gaudreau *et al*., 1997). Champagne *et al*. (2003) however found a systematic preference of

bakers' YE over brewers' YE was only encountered with *Lactobacillus casei*. Initial growth studies using *Bacillus thuringiensis* indicated considerable differences in biomass and byproduct formation between the brewer's- and the commercial YE (Saksinchai, Suphantharika, & Verduyn, 2001). Therefore, results from these studies show that the statement cannot be generalised, and that LAB react differently to the source of YE.

The general method for making YE for food products such as Vegemite and Marmite on a commercial scale is to add salt to a suspension of yeast, making the solution hypertonic, which leads to the cells shriveling up. This triggers autolysis, a process of self-destruction, where the yeast's digestive enzymes break their own proteins down into simpler compounds. The dying yeast cells are then heated to complete their breakdown, after which the husks (yeast with thick cell walls which would give poor texture) are separated. This method differs from YE prepared to be used as growth medium (Yoo, 2009).

The production process of YE used as bacterial growth medium is depicted in Figure 10. The yeast is harvested, washed, and resuspended in water, where it undergoes autolysis, i.e., self-digestion using the yeast's enzymes (Acumedia, 2004). Autolysis by endogenous enzymes occurs naturally in yeasts when they complete the cell growth cycle and enter the death phase. In autolysis or self-digestion, the intracellular enzymes break down proteins, glycogen, nucleic acids and other cell constituents. The autolytic process requires careful application and control of heat to kill cells without inactivating the yeast enzymes. This process is generally carried out under moderate agitation and temperatures ranging between 30 and 60 °C for 12-24 hours (Chae *et al*., 2001; Sommer *et al*., 1996). The duration of the autolysis depends on the type of yeast and to what extent the proteins have to be broken down. This causes the release of peptides, amino acids, vitamins and other yeast cell components. The autolytic activity is stopped by a heating step. The autolysis process is carefully controlled to preserve naturally occurring B-complex vitamins (Acumedia, 2004).

Hydrolysis is the most efficient method of solubilising yeast, and is carried out by hydrochloric acid or proteolytic enzymes. Despite a high production yield, acid hydrolysis is less attractive to manufacturers because of relatively high capital investment cost, high salt content and high probability of containing carcinogenic compounds such as monochloropropanol and dichloropropanol (Chae *et al*., 2001).

YE is the total soluble portion of this autolytic action (Acumedia, 2004) from which all insoluble components, such as the cell walls, are removed (Sommer *et al*., 1996). The resulting YE is concentrated and pasteurised. It is then either packaged in pails or drums for

the liquid and paste forms, or dried into a powder by spray drying where after it is packed into bags or boxes. The powder form of YE can either be in fine or granulated particles, or can even be oil coated to prevent the emission of dust (Acumedia, 2004).

Processing and filtration steps may decisively alter the vitamin and mineral contents and ratios. Through this, the subsequent growth of microorganisms can be affected considerably (Sommer *et al*., 1996).

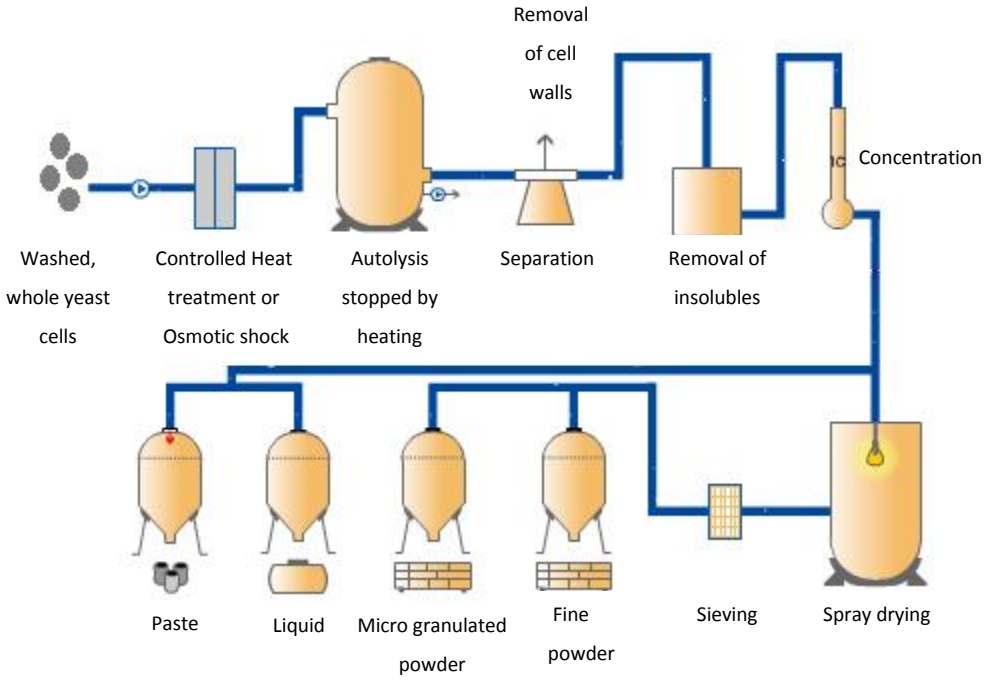


Figure 10 The production process of yeast extract (Figure adapted from European Association for Specialty yeast products).

1.5.4 YEAST EXTRACT COMPOSITION

An understanding of bacterial cation composition is of importance in devising nutrient media which are optimal, not only for growth but also for the formation of endo- and exocellular products. In many instances, a certain complex medium gives satisfactory results while others fail. Similarly, a medium from one commercial firm is satisfactory, whereas purportedly identical media from other firms are less favorable or are completely unsuitable (Bovallius & Zacharias, 1971). There is little information on how variations in the composition of YE influence their growth-promoting properties (Champagne *et al*. 2003).

Although microbial media from different manufacturers often carry the same name and thus give the impression of being identical, they may differ in important details i.e. they do not contain the same amount of measured cations. Thus one of the more commonly used media, YE, contains about four times more magnesium in the Difco product as compared to the Oxoid preparation. On the other hand, the Oxoid product contains about three times more iron than the Difco product (Bovallius & Zacharias, 1971). The same tendencies were noted for zinc as well as for calcium.

The above is an example of the variation in the amount of components in YE from different manufacturers, as well from different batches produced by the same manufacturer. This could affect the growth and the production of different quantities of organic acids, enzymes or other organic compounds by microbial organisms. Bovallius and Zacharias (1971) confirmed this by reporting that complex media from different manufacturers resulted in the production of different quantities of a cholinesterase-liberating factor from a *Cytophaga* sp. It could be demonstrated that the production of this factor was dependant on the magnesium content of the medium. Champagne *et al*. (2003) and Yoo (2009) suggested that significant variations in free amino acid and peptide contents are encountered in commercial YE. These research groups claimed that the differences in nitrogen content are probably related to the yeast strains as well as the autolysis procedure used. Loane, Mendel, Abele, Paull and Macka (2007) also stated that there is a large variety of YE available which can be accounted for by variations in environmental factors during yeast cell growth. The composition of YE is therefore incredibly complex and potentially highly variable. Edens *et al*. (2002) also stated that during their studies the results obtained with ion-pairing HPLC and 31PNMR methods indicate that YE is more complex than previously known.

It must therefore be kept in mind that although the amounts of components in different YE vary, the specific components are the same. Although it will be in varying concentrations, the accelerating compound(s) will therefore be present in all commercial YE.

It was however necessary to identify the accelerating compound by using a single batch of YE from the same manufacturer, in order to limit product inconsistency. The selected YE used in the current study is manufactured by Oxoid (Product Code LP0021). It is described by the manufacturer as a dried yeast autolysate which is a good source of amino-nitrogen and vitamins, particularly the water soluble B-complex vitamins. The use of it in many media or fermentation broths increases the yield of organisms and is recommended where rapid and luxuriant growth is required.

YE contains a wide range of minerals (Table 2) and amino acids, and glutamic acid is the amino acid present in YE in the highest concentration (Table 3). What must be noted about glutamic acid, is the fact that it is essential for the assimilation of other amino acids in microorganisms (Sommer *et al*., 1996).

Table 2 Typical Analysis (% w/w) of Yeast Extract LP0021 provided by manufacturer (Oxoid)

%							mg/100 g								
MO	ASH	AN	TN	K	NaCl	pH	Ca	Mg	Fe	Cu	Pb	Mn	Sn	Zn	Co
<5	13.3	5.3	10.9	7	0.3	7	155	205	52	2	0.7	1.3	3	94	3.1

% MO= % Moisture

% AN = % Amino nitrogen

% TN = % Total nitrogen

* = mg/100 g

Table 3 Typical Amino Acids Analysis (% w/w) of Yeast Extract LP0021 provided by manufacturer (Oxoid)

Ala	Arg	Asp	Cys	Glu	Glu	Ile	Leu	Lys	Met	Phe	Ser	Thr	Trp	Tyr	Val
0.91	3.31	7.07	0.76	13.49	5.95	4.81	6.04	5.40	0.80	3.78	3.42	2.73	0.85	4.95	1.00

The average protein content of a standard salt-free YE for the fermentation industry is 73 – 75 %. The protein content consists of 35-40 % free amino acids. Di-, tri- and tetrapeptides with molecular weights less than 600 Dalton contribute to 10-15 % of the total protein. With a total of 45 – 55 %, these two fractions are the largest portion of the YE protein (Sommer *et al*., 1996).

The portion second in size is the oligopeptides with molecular weight of 2-3 kDa. These oligopeptides amount to 40 – 45 % of the total protein. Oligopeptides with molecular weights of 3 – 100 kDa are the smallest fraction of only 2 – 5 % (Sommer *et al*., 1996).

Sodium and polysaccharide (mainly mannans and glucans) contents do not exceed 0.5 % or 5 %, respectively. Fat contents less than 0.5 % do not significantly affect the fermentation of microorganisms (Sommer *et al*., 1996).

1.5.5 THE SUPPLEMENTATION OF DIFFERENT MEDIA WITH YEAST EXTRACT

The supplementation of milk with YE and wheat embryos stimulates the multiplication process of bifidobacteria cells and their fermentation activity. YE is generally employed in concentrations ranging from 3 – 5 g/l and usually do not exceed 10 g/l (Acumedia, 2004; Barascu & Banu, 2003; Champagne *et al*., 2003; Gaudreau *et al*., 1997).

YE not only accelerates the yogurt fermentation process; due to the composition of YE, it will inadvertently increase the amount of vitamins, especially B-vitamins, and minerals present in the yogurt. It has been stated by Sommer *et al*. (1996) that in future the demand for YE with special components to support defined functions or reactions will increase rapidly.

Lactococci can assimilate peptides and do not require a complete hydrolysis of the proteins into free amino acids. Some strains even prefer peptides over free amino acids (Barrette *et al*., 2001). The mechanism of the stimulation of growth of other LAB by peptides has been postulated as the ability to serve as a means of increasing the penetration of some amino acid into the cells (Jones & Woolley, 1962). Peptides can therefore be superior to free amino acids as nitrogen sources for LAB because they can provide the cell with amino acids in a form that can be utilised more efficiently (Yoo, 2009).

In cases in which inefficient utilisation of amino acids had been observed previously, appropriate peptides containing the amino acid frequently were more effective in promoting growth than the free amino acid. Numerous instances are now known in which peptides supply limiting amounts of an essential amino acid to an auxotrophic microorganism more efficiently than equimolar amounts of the free amino acid. However, the superiority of the peptides to the free amino acid as a source of D-alanine for growth appear to result from the fact that these peptides are absorbed from the growth medium more readily than the free amino acid (Kihara, Ikawa, & Snell, 1961).

The following findings were observed by Kihara *et al*. (1961) and Yoo (2009):
(a) to serve as a sole source of an essential amino acid for growth, a peptide must be hydrolysed by the cell
(b) the growth-promoting activity of hydrolysable peptides may be greater than, equal to, or less than that of the free limiting amino acid they supply. This depends on whether the two independent processes of absorption and hydrolysis supply the limiting amino acid to the cell at a rate greater than, equal to, or less than that at which the free amino acid can be absorbed from the medium.

Jones and Woolley (1962) noted that *Lactobacilllus bulgaricus* had an absolute growth requirement for a suitable peptide, which was satisfied by trypsinised casein or hemoglobin. They further observed that the addition of peptide-containing digests, for example a trypsin digest of casein, to the growth medium of *Lactobacilllus* casei, shortens the lag phase and stimulates the growth rate of this organism (Jones & Woolley, 1962).

Although part of the growth-stimulatory activity of YE undoubtedly is due to nucleotides and nucleosides of uracil and cytosine, other unidentified stimulatory substances, among which peptides are undoubtedly present, must contribute to the effects of YE to explain its high activity relative to that of known compounds (Ikawa & O'Barr, 1956).

The effects of inoculum levels, temperature, pH, and the supplementation of whey ultrafiltrate with YE on cell growth and lactic acid production by *Lactobacilllus helveticus* were investigated by Kulozik and Wilde (1999). The objective was to accelerate fermentation. Addition of 10 g/l YE was required to optimise the volumetric productivity. Higher YE levels further improved cell growth, but lactic acid production remained unchanged. A temperature of 42°C and pH 5.8 were found to be optimal. pH values above 5.8 resulted in a decrease in productivity and yield, mainly because galactose was only partially utilised (Kulozik & Wilde, 1999).

During studies done by Gaudreau *et al*. (1997), lactic acid fermentation was carried out to evaluate the effects of high concentrations of YE on growth, acidification and diacetyl production by various lactic cultures including *Lactococcus lactic* ssp. *lactis, Lactobacilllus casei* ssp. *casei and Lactobacilllus rhamnosus.* At a constant pH of 5.0 maintained by 5N NaOH, an uncoupling effect between growth and acidification was suggested. Gaudreau *et al*. (1997) further stated that the uncoupling effect between growth and metabolism is well known with LAB.

The active components in YE are not yet known. Compositional analysis, ion-pairing HPLC and ^{31}P NMR done by Edens *et al*. (2002) provided a more complete picture of the components of YE, and fractions prepared from it, than was available previously. The HPLC-UV data suggested that the mixtures were enriched in purine- and pyrimidine containing compounds. The ^{31}P NMR data contained resonances consistent with a complex mixture of phosphorylated biochemicals, including phosphomonoesters, phosphodi-esters and polyphosphates; however, additional experimentation was required for specific compound identification. Examples of these classes of compounds, which have been previously reported as components of YE, are phosphorylated monosaccharides and mononucleotides, glycerol phosphodiesters and nucleoside triphosphates, respectively. The ^{31}P NMR data also suggested that YE contains inorganic phosphate. To reduce this complexity, YE was fractionated by open column chromatography to yield three fractions that differ in hydrophobicity, polarity and inorganic content. These three fractions were roughly equal in mass, but may be expected to have differing composition. Fraction 1, obtained from column flow-through, was relatively rich in minerals, salt, inorganic phosphate and hydrophilic organic compounds, whereas Fraction 3, eluted with 40 % acetonitrile, was more enriched in organic compounds that were more hydrophobic and less polar than those in Fraction 1. Fraction 2, eluted with ammonium acetate buffer, contained components found in both Fraction 1 and Fraction 3. Components common to Fractions 2 and 3 included complex nucleotides and peptides. Fraction 1 (least hydrophobic) was relatively inactive in glucose metabolism and lipolysis assays. Fraction 2 and Fraction 3 (more hydrophobic) were relatively more potent in these assays (Edens *et al*., 2002).

Utilising whey or ultra filtrated (UF) whey permeate as a medium for *Lactobacilllus bulgaricus* or *Streptococcus thermophilus* fermentation is a cheap and readily available source. In spite of this fact both media require supplementation of YE or another complex additive in order for LAB 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, Jelen, & Kimura, 1998).

In studies by Cox and MacBean (1977), *Lactobacilllus bulgaricus* was grown in continuous culture in cheese whey ultrafiltrate containing various levels of the YE and corn steep liquor. The supplements increased both the rate of acid production and the bacterial concentration

by up to six fold. YE was however more effective than corn steep liquor (Cox & MacBean, 1977).

The cultivation in the current study was done on milk, and not on whey, whey permeate or whey ultrafiltrate as was used by Amrane and Prigent (1998), Bury *et al*. (1998), Cox and MacBean (1977) and Kulozik and Wilde (1999).

Table 4 portrays the amount of free amino acids in milk and yogurt. It is clear from the table that when no additional peptides or amino acids are added, the amount of free amino acids present in yogurt is higher than in milk, except for the isolated case of glycine. This indicates that although free amino acids are used by the starter bacteria, the proteolytic action of the bacteria is responsible for the production of the additional amounts of amino acids.

Table 4 Free amino acid content (mg/100 ml) of milk and yogurt (Shihata, 2004).

Amino acid	**Milk**	**Yogurt**
Alanine	0.16-0.64	1.17-3.80
Arginine	0.16-0.96	0.70-1.39
Aspartic acid	0.23-0.52	0.70-1.20
Glycine	0.30-0.53	0.28-0.45
Glutamic acid	1.48-3.90	4.80-7.06
Histidine	0.11	0.80-1.70
Isoleucine	0.06-0.15	0.15-0.40
Leucine	0.06-0.26	0.70-1.82
Lysine	0.22-0.94	0.80-1.11
Methionine	0.05	0.08-0.20
Phenylalanine	0.05-0.13	0.17-0.61
Proline	0.12	5.40-7.05
Serine	0.08-1.35	1.50-2.90
Threonine	0.05-0.26	0.24-0.70
Tryptophan	Trace	0.2
Tyrosine	0.06-0.14	0.18-0.61
Valine	0.10-0.25	0.90-1.86
Total	3.29-10.31	18.77-33.06

1.5.6 EFFECT OF YEAST EXTRACT ON BACTERIAL GROWTH

YE is the common name for various forms of processed yeast products that are used as food additives or flavours. They are often used in the same way that monosodium glutamate (MSG) is used, and like MSG, often contain free glutamic acids (Yoo, 2009).

YE enhance lactic acid production rates by lactic acid bacteria, and seems to be a more convenient source of various peptides for LAB than casein hydrolysates or malt sprout extracts (Amrane & Prigent 1998; Barascu & Banu 2003; Champagne *et al*. 2003). Several studies show that lactic acid productivity of most lactobacilli is significantly improved by the addition of YE, amino acids, protein concentrates, hydrolysates and vitamins (Cheng *et al*. 1991; Amrane & Prigent 1998; Demirci *et al*. 1998). The nature of the stimulatory effect of YE was also reflected in results obtained by Ikawa and O'Barr (1956). That YE supply growth stimulatory substances rather than additional absolute growth requirements was shown by the fact that unsupplemented basal medium permitted maximum growth, only with longer incubation times.

YE powder (also known as yeastolate) contains an undefined mixture of amino acids, water soluble peptides, polysaccharides, vitamins, and minerals, making it one of the most complete nutritional supplements available (Edens *et al*., 2002; Loane *et al*., 2007; Sommer *et al*., 1996). YE has been successfully used as an ingredient in microbiological cell culture media for bacterial studies in milk and other dairy products (Acumedia, 2004). This is due to the presence of many vitamins, nitrogen, amino acids, nucleotides, carbon and other growth stimulating compounds which promote rapid propogation of the cells, enhance metabolism and stimulate the physiological activity of the cells (Gaudreau *et al*., 1997; Ghaly *et al*., 2003). Amino acids and peptides stimulate the LAB to synthesise protein and they have been shown to be partially responsible for YE stimulation of the growth of lactic cultures (Yoo, 2009).

Many efforts have been dedicated to screen yeastolate and to identify active components which support the cell growth and fermentation. However, yeastolate is a complex mixture and identification of the constituents responsible for cell growth promotion has not yet been achieved (Edens *et al*., 2002; Sommer *et al*., 1996; Yoo, 2009).

1.6 ACCELERATION OF THE YOGURT FERMENTATION PROCESS

Direct acidification using an acid such as lactic acid may be used as an alternative to biological acidification. It is used commercially to a significant extent in the manufacturing of cottage cheese and Feta-type cheese from ultrafiltration-concentrated milk. Direct acidification is more controllable than biological acidification, and, unlike starters, is not susceptible to phage infection (Fox, Guinee, Cogan, & McSweeney, 2000). However, in addition to acidification, the starter bacteria serve very important functions in yogurt fermentation, and therefore chemical acidification cannot be used in products where flavour is as important as texture.

The presence of starter bacteria is extremely important for yogurt fermentation as they produce important flavour and texture components such as acetaldehyde and exopolysaccharides, which cannot be produced if no viable lactic acid bacteria are added to the milk (U.S. Patent No. 20100015285 A1, 2010). It is therefore important to focus on accelerating the growth rate of the starter bacteria, or increasing their rate of lactic acid production, consequently decreasing yogurt fermentation time.

1.6.1 Possible Accelerating Effect Of Amino Acids On Yogurt Fermentation

The free amino acid content of cow's milk generally does not exceed 10 mg/100 ml (Table 4). In yogurt, the free amino acid pattern depends on the type of milk (animal species, season), its heat treatment, manufacturing techniques, bacterial strains used and storage conditions (Amer & Lammerding, 1983).

Each peptide has a distinct and characteristic solubility in a defined environment and any changes to those conditions (buffer or solvent type, pH, ionic strength, temperature, etc.) can cause peptides to lose the property of solubility and precipitate out of solution. The environment can be manipulated to bring about the separation of peptides (Yoo, 2009).

Of the essential amino acids required by most lactic streptococci, only methionine appears to be absent in free form from normal milk. However, methionine is present in low molecular weight peptides contained in the non-protein nitrogen (NPN) fraction. Comparison of the amino acid requirements for bacterial protein synthesis with the levels of free amino acids in milk indicated that most free amino acid levels are well below the minimum requirement for lactic acid bacteria growth to high cell densities (Shihata & Shah, 2000). Single and multiple amino acid omission analysis in studies by Iyer *et al*. (2010) showed that the number and

type of essential amino acids required for growth is strain-dependent, with some *Streptococcus thermophilus* strains exhibiting no absolute amino acid requirement.

Fermented milks containing probiotic microorganisms are generally considered as functional foods. Their consumption has the potential to improve lactose digestion (Lin, Savaiano, & Harlander, 1991) as well as to modulate immune function (Donnet-Hughes, Rochat, Serrant, Aeschlimann, & Schiffrin, 1999) and enteric flora (Nord, Lidbeck, Orrhage, & Sjöstedt, 1997). This leads to the prevention of some gastro-intestinal disorders in humans such as antibiotic-associated diarrhoea (Arvola *et al*., 1999), rotavirus gastroenteritis, traveller's diarrhoea (Juntunen, Kirjavainen, Ouwehand, Salminen, & Isolauri, 2001) and radiation-induced diarrhoea (Urbancsek, Kazar, Mezes, & Neumann, 2001). In order to provide health benefits, fermented milks should contain a minimum level of living probiotic bacteria at the use-by date. Milk supplementation with peptides and amino acids, which are abundant in YE, may also increase the viability of probiotic organisms (Lucas, 2004).

During research done by Abraham *et al*. (1993), the specific proteolytic activity of *Lactobacilllus bulgaricus* cell suspensions obtained from i) cultures grown in reconstituted skim milk naturally containing 7.5 mg of trichloroacetic acid (TCA)-soluble nitrogen as Tyr/100 ml, ii) reconstituted skim milk supplemented with casein hydrolysate containing 15.5 mg of TCA-soluble nitrogen as Tyr/100ml and iii) broth containing 10 g/l of tryptone, YE and lactose, respectively, were compared. Results indicated that the *Lactobacilllus bulgaricus* grown in the unsupplemented milk had a significantly higher (approximately twice as high) specific proteolytic activity than the cultures grown in the other two media. A high availability of peptides and amino acids can be postulated to suppress synthesis of proteolytic enzymes, affect the transformation of the enzyme to an active form, or inhibit the activity of the enzyme (Abraham *et al*., 1993). These results are in agreement with those obtained by Laloi, Atlan, Blanc, Gilbert and Portalier (1991) in which casein hydrolysis by *Lactobacilllus bulgaricus* grown in MRS broth was significantly lower than that observed with the bacteria grown in milk. Proteolytic activity was also affected by temperature. The specific proteolytic activity was high and constant at low concentrations of amino acids and peptides in milk growth media and at low temperatures between 34 and 38 °C. The specific activity decreased sharply when cells were grown between 38 and 40 °C. This low value was maintained at temperatures higher than 40 °C (Abraham *et al*., 1993).

From the results obtained by the two above mentioned research groups, it could be concluded that the proteolytic activity of *Lactobacilllus bulgaricus* may be regulated by amino acid and peptide content of the media as well as by the growth temperature. Abraham *et al*.

(1993) concluded that when bacteria are grown in milk at high temperatures, the basal level of proteases is sufficient to sustain bacterial growth because the enzyme is more active than at 37 °C.

Studies by Juille, Le Bars and Juillard (2005) concluded that *Streptococcus thermophilus* ST 8, which is a proteinase negative strain, required four amino acids for growth. These included histidine, methionine, glutamic acid and proline. On the other hand, Thomas and Mills (1981) reported that methionine is not required by *Streptococcus thermophilus* strains while cysteine and tryptophan are usually essential.

Mikhlin and Radina (1981) stated that the absence of cysteine negatively affected bacterial development. A key role for this particular amino acid is attributed to the disulphide bonds and sulphydrilic groups acting as one of the main redox potential donors in milk. Earlier work of Dave and Shah (1998) indicated that high levels of cysteine (250 or 500 mg/l) affected the growth of *Streptococcus thermophilus*. The cell morphology of *Streptococcus thermophilus* was adversely affected with increased concentration of cysteine (500 mg/l) as a result of the reduced redox potential.

The difference in findings could be due to the different media and strains used in each study. Variability between strains has been observed by many researchers (Accolas, Veaux, & Auclair, 1971; Bracquart, Lorient, & Alais, 1978).

1.7 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. One of the biggest problems experienced with yogurt fermentation is the fermentation time. When the heating of the milk, pasteurisation time, fermentation time and the time used for flavour addition steps are combined, the entire yogurt production process extends beyond one working shift. This results in difficulties arising owing to human error associated with change in personnel between two shifts.

Acceleration of the yogurt fermentation process will not only solve the problems associated with shift changes, but will also result in a greater amount of product being produced in the same time span while requiring no additional infrastructure.

Faster yogurt fermentation processes are necessary and literature as well as previous research conducted in our laboratories, indicated that YE has the potential to achieve this. The addition of YE however results in an unacceptable end product in terms of sensory properties, which necessitates the identification of the accelerating compound(s) present in YE. It will also have to be determined whether YE accelerate the rate of starter bacteria growth or the rate of lactic acid production.

The decrease in yogurt fermentation time 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. This formed the basis of the current study.

1.8 REFERENCES

Abraham, A. G., Antoni, G. L., & Anon, M. C. (1993). Proteolytic activity of *Lactobacillus bulgaricus* grown in milk. *Journal of Dairy Science, 76,* 1498–1505.

Accolas, J. P., Veaux, M., & Auclair, J. (1971). Study of interactions between various mesophillic and thermophilic lactic acid bacteria in relationship with the production of hard cheese. *Lait, 51,* 249–272.

Acumedia. (2004). Yeast Extract. Retrieved from http://www.neogen.com/acumedia/pdf/prodinfo/7184_pi.pdf.

Adamberg, K. (2003). The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. *International Journal of Food Microbiology*, *85*, 171–183.

Adams, D. M., Barach, J. T., & Speck, M. L. (1976). Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. *Journal of Dairy Science*, *59*, 823–827.

Aeschlimann, A., & Von Stockar, U. (1990). The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helveticus*. *Applied Microbiology and Biochemistry*, *32*, 398–402.

Amer, M. A., & Lammerding, A. M. (1983). Health maintenance benefits of cultured dairy products. *Cultured Dairy Products Journal*, *18*, 6–10.

Amrane, A., & Prigent, Y. (1998). Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. *World Journal of Microbiology and Biotechnology*, *14*, 529–534.

Angelov, M., Kostov, G., Simova, E., Beshkova, D., & Koprinkova-Hristova, P. (2009). Proto-cooperation factors in yogurt starter cultures. *Revue de Génie Industriel*, *3*, 4–12.

Arvola, T., Laiho, K., Torkkeli, S., Mykkänen, H., Salminen, S., Maunula, L., & Isolauri, E. (1999). Prophylactic *Lactobacillus GG* reduces antibiotic-associated diarrhea in children with respiratory infections : A Randomized Study. *Journal of the American Academy of Pediatrics*, *104*, 64–69.

Barascu, E. L., & Banu, C. (2003). Influence of yeast extract and wheat embryos on growth of bifidobacteria in milk. *The Annals of the University Dunarea de Jos of Galati, 6*, 10-13.

Barrett, N. E., Grandison, A. S., & Lewis, M. J. (1999). Contribution of the lactoperoxidase system to the keeping quality of pasteurized milk. *The Journal of Dairy Research*, *66*, 73–80.

Barrette, J., Champagne, C. P., & Goulet, J. (2001). Growth-promoting properties of yeast extracts produced at different pH values, with different autolysis promoters and bacterial populations. *Journal of Chemical Technology & Biotechnology*, *76*, 203–209.

Boeneke, C. A. Ã., & Aryana, K. J. (2008). Effect of folic acid fortification on the characteristics of lemon yogurt. *Lebensmittel-Wissenschaft & Technologie*, *41*, 1335–1343.

Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S., Kulakauskas, S., Lapidus, A., *et al*. (2004). Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nature Biotechnology*, *22*, 1554–1558.

Bouzar, F., Cerning, J., & Desmazeaud, M. (1997). Exopolysaccharide production and texture-promoting abilities of mixed-strain starter cultures in yogurt production. *Journal of Dairy Science*, *80*, 2310–2317.

Bovallius, A., & Zacharias, B. (1971). Variations in the metal content of some commercial media and their effect on microbial growth. *Applied Microbiology*, *22*, 260–262.

Bracquart, P., Lorient, D., & Alais, C. (1978). Effect of amino acids on the growth of five *Streptococcus thermophilus* strains. *Milchwissenschaft*, *33*, 341–344.

Bury, D., Jelen, P., & Kimura, K. (1998). Whey protein concentrate as a nutrient supplement for lactic acid bacteria. *International Dairy Journal*, *8*, 149–151.

Cais-Sokolinska, D., Michalski, M., & Pikul, J. (2004). Role of the proportion of yoghurt bacterial strains in milk souring and the formation of curd qualitative characteristics. *Bulletin of The Veterinary Institute in Pulawy*, *48*, 437–441.

Ceballos, L. S., Morales, E. R., Adarve, G. T., Castro, J. D., Martinez, L. P., & Sampelayo, M. R. S. (2009). Composition of goat and cow milk produced under similar conditions and analyzed by identical methodology. *Journal of Food Composition and Analysis*, *22*, 322–329.

Chae, H. J., Joo, H., & In, M. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, *76*, 253–258.

Champagne, C., Gaudreau, H., & Conway, J. (2003). Effect of the production or use of mixtures of bakers' or brewers' yeast extracts on their ability to promote growth of lactobacilli and pediococci. *Electronic Journal of Biotechnology*, *6*, 185–197.

Chavarri, F., Santisteban, A., Virto, M., & de Renobales, M. (1998). Alkaline phosphatase, acid phosphatase, lactoperoxidase, and lipoprotein lipase activities in industrial ewe's milk and cheese. *Journal of Agricultural and Food Chemistry*, *46*, 2926–2932.

Cheng, P., Mueller, R. E., Jaeger, S., Bajpai, R., & Lannotti, E. L. (1991). Lactic acid production from enzyme-thinned cornstarch using *Lactobacillus amylovorus*. *Journal of Industrial Microbiology*, *7*, 27–34.

Chervaux, C., Ehrlich, S. D., & Maguin, E. (2000). Physiological study of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains in a novel chemically defined medium. *Applied and Environmental Microbiology*, *66*, 5306–5311.

Codex standard for fermented milk (2008). Retrieved from http://www.codexalimentarius.-net/input/download/standards/400/CXS_243e.pdf.

Cogan, T., & Hill, C. (2004). Cheese Starter Cultures. In P. Fox (Ed.), *Cheese: Chemistry, Physics and Microbiology* (pp. 193–255). London, UK: Chapman & Hall.

Collado, M., & Hernandez, M. (2007). Identification and differentiation of *Lactobacillus, Streptococcus* and *Bifidobacterium* species in fermented milk products with bifidobacteria. *Microbiological Research*, *162*, 86–92.

Considine, T., Patel, H. A., Anema, S. G., Singh, H., & Creamer, L. K. (2007). Interactions of milk proteins during heat and high hydrostatic pressure treatments - A Review. *Innovative Food Science & Emerging Technologies*, *8*, 1–23.

Cousin, M. A. (1982). Presence and activity of psychrotrophic microorganisms in milk and dairy products. *Journal of Food Protection*, *45*, 172–207.

Cousin, M. A., & Marth, E. H. (1977). Cottage cheese and yogurt manufacture from milks precultured with psychrotrophic bacteria. *Cultured Dairy Products Journal*, *12*, 15–17.

Cox, G. C., & MacBean, R. D. (1977). Lactic acid production by *Lactobacillus bulgaricus* in supplemented whey ultrafiltrate. *Australian Journal of Dairy Technology*, *32*, 19–22.

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology*, *80*, 217–222.

Dalgleish, D. (2004). The Enzymatic Coagulation of Milk. In P. Fox (Ed.), *Cheese: Chemistry, Physics and Microbiology* (pp. 69–90). London, UK: Chapman & Hall.

Dave, R. I., & Shah, N. P. (1996). Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbruekii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, and Bifidobacteria. *Journal of Dairy Science*, *79*, 1529–1536.

Dave, R. I., & Shah, N. P. (1998). Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *Journal of Dairy Science*, *81*, 2804–2816.

De Brabandere, A. G., & de Baerdemaeker, J. G. (1999). Effects of process conditions on the pH development during yogurt fermentation. *Journal of Food Engineering*, *41*, 221–227.

De Giori, G., de Valdez, G., Holgado, A., & Oliver, G. (1985). Effect of pH and temperature on the proteolytic activity of lactic acid bacteria. *Journal of Dairy Science*, *68*, 2160–2164.

De Kruif, C., & Holt, C. (2003). Casein micelle structure, functions and interactions. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry* (3rd ed., pp. 234–276). New York: Springer.

De Lima, C. J. B., Coelho, L. F., & Contiero, J. (2010). The use of response surface methodology in optimization of lactic acid production: Focus on medium supplementation, temperature and pH control. *Food Technology and Biotechnology*, *48*, 175–181.

Deeth, H. C., & Fitz-Gerald, C. H. (2006). Lipolytic enzymes and hydrolytic rancidity. In P. F. Fox & P. McSweeney (Eds.), *Advanced Dairy Chemistry* (2nd ed., pp. 481–556). New York: Springer.

Delorme, C. (2007). *Streptococcus thermophilus*. *International Journal of Food Microbiology*, *126*, 274–277.

Demirci, A., Pouetto, L., Lee, B., & Hinz, P. (1998). Media evolution in repeated batch lactic acid fermentation with *Lactobacillus plantarum* and *Lactobacillus casei*. *Journal of Agriculture and Food Chemistry*, *46*, 4771–4774.

Donnet-Hughes, A., Rochat, F., Serrant, P., Aeschlimann, J. M., & Schiffrin, E. J. (1999). Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. *Journal of Dairy Science, 82*, 863-869.

Edens, N. K., Reaves, L. A., Bergana, M. S., Reyzer, I. L., Mara, P. O., Baxter, J. H., & Snowden, M. K. (2002). Yeast extract stimulates glucose metabolism and inhibits lipolysis in rat adipocytes in vitro. *Journal of Nutrition*, *6*, 1141–1148.

Ekinci, F., & Gurel, M. (2008). Effect of using propionic acid bacteria as an adjunct culture in yogurt production. *Journal of Dairy Science*, *91*, 892–899.

Elli, M., Zink, R., Reniero, R., & Morelli, L. (1999). Growth requirements of *Lactobacillus johnsonii* in skim and UHT milk. *International Dairy Journal*, *9*, 507–513.

Fajardo-Lira, C., Garcia-Garibay, M., Wacher-Rodarte, C., Farres, A., & Marshall, V. (1997). Influence of water activity on the fermentation of yogurt made with extracellular polysaccharide-producing or non-producing starters. *International Dairy Journal*, *7*, 279–281.

Farkye, N., & Vedamuthu, E. (2002). Microbiology of soft cheeses. In R. K. Robinson (Ed.), *Dairy Microbiology Handbook* (3rd ed., pp. 479–514). New York: Wiley-Interscience.

Farrell, H. M., Brown, E. M., Hoagland, P. D., & Malin, E. L. (2003). Higher order structure of the caseins. In P. F. Fox & P. L. H. McSweeney (Eds.), *Advanced Dairy Chemistry* (3rd ed., pp. 203–231). New York: Kluwer Academic.

Farrell, H. M., Jimenez-Florez, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hocks, C. L., *et al*. (2004). Nomenclature of proteins of cows' milk: Sixth revision. *Journal of Dairy Science*, *87*, 1641–1674.

Fernandez-Garcia, E. (1994). Determination of organic acids during the fermentation and cold storage of yogurt. *Journal of Dairy Science*, *77*, 2934–2939.

Fitzpatrick, J. J., & O'Keeffe, U. (2001). Influence of whey protein hydrolysate addition to whey permeate batch fermentations for producing lactic acid. *Process Biochemistry*, *37*, 183–186.

Fox, P. F. (2004). Cheese: An Overview. In P. F. Fox (Ed.), *Cheese: Chemistry, Physics and Microbiology* (pp. 1–35). London, UK: Chapman & Hall.

Fox, P. F., & Brodkorb, A. (2008). The casein micelle: Historical aspects, current concepts and significance. *International Dairy Journal*, *18*, 677–684.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2000). *Fundamentals of cheese science* (pp. 10–18). Maryland, USA: Aspen Publishers.

Fox, P. F., & Kelly, A. (2004). The Caseins. In R. Y. Yada (Ed.), *Proteins in Food Processing* (pp. 29–55). Cambridge, UK: Woodhead Publishing.

Fox, P. F., & Kelly, A. (2006). Indigenous enzymes in milk : Overview and historical aspects — Part 1. *International Dairy Journal*, *16*, 500–516.

Gardini, F., Lanciotti, R., Guerzoni, M., & Torriani, S. (1999). Evaluation of aroma production and survival of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* in fermented milks. *International Dairy Journal*, *9*, 125–134.

Gassem, M. A., & Frank, J. F. (1991). Physical properties of yogurt made from milk treated with proteolytic enzymes. *Journal of Dairy Science*, *74*, 1503–1511.

Gaudreau, H., Champagne, C. P., Goulet, J., & Conway, J. (1997). Lactic fermentation of media containing high concentrations of yeast extracts. *Journal of Food Science*, *62*, 1072–1075.

Ghaly, A. E., Tango, M. S. A., & Adams, M. A. (2003). Enhanced lactic acid production from cheese whey with nutrient supplement addition. *Agricultural Engineering International: the CIGR Journal of Scientific Research and Development*, *5*, 1–20.

Goff, D. (1999). Dairy science and technology education. Retrieved from www.foodsci.uoguelph.ca/dairyedu/home.html

Gueguim-Kana, E., Oloke, J., Lateef, A., & Zebaze-Kana, M. (2007). Novel optimal temperature profile for acidification process of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yoghurt fermentation using artificial neural network and genetic algorithm. *Journal of Industrial Microbiology and Biotechnology*, *34*, 491–496.

Guzel-Seydim, Z., Sezgin, E., & Seydim, A. (2005). Influences of exopolysaccharide producing cultures on the quality of plain set type yogurt. *Food Control*, *16*, 205–209.

Haider, T., & Husain, Q. (2009). Hydrolysis of milk / whey lactose by β galactosidase: A comparative study of stirred batch process and packed bed reactor prepared with calcium alginate entrapped enzyme. *Chemical Engineering and Processing*, *48*, 576–580.

Hassan, A. N., Frank, J. F., & Shalabi, S. I. (2001). Factors affecting capsule size and production by lactic acid bacteria used as dairy starter cultures. *International Journal of Food Microbiology*, *64*, 199–203.

Hickey, M., Hillier, A., & Jago, G. (1986). Transport and metabolism of lactose, glucose, and galactose in homofermentative Lactobacilli. *Applied and Environmental Microbiology*, *51*, 825–831.

Horiuchi, H., & Sasaki, Y. (2012). Short communication: Effect of oxygen on symbiosis between *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. *Journal of Dairy Science*, *95*, 2904–2909.

Horne, D. S. (2002). Casein structure, self-assembly and gelation. *Current Opinion in Colloid and Interface Science*, *7*, 456–461.

Huppertz, T., Fox, P. F., de Kruif, K., & Kelly, A. (2006). High pressure-induced changes in bovine milk proteins: A review. *Biochemica et Biophysica Acta*, *1764*, 593 – 598.

Ikawa, M., & O'Barr, J. (1956). The nature of some growth stimulatory substances for *Lactobacillus delbruekii*. *Journal of Bacteriology*, *71*, 401–405.

Iyer, R., Tomar, S. K., Maheswari, T. U., & Singh, R. (2010). *Streptococcus thermophilus* strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*, 133–141.

Iyer, R., Tomar, S. K., Singh, R., & Sharma, R. (2009). Estimation of folate in milk by microbiological assay using trienzyme extraction method. *Milchwissenschaft*, *64*, 125–127.

Jones, K. M., & Woolley, D. W. (1962). Peptides required for the growth of *Lactobacillus bulgaricus*. *Journal of Bacteriology*, *83*, 797–801.

Juille, O., Le Bars, D., & Juillard, V. (2005). The specificity of oligopeptide transport by *Streptococcus thermophilus* resembles that of *Lactococcus lactis* and not that of pathogenic streptococci. *Microbiology*, *151*, 1987–1994.

Juntunen, M., Kirjavainen, P. V., Ouwehand, A. C., Salminen, S. J., & Isolauri, E. (2001). Adherence of probiotic bacteria to human intestinal mucus in healthy infants and during rotavirus infection. *Clinical and Diagnostic Laboratory Immunology*, *8*, 293–296.

Kalab, M. (1997). From milk to yogurt - An electron microscope story. Retrieved from http://www.medicinalfoodnews.com/vol01/issue5/kalab.html

Kang, O., Vezinz, L., Laberge, S., & Simard, R. (1998). Some factors influencing the autolysis of *Lactobacillus bulgaricus* and *Lactobacillus casei*. *Journal of Dairy Science*, *81*, 639–646.

Khan, M., Hussain, M., Wajid, A., & Rasool, S. A. (2008). Microbial population load and enzyme production of indigenously isolated yeast. *Pakistan Journal of Botany*, *40*, 2225–2230.

Kihara, H., Ikawa, M., & Snell, E. (1961). Peptides and bacterial growth. *Journal of Biological Chemistry*, *236*, 172–176.

Kotz, C., Furne, J., Savaiano, D., & Levitt, M. (1994). Factors affecting the ability of a high β-Galactosidase yogurt to enhance lactose absorption. *Journal of Dairy Science*, *77*, 3538–3544.

Kulozik, U., & Wilde, J. (1999). Rapid lactic acid production at high cell concentrations in whey ultrafiltrate by *Lactobacillus helveticus*. *Enzyme and Microbial Technology*, *24*, 297–302.

Laloi, P., Atlan, D., Blanc, B., Gilbert, C., & Portalier, R. (1991). Cell-wall-associated proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: differential extraction, purification and properties of the enzyme. *Applied Microbial Biotechnology*, *36*, 196–204.

Lee, W. J., & Lucey, J. A. (2010). Formation and physical properties of yogurt. *Asian-Australasian Journal of Animal Sciences*, *23*, 1127 – 1136.

Lin, M. Y., Savaiano, D., & Harlander, S. (1991). Influence of nonfermented dairy products containing bacterial starter cultures on lactose maldigestion in humans. *Journal of Dairy Science, 74*, 87–95.

