Physical Properties of Functional Fermented Milk Produced with Exopolysaccharide-Producing Strains of *Streptococcus thermophilus*

A thesis submitted for the degree of
Doctor of Philosophy

By
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Dedication

"Say: Truly, my prayer and my service of sacrifice, my life and death are (all) for Allah, The Cherisher of the Worlds"

Al Qur'an 6:1

Abstract

This thesis focused on the study of the influence of different exopolysaccharide types produced by two strains of *Streptococcus thermophilus* on the physical properties of fermented milk. First, the fermentation factors affecting EPS production were studied to ascertain required carbon source and environmental conditions which would support their production. Higher fermentation temperature (42°C) resulted in a greater cell growth and EPS production. EPS production was growth associated in glucose or lactose-containing M17 medium. The examined strains appeared to be able to utilize galactose for the EPS assembly and produced comparable amounts of EPS, albeit restrictive cell growth. The EPS production of the two strains was comparable, ranging from ~100 to ~600 mg/L. Secondly, the EPS were rheologically characterized to show their resistance to deformation. Influence of temperature, pH and concentration on the flow behaviour of these EPS was also assessed. Under acidic conditions, capsular-ropy EPS was less responsive to temperature with a higher zero shear viscosity η_o (14.36 to 150.82 mPa s) than capsular EPS (93.72 to 9.24 mPa s), and slightly higher relaxation time τ (0.43 to 15.82 s for capsular-ropy EPS and 0.72 to 9.36 s for capsular EPS). The opposite behavior was observed under neutral pH. EPS concentration did not give significant effect ($P>0.05$) on η_o and τ.

The second study examined the effects of types of EPS on yoghurt texture under selected conditions. Fermented milk made using capsular-ropy EPS showed greater resistance to flow with less solid-like behaviour. It also had greater water holding capacity although the milk gel was less compact and brittle compared to fermented milk with capsular EPS. The EPS production in milk during fermentation between the two strains was comparable with maximum concentration was 840 ± 47.5 mg EPS/kg fermented milk. Syneresis was lower in fermented milk incubated in low temperature, was ranging from 4.1-2.4 g/100 g fermented milk with capsular-ropy-EPS, and 10.9-26.6 g/100 g in fermented milk

with capsular EPS. G' was 23.8-365.1 Pa and 57.6-1040 Pa for fermented milk with capsular ropy and capsular EPS, respectively.

The third study examined the involvement of EPS in the texture creation of fermented milk supplemented with calcium and/or sucrose, or calcium and whey proteins. Calcium addition to milk base resulted in increased acidity and greater syneresis (~20-30 g/100 g in fermented milk with capsular-ropy EPS and ~30-50 g/100 g in fermented milk with capsular EPS) and thixotropy of fermented milk, as compared to fermented milk without added calcium. Sucrose affected the parameters in opposite manner. EPS production did not differ from that of the control fermented milk. Storage modulus (G') was 96-230.4 Pa, and 502.8-1143.5 for fermented milk with capsular ropy and capsular EPS, respectively.

The effect of heat-untreated whey protein isolate or whey protein concentrate on calcium-fortified fermented milk was studied using capsular ropy EPS producer. Result showed that combined effect of both supplement was detrimental to texture of fermented milk to make it resemble that of drinking yoghurt. Syneresis was up to ~50 g/100 g, while G' was only around 4 mPa.

The next experiment studied the effect of heat-treated whey protein isolate addition on fermented milk texture. Results showed that heat-treatment applied to added whey protein preserved the G' and syneresis with the values close to those of normal fermented milk. However, at high concentration of added heat-treated whey protein (whey protein:casein 3:1), the texture became very hard with 0 m^2 permeability. Gelation was started very early in fermented milk added with heat-denatured whey protein. Whey protein addition induced the beginning of gelation. Supplemented fermented milk made using capsular-ropy EPS producer consistently showed lower G', lower syneresis, and more shear-resistant compared to that made using capsular EPS.

In conclusion, capsular ropy EPS, both in dispersion and in fermented milk with or without different supplementation, exhibited less solid-like properties and more shear-resistant behavior compared to capsular EPS.

Declaration

"I, Umi Purwandari, declare that the PhD thesis entitled "Physical Properties of Functional Fermented Milk Produced with Exopolysaccharide-Producing Strains of Streptococcus thermophilus" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature: Date: 13 April 2009

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List of Publications

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1 Introduction

1.1 Background

Yoghurt market has shown a marked increase in recent years in the European countries and US. Moreover, yoghurt has also entered the market in Asian and Middle Eastern countries. Although nearly every culture in the world recognised this product in the past (Vasiljevic and Shah, 2007), its largest market currently has been Europe, followed by the US (Defra, 2007). The world market volume is now around 14.5 millions tonnes, with market growth around 5% (Defra, 2007). The per capita consumption of fermented milk has been continuously increasing even in European countries and the US, and has reached around 10-15 pounds in Canada and UK, 5.4 pounds in the US, and 30 pounds in some European countries (Cook, 2003).

The health benefits of yoghurt were frequently cited very early, especially at the beginning of the last century. Nowadays, it is an important part of every day diet for the maintenance and improvement of health status in the society. The health benefits of yoghurt are even more improved due to its ability to be a vehicle for probiotics and nutrient supplementation. Since yoghurt has also gained a very good acceptance in children and the elderly, it is now an important element of the health improvement strategies for these groups. Calcium intake has been declining (Perales et al., 2006), partly due to lower consumption of milk. This may lead to several potential health problems associated with calcium deficiency such as hypovitaminosis and related diseases (Calvo et al., 2004). On the other hand, milk and milk products including yoghurt provide good environment for a better calcium bioavailability (Kitts and Kwong, 2004). Milk phosphopeptides enhance the availability calcium added in milk or milk products. Yoghurt has been recommended for athletes who experienced bone fractures and stress to increase the calcium intake (Cook, 2003).

Although health-related attributes are likely the main characteristic driving the yoghurt acceptance, sensory properties such as flavour and texture are also important. Despite the high attractiveness of non-fat yoghurt for its consumption, the desirable texture has the 'creamy' mouthfeel (Jaworska et al., 2005). Therefore, the texture creation of yoghurt is an important facet in designing any new yoghurt product(s) with additional health advantages. The texture of yoghurt is determined by fermentation conditions and interaction of yoghurt components, especially casein, whey protein, and exopolysaccharide (EPS) produced by yoghurt starter cultures (Lucey et al., 1998). Temperature, for example, affects protein-protein arrangement within the three-dimensional structure of yoghurt. EPS, as a member of hydrocolloid groups potentially gives 'creamy' characteristic in yoghurt (Cayot et al., 2008). Although it is well-known for its very limited amount produced in yoghurt, it plays an important role in texture creation of the final product. The types of EPS vary the yoghurt texture from shiny to dull, from ropy to crumble or brittle (Cayot et al., 2008). In regard to this characteristic, EPS are categorised into two main groups, ropy and non-ropy or capsular EPS. To add to the diversity, some strains produce a mixture of the two types of EPS with various proportion (Zisu and Shah, 2002, 2003). Their effect on the yoghurt texture is not easily predicted. Sucrose is a very common additive in yoghurt manufacturing and drives the flavour perception (Perez et al., 1994). It potentially affects the yoghurt texture through its interactions with either milk proteins (Braga and Cunha, 2004) or EPS (Yanes et al., 2002). Other supplements such as calcium (Achanta et al., 2007) and whey proteins (Guggisberg et al., 2007, Oliveira et al., 2001, Patocka et al., 2004) may also have a significant influence on the texture of yoghurt through their possible interactions with caseins and/or exopolysaccharides.