Ling, H. (2010). U.S. Patent No. 20100015285 A1. Washington, DC: U.S. Patent and Trademark Office.

Liu, Y., & Guo, R. (2008). Aggregation properties of aqueous casein hydrolysate solutions at different pH. *International Dairy Journal*, *18*, 1022–1027.

Loane, M., Mendel, E., Abele, S., Paull, B., & Macka, M. (2007). The potential of capillary electrophoresis for the analysis of yeast extracts. *Analytical Research Forum Royal Society of Chemistry*. Glasgow, Scotland.

Lucas, A. (2004). Probiotic cell counts and acidification in fermented milks supplemented with milk protein hydrolysates. *International Dairy Journal*, *14*, 47–53.

Mahdian, E., & Tehrani, M. M. (2007). Evaluation the effect of milk total solids on the relationship between growth and activity of starter cultures and quality of concentrated yoghurt. *American-Eurasian Journal of Agriculture & Environmental Sciences*, *2*, 587–592.

Malbaša, R. V., Vitas, J. S., Lončar, E. S., & Milanović, S. D. (2012). Physical and textural characteristics of fermented milk products obtained by kombucha inoculums with herbal teas. *Acta Periodica Technologica*, *342*, 51–59.

Matalon, M. E., & Sandine, W. E. (1986). *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and yogurt: A review. *Cultured Dairy Products Journal*, *21*, 6–12.

McKinley, M. (2005). The nutrition and health benefits of yoghurt. *International Journal of Dairy Technology, 58,* 1–12.

Mikhlin, E. D., & Radina, V. P. (1981). Activation of lactic acid bacteria. *Applied Biochemistry and Microbiology, 17*, 253–267.

Mozzi, F., de Giori, G. S., Oliver, G., & de Valdez, G. F. (1994). Effect of culture pH on the growth characteristics and polysaccharide production by *Lactobacillus casei*. *Milchwissenschaft*, *49*, 667–670.

Mullan, W. (2001). Microbiology of starter cultures. *Dairy Science and Food Technology*, *32*, 4–6.

Nakada, M., Dosako, S., Hirano, R., Oooka, M., & Nakajima, I. (1996). Lactoperoxidase suppresses acid production in yoghurt during storage under refrigeration. *International Dairy Journal*, *6*, 33–42.

Nakajo, Y., & Sano, H. (2002). US Patent No. 6,344,231 B1. Tokyo, Japan: Nihon Tobacco Inc.

Nelson, F. E. (1981). The Microbiology of Market Milk. In R. K. Robinson (Ed.), *Dairy Microbiology* (pp. 195–197). Essex, UK: Applied Science Publishers.

Nord, C. E., Lidbeck, A., Orrhage, K., & Sjöstedt, S. (1997). Oral supplementation with lactic acid-producing bacteria during intake of clindamycin. *Clinical Microbiology and Infection*, *3*, 124–132.

Oliveira, M., Sodini, I., Remeuf, F., & Corrieu, G. (2001). Effect of milk supplementation and culture composition on acidification, textural properties and microbiological stability of fermented milks containing probiotic bacteria. *International Dairy Journal*, *11*, 935–942.

Ouwehand, A., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek*, *82*, 279–289.

Ozer, B., & Atasoy, F. (2002). Effect of addition of amino acids, treatments with β-Galactosidase and use of heat-shocked cultures on the acetaldehyde level in yogurt. *International Journal of Dairy Science*, *55*, 166–170.

Park, K., & Oh, S. (2007). Production of yogurt with enhanced levels of gamma-aminobutyric acid and valuable nutrients using lactic acid bacteria and germinated soybean extract. *Bioresource Technology*, *98*, 1675–1679.

Pastink, M. I., Teusink, B., Hols, P., Visser, S., de Vos, W. M., & Hugenholtz, J. (2009). Genome-scale model of *Streptococcus thermophilus* LMG18311 for metabolic comparison of lactic acid bacteria. *Applied and Environmental Microbiology*, *75*, 3627–3633.

Penna, A., Baruffaldi, R., & Oliveira, M. (1997). Optimization of yogurt production using demineralized whey. *Journal of Food Science*, *62*, 846–850.

Petry, S., Furlan, S., Crepeau, M., Cerning, J., & Desmazeaud, M. (2000). Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. *Applied and Environmental Microbiology*, *66*, 3427–3431.

Phadungath, C. (2005a). Casein micelle structure: a concise review. *Songklanakarin Journal of Science and Technology*, *27*, 201–212.

Phadungath, C. (2005b). The mechanism and properties of acid-coagulated milk gels. *Songklanakarin Journal of Science and Technology*, *27*, 433–448.

Quiberoni, A., Auad, L., Binetti, A., Suarez, V., Reinheimer, J., & Raya, R. (2003). Comparative analysis of *Streptococcus thermophilus* bacteriophages isolated from a yogurt industrial plant. *Food Microbiology*, *20*, 461–469.

Radke-Mitchell, L., & Sandine, W. E. (1984). Associative growth and differential enumeration of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*: a review. *Journal of Food Protection*, *47*, 245–248.

Radke-Mitchell, L., & Sandine, W. E. (1986). Influence of temperature on associative growth of *Streptococcus thermophillus* and *Lactobacillus bulgaricus*. *Journal of Dairy Science*, *69*, 2558–2568.

Rajagopal, S., & Sandine, W. (1990). Associative growth and proteolysis of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in skim milk. *Journal of Dairy Science*, *73*, 894–899.

Rawson, H. L., & Marshall, V. M. (1997). Effect of "ropy" strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* on rheology of stirred yogurt. *International Journal of Food Science and Technology*, *32*, 213–220.

Renan, M., Guyomarc'h, F., Arnoult-Delest, V., Paquet, D., Brule, G., & Famelart, M.H. (2009). Rheological properties of stirred yoghurt as affected by gel pH on stirring, storage temperature and pH changes after stirring. *International Dairy Journal*, *19*, 142–148.

Robinson, R. K. (2001). *Dairy Microbiology* (3rd ed.). New York: Wiley Interscience.

Robinson, R. K., Tamime, A. Y., & Wszolek, M. (2004). Microbiology of fermented milks. In R. Robinson (Ed.), *Dairy Microbiology Handbook* (pp. 367–430). New York: Wiley-Interscience.

Robitaille, G., Tremblay, A., Moineau, S., St-Gelais, D., Vadeboncoeur, C., & Britten, M. (2009). Fat-free yogurt made using a galactose-positive exopolysaccharide-producing recombinant strain of *Streptococcus thermophilus*. *Journal of Dairy Science*, *92*, 477–482.

Rojan, J. P., Anisha, G. S., Nampoothiri, K. M., & Ashok, P. (2009). Direct lactic acid fermentation: Focus on simultaneous saccharification and lactic acid production. *Biotechnology Advances*, *27*, 145–152.

Saksinchai, S., Suphantharika, M., & Verduyn, C. (2001). Application of a simple yeast extract from spent brewer's yeast for growth and sporulation of *Bacillus thuringiensis* subsp. *kurstaki*: a physiological study. *World Journal of Microbiology and Biotechnology*, *17*, 307–316.

Salminen, S., Bouley, M., & Boutron-Rualt, M. (1998). Functional food science and gastrointestinal physiology and function. *The British Journal of Nutrition*, *80*, 147–171.

Salminen, S., Kenifel, W., & Ouwehand, A. C. (2011). Probiotics, applications in dairy products. In J. W. Fuquay, P. F. Fox, & P. L. H. McSweeney (Eds.), *Encyclopedia of Dairy Science* (pp. 412–419). United States: Academic Press.

Salminen, S., Ouwehand, A., Benno, Y., & Lee, Y. (1999). Probiotics: How should they be defined? *Trends in Food Science & Technology*, *10*, 107–110.

Shaker, R., Jumah, R., & Abu-Jdayil, B. (2000). Rheological properties of plain yogurt during coagulation process: impact of fat content and preheat treatment of milk. *Journal of Food Engineering*, *44*, 175–180.

Shihata, A. (2004). *The proteolytic activity of yoghurt and probiotic bacteria for the improved viability of probiotic bacteria in fermented milk products* (Doctoral dissertation, Victoria University, Australia). Retrieved from http://vuir.vu.edu.au/15367.

Shihata, A., & Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal*, *10*, 401–408.

Silanikove, N., Merin, U., & Leitner, G. (2006). Physiological role of indigenous milk enzymes: An overview of an evolving picture. *International Dairy Journal*, *16*, 533–545.

Simov, Z., Simova, E., & Beshkova, D. (2006). Impact of two starter cultures on proteolysis in Kashkaval cheese. *World Journal of Microbiology & Biotechnology*, *22*, 147–156.

Singh, H., & Bennett, R. (2002). Milk and Milk Processing. In R. K. Robinson (Ed.), *Dairy Microbiology Handbook* (pp. 5–9). New York: Wiley-Interscience.

Smith, J. S., Hillier, A. J., & Lees, G. J. (1975). The nature of the stimulation of the growth of Streptococcus lactis by yeast extract. *Journal of Dairy Research*, *42*, 123–138.

Sodini, I., Latrille, E., & Corrieu, G. (2000). Identification of interacting mixed cultures of lactic acid bacteria by their exclusion from a model predicting the acidifying activity of non-interacting mixed cultures. *Applied Microbial Biotechnology*, *54*, 715–718.

Sodini, I., Montella, J., & Tong, P. (2005). Physical properties of yogurt fortified with various commercial whey protein concentrates. *Journal of Science of Food and Agriculture*, *85*, 853–859.

Sommer, R., Haider, T., Cabaj, A., Heidenreich, E., & Kundi, M. (1996). Increased inactivation of Saccharomyces cerevisiae by protraction of UV irradiation. *Applied and Environmental Microbiology*, *62*, 1977–1983.

Speck, M. L., McAnelly, J. K., & Wilbur, J. D. (1958). Variability in response of lactic Streptococci to stimulants in extracts of pancreas, liver, and yeast. *Journal of Dairy Science*, *41*, 502–508.

Stanton, C., Gardiner, G., Meehan, H., Collins, K., Fitzgerald, G., Lynch, P. B., & Ross, R. P. (2001). Market potential for probiotics. *The American Journal of Clinical Nutrition*, *73*, 476–483.

Stiles, M. E., & Holzapfel, W. H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, *36*, 1–29.

Surono, S., & Hosono, A. (2011). Starter cultures. *Encyclopedia of Dairy Sciences*.

Tabasco, R., Paarup, T., Janer, C., Peláez, C., & Requena, T. (2007). Selective enumeration and identification of mixed cultures of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium lactis* in fermented milk. *International Dairy Journal*, *17*, 1107–1114.

Tagg, J. R., Wescombe, P. A., & Burton, J. P. (2011). Streptococcus: A brief update on the current taxonomic status of the genus. In A. von Wright (Ed.), *Lactic Acid Bacteria: Microbiological and Functional Aspects* (pp. 123–146). New York: CRC Press.

Tamime, A. Y. (2002). Microbiology of starter cultures. In R K. Robinson (Ed.), *Dairy Microbiology Handbook* (pp. 261–366). New York: Wiley-Interscience.

Tamime, A. Y. (2005). Microbiology of starter cultures. In R K. Robinson (Ed.), *Dairy Microbiology: The microbiology of milk products* (3rd ed., pp. 261–366). London: Elsevier Applied Science.

Tamime, A. Y., & Robinson, R. K. (1999). Background to Manufacturing Practice. *Yogurt: Science and Technology* (pp. 6–47). Cambridge, UK: Woodhead Publishing.

Tari, C., Ustok, F. I., & Harsa, S. (2009). Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology. *International Dairy Journal*, *19*, 236–243.

Thomas, T., & Mills, O. (1981). Proteolytic enzymes of starter bacteria. *Netherlands Milk and Dairy Journal*, *35*, 255–273.

Torriani, S., Vescovo, M., & Scolari, G. (1994). An overview on Lactobacillus helveticus. *Annali di Microbiologia e Enzimologia, 44,* 163-191.

Urbancsek, H., Kazar, T., Mezes, I., & Neumann, K. (2001). Results of a double-blind, randomized study to evaluate the efficacy and safety of Antibiophilus in patients with radiation-induced diarrhoea. *European Journal of Gastroenterology & Hepatology, 13,* 391–396.

Van der Walt, J. P. (1971). Criteria and methods used in classification. In J. Lodder (Ed.), The yeasts: A Taxonomic study (pp. 84–113). Amsterdam: North-Holland Publishing Company.

Vandeweghe, P., Norquist, P., & Flynn, E. (2002). U.S. Patent No. 6,399,122 B2. Washington, DC: U.S. Patent and Trademark Office.

Vasiljevic, T., & Jelen, P. (2001). Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. *Innovative Food Science & Emerging Technologies*, *2*, 75–85.

Venkatesh, K. V., Okos, M. R., & Wankat, P. C. (1993). Kinetic model of growth and lactic acid production from lactose by Lactobacillus bulgaricus. *Process Biochemistry*, *28*, 231–241.

Vázquez, J. A., & Murado, M. A. (2008). Enzymatic hydrolysates from food wastewater as a source of peptones for lactic acid bacteria productions. *Enzyme and Microbial Technology*, *43*, 66–72.

Weinbrenner, D. R., Barefoot, S. F., & Grinstead, D. A. (1997). Inhibition of yogurt starter cultures by Jenseniin G , a *Propionibacterium* Bacteriocin. *Journal of Dairy Science*, *80*, 1246–1253.

Yoo, S. M. (2009). *Fractionation of non-animal protein hydrolysates for use in CHO (Chinese Hamster Ovary) cell media* (Master's thesis, University of Waterloo, Canada). Retrieved from http://uwspace.uwaterloo.ca/handle/10012/4986?mode=full.

CHAPTER 2

STANDARDISATION OF YOGURT FERMENTATION PROCESS

2.1 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 thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* are used as mixed cultures in yogurt production (Sodini, Latrille, & Corrieu, 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 (U.S. Patent No. 6,399,122 B2, 2002). The growth association that exists between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* enhances acid production in comparison to the two organisms used individually (Sodini *et al*., 2000; Tamime, 2002; Tari, Ustok, & Harsa, 2009).

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 variation in starter culture loads used in independent experiments is another factor which 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 as well as the fermentation medium used for yogurt fermentation. It was also essential to determine the effect of various concentrations of YE on individual yogurt fermentations.

2.2 MATERIALS AND METHODS

2.2.1 YOGURT PRODUCTION PROCESS

2.2.1.1 REHYDRATION OF SKIM MILK POWDER

Due to the daily variation of fresh milk composition (supplied by Dairy Corporation, Bloemfontein, RSA) influencing repeatability, reconstituted skim milk powder was used to prepare yogurt. Low heat-treated milk powder was used as it is nutritionally superior to high heat-treated milk powder. Skim milk powder (Nestlé, RSA) was reconstituted with distilled water (10% w/v) by heating to 50 °C for 60 min in a 500 ml Erlenmeyer flask with a cotton wool plug.

2.2.1.2 STARTER BACTERIA

A thermophilic yogurt culture, FD-DVS YF-L812 Yo-Flex, (Chr. Hansen, Regal Fruits, RSA) was used throughout all experiments.

The starter culture was received as freeze-dried granules and ground with a mortar and pestle to reduce particle size. This resulted in uniform distribution of the starter bacteria and also simplified the weighing process in comparison to weighing the whole granules.

It has been reported by Surono and Hosono (2011) that each starter culture pouch has a starter population of approximately 10^{11} cells/g. A starter culture inoculum load of 0.104 g/l was used in all yogurt fermentations.

2.2.1.3 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, Michigan, USA) prior to use. The pH decrease was used as an indication of the rate of lactic acid production.

2.2.1.4 YOGURT MANUFACTURE

Skim milk powder was rehydrated as described in Section 2.2.1.1. The milk was heated to 90 °C for 8-10 min to pasteurise and was subsequently cooled to 42 °C. Similar heating conditions were also used by Arkbage (2003) and Tamime and Robinson (1999). The starter culture inoculum load was 0.104 g/l as stated in Section 2.2.1.2. Yogurt fermentation was done in a water bath and was stopped at pH 4.6, the iso-electric point (Fox & Brodkorb, 2008) of casein proteins by cooling the yogurt. No stabilisers were added during any

experiments. Yogurt prepared in this way served as control in all yogurt experiments. The percentage increase or decrease in fermentation times reported in this study refers to the difference in times to reach pH 4.6 in comparison to the control.

Statistical experimental design

All fermentations were carried out in independent triplicate experiments (standard deviations were indicated by error bars). All yogurt fermentations in this study were carried out in this manner.

2.2.1.5 Sensory evaluation

A bench level sensory evaluation involving a few persons was conducted.

2.2.1.6 Effect of different concentrations of yeast extract on yogurt fermentation

A stock solution of 15 g YE (LP0021, Oxoid, Hampshire, England) per litre of milk was prepared. Separate batch fermentations containing 0.4, 1, 2, 3, 4, 6, 8, 10 and 15 g/l YE, respectively, were carried out in order to determine whether it will result in various effects on the yogurt fermentation time. Fermentations were performed using the 2 litre yogurt fermentation tubes. The yogurt fermentation with 0.4, 2, 4, 6 and 10 g/l YE concentrations was also carried out in 15 ml test tubes in order to determine whether the fermentations could be scaled down successfully.

2.2.1.7 Viscosity determinations

Viscosity determinations were performed using a Brookfield Digital Viscometer (Middleboro, USA) Model DV-II. The viscosity of the positive and negative control were determined at time 0 of fermentation as well as when the respective controls reached pH 4.6.

2.3 RESULTS AND DISCUSSION

Before research could commence, two key aspects, which do not pose problems on industrial scale (5000 l) but influence yogurt production on laboratory scale (15 ml), had to be addressed. The first was uniformity of starter culture granules, and the second was the variation in fresh milk composition between different milk batches.

2.3.1 YOGURT PRODUCTION PROCESS

2.3.1.1 STARTER CULTURE STANDARDISATION

Starter culture is provided as unevenly distributed granules (Figure 11). This complicates the weighing of small amounts due to the two starter organisms not being equally represented in individual yogurt fermentations. By grinding the starter culture, uniform distribution of the two organisms is ensured and consistency can be maintained between independent fermentations. This ground starter culture was used in all fermentations in this study.

Figure 11 Starter culture granules as provided by supplier (A) and ground starter culture (B)

2.3.1.2 VARIATION IN FRESH MILK COMPOSITION

The use of fresh milk for yogurt fermentation may not pose a problem for industrial use as an entire sachet of starter culture is used to prepare one batch of yogurt. When used on laboratory scale, however, the variation in the composition of fresh milk influenced 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 12. The time to reach pH 4.6 varied between 195 and 345 minutes, a relative difference of 43 %. A variation in the initial pH of fresh milk, which ranged between pH 6.3 and pH 6.6, was also observed. This variation indicated that fresh milk is not a reliable or trustworthy source to be used for yogurt

fermentation experiments. Vercet, Oria, Marquina, Crelier and Lopez-Buesa (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. 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 13.

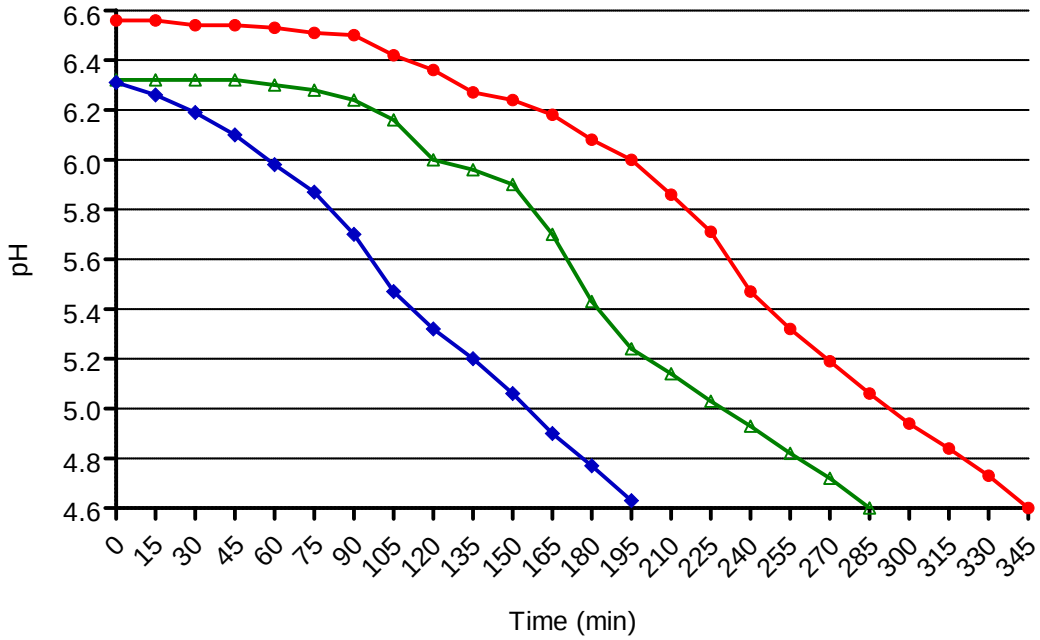


Figure 12 The variation in control yogurts prepared with three different batches of fresh milk

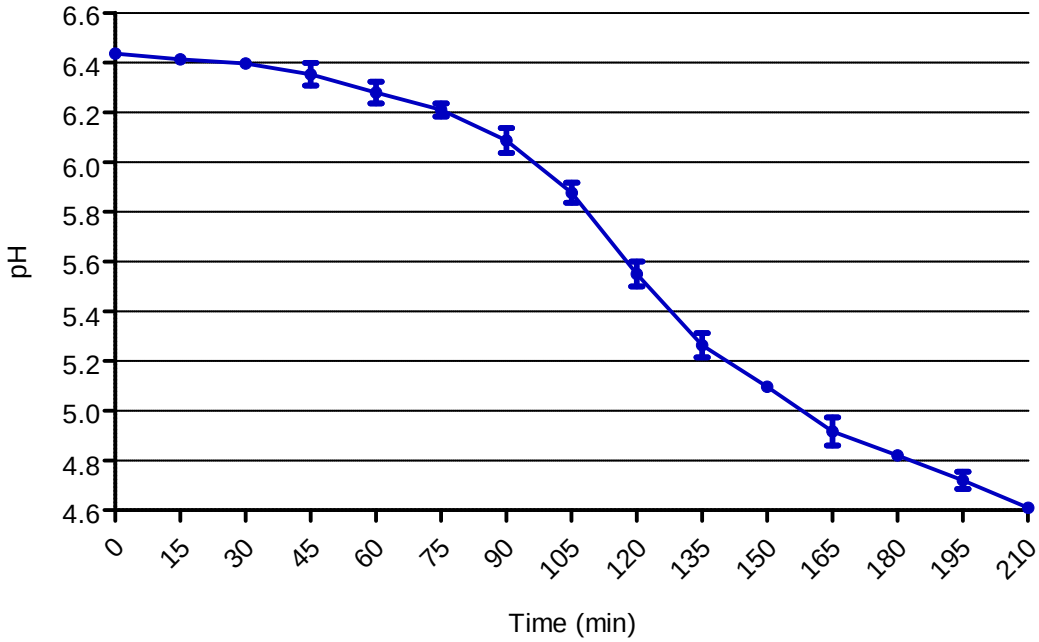


Figure 13 Control yogurts prepared with different batches of skim milk powder

2.3.1.3 Effect of different concentrations of yeast extract on yogurt fermentation

Due to many reports (Barascu & Banu, 2003; Champagne *et al*., 2003; Gaudreau *et al*., 1997; Sommer *et al*. 1996; Yoo, 2009) on the enhancement of bacterial growth and lactic acid production by YE, it was evaluated as supplementation to yogurt fermentation. The effect of various YE concentrations on the fermentation process was assessed due to reports that the rate of lactic acid production is related to the YE concentration.

Cogan, Gilliland and Speck (1968) found YE to be a good stimulant for *Lactobacilllus delbrueckii* growth. Studies done by Elli, Zink, Reniero and Morelli (1999) concurred with the finding that stated that bacterial growth can be improved by the addition of substances such as YE or peptones (of various origins) to the growth medium. The stimulatory role of substances such as YE and peptones on bacterial growth in milk has been related to their nucleotide content (Elli *et al*., 1999).

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

From the results obtained in Figure 14 it was evident that there were no differences in the yogurt fermentation profile or fermentation time when YE was used in concentrations ranging from 1-10 g/l. Relative to the control, a decrease in fermentation time of 21 % (45 min) was noted for YE supplementation of 1-10 g/l in comparison to the negative control which contained no YE. A YE concentration of 0.4 g/l decreased fermentation time by 7 % (15 min) in comparison to the control, although it was 14 % (30 min) longer than the higher concentrations that were evaluated. In contrast, the highest evaluated concentration, 15 g/l, increased fermentation time. This increase could most likely be ascribed to the initial high pH, which consequently influenced starter bacteria growth and subsequent lactic acid production. As the YE concentrations increased, the sliminess of the yogurt consequently increased, i.e. the yogurt supplemented with 8 g/l had a slimier consistency than that of the 2 g/l, which in turn was slimier than the 0.4 g/l YE supplemented yogurt. The highest degree of sliminess was reached with 15 g/l YE.

The final products that were produced with YE supplementation had an undesirable flavour associated with YE, which would be unacceptable to the consumer. The abundance of peptides naturally present in YE was most likely the cause of this unwanted flavour which then activated the human bitter receptors, hTAS2Rs, which is strongly activated by bitter peptides (Maehashi *et al*., 2008). 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 YE and soy, render these substances less suitable for industrial production of commercial dairy products.

From the above results it is clear that yogurt supplementation by YE between 1-10 g/l yields similar results in terms of fermentation time and profile. Any concentration between 1-10 g/l would therefore result in the same decrease in fermentation time.

These results do not correspond to those obtained by Hujanen and Linko (1996) and Amrane and Prigent (1998). In studies performed by Amrane and Prigent (1998), *Lactobacilllus helveticus* was cultivated batchwise on whey permeate supplemented with 2-30 g/l of YE. For low supplementation levels growth was slow. As the amount of supplemented media increased, the final lactic acid concentration was constant, while fermentation time decreased significantly. Hujanen and Linko (1996) established that addition of YE had a significant effect on lactic acid production during the lag phase of bacterial growth. An increase in YE level lead to a linear increase in lactic acid concentration.

Aeschlimann and Von Stockar (1990) and Ghaly, Tango and Adams (2003) stated that supplementation of whey permeates with 10 g YE per litre appeared to be reasonable and gave the highest cell growth, lactose utilisation and lactic acid yield. For this reason 10 g/l was the maximum YE concentration that was evaluated in our studies.

A positive control containing 10 g/l YE was used from this point on due to the fact that it was the highest evaluated YE concentration that considerably accelerated yogurt fermentation in comparison to the negative control containing no YE supplementation.

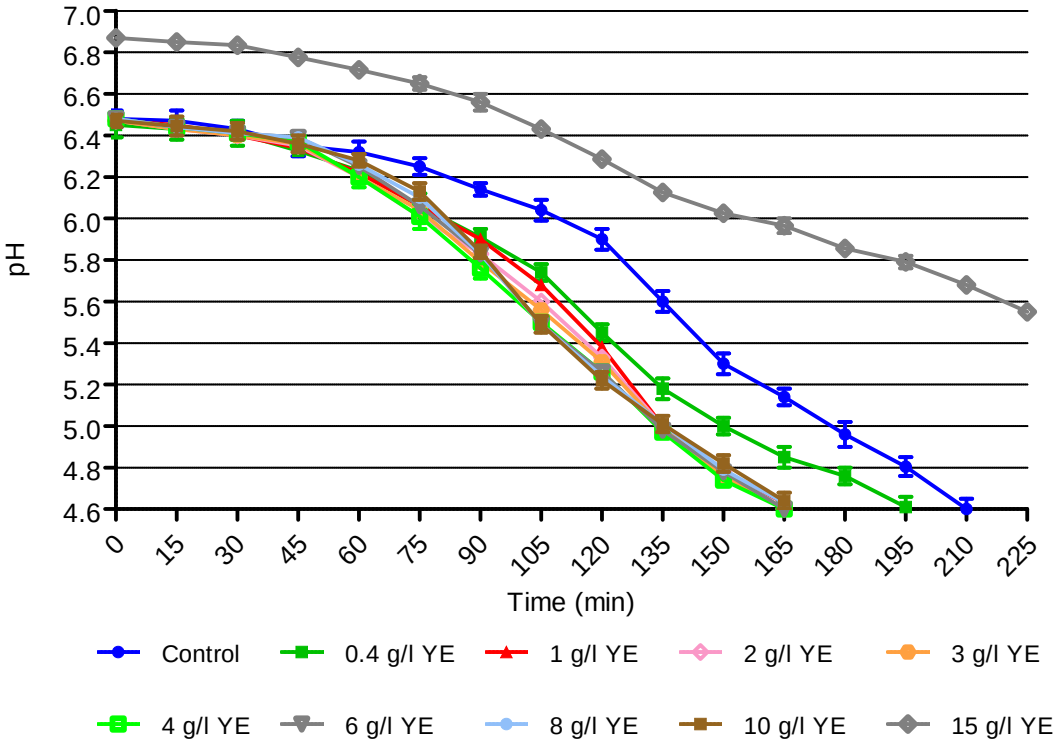


Figure 14 The effect of different yeast extract concentrations on the yogurt fermentation process

2.3.1.4 Reduction of fermentation volumes

After determining that the YE concentration between certain ranges does not influence yogurt fermentation, the next aspect that had to be addressed was whether the fermentation volumes could successfully be reduced. The fermentation profiles of the original 2 litre volumes were compared with 15 ml volumes. From Figure 15 it was evident that the reduction of fermentation volumes from 2 litres to 15 ml had no effect on the fermentation process. Due to very small sample amounts and in order to carry out more individual fermentations in a single experiment, the fermentation volume was reduced to 15 ml in all subsequent experiments (unless otherwise stated).

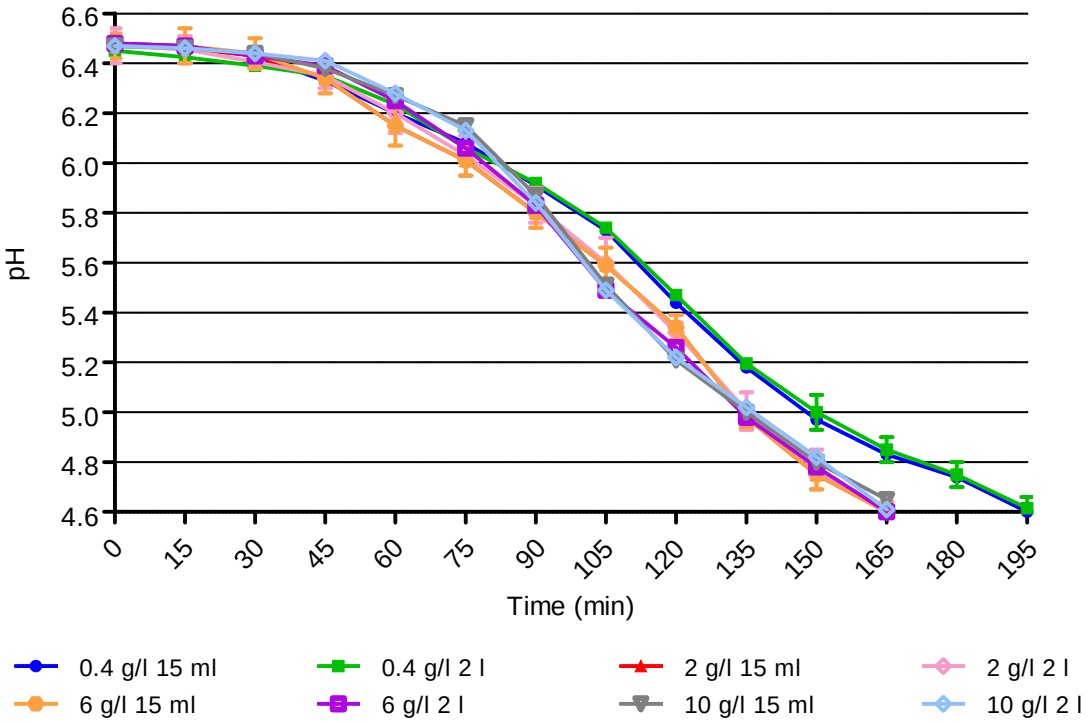


Figure 15 Yogurt fermentation using different volumes of substrate

2.3.1.5 VISCOSITY DETERMINATIONS

The viscosity of the positive and negative control were determined at the beginning of fermentation (Time 0) as well as when the respective controls reached pH 4.6. The determination of the viscosity of yogurt posed difficulties in terms of repeatability. Similar problems were experienced by Shihata (2004) who noted that yogurt, being a non-newtonian fluid, gave no stable reading of viscosity. This can be ascribed to the fact that the yogurt coagulum is disturbed by the mechanical action of the viscosity meter, subsequently reducing the viscosity.

To overcome this problem, triplicate readings were obtained for each sample. The initial viscosity of both the negative control yogurt and positive control was 128 cps. The fact that there was no difference in viscosity between the two controls indicated that the addition of YE did not influence the initial viscosity of milk.

At pH 4.6 the viscosity of the negative control was 310 cps and that of the positive control was 2300 cps. This indicated that the addition of YE to milk increased the final viscosity of yogurt almost 7.5 times.

2.4 CONCLUSIONS

The yogurt fermentation process had to be standardised before the effect of YE supplementation could be established. This was done by addressing a few main areas.

Irregular distribution of starter culture granules influenced the ratio of the two organisms during the weighing process and consequently resulted in unequal amounts being present between individual fermentations. This problem was resolved by grinding the starter culture granules prior to weighing.

The use of different batches of fresh milk as medium for yogurt fermentations also influenced the yogurt fermentation process. Respective times to reach pH 4.6 varied up to 43 %. The substitution of fresh milk with milk powder eliminated this problem and ensured consistent fermentation times between respective fermentation runs.

The supplementation of milk with YE decreased yogurt fermentation time by up to 45 min, i.e. 21 %, relative to the unsupplemented yogurt. Different YE concentrations ranging between 1 g/l and 10 g/l had similar effects on fermentation time, indicating that the significance of the accelerating effect was not dependent on the amount of YE supplementation between these ranges.

During the examination of fermentation volumes, it was apparent that 2 litre fermentation volumes could successfully be scaled down to 15 ml fermentations, without any deviations in the fermentation profile.

After the yogurt fermentation process had been standardised according to the above criteria, the fractionation of YE and the effect of the various fractions on yogurt fermentation could proceed.

The addition of YE to milk did not influence the initial viscosity, although a difference was noted at the end of fermentation. At a pH of 4.6, the positive control yogurt had a much higher viscosity than the negative control. The addition of YE therefore either resulted in higher amounts of exopolysaccharides formed by the starter bacteria, or directly influenced the structure of the milk, therefore increasing the viscosity.

Although results obtained in this chapter indicated that YE decreases yogurt fermentation time, it delivered an end product with unacceptable texture and undesirable flavour which

would not be acceptable to the consumer. The main aim of Chapter 3 was therefore to identify and isolate the component(s), present in YE, responsible for the acceleration. It was anticipated that, when used in isolation, it would not affect the flavour or texture of yogurt.

2.5 REFERENCES

Aeschlimann, A., & Von Stockar, U. (1990). The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helveticus*. *Applied Microbiology and Biochemistry*, *32*, 398–402.

Amrane, A., & Prigent, Y. (1998). Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. *World Journal of Microbiology and Biotechnology*, *14*, 529–534.

Arkbage, K. (2003). *Vitamin B12, Folate and Folate-binding Proteins in Dairy Products*. Swedish University of Agricultural Sciences.

Cogan, T. M., Gilliland, S. E., & Speck, M. L. (1968). Identification of stimulants for *Lactobacillus bulgaricus* in tomato juice. *Applied Microbiology*, *8*, 1215–1219.

Elli, M., Zink, R., Reniero, R., & Morelli, L. (1999). Growth requirements of *Lactobacillus johnsonii* in skim and UHT milk. *International Dairy Journal*, *9*, 507–513.

Fox, P. F., & Brodkorb, A. (2008). The casein micelle: Historical aspects, current concepts and significance. *International Dairy Journal*, *18*, 677–684.

Ghaly, A. E., Tango, M. S. A., & Adams, M. A. (2003). Enhanced lactic acid production from cheese whey with nutrient supplement addition. *Agricultural Engineering International: the CIGR Journal of Scientific Research and Development*, *5*, 1–20.

Goff, D. (1999). Dairy science and technology education. Retrieved from www.foodsci.uoguelph.ca/dairyedu/home.html

Hujanen, M., & Linko, Y. Y. (1996). Effect of temperature and various nitrogen sources on L(+)-lactic acid production by *Lactobacillus casei*. *Applied Microbiology and Biotechnology*, *45*, 307–313.

Maehashi, K., Matano, M., Wang, H., Vo, L. A., Yamamoto, Y., & Huang, L. (2008). Bitter peptides activate hTAS2Rs, the human bitter receptors. *Biochemical and Biophysical Research Communications*, *365*, 851–855.

Shihata, A. (2004). *The proteolytic activity of yoghurt and probiotic bacteria for the improved viability of probiotic bacteria in fermented milk products* (Doctoral dissertation, Victoria University, Australia). Retrieved from http://vuir.vu.edu.au/15367.

Sodini, I., Latrille, E., & Corrieu, G. (2000). Identification of interacting mixed cultures of lactic acid bacteria by their exclusion from a model predicting the acidifying activity of non-interacting mixed cultures. *Applied Microbial Biotechnology*, *54*, 715–718.

Surono, S., & Hosono, A. (2011). Starter cultures. *Encyclopedia of Dairy Sciences*.

Tamime, A. Y. (2002). Microbiology of starter cultures. In R. K. Robinson (Ed.), *Dairy Microbiology Handbook* (pp. 261–366). New York: Wiley-Interscience.

Tamime, A. Y., & Robinson, R. K. (1999). Background to Manufacturing Practice. *Yogurt: Science and Technology* (pp. 6–47). Cambridge, UK: Woodhead Publishing.

Tari, C., Ustok, F. I., & Harsa, S. (2009). Optimization of the associative growth of novel yoghurt cultures in the production of biomass, β-galactosidase and lactic acid using response surface methodology. *International Dairy Journal*, *19*, 236–243.

Vandeweghe, P., Norquist, P., & Flynn, E. (2002). U.S. Patent No. 6,399,122 B2. Washington, DC: U.S. Patent and Trademark Office.

Vercet, A., Oria, R., Marquina, P., Crelier, S., & Lopez-Buesa, P. (2002). Rheological properties of yoghurt made with milk submitted to manothermosonication. *Journal of Agricultural and Food Chemistry*, *50*, 6165–6171.

CHAPTER 3

FRACTIONATION AND CHARACTERISATION OF YEAST EXTRACT

3.1 INTRODUCTION

Given the importance of YE as a feedstock in various industrial biotechnology production processes, its characterisation and quality assurance are highly desirable (Loane, Mendel, Abele, Paull, & Macka, 2007). Although YE is generally less expensive than protein hydrolysates, it still remains a relatively expensive material for large-scale fermentation processes (Champagne, Gaudreau, & Conway, 2003). As reported in Chapter 2, the addition of YE to the yogurt fermentation process resulted in a sensorial unacceptable product which had an undesirable flavour. A report by Elli, Zink, Reniero and Morelli (1999) that changes in flavour and colour are usually observed in fermented dairy products supplemented with YE, confirmed these findings. Due to these problems with the end product, it was decided to attempt to identify the specific component present in YE that is responsible for the decrease in yogurt fermentation time. It was anticipated that when used in isolation, the accelerating compound would optimistically not influence the taste and flavour of the final product.

Due to results in Chapter 2 indicating that YE decreases yogurt fermentation time, the goal of this part of the study was to reduce the number of components that could be responsible for the acceleration of fermentation. In order to accomplish this, YE was fractionated, retaining, if not concentrating, the activity of components in the defined fractions.

3.2 MATERIALS AND METHODS

Preparative and analytical fractionation methods were used to fractionate and characterise YE. Preparative fractionation included dialysis, ultrafiltration and size exclusion chromatography, after which analytical fractionation methods followed (Figure 16).

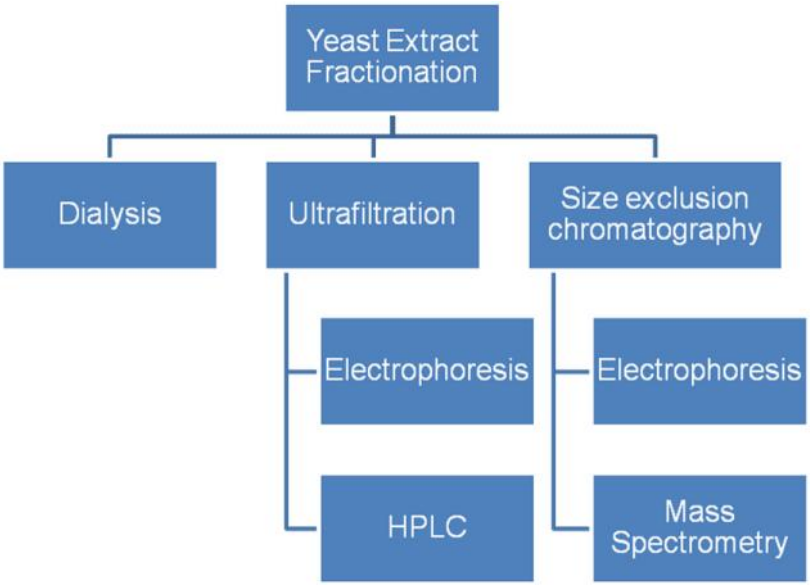


Figure 16 Preparative and analytical fractionation of yeast extract

3.2.1. DIALYSIS OF YEAST EXTRACT

Two dialysis membranes, both supplied by Labretoria, Pretoria, RSA, with molecular weight cut-off (MWCO) of 12-14 kDa and 3.5 kDa respectively, were used for YE fractionation. A calculated length (±15 cm) was cut from each roll where after the membranes were boiled in distilled water before use. Ends were closed with knots. In each of the membrane tubes, 10 g YE, dissolved in 30 ml distilled water was loaded and dialysis was performed at 4 °C in 5 litres of distilled water, stirred with a magnetic stirrer. The distilled water was replaced once after 12 hours. After another 12 hours, 20 ml of each concentrated retentate was used as supplement in two separate yogurt fermentations.

3.2.1.1 YOGURT PREPARATION USING DIALYSIS RETENTATE

After 24 hours of dialysis, 20 ml of the concentrated YE solution was added to 230 ml milk, after which it was pasteurised at 90 °C for 8-10 min, cooled to 42 °C, then inoculated with starter culture (Section 2.2.1.2).

3.2.2 ULTRAFILTRATION OF YEAST EXTRACT

3.2.2.1 PREPARATION OF YEAST EXTRACT STOCK SOLUTION

YE (20 % (w/v)) prepared and stirred for 5 min prior to ultrafiltration.

Dead-end Ultrafiltration

Stirred dead-end ultrafiltration was performed using an Amicon ultrafiltration cell (model 8200, Amicon Inc., Massachusetts, USA). The maximum volume of the ultrafiltration cell was 200 ml with a membrane area of 19.6 cm^2. Nitrogen gas was applied at a pressure of 55 psi. Four different membranes with various MWCO were used successively in this study: 30 kDa, 10 kDa, 3 kDa and 1 kDa.

Before each filtration, each membrane was soaked in distilled water for 90 min with the skin (glossy) side down. The water was changed three times to remove the glycerine coating (which was added by the manufacturer to prevent drying).

Undialised YE was fractionated into 9 fractions using the 1, 3, 10 and 30 kDa MWCO membranes. The 20 % (w/v) YE unfractioned stock solution was used as Fraction 1. All ultrafiltration procedures were carried out at 4 °C. The 30 kDa MWCO membrane was used first. The retentate was collected as Fraction 2, and an aliquot of the filtrate from the 30 kDa filtration was stored, to be used as Fraction 3. The remaining 30 kDa filtrate was further fractionated using the 10 kDa MWCO membrane. The retentate was collected as Fraction 4, and an aliquot of the filtrate was stored as Fraction 5. This procedure was repeated with the 3 kDa and 1 kDa MWCO membranes respectively, during which Fractions 6-9 were obtained. A schematic diagram of the ultrafiltration sequence is depicted in Figure 17.

After the collection of all 9 fractions, protein determinations were performed using a Bicinchoninic acid (BCA) protein assay (See Section 3.2.3).

From each of the 9 fractions obtained during ultrafiltration, 0.5 ml was added to respective batches of 15 ml of milk prior to pasteurisation. The effect of each fraction on the yogurt fermentation process was then subsequently determined.

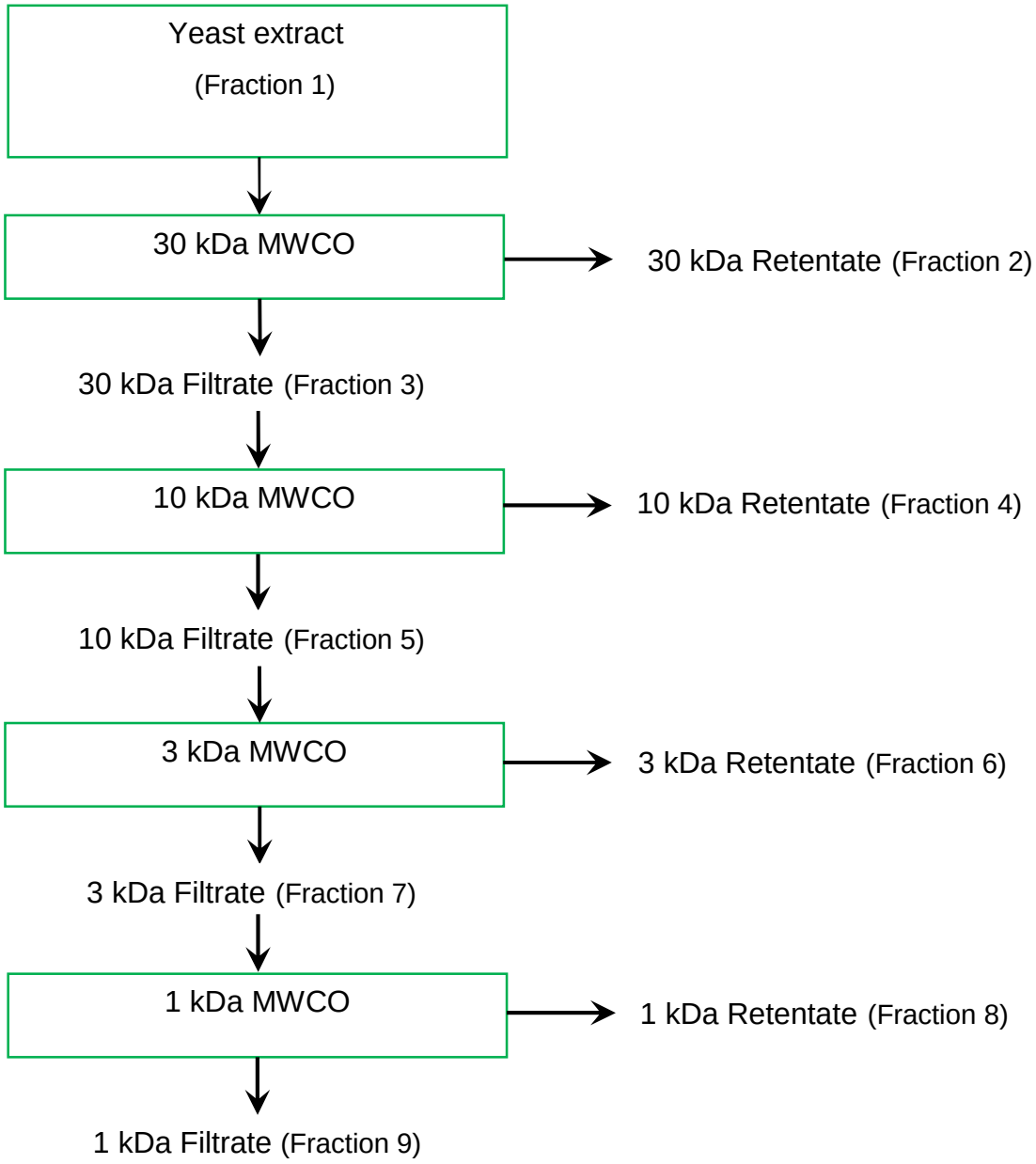


Figure 17 Schematic diagram of the ultrafiltration sequence of yeast extract using 4 individual molecular weight cut-off membranes

3.2.3. PROTEIN ASSAYS OF YEAST EXTRACT ULTRAFILTRATES

A Pierce BCA Protein Assay Kit (Thermo Scientific, Massachusetts, USA) was used for all protein assays. The working reagent (WR) was prepared by combining 12 ml BCA Reagent A with 240 µl BCA Reagent B. Aliquots of 50 µl samples were added to 1 ml WR. The 1.05 ml mixture was then incubated at 37 °C for 30 min. The absorbance of each sample was measured at 562 nm.

3.2.3.1 SPECTROPHOTOMETRY

A Cecil CE2021 2000 series (Lasec, Cape Town, RSA) spectrophotometer was used in all absorbance determinations. Quartz, and where suitable, glass cuvettes with a volume of 1 ml, were used in all experiments.

3.2.4 SUPPLEMENTATION OF YOGURT WITH YEAST EXTRACT ULTRAFILTRATES

Respective yogurt fermentations were carried out using the 9 fractions obtained during ultrafiltration. These fractions included the unfractionated YE as well as the retentates and filtrates from the 30 kDa, 10 kDa, 3 kDa and 1 kDa membranes. Aliquots of 0.5 ml of each of the 9 fractions were added to respective batches of 15 ml milk, prior to pasteurisation. Yogurt fermentations were further carried out as described in Section 2.2.1.5.

3.2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) OF YEAST EXTRACT FRACTIONS

A Fisher Scientific Surveyor HPLC system with quaternary pump, auto sampler and photo diode detector was used. The column was a Phenomenex Jupiter C18 (Thermo Scientific) with particle and pore size of 5 µm and 300 Å, respectively. Column temperature was maintained at 40 °C and that of the autosampler tray at 2 °C. Aliquots of 100 µl sample were injected. The solvents used were HPLC-grade acetonitrile (Merck, Johannesburg, RSA), trifluoroacetic acid (Merck) and deionised distilled water. Gradient elution was performed with solvent A: 5 % aqueous acetonitrile and solvent B: 75% aqueous acetonitrile, both containing 0.1 % trifluoroacetic acid. The initial concentration of Solvent B was 0 %, increased to 50 % in 70 min and then increased to 100 % in 15 min and held for 15 min, after which it was returned to 0 % and held for 15 min for column re-equilibration. All 9 fractions obtained during YE ultrafiltration were analysed using this method. Fractions were collected in 10 ml glass tubes with a Biorad 2110 fraction collector (Biorad, Johannesburg, RSA) and frozen until further analysis.

The 1 kDa filtrate fraction obtained by ultrafiltration (Section 3.2.2) was further fractionated with HPLC. This fraction, as well as an unfractionated YE sample, was precipitated using 24 % (w/v) trichloroacetic acid (TCA) (Merck). Aliquots of 0.5 ml TCA was added to 1.5 ml sample. The samples were then centrifuged for 5 min at 10000 g, after which the supernatant was separated from the precipitate. The precipitate was resuspended in 50 µl distilled water. HPLC was then performed on the supernatant, the precipitate as well as one sample of each of the two fractions which did not undergo any precipitation steps i.e. six samples in total. For these specific samples the HPLC conditions were adjusted to the following: the program began with 5 % acetonitrile which was increased to 40 % in 70 min, where after it was increased to 75 % in 15 min and held for 15 min. After 15 min the concentration was decreased to 5 % in 10 min and held for 20 min before the next sample was injected.

3.2.5.1 PREPARATION OF 1 KDA FILTRATE FRACTION POOLED DURING HPLC ANALYSIS

Further HPLC was performed on the 1 kDa filtrate fraction using the same method as described above. Due to the 1 kDa filtrate accelerating the yogurt fermentation process the most in comparison to the other fractions, only this fraction was further analysed using HPLC. A fraction collector was used, and the eluate was collected in three time intervals: 1-24, 25-72 and 73-108 min. This process was repeated 24 times in order to collect enough protein material to be used as supplements in individual yogurt fermentations.

3.2.5.2 EVAPORATION OF 1 KDA FILTRATE FRACTION POOLED DURING HPLC ANALYSIS

The pooled fractions obtained in Section 3.2.5.1 were evaporated to reduce the sample volume in order to be able to use the fractions in individual yogurt fermentations. A Büchi Waterbath B-480, Büchi Rotavapor R-114 and Büchi Vacuumpump V-700 (all supplied by Sigma-Aldrich, Missouri, USA) were used. The evaporations were performed at 45 °C using 200 Mbar vacuum. This evaporating equipment was used for all samples. Round bottom flasks (250 ml) were used during all evaporations.

3.2.6 SDS-PAGE OF YEAST EXTRACT ULTRAFILTRATES

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in order to assess the sizes of the different YE fractions obtained during ultrafiltration. The “Mighty small” miniature slab gel electrophoresis unit SE 200 was used according the manufacturers’ (Hoefer Scientific Instruments, Massachusetts, USA) specifications. The relative molecular mass of the bands were estimated by comparing the electrophoretic

mobility with those of known proteins. A 15 % resolving gel containing 30 % Acrylamide/ 0.8 % Bisacrylamide solution, 3 M Tris-HCl pH 8.8 solution, 10 % SDS, 10 % Ammonium Persulphate (APS), N, N, N', N'-tetramethylethylenediamine (TEMED) and distilled water was used (All supplied by Merck). The 4 % stacking gel consisted of 30 % Acrylamide/0.8 % Bisacrylamide solution, 0.5 M tris-HCl pH 6.8 solution, 10 % SDS, 10 % APS, TEMED and distilled water (Laemmli, 1970).

The resolving gel was prepared in the Hoefer casting chamber and layered with 1 ml of H_2O saturated tetra-amyl alcohol before polymerisation. The gel was solidified for approximately 20 min at room temperature and the H_2O saturated tetra-amyl alcohol discarded. Any residual H_2O saturated tetra-amyl alcohol was removed by flushing the top end of the resolving gel with 10 ml distilled water.

Once the H_2O saturated tetra-amyl alcohol was completely removed, the stacking gel was added to the top the resolving gel. A 10 well comb was inserted before polymerisation for approximately 15 min at room temperature.

The SDS-PAGE electrophoresis buffer contained 30.28 g/l tris-HCl, 144 g/l glycine (Merck) and 10 g/l SDS (Merck) adjusted to pH 8.5.

3.2.6.1 Sample Preparation

The sample was prepared as follows: 24 µl loading buffer (24 g/l tris-HCl, 1.8 g/l EDTA, 0.34 sucrose and 0.1 g Bromophenol blue at pH 8.8 and 6 µl denaturing buffer (100 g/l SDS and 7.71 g/l dithiothreitol (DTT)) was added to the freeze dried YE fractions and mixed. The samples were transferred to 1 ml eppendorf tubes and heated for 5 min at 96 ºC, after which 25 µl (unless otherwise stated) of each of the samples were loaded into different wells. A volume of 5 µl of the Precision Plus Protein™ Standards All Blue pre stained protein ladder (Biorad) comprising of protein standards ranging from 250 000 Da to 10 000 Da, was loaded in the first well. The Hoefer mini VE Vertical Electrophoresis chamber was connected to an external power supply (Elite 300 Plus, Wealtec, Nevada, USA) and the gel was run at 100 Volts for approximately 1.5 hours.

The gel was stained with Coomassie® Brilliant Blue (CBB) R250 (BioRad) using the Fairbanks method (Fairbanks, Steck, & Wallach, 1971). Once electrophoresis was complete the SDS-PAGE gel was stained overnight with 100 ml Fairbanks buffer A (25 % (v/v) isopropanol (Merck), 10 % (v/v) acetic acid (Merck) and 0.5 g/l Coomassie Brilliant Blue R-250 (Merck)) with gentle stirring. The following day the gel was brought to boiling

temperature and left to cool for 10 min at which point the remaining Fairbanks buffer A was discarded. The gel was then rinsed in 50 ml distilled water and transferred to 100 ml of Fairbanks buffer B (10 % (v/v) isopropanol, 10 % (v/v) acetic acid and 0.05 g/l Coomassie Brilliant Blue R-250). The gel was then briefly boiled for a second time and left to cool for 10 min. The remaining Fairbanks buffer B was discarded and the gel rinsed in 50 ml distilled water. After rinsing, the gel was transferred to 100 ml of Fairbanks buffer C (10 % (v/v) acetic acid and 0.02 g/l Coomassie Brilliant Blue R-250), boiled briefly, left to cool for 10 min, the remaining Fairbanks buffer C was discarded and the gel rinsed with 50 ml distilled water. The gel was then transferred to 100 ml of Fairbanks buffer D (10 % (v/v) acetic acid), boiled briefly and left to cool for 10 min. The remaining Fairbanks buffer D was discarded and the gel placed in 100 ml distilled water.

The above described SDS-PAGE protocol was used in all SDS-PAGE analyses.

A SDS-Page gel was run using all 9 fractions obtained by ultrafiltration.

After staining and subsequent destaining of the gel as described above, the gel was stained using the silver stain technique. The gel was submerged in 1.5 mM sodiumdithionite solution (Merck) for 1 min after which it was washed 3 times for 30 seconds with distilled water. It was then left in 0.2 % silver nitrate for 20-25 min and once again washed in distilled water for 5 min, followed by 5 min in developer (6 % sodium carbonate (Sigma-Aldrich), 2 µM anhydrous sodium thiosulfate (Merck)). The developer was discarded and the gel was placed in 50 ml developer to which 200 µl formaldehyde (Merck) had been added. The reaction was stopped by adding 5 % acetic acid solution directly to the developer mixture. The gel was then washed with distilled water to remove the background.

The SDS-PAGE gel containing the 9 fractions obtained by ultrafiltration was subjected to the silver staining technique after it was stained and de-stained as described in the previous section.

The SDS-PAGE gel containing only the 1 kDa filtrate sample was not subjected to silver staining, as gels stained with this technique cannot be analysed by MS.

3.2.7 Concentration of 1 kDa filtrate sample

After no visible protein bands could be observed during the SDS-PAGE analysis of the 9 fractions obtained by ultrafiltration, the 1 kDa filtrate sample was concentrated. This specific

fraction was used as it contained the smallest components, as well as the fermentation accelerant. Three individual methods were used in order to determine the most effective one: ethanol precipitation, TCA precipitation and isopropanol precipitation.

3.2.7.1 Concentration of 1 kDa filtrate sample by ethanol precipitation

An aliquot of 100 µl 1 kDa filtrate was added to 900 µl of cold ethanol (100 %) and stored at -20 °C overnight. The sample was centrifuged for 15 min at maximum speed (15000 rpm) at a temperature of 4 °C. The supernatant was carefully removed to retain the pellet. The tube was dried by inversion on tissue paper for 15 min after which the pellet was washed with 950 µl 90 % cold ethanol. It was then mixed using a vortex for 10 seconds and centrifuged for 5 min at 15000 g. The wash step was repeated with 950 µl 80 % ethanol. The sample was dried using a Speed Vac (Eppendorf Concentrator 5301, Hamburg, Germany) at speed of 240 g at 65 °C to eliminate any ethanol residue (Wessel & Flugge, 1984). The sample was then resuspended in sample buffer and denatured at 95 °C for 5 min.

3.2.7.2 Concentration of 1 kDa filtrate sample by TCA precipitation

An aliqout of 500 µl 60 % (w/v) TCA was added to 1 ml of the 1 kDa filtrate. The sample was left on ice for 10-20 min. It was subsequently centrifuged for 15 min at 4 °C @ 10000 g. The sample was then rinsed with 500 µl ice cold isopropanol, where after it was centrifuged for 5 min at the previous conditions. The supernatant was removed, and the pellet was dried for 30 min (or until isopropanol had evaporated) using a Speed Vac. The sample was then resuspended in SDS loading dye and denatured at 95 °C for 5 min.

3.2.7.3 Concentration of 1 kDa filtrate sample by isopropanol precipitation

An aliquot of 100 µl of the 1 kDa filtrate fraction was suspended in a ratio of 1:3 with isopropanol, after which it was stored at -20 °C for 30 min. It was centrifuged for 15 min at 4 °C @ 10000-15000 g, where after the supernatant was removed. The pellet was then gently rinsed with 70 % ethanol and recentrifuged. The liquid was removed where after the sample was dried using a Speed Vac for 5-20 min. The sample was resuspended in SDS loading dye and denatured at 95 °C for 5 min.

After the three precipitation methods were used on three different 1 kDa filtrate samples, the samples were loaded on a SDS-PAGE gel.

3.2.8 FRACTIONATION OF YEAST EXTRACT BY SIZE EXCLUSION CHROMATOGRAPHY

Sephadex (Sigma-Aldrich) beads with particles size 20-80 µ and a fractionation range of 1-70 kDa were used during all size exclusion chromatography experiments. Blue Dextran (Merck) and β-mercaptoethanol (Merck) were used to determine the void and final volumes of the column.

The peristaltic pump (BT 100-1J, MRC, Israel) was set to 4 rpm which resulted in a flow rate of 30 ml/h.

Two millilitres of a 1 g/ml YE stock solution was loaded onto the column. It was found that this was an overload. The volume loaded was subsequently reduced to 200 µl. Aliquots of 5 ml were collected in test tubes using a fraction collector (Retriever 500, Teledyne Isco, Nebraska, USA).

The OD of the eluate in each of the test tubes was determined at 230 nm and 280 nm. The 4 main peaks were subsequently identified and the tubes containing the respective peaks were pooled individually and evaporated in a Büchi evaporator (Section 3.2.5.2).

Each evaporated fraction was resuspended in 1 ml distilled water to facilitate transfer to cryogenic tubes and subsequently freeze dried using a Labconco FreeZone 6Plus (Vacutec, Johannesburg, RSA) freeze dryer at 0.01 mBar kept at -89 °C.

3.2.9 EFFECTS OF YEAST EXTRACT FRACTIONS OBTAINED BY SIZE EXCLUSION CHROMATOGRAPHY ON YOGURT FERMENTATIONS

Individual yogurt fermentations were performed using the 4 pooled fractions obtained from size exclusion chromatography. The fractions were added to the milk prior to pasteurisation. To confirm that the combination of the 4 fractions after size exclusion chromatography had the same or similar effect as the unfractionated YE before size exclusion chromatography, aliquots of the four fractions were combined. This combination of fractions was added to an individual milk batch prior to pasteurisation.

3.2.10 Characterisation of Yeast extract fractions obtained by Size exclusion chromatography by Electrophoresis

After repeating the size exclusion chromatography, evaporation and freeze drying of YE, loading buffer (30 µl) was added to each of the 4 freeze dried fractions which were subsequently loaded onto the gel. After it was evident that bands of the accelerating fraction were too faint, the SDS-PAGE was repeated using 70 µl each of the specific fractions as loading volume.

3.2.11 Analysis of Accelerating Fraction by Mass Spectrometry (MS)

Using the SDS-PAGE gel on which 70 µl of the accelerating fraction (Fraction 4) was loaded, the area where the smallest band was initially observed was cut out and MS analysis was performed on this sample. A corresponding region of the exact same size on the same gel was taken from a clean lane in which no sample was loaded to serve as control to eliminate any “disturbances” found during MS analysis.

The protein sample was separated by SDS-PAGE, where after the band of interest was sliced out. The sample was not digested with trypsin due to the fact that it did not contain intact proteins. The peptides generated were extracted for Nano LC/MS/MS analysis. Aliquots of 5 µl of each digest was individually injected and concentrated on a C18 reverse phase trapping column and then eluted onto and separated by a custom packed C18 reverse phase column. The peptides were separated and eluted off the column with a 10 to 25 % Acetonitrile and 5 % formic acid gradient over 60 min. The eluting peptides were analysed on an AB SCIEX 4000 QTRAP hybrid triple quadruple ion trap mass spectrometer with a nanospray source of 350 nl/min using an Agilent Nano HPLC. A survey scan between 400 and 1200 Da was performed looking for eluting peptides. An enhanced resolution scan was performed on peptides to determine the charge state of each peptide before fragmenting the peptides in the collision cell. The peptide sequence information obtained from this MS/MS experiment was analysed by an in-house Mascot server using the latest Swissprot database.

3.2.12 Preparation of Vitamin-, Mineral- And Amino Acid Solutions To Determine Origin Of Accelerator

To establish the nature of the accelerant present in Fraction 4 obtained after size exclusion chromatography of YE, a vitamin- mineral and amino acid solution was prepared respectively. The content of each solution was based on the Oxoid Product Information Leaflet (2009) on YE LP0021 (Tables 2 and 3) reporting the composition of this specific YE.

A mineral solution generally used in fermentations (du Preez & van der Walt, 1983) was prepared and its effect on the yogurt fermentation process was established. All the elements and minerals present in YE according to the Oxoid Product Information Leaflet (2009) were present in this prepared solution. This solution contained iron sulphate (35 mg/l), iron chloride (6 mg/l), zinc sulphate (11 mg/l) (Kanto Chemical Co Inc.), manganese sulphate (7 mg/l), calcium chloride (500 mg/l), magnesium sulphate (200 mg/l), tin(II)chloride (10 mg/l), aluminum sulphate (1.6 mg/l), copper sulphate (1 mg/l), cobalt(II)chloride (2 mg/l), potassium iodide (0.4 mg/l) and sodium molybdate (1.3 mg/l). Unless otherwise stated, all minerals were supplied by Merck.

Mineral analysis was done on the accelerating fraction (Fraction 4) according to the technique used by Hesse (1971). This was done in order to determine whether the same minerals present in YE (reported in the Oxoid Product Information Leaflet (2009)), was also present in the accelerating fraction (Fraction 4). The minerals reported in the Oxoid Product Information Leaflet (2009) included calcium, magnesium, iron, potassium, sodium chloride, copper, tin, zinc, manganese, lead and cobalt. The mineral analysis indicated that the accelerating fraction contained all the minerals present in the prepared mineral solution (prepared according to du Preez and van der Walt (1983)), amongst others. The respective effects of the prepared mineral solution and the minerals present in the accelerating fraction on the fermentation process were evaluated.

The prepared vitamin solution was high in B vitamins due to literature reporting that YE is a good source of specifically these water soluble vitamins (Chae, Joo, & In, 2001; Gaudreau, Champagne, Goulet, & Conway, 1997). The prepared solution contained D-biotin (0.025 mg/l), calcium panthotenate (0.5 mg/l), nicotinic acid (0.5 mg/l), aminobenzoic acid (0.1 mg/l), pyrodoxine (0.5 mg/l), thiamine (0.5 mg/l), folic acid (0.004 mg/l), niacin (0.6 mg/l), riboflavin (0.4 mg/l) and inositol (12.5 mg/l). All vitamins were supplied by Sigma-Aldrich.

The amino acid solution contained 50 mg/l of each of the following (all supplied by Merck): adenine hemisulfate salt, L-alanine, L-argininine monohydrochloride, L-asparagine monohydrate, L-aspartic acid sodium salt monohydrate, L-cysteine hydrochloride monohydrate, L-glutamic acid hydrochloride, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine monohydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, uracil and L-valine. A stock solution of all components except the leucine and cysteine was prepared. The pH was then adjusted to pH 11 with NaOH (Merck), whereafter the leucine (400 mg/l) and cysteine (50 mg/l) were added. In cases where there was difficulty in leucine and cysteine solubility, KOH was added until successfully dissolved.

Each of these solutions was fractionated by size exclusion chromatography in order to determine the respective elution times.

3.2.13 FREE AMINO GROUPS PRESENT IN YEAST EXTRACT

The amount of free amino groups in the four identified peaks obtained from size exclusion chromatography (Section 3.2.8) was determined in triplicate by the Cd-ninhydrin method according to Folkertsma and Fox (1992). An aliqout of 20 µl sample was diluted to 1 ml in distilled water to which 2 ml Cd-ninhydrin reagent (0.8g ninhydrin dissolved in a mixture of 80 ml 99.5 % ethanol and 10 ml acetic acid, followed by the addition of 1 g $CdCl_2$, dissolved in 1 ml distilled water) was added. The mixture was heated to 84 °C for 5 min and cooled to room temperature whereafter the absorbance was determined on a Genesys 10 Vis Thermo Spectronic spectrophotometer at 570 nm. The results were expressed as mg leucine/g using a standard curve. The standard curve was prepared by using a stock solution of 10 mM of leucine in distilled water. The stock solution was diluted to a range of 0.5-10 mM in distilled water.

3.2.14 AMINO ACID ANALYSIS OF THE ACCELERATING FRACTION (FRACTION 4) POOLED AFTER SIZE EXCLUSION CHROMATOGRAPHY

After it was determined that the accelerant present in Fraction 4 might be of amino acid origin, the amino acid composition of Fraction 4 obtained after size exclusion chromatography of YE (Section 3.3.9) was determined by using a Waters AccQ Tag Ultra Derivitisation Kit. An aliquot of 10 µl of the diluted sample was added to the Waters AccQ Tag Kit constituents and placed in a heating block at a temperature of 55 °C for 10 minutes,

after which it was analysed using a Waters API Quattro Micro API (Thermo Scientific). The inlet method that was used was as follows:

Solvents A1 and A2: Eluent A2 (100 ml Eluent A concentrate and 900ml Water)

Solvents B1 and B2: Eluent B (Supplied by AccQ Tag Kit)

Column: AccQ Tag C18, 1.7 µm, 2.1x100 mm

Injection: 1 µl

MS conditions: Capillary voltage: 3.5 kV Cone voltage: 15 V Source: 120 ºC

Desolvation temp: 350 ºC Desolvation gas: 350 l/h Cone gas: 50 l/h

3.2.14.1 AMINO ACID HYDROLYSIS

Before hydrolysis, the tubes were washed with a mixture of HCl and HNO_3 in order to remove any residues present. After this mixture was left in the tubes for ±1 hour, the tubes were rinsed with potable water, and thereafter with distilled water. Known concentrations of each sample were then carefully added into each tube. The samples were then dried using a Speed Vac (Eppendorf Concentrator 5301, Hamburg, Germany) at a speed of 240 g at 65 °C to eliminate any ethanol residue after which 6 N HCl, containing ±1% phenol (Merck), was added. The phenol was added to protect the tyrosine in the sample by acting as an oxygen scavenger. Vacuum was applied while the samples were kept on ice in an ultrasonic bath (UMC 5, Integral Systems, Johannesburg, RSA) in order to degas the samples. The samples were then heated to 105-110 °C for 24 hours. The samples were then removed and cooled to room temperature for 30 min. The samples were once again dried using a Speed Vac.

3.3 RESULTS AND DISCUSSION

3.3.1 DIALYSIS OF YEAST EXTRACT

YE was dialysed as it was anticipated that the accelerating component would be retained by the MWCO membranes used. In order to determine in which size range the accelerating compound in YE lies, a dialysis membrane with MWCO of 12-14 kDa was firstly used. The preparation of yogurt with the dialysed retentate (Figure 18) indicated no difference in fermentation time in comparison to the negative control which contained no YE. The positive control which contained 0.4 g/l YE however resulted in a 27 % decrease in fermentation time. These results indicated that the accelerating component in YE was not present in the retentate. It could therefore be concluded that it diffused out into the distilled water indicating the molecular weight of the component was smaller than 12-14 kDa.

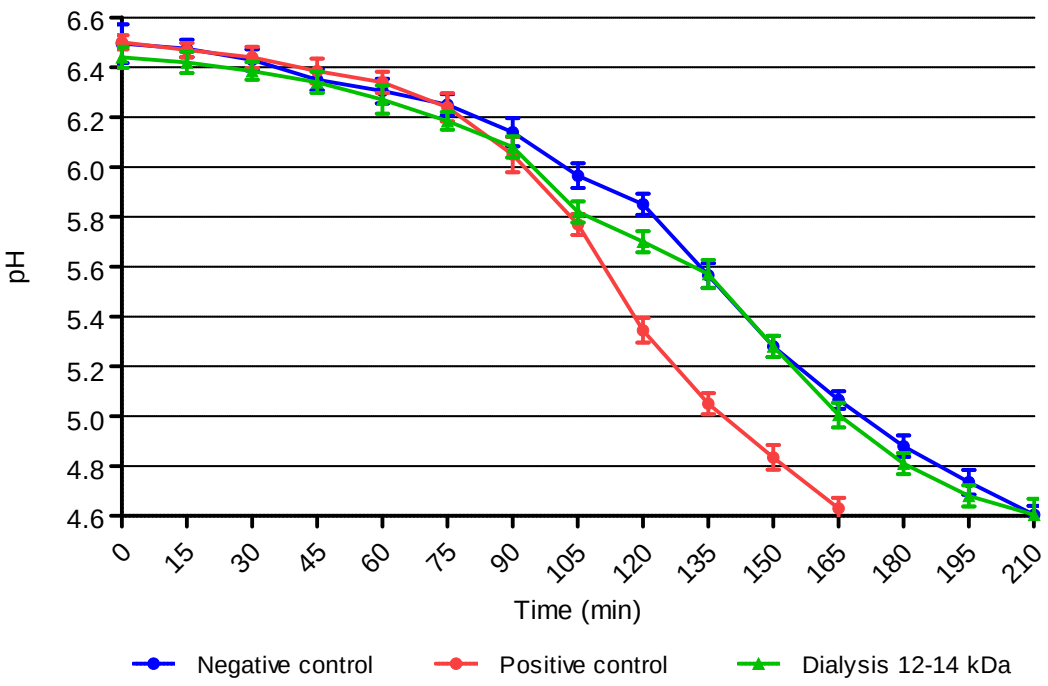


Figure 18 Comparison of yogurt supplemented with dialysed yeast extract (12-14 kDa membrane) with positive and negative controls

The use of a 3.5 kDa dialysis membrane however, delivered different results. The positive control once again resulted in a 27 % decrease in fermentation when compared to the negative control. The retentate obtained by 3.5 kDa dialysis decreased yogurt fermentation time by 14 %. This was an improvement on the negative control, although the unfractionated YE showed a further 14 % improvement (Figure 19).

The fact that the decrease in fermentation time resulting from the dialysed supernatant supplementation was not equal to that of the negative control indicated that the accelerating component present in YE has a molecular weight lower or equal to 3.5 kDa. If the component was larger than 3.5 kDa, acceleration similar to that of unfractionated YE would have been observed, which was not the case. However, another possible explanation for the limited decrease in fermentation time could be that some larger peptides containing the accelerant were retained and did not diffuse into the exterior dialysis environment as was the case for the pure accelerating fraction.

Although dialysis removed the salt from YE (Park & Lee, 1999), the low molecular weight components present in YE were also removed. This was problematic due to the possibility that the decrease in yogurt fermentation time observed with YE supplementation was owing to the presence of certain low molecular weight components. The aim was therefore to retain all YE fractions by using other fractionation methods, such as ultrafiltration.

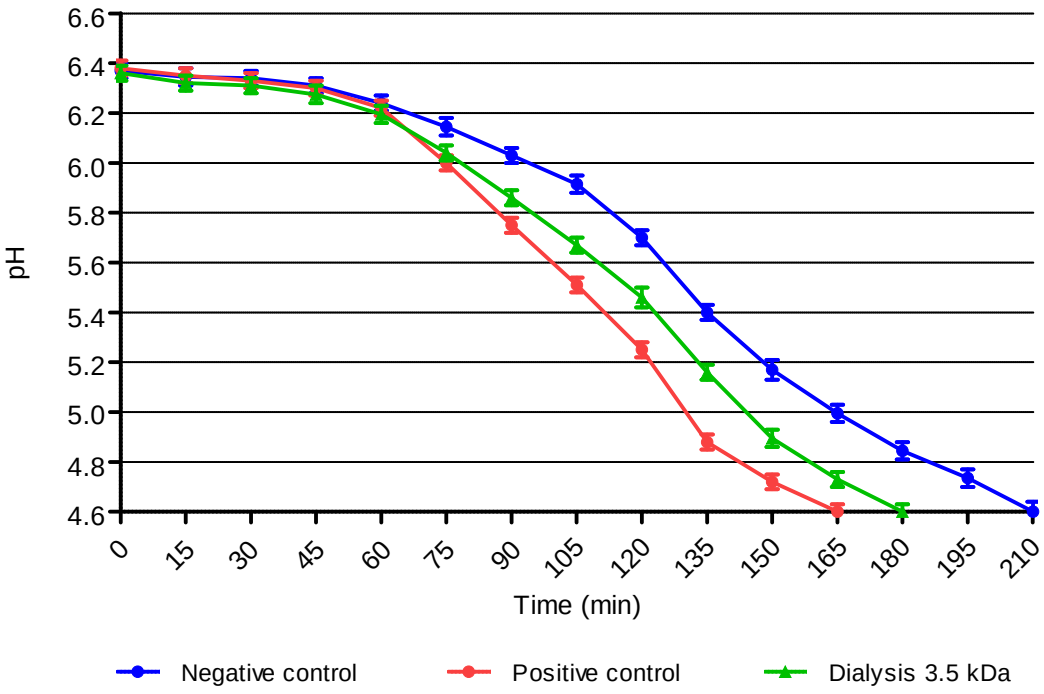


Figure 19 Comparison of yogurt supplemented with dialysed yeast extract (3.5 kDa membrane) with positive and negative controls

3.3.2 SUPPLEMENTATION OF YOGURT WITH YEAST EXTRACT ULTRAFILTRATES

After determining during dialysis that most of the accelerant diffused out of the smallest evaluated membrane (3.5 kDa), it was decided to move to a smaller exclusion, using ultrafiltration. Undialised YE was fractionated into 8 fraction size groups using 4 different MWCO membranes. This was done by passing the solution, successively, through membranes of decreasing pore size. The largest proteins were retained by the membrane with the largest pore size. The initial YE concentration was 200 mg/ml.

3.3.2.1 PROTEIN ASSAYS OF YEAST EXTRACT ULTRAFILTRATES

The protein content of each fraction was determined using a BCA protein assay kit.

Using BSA (Bovine Serum Albumin), a standard curve was determined as OD = 0.001019x + 0.009307 (Figure 20).

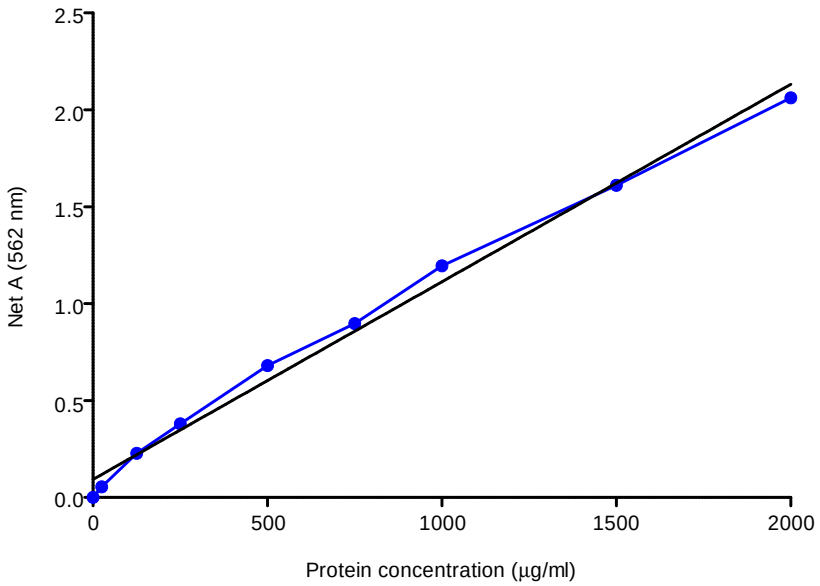


Figure 20 Colour response curve for bovine serum albumin using the Standard Test Tube Protocol (30 min at 37 °C incubation)

Table 5 Protein content of yeast extract fractions obtained during ultrafiltration

Ultrafiltration fractions	Protein concentration (mg/ml)
Unfractionated YE	96±3.23
30 kDa Retentate	96±2.7
30 kDa Filtrate	97±2.5
10 kDa Retentate	61.13±1.41
10 kDa Filtrate	60.44±3.6
3 kDa Retentate	58.05±3.4
3 kDa Filtrate	56±2.8
1 kDa Retentate	48.06±3.4
1 kDa Filtrate	38.93±2.85

*All values are means of triplicate values

It is clear from the protein assay results (Table 5) that the protein content of the various fractions decreases as the size of the MWCO membrane subsequently decreases. The unfractionated YE, 30 kDa retentate and 30 kDa filtrate contained the same amount of protein. From 10 kDa retentate the protein content decreases up until 1 kDa filtrate. The 1 kDa filtrate fraction still contained 38.9 % of the total protein.

Interestingly, Yoo (2009) noted similar low growth patterns of the different fractions could not be related to the different amounts of total peptide estimated in the various fractions.

3.3.2.2 INDIVIDUAL YOGURT FERMENTATIONS USING ULTRAFILTRATION FRACTIONS

The effect of supplementing yogurt with the 9 fractions (each containing a mixture of components) obtained from ultrafiltration was assessed.

From Figure 21 it was evident that the unfractionated YE and all the retentate and filtrate fractions decreased yogurt fermentation time by 21.4 %.

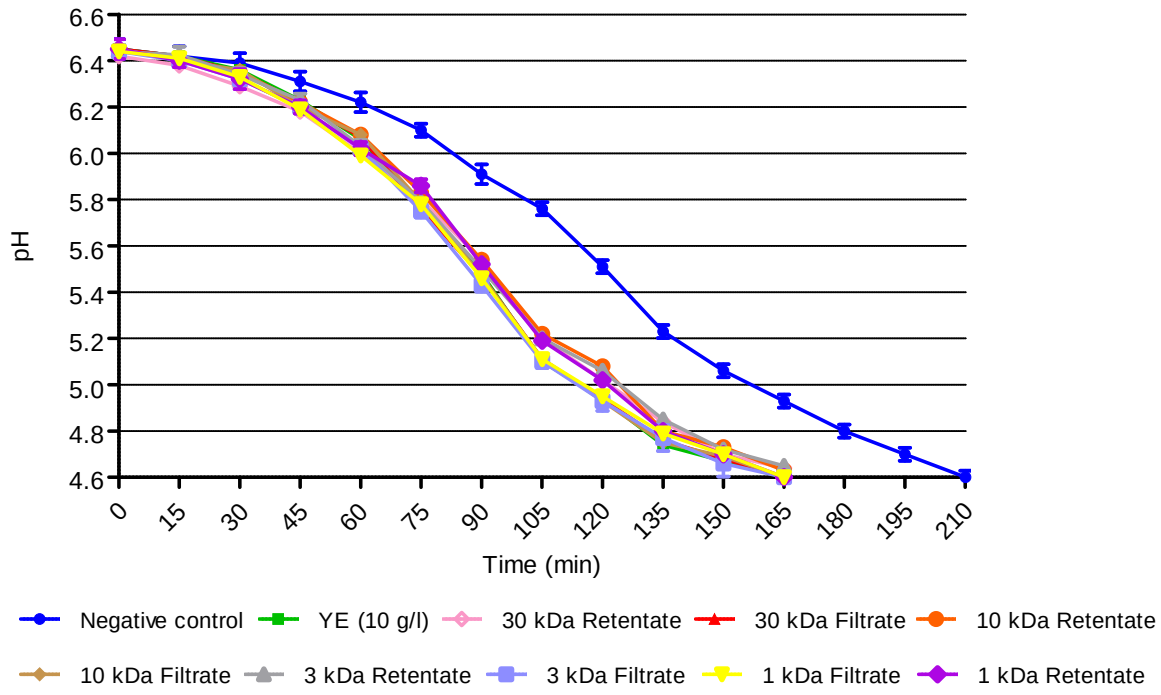


Figure 21 Effects of fractions obtained during yeast extract ultrafiltration on yogurt fermentation time

Owing to the fact that all the YE fractions obtained from ultrafiltration decreased yogurt fermentation time, it could be assumed that the accelerating component was present in all fractions and was therefore smaller than 1 kDa. If it is assumed that the accelerator is of low molecular weight, the retentates obtained by the high MWCO membranes should theoretically not decrease the fermentation time. A possible explanation for this phenomenon could be that not all liquid could be filtered through the various membranes and a small remaining amount was used as the respective retentate fractions. Some of the accelerant would therefore still be present in the retentate fraction, although it was small enough to move through the respective MWCO membrane. For this reason the high molecular weight retentate fractions had the same effect on respective yogurt fermentation times as did the lower molecular weight filtrate fractions. The protein concentrations reported in Table 5 are therefore only estimates of the total protein present in each fraction. It was however important to note that the higher molecular weight components could not be eliminated with certainty and therefore other fractionation methods had to be investigated i.e. HPLC and size exclusion chromatography.

If the assumption that the accelerant had a low molecular weight is accepted as correct, then the 1 kDa filtrate fraction contained the purest form of the accelerating compound due to the fact that most peptides larger than 1 kDa had been removed during ultrafiltration.

The above conclusions are in accordance with a study by Smith, Hillier and Lees (1975) which indicated that YE contains peptides of small size which have potential as growth enhancers. Yoo (2009) stated that approximately 50 % of the amino acids present in YE are smaller than 3 kDa and occur as important nutrients for cells in culture.

During studies done by Yoo (2009) using Chinese Hamster Ovary cells in serum free basal medium as growth medium, YE showed no growth enhancement for any of the fractions (50 kDa filtrate, 50 kDa retentate, 3 kDa filtrate, 3 kDa retentate, 1 kDa filtrate, 1 kDa retentate) when compared to a culture supplemented with the crude non-fractionated YE. Supplementation of the serum free basal medium with the various YE fractions was less growth-promoting compared to the YE negative control. This suggested that the YE fractions may have contained growth inhibitory elements in higher amounts than in the crude unfractionated YE (Yoo, 2009). Contradictory to the results reported by Yoo (2009), when using milk as medium in the current study, results indicated that all 9 the ultrafiltrated YE fractions, including the unfractionated YE, resulted in the same decrease in yogurt fermentation time.

Gaudreau *et al*. (1997) have compared the effect of ultrafitration of YE on their ability to promote LAB growth. Five types of YE, of which three were from bakers' yeasts and two from brewers' yeasts origin were fractionated by ultrafiltration (UF) with 1, 3, and 10 kDa MWCO membranes. The fractions were concentrated by freeze-drying and the resulting powders of YE filtrates (YEF) were evaluated for their growth-promoting properties on 9 cultures of LAB. The source of YE i.e. bakers' or brewers' yeast had a much greater effect on the growth of LAB than the respective ultrafiltrate fractions. Three *Lactobacilllus casei* strains evaluated by Gaudreau *et al*. (1997) did not grow as well in media containing the YE filtrates obtained after filtering with 1 and 3 kDa membranes. In the above study a single bacterium strain was used, and not a mixed culture as in the case of the current study.

In studies done by Smith *et al*. (1975) it was found that after YE had been fractionated, the fraction most stimulatory to the growth of *Streptococcus lactis* contained over 70 % of the amino nitrogen present in the YE. This fraction consisted of a wide variety of free amino acids and a small amount of peptide material. Examination of possible replacement factors for this fraction revealed that the amino acid material present was largely responsible for the stimulation of *Streptococcus lactis.* It appeared that YE and the other amino acid supplements prevented an observed inhibition of the growth of the slow strains below pH 6.0. This was apparently due to the YE satisfying a nutritional deficiency caused by a drop in pH (Smith *et al*., 1975). These results correspond to the results obtained in the current study

where it was clear that the smallest fraction, 1 kDa, in which all the free amino acids and small peptides would be present, contained the accelerant.