1.2 Aims

The main aims of this research were to study the effect of exopolysaccharide producing strains of *Streptococcus thermophilus* on the rheological and physical properties of various set type fermented milk products including fermented milk

fortified with calcium and/or whey proteins. A special discussion will focus on the role of exopolysaccharide in determining the final texture of set type fermented milk. Set-type fermented milk was selected due to its intact texture that would better describe various interactions that would take place during fermentation and supplementation with various adjuncts. It was expected to achieve texture of fermented milk as semi solid gel with minimum syneresis, and shear-resistant character.

The specific aims of this research are to study:

1. the effect of carbon source, temperature and fermentation time on the growth and EPS production of two strains of *S. thermophilus* one produces mixed of capsular and ropy EPS, and the other produces capsular EPS.
2. the characteristics of two types of EPS determined using rheological methodology
3. the effect of incorporation of two strains of *S. thermophilus* producing different types of EPS in production of low-fat fermented milk on the final texture as affected by fermentation temperature and storage time
4. the effect of calcium or/and sucrose addition and EPS-producing strains on the texture of low-fat fermented milk
5. the effect of addition of calcium and whey protein isolate or whey protein concentrate on the texture of low-fat fermented milk prepared using capsular-ropy strain of *S. thermophilus*
6. the effect of supplementation of non-heat-treated and heat-treated whey proteins and two types of EPS on the gelation properties of low-fat fermented milk.

1.3 Thesis outline

Chapter two presents a review of the literature on the health benefit of fermented milk, fermented milk quality acceptance and textural quality, yoghurt starter cultures and their growth in fermented milk, exopolysaccharide production in fermented milk and their effects on final texture, rheological method in

analysing fermented milk texture, and the effect of calcium or whey protein supplementation on fermented milk texture.

Throughout the study, the strains used are *Streptococcus thermophilus* ST 1275 and ST 285. The *S. thermophilus* ST 1275 produces both ropy and capsular EPS, but mostly ropy type. Therefore, we called this strain as capsular-ropy EPS producer. Whilst, strain ST 285 produces both type of EPS, but mostly capsular type of EPS. The study on the characterization of EPS of these strains was carried out by Zisu & Shah (2003, 2005).

Chapter three reports a study on the production of EPS by two strains of *Streptococcus thermophilus* which produced capsular-ropy (strain ST 1275) and capsular EPS (strain ST 285) respectively, as the effect of carbon source and temperature. The medium used was M17 containing glucose, galactose or lactose at 2% sugar concentration. The incubation temperatures were set at 30, 37 and 42 °C. A particular attention was given to the conditions to give the highest amount of EPS produced. The rheological and physico-chemical characterization of capsular-ropy or capsular EPS was then carried out. The behaviour of EPS dispersion during shearing as the effect of concentrations was studied. The mobility of EPS molecules in the dispersion due to hydration and enlargement was predicted using Cross model. Subsequently, degree of entanglement was also predicted.

Chapter four reveals the results from the experiment on the physico-chemical and textural characteristics of low-fat set-styled fermented milk as affected by the two strains of *S. thermophilus* producing different type of EPS and fermentation temperatures. The effect of EPS was examined by using artificial acidulant glucono-δ-lactone. The temperatures applied were 30, 37, and 42 °C. The total solid was 14%, and bacterial inoculation was set at 2%.

Chapter five deals with the study on calcium supplementation in low-fat fermented milk in the presence of sucrose, and fermented by the two strains of *S. thermophilus.* The fermentation temperature was set at 42 °C, and calcium chloride concentration varied from 0 to 9 mM. Sucrose concentrations were 0, 15, 30, 45 mM. The total solid and inoculum level were 14 and 2%, respectively.

Chapter six presents the report on the combined effects of calcium and whey protein concentrate or whey protein isolate on the physico-chemical characteristics of low-fat fermented milk produced using capsular-ropy strain of *S. thermophilus*, fermented at 42 °C. The experimental design and data analysis were carried out using the response surface methodology. Optimum combinations between concentrations of calcium chloride and whey protein concentrate or isolate were explored.

Chapter seven demonstrates the gelation behaviour of fermented milk prepared using the two strains of *S. thermophilus* differing in their EPS type, as affected by whey protein addition in native or heat-treated form. The structural development was followed by rheological method, revealing the changes in storage moduli over time. The final textural properties were assessed by both physico-chemical and rheological methods.

Chapter eight provides the summary of the results of all experiments carried out in this study, and underlines important findings. This chapter also presents a possible future research to further explore the possibilities of producing yoghurt with additional health benefits, without impairing physical and sensory quality.

2 Literature Review

2.1 Yoghurt - consumption and health benefits

Before Ellie Metchnikoff in 1910 related health benefits of yoghurt to longevity of the Bulgarian peasants, the attention paid to this product was rather minor. Around the mid of the last century an extensive industry-driven research started to take place in an attempt to reveal all the benefits related to this product (Vasiljevic and Shah, 2007). As a result, more recently yoghurt started to be considered as an important part of a healthy diet in order to improve overall well being. It is one among the recommended foods for infants and toddlers to improve health and eating habit in the US (Fox et al., 2004). The aggressive marketing campaign that utilized the health potential resulted in the good response and lead to an increase (Sloan, 2004). At present, the world market for this product increased around 5%, projected to reach 14.5 million tonnes consisting of various types of yoghurts (Defra, 2007). The largest market is European Union, and the fastest growing markets (8% per year) are Latin America, Africa and the Middle East. Consumers are attracted by the 'healthiness' attribute of yoghurt, rather than desirable flavour or pleasure (Valli and Traill, 2005), although some others perceived yoghurt as 'slimmy' and 'healthy' product with pleasant flavour (Kim et al., 1997). It is one of the 'healthy' food choice in 'health conscious group' to reduce their fat intake (Bowman and Davis, 1996). Yoghurt consumption is also influenced by country, knowledge about yoghurt, as well as socio-demographic factors (Valli and Traill, 2005). It consumed as a snack, popular for young and elderly in Europe (Valli and Traill, 2005), and children in the US (Sloan, 2006).

The health benefits of yoghurt were originally associated with microorganisms involved in yoghurt fermentation, and/or its fermentation products. However, more recently many other physiological benefits have been recognized that originated from other yoghurt components. Two main species of bacteria used as starter culture in the production of yoghurt are *Lactobacillus*

delbrueckii subsp. *bulgaricus,* and *Streptococcus thermophilus*. Consumption of yoghurt also presents a way of administration of living yoghurt bacteria into the gastrointestinal tract. By surviving low acid environment and presence of bile acid, this culture may eventually reach intestine where native community of microflora is already established. The mutual interaction with the gut microflora may further lead to several health benefits to the host, depending on several factors such as the ability and duration of the yoghurt bacteria to reside in the gut. Among the well-known health advantages of yoghurt is the ability of yoghurt cultures to improve lactose digestion by making it metabolically available for people with lactose-intolerance (Guarner et al., 2005). Accordingly, better lactose metabolism reduced the extent of flatulence, abdominal pain and diarrhea (Hertzler and Clancy, 2003). Yoghurt contained microbial metabolic product which may benefit human health, such as lactic acid, free amino and fatty acids (Gurr, 1987), as well as low-calorie sugars and B vitamins (Hugenholtz and Smid, 2002).