3.3.3 CHARACTERISATION OF YEAST EXTRACT AND YEAST EXTRACT ULTRAFILTRATES BY HPLC

HPLC analysis was done in order to determine whether a specific peak present in all 9 fractions obtained from YE ultrafiltration, could be identified.

It was evident from the 9 HPLC profiles that as the MWCO of the various membranes decreased, the base line in the chromatograms subsequently decreased (Figures 22-30). It was also apparent that certain peaks became more prominent as the MWCO of the membranes decreased. This was especially noticeable in the initial part (first 20 min) of the separations. Most of the peaks in all 9 HPLC graphs eluted between 0-35 min. In the chromatograms of the smallest fractions i.e. 1 kDa filtrate, 1 kDa retentate, 3 kDa filtrate and 3 kDa retentate, the main peaks all eluted before 25 min. Two distinctive peaks which eluted at ±15 min and ±17 min, respectively, were present in all 4 these fractions.

After determining the above, HPLC analysis was done using only the 1 kDa filtrate fraction as it was evident from results discussed in Section 3.3.2.2 that the accelerating compound had a low molecular weight. The 1 kDa filtrate fraction was precipitated using TCA. This was done due to the fact that TCA is significantly less effective in precipitating unfolded or denatured proteins (Rajalingam, Loftis, Xu, Kumar, & Krishnaswamy, 2009). Most peptides would therefore still be in solution.

It was anticipated that by analysing the precipitate and supernatant (obtained after precipitation of the 1 kDa filtrate) with HPLC, different peaks would be observed between the aforementioned fractions. The original 1 kDa filtrate fraction (which had not been precipitated with TCA) was used as control. There were therefore 3 samples in total of which the results can be seen in Figures 31-33. The chromatogram of the 1 kDa filtrate (Figure 31) did not correspond with that obtained during initial HPLC analysis (Figure 22) where the same fraction was used. This is due to the fact that the running conditions of the two groups of experiments were different. During the initial HPLC analysis the acetonitrile concentration ranged from 0 % which was increased to a final concentration of 100 %, whereas during the analysis on the TCA precipitated fractions, the initial acetonitrile concentration was 5 %

which was increased to a final concentration of 75 %. By stretching the acetonitrile gradient the peaks in the respective chromatograms were more distinguishable.

Theoretically, combined, the precipitate and supernatant HPLC chromatographs must have a combined profile of the chromatograph of the unprecipitated 1 kDa filtrate fraction. As can be seen in Figure 31, this was not the case. Although the presence of TCA in the samples prevented determining their effects on individual yogurt fermentations, the HPLC profiles of the 3 samples discussed above were determined. No distinctive peaks could however be identified when comparing the precipitate and supernatant fractions. Further research using TCA precipitation was therefore not done.

The chromatogram obtained during HPLC analysis of the 1 kDa filtrate fraction (Figure 31) was used as a guideline during the determination of which part of this fraction was responsible for the decrease in yogurt fermentation time. Only this fraction was used due to the fact that it was evident from previously described results that it contained the accelerating fraction. From the peaks observed in the chromatogram depicted in Figure 31, it was evident that the components present in the samples eluted in three main groups. HPLC analysis of the 1 kDa filtrate fraction was therefore repeated during which three different aliquots were collected based on their elution times. Elution time 1-24 min formed the first group and elution time 25-72 min the second group. The third time interval that was collected was the components which eluted between 73 and 108 min.

The effects of the three pooled aliquots on independent yogurt fermentation processes are depicted in Figure 34. The components present in the first elution time interval (1-24 min), which contained most of the hydrophilic components present in the sample, increased the yogurt fermentation time (7 %) in comparison to the negative control. The second pooled fraction (25-72 min) increased fermentation time by 45 min (21 %), and the third pooled fraction increased time to reach pH 4.6 by 60 min (28 %) in comparison to the negative control. The separation of the 1 kDa filtrate by HPLC analysis was repeated several times in order to obtain a substantial amount of sample with which the individual yogurt fermentations could be performed. However, no positive effect on the fermentation process was observed after supplementation with any of the 3 individually pooled fractions. This could be attributed to the presence of trifluoroacetic acid (TFA) in the samples used during HPLC analysis as an ion pairing agent. The acid (TFA) most probably influenced the acid production ability of the starter bacteria which then subsequently resulted in an increase in yogurt fermentation time. The absence of any accelerating effect could also be due to a possible synergistic effect exhibited by the combination of the 3 pooled fractions.

The aim of all of the above HPLC analyses was to determine whether any distinctive peaks were present in more than one of the 9 fractions as all 9 fractions decreased yogurt fermentation time equally. The HPLC chromatograms showed an abundance of peaks, indicating a huge amount of proteins and peptides present in all fractions.

In an attempt to obtain more conclusive data, the profiles of the 9 fractions obtained from ultrafiltration of YE were further analysed by using SDS-PAGE.

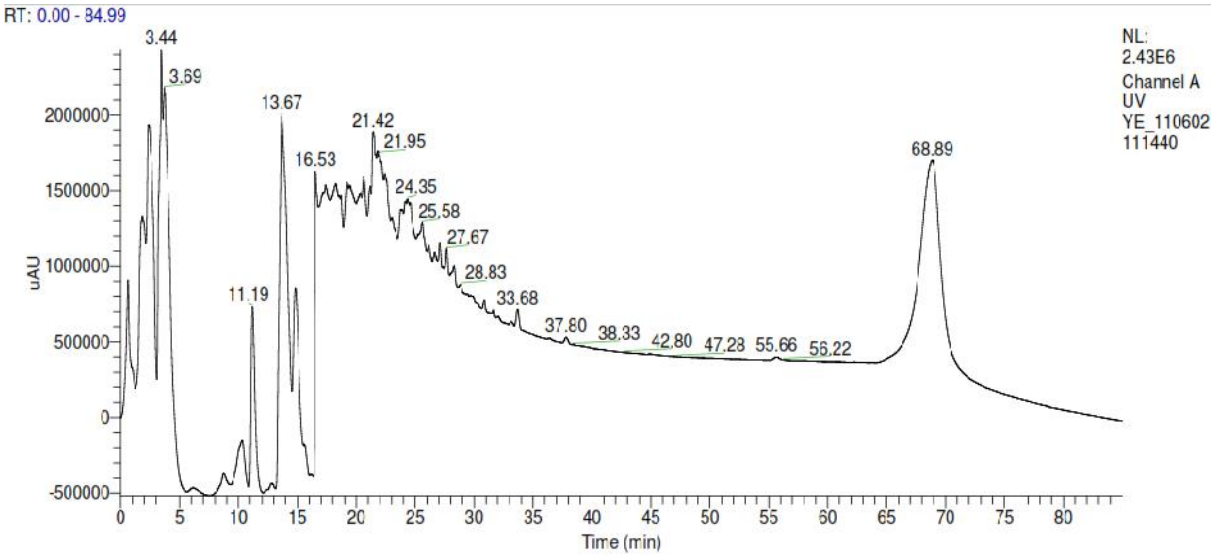


Figure 22 HPLC chromatogram of unfractionated yeast extract

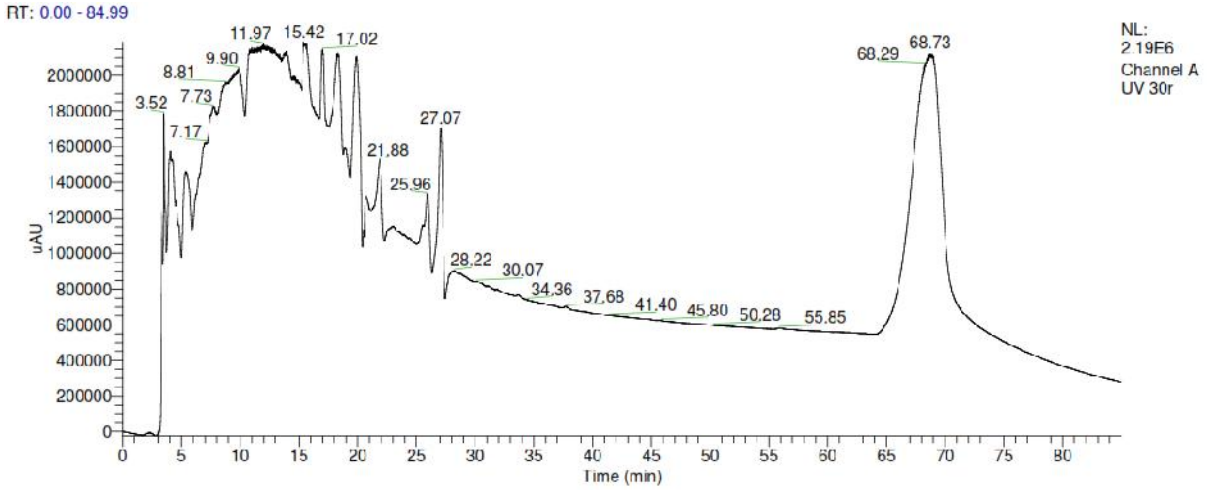


Figure 23 HPLC chromatogram of the 30 kDa retentate fraction obtained by ultrafiltration of yeast extract

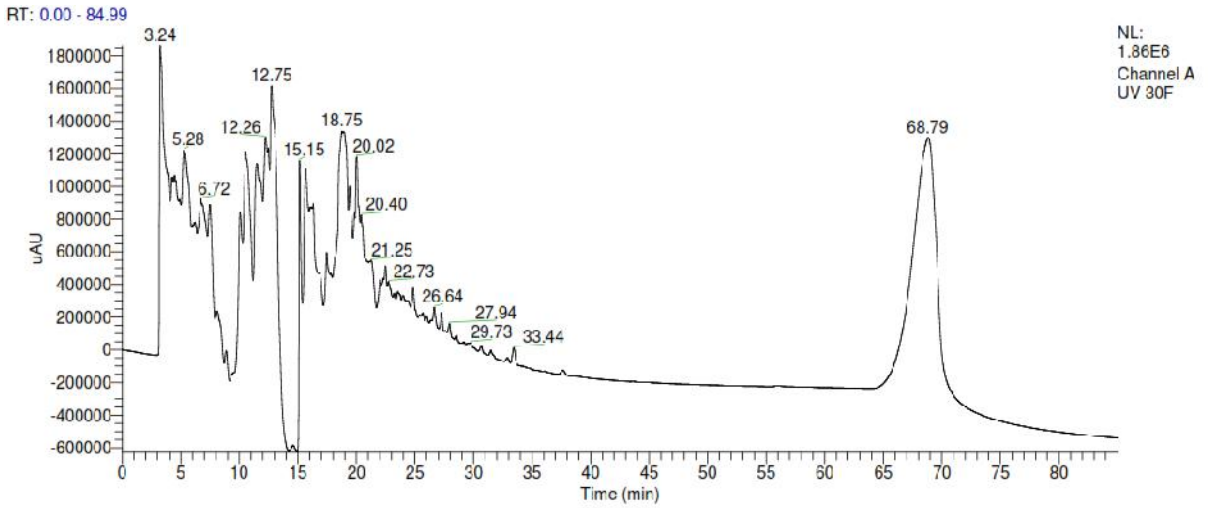


Figure 24 HPLC chromatogram of the 30 kDa filtrate fraction obtained by ultrafiltration of yeast extract

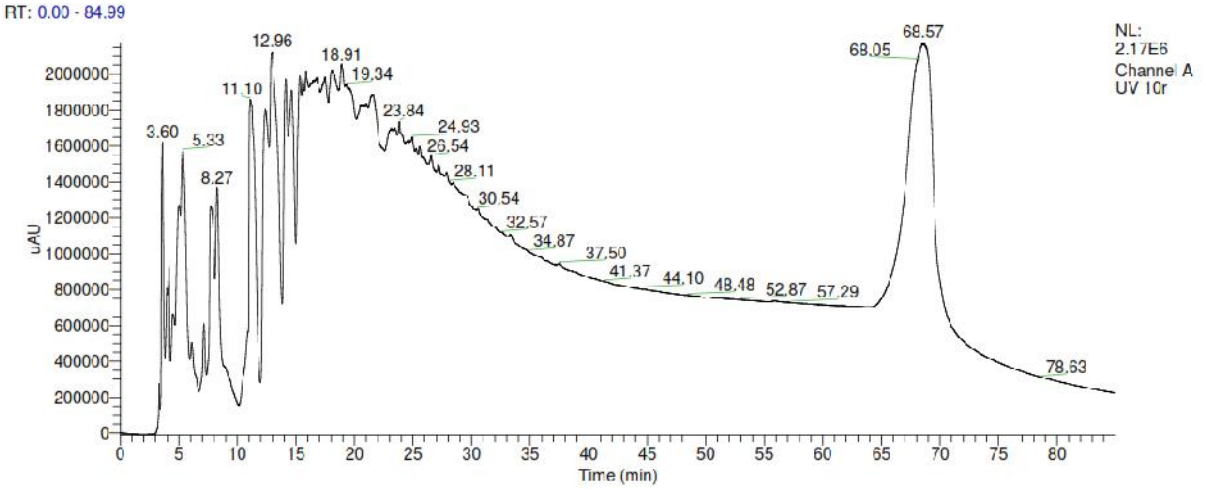


Figure 25 HPLC chromatogram of the 10 kDa retentate fraction obtained by ultrafiltration of yeast extract

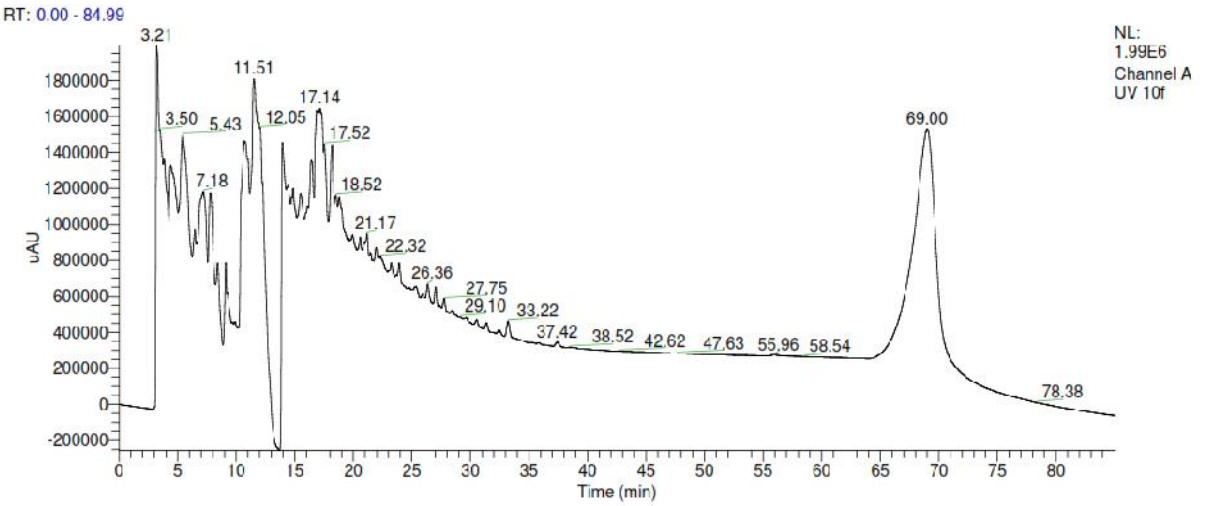


Figure 26 HPLC chromatogram of the 10 kDa filtrate fraction obtained by ultrafiltration of YE

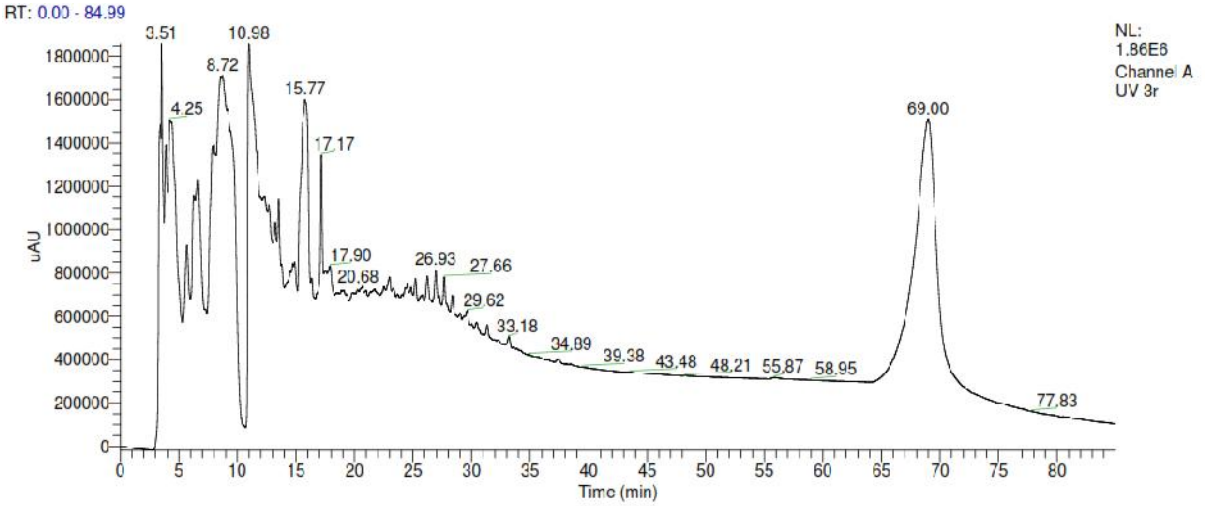


Figure 27 HPLC chromatogram of the 3 kDa retentate fraction obtained by ultrafiltration of yeast extract

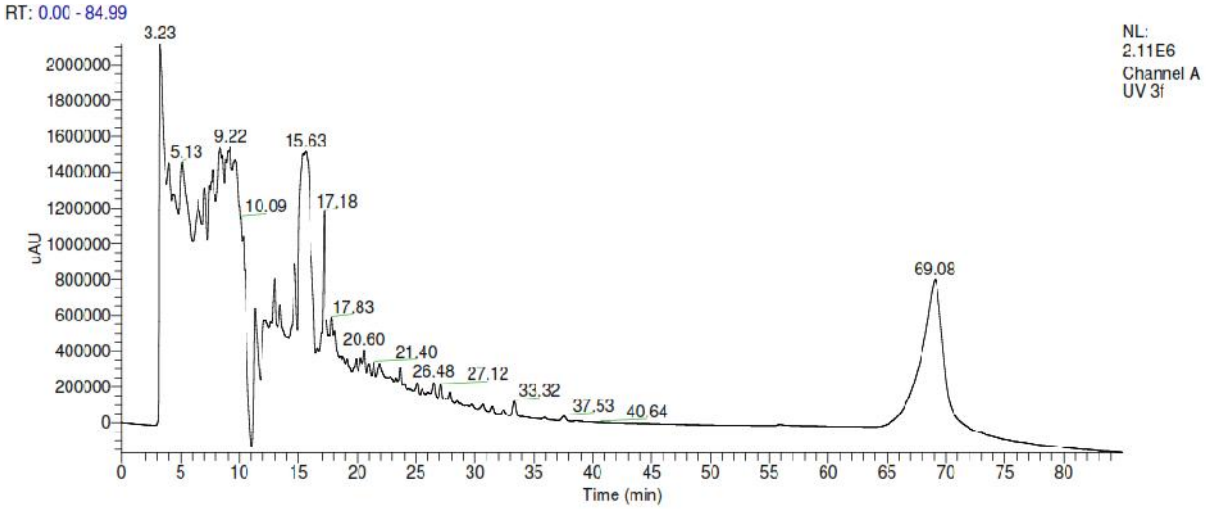


Figure 28 HPLC chromatogram of the 3 kDa filtrate fraction obtained by ultrafiltration of yeast extract

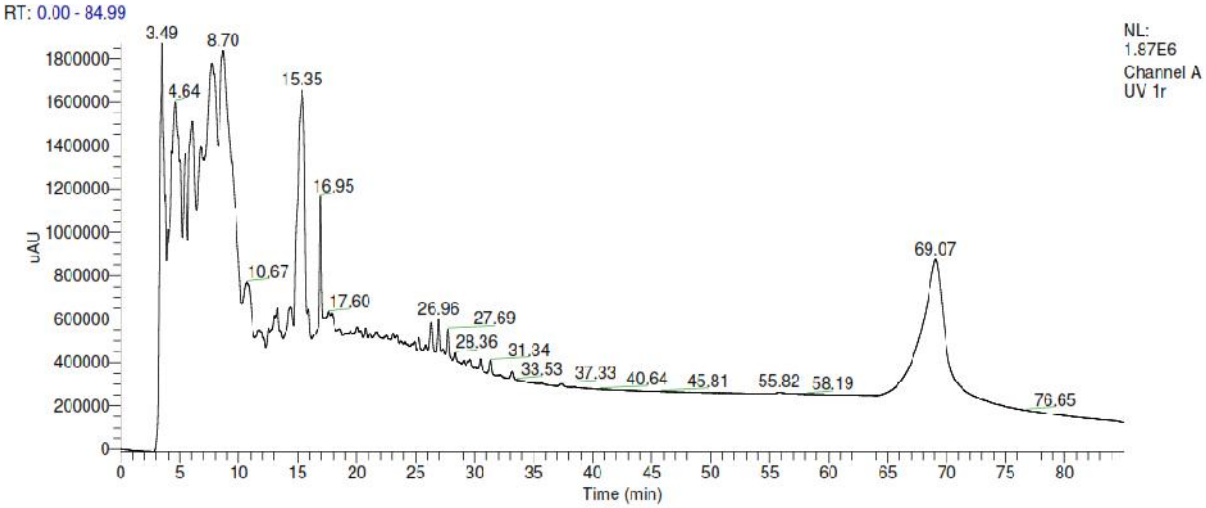


Figure 29 HPLC chromatogram of the 1 kDa retentate fraction obtained by ultrafiltration of yeast extract

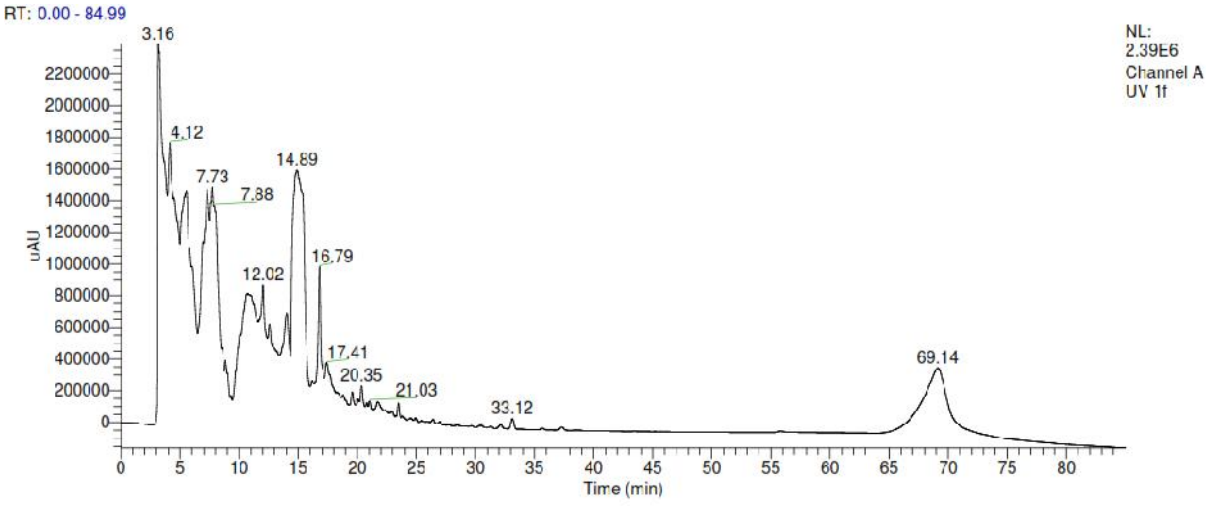


Figure 30 HPLC chromatogram of the 1 kDa filtrate fraction obtained by ultrafiltration of yeast extract

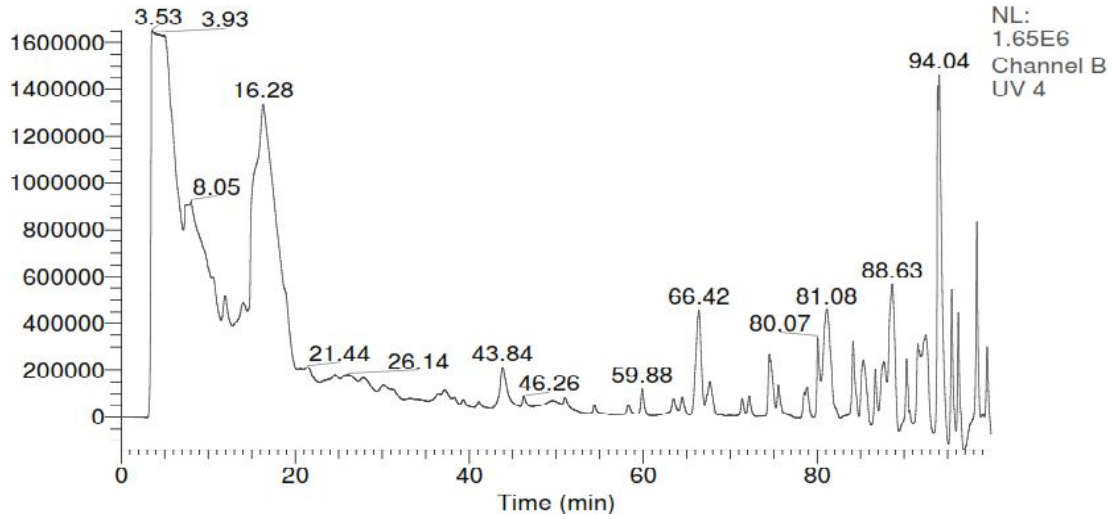


Figure 31 HPLC chromatogram of 1 kDa filtrate fraction obtained by ultrafiltration of yeast extract with adapted acetonitrile conditions 5%-75%-5%.

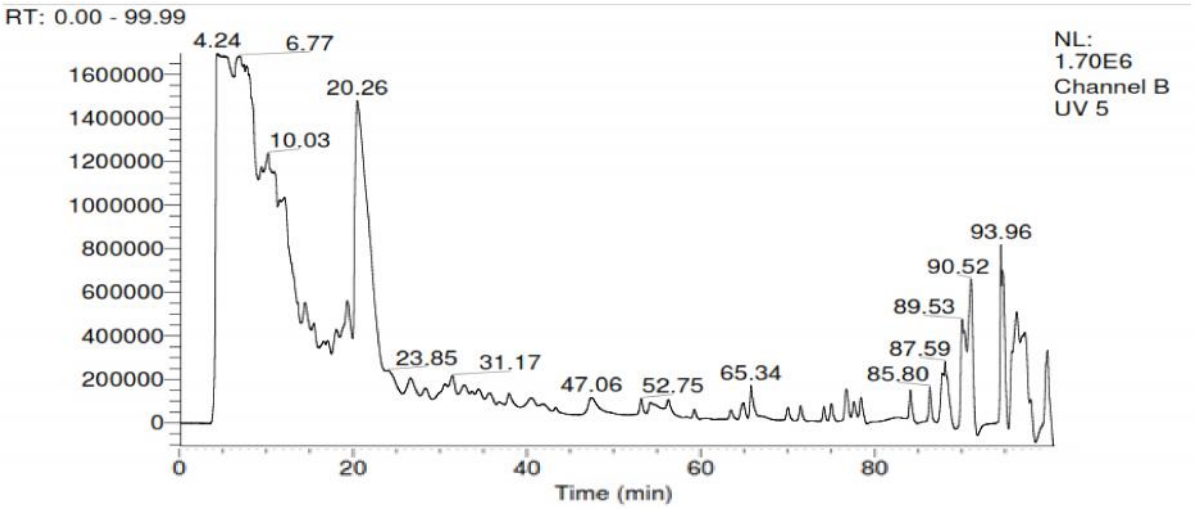


Figure 32 HPLC chromatogram of the supernatant obtained by TCA precipitation of 1 kDa filtrate fraction obtained by ultrafiltration of yeast extract

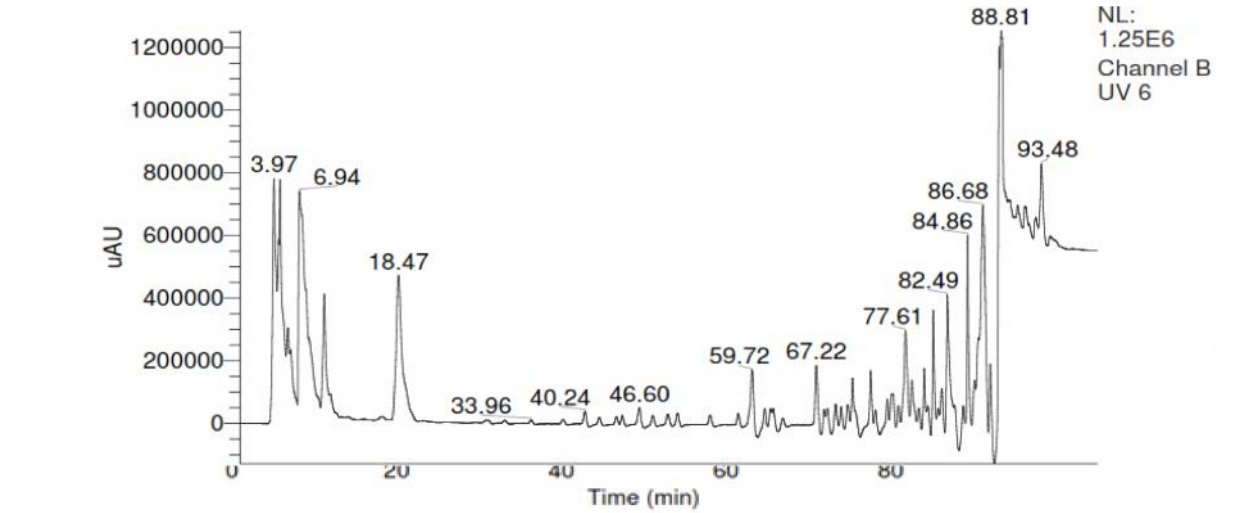


Figure 33 HPLC chromatogram of the precipitate obtained by TCA precipitation of 1 kDa filtrate fraction obtained by ultrafiltration of yeast extract

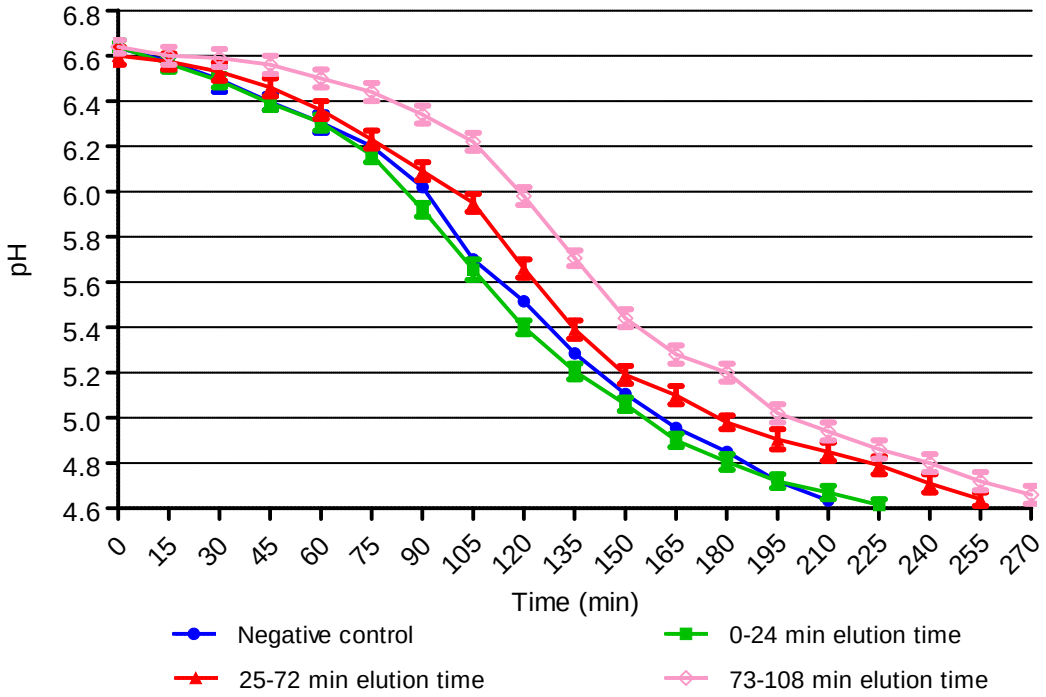


Figure 34 Yogurt fermentations with HPLC pooled fractions of 1 kDa filtrate sample

3.3.4 CHARACTERISATION OF YEAST EXTRACT AND YEAST EXTRACT ULTRAFILTRATES BY SDS-PAGE

SDS-PAGE analysis was done on the 9 samples obtained during ultrafiltration of YE in order to determine whether a band common to all 9 fractions could be identified. From Figure 35 it was evident that the visible bands of all the filtrate fractions (30 kDa, 10 kDa, 3 kDa and 1 kDa) had similar molecular weights. With exception of the 30 kDa retentate where a smear was visible (most probably due to overloading of sample), the visible protein bands of the retentates decreased as the molecular weight of the various fractions decreased. A band visible at ±12 kDa could be observed in all of the fractions except in the lane loaded with the 1 kDa filtrate fraction.

Although the 10 kDa filtrate fraction was used to load onto the 3 kDa MWCO membrane during ultrafiltration, the bands of the 3 kDa retentate were not equal to the sum of the 10 kDa filtrate plus 3 kDa filtrate fractions. The higher molecular weight band of the 3 kDa retentate in comparison to the 10 kDa filtrate can possibly be ascribed to the fact that the higher molecular weight components present in the 3 kDa retentate fraction were highly concentrated and therefore more visible on the electrophoretogram. No bands were visible in the lane loaded with the 1 kDa filtrate sample. Shihata (2004) reported similar findings which included that the detection of small molecules like peptides and amino acids are impossible to visualise in PAGE because of problems with fixing and staining. After staining with CBB, the gel in Figure 35 was subjected to the silver staining technique. The only additional information provided by this technique was the slight increase in visibility of the two smallest bands of the 10 kDa retentate sample below the 10 kDa area (indicated with a red block in Figure 36), which were stained by silver staining and not by CBB. It was hypothesised that one of these bands could contain the accelerator although these bands were not visible in any of the other samples.

If the lane containing the 10 kDa retentate fraction had not been overloaded (as was the case), the two bands smaller than 10 kDa would not have been visible after silver staining. A ±22 kDa molecular weight band was visible in the 10 kDa retentate fraction but not visible in either the unfractionated YE or the 30 kDa retentate fractions. This could be due to overloading of the latter two fractions which resulted in a smear. The overloading subsequently prevented the visualisation of individual high molecular weight bands. The overloading was therefore necessary to visualise bands with low molecular weight, as can be seen below the 10 kDa region of the 10 kDa retentate fraction. It could also be concluded that the proteins present in the 10 kDa retentate fraction were highly concentrated though

still containing low molecular weight components as can be seen in the red block in Figure 36. Although the lane containing the 30 kDa retentate fraction produced mostly a smear, bands were visible between the 17 and 25 kDa region as well as below the 10 kDa region.

As was the case after CBB staining, no bands were visible in the 1 kDa filtrate lane after silver staining.

What the above described results clearly indicate is the difficulty encountered during the determination of the correct sample concentration. A fine balance had to be established between visualising low molecular weight bands and sample overload. Sharply defined protein bands of all proteins in all the molecular weight sizes could not be achieved. This indicated that loading of the correct amount of protein in order to achieve sharply defined protein bands, would have lead to a loss of information. It seemed as if, in this case, the limits of the technique had been reached to solve the complexity of the current problem.

The fractions obtained during ultrafiltration of YE therefore did not follow the expected profile according to the decrease in molecular weight of the peptides.

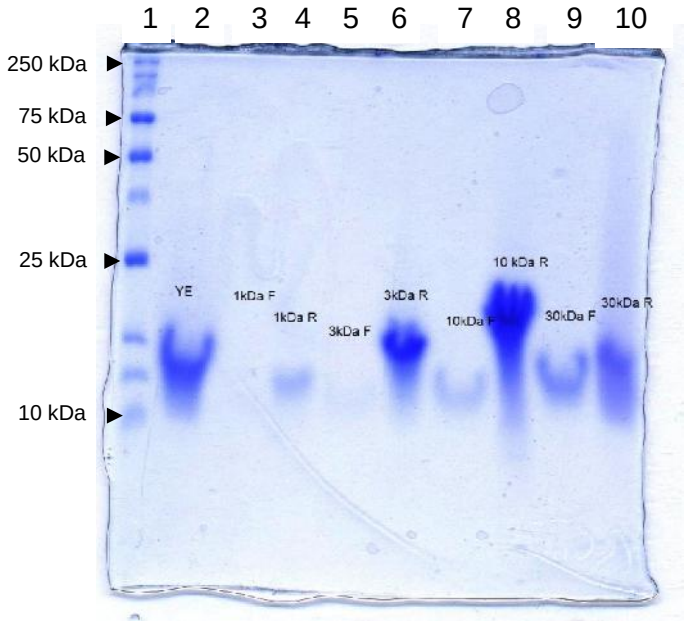


Figure 35 SDS-PAGE of the 9 fractions obtained during ultrafiltration of yeast extract
Lane 1=Protein standard, Lane 2=Unfractionated YE, Lane 3=1 kDa Filtrate, Lane 4=1 kDa Retentate, Lane 5=3 kDa Filtrate, Lane 6=3 kDa Retentate, Lane 7=10 kDa Filtrate, Lane 8=10 kDa Retentate, Lane 9=30 kDa Filtrate, Lane 10=30 kDa Retentate.

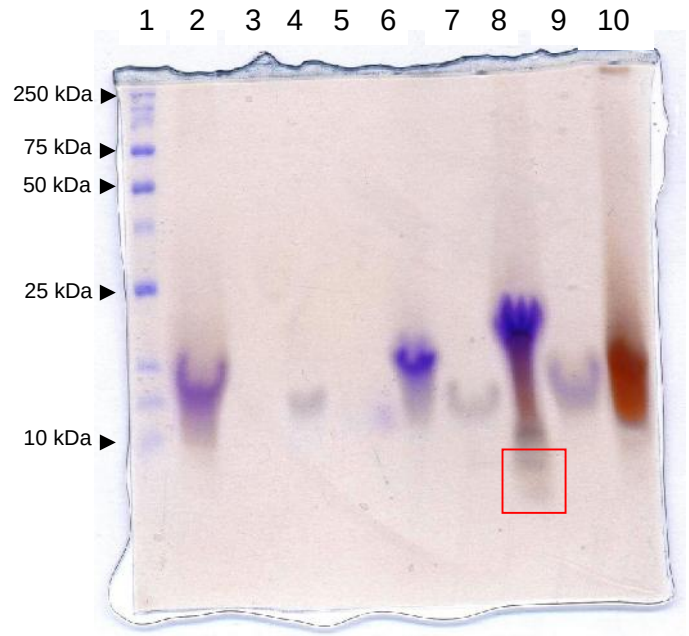


Figure 36 Silver stained SDS-PAGE of the 9 fractions obtained during ultrafiltration of yeast extract.
Lane 1=Protein standard, Lane 2=Unfractionated YE, Lane 3=1 kDa filtrate, Lane 4=1 kDa retentate, Lane 5=3 kDa filtrate, Lane 6=3 kDa retentate, Lane 7=10 kDa filtrate, Lane 8=10 kDa retentate, Lane 9=30 kDa filtrate, Lane 10=30 kDa retentate, The red block indicates the two bands in the lane containing the 10 kDa retentate fraction which became visible after silver staining

3.3.4.1 SAMPLE CONCENTRATION

Due to no visible bands present in the lane loaded with the 1 kDa filtrate, the original sample obtained during ultrafiltration was concentrated. Three different concentration techniques were used in an attempt to improve the detection of possible bands. Three separate 1 kDa filtrate samples were prepared by ethanol-, TCA- and isopropanol precipitation and the protein profiles of the samples were analysed by SDS-PAGE. The sample that was treated with the ethanol precipitation method was loaded in three different concentrations: an aliquot of 50 µl of sample buffer was added to the first ethanol precipitated fraction, 100 µl to the second fraction and 150 µl to the third. This was done in order to determine whether the visibility of the bands on the gel varied between the lowest and the highest loaded concentration. Aliquots of 10 µl of each concentration were loaded onto the gel.

For the samples concentrated with the TCA and isopropanol protein concentration techniques, an even smaller sample buffer volume of 20 µl was used as solvent in order to obtain the highest possible sample concentration, of which 10 µl aliquots were loaded onto the gel.

It was evident from the results (data not shown) that no bands were visible on the gel of any of the concentrated samples. When done in triplicate similar results were obtained. Proteins

precipitate out when large amounts of a water-miscible solvent such as ethanol or acetone are added to a protein solution. The conventional wisdom is that this is due to a decrease of the dielectric constant, which would make interactions between charged groups on the surface of proteins stronger (van Oss, 1989). Although Wessel and Flugge (1984) stated that ethanol precipitation removes salts more effectively than other precipitation techniques, this had no influence on the visibility of the bands in the current study.

A preparative scale of YE fractionation was evaluated next, where the fractions could be evaluated when water was used as eluate.

3.3.5 Fractionation of Yeast extract by Size exclusion chromatography

3.3.5.1 Size exclusion chromatography of unfractionated yeast extract

The elution time of Blue Dextran was determined to be 4:20 hours at a volumetric flow rate of 30 ml/h. The elution volume of Mercaptoethanol at the same flow rate was obtained after 9:40 hours. All YE fractions therefore eluted between these two time 'frames' (Figure 37).

The linear flow rate (Dennison, 2002) was calculated as:
Volumetric flow rate = πr^2 x linear flow rate
30 ml/h = π $(0.75)^2$ x linear flow rate
Linear flow rate = 17 cm/h
With a volumetric flow rate of 30 ml/h in a 1.5 cm diameter column, the linear flow rate was 17 cm/h.

Because of its simplicity, UV absorption is the method favoured for continuous (semi-quantitative) monitoring of the protein concentration in the eluate from chromatography columns (Dennison, 2002). This method was therefore used for all continuous determinations of eluates obtained from size exclusion chromatography columns. The protein concentrations of specific samples before and after size exclusion chromatography (not continuous) were determined by a specific protein assay (BCA assay) described in Section 3.2.3. The separation of proteins in YE was monitored by collecting the eluate of the entire run in fractions in test tubes, where after the absorbance was measured at 280 nm.

The results were then used to construct an elution profile (Figure 37).

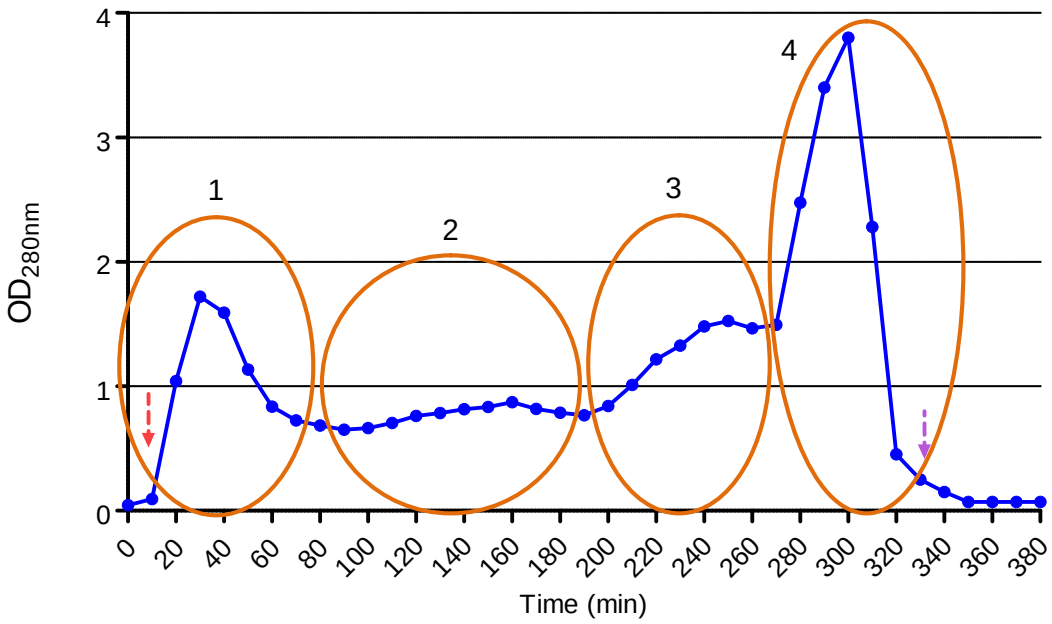


Figure 37 Size exclusion chromatogram of yeast extract with 4 identified regions 1=Fraction 1, 2=Fraction 2, 3=Fraction 3, 4=Fraction 4. The red arrow represents blue dextran elution time and purple arrow represents mercaptoethanol elution time, the void- and total volumes of the column, respectively. The above graph is the means of triplicate values.

The different peaks observed in Figure 37 were divided into 4 fractions based on the elution times:

Fraction 1: 10-70 min

Fraction 2: 80-180 min

Fraction 3: 190-260 min

Fraction 4: 270-320 min

3.3.5.2 YOGURT FERMENTATION USING THE 4 FRACTIONS OBTAINED DURING SIZE EXCLUSION CHROMATOGRAPHY AS SUPPLEMENTS

The 4 fractions were pooled and evaporated separately. Individual yogurt fermentations were prepared with each fraction (Figure 38).

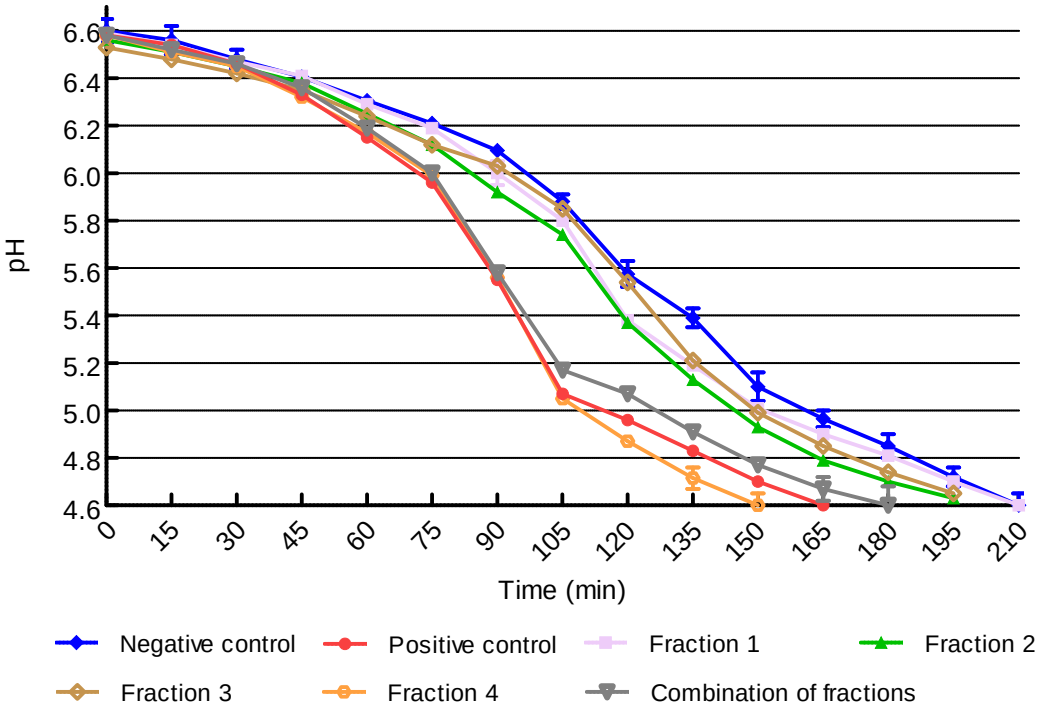


Figure 38 Yogurt fermentations with 4 individually pooled size exclusion fractions

From Figure 38 it was evident that all the YE fractions, except Fraction 1, accelerated yogurt fermentation time in comparison to the negative control. Fraction 1 collected after size exclusion chromatography did not have any effect on the yogurt fermentation process when compared to the control which reached pH 4.6 after 210 min. Fractions 2 and 3 both decreased the fermentation time by 7 % in comparison to the control. Fraction 4 however displayed the most significant acceleration, and decreased the time to reach pH 4.6 by 28.6 % (60 min), which is an even more significant acceleration than that of the positive control (10 g/l YE), which decreased fermentation time by 21 % (45 min). A possible explanation for the fact that Fraction 2 and 3 did not decrease yogurt fermentation time as much as Fraction 4, could be that although both Fractions 2 and 3 contained the accelerating component which formed part of larger peptides, it was not as easily accessible as the smaller peptides present in Fraction 4. Due to the effect of Fraction 4 on yogurt fermentation time being the most noteworthy, further investigation on isolation of the accelerating component was done

only on this fraction. Fraction 4 was from this point on referred to as the accelerating peak/fraction.

In order to establish whether the combination of the 4 individual fractions had the same effect on the yogurt fermentation process as the positive control, aliquots of each fraction were combined and the effect was evaluated in order to prepare a reconstituted YE. Results indicated that the fermentation profile of the positive control and the combined fractions were very similar and differed by only 15 min (7 %). This difference may possibly be ascribed to the re-combined mixture not being a complete re-creation of the original YE.

The fermentation profile of the positive control was similar to that of Fraction 4 up until 105 min, after which the lactic acid production rate decreased which resulted in a 15 min increase in the total fermentation time i.e. 165 min.

The prominent decrease in lactic acid production at 105 min in the cases of the positive control, the combination of fractions and Fraction 4 were investigated, but no concrete explanation could be given for this phenomenon.

After the 4 fractions had been pooled, evaporated and freeze dried, the fractions were further analysed using SDS-PAGE.

3.3.5.3 CONCENTRATION OF THE 4 INDIVIDUALLY POOLED FRACTIONS

The 4 pooled fractions obtained from size exclusion chromatography were evaporated in order to reduce the volume. Only the water was removed and all the non volatiles were retained during evaporation. After evaporation a small amount of distilled water was added to each of the evaporated fractions, transferred to cryogenic tubes and subsequently freeze dried, which preserved the samples for future analysis by SDS-PAGE, HPLC, MS and amino acid analysis.

3.3.5.4 SDS-PAGE OF THE 4 INDIVIDUALLY POOLED FRACTIONS

All 4 fractions obtained from size exclusion chromatography of YE were loaded onto an SDS-PAGE gel (Figure 39). It was evident that the smallest bands of the accelerating fraction (Fraction 4) were too faint. The SDS-PAGE was repeated using 70 µl each of the 4 fractions as loading volume and not 30 µl as was previously used. Similar results were obtained in Fraction 1-3. After staining the gel (Figure 39), a band could be observed slightly above the front in the lane loaded with the Fraction 4 sample. It was anticipated that the

accelerant could be present in this band. After de-staining the gel, the band had disappeared. Re-staining of the gel did not bring out this low molecular weight protein band.

The disappearance of the low molecular weight protein band could be due to two causes. The proteins present in this band either do not react with the CCB stain, or are so small in size that they were not fixed in the gel and diffused from the gel into the buffer. Ahmad and Saleemuddin (1985) stated that the determination of microgram quantities of immobilised proteins is based on the property of CCB blue G-250 to bind strongly yet reversibly to proteins. This reversibility could therefore also not be excluded as possible explanation for the disappearance of this band.

The peak containing the accelerating component (Fraction 4) was freeze dried and directly analysed by MS.

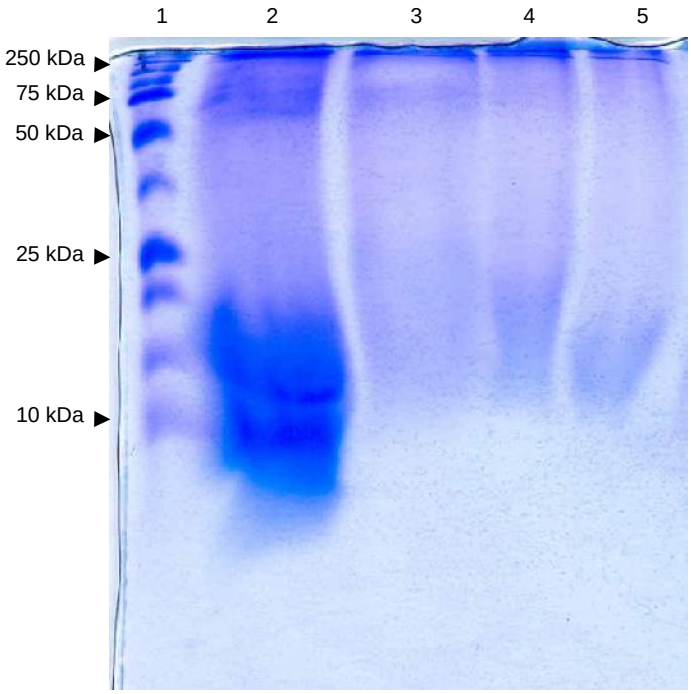


Figure 39 SDS-PAGE of the 4 fractions obtained during size exclusion chromatography of yeast extract
Lane 1= Protein standard, Lane 2= Fraction 1, Lane 3= Fraction 2, Lane 4= Fraction 3, Lane 5= Fraction 4.

3.3.6 Mass Spectrometry of fraction 4

The MS results of the electrophoretogram region where the low molecular protein band was visible shortly after staining were inconclusive due to too little protein being present, although the maximum amount of sample was loaded onto the initial gel from which the sample was cut. This confirmed once again that SDS-PAGE was not the most suitable technique for the identification of the accelerant and that other methods should be found.

MS analysis was repeated directly on Fraction 4 obtained after size exclusion chromatography of YE. Results were compared to the database of *Saccharomyces cerevisiae* due to this yeast being used to prepare YE. According to the database used as reference for the MS analysis, 4 enzymes present in *Saccharomyces cerevisiae* were identified as possible sources of some of the peptides:

- **G3P3_YEAST** Glyceraldehyde-3-phosphate dehydrogenase 3 OS=*Saccharomyces cerevisiae* (strain ATCC 204508/S288c)

  13 Matched peptides:

  - S.AGIQL.S
  - E.RDPANLPW.G
  - L.PWGSSNVDI.A
  - K.ETTYDEIK.K
  - S.TRVVDLVEH.V
  - E.RDPANLPWGSS.N
  - E.RDPANLPWGSS.N
  - E.RDPANLPWGSSN.V
  - M.FKYDSTHGRYAGE.V
  - T.VDGPSHKDWRGGRTA.S
  - L.VSWYDNEYGYSTR.V
  - H.KDWRGGRTASGNIIPS.S
  - K.TVDGPSHKDWRGGRTA.S

- **ENO1_YEAST** Enolase 1 OS=*Saccharomyces cerevisiae* (strain ATCC 204508/S288c)

  7 Matched peptides:

  - D.EGGVA.P
  - T.AGIQI.V
  - D.KSKWLTGPQ.L

- F.KNPNSDKSKWLTGPQ.L
- D.FKNPNSDKSKWLTGPQ.L
- S.HAGGALALQEFMIAP.T
- H.AGGALALQEFMIAPTGA.K

- **MAL12_YEAST** Alpha-glucosidase MAL12 OS=*Saccharomyces cerevisiae* (strain ATCC 204508/S288c)

  8 Matched peptides:

  - T.KEYED.K
  - E.HEWFKE.S
  - F.TGPDVKPW.F
  - E.GKPIPPNNW.K
  - L.DHGVDGFRIDT.A
  - E.GKPIPPNNWKS.F
  - S.KLQHPNWGSHNGPR.I
  - M.KNRVKDGREIMTVGE.V

- **IF4A_YEAST** ATP-dependent RNA helicase eIF4A OS=*Saccharomyces cerevisiae* (strain YJM789)

  1 Matched peptide:

  - G.RVFDNIQR.R

If the significance threshold had been set higher than 0.05, it is possible that a fit with more than four proteins/enzymes could have been possible. The certainty of the presence of the peptides in the additional identified enzymes would however not have been distinct.

A further 909 peptides matches not assigned to any protein hits were identified (See Appendix A). These ranged in length from 5 to 26 amino acids. With such a large amount of peptides present, it would be difficult to fit the amino acid composition of the accelerating peak with one or a small number of the unmatched peptides. A possible explanation for the presence of all these unmatched peptides can be that these short peptides may be homologous to a large number of proteins. Another reason for their presence might be that the plastein reaction, a synthesis reaction by proteolytic enzymes, possibly takes place during YE production. The term ‘plastein reaction’ is commonly used to refer to the protease-catalysed process involved in the formation of a plastein (polypeptides) from a protein hydrolysate or an oligopeptide mixture by a reversal of the usual peptide bond hydrolysis to

produced “resynthesised” protein or polypeptides (Watanabe & Arai, 1992; Zhou & Feng, 2006).

The formation of the plastein reaction is due largely to protease-catalysed transpeptidation under conditions favouring peptide bond synthesis. Three criteria have been established for the efficient formation of a plastein (McEvily & Zaks, 1991):

1. The molecular weight of the substrate for synthesis should be approximately 500-1000 Da
2. Substrate concentration should be 20 to 40 %
3. The pH should be in the range of 4 to 7

These criteria coincide with conditions applied during YE manufacture, confirming the occurance of the plastein reaction to result in non-sense sequences which cannot be identified as being derived from specific proteins.

The fact that the manufacturers of YE do not disclose which specific *Saccharomyces cerevisiae* strain is used for YE manufacture, further complicated the identification and matching of peptides.

From the results obtained (Appendix A) it was evident that an abundance of peptides are present in the sample. These results are in accordance to the large number of peaks observed in the HPLC chromatograms (Section 3.3.3).

It may therefore be concluded that the component responsible for acceleration of yogurt fermentation is of low molecular weight and possibly a peptide or a large number of small peptides of different sequences. Since Fraction 4 eluted with the total volume during size exclusion chormatography, it may not be excluded that the fraction could also contain small molecules such as free amino acids, vitamins and salts, all of which may benefit the metabolism of the yogurt cultures and therefore accelerate the fermentation process. An in depth characterisation of this Fraction 4 was therefore necessary.

Characterisation of the low molecular weight peptide material from Fraction 4 will be described in Chapter 4.

3.3.7 PREPARATION OF VITAMIN-, MINERAL- AND AMINO ACID SOLUTIONS TO DETERMINE ORIGIN OF ACCELERANT

The concentrations in which each of the vitamin-, mineral and amino acid solutions were evaluated in, were based on the product information leaflet provided by Oxoid on YE LP0021 (2009) indicating the amount of each present in 100 g of YE. This was referred to as the 1x concentration of each solution. Each was fractionated by size exclusion chromatography (Figures 40 and 41). When the respective elution times of the accelerating fraction (Fraction 4) was compared to that of the vitamin-, mineral- as well as amino acid solution with size exclusion chromatography, they were very similar. This indicated that at this stage none of these components could be eliminated as possible accelerators present in Fraction 4 and it was therefore important to determine whether the acceleration by YE was due to amino acids, minerals or vitamins present in YE (to be determined in Chapter 4).

The minerals reported in the Oxoid Product Information leaflet present in YE LP0021 was used as reference for the mineral analysis on the accelerating fraction (Table 2). These included calcium, magnesium, iron, potassium, sodium, copper, tin, zinc, manganese, lead and cobalt. As can be seen from Table 6, no copper, tin, manganese, lead or cobalt was detected in the accelerating fraction. From the mineral analysis performed on Fraction 4 it was evident that all the minerals present in Fraction 4 were mentioned in the Oxoid Product Information Leaflet (2009) on YE. As expected, all minerals in Fraction 4 were present in lower amounts than in unfractionated YE according to Oxoid Product Information Leaflet (2009).

Table 6 Mineral composition of the accelerating fraction (Fraction 4)

Ca (%)	Mg (%)	K (%)	Na (%)	Fe (%)	Zn (%)
0.056	0.0254	2.3	0.1214	0.006	0.008

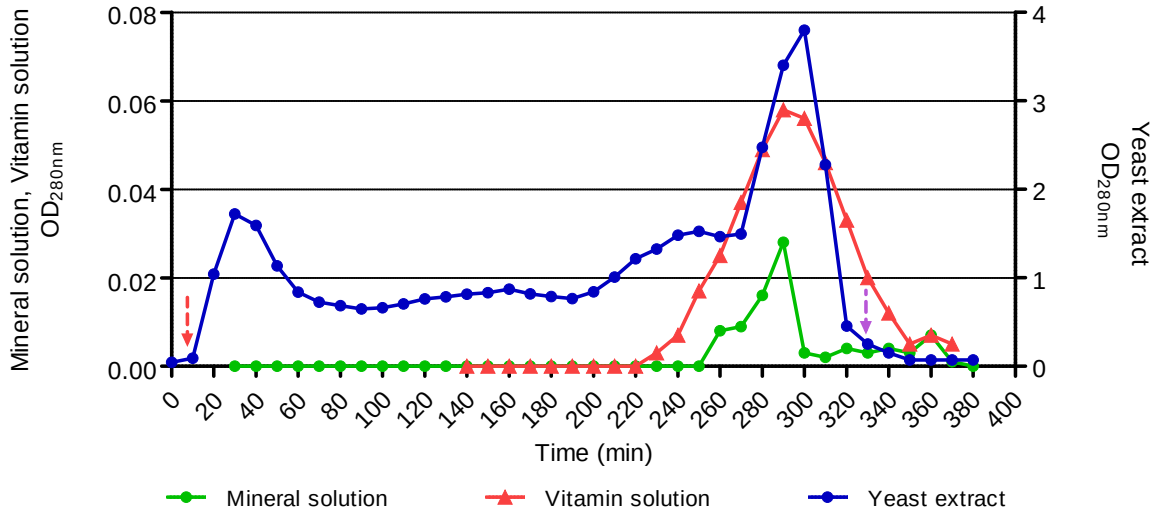


Figure 40 Size exclusion chromatogram of the mineral solution, vitamin solution and yeast extract. The red and purple arrows represent the elution times of blue dextran and mercaptoethanol, indicating the void- and total volumes of the column, respectively.

3.3.8 FREE AMINO GROUPS PRESENT IN YEAST EXTRACT

The Cd-ninhydrin reacts with free amino acids present in the sample (Folkertsma & Fox, 1992). From Figure 41 it was evident that the free amino acids present in YE eluted between 270 min and 330 min from the size exclusion column, and coincide with the elution of small peptides of Fraction 4. The bulk of amino acids (270-300 min) eluted earlier than the bulk of the peptides (280-320 min), which is possible, due to interactions with the column material (Hahn, Deinhofer, Machold, & Jungbauer, 2003). It was therefore not possible to separate the free amino acids completely from the small peptides with this separation method. This supports the data obtained from mass spectrometry (Section 3.3.6) that the accelerating peak contained free amino acids, as well as peptides. The standard curve obtained during Cd-ninhydrin analysis with the equation is attached in Appendix B.

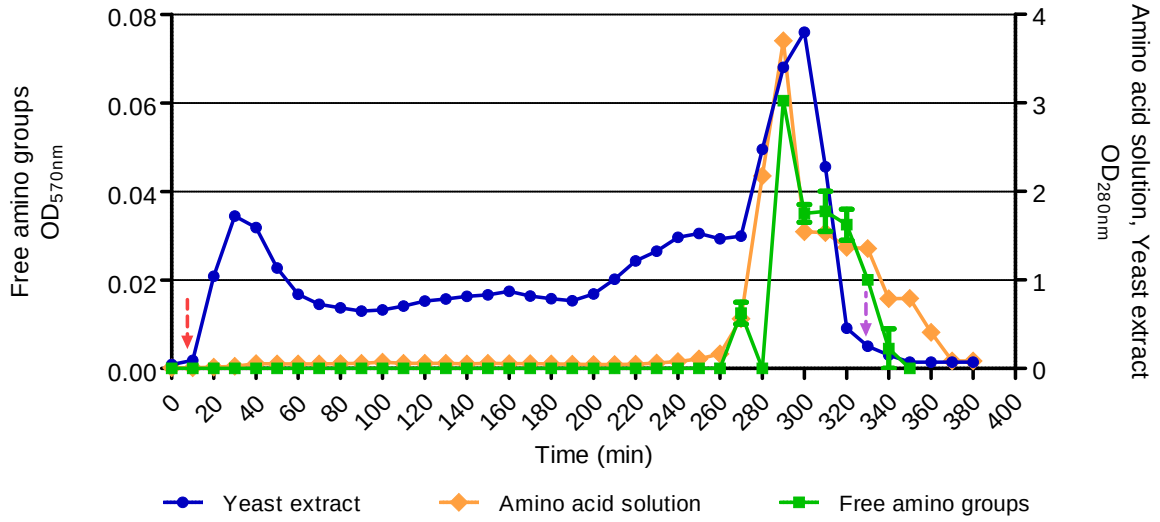


Figure 41 Size exclusion chromatograms of yeast extract, the amino acid solution and the free amino groups present in yeast extract. The red and purple arrows represent the elution times of blue dextran and mercaptoethanol, indicating the void- and total volumes of the column, respectively.

3.3.9 Amino acid analysis of the accelerating fraction (Fraction 4) pooled after size exclusion chromatography

In Chapter 3, Fraction 4 obtained after size exclusion chromatography of YE, was identified as the fraction containing the accelerating component. Due to the fact that Fraction 4 contained the lowest molecular weight components and that approximately 50 % of the amino acids and small peptides present in YE are smaller than 3 kDa (Yoo, 2009) the amino acid content of Fraction 4 was determined (Table 7).

Table 7: Amino acid content of accelerating fraction (Fraction 4) expressed in g/100 g (%) of total amino acids

Name	g/100 g (%)
L-Histidine	1.24
L-Serine	6.87
L-Arginine	5.2
L-Glycine	5.33
L-Aspartic acid	8.66
L-Glutamic acid	18.86
L-Threonine	6
L-Alanine	9.48
L-Proline	3.82

L-Cysteine	0.19
L-Lysine	6.95
L-Tyrosine	0.91
L-Methionine	2.47
L-Valine	8.49
L-Iso-Leucine	5.96
L-Leucine	8.49
L-Phenylalanine	1.07
Total amino acid % in Fraction 4	**100**

When comparing Table 3 with Table 7 it was evident that the amino acid composition of YE according to Oxoid Product Information Leaflet (2009) corresponded to the amino acids present in Fraction 4 qualitatively. The only exceptions were: 1) L-tryptophan which was reported to be present in YE although it was not present in the accelerating fraction and 2) L-histidine, which according to the information leaflet is not present in YE, although amino acid analysis indicated that it was present in the accelerating fraction.

When comparing the amino acids present in the accelerating fraction (Fraction 4) with those reported by Shihata (2004) to be present in milk, all except 2 amino acids corresponded i.e. L-tryptophan and L-cysteine. Shihata (2004) reported L-tryptophan to be present in trace amounts in milk although this amino acid was not present in Fraction 4 of the current study. Amino acid analysis of the accelerating fraction showed that (although in very low amounts), L-cysteine was present, which according to Shihata (2004) is not present as a free amino acid in milk.

3.4 CONCLUSIONS

YE was fractionated by ultrafiltration into 9 fractions ranging in size from 30 kDa to smaller than 1 kDa. Yogurt fermentations were prepared with each of the fractions and were observed to be similar regarding the time it took for the pH to reach pH 4.6. These results indicated that the accelerant present in YE was present in all fractions and was therefore a component smaller than 1 kDa.

With SDS-PAGE analysis of the various YE fractions obtained from ultrafiltration it was evident that the accelerating component was too small to visualise on an electrophoretogram. Other fractionation and characterisation techniques were therefore investigated.

Fractionation of YE was also performed by size exclusion chromatography with which four distinct fractions were obtained. After supplementing individual milk batches with each of these four fractions, it was evident that the fraction that eluted last, containing components with smallest molecular mass, decreased yogurt fermentation time the most. This fraction decreased fermentation time by more than 71 %, or 60 min, in comparison to the negative control, which was even more than the YE itself. This once again indicated that the accelerant had a low molecular weight.

With mass spectrometry analysis on the accelerating fraction of yogurt fermentation (Fraction 4) some peptide sequences were identified that could be matched to specific proteins or enzymes of *Saccharomyces cerevisiae* in a protein database. The four enzymes that were identified as possible sources of the peptide sequences were: Glyceraldehyde-3-phosphate dehydrogenase, Enolase, Alpha-glucosidase and ATP-dependent RNA helicase. The first 3 identified enzymes originated from *Saccharomyces cerevisiae* strain ATCC 204508/S288c whereas the ATP-dependent RNA helicase was associated with *Saccharomyces cerevisiae* strain YJM789. A further 909 peptide sequences, which did not match any peptides in the database, were also identified. This abundance in unmatched peptides was described as having a homology with too many proteins, or that they could have been formed by the plastein reaction during the YE manufacture, and would therefore not match peptide sequences from *Saccharomyces cerevisiae* proteins.

Although the yogurt fermentation accelerating fraction could be one peptide or a group of peptides, the peptides could not be separated from other small molecules such as free

amino acids, vitamins and minerals. The effect of individual free amino acids on yogurt fermentation therefore had to be determined, which was the subject of Chapter 4.

3.5 REFERENCES

Ahmad, H., & Saleemuddin, M. (1985). A Coomassie blue-binding assay for the microquantitation of immobilized proteins. *Analytical Biochemistry*, *148*, 533–541.

Chae, H. J., Joo, H., & In, M. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, *76*, 253–258.

Champagne, C., Gaudreau, H., & Conway, J. (2003). Effect of the production or use of mixtures of bakers' or brewers' yeast extracts on their ability to promote growth of lactobacilli and pediococci. *Electronic Journal of Biotechnology*, *6*, 185–197.

Dennison, C. (2002). *A guide to protein isolation*. New York: Kluwer Academic Publishers.

Du Preez, J. C., & van der Walt, J. P. (1983). Fermentation of D-xylose to ethanol by a strain of *Candida shehatae*. *Biotechnology Letters*, *5*, 357–362.

Elli, M., Zink, R., Reniero, R., & Morelli, L. (1999). Growth requirements of *Lactobacillus johnsonii* in skim and UHT milk. *International Dairy Journal*, *9*, 507–513.

Fairbanks, G., Steck, T. L., & Wallach, D. F. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*, *10*, 2606–2617.

Folkertsma, B., & Fox, P. F. (1992). Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. *Journal of Dairy Research*, *59*, 217–224.

Gaudreau, H., Champagne, C. P., Goulet, J., & Conway, J. (1997). Lactic fermentation of media containing high concentrations of yeast extracts. *Journal of Food Science*, *62*, 1072–1075.

Hahn, R., Deinhofer, K., Machold, C., & Jungbauer, A. (2003). Hydrophobic interaction chromatography of proteins. II. Binding capacity, recovery and mass transfer properties. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences, 790*, 99.

Hesse, P. R. (1971). *A textbook of soil chemical analysis* (pp. 326–327). London, UK: John Murray Ltd.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Loane, M., Mendel, E., Abele, S., Paull, B., & Macka, M. (2007). *The potential of capillary electrophoresis for the analysis of yeast extracts.* Poster presented at the Analytical Research Forum Royal Society of Chemistry, Glasgow, UK.

McEvily, A. J., & Zaks, A. (1991). Emulsifiers and Surfactants. In I. Goldberg & R. Williams (Eds.), *Biotechnology and Food Ingredients* (pp. 202–207). New York: Springer.

Park, C. B., & Lee, S. B. (1999). Cultivation of the hyperthermophilic archaeon *Sulfolobus solfataricus* in low-salt media. *Biotechnology and Bioprocess Engineering*, *4*, 21–25.

Rajalingam, D., Loftis, C., Xu, J. J., Kumar, T., & Krishnaswamy, S. (2009). Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. *Protein Science*, *18*, 980–993.

Shihata, A. (2004). *The proteolytic activity of yoghurt and probiotic bacteria for the improved viability of probiotic bacteria in fermented milk products* (Doctoral dissertation, Victoria University, Australia). Retrieved from http://vuir.vu.edu.au/15367.

Smith, J. S., Hillier, A. J., & Lees, G. J. (1975). The nature of the stimulation of the growth of *Streptococcus lactis* by yeast extract. *Journal of Dairy Research*, *42*, 123–138.

Van Oss, C. J. (1989). On the mechanism of the cold ethanol precipitation method of plasma protein fractionation. *Journal of Protein Chemistry*, *8*, 661–668.

Watanabe, M., & Arai, S. (1992). The plastein reaction: Fundamentals and applications. In B. J. F. Hudson (Ed.), *Biochemistry of Food Proteins* (pp. 271–305). Bath, UK: Springer.

Wessel, D., & Flugge, U. (1984). A method to concentrate proteins and removal of guanidine hydrochloride before PAGE-SDS. *Analytical Biochemistry*, *138*, 141–143.

Yoo, S. M. (2009). *Fractionation of non-animal protein hydrolysates for use in CHO (Chinese Hamster Ovary) cell media* (Master's thesis, University of Waterloo, Canada). Retrieved from http://uwspace.uwaterloo.ca/handle/10012/4986?mode=full

Zhou, Z., & Feng, Z. (2006). Progress and application of plastein reaction in food proteins. *Journal of Northeast Agricultural University, 13,* 81-84.

CHAPTER 4

EFFECT OF AMINO ACIDS PRESENT IN THE ACCELERATING FRACTION ON YOGURT FERMENTATION TIME

4.1 INTRODUCTION

It has long been recognised that peptides are of great nutritional value for many types of bacteria and that bacteria usually hydrolyse peptides and use the amino acids (amino acids) either directly for protein synthesis, or after further breakdown, as sources of nitrogen, carbon, or sulphur (Shihata, 2004).

During evaluation of YE by Speck, McAnelly and Wilbur (1958) there were indications of an inverse relationship between the growth rate of a culture and the number of peptides in YE to which the culture showed response. Possibly, this was a reflection of the proteolytic activities of the culture. Faster cultures presumably obtain required peptides by hydrolysis of milk proteins and thus show a response to fewer preformed peptides furnished from external sources (Speck *et al*., 1958).

There are certain practical implications of the above described study with respect to the choice of cultures for commercial uses. It would appear that cultures with less dependence on the presence of preformed peptides in milk would be capable of improved growth and should be chosen for the making of fermented milk products. However, there may be certain qualities of a culture which would make its use as a starter desirable, in spite of its greater dependence on preformed peptides for most rapid growth. In such an instance, it would be advantageous to supplement milk with a source of the stimulatory peptides, in order to obtain the qualities desired of the culture and to promote its rapid growth (Speck *et al*., 1958).

Proteinase activity plays a role in the nutrition of lactic acid bacteria, since *Lactobacilllus bulgaricus* and *Streptococcus thermophilus* are auxotrophic for a number of amino acids. The principal substrate for proteolysis is casein. The principal casein protein in milk hydrolysed by proteolytic enzymes of yogurt cultures is β-casein, although a_{s1}- and κ-casein are degraded to a lesser extent (Shihata, 2004). There have also been reports that whey proteins are degraded to a limited extent by these bacteria (Chandan, Argyle, & Mathison, 1982; Khalid, El Soda, & Marth, 1991; Shihata, 2004). The net effect of this proteolytic activity is that fermented milks have a higher content of peptides and free amino acids, especially valine, histidine, serine and proline, than milk. *Lactobacilllus bulgaricus* appears to be the main species responsible for these changes (Shihata & Shah, 2000).

Some amino acids, such as glutamic acid, proline and, to a lesser degree, alanine and serine, are presumably not required by the yogurt starter bacteria, and thus accumulate in

the product. The remaining amino acids are utilised by *Streptococcus thermophilus* and *Lactobacilllus bulgaricus* during growth and/or fermentation and therefore accumulate less in the medium (Shihata, 2004).

Lactic acid bacteria are nutritionally fastidious, needing an exogenous supply of amino acids to initiate growth (Iyer, Tomar, Maheswari, & Singh, 2010). Jones and Woolley (1962) confirmed this by their observation that *Lactobacilllus bulgaricus* cultivated in a basal medium is unable to grow in the absence of added peptide. In the current study it was investigated whether this could be verified when the same organism is cultivated together with *Streptococcus thermophilus* as a co-culture in milk.

Most work on proteolytic activity of lactobacilli has been conducted with strains grown in culture media in which the concentration of free amino acids was high enough to sustain growth without the organisms having to produce proteolytic enzymes (Abraham, Antoni, & Anon, 1993). In the manufacture of dairy products however, milk is used without the addition of free amino acids. It was therefore important to study the response of starter bacteria grown in milk supplemented with free amino acids.

When considering the contradictory reports on the effect of various amino acids on starter culture growth and lactic acid production, it was clearly essential to determine the effect of the individual amino acids present in the accelerating fraction (identified in Chapter 3) on the yogurt fermentation process. This will be addressed in the present chapter.

4.2 MATERIALS AND METHODS

4.2.1 EFFECT OF VITAMIN-, MINERAL- AND AMINO ACID SOLUTIONS ON YOGURT FERMENTATION TIME

The three solutions prepared in Chapter 3 Section 3.3.7 were used to determine their effect on individual yogurt fermentations. Approximate amounts to that reported by Oxoid Product Information Leaflet (2009) to be present in 100 g of YE, were used as supplement loads.

4.2.2 PREPARATION OF A COCKTAIL CONTAINING ONLY THE AMINO ACIDS PRESENT IN THE ACCELERATING FRACTION (FRACTION 4)

The individual amino acids present in the accelerating fraction derived from YE were combined in the same concentrations in which they were present in the original fraction (based on the amino acid analysis of Fraction 4 in Section 3.3.9).

All amino acids, except L-leucine and L-cysteine, were combined in the following concentrations: L-alanine (0.34 g/l), L-arginine (0.19 g/l), L-aspartic acid (0.315 g/l), L-glutamic acid (0.686 g/l), L-glycine (0.19 g/l), L-histidine (0.045 g/l), L-iso-leucine (0.217 g/l), L-lysine (0.253 g/l), L-methionine (0.09 g/l), L-phenylalanine (0.04 g/l), L-proline (0.14 g/l), L-serine (0.25 g/l), L-threonine (0.218 g/l), L-tyrosine (0.033 g/l), and L-valine (0.3 g/l).

In order to solubilise the amino acids, the pH was adjusted to 11 using 0.1 N NaOH (Merck), where after the L-leucine (0.31 g/l) and L-cysteine (0.007 g/l) were added. Potassium hydroxide was added until the two last mentioned amino acids dissolved. The cocktail was then stored at 4 °C overnight, or until needed, before the pH was adjusted to that of fresh milk (± pH 6.5) using HCl (Merck). This final cocktail was referred to as “the amino acid cocktail” in all further discussions.

4.2.3 SIZE EXCLUSION CHROMATOGRAPHY OF THE PREPARED AMINO ACID COCKTAIL

The same size exclusion chromatography conditions were used as described in Chapter 3 Section 3.2.8). In this case the column was loaded with 200 µl of 1x amino acid cocktail.

4.2.4 ESTABLISHMENT OF THE OPTIMAL AMINO ACID COCKTAIL CONCENTRATION

In order to establish the specific amino acid cocktail concentration decreasing yogurt fermentation time the most significantly, a stock solution of the amino acid cocktail prepared in Section 4.2.2 was used. Various concentrations of the prepared stock solution were evaluated within the range of 1x, 5x and 10x of the original amino acid cocktail concentration.

4.2.5 EFFECT OF INDIVIDUAL AMINO ACIDS PRESENT IN AMINO ACID COCKTAIL ON YOGURT FERMENTATION

The effect of the individual amino acids present in the accelerating fraction (Fraction 4) on individual yogurt fermentations was evaluated. The amino acids were used in the concentrations as determined during amino acid analysis of Fraction 4 (Section 3.3.9).

The effect of L-tryptophan, which was reported to be present in YE according to the Oxoid Product Information Leaflet (2009) but not detected in Fraction 4, was also determined using the concentration stated in the Oxoid Leaflet. Neither L-asparagine nor L-glutamine was evaluated as neither were detected in Fraction 4 or reported by the Oxoid Product Information Leaflet (2009) to be present in YE. The effects of the following amino acids were evaluated: L-alanine (0.34 g/l), L-arginine (0.19 g/l), L-aspartic acid (0.315 g/l), L-cysteine (0.007 g/l), L-glutamic acid (0.686 g/l), L-glycine (0.19 g/l) L-histidine (0.045 g/l), L-iso-leucine (0.217 g/l), L-leucine (0.31 g/l), L-lysine (0.253 g/l), L-methionine (0.09 g/l), L-phenylalanine (0.04 g/l), L-proline (0.14 g/l), L-serine (0.25 g/l), L-threonine (0.218 g/l), L-tryptophan (0.085 g/l), L-tyrosine (0.033 g/l), and L-valine (0.3 g/l). All amino acids were supplied by Sigma-Aldrich.

As none of the individual amino acids decreased yogurt fermentation in the above concentrations, the specific concentrations stated in the Oxoid Product Information Leaflet (2009) were also evaluated.

4.2.6 EFFECT OF AMINO ACIDS ON PH DURING YOGURT FERMENTATION

Individual amino acids have various effects on the initial pH of milk when added to milk (Amend & Helgeson, 1997). Certain amino acids had the same initial pH as milk (pH 6.3-6.7) whereas others altered the initial pH of milk and consequently influenced yogurt fermentation (Table 8). It was therefore essential to determine 1) the effect of the amino acids which alter

milk pH when titrated to ±pH 6.5 prior to fermentation 2) the effect of the chemicals used during titration, on the fermentation process.

Table 8 Solubility and pH of the amino acids present in the accelerating fraction (Fraction 4) (Amend & Helgeson, 1997)

Amino Acid	Solubility in water (g/l at 20 °C)	pH
L-Cysteine hydrochloride monohydrate	650	0.8-1.2 (100 g/l water)
L-Valine	85	5.5-6.5 (100 g/l)
L-Tyrosine	0.38	6.5 (0.1 g/l)
L-Threonine	90	5-6 (100 g/l)
L-Serine	270	5-6 (50 g/l)
L-Proline	1500	5-7 (100 g/l)
L-Phenylalanin	27	5.4-6 (10 g/l)
L-Methionine	48	5-7 (10 g/l)
L-Lysine monohydrochloride	420	5.5-6 (100 g/l)
L-Leucine	24	5.5-6.5 (20 g/l)
L-Isoleucine	40	5.5-6.5 (40 g/l)
L-Histidine	38.2	~7.7 (10 g/l)
L-Glycine	225	5.9-6.4 (50 g/l)
L-Glutamic acid hydrochloride	490	3-3.5 (8.6 g/l)
L-Aspartic acid sodium salt monohydrate	100	2.5-3.5 (4 g/l)
L-Arginine monohydrochloride	730	~5.7 (100 g/l)
L-Alanine	166.5	5.5-6.5 (100 g/l)

Two different amino acid preparations were prepared. The first preparation contained amino acids which, when used in the concentrations at which they are present in the accelerating fraction (Fraction 4), had the same pH range as fresh milk (± pH 6.5). This included L-valine, L-threonine, L-serine, L-proline, L-phenylalanine, L-methionine, L-lysine, L-iso-Leucine, L-leucine, L-tyrosine, L-alanine and L-glycine. The second preparation contained all the amino acids present in the first cocktail, plus the amino acids which did not have the same pH of milk namely L-cysteine, L-glutamic acid, L-histidine, L-aspartic acid and L-arginine. The pH of the second preparation therefore had to be adjusted to pH 6.5.

4.2.7 EFFECT OF VARIOUS COMBINATIONS OF AMINO ACIDS PRESENT IN AMINO ACID COCKTAIL ON THE YOGURT FERMENTATION PROCESS

Due to lack of apparent effect on acceleration of yogurt fermentation by any amino acids when evaluated singly, the amino acids present in the amino acid cocktail was divided in various combinations. Initially it was contemplated to evaluate the effect of all possible combinations of the 17 amino acids present in Fraction 4. This however would have resulted in a large number of different combinations. The number of amino acid combinations was therefore narrowed down and divided into various groups based on their amounts present in Fraction 4 determined by amino acid analysis in Chapter 3.

The same concentrations in which the individual amino acids are present in the 1x amino acid cocktail were used. The combinations were as follows:

1. Full amino acid cocktail
2. Full amino acid cocktail without L-leucine and L-cysteine
3 Amino acids which contribute to 3 % or more of the total amino acid content in the accelerating fraction: L-aspartic acid, L-glutamic acid, L-alanine and L-valine
4. Amino acids which contribute to less than 3 % of the total amino acid content in the accelerating fraction: L-histidine, L-serine, L-arginine, L-glycine, L-threonine, L-proline, L-lysine, L-tyrosine, L-methionine and L-phenylalanine
5. The 4 amino acids present in the lowest concentrations in the accelerating fraction: L-histidine, L-tyrosine, L-methionine and L-phenylalanine
6. The 10 amino acids present in the highest concentrations in the accelerating fraction: L-Serine, L-arginine, L-glycine, L-threonine, L-proline, L-lysine, L-aspartic acid, L-glutamic acid, L-alanine and L-valine

Due to the fact that KOH had to be added in order to solubilise L-leucine and L-cysteine, and also because both these amino acids increased fermentation time significantly when evaluated individually, they did not form part of any of the combinations, except for the full amino acid cocktail.

4.2.8 EVALUATION OF THE EFFECT OF VARIOUS AMINO ACID COMBINATIONS, BASED ON LITERATURE ON THE YOGURT FERMENTATION PROCESS

The effect of 10 different amino acid combinations on the yogurt fermentation process was evaluated. Combinations 1, 2, 3, 5, 7 and 9 were used as stated in literature (Table 9). In the light of the above mentioned combinations in which the three amino acids leucine, iso-leucine and cysteine were present, these three were omitted in amino acid combinations of

this section as they increased yogurt fermentation time (Section 4.3.7). These were then referred to as Combinations 4, 6, 8 and 10.

Table 9 Amino acid combinations reported in literature to stimulate *Streptococcus thermophilus* growth

Combination	**Amino acids present in combination**	**Medium used in literature**	
		Milk	**Chemically defined medium**
1	Glu, Met, His	(Letort & Juillard, 2001)	
2	Glu, Cys, Met, His	(Iyer *et al*., 2010)	
3	Glu, Cys, Met, His, Val, Leu	(Robinson, 2001)	
4	Glu, Met, His, Val		
5	Glu, Cys, Met, Val, Leu,Ile		(Letort & Juillard, 2001)
6	Glu, Met, Val		
7	Glu, Cys, Met, His, Val, Leu, Ile, Trp, Arg, Tyr	(Shihata, 2004)	
8	Glu, Met, His, Val, Trp, Arg, Tyr		
9	Glu, Cys, Met, Val, Leu, Gln		(Letort & Juillard, 2001)
10	Glu, Met, Val, Gln		

4.3 RESULTS AND DISCUSSIONS

Although results reported in Chapter 3 indicated that the accelerating component was present in Fraction 4 of YE fractionated by size exclusion chromatography, and that it contained minerals, vitamins, free amino acids and peptides, it was still not determined which one, or more, of these four is the active component responsible for the acceleration of yogurt fermentation.

4.3.1 EFFECT OF VITAMIN-, MINERAL- AND AMINO ACID SOLUTIONS ON YOGURT FERMENTATION TIME

In order to determine which type of component was responsible for the accelerating effect observed when yogurt is supplemented with YE, the respective effects of the vitamin-, mineral- and amino acid solutions prepared in Chapter 3 Section 3.3.7 on yogurt fermentation time were evaluated.