Yoghurt cultures commonly used are strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. *L. bulgaricus* may impart specific sensory properties due to 'post-acidification' (Oliveira et al., 2001). Alternatively, yoghurt may be produced by a single strain culture of *S. thermophilus,* which results in a mild flavour. This approach has been accommodated by several regulatory bodies. In Australia, yoghurt is defined as a fermented milk produced by fermentation with lactic acid producing microorganisms (FSANZ, 2003). This particular strain also gives a desirable body to yoghurt due to its production of exopolysaccharides (Hassan et al., 1996a). The presence of EPS in fermented products influences several important sensory properties, including mouth thickness, shininess, clean cut, ropiness and creaminess (Folkenberg et al., 2005). Due to discrepancy in regulations worldwide, the products produced from a yoghurt base and fermented by a single strain of a mixed yoghurt culture or any other lactic acid bacterium are generally termed fermented milks.

Yoghurt starter cultures exhibited probiotic effect, although it is weaker than those of probiotic species, based on the resistance to gastric juice and bile acid, β-

galactosidase activity, and hydrophobicity (Vinderola et al., 2005). Consumption of yoghurt is adversely related to the incidence of vascular disease due to reduction of serum tHcy (Ganji and Kafai, 2004) and greater protective effect against stroke (Bernal-Pacheco and Roman, 2007). Reduced serum cholesterol level was observed after 42 days consumption (Canzi et al., 2000). Yoghurt containing living or dead bacteria inhibited activity of α-amylase, α-glucosidase, pancreatic α-amylase, and angiotensin-converting-enzyme-I (ACE-I), rendering its consumption suitable for diabetics and people with high risk of cardiac disease (Apostolidis et al., 2006).

Both yoghurt cultures potentially survive harsh environment in the gut (Mater et al., 2005), and may alter gut microbiota composition (Alander et al., 1999, Canzi et al., 2000). Their residing in the gut may suppress the growth of pathogenic bacteria such as *Listeria monocytogenes* (Benkerroum et al., 2002). *S. thermophilus* produced bacteriocin with anti-listeria activity (Villani et al., 1995). There was a possible immunomodulation effect of yoghurt cultures. The immune enhancing effect of consumption of yoghurt fermented using *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* was observed, but may be absent depending on the strains (Makino et al., 2006). When consumed as live bacteria, the systemic immune response can be enhanced through stimulation of anti-inflammatory cytokines (TNF-α, IFN-γ, or IL-12) (Perdigon et al., 2003). *L. delbrueckii* subsp. *bulgaricus* exhibited good adherence to intestinal mucus, thus potentially provided a health benefit by modulation of immune system as well as reduction of allergy in infants (Ouwehand et al., 1999). Both yoghurt cultures induced proliferation of Payer's patch and splenic B cells, to give immunostimulatory effect (Kitazawa et al., 2003). Result on the effect of yoghurt starter culture on alleviation of food allergy was not consistent, as they did not show any effect, except when supplemented with probiotic (Cross et al., 2001).

S. thermophilus exhibited lesser probiotic effect compared to *L. delbrueckii* subsp *bulgaricus* (Vinderola et al., 2006). However, *S. thermophilus* may provide certain health benefits through its production of exopolysaccharides (EPS), a carbohydrate essential for yoghurt textural building. Some strains of *L. delbrueckii* subsp *bulgaricus* are also EPS-producers (Welman and Maddox,

2003). Exopolysaccaharides may appear in two forms: as capsular or slimy (extracellular) EPS (Wicken et al., 1983a). Capsular EPS is a thick outermost layer covalently attached to cell wall, while 'slimy' EPS is excreted into the medium. Nevertheless, some strains may have both types of EPS with various proportions between the two (Zisu and Shah, 2005b, 2007). From a technological point of view, ropy EPS give a 'ropy' appearance to yoghurt, a continuous strand-like structure. When this yoghurt is probed by a stick which is lifted or pulled up to a certain distance before the strand is broken (Folkenberg et al., 2006a). Thus, ropiness is a non-oral parameter of yoghurt. On the other hand, EPS in yoghurt that do not show strand-like structure are categorised as 'non-ropy' EPS. Ropy EPS fill the pores in the protein network in yoghurt, with more ropy EPS showing a more compact strand-like structure than less ropy EPS (Hassan et al., 2003a). Carbohydrate components attached on the surface of lactic acid bacteria may facilitate colonization of mucus in the colon (Vandevoorde et al., 1992), and subsequently inhibit pathogens' colonization in the area (Spencer and Chesson, 1994). Although the role of carbohydrates on the cell surface on binding appeared to be inferior to that of proteinaceous portion (Rojas et al., 2002), carbohydrate-rich cell surface was also adhesive (Schaer-Zammaretti and Ubbink, 2003). On the other hand, carbohydrate component was apparently important in facilitating aggregation and co-aggregation processes (Spencer and Chesson, 1994). Aggregation was a characteristics related to cell persistence in the gut, allowing sufficient time to produce a probiotic effect (Cesena et al., 2001). On the other hand, co-aggregation was related to cell binding to pathogens. Some *Lactobacillus* strains shared carbohydrate-binding specificity with pathogenic bacteria. Some lactobacilli co-aggregated to *Escherichia coli* and *Salmonella enteridis*, and consequently reduced the growth of the pathogens through competitive or exclusion mechanism (Drago et al., 1997). Excreted EPS may also support growth of probiotic bacteria by providing carbon source (Chung and Day, 2002). Acidic fraction of high molecular weight EPS produced in yoghurt fermented with both yoghurt starter cultures was related to immunomodulation effect, specifically by stimulation of production of interferon-γ and increased the activity of natural killers (Makino et al., 2006). It was proposed that the difference in the ability to

modulate immune response among bacteria may originate from the surface properties (Perdigon et al., 2003).

In order to improve health benefits of yoghurt, probiotic bacteria are frequently included in the product during yoghurt manufacture. The resistance to extremely low acid and bile acid of probiotic bacteria can overcome the problem of the lack of these characteristics in yoghurt starter cultures, as well as proved more pronounced health-improving effects including reduction of serum cholesterol and anti-carcinogenic activities (Lourens-Hattingh and Viljoen, 2001). In this case, the survival and viability of probiotic during yoghurt fermentation and storage are important attributes. Probiotic bacteria may be antagonistic to yoghurt starter culture. *L. acidophilus* produces anti-bacterial substances active against *L. delbrueckii* subsp *bulgaricus*. On the other hand, *L. delbrueckii* subsp. *bulgaricus* also produces hydrogen peroxide, which is toxic to *L. acidophilus.* In contrast, *Bifidobacterium bifidus* grows in synergy with *S. thermophilus*. *S. thermophilus* is capable of utilising oxygen in a significant amount, thus providing anaerobic environment supportive for the growth of *B. bifidum*. Another important consideration is avoiding suppression of the growth of probiotic bacteria due to the over dominance of yoghurt culture, by controlling the culture inoculum level, cell number during fermentation and storage, as well as production of metabolites. The minimum concentration of probiotics to result in a health effect (therapeutic minimum level) varies from 10^6 (Rybka and Kailasapathy, 1995).

2.2 Yoghurt microorganisms

Two yoghurt cultures, *Lactobacillus delbrueckii* subsp *bulgaricus* and *Streptococcus thermophilus*, grow symbiotically in yoghurt and support each other's growth and subsequently produce lactic acid (Tamime and Robinson, 1985). Proteolytic activity is also important for survival and growth of bacteria in milk. However, *S. thermophilus* is described as a poor proteolytic species, thus requires several essential amino acids for its growth with valine apparently the most important (Tamime and Robinson, 1985). *L. delbrueckii* subsp *bulgaricus*

provides the amino acids through its proteolytic activity. On the other hand, the growth of *Lactobacillus* is promoted by organic acids and carbon dioxide produced by *S. thermophilus*. This species also produces vitamins, including folic acid, required for the growth of *L. delbrueckii* subsp *bulgaricus*. During fermentation, *L. delbrueckii* subsp *bulgaricus* first releases catabolic products form milk proteins which are then used by *S. thermophilus* to grow profusely and consequently produce lactic acid and other organic acids including formic acid essential for the growth of *L. delbrueckii* subsp *bulgaricus*. The synergistic interaction between the two is also shown in their EPS production and eventually increase viscosity of yoghurt (Moreira et al., 2000). The geometry of the two bacteria affected their metabolic performance (Ginovart et al., 2002). In this case, the ratio of surface and biomass (S/M ratio) is larger in *S. thermophilus*, thus provided the cells with better absorption of substrate and faster growth. However, the decline in growth was also earlier in this species, due to high lactic acid concentration. Whilst *S. thermophilus* cease to grow at low pH, the cells of *L. delbrueckii* subsp *bulgaricus* continue growing.

Sugar utilization by yoghurt cultures is initiated by its transport across cell wall into the cytoplasm. It is commonly recognised that there are *primary* and *secondary transport systems* (Poolman, 2002a). The energy for the first type is drawn from the ATP pool, while the second type uses the energy generated by the proton motive force derived from the difference in the internal and external pH and transmembrane potential. The primary transport system is common in lactic acid bacteria for the accumulation of substrates and compatible solutes and expulsion of unwanted metabolic products. The secondary systems, also called *group translocation*, mostly use carbohydrates and alditols as substrates. Some of lactic acid bacteria transport lactose via the secondary transport system involving phosphotranferase phosphoenolpyruvate-dependent system (Tamime and Robinson, 1985). However, the operons expressing this system are absent in yoghurt culture (Poolman, 2002a). They rather utilize a specific permease for the lactose uptake with concomitant excretion of galactose (Tamime and Robinson, 1985). Subsequently, lactose is activate and converted into lactose-P (glucosyl β-(1,4)-galactoside-6P) which is then further cleaved into D-glucose and β-D-

galactose via glycolysis. The glucose passes through the Embden-Meyerhoff-Parnas pathway to produce pyruvate as the end product. The pyruvate is metabolised to produce various metabolites, with lactic acid as the main product. Several other products includes organic acids such as formic and acetic acid, flavour compounds such as acetaldehyde, diacetyl and ethanol, and amino acid such as alanine (Kleerebezem et al., 2000). The conversion of pyruvate to lactic acid is facilitated by lactate dehydrogenase (LDH). In lactic acid bacteria, the activity of this enzyme is typically high, leads to high rate and less efficient metabolism (Hugenholtz and Kleerebezem, 1999). As a consequence, the growth is very abundant in a nutrient rich environment, makes these bacteria potentially inhibit growth of other bacteria by outgrowing them or rapid reduction of pH through lactic acid production.

The galactose moity is excreted back into the medium, and can be incorporated into other metabolic pathway, depending on the ability of strain to metabolise galactose (Poolman, 2002a). It became apparent that certain portion of galactose may not be excreted, and may take part in the synthesis of exopolysaccharides. The bacterial utilization of galactose in lactic acid fermentation is of technological interest, since it improves cell metabolic economy especially in carbon source utilization. Whilst most of lactobacillus strains are capable of utilising galactose, most strains of *S. thermophilus* have limited capacity to ferment it. Galactose fermenting ability of this species varies from completely unable to complete ability to utilise galactose (de Vin et al., 2005). Some strains loose this ability after several hours. Galactose may be incorporated in both energy-generating and synthesis metabolism (Boels et al., 2001). The catabolic pathway for this sugar is tagatose-6-P pathway, while the synthesis is via Leloir pathway. In *S. thermophilus*, apparently the energy-generating system in the presence of galactose operates at a slower rate resulting in a slower cell growth, compared to that of other sugars. In this species, the main product of Leloir pathway is sugar nucleotide, a monomer for EPS polymer building. In *L. bulgaricus*, however, the presence of galactose metabolising enzymes and Leloir pathway may not always relate to EPS production (Marshall et al., 2001b). In general, strains of *S. thermophilus* possess the Leloir pathway, however the

system varies in the activity rate. In Gal^- strains, the enzymes in the system are less active than in Gal^+ strains (Degeest and de Vuyst 2000).

The rate of sugar uptake and metabolism in lactic acid bacteria varies and partly depends on a type of sugar available. In this case, a catabolic repression mechanism regulates the sequence of sugar types to be metabolised (Degeest and de Vuyst 2000). The preferred sugar is metabolised first, which can cause the inhibition of metabolism of other sugar(s). It may also mean a higher uptake rate of preferred sugar (Poolman, 2002a). The catabolic repression is observed when cells are grown in a mixture of sugars. For instance, *S. thermophilus* strains, unlike other gram-positive bacteria, slowly metabolise glucose, but rapidly utilise sucrose and lactose (Poolman, 2002a). Thus, in the mixture of galactose and lactose, the smaller lactose portions in the mixture, the slower the uptake rate as well as glycolysis activity. Similarly, in the later stage of growth, the more galactose is accumulated, the slower metabolic rate.