The concentrations at which each of the three solutions was evaluated, were based on the product information leaflet provided by Oxoid on YE LP0021 (2009) indicating the amount of each present in 100 g of YE. This was referred to as the 1x concentration of each solution. A solution which contained a combination of 1x of each of the mineral-, vitamin- and amino acid solutions were prepared to evaluate their combined effect on the yogurt fermentation process.

From the mineral analysis performed on Fraction 4 (Chapter 3 Table 6) it was evident that all the minerals present in Fraction 4 were mentioned in the Oxoid Product Information Leaflet (2009) of the YE (Table 2). There were, however, additional minerals mentioned in the Product Information which were not detected in Fraction 4. It was therefore important to evaluate the effect of the minerals present in Fraction 4, as well as the effect of the minerals reported in the Oxoid Product Information, respectively, on yogurt fermentation.

The vitamin solution that was used as supplement to yogurt fermentation contained a wide range of B vitamins as YE contain high amounts of the vitamins (Chae, Joo, & In, 2001; Gaudreau, Champagne, Goulet, & Conway, 1997).

From Figure 42 it was clear that all the evaluated solutions of minerals and vitamins either reached pH 4.6 in the same time span, or increased the yogurt fermentation time in

comparison to the negative control. This included the 1x mineral-, 10x mineral-, 1x vitamin-, 5x vitamin- and the 10x vitamin solution concentrations.

The only exception was the 5x minerals solution, which reached pH 4.6 17 % faster than the negative control. The reduction in initial pH from 6.5 to 6.1 may be responsible for this decrease in fermentation time, and was therefore not considered as viable acceleration. Due to the significant reduction in initial pH, the use of the 10x mineral solution concentration as supplementation was not further investigated. The highest evaluated concentration of the mineral cocktail (10 times that of the original) decreased the initial pH even more significantly from pH 6.5 to below pH 5.8. It could be concluded that the increase in mineral concentration also decreased the initial pH of milk.

When comparing these results to literature, the only minerals that were found by Letort and Juillard (2001) to be essential for *Streptococcus thermophilus* growth were magnesium and calcium. Growth rates in a chemically defined medium (CDM) deprived of $CaCl_2$ ranged between 47 and 66 % of those obtained in the complete CDM. In contrast, the removal of iron, zinc, cobalt, copper and manganese did not significantly affect the growth rate (Letort & Juillard, 2001). The current study indicated that neither calcium nor magnesium had any positive effect on yogurt fermentation time.

Interesting conclusions could be drawn when examining the fermentation profiles of 1x minerals solution, 1x, 5x and 10x vitamin solutions, 1x amino acid solution and the combined solution in Figures 42 and 43 up to pH 5.2, which is the coagulation pH of yogurt. It was clear that at pH 5.2, all vitamin and mineral solutions exhibited similar fermentation profiles to the negative control as all reached pH 5.2 after 150 minutes. The 1x amino acid solution and the solution containing the 3 combined solutions, however, reached pH 5.2 after ±110 min, which was ±25 % faster than the negative control and close to the coagulation pH of the positive control.

The overall decreases of the fermentation curves of all mineral and vitamin concentrations evaluated corresponded to that of the negative control. This indicated that, although the initial pH differed between the independent fermentations, the mineral cocktails all had similar effects on the fermentation process as the negative control. The accelerant present in the accelerating fraction was therefore not likely to be of mineral or vitamin origin.

The increase in rate of pH decrease between 45 and 105 minutes in the positive control was not visible in any of the fermentations in Figure 42, except for the solution prepared from a combination of the vitamin-, mineral- and amino acid solutions. In the results depicted in Figure 43 however, the yogurt which had been supplemented with the 1x amino acid and 5x amino acid concentrations as well as the combined solution all followed the same yogurt fermentation profile as the positive control.

This indicated that the amino acids present in the combined solution were responsible for the tendency, and not the minerals or vitamins. What was also evident from Figure 43 was that, as the concentration of the amino acid solution increased, the initial pH of the milk correspondingly increased. Interestingly, the yogurt supplemented with the 10x amino acid solution concentration did not produce any lactic acid over a 4 hour period and the pH at the end of this interval was very similar to the initial pH.

Due the the results reported above, it was decided to continue research only on amino acids. No further research was done using any of the minerals or vitamins present in YE.

Visual observations of end products:
The respective yogurt batches supplemented with various concentrations of the mineral solution all exhibited an uneven consistency with grittiness and syneresis. This was not the case with the end products which had been supplemented with the vitamin solution which had a smooth texture and very little syneresis. The yogurts supplemented with the different amino acid solution concentrations were all as smooth as the positive control without syneresis, except the product delivered with the 20x amino acid concentration solution which closely resembled milk. The positive control was smooth and had the least amount of whey (syneresis) present, whereas the negative control, although also having a smooth texture, contained the most whey (syneresis). What was interesting was that the whey of the positive control was yellow in colour, which was not the case for the negative control. This was most probably due to the nature and colour of YE itself. The visual observations of the negative and positive controls will not be given further attention, unless when compared to observations of other end products.

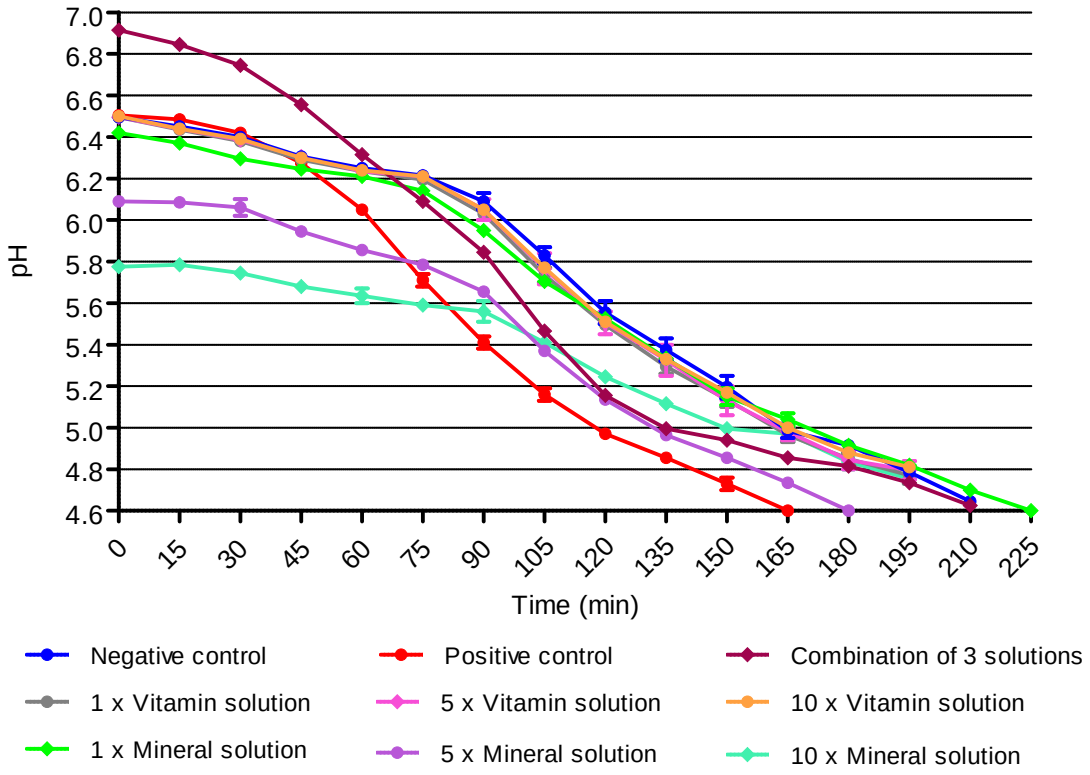


Figure 42 The effect of mineral- and vitamin solutions on the yogurt fermentation process

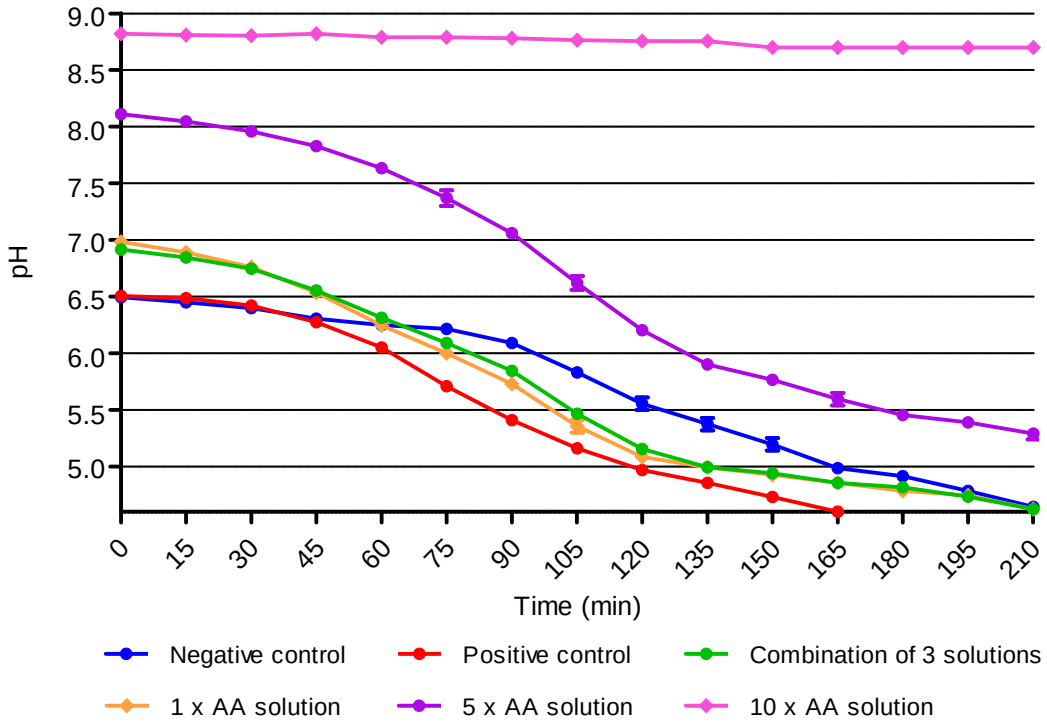


Figure 43 The effect of amino acid solutions on the yogurt fermentation process

4.3.2 PREPARATION OF A COCKTAIL CONTAINING ONLY THE AMINO ACIDS PRESENT IN THE ACCELERATING FRACTION (FRACTION 4)

The amino acids present in the accelerating fraction (Fraction 4) were combined based on its amino acid analysis (Section 3.3.9). This “simulation” of the accelerating fraction was from this point on referred to as the amino acid cocktail.

In cases where the pH of the amino acid cocktail was adjusted to 6.5 immediately after adding KOH, the solution became turbid. It was therefore important that the amino acid cocktail be stored overnight at 4 °C in order for the amino acids to completely solubilise in the solution before adjusting the pH. Furthermore, the pH had to be adjusted to pH 6.5 just before use, as the clear cocktail turned turbid when it was placed at 4 °C after titration.

4.3.2.1 ESTABLISHMENT OF THE OPTIMAL AMINO ACID COCKTAIL CONCENTRATION

Varying concentrations of the amino acid cocktail were used as supplements to independent yogurt fermentations in order to determine the optimal concentration needed for the decrease of yogurt fermentation time.

The results of Chapter 2 indicated that the supplementation of yogurt with YE concentrations ranging between 1 and 10 g/l had the same effect on yogurt fermentation time. From this data it was, however, evident that when the YE concentration exceeded 10 g/l the initial pH of the milk was increased. The higher YE concentration (100 g/l) also resulted in a significant increase in fermentation time when compared to a lower YE concentration (10 g/l) used as positive control throughout this study. This was in accordance with results obtained by Amrane and Prigent (1998) who found that YE concentrations in excess of 20 g/l led to lower lactic acid production rates. This could be attributed to the fact that it had previously been suggested that high YE concentrations might be toxic (Aeschlimann & Von Stockar 1990; Amrane & Prigent 1998). Ghaly, Tango and Adams (2003) confirmed this by stating that microorganisms require micronutrients and macronutrients only in trace quantities, as excess amounts result in retarding the synthesis of cellular material and enzymatic reactions.

From Figure 44 it was apparent that the 10x amino acid cocktail concentration increased the initial pH to 9.4 and resulted in only a slight decrease in pH over 225 min. The 5x amino acid cocktail increased the initial pH to 8.3 and resulted in a 7 % increase in fermentation time in comparison to the negative control. The lowest evaluated amino acid cocktail concentration

(1x) slightly increased the initial pH of milk to 6.8. However, it reached pH 4.6 after 195 min, which was 7 % faster than the negative control.

Results depicted in Figure 44 indicated that the initial pH of milk correspondingly increased as the amino acid cocktail concentration increased. In this stage of the study it was assumed that the high concentration of amino acid cocktail was the reason for the complete inhibition in lactic acid production in the case of the 10x amino acid cocktail concentration. The high initial pH would be problematic to the dairy industry, as it will be costly and time consuming to adjust the pH of a 5000 l batch of milk. It was therefore necessary to adjust the initial pH of the amino acid cocktail to that of the respective controls of ± 6.5.

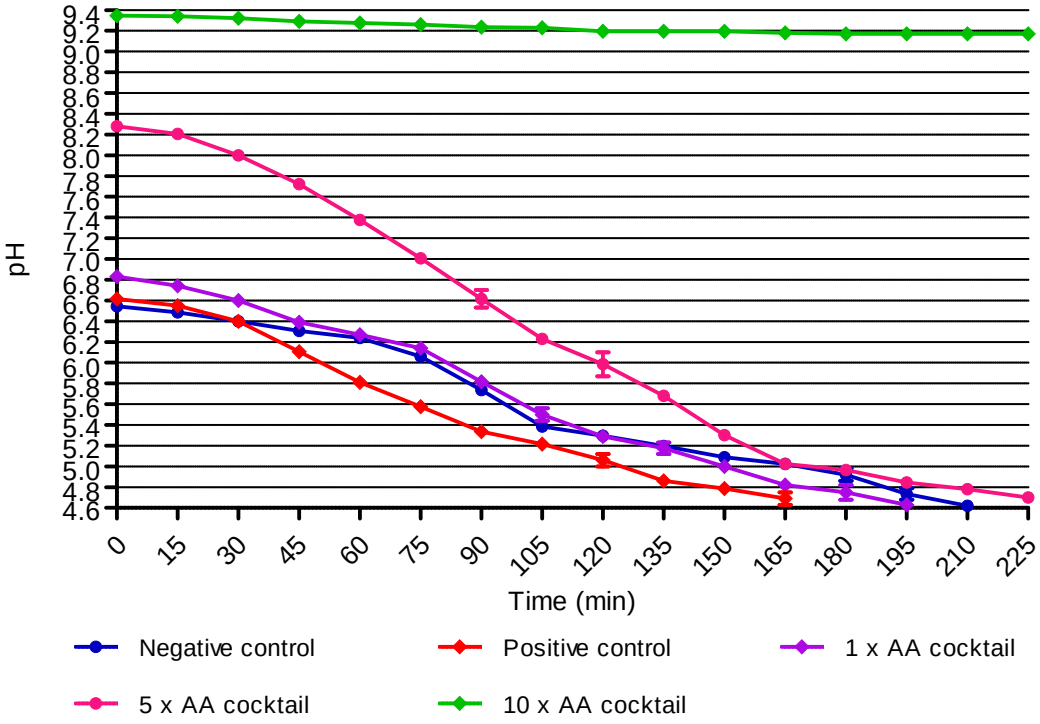


Figure 44 The effect of various concentrations of the amino acid cocktail on the yogurt fermentation process

4.3.2.2 Effect of various amino acid cocktail concentrations of which the initial pH had been adjusted

The first aspect that had to be investigated during further amino acid experiments was the fact that increasing concentrations of the amino acid cocktail increased the initial pH of milk. Due to the fact that pH decrease was used as indication of the rate of fermentation, it was essential to standardise the initial pH of all fermentations.

After adjusting and standardising the initial pH of the different amino acid cocktail concentrations (1, 5 and 10x) to ± pH 6.5 by titration with HCl and NaOH, the fermentation

curves were more consistent (Figure 45). It was apparent that when all 3 the amino acid cocktail concentrations had a similar initial pH, the varying concentrations had the same effect on the fermentation process. These data coincided with results discussed in Chapter 2 where different YE concentrations ranging between 0.2 – 10 g/l had the same effect on yogurt fermentation time. The accelerating component therefore was not concentration dependent between certain ranges.

Although the evaluated amino acid cocktail concentrations increased the time to reach pH 4.6 by 14 % in comparison to the positive control and 23 % in comparison to the pure accelerating fraction (Fraction 4), it decreased the time by 7 % when compared to the negative control. The accelerating component therefore was present in the amino acid cocktail, only not in the optimal combination or -form as in unfractionated YE. A possible explanation for the more significant decrease in fermentation time observed with the positive control and the pure accelerating fraction could be due to these two fractions containing peptides, and not only free amino acids as in the case of the amino acid cocktail.

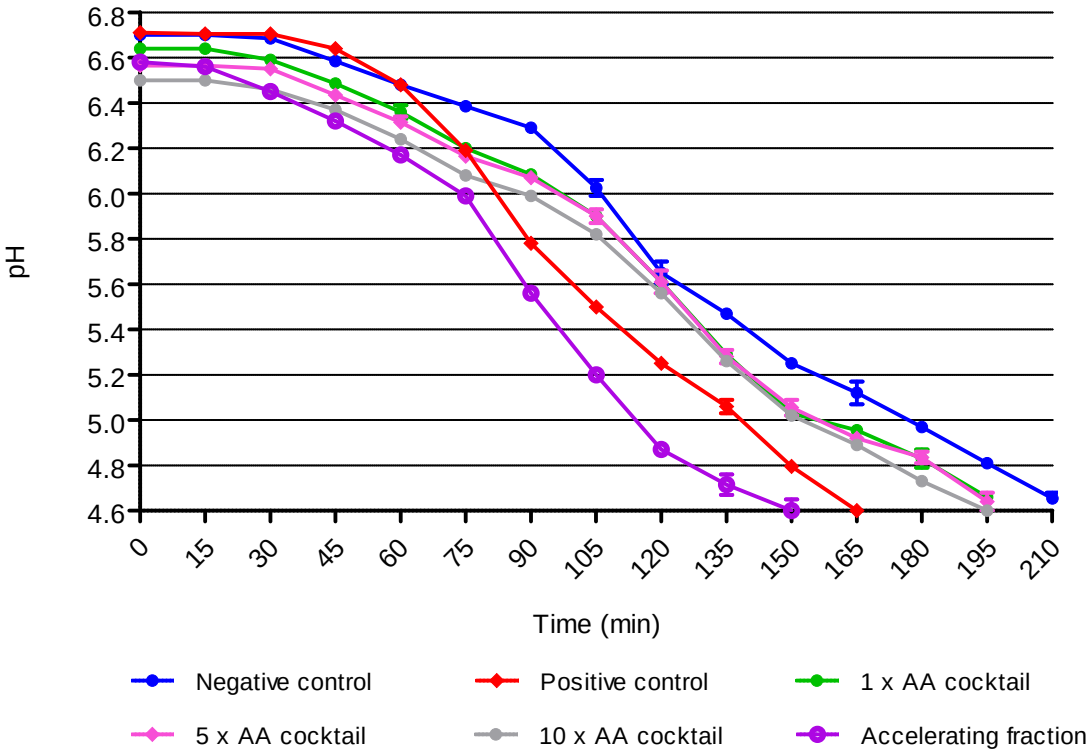


Figure 45 Various amino acid cocktail concentrations with the initial pH adjusted

It was clear from Figure 45 that although it was initially thought that the high concentration of amino acids was the reason for the inhibition in lactic acid production (Figure 44), this was

not the case. The fact that the different amino acid cocktail concentrations (which had been adjusted to an initial pH of 6.5) all delivered similar fermentation curves, indicated that amino acid cocktail concentrations ranging between 0.1-1x had the same effect on fermentation time. The inhibition in lactic acid production observed in Figure 44 was therefore not due to substrate inhibition, but due to the fact that the starter cultures were unable to grow and produce lactic acid at the high initial pH of the specific milk batches.

Visual observations of end products:
The yogurt supplemented with 1x amino acid cocktail contained less whey than the negative control. The 5x cocktail had 2 whey layers: the top layer containing very little whey, the bottom layer containing more whey. When combining the 2 layers, the amount of whey was the same as that of the 1x amino acid cocktail. The 10x cocktail contained even less whey than the 1x cocktail. The negative control exhibited the most syneresis in comparison to the other fermentations.

4.3.3 SIZE EXCLUSION CHROMATOGRAPHY OF THE PREPARED AMINO ACID COCKTAIL

In order to compare the elution time of the amino acid cocktail with that of the accelerating fraction (Fraction 4), the amino acid cocktail was analysed using size exclusion chromatography. The chromatogram obtained was very similar to that of the amino acid solution depicted in Chapter 3, Figure 41, and it was clear that the peaks of both the amino acid cocktail- and the chromatogram of the accelerating fraction were superimposed. Due to the peaks in the two chromatograms correlating so well, it was clear that the amino acid cocktail, which produced a similar fermentation profile as the positive control, eluted after the same time interval as the accelerating fraction. It could therefore be concluded that the decrease in yogurt fermentation time observed during the addition of YE was most probably due to the presence of amino acids which were assimilated by the starter bacteria either as free amino acids or as di-, tri- or poly peptides.

The fact that the positive control which contained YE decreased fermentation time more significantly than the addition of free amino acids to milk, could possibly be attributed to YE containing free amino acids as well as peptides. This is supported by other researchers, that certain organisms, which include LAB, assimilate peptides more efficiently than free amino acids (Barrette, Champagne, & Goulet, 2001; Jones & Woolley, 1962) which was most likely the reason for the differences in fermentation times. Studies by Barrette *et al*. (2001)

indicated that lactococci can assimilate peptides and do not require a complete hydrolysis of the proteins into free amino acids. This research group also observed that some strains even prefer peptides over free amino acids. The mechanism of the stimulation of growth of other lactic acid bacteria by peptides has been postulated as the ability to serve as a means of increasing the penetration of some amino acids into the cells (Jones & Woolley, 1962). Peptides can therefore be superior to free amino acids as nitrogen sources for LAB because they can provide the cell with amino acids in a form that can be utilised more efficiently (Yoo, 2009).

Based on this information, the fact that the amino acid cocktail in the current study did not accelerate the fermentation process as significantly as the positive control indicated that small peptides could probably be responsible for the accelerating effect. This assumption was based on the fact that, if the acceleration was due to free amino acids only, the amino acid cocktail would result in the same acceleration as the positive control, which according to the results, was clearly not the case.

4.3.4 Effect of free amino acids on yogurt fermentation

The fact that YE is rich in amino acids and peptides can be used as a possible explanation for the accelerating effect observed in fermentation time when milk is supplemented with YE. It was hypothesised that this effect could possibly be attributed to increased *Streptococcus thermophilus* growth. Due to the poor proteolytic ability of *Streptococcus thermophilus* discussed in Chapter 1, the increased amount of amino acids and peptides provided during the addition of YE, stimulated growth. As it was clear from results obtained in Chapter 3, the free amino acids and the peptides present in the accelerating fraction could not be separated. Focus was therefore placed on the effect of free amino acids, singularly as well as in various combinations, on the yogurt fermentation process.

Due to the prepared amino acid cocktail not decreasing yogurt fermentation time as much as Fraction 4, although both theoretically had the same amino acid content, it was decided to evaluate the effect of the individual amino acids (Figures 46A-46C).

Figures 46A and 46B indicated the supplementation of independent yogurt batches with 0.007 g/l L-cysteine and 0.31 g/l L-leucine increased the yogurt fermentation process significantly. These results were in contrast with the reports of Thomas and Mills (1981) and Mikhlin and Radina (1981), which indicated that cysteine was essential for *Streptococcus thermophilus* growth.

Supplementation of yogurt with 0.217 g/l iso-leucine (Figure 46C) resulted in an almost complete inhibition of lactic acid production. Although the addition of 0.686 g/l L-glutamic acid (Figure 46B) resulted in a decrease in the initial pH of the milk, it had no effect on the yogurt fermentation process in comparison to the negative control. Supplementation of yogurt with 0.218 g/l L-threonine and 0.3 g/l L-valine (Figure 46A), 0.34 g/l alanine and 0.19 g/l L-glycine (Figure 46C) all respectively resulted in a 7 % increase in fermentation time, when compared to the negative control. When compared to the negative control, 0.25 g/l serine increased the fermentation time by 14 %. The addition of all other evaluated single amino acid s including L-methionine (0.09 g/l), L-tyrosine (0.033 g/l) (Figure 46B), 0.315 g/l L-aspartic acid, 0.085 g/l L-tryptophan, 0.04 g/l L-phenylalanine and 0.045 g/l L-histidine (Figure 46A), 0.19 g/l arginine, 0.253 g/l lysine and 0.14 g/l proline (Figure 46C), neither had any effect on the rate of lactic acid formation, and pH 4.6 was reached in all instances in the same time span as the negative control. As was the case for all other fermentations in this study, the positive control decreased the time to reach pH 4.6 by about 21 %.

An interesting observation was that all the hydrophobic aliphatic amino acids, which included valine, leucine, iso-leucine, glycine and alanine, increased the fermentation process in comparison to the negative control.

As none of the individual amino acids decreased yogurt fermentation time in the concentrations present in the accelerating fraction, the specific amino acid concentrations listed on the Oxoid Product Information Leaflet (2009) of YE, were also evaluated (data not shown). Similar fermentation profiles as observed in Figures 46A-46C were observed and no decrease in fermentation times were observed in comparison to the positive control.

Although using different conditions, the above results are in agreement with those obtained by Letort and Juillard (2001). This research group found that, when *Streptococcus thermophilus* was cultivated using CDM, most strains exhibited no absolute amino acid requirement. The removal of individual amino acids from the complete CDM also did not prevent *Streptococcus thermophilus* growth.

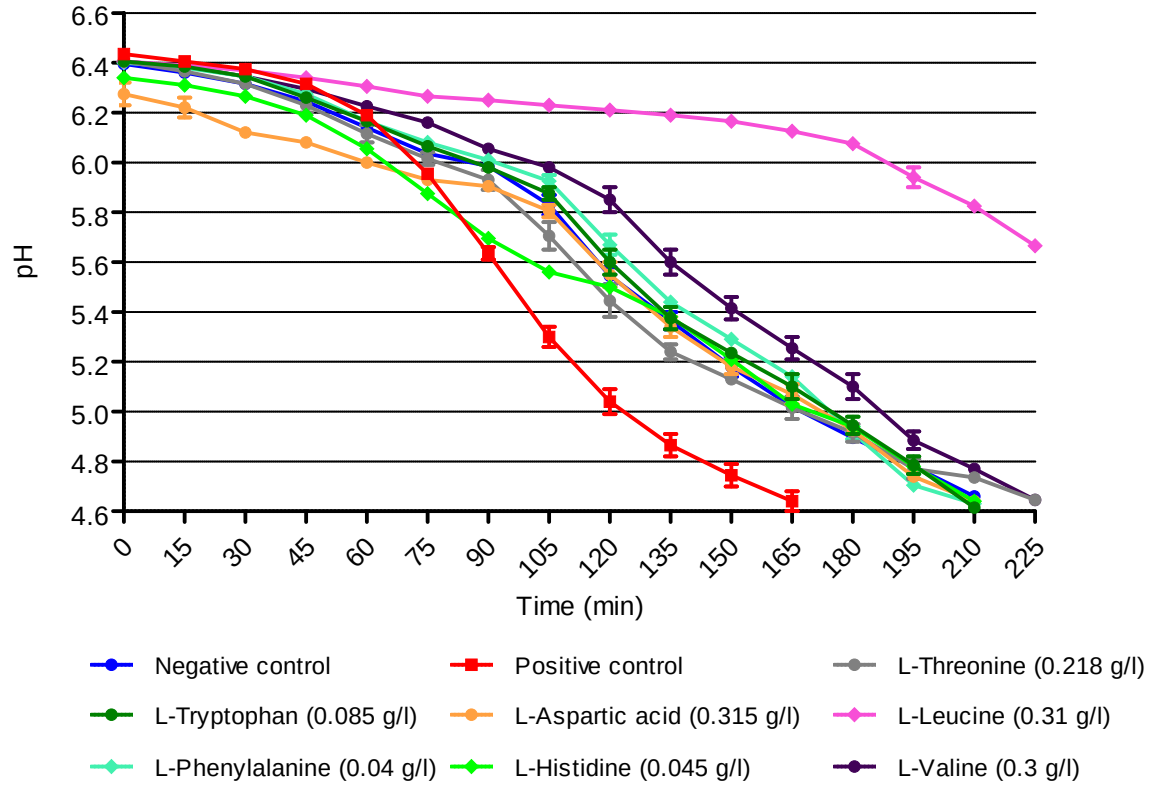


Figure 46A The effect of individual amino acids on the yogurt fermentation process

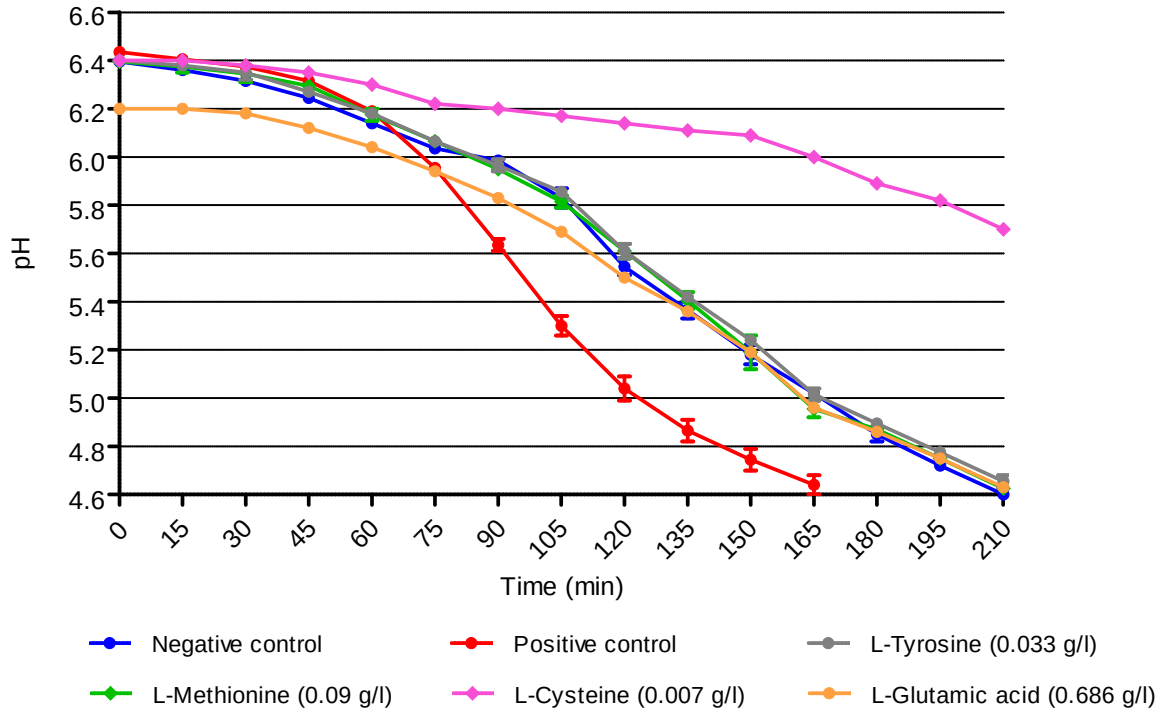


Figure 46B The effect of individual amino acids on the yogurt fermentation process

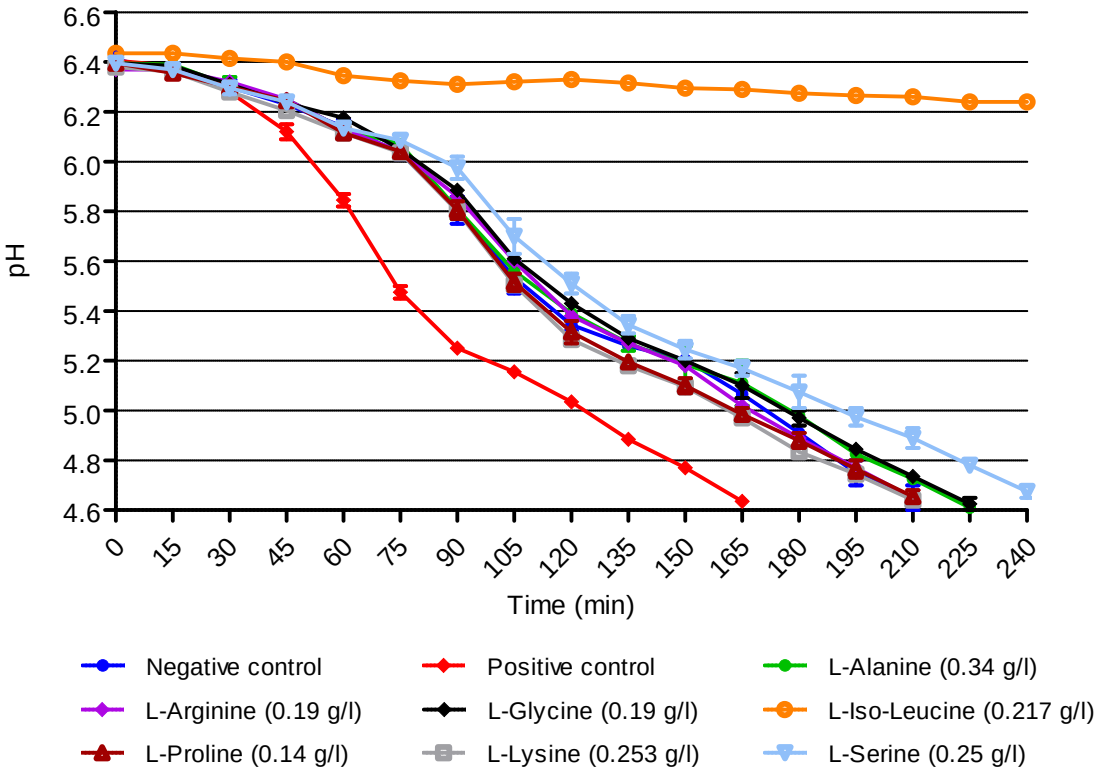


Figure 46C The effect of individual amino acids on the yogurt fermentation process

The initial acceleration in acid production of the yogurt supplemented with L-histidine at 75-120 min (Figure 46A) displayed potential. It was thought possible that the decrease in acid production rate after 120 minutes was due to depletion in substrate. L-Histidine supplementation was therefore evaluated at higher concentrations (1 g/l and 2 g/l). Results clearly indicated that the higher loads decreased the initial pH of milk and had the same effect on fermentation time as the original 0.2 g/l (data not shown). Substrate deficiency was therefore not the cause of the increase in acid production rate between 75 and 120 min and further fermentations with histidine were therefore not done.

Visual observations of end products produced by supplementing yogurt with individual amino acids:

The yogurts supplemented with L-aspartic acid, L-arginine, L-phenylalanine, L-methionine, L-valine, L-tyrosine, L-threonine, L-glutamic acid and the negative control were smooth and slightly thick, L-histidine and L-lysine products were both watery, whereas the yogurts supplemented with L-glycine and L-tryptophan was watery and smooth. The positive control was smooth and thick and the L-leucine supplemented yogurt was very slimy. The products delivered with L-alanine and L-proline and L-serine all had a smooth texture with very little

whey, whereas the yogurts supplemented with L-iso-leucine and L-cysteine both exhibited no syneresis and were very slimy.

4.3.5 AMINO ACID PREPARATIONS WHICH HAD NO EFFECT ON INITIAL MILK PH AND PREPARATIONS WHICH ALTERED INITIAL MILK PH

Due to the HCl, NaOH and KOH used during pH adjustments by titrations, it had to be determined whether the presence of these chemicals influenced the yogurt fermentation process. This was done in two experiments: First supplementing yogurt with only the amino acids which had the same pH as milk when used in the concentrations at which they are present in the accelerating fraction (±pH 6.5). No pH adjustments were required and therefore no additional acids or bases had to be added. A second preparation contained the amino acids present in the first preparation, plus the amino acids which altered the initial pH of milk. Chemicals used during titrations were therefore present in the second preparation.

The results obtained (not shown) indicated that the addition of acids or bases used during pH adjustments by titrations did not influence the fermentation process when compared to the preparation without them. Adjusting the pH of the various cocktails and supplements could therefore be done without concern that it would influence the yogurt fermentation process. The profiles of the fermentation curves of the two preparations were very similar and did not differ significantly from the various amino acid cocktail profiles in Figure 45. Two main conclusions could be drawn from these results: Firstly the presence of acids or bases due to titrations prior to the fermentation process did not significantly influence the yogurt fermentation process. Secondly, it was apparent that the additional 5 amino acids added to the second preparation, namely L-cysteine, L-glutamic acid, L-aspartic acid, L-histidine and L-arginine, did not have any effect (positive or negative) on the fermentation process when compared to the influence of the first amino acid preparation. It could therefore be assumed that none of these 5 last mentioned amino acids was responsible for the accelerating effect on fermentation time when used singly.

Visual observations of end products:
The respective yogurts supplemented with the amino acid preparations which had no effect on initial pH, as well as the preparations which altered the initial pH, exhibited no syneresis and had a smooth texture.

A possible explanation for the absence of syneresis in the respective yogurt fermentations supplemented with the two amino acid preparations can be that the specific amino acids used in this instance interacted with the casein proteins in milk, forming a matrix in which whey proteins were entrapped, thus preventing syneresis.

4.3.6 EFFECT OF VARIOUS COMBINATIONS OF AMINO ACIDS PRESENT IN THE ACCELERATING FRACTION ON THE YOGURT FERMENTATION PROCESS

Since the above results demonstrated that individual amino acids alone did not result in the same decrease of yogurt fermentation as the positive control or as the amino acid cocktail, it was decided to use the amino acids present in the amino acid cocktail in various combinations. The same concentrations in which the amino acids were present in the amino acid cocktail were used.

Initially it was intended to evaluate all the possible combinations of the 17 amino acids present in the accelerating fraction. After consultation with a mathematical statistician however, it was established that this will require a very large number of combinations. The possible combinations were therefore narrowed down stepwise, starting with the following five combinations:

- The amino acids which contribute to less than 3 % of the total amino acids present in the accelerating fraction
- The amino acids which contribute to more than 3 % of the total amino acids present in the accelerating fraction
- The 4 amino acids present in the lowest concentrations in the accelerating fraction
- The 10 amino acids present in the lowest concentrations in the accelerating fraction
- The amino acid cocktail without L-leucine, L-iso-leucine and L-cysteine

Based on results in Figures 46A, 46B and 46C which indicated the inhibiting effects of L-iso-leucine, L-leucine and L-cysteine, these 3 amino acids were not used in any of the combinations, except the full amino acid cocktail.

The results shown in Figure 47 indicated that none of the evaluated amino acid combinations decreased the fermentation time to reach pH 4.6 in comparison to the respective controls. From all the amino acid combinations, the 4 amino acids present in the lowest concentrations in the accelerating fraction resulted in the fastest fermentation time, which was still an increase of 14 % in comparison to the negative control. The amino acid

combinations where amino acids which contribute to 3 % or more of the total amino acid content, and the 10 amino acids present in the highest concentrations in the accelerating fraction, respectively, both resulted in a 21 % increase in fermentation time. The amino acid cocktail without the L-leucine and L-cysteine resulted in an even more significant increase in fermentation time of 42.9 %. The amino acids which contributed to less than 3 % of the total amino acid content in the accelerating fraction reached pH 4.6 in the longest time interval, and resulted in a 63.6 % increase in comparison to the negative control.

What can be noted when examining the effect of the amino acids which contribute more than 3 % and the 4 amino acids lowest in concentration, is that these 2 combinations were the only 2 which did not contain L-iso-leucine. This may be significant since these 2 combinations reached pH 4.6 in the shortest time span in comparison to the other combinations. This corresponds to results discussed in Figure 46C which indicated that free L-iso-leucine clearly increased yogurt fermentation time significantly. When examining the effect of the 1x amino acid cocktail which contained L-iso-leucine, however, it is clear that, as previously observed, the fermentation process was accelerated. It was therefore hypothesised that although L-iso-leucine (which clearly increased fermentation time when evaluated individually) was present in the amino acid cocktail, it either interacted with other amino acids to form an accelerating peptide, or the accelerating effect of other amino acids present in the amino acid cocktail overpowered its inhibiting effect.

What was interesting was that although the addition of free L-leucine and L-cysteine (Figures 46A and 46B) resulted in significant increases in the time to reach pH 4.6, when an amino acid cocktail was prepared without L-leucine and L-cysteine, the fermentation time was increased by nearly 43 % (Figure 47). The reason for this phenomenon was not clear, although the presence of L-iso-leucine may have played a possible role.

Although the combination containing the 4 amino acids lowest in concentration (L-histidine, L-tyrosine, L-methionine and L-phenylalanine) did not accelerate yogurt fermentation, the results were still in accordance with results obtained by Letort and Juillard (2001) that indicated that when L-leucine, L-iso-leucine and L-valine were removed from complete CDM, *Streptococcus thermophilus* was still able to grow.

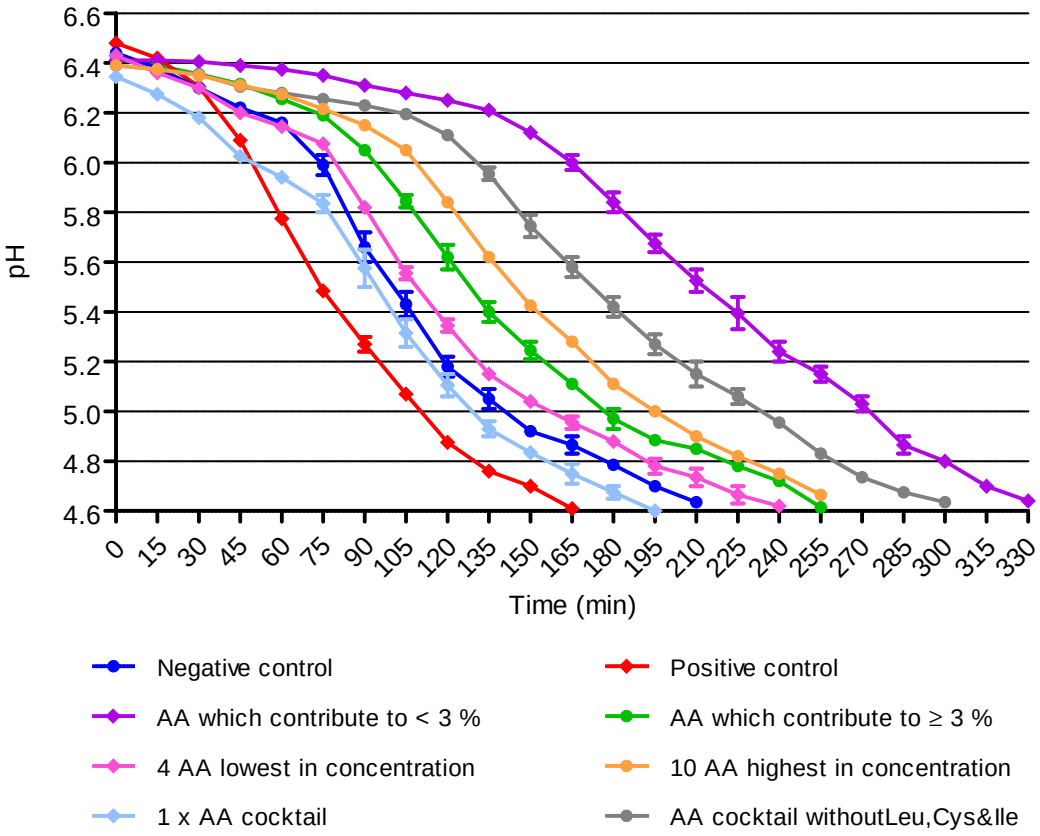


Figure 47 The effect of various combinations of the amino acids present in amino acid cocktail on the yogurt fermentation process

Visual observations of end products:

During research on the addition of amino acids in various combinations to milk, it seems that different combinations influenced the texture and viscosity of the yogurt in different ways. The addition of the full amino acid cocktail delivered a very smooth product with the desired consistency required for a high quality yogurt. The YE supplemented yogurt which served as positive control, as well as the yogurt supplemented with the 11 amino acids highest in concentration, produced a smooth yogurt. Yogurt supplemented with the 4 amino acids lowest in concentration was uneven in texture. The yogurt supplemented with the full amino acid cocktail without L-leucine and L-cysteine produced the highest quality yogurt in terms of smoothness. The combination containing the amino acids which contribute to 3 % or more of the total amino acid content in the accelerating fraction delivered a yogurt smoother in consistency and thicker than that of amino acids which contribute to less than 3 %. It was however not as desirable a texture and consistency as that delivered by the amino acid cocktail, the cocktail without L-leucine and L-cysteine, the 11 amino acids present in the highest concentrations and the positive control.