2.3 Exopolysaccharide production

In yoghurt, the production of exopolysaccharides by the culture is very low, maximum of ~500 mg/L, but it plays a major role in the development of texture of the final product (Bouzar et al. 1997). *S. thermophilus* is considered to be responsible for the EPS production, although some strains of *L. delbrueckii* subsp. *bulgaricus* are also EPS-producers. A mutual interaction between the two species of yoghurt starter cultures has been noticed leading to a higher EPS production when both grew together (Bouzar et al. 1997). The EPS production of both yoghurt cultures is growth-associated (Bouzar et al. 1997, Kimmel and Roberts, 1998). Therefore, the optimum EPS production occurs during the maximum of the cell production (Petry et al. 2000). In general, the EPS production starts during the exponential phase and may reach the maximum in the stationary phase (Petry et al. 2000). In the later stage of growth, the EPS degradation may take place (Deegest and de Vuyst, 2000, Pham et al. 2000) likely due to activation of the EPS-degrading enzyme(s) with relatively low molecular weight of 50,000-10,000

Da (Degeest et al., 2002). Both endo- and exo-enzymes are found to breakdown the EPS into low molecular weight polymeric compounds, but the activity of endoenzymes is apparently more dominant (Degeest et al., 2002). It was suggested that glycohydrolases were responsible for the EPS degradation. The EPS may be synthesized either from external sugars in the medium or internal sugars via Leloir pathway (Boels et al. 2001), with the latter pathway likely occurring in yoghurt. As a consequence, the EPS production is less stable and easily altered by external factors such as sugar type, temperature and other supplements. In *L. delbrueckii* subsp. *bulgaricus,* lactose supported the EPS production poorly, while the greater concentrations were achieved in the presence of glucose (Petry et al. 2000). Sucrose addition into lactose containing medium increased the EPS production in the lactose-grown cells (Gancel and Novel, 1994). Similarly, lactose supplementation during growth of sucrose-grown cells also enhanced the EPS production by *S. thermophilus*. Moreover, there was a synergistic effect when both sugars were present in the medium. A sugar concentration may or may not enhance the EPS production and is dependent on the kind of strain (Petry et al. 2000). In some strains, an excess of sugar concentration increased the EPS production, while in other strains it did not. Lower fermentation pH, around 5.8 or 6.0, resulted in a greater EPS concentration than higher pH (Kimmel and Roberts, 1998, Petry et al., 2000). In mesophilic strains, lower fermentation temperatures around 15-25 °C increased the EPS and EPS specific production. Whilst, in *S. thermophilic* strains, higher temperature around 42 °C is more supportive for the EPS production (De Vuyst and Degeest, 1999). Some nutrients such orotic acid, thiamine, adenine and xanthine (Petry et al., 2000a), other members of vitamin B (Chervaux et al., 2000) increased the growth and subsequently the EPS production. However, in some other strains, addition of some amino acids and certain types of carbohydrates into the medium did not enhance its production, since they were not taken up by the cells (Degeest et al., 2002). The excessive protein addition may reduce the EPS yield (Kimmel and Roberts, 1998). Similarly, addition of whey permeate in low concentration was supportive to the EPS production (Macedo et al., 2002). A complex medium high in nutrients was found to improve the EPS yield, likely due to vitamin and

nucleic acids (purin and pirimidin) content (Degeest et al., 2002). Depletion of oxygen was reported to positively affected EPS production (Petry et al., 2000).

The EPS formation in yoghurt is mostly considered as a product of Leloir pathway which is expressed in most of *S. thermophilus* strains. However, the enzymes are not always in an active stage (Poolman, 2002a). Similarly, all *S. thermophilus* studied contained *eps* operon, responsible for the production of EPS (Mozzi et al., 2006). The pathway utilises galactose as a substrate which is taken up from the medium via LacS protein (Poolman et al., 1989). LacS protein can function either as lactose-glucose antiporter or proton symporter. In *S. thermophilus* strains, galactose utilisation mainly relates to the EPS production, while certain portion is used for the cell wall development (Levander and Radstrom, 2001). Only minor quantity was incorporated into the energy-generating metabolism. Galactose metabolising enzyme in this system is phosphoglucomutase which is responsible for both EPS and lactate production (Levander and Radstrom, 2001). Only ~10% of the strain population of *L. delbrueckii* subsp. *bulgaricus* studied expressed the *eps* genes (Mozzi et al., 2006). For *Lactobacillus* strains, the EPS production in this pathway is in particular related to the activity of galactokinase which is absent in non-EPS producing strain (Mozzi et al., 2001). This system metabolises galactose better than glucose, and resultes in a slower cell growth (Poolman, 2002a), but greater EPS yield (Mozzi et al., 2001). Therefore, decreasing galactose concentration in the medium without observable galactokinase activity at the later stage of fermentation may indicate that the cells metabolism is switched into tagatose pathway.

Molecular weight of EPS varies greatly, ranging from ~10 to >5000 kDa (Mozzi et al., 2006) and affects the rheological properties of EPS. Molecular mass of EPS may or may not be affected by growth medium, and is mainly strain dependant (Vaningelgem et al., 2004b). Molecular weight and intrinsic viscosity of EPS is higher when cells are grown in milk compared to those in a chemically defined medium (Petry et al., 2003). High molecular mass is an important characteristic since it relates to ropiness (Mozzi et al., 2006), a characteristic that greatly influences the yoghurt texture. The ropy EPS consists of mainly high

molecular weight EPS as opposed to non-ropy EPS composed of low molecular weight EPS (Petry et al., 2003). The molecular weight of EPS potentially influences the EPS-protein interactions by creating depletion flocculation. Some of the strains also produce 'floating' or 'non-floating' EPS or mixture of both, but this characteristic is apparently independent of the molecular weight (Vaningelgem et al., 2004b). The EPS type is influenced by medium, depending on the strains. Floating EPS potentially made isolation difficult, due to loss during precipitation. Main sugar monomers creating the EPS building blocks are glucose and galactose (Degeest et al., 2002, Marshall et al., 2001a, Petry et al., 2003) and, in lesser amount, other sugars or their derivatives such as rhamnose (Marshall et al., 2001a, Petry et al., 2003), and N-acetyl glucosamine (Degeest et al., 2002) or N-acetyl galactosamine (Vaningelgem et al., 2004b). Galactose is the most common sugar monomer constructing many types of the EPS secreted by *S. thermophilus* (Mozzi et al., 2006, Vaningelgem et al., 2004b). On the other hand, rhamnose is rarely found in the EPS (Mozzi et al., 2006). The EPS yield varies among strains and is affected by several external factors. However, the sugar monomers (Degeest and de Vuyst, 2000a) and their ratio (Mozzi et al., 2006) tend to remain unchanged. Sugar composition in EPS appears unstable during early stages of formation, and stabilizes at the later stage. Consequently, the composition differences among strains frequently diminish at the end of fermentation.

2.4 Rheological characteristics of EPS

As a hydrocolloid, the EPS exhibit viscoelastic properties of solution. However, the correlation between EPS and viscosity is very complex, since it is influenced by concentration, molecular mass, and environmental pH (Vaningelgem et al., 2004b). Although high molecular weight tends to result in a higher viscosity, it is not the only governing factor (Petry et al., 2003). For example, solution containing high concentration of low molecular mass EPS may have similar viscosity as low concentration of high molecular mass EPS

(Vaningelgem et al., 2004a). Another possible factor is the higher proportion of glucose monomers in the EPS backbone (Petry et al., 2003). Similarly, consistency of EPS solution is influenced by interaction of the concentration and molecular mass as well as the molecular structure (Vaningelgem et al., 2004b). Stiff polymer chain results in a higher consistency. The EPS containing β-linkage are mostly forming stiff chain (Vaningelgem et al., 2004b). On the other hand, α-linkage gives flexible polymer characteristics, with less stiffness and lower consistency. Nevertheless, when the EPS are incorporated into yoghurt manufacturing, they may behave differently from pure EPS solution due to their possible interactions with other yoghurt constituents.