4.3.7 EVALUATION OF THE EFFECT OF VARIOUS AMINO ACID COMBINATIONS (BASED ON LITERATURE) ON THE YOGURT FERMENTATION PROCESS

Due to the very little conclusions being drawn from the results in Figure 47, different amino acid combinations were once again investigated. In this case the combinations were based on amino acid combinations described in literature (Table 9) to stimulate *Streptococcus thermophilus* growth. Focus was placed on the growth of *Streptococcus thermophilus* due to this organism being the growth limiting organism between the 2 starter organisms used for yogurt fermentation.

Researchers reported different amino acids to stimulate *Streptococcus thermophilus* growth. Letort and Juillard (2001) reported that, when cultivated in milk, the exponential growth of *Streptococcus thermophilus* required L-glutamic acid, L-methionine and L-histidine. When cultivated in a minimal CDM however, L-glutamic acid, L-cysteine, L-methionine, L-valine, L-leucine and L-iso-leucine are required. Further reports indicated that during cultivation in CDM without L-glutamic acid, L-cysteine, L-methionine, L-valine and L-leucine, the growth of *Streptococcus thermophilus* was very low, and even completely abolished in some strains (Letort & Juillard, 2001).

All the combinations reported by various researchers listed in Table 9 were evaluated. Due to L-cysteine, L-iso-leucine and L-leucine increasing yogurt fermentation time so significantly, these 3 amino acids were removed from all combinations in Table 9 to constitute new combinations for additional evaluation.

When comparing the effects of Combinations 1 and 4 on the fermentation process, it was clear that, although both resulted in the increase in fermentation time when compared to the negative control, Combination 4 increased the fermentation process even more than Combination 1 (Figure 48A). The only logical explanation for this phenomenon was the presence of L-valine in Combination 4, which resulted in the further increase in fermentation time. These results were in accordance with reports by Bautista, Dahiya and Speck (1966), who did not observe stimulation of *Streptococcus thermophilus* growth by L-valine. Accolas, Veaux and Auclair (1971) however, observed stimulation of *Streptococcus thermophilus* by various mixtures of L-valine, L-leucine, L-histidine and L-iso-leucine. In similar studies, Shihata (2004) concluded that omission of L-glutamic acid, L-valine, L-leucine, L-histidine and L-tryptophan from the growth medium reduced the stimulation of *Streptococcus thermophilus* growth by 50 %. Similar results were obtained by Beshkova, Simova, Frengova, Somov and Adilov (1998) who indicated that *Streptococcus thermophilus* required

L-glutamic acid, L-histidine, L-methionine, L-cysteine, L-valine, L-leucine and L-tyrosine for optimal growth. According to these results, one would expect Combination 7, which contained all of these amino acids, to decrease yogurt fermentation time. When comparing the above with the results obtained in Figure 48B however, Combination 7 clearly increased the fermentation process in comparison to the negative control. The other amino acids present in Combination 7, not mentioned by the above researchers, as well as the fact that milk was used as cultivation medium and not microbial broth, could all contribute to the conflicting results.

The influence of fortifying milk with some amino acids on growth and lactic acid production by *Streptococcus thermophilus* 2002, *Streptococcus thermophilus* 2014, *Lactobacillus bulgaricus* 2501, *L. acidophilus* 2405, and *Bifidobacterium* ssp. 20210 was studied by Shihata (2004). Both *Streptococcus thermophilus* strains (2002 and 2014) had utilised the amino acids L-aspartic acid, L-threonine, L-serine, L-glutamic acid, L-alanine, L-valine, L-leucine, and L-lysine. Bacterial cultures were also grown in the presence of amino acids, in particular, those containing sulphur, i.e. L-cysteine and L-methionine to assess their influence on cell growth and lactic acid production. The differences in the log cell counts among the yogurt bacteria when supplemented with the amino acids were lower when compared to the control group (not supplemented with amino acids) (Shihata, 2004).

None of the 10 various combinations evaluated in Figures 48A and 48B decreased the yogurt fermentation time in comparison to the negative control or the yogurt containing the amino acid cocktail.

Results obtained by Shihata (2004) however indicated growth stimulation by the specific 10 amino acids (Table 9). The differences between the results obtained in the current study and that of Shihata (2004) could possibly be attributed to the varying conditions of the two studies. In studies by Shihata (2004) all 10 amino acids were filter sterilised and combined to give final concentrations of 0.05 % (w/v). Incubation was carried out at 37 °C. Samples were taken at 0, 6, 12 and 24 hours. Development of acidity was determined by measuring the pH (at 0, 6, 12, and 24 hours) of bacterial culture by use of a standard pH meter.

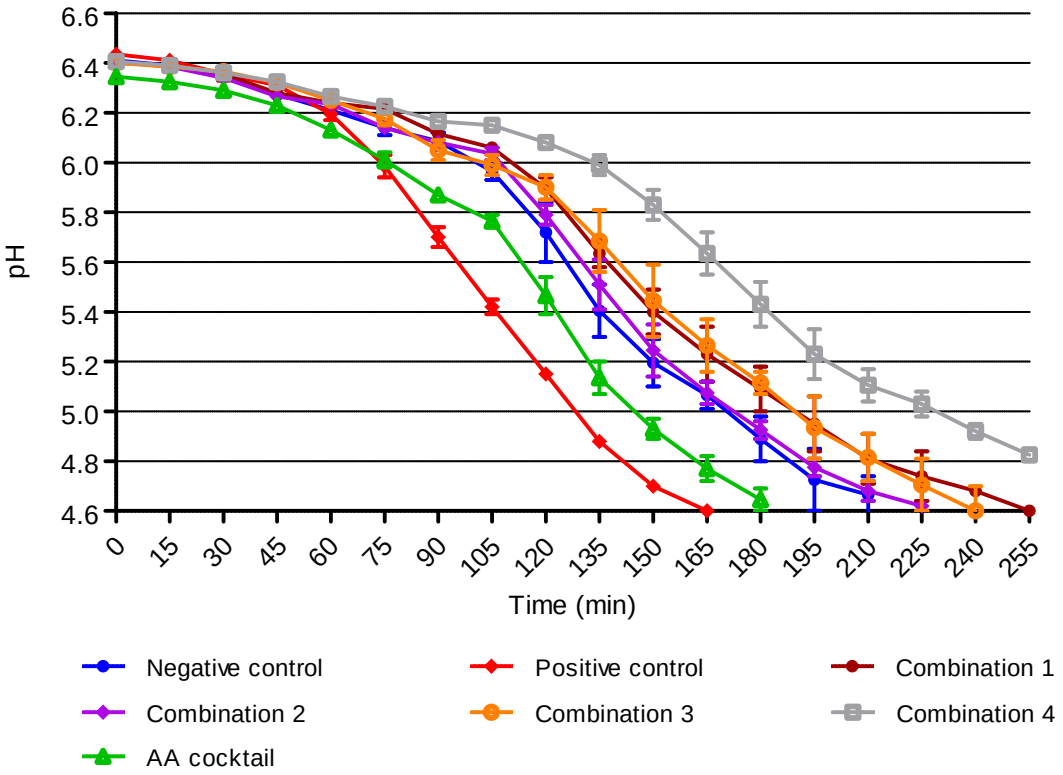


Figure 48A Effect of combinations of free amino acids on yogurt fermentation according to literature Combination 1: Glu, Met, His, Combination 2: Glu, Cys, Met, His, Combination 3: Glu, Cys, Met, His, Val, Leu, Combination 4: Glu, Met, His, Val.

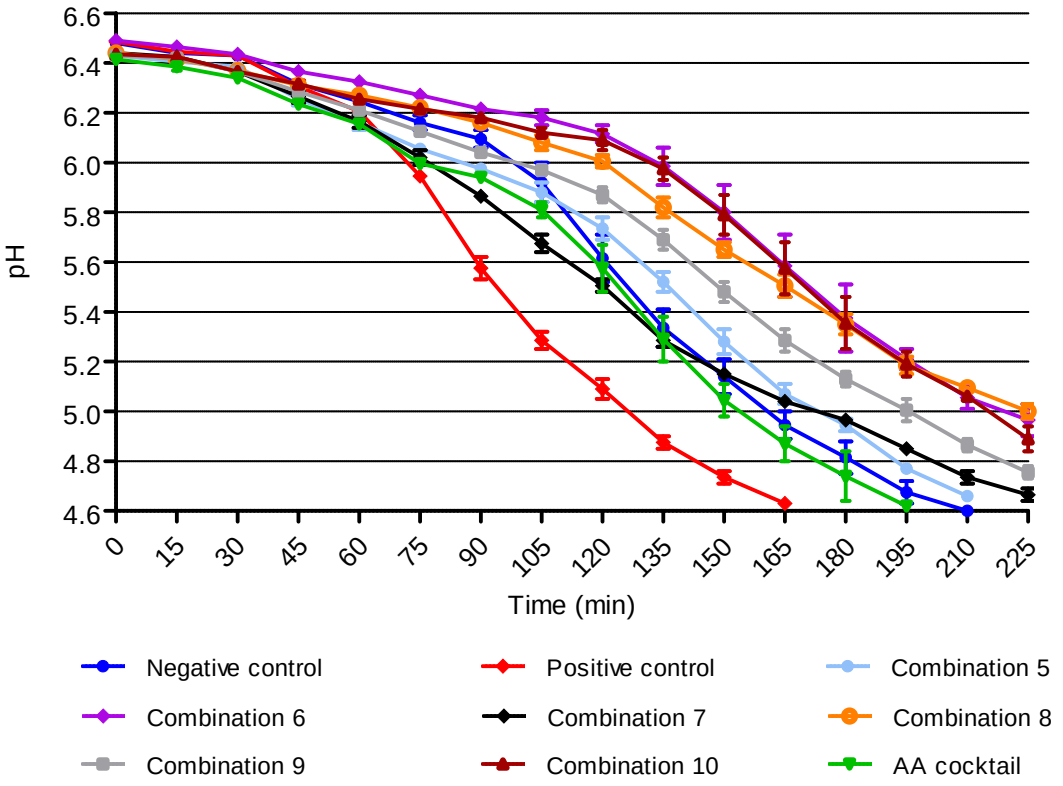


Figure 48B Effect of combinations of free amino acids on yogurt fermentation according to literature Combination 5: Glu, Cys, Met, Val, Leu,Ile, Combination 6: Glu, Met, Val, Combination 7: Glu, Cys, Met, His, Val, Leu, Ile, Trp, Arg, Tyr, Combination 8: Glu, Met, His, Val, Trp, Arg, Tyr, Combination 9: Glu, Cys, Met, Val, Leu, Gln, Combination 10: Glu, Met, Val, Gln.

Visual observations of end products:

The yogurt supplemented with Combination 1 had approximately the same amount of whey than the positive control, while combination 2 had the same amount of whey as the negative control. The presence of L-cysteine therefore increased the amount of whey present on top of the final product. Combination 3 had the most whey in comparison to the other fermentations. The yogurt produced with the supplementation of combination 4 formed whey pockets throughout the product and whey did not form a layer on top of the products as in the case of the other fermentations. The total amount of whey present in the product was comparable to the positive control. Combinations 5, 6, 8, 9 and 10 exhibited more whey than the negative control. Combination 7 and the yogurt containing the amino acid cocktail had the same amount of whey as the negative control.

Concerning the texture of the products, Combinations 1 and 4 had coarse textures, whereas Combination 2 had a much smoother texture. Combination 3 also had a coarse texture to some extent, but the end product was thicker than that of combination 4. Combination 5 had

a smooth texture, with combination 6 resulting in a coarse texture with little slime production. The product produced with the addition of combination 7 was smooth and exhibited high slime production. Combinations 8 and 9 were both smooth, with combination 9 containing more slime than combination 8. The combination 10 yogurt had a coarse texture with little slime present. The cocktail yogurt was relatively smooth with little slime production, although it contained some lumps. The positive control had the smoothest texture of all the end products.

The fact that (as previously described) some LAB can assimilate peptides better than free amino acids and in some instances even prefer peptides (Barrette *et al*., 2001; Yoo, 2009), can serve as a possible explanation for the different results observed in Figures 48A and 48B. It is possible that, as the amino acid combinations differed, the assimilation of the various amino acids by the starter organisms also differed, resulting in different effects on the fermentation time.

It must be noted that no stabilisers were used during any yogurt fermentation studies. Reference to texture and consistency was therefore directly due to the addition of the various supplements.

When the amino acids present in Fraction 4 were evaluated individually or in various combinations, no accelerating effect was observed. This indicated that the acceleration by Fraction 4 may be due to the presence of a peptide or polypeptide, and not only single amino acids.

4.4 CONCLUSIONS

During the evaluation of the effect of amino acid-, vitamin- and mineral solutions respectively on the yogurt fermentation process, it became clear that the accelerating component present in YE was not a mineral or vitamin, but could be of amino acid origin. Although it was found that YE accelerated the yogurt fermentation process, when amino acids were evaluated individually or in various combinations, the extent of acceleration as observed with the YE was not attained. This indicated that at least one essential nutrient was lacking despite that it was not detected using the combinations evaluated. It may therefore be concluded that the component responsible for accelerated yogurt fermentation may be a peptide, possibly in combination with free amino acids.

An interesting question to be asked at this stage is: what is the specific mechanism with which the accelerating components in YE decrease yogurt fermentation time. This was studied in Chapter 5.

4.5 REFERENCES

Abraham, A. G., Antoni, G. L., & Anon, M. C. (1993). Proteolytic activity of *Lactobacillus bulgaricus* grown in milk. *Journal of Dairy Science*, *76*, 1498–1505.

Accolas, J. P., Veaux, M., & Auclair, J. (1971). Study of interactions between various mesophillic and thermophilic lactic acid bacteria in relationship with the production of hard cheese. *Lait*, *51*, 249–272.

Aeschlimann, A., & Von Stockar, U. (1990). The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helveticus*. *Applied Microbiology and Biochemistry*, *32*, 398–402.

Amend, J., & Helgeson, H. (1997). Solubilities of the common L-amino acids as a function of temperature and solution pH. *Pure and Applied Chemistry, 69*, 935-942.

Amrane, A., & Prigent, Y. (1998). Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. *World Journal of Microbiology and Biotechnology*, *14*, 529–534.

Barrette, J., Champagne, C. P., & Goulet, J. (2001). Growth-promoting properties of yeast extracts produced at different pH values, with different autolysis promoters and bacterial populations. *Journal of Chemical Technology & Biotechnology*, *76*, 203–209.

Bautista, E. S., Dahiya, R. S., & Speck, M. L. (1966). Identification of compounds causing symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk. *Journal of Dairy Science*, *33*, 299.

Beshkova, D. M., Simova, E. D., Frengova, G. I., Somov, Z. I., & Adilov, E. F. (1998). Production of amino acids by yoghurt bacteria. *Biotechnology Progress Journal*, *14*, 963–965.

Chae, H. J., Joo, H., & In, M. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid and protein recovery and flavor characteristics. *Bioresource Technology*, *76*, 253–258.

Chandan, R. C., Argyle, P. J., & Mathison, G. E. (1982). Action of *Lactobacillus bulgaricus* proteinase preparations on milk proteins. *Journal of Dairy Science*, *65*, 1408–1413.

Gaudreau, H., Champagne, C. P., Goulet, J., & Conway, J. (1997). Lactic fermentation of media containing high concentrations of yeast extracts. *Journal of Food Science*, *62*, 1072–1075.

Ghaly, A. E., Tango, M. S. A., & Adams, M. A. (2003). Enhanced lactic acid production from cheese whey with nutrient supplement addition. *Agricultural Engineering International: the CIGR Journal of Scientific Research and Development*, *5*, 1–20.

Iyer, R., Tomar, S. K., Maheswari, T. U., & Singh, R. (2010). *Streptococcus thermophilus* strains: Multifunctional lactic acid bacteria. *International Dairy Journal*, *20*, 133–141.

Jones, K. M., & Woolley, D. W. (1962). Peptides required for the growth of *Lactobacillus bulgaricus*. *Journal of Bacteriology*, *83*, 797–801.

Khalid, N. M., El Soda, M., & Marth, E. H. (1991). Peptide hydrolases of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Journal of Dairy Science*, *74*, 29–45.

Letort, C., & Juillard, V. (2001). Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. *Journal of Applied Microbiology*, *91*, 1023–1029.

Mikhlin, E. D., & Radina, V. P. (1981). Activation of lactic acid bacteria. *Applied Biochemistry and Microbiology, 17*, 253–267.

Robinson, R. K. (2001). *Dairy Microbiology* (3rd ed.). New York: Wiley Interscience.

Shihata, A. (2004). *The proteolytic activity of yoghurt and probiotic bacteria for the improved viability of probiotic bacteria in fermented milk products* (Doctoral dissertation, Victoria University, Australia). Retrieved from http://vuir.vu.edu.au/15367.

Shihata, A., & Shah, N. P. (2000). Proteolytic profiles of yogurt and probiotic bacteria. *International Dairy Journal*, *10*, 401–408.

Speck, M. L., McAnelly, J. K., & Wilbur, J. D. (1958). Variability in response of lactic Streptococci to stimulants in extracts of pancreas, liver, and yeast. *Journal of Dairy Science*, *41*, 502–508.

Thomas, T., & Mills, O. (1981). Proteolytic enzymes of starter bacteria. *Netherlands Milk and Dairy Journal*, *35*, 255–273.

Yoo, S. M. (2009). *Fractionation of non-animal protein hydrolysates for use in CHO (Chinese Hamster Ovary) cell media*. University of Waterloo.

CHAPTER 5

GROWTH RATES AND LACTIC ACID PRODUCTION OF YOGURT STARTER CULTURES

5.1 INTRODUCTION

The activity of yogurt starter bacteria can be measured either by a decrease in pH or a rise in titratable acidity. When lactic acid is not produced by a starter culture at a desired rate, the culture is called 'slow'. The slowness can be due to either the genetic makeup of the strains or external factors. The latter may include:

- Bacteriophage
- Residues of antibiotics and sanitising agents
- Inhibitory compounds naturally present in milk
- Variations in milk composition due to mastitis or seasonal factors
- Metabolites of spoilage bacteria
- Irregular culture transfers
- Fluctuations in incubation temperature
- Over acidification

  (Surono & Hosono, 2011)

Champagne, Gaudreau and Conway (2003) stated that, since μ_{max} occurs at the beginning of the growth curve, the effect on growth is related to the mere presence of essential as well as stimulatory components, and high concentrations are not required at this point. However, for OD_{max}, the concentration of all the essential growth factors is of utmost importance, since growth will stop when only one essential factor has been depleted in the medium. In their studies they found a correlation between OD_{max} and μ_{max} values.

Although *Lactobacilllus delbruekii bulgaricus* growth flourishes in media of defined composition, the time required for heavy growth from small inocula in such media is reduced considerably by the addition of crude natural materials such as YE. These supplements reduce the length of the lag phase when cultivated in chemically defined media, but do not increase the growth rate materially (Ikawa & O'Barr, 1956). This had to be investigated in detail in the current study where *Lactobacillus bulgaricus* was used together with *Streptococcus thermophilus* as a co-culture, using milk as medium.

When appropriate levels of acidity is reached after fermentation i.e. pH 4.6 has been reached, ±70 % of the original amount of lactose still remains in the milk (Shihata, 2004; U.S. Patent No. 6,399,122 B2, 2002). This indicates that lactose is in abundance in milk and is not a limiting factor during yogurt fermentation.

Due to the fact that it was not clear whether the supplementation of milk with YE prior to yogurt fermentation increased the rate of cell growth or lactic acid production, both these parameters were investigated.

Growth responses of the starter culture in milk used for yogurt fermentation were measured by adding 10 g YE per liter of milk prior to pasteurisation. This was compared to growth responses in unsupplemented milk.

From literature it is evident that Elliker's broth promotes superior recovery of *Lactobacilllus bulgaricus* and *Streptococcus thermophilus* (Barach, 1979; Moon, Hamann, & Reinbold, 1974). Studies by Moon *et al*. (1974) showed that Elliker's broth at pH 6.8 was superior to other media with regard to *Lactobacilllus bulgaricus* and *Streptococcus thermophilus* recovery, range and colony morphology. For this reason this specific medium was used to enumerate the two bacteria used as yogurt starter culture in the current study.

In order to determine the specific mechanism with which YE decreased yogurt fermentation time, three hypotheses were investigated:

1. Does YE addition increase starter culture growth rate (lactic acid production per cell unchanged, cell amount increased)?;

2. Does YE addition meet the growth requirements of starter bacteria resulting in increased levels of lactic acid production (amount of cells constant, lactic acid production optimised)?; and,

3. Does YE addition decrease the lag phase of lactic acid production (shorter lag phase, fermentation more efficient)?

5.2 MATERIALS AND METHODS

5.2.1 GROWTH STUDIES OF STARTER BACTERIA USED FOR YOGURT FERMENTATION

5.2.1.1 METABOLIC ACTIVITY OF STARTER BACTERIA

The metabolic activity of the starter culture was determined using resazurin tablets (Merck) which had been certified by the Commission of Standardisation of Biological Stains to contain approximately 11 mg dye per tablet. Yogurt was manufactured as described in Chapter 2. The difference in metabolic activity between the control yogurt and the YE supplemented (10 g/l) yogurt was evaluated. A batch solution containing one resazurin tablet dissolved in 200 ml distilled water was prepared. Aliquots of 9 ml of the YE supplemented, control yogurt as well as pasteurised reconstituted skim milk (Section 2.2.1.1), respectively, were taken every 30 min and added to 1 ml of resazurin solution. The temperature of the samples was brought to 35 °C within 10 minutes. As soon as the temperature reached 35 °C, the tubes were inverted a few times to ensure uniform distribution of the resazurin solution. This time was recorded as the beginning of the incubation period. The time that it took to change from white to pink was noted and was used as an indication of the metabolic activity (Moyer & Campbell, 1963).

5.2.1.2 ENUMERATION OF YOGURT STARTER BACTERIA

Determination of suitable dilution levels

An initial experiment was performed in order to determine which sample dilutions should be used to evaluate the different media. Elliker's broth (Sigma-Aldrich) containing 1.5 % bacteriological agar (Oxoid) was adjusted to pH 6.8 and used for this preliminary experiment.

The two starter bacteria, *Streptococcus thermophilus* and *Lactobacilllus bulgaricus*, were enumerated independently by aseptically blending 1 ml yogurt samples in 9 ml of chilled 0.1 % peptone (Merck) diluent at high speed for 60 seconds to break up the rod and coccus chains for more accurate analysis (Hassan, Frank, & Shalabi, 2001). Serial dilutions (10^{-1} – 10^{-7}) were plated by using the pour plate technique (Ozer, Kirmaci, Oztekin, Hayaloglu, & Atamer, 2007). Incubation was carried out at 37 °C for 48 hours. The dilutions most suitable for starter culture enumeration were identified as 10^{-4}, 10^{-5} and 10^{-6} for YE supplemented yogurt as well as for the control yogurt.

Preparation of media

After the optimum dilution levels for enumeration had been determined, the most suitable enumeration medium had to be identified.

Elliker's broth was used to enumerate yogurt starter bacteria, i.e. *Streptococcus thermophilus* and *Lactobacilllus bulgaricus* according to methods used by (Barach, 1979; Matalon & Sandine, 1986; Moon *et al*., 1974). Due to various reports on the lack of certain substances in Elliker's broth as well as the effect of various supplementations on starter bacteria enumeration, several variations were prepared to identify the medium which visually differentiated the two starter organisms the best. Each variation is discussed below.

1. The first medium contained only 4.8 % (w/v) Elliker's broth with 1.5 % bacteriological agar. The pH was adjusted to 6.8 after which the medium was autoclaved at 121 °C for 15 min.

2. A batch of Elliker's broth (4.8 % (w/v)) was supplemented with 0.1 % Tween 80 (Merck) and gently heated with continuous stirring to dissolve, after which the pH was adjusted to 6.8. Bacteriological agar was then added at 1.5 % and the medium was autoclaved at 121 °C for 15 min. An aliquot of 50 µg/ml filter-sterilised triphenyltetrazolium chloride (TTC, Merck) was added after the medium had cooled to 47 °C. The medium was then gently mixed avoiding the incorporation of excessive air (Matalon & Sandine, 1986).

3. In the third Elliker's broth variation, the TTC from the second variation was substituted with 7 % (v/v) sterile (121 °C for 15 min) reconstituted skim milk (11 % solids, (w/v)). As in the second variation, Elliker's broth was supplemented with 0.1 % Tween 80 (Merck) and gently heated with continuous stirring to dissolve, after which the pH was adjusted to 6.8. Bacteriological agar was then added at 1.5 % and the medium was autoclaved at 121 °C for 15 min. The reconstituted skim milk was sterilised separately. To avoid partial solidification of the agar, the sterilised milk was reheated to 47 °C before addition to the sterilised agar medium.

The following media were used to determine the medium most suitable for starter bacteria enumeration studies:

- Yogurt not supplemented with YE (negative control)
- Yogurt supplemented with 10 g/l YE (positive control)
- Milk containing no starter culture or YE

All three Elliker's broth growth media variations were used with the different yogurt fermentations using the dilution levels 10^{-4}, 10^{-5} and 10^{-6}. Yogurt samples were extracted at the beginning of fermentation, after 80 min, and at the end of fermentation i.e. pH 4.6. All experiments were completed in triplicate.

The first medium containing only the Elliker's broth with bacteriological agar was identified as the optimum starter culture enumeration media. Only this medium was used in further enumeration studies.

After the most suitable enumeration medium and optimal dilution levels had been determined, samples from the positive control, negative control and the reconstituted skim milk (containing no starter culture or YE) were extracted every 30 min until pH 4.6 was reached in each case (except for the reconstituted skim milk where samples were extracted every 30 min in order to confirm that the base medium was uncontaminated). Enumeration studies were done using each sample to determine the growth profiles of both starter organisms in each sample.

5.2.2 LACTIC ACID PRODUCTION BY YOGURT STARTER BACTERIA

5.2.2.1 ARTIFICIAL ACIDIFICATION OF MILK USING LACTIC ACID

Lactic acid (Merck) was added in 50 µl aliqouts to 100 ml skim milk in order to determine whether the buffer capacity of milk will influence the rate of pH decrease. Sixteen aliquots in total were added. The container was inverted after each addition in order to distribute the lactic acid evenly throughout the milk. The pH of the initial milk was taken, and subsequent pH measurements were done after each aliquot of lactic acid had been added. The final amount of lactic acid added to the milk was 750 µl.

5.2.2.2 DETERMINATION OF ACIDITY

A 10 g yogurt sample was added into a 100 ml beaker using a pipette. The pipette was rinsed with 20 ml of water and added to the sample in the beaker. The contents were mixed gently but thoroughly using a magnetic stirrer. A 1 % phenolphthalein (Merck) solution was prepared using 95 % ethanol as solvent. An aliquot of 0.5 ml of the phenolphthalein solution was added to the sample mixture after which it was titrated using 0.1 N NaOH. The titration was continued until the first permanent colour change to pink was observed for 30 seconds (Bradley *et al*., 1992).

The % acidity (lactic acid) was determined as follows:

$$\% \text{ acidity} = \frac{(\text{ml NaOH}) \times (\text{Normality of NaOH}) \times 9^{\dagger}}{\text{Mass in grams of reconstituted sample titrated}}$$

† Constant (Bradley *et al*., 1992)

5.3 RESULTS AND DISCUSSION

5.3.1 GROWTH STUDIES OF STARTER BACTERIA USED FOR YOGURT FERMENTATION

To establish whether YE increased the levels of lactic acid production or increased the rate of cell growth (therefore increasing the netto lactic acid produced), the effect of YE on pH decrease and lactic acid production was evaluated first.

5.3.1.1 METABOLIC ACTIVITY OF STARTER BACTERIA

The resazurin test was used to determine the microbial metabolic activity. Similar techniques were described by Atherton and Newlander (1977) and Shahidi, Ho and van Chuyen (1998). It is visualised that zones of high hydrogen ion activity occur at the surface of bacteria. Resazurin diffusing into these zones in the presence of ascorbate becomes reduced and remains reduced onto diffusion back into the milk plasma. The rate of reduction would therefore depend on the pH and the number of the zones (Moyer & Campbell, 1963).

Table 10 Determinations of metabolic rates of the positive and negative controls

Time (min)	pH Negative control	pH Positive control	Positive control time (in min)*	Negative control time (in min)**	Pasteurised skim milk
0	6.6	6.62	56	56	blue
30	6.48	6.53	52	52	blue
60	6.36	6.3	41	45	blue
90	6.18	5.72	24	30	blue
120	5.59	5.23	20	25	blue
150	5.16	4.73	15	24	blue
180	4.88	4.56	15	24	blue
210	4.65		15	24	blue

*Time for YE supplemented yogurt to change colour to pink

**Time for control yogurt to change colour to pink

From the results depicted in Table 10 it was evident that as the pH decreased, the YE supplemented yogurt turned pink after shorter time intervals than the control yogurt. The metabolic activity of the positive control yogurt therefore increased at a faster rate than that of the negative control. It appeared according to the results obtained that the metabolic activity remained constant after the onset of coagulation of the yogurt at about pH 5.2 (120 min in positive control and 150 min in negative control). This can possibly be due to a restriction in movement of not only molecules as previously mentioned, but also of resazurin

to the cell surface. The blue colour present in the pasteurised skim milk throughout the time span of yogurt fermentation indicated that no metabolic activity was present and that the milk used for this experiment was therefore pasteurised effectively.

5.3.1.2 ENUMERATION OF YOGURT STARTER BACTERIA

Determination of suitable dilution levels

An initial experiment was performed in order to determine which dilutions should be used for starter culture enumeration on the different media. In this initial experiment only the first variation containing Elliker's broth and bacteriological agar adjusted to pH 6.8 was used. This could be done due to the fact that no difference in cell numbers will be obtained between the different media variations, only differences in ease of enumeration. The use of peptone water as diluent was preferred, given that exposure to phosphate in diluents may reduce cell recovery (Bradley *et al*., 1992).

After the dilution levels 10^{-1} – 10^{-7} were evaluated, dilutions 10^{-4}, 10^{-5} and 10^{-6} were identified as the optimal levels for successful starter culture enumeration. Identification of the most suitable enumeration medium could then proceed.

Preparation of media

Due to various reports on the lack of certain substances in Elliker's broth as well as the effect of various supplementations on starter bacteria enumeration, several variations were prepared to identify the most suitable medium.

The first medium contained only Elliker's broth with 1.5 % bacteriological agar (Merck). This medium was referred to as Elliker's lactic agar.

For the second medium, a batch of Elliker's broth was prepared with the addition of 0.1 % Tween 80 (Merck) and 50 µg/ml TTC. According to Matalon and Sandine (1986) the addition of TTC results in *Streptococcus thermophilus* producing small, red colonies and *Lactobacilllus bulgaricus* producing larger, white colonies. This medium was referred to as Modified Elliker's agar.

In the third Elliker's broth variation, the TTC was substituted with 7 % (v/v) sterile (121 °C for 15 min) reconstituted skim milk (11 % solids, w/v). This medium was referred to as Yogurt lactic agar.

Results indicated that, although the Yogurt lactic agar allowed good rod-coccus differentiation, the colonies were not as easily distinguishable due to the milk causing the agar to turn opaque. Studies by Matalon and Sandine (1986) coincided with results reported in Table 11 that *Lactobacilllus bulgaricus* appeared as large white colonies surrounded by a cloudy halo and *Streptococcus thermophilus* as smaller white colonies devoid of a surrounding halo.

The Elliker's lactic agar and the Modified Elliker's agar delivered similar results in terms of enumeration efficiency. It was however decided to use Elliker's lactic agar for all enumeration studies due to superior recovery, colony morphology and enumeration efficiency (Figure 49). The cloudy halos observed around certain colonies were due to casein precipitation by the more acidogenic *Lactobacilllus bulgaricus* colonies.

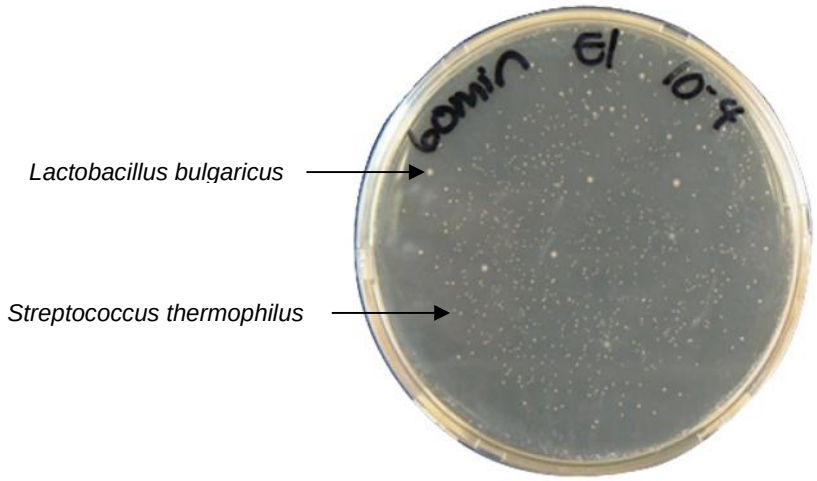


Figure 49 Depiction of enumeration efficiency of Elliker's lactic agar incubated at 37 °C for 48 hours. The larger, white cloudy colonies represent *Lactobacillus bulgaricus* whereas the smaller colonies represent *Streptococcus thermophilus.*

Table 11 Appearance of colonies observed on three different variations of Elliker's broth

Medium	***Streptococcus thermophilus***	***Lactobacillus bulgaricus***
1. Elliker's lactic agar	Small, white, no halo	Large, white, cloudy halo
2. Yogurt lactic agar 7 % skim milk (11 % solids)	Small, white, no halo	Large, white, cloudy halo
3. Modified Elliker's agar 50 µg/ml TTC	Small, red, no halo	Large, red, cloudy halo

The positive and negative control yogurts were both used for the determination of the optimum enumeration medium. All 3 Elliker's broth variations described above were used. Samples were extracted at the beginning of fermentation, ±80 min and at the end of fermentation i.e. pH 4.6. At each time interval samples were extracted and 10^{-4} – 10^{-6} dilutions were prepared, after which the plates were prepared using the pour plate technique.

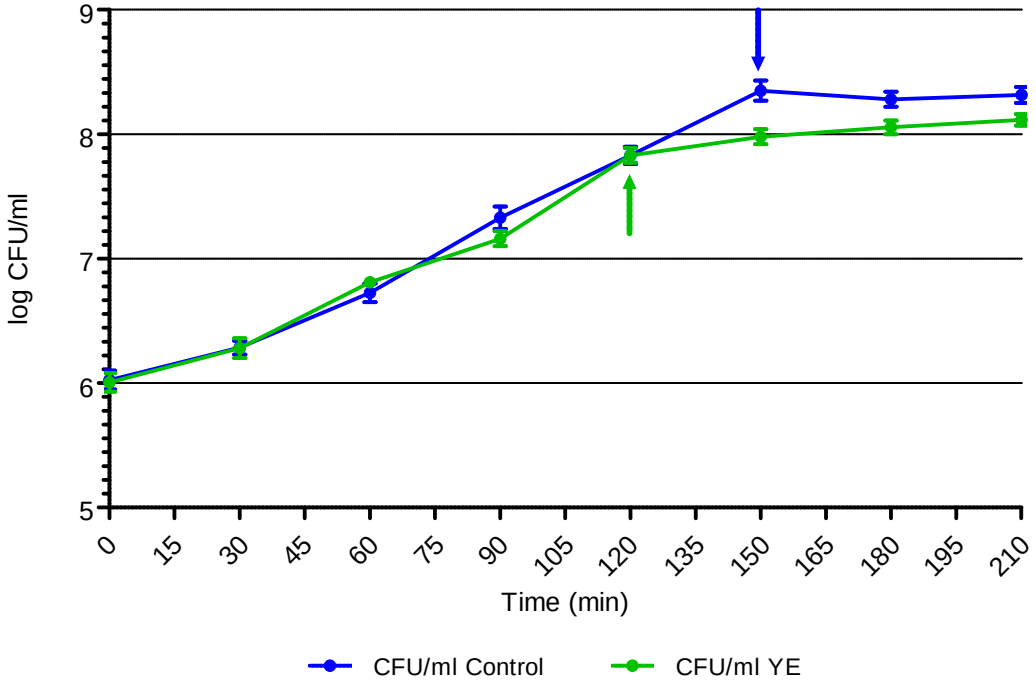


Figure 50 Cell counts of yogurt starter bacteria in YE supplemented and unsupplemented (negative control) yogurt. The green and blue arrows represent the respective coagulation points (pH 5.2) of YE supplemented yogurt and negative control yogurt.

When examining the effect of YE on the combined growth of *Streptococcus thermophilus* and *Lactobacilllus bulgaricus*, it was observed from Figure 50 that the addition of YE had no significant effect on the amount of cfu/ml up until 120 min. After 120 min however, the rate of cell growth of the YE supplemented yogurt moves into stationary phase whereas the negative control continues exponential growth for another 30 min before reaching stationary phase.

These results partially coincide with Ikawa and O'Barr (1956) who observed that growth rates on YE supplemented and unsupplemented media (not yogurt) were very similar. The only favourable effect of supplementation with YE observed by these researchers was a pronounced reduction in the lag phase of bacterial growth, which was not observed in Figure 50.

Results obtained by Ghaly, Tango and Adams (2003) however did not support the findings in the current study. The maximum cell numbers obtained in cheese whey by these researchers with the addition of YE substantially increased the maximum number of cells by 96 %. A possible explanation for the conflicting results can possibly be that the experiments by Ghaly *et al*. (2003) were conducted under controlled pH and temperature conditions of 5.5 and 42 °C, respectively. YE was also used as nutrient during batch fermentation experiments of lactic acid production from cheese whey. In the current study pH was not controlled, and milk was used as substrate instead of cheese whey.

Also, Aeschlimann and Von Stockar (1990) observed that supplementation of whey with lower concentrations of nutrients greatly enhances the fermentation by *Lactobacilllus helveticus*, but higher concentrations diminish the cell concentration as a result of toxicity.

When referring to Figure 50, the growth rate of YE supplemented yogurt at 0-120 min was 0.0152 per min and that of the negative control between 0 and 150 min was 0.0155 per min. This indicated that supplementing yogurt with YE did not have an effect on the μ_{max}. After 120 min the negative control continued linear cell growth whereas the rate of cell growth in the YE supplemented yogurt decreased and started to enter the stationary phase. After 150 min the starter culture cells in the negative control entered stationary phase. It was therefore clear that, although the addition of YE to yogurt decreased the time to reach pH 4.6, the μ_{max} in the respective exponential regions of the YE supplemented and positive control yogurt was the same. It could therefore be concluded that YE supplementation had no effect on growth rate of the two combined starter bacteria.

Ikawa and O'Barr (1956) reported similar findings that, during *Lactobacilllus delbruekii* growth in media of defined composition, the growth rate did not increase materially. However, the length of the lag phase was reduced considerably by the addition of crude natural materials such as YE. No effect on the lag phase of growth was however observed in the current study.

The first hypothesis, that YE addition increased the starter culture growth rate, could therefore be rejected as reason for the decrease in yogurt fermentation time. It was however important to establish the influence of YE on the two starter bacteria, individually.

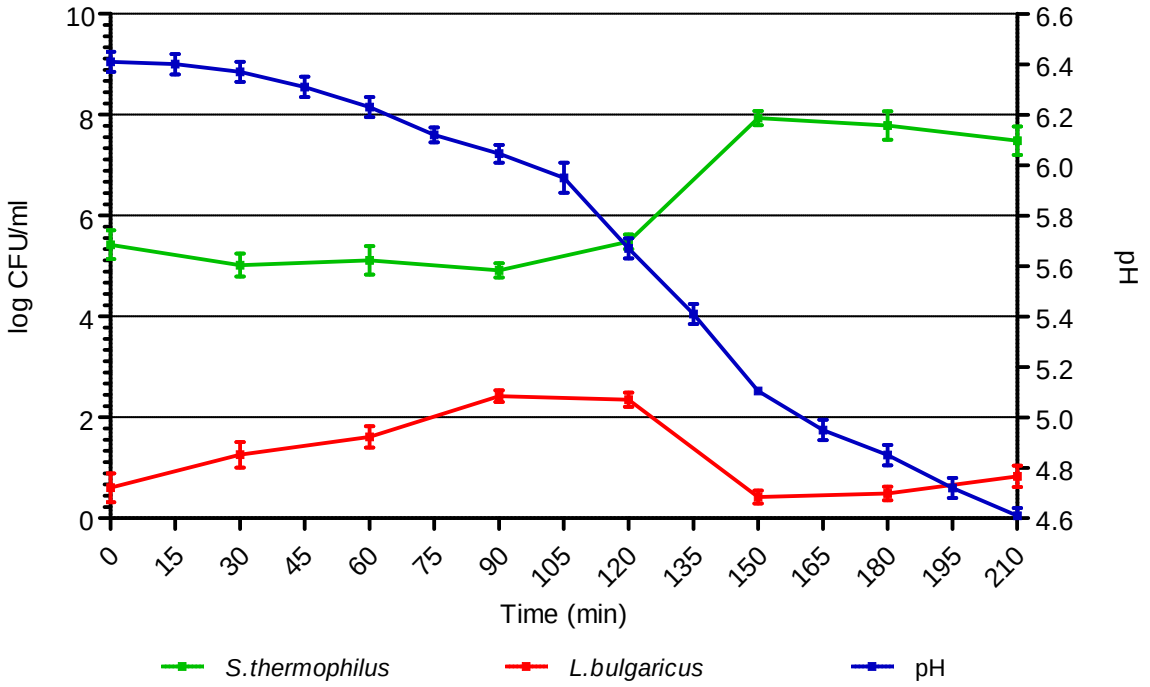


Figure 51A Cell counts of negative control yogurt

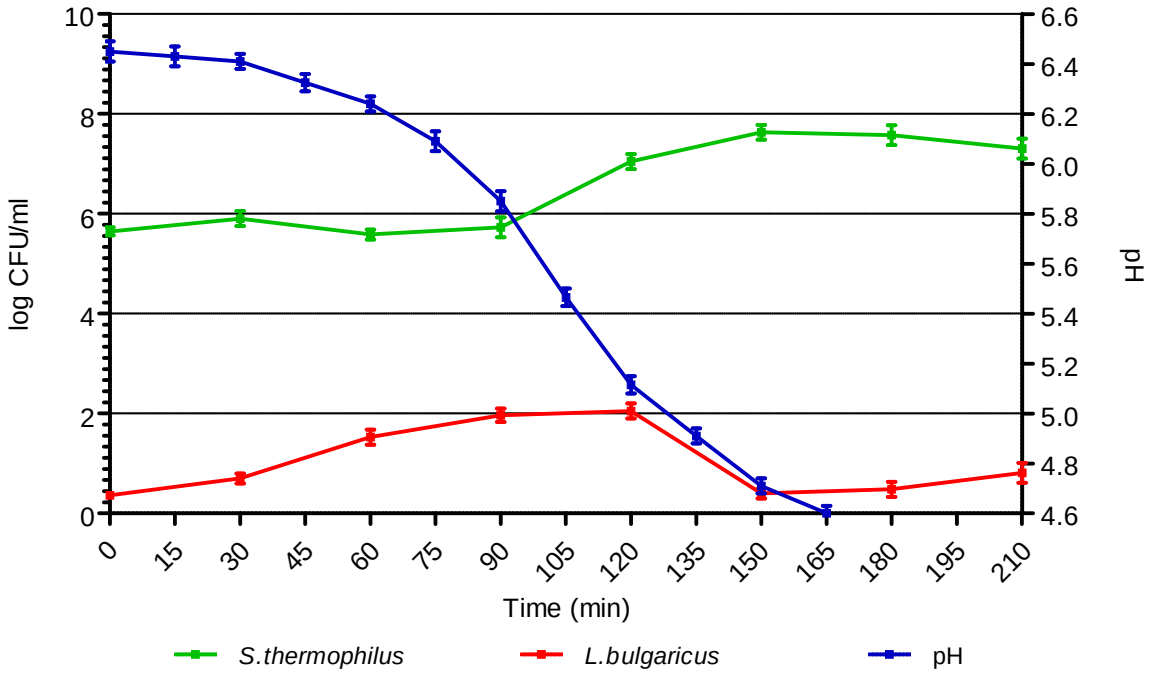


Figure 51B Cell counts of yeast extract supplemented yogurt

From Figure 51A it was evident that when studying the ratios of the two starter bacteria, *Streptococcus thermophilus* was present in much larger numbers than *Lactobacilllus bulgaricus.* Although it is stated in the literature that the ratio between *Streptococcus thermophilus* and *Lactobacilllus bulgaricus,* when used as a mixed yogurt starter culture

(irrespective of whether a freeze dried culture or a liquid bulk starter is used), should be around 1:1 (Cais-Sokolinska, Michalski, & Pikul, 2004; Gueguim-Kana, Oloke, Lateef, & Zebaze-Kana, 2007; Penna, Baruffaldi, & Oliveira, 1997), the low *Lactobacillus bulgaricus* counts observed in the current study are explained by Tamime and Robinson (1999). Confirmation of the ratio of the two starter organisms in a specific starter culture batch can be obtained by selective enumeration on appropriate media. However, as the long chains of *Streptococcus thermophilus* may consist of 15-20 individual cells compared with chains of 3-4 cells for *Lactobacilllus bulgaricus,* the breakup of the chains during dilution and plating alters the anticipated 1:1 ratio quite dramatically. A different colony count may well indicate that *Streptococcus thermophilus* accounts for some 75-85 % of the viable cells present in the yogurt culture (Tamime & Robinson, 1999). Tari, Ustok and Harsa (2009) confirmed this by stating that 70–80 % of *Streptococcus thermophilus* and 20–30 % of *Lactobacilllus bulgaricus* contribute to biomass, lactic acid and β-galactosidase activity. Aguirre-Ezkauriatza *et al*. (2008) agreed that, throughout the fermentation process, the *Streptococcus thermophilus* population is always greater than the *Lactobacilllus bulgaricus* population. This was also the case in the current study (Figures 51A and 51B).

When Figure 51A was compared to Figure 51B, it was clear that from Time 0-120 min, the average *Lactobacilllus bulgaricus* values were lower and the average *Streptococcus thermophilus* cell counts were considerably higher in YE supplemented yogurt than in the negative control yogurt. The maximum *Lactobacilllus bulgaricus* cell numbers (OD_{max}) were also lower in the positive control. These findings are in accordance with Ikawa and O'Barr (1956) who reported that, when cultivated in chemically defined media, supplements such as YE do not increase the growth rate of *Lactobacillus bulgaricus* materially.

Streptococcus thermophilus counts decreased immediately (0-30 min) in the negative control yogurt, which was not the case in the YE supplemented yogurt. It was clear from Figures 51A and 51B that as soon as the pH reached ±5.1, the growth rate of both bacteria in both the negative and positive controls decreased. This was particularly apparent in the growth rate of *Streptococcus thermophilus* in the negative control and the *Lactobacillus bulgaricus* in the positive control. In the case of the *Streptococcus thermophilus* cell counts in the positive control, however, they continued to proliferate, although at a slower rate.

The increased numbers of *Streptococcus thermophilus* cells in YE supplemented yogurt up to 120 min can possibly be ascribed to the following: During the proto-cooperation between the two starter bacteria, *Streptococcus thermophilus* firstly produces pyruvic acid, formic acid and lactic acid. The oxygen present in the milk is also assimilated. Galactose in the medium

is taken up by *Streptococcus thermophilus* and transformed into lactic acid by the Leloir metabolic pathway. The anaerobic conditions created by *Streptococcus thermophilus* stimulate the growth of *Lactobacilllus bulgaricus.* Due to the protease in the cell walls, *Lactobacilllus bulgaricus* is able to produce amino acids and small peptides from the milk caseins. The production of these small peptides and amino acids in turn stimulate *Streptococcus thermophilus* growth. *Streptococcus thermophilus* has intracellular peptidases involved in the hydrolysis of casein-derived peptides and due to the lack of protease in the bacterial wall often has low proteolytic activity. As a result *Streptococcus thermophilus* growth is restricted due to the lack of enough amino acids and peptides in the milk. The amino acids and small peptides produced by *Lactobacilllus bulgaricus* can however be used by *Streptococcus thermophilus* and are metabolised by the intracellular peptidases (Angelov, Kostov, Simova, Beshkova, & Koprinkova-Hristova, 2009; Tamime, 2005).

According to the above, *Streptococcus thermophilus* growth is dependent on the rate of proteolytic activity of *Lactobacilllus bulgaricus*. Only when short peptides and amino acids have been produced by *Lactobacilllus bulgaricus* can *Streptococcus thermophilus* growth continue. When using this to interpret the results in Figure 51B, it can be assumed that the addition of YE to milk provided small peptides and amino acids required by *Streptococcus thermophilus*. The nitrogen sources in YE are easy to absorb and do not depend on proteolytic activity by *Lactobacilllus bulgaricus* and subsequently *Streptococcus thermophilus* growth can therefore continue immediately. This possibly explains the increased amount of *Streptococcus thermophilus* cells in YE supplemented yogurt up to 120 min in Figure 51B in comparison to levels in Figure 51A.

It was evident from the interval 120-150 min of Figures 51A that the continuation of overall cell growth 2 was due to a dramatic increase in the growth rate of *Streptococcus thermophilus*. A similar phenomenon, however much more subtle, could be observed at time interval 90-120 min of the YE supplemented yogurt (Figure 51B). The overlapping factor in both cases was that this high growth rate of *Streptococcus thermophilus* ended as soon as ±pH 5.2 was reached.

Between 150-210 min in the negative control (Figure 51A), a slight increase in *Lactobacilllus bulgaricus* cell counts was observed, with a corresponding decline in *Streptococcus thermophilus* counts. A similar tendency could be observed in the YE supplemented yogurt (Figure 51B) in the same time span. When comparing the rate of linear pH decrease with the respective starter bacteria growth rates in the negative control (Figure 51A) at 150 min, an interesting observation can be made. As the pH reached ±pH 5.2, the maximum

Streptococcus thermophilus and the minimum *Lactobacilllus bulgaricus* cell counts are reached respectively. This is similar to observations by Tari *et al*. (2009) and which may be explained as *Streptococcus thermophilus* being acid-sensitive and consequently does not grow as well as *Lactobacilllus bulgaricus* at a pH below ±pH 5.2. When examining the association between the two starter bacteria, the cell counts of *Lactobacilllus bulgaricus*, due to its acid-tolerance, therefore increased after 150 min although the pH dropped below 5.2.

After the linear reduction in pH in Figures 51A and 51B i.e. 120 min in YE and 150 min in the negative control curve, the rate of pH decrease clearly slows down. Both these two points are at ±pH 5.2, the coagulation point of milk. What is interesting when compared to the cell counts is the fact that the moment the pH reaches ±5.2, the rate of cell growth decreases. A possible explanation similar to the one described in Section 5.3.1.1 can be given for this tendency: Due to milk coagulating at pH 5.2, nutrients are captured in the gel and cannot move around freely in the medium as before. This results in nutrients not being as readily available to the starter bacteria as in the fluid state of milk. The rate of cell growth therefore decreased due to a lack in nutrient attainability.

When comparing the highest increase in *Streptococcus thermophilus* numbers in Figure 51A (120-150 min) and Figure 51B (90-120 min) to their respective pH decrease profiles, it was evident that both growth regions correspond with the regions in which the rate of pH decrease was the highest.

When comparing studies done by Aguirre-Ezkauriatza *et al*. (2008) to the current study, the cell counts of *Streptococcus thermophilus* obtained by this research group were more comparable to that of the positive control than that of the negative control. Although similar *Streptococcus thermophilus* counts were obtained in both the current study and that performed by Aguirre-Ezkauriatza *et al*. (2008), their approach was different. Aguirre-Ezkauriatza and co-workers did not use a water bath as heat source as in the current study, but rather a fermentor heated through a jacketed metal-dished bottom. The difference is that the fermentor heated from beneath would not ensure such a constant uniform incubation temperature as the water bath.

5.3.2 LACTIC ACID PRODUCTION BY YOGURT STARTER BACTERIA

Titrations are relatively time-consuming compared to other methods. For this reason it was important to establish the relationship between pH and titratable acidity in order to determine whether pH can be used as lactic acid indicator. This was done by titrating milk with added lactic acid without the presence of any starter bacteria.

5.3.2.1 ACIDIFICATION OF MILK USING LACTIC ACID

Lactic acid was added in increasing concentrations to skim milk in order to determine whether the buffer capacity of milk will influence the rate of pH decrease (Figure 52). It was evident that no buffering effect was observed and a R^2 value of 0.98 indicated a good fit which indicated that an increase in lactic acid concentration would directly result in a decrease in milk pH. In a biological system, i.e. yogurt fermentation, this direct correlation could however not be made due to metabolic activity which can attribute to higher proton amounts, subsequently lowering pH. The decrease in pH during yogurt fermentation can therefore be directly due to lactic acid production, as well as an increase in other metabolic activity.

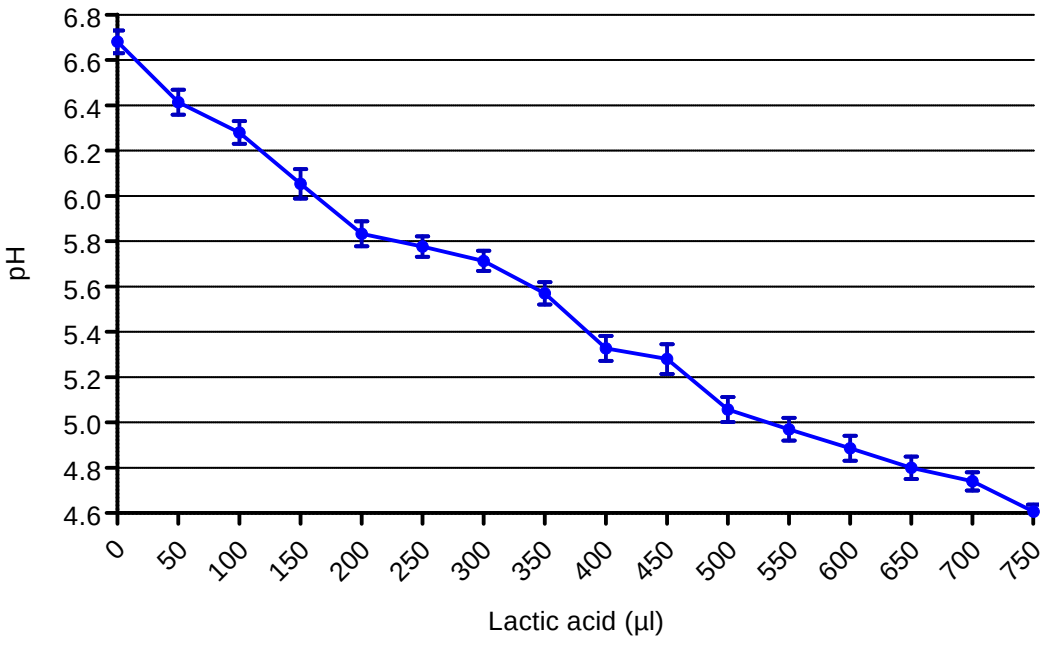


Figure 52 Effect of lactic acid addition to reconstituted skim milk on pH

5.3.2.2 Production of Lactic acid during yogurt fermentation by lactic acid bacteria

The pH profile (Figure 53) shows that YE supplementation decreased yogurt fermentation time by 45 min in comparison to the control which reached pH 4.6 after 210 min. In the YE supplemented yogurt, the pH started to markedly decrease after 75 min whereas the control only started to show significant decrease in pH after 105 min.

Although the positive control reached pH 4.6 after 165 min of fermentation, the titratable acidity determinations were continued up until 210 min, which is when the negative control reached pH 4.6, in order to be compared with the negative control (Figure 53).

When examining the slopes of the pH curves of the negative and positive control it appears that they were very similar. The slope of the negative control yogurt was -0.019 in its linear region (105-150 min) while that of the YE supplemented yogurt (positive control) was -0.022 (75-120 min). The slope indicated the amount of pH units the milk decreased per minute. It therefore appeared as though the addition of YE did not increase the rate of pH decrease considerably.

From Figure 53 it was evident that after 120 min in the YE supplemented yogurt and after 150 min in the control yogurt, respectively, the linear decrease in pH was followed by a reduced rate of pH decrease. The rate of pH decrease in both pH curves decreased at pH 5.2, the coagulation point of yogurt, was reached. A possible explanation for the decrease in lactic acid production rate can be that, due to coagulation, nutrients are captured in the gel structure, making them less accessible for use by starter bacteria. This then resulted in the rate of lactic acid production declining.

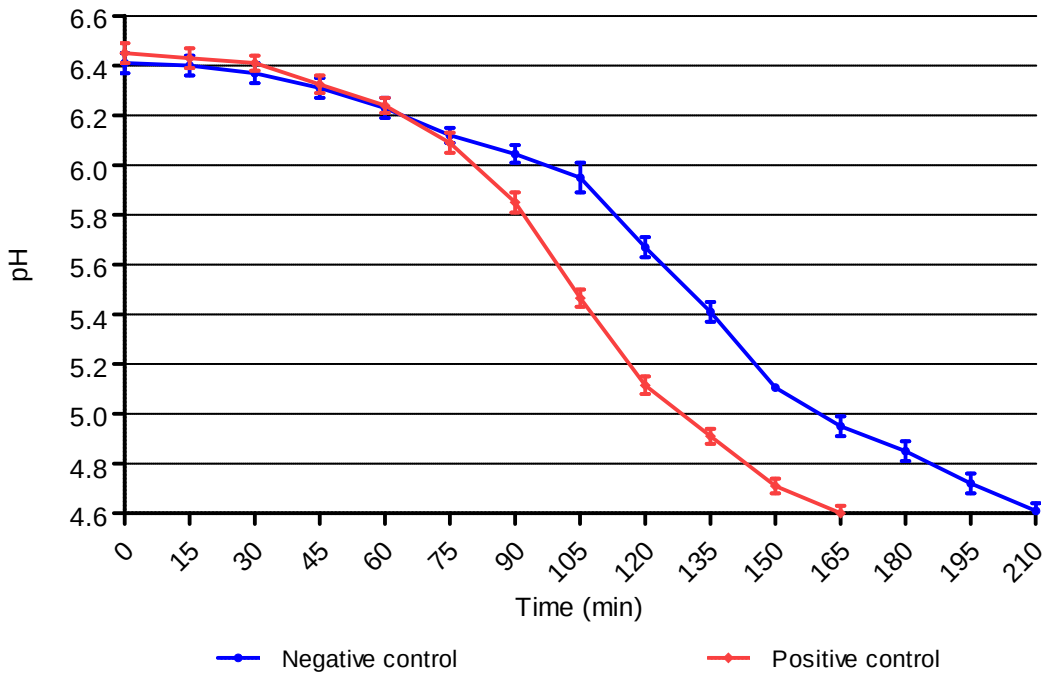


Figure 53 pH profile of unsupplemented yogurt (negative control) in comparison to yeast extract supplemented yogurt (positive control)

It was clear from Figure 54 that lactic acid production started about 30 min earlier in the YE supplemented yogurt in comparison to the negative control. This reduction in lag phase could possibly be ascribed to, as previously discussed during bacterial growth studies (Section 5.3.1), the addition of YE to milk providing small peptides and amino acids required by *Streptococcus thermophilus*. The nitrogen sources in YE are easy to absorb and do not depend on proteolytic activity by *Lactobacilllus bulgaricus*. *Streptococcus thermophilus* growth can therefore immediately continue.

From Figure 54 it was evident that the lactic acid production levels by the positive control was considerably higher than that of the negative control. The initial lactic acid levels and production rate in the first 60 min was similar in the supplemented (positive control) and unsupplemented yogurts (negative control). After 60 min however the rate of lactic acid production in the positive control increased in comparison to the negative control.

When comparing the lactic acid content of the positive and negative control, the lactic acid content of the positive control was 22 % higher (8.1 mg/ml) at 165 min than that of the negative control at 210 min (6.9 mg/ml) when pH 4.6 was reached in the respective yogurts. This corresponds to the brief discussion in Section 5.3.2.1 that pH decrease cannot be

directly correlated to lactic acid production in a biological system such as yogurt fermentation.

During examination of lactic acid production of the positive and negative control, the linear phase of the slopes were determined as 0.068 for the positive control (between 90 and 150 min) and 0.041 for the negative control (between 90 and 180 min), respectively. These results were in accordance to those observed during metabolic activity determinations by resazurin in Section 5.2.1.1 where it was observed that YE increased the metabolic activity. It could therefore be concluded at this point that although the metabolic activity was increased with YE addition, it was the lactic acid production that was increased and not the bacterial growth rate.

Figure 54 shows that YE supplementation increased the amount of lactic acid formation, as well as starting lactic acid production earlier in the fermentation process as previously hypothesised in Section 5.1. The initial lactic acid content in milk supplemented with YE was slightly higher at 2.160 mg/ml than that of the negative control which contained 1.950 mg/ml at time 0. It was also evident that the pH correspondingly decreased indirectly proportionally as the amount of lactic acid increased.

Figure 54 indicated that 6.3 mg/ml lactic acid levels were produced in the negative control yogurt after 180 min (3 hours). Tari *et al*. (2009) reported that a mixed culture containing *Streptococcus thermophilus* and *Lactobacilllus bulgaricus* produced about 7 mg/ml lactic acid after 3 hours of incubation in a bioreactor. Although their experimental conditions varied from the current study, similar lactic acid levels were reached using the same species of bacteria.

According to Amrane and Prigent (1998) the rate of lactic acid production by *Lactobacilllus helveticus* during whey permeate fermentation increased largely from 1.5 to 8 g/l YE supplementation although the rate decreased at a supplementation level above 8 g/l. On the contrary, YE concentration had no effect on lactic acid yield in the study of Amrane and Prigent (1998).

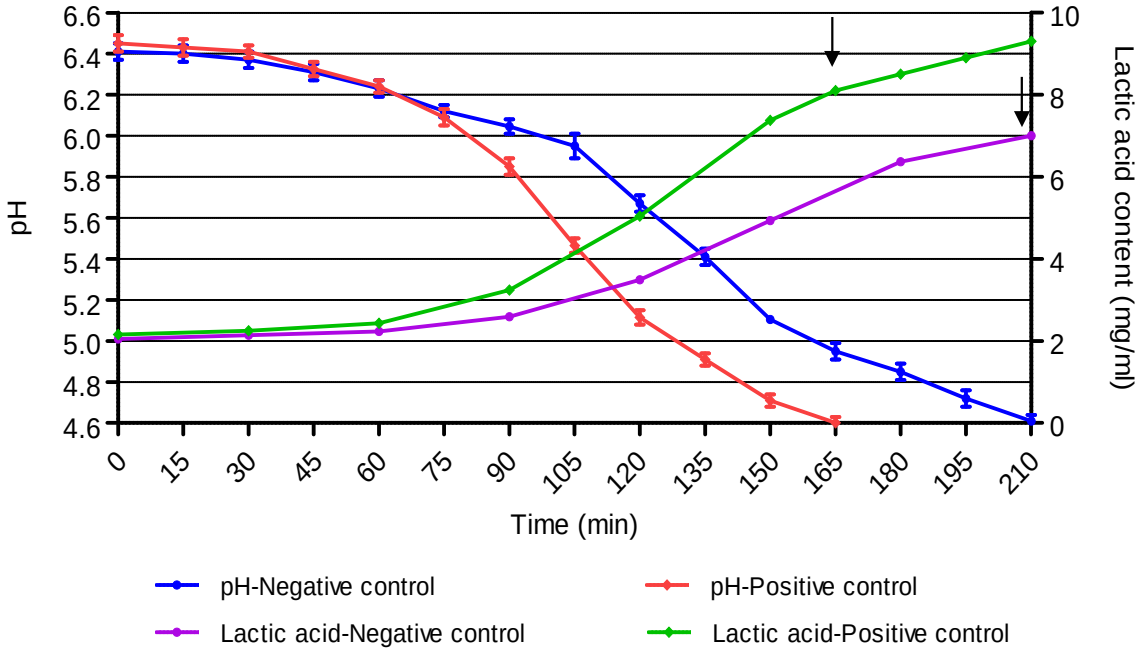


Figure 54 pH and lactic acid content of YE supplemented and the control yogurt. The arrows indicate the point at which pH 4.6 was reached in the two fermentations, respectively.

In reference to the three hypotheses as to determine the specific mechanism with which YE decreases yogurt fermentation time, it could be concluded that the second hypothesis, that YE addition supplied the growth requirements of starter bacteria resulting in increased levels of lactic acid production, as well as the third hypothesis, that YE addition slightly reduced the lag phase of lactic acid production, could both be accepted.

Evidently from the results obtained, the increase in lactic acid production was not provided by a growth-linked production process. It was noteworthy that although YE supplementation influenced the rate of lactic acid production considerably, it also shortened the lag phase for lactic acid production.

5.4 CONCLUSIONS

After determination of the effect of YE on starter bacteria growth, it was evident that when examining the effect of YE on the combined starter growth, it had no positive effect. Surprisingly, supplementing yogurt with YE resulted in the starter bacteria entering stationary phase before that of the negative control and consequently decreased the final amount of bacterial cells present in the yogurt in comparison to the control. A simple solution to this problem however, is adding more probiotics to the yogurt. The addition of probiotic species such as *Lactobacilllus acidophilus* or *Bifidobacterium lactis* would increase the amount of probiotics in the final product.

During examination of the effect of YE on the two starter organisms individually, however, it was interesting to observe that YE increased *Streptococcus thermophilus* growth in comparison to the negative control. This was attributed to the fact that YE is high in amino acids and peptides which, when added to the milk at the beginning of fermentation, are readily available to *Streptococcus thermophilus*. This organism could then immediately commence with lactic acid formation and did not have to wait for the more proteolytic *Lactobacillus bulgaricus* for providing these essential growth requirements.

It was evident that YE increased the total amount of lactic acid produced at pH 4.6 by 1.2 mg/ml in comparison to the negative control. The fact that YE supplemented yogurt had a higher lactic acid production rate than the negative control after 60 minutes indicated that YE not only increased lactic acid levels, but also increased the rate of lactic acid production. Lactic acid was also produced earlier in the fermentation process, resulting in higher lactic acid levels earlier in the process.

5.5 REFERENCES

Aeschlimann, A., & Von Stockar, U. (1990). The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helveticus*. *Applied Microbiology and Biochemistry*, *32*, 398–402.

Aguirre-Ezkauriatza, E. J., Galarza-González, M. G., Uribe-Bujanda, A. I., Ríos-Licea, M., López-Pacheco, F., Hernández-Brenes, C. M., & Alvarez, M. M. (2008). Effect of mixing during fermentation in yogurt manufacturing. *Journal of Dairy Science*, *91*, 4454–4465.

Amrane, A., & Prigent, Y. (1998). Influence of yeast extract concentration on batch cultures of *Lactobacillus helveticus*: growth and production coupling. *World Journal of Microbiology and Biotechnology*, *14*, 529–534.

Angelov, M., Kostov, G., Simova, E., Beshkova, D., & Koprinkova-Hristova, P. (2009). Proto-cooperation factors in yogurt starter cultures. *Revue de Génie Industriel*, *3*, 4–12.

Atherton, H., & Newlander, J. (1977). *Chemistry and testing of dairy products* (4th ed.). Connecticut: AVI Publishing Company.

Barach, J. T. (1979). Improved enumeration of lactic acid Streptococci on Elliker agar containing phosphate. *Applied and Environmental Microbiology*, *38*, 173–174.

Bradley, R. L., Arnold, E., Barbano, D. M., Semerad, R. G., Smith, D. E., & Vines, B. K. (1992). Chemical and Physical Methods. In R. Marshall (Ed.), *Standard methods for the examination of dairy products* (16th ed.). Washington, DC.

Cais-Sokolinska, D., Michalski, M., & Pikul, J. (2004). Role of the proportion of yoghurt bacterial strains in milk souring and the formation of curd qualitative characteristics. *Bulletin of The Veterinary Institute in Pulawy*, *48*, 437–441.

Champagne, C., Gaudreau, H., & Conway, J. (2003). Effect of the production or use of mixtures of bakers' or brewers' yeast extracts on their ability to promote growth of lactobacilli and pediococci. *Electronic Journal of Biotechnology*, *6*, 185–197.

Ghaly, A. E., Tango, M. S. A., & Adams, M. A. (2003). Enhanced lactic acid production from cheese whey with nutrient supplement addition. *Agricultural Engineering International: the CIGR Journal of Scientific Research and Development*, *5*, 1–20.

Gueguim-Kana, E., Oloke, J., Lateef, A., & Zebaze-Kana, M. (2007). Novel optimal temperature profile for acidification process of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in yoghurt fermentation using artificial neural network and genetic algorithm. *Journal of Industrial Microbiology and Biotechnology*, *34*, 491–496.

Hassan, A. N., Frank, J. F., & Shalabi, S. I. (2001). Factors affecting capsule size and production by lactic acid bacteria used as dairy starter cultures. *International Journal of Food Microbiology*, *64*, 199–203.

Ikawa, M., & O'Barr, J. (1956). The nature of some growth stimulatory substances for *Lactobacillus delbruekii*. *Journal of Bacteriology*, *71*, 401–405.

Matalon, M. E., & Sandine, W. E. (1986). Improved media for differentiation of rods and cocci in yogurt. *Journal of Dairy Science*, *69*, 2569–2576.

Moon, N. J., Hamann, A. C., & Reinbold, G. W. (1974). Recovery of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* on nine commonly used agar media. *Applied Microbiology*, *28*, 1076–1078.

Moyer, R., & Campbell, J. (1963). Mechanism of resazurin reduction in milk of low bacterial content. *Journal of Dairy Science*, *46*, 897–906.

Ozer, B., Kirmaci, H., Oztekin, S., Hayaloglu, A., & Atamer, M. (2007). Incorporation of microbial transglutaminase into non-fat yogurt production. *International Dairy Journal*, *17*, 199–207.

Penna, A., Baruffaldi, R., & Oliveira, M. (1997). Optimization of yogurt production using demineralized whey. *Journal of Food Science*, *62*, 846–850.

Shahidi, F., Ho, C., & van Chuyen, N. (1998). *Process-induced chemical changes in food*. New York: Springer.

Shihata, A. (2004). *The proteolytic activity of yoghurt and probiotic bacteria for the improved viability of probiotic bacteria in fermented milk products* (Doctoral dissertation, Victoria University, Australia). Retrieved from http://vuir.vu.edu.au/15367.

Surono, S., & Hosono, A. (2011). Starter cultures. *Encyclopedia of Dairy Sciences*.

Tamime, A. Y. (2005). Microbiology of Starter Cultures. In R. K. Robinson (Ed.), *Dairy Microbiology: The microbiology of milk products* (3rd ed., pp. 261–366). London: Elsevier Applied Science.

Tamime, A. Y., & Robinson, R. K. (1999). Background to Manufacturing Practice. *Yogurt: Science and Technology* (pp. 6–47). Cambridge, UK: Woodhead Publishing.

Tari, C., Ustok, F. I., & Harsa, S. (2009). Optimization of the associative growth of novel yoghurt cultures in the production of biomass , β-galactosidase and lactic acid using response surface methodology. *International Dairy Journal*, *19*, 236–243.

Vandeweghe, P., Norquist, P., & Flynn, E. (2002). U.S. Patent No. 6,399,122 B2. Washington, DC: U.S. Patent and Trademark Office.

CHAPTER 6

GENERAL CONCLUSIONS

The decrease of yogurt fermentation time will be to the advantage of the dairy industry as well as to the consumer. The effect of a range of supplements and physical conditions on the duration of the yogurt fermentation process was therefore evaluated in earlier studies (M.Sc). These studies identified YE as the only potential accelerator. The addition of YE to the yogurt fermentation process however resulted in a product with unacceptable flavour and other sensory properties. It was therefore attempted to identify the specific component present in YE that is responsible for the decrease in yogurt fermentation time. It was anticipated that when used in isolation, the accelerating compound would have no influence on the taste and flavour of the final product.

Due to inconsistencies between the controls of independent fermentations, it was essential to standardise the fermentation medium (milk) and the yogurt starter cultures. Irregular distribution of starter culture granules influenced the ratio of the two organisms during the weighing process and consequently resulted in unequal amounts being present between individual fermentations. This problem was resolved by grinding the starter culture granules prior to weighing.

The use of different batches of fresh milk as medium for yogurt fermentations also influenced the yogurt fermentation process. Respective times to reach pH 4.6 varied up to 43 %. The substitution of fresh milk with milk powder eliminated this problem and ensured consistent fermentation times between respective fermentation runs.

After the yogurt fermentation process had been standardised according to the above criteria, the fractionation of YE and the effect of the various fractions on yogurt fermentation could proceed.

The goal of this part of the study was to reduce the number of components that could be responsible for the positive effect of YE on yogurt fermentation time. After the fractionation of YE using ultrafiltration cut-off membranes ranging between 30 and 1 kDa, respective yogurt batches were prepared using the various fractions. Results indicated that the accelerant was present in all fractions, and was therefore of low molecular weight (<1 kDa). At this stage the accelerant could have been of amino acid-, mineral- or vitamin origin. In order to evaluate this, the effect of amino acid-, vitamin- and mineral solutions respectively on individual yogurt

fermentations was determined. It became clear that the accelerating component present in YE was not a mineral or vitamin, but was of amino acid origin.

Fractionation of YE was also performed by size exclusion chromatography with which four distinct fractions were obtained. After supplementing individual milk batches with each of these four fractions, it was evident that the fraction that eluted last, containing components with smallest molecular mass, decreased yogurt fermentation time the most. This fraction decreased fermentation time by more than 60 min (71 %), compared to the negative control, which was even more than the YE itself. This once again indicated that the accelerant had a low molecular weight.

With mass spectrometry analysis on the accelerating fraction of yogurt fermentation (Fraction 4) some peptide sequences were identified that could be matched to specific proteins or enzymes of *Saccharomyces cerevisiae* in a protein database. The four enzymes that were identified as possible sources of the peptide sequences were: Glyceraldehyde-3-phosphate dehydrogenase, Enolase, Alpha-glucosidase and ATP-dependent RNA helicase. The first 3 identified enzymes originated from *Saccharomyces cerevisiae* strain ATCC 204508/S288c whereas the ATP-dependent RNA helicase was associated with *Saccharomyces cerevisiae* strain YJM789. A further 909 peptide sequences, which did not match any peptides in the database, were also identified. This abundance in unmatched peptides was described as having a homology with too many proteins, or that they could have been formed by the plastein reaction during the YE manufacture, and would therefore not match peptide sequences from *Saccharomyces cerevisiae* proteins.

Although it was found that YE accelerated the yogurt fermentation process, the same acceleration as observed with the YE was not obtained when amino acids present in the accelerating fraction were evaluated individually or in various combinations. The acceleration was therefore not due to a single free amino acid and could most probably be attributed to the presence of a peptide.

After determination of the effect of YE on starter bacteria growth, it was evident that when examining the effect of YE on the combined starter growth, it had no positive effect. Surprisingly, supplementing yogurt with YE resulted in the starter bacteria entering stationary phase before that of the negative control and consequently decreased the final amount of bacterial cells present in the yogurt in comparison to the negative control.

During examination of the effect of YE on the two starter organisms individually, however, it was interesting to observe that YE increased *Streptococcus thermophilus* growth in comparison to the negative control.

It was evident that YE increased the total amount of lactic acid produced at pH 4.6 by 1.2 mg/ml in comparison to the negative control. The fact that YE supplemented yogurt had a higher lactic acid production rate than the negative control after 60 minutes indicated that YE not only increased lactic acid levels, but also increased the rate of lactic acid production. Lactic acid was also produced earlier in the fermentation process, resulting in higher lactic acid levels earlier in the process.

From the various results obtained in this study it could be concluded that YE can be fractionated with size exclusion chromatography to yield a fraction which accelerates yogurt fermentation time even more than the unfractionated YE. Although the accelerating component is not pure but part of the pooled fraction, it produced a product with improved flavour in comparison to unfractionated YE.

The supplementation of yogurt with YE does not necessarily provide additional nutrients to those already present in milk. YE supplementation rather provides peptides already present in milk, but in a readily available form at the beginning of fermentation.

After the current study had been concluded, various new questions emerged, which could be investigated in future studies:

- The sensory evaluation of yogurt containing the accelerating fraction in comparison to a control yogurt sample.

- Determine the effect of yogurt coagulation on the mobility of nutrients in milk.

- Determine the effect of YE on exopolysaccaride production.

- Determine which specific peptides are utilised by starter culture which subsequently results in acceleration of yogurt fermentation.

- Determine the impact of YE on fermentations other than yogurt fermentation for instance cheese, wine and bread.

CHAPTER 7

SUMMARY

In order to study the decrease of yogurt fermentation time, the effects of a wide range of supplements on yogurt fermentation time were evaluated. YE was identified as the only supplement which showed potential. Unfortunately it resulted in a product with an unacceptable flavour. It was therefore important to identify and isolate the specific component responsible for the decrease in yogurt fermentation time.

YE was fractionated with size exclusion chromatography and it was subsequently determined that the accelerating fraction had a low molecular weight.

It was important to establish whether the accelerating component was of mineral, vitamin or amino acid origin. Three cocktails containing the most common minerals, vitamins and amino acids were prepared and their respective effects on yogurt fermentation were determined. Results indicated that when compared to the respective controls, no decrease in fermentation time was observed with the mineral and vitamin cocktails. A decrease in fermentation time was observed with the amino acid cocktail, indicating that the accelerating component present in YE was of amino acid origin. It was however not clear whether it was a single amino acid or a peptide.

The accelerating fraction was further analysed by SDS-PAGE and due to no visible bands in the respective region (<1kDa), it could not be analysed with mass spectrometry. The fraction obtained directly after size exclusion chromatography was however analysed by using mass spectrometry in order to determine the total amino acid content of the accelerating fraction after which it was evident that the fraction containing the accelerant contained an abundance of peptides.

The individual effects of the 17 identified amino acids were determined in respective yogurt fermentations. Results indicated that no single amino acid was responsible for the decrease in yogurt fermentation time. Although a combination of the 17 amino acids in one fermentation run resulted in a decrease in fermentation time in comparison to unsupplemented yogurt, the decrease was not as considerable as that of the accelerating fraction obtained after size exclusion chromatography.

Due to the fact that it was not practical to evaluate the effect of all possible combinations of the 17 amino acids, the combinations that were evaluated were based on literature reports on stimulation of *Streptococcus thermophilus* growth. Focus was placed on the growth of *Streptococcus thermophilus* due to this organism being the growth limiting organism between the two starter organisms used for yogurt fermentation. None of the evaluated amino acid combinations decreased yogurt fermentation time, and it was therefore concluded that the accelerating component was a peptide and not a free amino acid.

In order to establish the mechanism of acceleration of the isolated YE fraction, it was important to determine whether the addition of YE to milk increased the rate of starter bacteria growth or whether it completed the growth requirements of starter bacteria. The latter could either result in earlier initiation of lactic acid production or in increased levels of lactic acid production. Results indicated that YE had no effect on the total growth rate of the starter bacteria. However, when examining the effect on the respective bacteria individually, it became clear that YE increased *Streptococcus thermophilus* cell numbers in comparison to the unsupplemented control. Using resazurin, it seems YE increased the metabolic rate of the starter bacteria.

Although supplementation of yogurt with YE did not influence combined starter bacteria growth, it influenced lactic acid production. The addition of YE to yogurt resulted in increased lactic acid levels as well as an increase in lactic acid production rate. Lactic acid was also initiated earlier in the fermentation process, resulting in higher lactic acid levels in comparison to unsupplemented yogurt.

It was therefore evident from this study that YE did not provide nutrients that are not already present in milk, but rather provided nutrients in a readily available form at the beginning of the yogurt fermentation process resulting in the reduction of the lag phase of lactic acid production.

OPSOMMING

Ten einde die afname van jogurt fermentasie tyd aan te spreek, is die effek van ’n wye verskeidenheid van aanvullings op jogurt fermentasie tyd geëvalueer. Gisekstrak is geïdentifiseer as die enigste aanvulling wat potensiaal getoon het alhoewel dit ongelukkig gelei tot ’n produk met onaanvaarbare smaak. Dit was dus noodsaaklik om die spesifieke komponent wat verantwoordelik is vir die afname in jogurt fermentasie tyd, te identifiseer en te isoleer.

Gisekstrak is gefraksioneer met grootteuitsluitingschromatografie en dit is daarna vasgestel dat die versnellende fraksie ’n lae molekulêre massa het. Die versnellende fraksie is verder ontleed deur SDS-PAGE en as gevolg van geen sigbare bande in die onderskeie area (< 1kDa), kon dit nie met massaspektrometrie geanaliseer word nie. Die fraksie wat direk na die grootteuitsluitingschromatografie opgevang is, is egter geanaliseer deur die gebruik van massaspektrometrie waarna dit was duidelik was dat die versnellende fraksie ’n oorvloed van peptiede bevat het.

Dit was belangrik om vas te stel of die versnellende komponent van mineral-, vitamien- of aminosuur oorsprong is. Drie mengsels met die mees algemene minerale, vitamiene en aminosure is voorberei en hul onderskeie effekte op jogurt fermentasie is bepaal. Resultate het aangedui dat in vergelyking met die onderskeie kontroles, die versnellende komponent teenwoordig in gisekstrak van aminosuur oorsprong is. Wat egter nie duidelik was nie, is of dit ’n enkele aminosuur of ’n peptied is.

Ten einde dit te bevestig is die versnellende fraksie verkry na grootteuitsluitingschromatografie gehidroliseer en die aminosuurinhoud is bepaal. Die individuele effekte van die 17 aminosure geïdentifiseer is bepaal in onderskeie jogurt fermentasies. Resultate het aangedui dat geen enkele aminosuur verantwoordelik was vir die afname in jogurt fermentasie tyd nie. Hoewel ’n kombinasie van die 17 aminosure in een fermentasie lopie gelei het tot ’n afname in die fermentasie tyd in vergelyking met gewone jogurt, was die afname in tyd nie so groot soos dié van die versnelling fraksie verkry na grootteuitsluitingschromatografie nie.

As gevolg van die feit dat dit prakties nie uitvoerbaar was om die effek van alle moontlike kombinasies van die 17 aminosure te evalueer nie, is die kombinasies wat geëvalueer is gebaseer op literatuurverslae oor die stimulasie van *Streptococcus thermophilus* groei. Die fokus is op die groei van *Streptococcus thermophilus* geplaas as gevolg van die feit dat

hierdie organisme die groei beperkende organisme tussen die twee suurselorganismes is. Nie een van die geëvalueerde kombinasies het jogurt fermentasie verkort nie, en daar is dus tot die gevolgtrekking gekom dat die versnellende komponent ’n peptied en nie ’n aminosuur is nie.

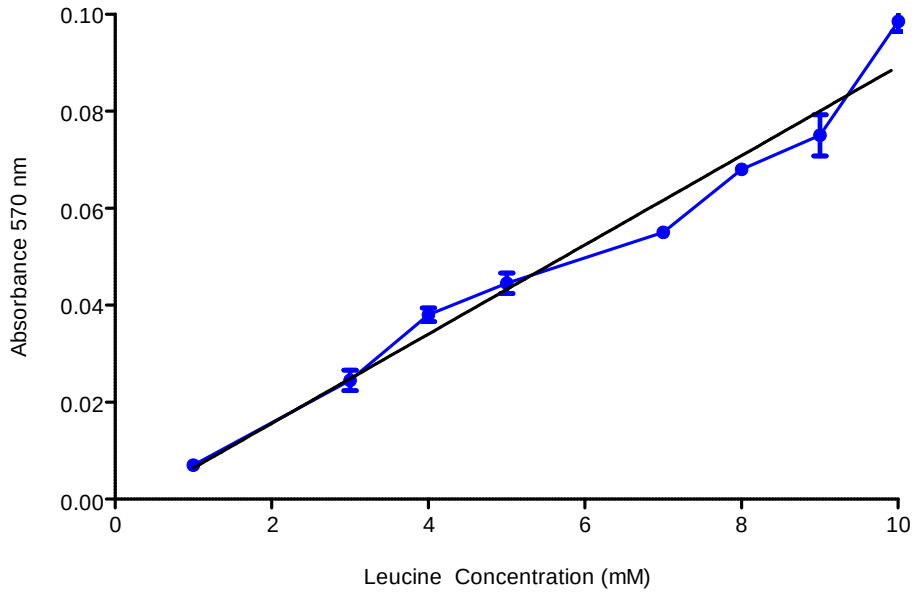
Ten einde die meganisme van gisekstrakversnelling te vestig, was dit belangrik om te bepaal of die toevoeging van gisekstrak tot melk die tempo van groei van bakterieë verhoog en of dit die groeivereistes van suurselbakterieë wat lei tot verhoogde vlakke van melksuur produksie aanvul. Resultate het aangedui dat gisekstrak geen effek op die totale groeitempo van die suurselbakterieë het nie. Maar, tydens die ondersoek van die uitwerking op die onderskeie individuele bakterieë was dit duidelik dat gisekstrak die *Streptococcus thermophilus* selgetalle verhoog het in vergelyking met die gewone kontrole. Dit was ook duidelik dat gisekstrak die metaboliese tempo van die suurselbakterieë verhoog het.

Hoewel die aanvulling van jogurt met gisekstrak nie die groei van gekombineerde suursel bakterie beïnvloed het nie, het dit die melksuurproduksie beïnvloed. Die byvoeging van gisekstrak tot jogurt het gelei tot verhoogde melksuur vlakke, sowel as ’n toename in melksuur produksietempo. Melksuur is ook vroeër gedurende die fermentasie proses geïnisieer wat gelei het tot hoër vlakke melksuur as in die gewone jogurt.

Dit was dus duidelik uit hierdie studie dat gisekstrak nie voedingstowwe verskaf wat nie in melk teenwoordig is nie, maar eerder voedingstowwe in ’n geredelik beskikbare vorm verskaf aan die begin van die jogurt fermentasie proses, wat dan die sloerfase van melksuurproduksie verkort.

APPENDIX A

APPENDIX B



Standard curve of Cd-ninhydrin analysis Y= 0.009144x – 0.002960 with R^2=0.96