The characterization of EPS can be carried out by several methods, including rheological methodology. Common rheological models for general viscous liquids include the Power law, Cross, Yasuda, Carreau, and Ellis models (Macosko, 1994). Modified Power Law model for shear rate against viscosity is expressed as:

$$\eta = K\dot{\gamma}^{(n-1)}$$

This model displays shear thinning or shear thickening behaviour, and covers a wide range of the shear rates. However, it poorly describes a Newtonian flow commonly encountered at very low shear rate. Hence, two power law indices are derived, those are K and n. K is consistency index and denotes the viscosity at shear rate 1/s (Rao, 1999). The exponent n is the flow behaviour index, which indicates the extent of deviation of material from the Newtonian flow (n = 1). Shear thinning is indicated by $n < 1$, while shear thickening is shown by $n > 1$.

Similarly, the two indices can also be derived from the Power Law model for shear stress plotted against shear rate (Rao, 1999):

$$\tau = K\dot{\gamma}^{n}$$

where τ is shear stress (Pa s), $\dot{\gamma}$ is shear rate (1/s), K is consistency index (Pa s^n), and n is the flow behaviour index (dimensionless).

The Cross model is often employed to describe the pseudoplasticity of the polymer dispersion (Rao, 1999). This model is expressed as:

$$\eta = \frac{\eta_0}{1+(\tau\dot{\gamma})^m}$$

where K, n, η, $\dot{\gamma}$ are consistency index (mPa s^n), flow behaviour index (dimensionless), apparent viscosity (mPa s) and shear rate per second (1/s), respectively. η_0 is viscosity at shear rate 0/s, τ relaxation time (s), and m is limiting slope (dimensionless). Yasuda model is expressed as:

$$\frac{\eta-\eta_\infty}{\eta^\circ-\eta_\infty} = \frac{1}{\left[1+\lambda^a|\mathrm{II}_{2D}|\right]^{(1-n)/a}}$$

This model is similar to the Cross model, but with additional fifth fitting parameter *a* (Macosko, 1994). Another equivalent model to Cross and Yasuda is the Carreau model. The Carreau model is characterised by the *a* value equals to 2 (Macosko, 1994). It is expressed as (Rao, 1999):

$$\eta_a = \eta_\infty + \frac{\eta_0-\eta_\infty}{\left[1+(\lambda_c\dot{\gamma})^2\right]^N}$$

The Ellis model is used when the limiting viscosity η_∞ is set at zero (Macosko, 1994). As a function of stress invariant, it is expressed as:

$$\frac{\eta_o}{\eta} = 1+\left(\frac{\mathrm{II}\,\tau}{k}\right)^{a-1}$$

A frequency sweep (dynamic oscillatory) analysis at 0.01-10 Hz at strain of 5% (in linear viscoelastic region) can be used to determine the dominance of solid-like (storage modulus, G') or liquid-like (loss modulus, G") characteristics of a EPS solution (Lambo-Fodje et al., 2007). The operation at linear region of viscoelasticity is important in order to avoid interference with the microstructure. This technique is also often employed to determine the frequency at which the intersection between G' and G" occurred, commonly called as cross-over frequency. Furthermore, G'_{max}, G' at plateau of the curve can also be derived. The reduction in this value indicates the collapse of polymer chain to form more compact and aggregated structure. The cross-over frequency designates a frequency when transition between solid- and liquid-like takes place. Furthermore,

the cross-over time can also be calculated using the equation (Lambo-Fodje et al., 2007):

$$t_{G'=G''} = \frac{2\pi}{f_{G'G''}}$$

The temperature dependence of EPS can be calculated using Arrhenius model (Rao, 1999):

$$\eta_a = \eta_{\infty A} \cdot e^{\frac{E_a}{RT}}$$

where η_a is viscosity at a particular shear rate (Pa), $\eta_{\infty A}$ frequency factor (Pa), E_a activation energy (J/mol), R gas constant (J/mol· K), and T absolute temperature (K).

Intrinsic viscosity $[\eta]$ is the viscosity as infinite dilution where the effect of the different particles are independent of each other (Lambo-Fodje et al., 2007). This characteristic positively correlates to the chain length, branching or stiffness. Intrinsic viscosity is derived by extrapolation to zero-concentration of a Kraemer (Lambo-Fodje et al., 2007) or Huggins and Kramer (Gorret et al., 2003) plot of ln $(\eta_{rel})/c$, with c is the concentration of the polymer dispersion.

2.5 Important aspects of the yoghurt texture

There are different types of yoghurt according to the process, such as set-type, stirred type, drinking, butter, and dried yoghurt (Tamime & Robinson 1985). They also differ in the texture. Set-type and stirred-type yoghurt belong to custard-like yoghurt, which has a semisolid character. However, set-type yoghurt tends to have firmer texture, since it is made by fermentation of yoghurt ingredients in individual cup or package. This type of processing allows very limited physical disturbance to enable restoring the original texture. In contrast, stirred-type yoghurt is made by fermentation in a large tank, the final product is then filled into individual package. During the filling process, a severe force is applied to the yoghurt and breaks the texture. The broken texture is redeveloped

when the yoghurt is kept in the package. The new texture is usually weaker than the original texture before filling process.

There appears to be a long discussion on the definition of texture in food. The first point of disagreement was whether it was a physical or sensory attribute. As a consequence, texture comprises of both physical and sensory characteristics. Although this common concept is accepted in general, it seems that there is no exact definition to satisfy all parties coming from different backgrounds. This leads to several different definitions proposed by experts or organizations. Nevertheless, some basic characteristics of food texture have been underlined, that texture is a set of physical structures of food, consisting either mechanical or rheological properties which are connected to the feeling of touch in the mouth or other parts of body, but do not relate to taste or odor. Texture is objectively measured by dimensions derivative of mass, distance, and time (Bourne, 2002).

There is a various and broad range of vocabulary used by different nations to describe texture of food (Meullenet, 2002). In principle, the sensory element of texture is perceived by touching, although those are perceived by hearing, vision and tasting also affect the evaluation (Kilcast, 2004). Furthermore, the response to touching can be devided into two modes. Firstly, it is texture as a surface response of skin (proprioception), and secondly, the deep response from muscle and tendon (somesthesis). Such responses can also be observed indirectly by using utensils, as well as organs in the mouth such as tounge, palate and teeth. Although sensory evaluation is the best method to describe consumer perception of yoghurt texture, it needs a carefully-designed procedure (Bourne, 2002). The result of sensory evaluation is, therefore, more meaningful illustration of yoghurt texture than physical assessment. However, sensory evaluation takes a long time to be carried out, and it is costly. In order to best describe the texture of food, a procedure called Texture Profiling Analysis (TPA) has been developed by a group led by Dr. Szczesniak to give the basic method in conducting sensory evaluation (Bourne, 2002). The use of trained panels and intensity test gives better and more reliable result than untrained panels or hedonic test. The procedure in TPA comprises several main stages: selection and training of panels, establishment of standard rating scales, development of TPA rating sheet, and developing comparative TPA

for every commodity. Physical and sensory properties are not only the main elements in texture, they are also closely interdependence. The influence of flavour and food texture such as viscosity and melting in the mouth is one of the examples.

Sensory properties which are determined by the consumer preference on yoghurt texture, are influenced by sexes, ages, and health concern (Kahkonen and Tuorila, 1999). Young people prefer fat-containing yoghurt, in contrast to the elderly. Smooth texture is important attribute for both young and elderly consumers (Kalviainen et al., 2003). Therefore, thickness (Jaworska et al., 2005) of yoghurt is important determinant of yoghurt texture, although it appears second to flavour in sensory perception of yoghurt attributes (Kalviainen et al., 2003).

Among textural characteristic characteristics, 'creamy texture' is the most preferred (Jaworska et al., 2005, Ward et al., 1999), although creaminess is not a sole textural property since it also involves other sensory and physical properties (Frost and Janhoj, 2007). In another research, creamy (smoothness) was described as a flavour paramater (Jaworska et al., 2005), and was the most important flavour component. More specifically, creaminess in set plain yoghurt is related to textural and mouthfeel descriptors, while in stirred plain yoghurt it is associated with relatively high viscosity, fat-related flavour, smooth mouthfeel and fatty after mouthfeel. Smoothness is also correlated to creaminess (Cayot et al., 2008).

The particle size yoghurt ranging from 100 to 150 μm enhances the sensation of product creaminess, but particles greater than 250 μm provide for a very thick texture (Cayot et al., 2008). A positive correlation between creaminess of yoghurt and thickness and smoothness has been previously established (Kokini, 1987) as:

$$\text{Creaminess} = \text{thickness}^{0.54} \times \text{smoothness}^{0.84}$$

The direct measurement of creaminess is very difficult due to the complex nature of this attribute. In custard, creaminess is predicted by initial rheological and mechanical breakdown properties (de Wijk et al., 2006). The rheological parameters included G' at 1 Pa, and critical stress or strain. Creaminess correlates positively to initial G' and low critical stress or strain. Very high values of storage

modulus reduce creaminess as the gel structure is hard to break down. Several physical parameters are used to measure creaminess including consistency, granulation, surface adhesion, surface angle measurement, viscosity and complex modulus (Cayot et al., 2008). Low viscosity and low complex modulus are attributes of a thick and creamy texture. In this case, creaminess is correlated to viscosity measured at shear rate 50/s (Frost and Janhoj, 2007). The thick and creamy sensation is originated from covering of the tongue and delayed the cleaning of the mouth (Cayot et al., 2008). The susceptibility to become liquid contributes to creamy perception.

In addition to creaminess, there are several other textural properties that affect yoghurt acceptance, including smoothness, viscosity, meltdown rate, astringent, fatty after mouthfeel and dry after mouthfeel (Frost and Janhoj, 2007). Physically, desirable yoghurt texture is depicted by the shear thinning behaviour, quick recovery after shearing and less syneresis or whey expulsion. Ropy EPS tend to exhibit high cohesion of yoghurt, and difficult to break into smaller size of particles, thus give thick characteristic. Texture of yoghurt is interrelated with flavour. Yogurt with one type of flavour results in a thick, sticky and less smooth texture, while yoghurt containing mixtures of flavours is perceived as less thick with smoother and less sticky texture (Saint-Eve et al., 2004). Furthermore, yoghurt made by additions of fatty flavours such as coconut and butter is perceived as thicker, while green apple or almond flavour provides for a sensation of a smoother texture.

Physically, desirable yoghurt texture is depicted by the shear thinning behaviour, quick recovery after shearing and less syneresis or whey expulsion. Syneresis results from a low water holding capacity of the acid formed gel, extensive large pores of gel, and can be induced by physical disturbance such as stirring. Several factors during yoghurt processing which lead to syneresis are high incubation temperature, low total solid content, types of EPS, and high casein to whey protein ratio. High heating temperature at 80-90 °C results in yoghurt with large cracks and rough texture, visible after gel development (Lucey et al., 1998). The structural breakdown and subsequent syneresis is positively correlated to brittleness and less flexible protein-protein bonds, rather than

porosity. Unlike textural properties and syneresis, porosity may not (Lucey et al., 1998) or be (Lee and Lucey, 2004b) affected by the heat treatment, apparently depending on the nature of the milk proteins. Higher temperatures increase significantly porosity and consequently also syneresis (Lee and Lucey, 2004b). The fracture of gel may also be caused by local pressure built up during continuing casein aggregation, which eventually exceeds the pressure resistance of the gel. Thick strand of yoghurt network can give good resistance to the break-down (Lucey et al., 1998). This condition can also be achieved by low incubation temperature. Moreover, high storage modulus and small tan δ value are indication of a high counter pressure towards the gel shrinkage, and results in less syneresis (Lee and Lucey, 2004b). Syneresis can also be reduced by increasing inoculation rate, which consequently leads into improvement of elastic properties of the gel (Lee and Lucey, 2004b). A lower yield stress derived from lower levels of inoculation was correlated to larger but weak pores within yoghurt network. During cold storage, syneresis may decline (Guzel-Seydim et al., 2005). Low temperatures during storage weaken hydrophobic bonds among casein particles in yoghurt, leading to less compact structure, more inter-particle bonds and better solid-like behavior and reduced syneresis (Van Vliet et al., 1989).

2.6 The role of milk proteins in yoghurt texture

Yoghurt gel is the result of aggregation of milk proteins following the collapse of casein micelles. There are several models proposed for the shape and functionality of the casein micelle. However, it appears that none of them provides thorough and complete explanation for the behaviour of the casein micelle in different environments. The model proposed by Fox, describes micelle as a cluster of submicelles in the form of κ-casein–rich fraction on the exterior and non-κ-casein fractions in the interior of micelle (Horne, 2006). The micelles are connected to the others by the colloidal calcium phosphate (CCP). Another model, based on the electron microscopy image, suggested a smooth or raspberry surface of the casein micelle (McMahon and McManus, 1998). Later, this model

was corrected by suggesting a non-smooth surface with tubular attachments on the micelle surface protruding from the centre of the micelle and creating gaps in between two adjacent tubular bodies (Dalgleish et al., 2004). The Holt model suggests that the micelle consists of nanoclusters of calcium phosphate which are assembled into a loop-like arrangement (Horne, 2006). This model seems to lack in a micelle growth-terminating mechanism. Nevertheless, all these models share a similar principle, in which the micelle consists of all types of casein: α-, β-, and κ-casein, and they self-associated in a special dynamic arrangement (Horne, 1998). The caseins are connected by inorganic nanoclusters of colloidal casein phosphate (CCP) (Horne, 2002) and thus responsible for the integrity of casein micelle. However, it was found that the collapse of CCP did not result in dissolution of micelle, thus suggesting other mechanisms are also involved in stabilizing the micellar integrity (Horne, 2006). At natural pH of milk, the α- or β-casein are linked by hydrophobic bonds to form a larger body, while κ-casein form a densely charged appendages on the surface of micelle (Horne, 1998). Individual aggregate of α-casein is a worm-like structure in a linear 'parallel/anti-parallel' order, with the anchor points as the hydrophobic components at every end of single particle. The aggregates of β-casein, on the other hand, are a 'hedgehog'-like shape, in which the hydrophobic components join to form an ellipsoidal central core and hairy structure sticking out at every end. κ-Casein structure is a mirror image of β-casein. However, sinceκ-casein lacks of a cluster of phosphoserine residue in its chain, it is unable to continue growing. Therefore, it terminates the growth of micelle. On the other hand, α- and β-casein play a part in the development and enlargement of casein micelle structure. The association of every type of casein has their equilibrium between monomer and micelle. While the equilibrium of α- or β-casein is affected by temperature, that of κ-casein was independent of temperature. When pH is lowered such as during yoghurt fermentation, negative charges are reduced, causing electrostatic repulsions to be shielded which consequently results in the increase in polymer size of both α- and β-casein (Horne, 1998). On the contrary, when pH is higher than natural pH of milk, the repulsive forces prevail over electrostatic attractions and polymer size of α- and β-casein is reduced. The strength of the interaction in

a micelle is a resultant of hydrophobic and repulsive forces. As the hydrophobic force supports the growth of micelle, the repulsive force limits it. κ-casein contributes not only to control of the micelle growth but also functions as a sterically stabilising brush, to prevent micelles from aggregating (Horne, 2002). Thus, the size of micelle is governed by the balance between attractive and repulsive forces. The collapse of κ-casein layer on the micelle surface during renneting triggers the casein aggregation.

Pre-heating of milk alters the balance of forces within milk environment (Horne, 1998). Increasing the temperature up to 35-40 °C enhances the strength of hydrophobic bond, and calcium ion is more strongly attached to the micelle. However, when temperature is above 40 °C, CCP precipitates, and the calcium components are detached from the casein, leading to the loosening of bonds and weakening the gel structure. Heating at 90 °C induces the complex formation of κ-casein and β-lactoglobulin via disulfide bridges (Horne, 1998). As a result, the gel strength is improved. Pre-heating of milk is also related to the denaturation of whey protein. Denatured whey protein can increase casein aggregate size as a result of enhanced covalent (disulfide-, and sulfhydrile bond) as well as cross-linking of intermolecular disulfide binding. Complete denaturation of milk whey protein can be attained by pre-heating milk at 95 °C for 5 mins (Sodini et al., 2004).

Pre-heated milk has a very important influence on yoghurt texture, especially as expressed by storage modulus (Xu et al., 2008). The difference of 1 °C in pre-heating temperature can have substantial implications. The association of heat-denatured whey protein into micelles elevates the G' to form a 'shoulder' at the beginning of the curve of G' against time. After that, a plateau is reached to indicate the solubilization of colloidal calcium phosphate. This is followed by a further G' increase reflecting extensive coagulation of casein aggregates. A more detailed information is provided on this phenomenon (Anema, 2008). Denatured 'non-sedimentable' whey proteins are able to bind to κ-casein. Thus, at neutral pH when colloidal calcium phosphate (CCP) is still intact, the whey proteins are mostly in the solution. As pH declines through the fermentation, the CCP is

solubilized and free colloidal calcium can bind the 'soluble' fraction of denatured whey proteins. This association forms a strand-like body which later functions as bridging appendages among casein particles via disulfide bonding. As a result, the casein particles become larger in size and subsequently increase the storage modulus. The extent of this association is influenced by pre-heating pH. While heating milk at its natural or higher pH leads to increase in the gel storage modulus, heating it at lower than its natural pH gives the contrasting effect. Heating milk at acidic environment results in less denatured whey proteins, including the portion of the soluble denatured component. The available denatured whey proteins tend to bind casein micelle rather than κ-casein. Consequently, there was less number of connecting body for casein particles, resulted in smaller aggregate size and less firm texture. Milk protein concentration adversely but only insignificantly affects denaturation of whey proteins (Anema, 2008). However, increasing milk protein concentration increases breaking stress and breaking strain, which reflects the number of covalent bonds.

As caseins are the main component in milk gel, the higher their concentration, the firmer (expressed as storage modulus G') the final texture of yoghurt (Anema, 2008). Heating of yoghurt base mixture (Lucey et al., 2001) as well as high fermentation temperature are known to increase viscosity, firmness and reduce syneresis (Shaker et al., 2000). The increase in viscosity during acidification exhibits three steps (Shaker et al., 2000). First, at the beginning of the gelation, there is a slight increase in viscosity, but the size of casein particles is still unaltered. Secondly, co-aggregation of casein particles into larger aggregates starts indicated by a sharp increase in viscosity to eventually reach a plateau. The size and shape of the aggregates vary with no apparent trend. Finally, by the decrease in pH at the later stage of acidification, the aggregate size is reduced to produce small particles which are connected into the gel network.

Other technique to improve yoghurt texture is application of high pressure homogenisation. This method is designated to avoid the use of additive(s) which can give off-flavours. High pressure (more than 100 MPa) applied during homogenisation breaks the casein particles (Penna et al., 2007) or fat globules (Serra et al., 2007), further resulting in altered physical and rheological properties

of yoghurt. The higher the pressure applied, the firmer the yoghurt gel resulted. High pressure induces denaturation of whey proteins to a certain extent (Serra et al., 2007). At lower pH when CCP is solubilized, the smaller size of casein particles is connected by a bridging material consisting of κ-casein and denatured whey proteins associated by disulfide bonds (Penna et al., 2007). Large particles of caseins are subsequently produced, as their aggregation is hindered by the bridging body. The result is a firmer texture with less serum expulsion. In this case, firmer gel as indicated by higher storage modulus is negatively correlated with syneresis (Serra et al., 2007). In the presence of fat, high pressure treatment increases the active surface of fat globules as the globular size is reduced. This would in turn facilitate their association in the development of three dimensional gel network and improve the aggregation rate. Clusters of possibly cross-linked proteins and lipids may be formed during this treatment leading to a higher storage modulus.

2.7 The role of EPS in yoghurt texture

The exopolysaccharides (EPS) content in yoghurt affects the yoghurt texture. In general, they reduce firmness of yoghurt as measured by storage modulus (G') (Folkenberg et al., 2006a). Ropy type of EPS gives rise in ropiness of yoghurt, as well as mouth thickness and creaminess. A mechanism for fat-replacer capacity of EPS has been suggested, involving the ability of EPS to cover the tongue and delay the cleaning of mouth by saliva (Cayot et al., 2008) resembling the action of fat in the mouth cavity during chewing. The thickness character is raised from a high cohesion which prevents yoghurt particles to segregate into smaller particles, resulting in a more elastic material. Mouth thickness is correlated with viscosity at shear rate of 241/s (Folkenberg et al., 2006a). Although ropiness of EPS is related to creaminess, the EPS produced by some non-ropy strains are also able to exhibit creaminess in yoghurt (Folkenberg et al., 2006a). The effect of the EPS on the textural properties also varies with different strains. The EPS from *L. delbrueckii* subsp. *bulgaricus*, for example,

influenced firmness, while that of *S. thermophilus* gave creaminess, mouth thickness and ropiness of the final product. A yoghurt culture called strain YC180 gave creamy characteristics similar to a fat-containing yoghurt, including creamy flavour, mouthfeel, sweet taste with reduced astringency, bitter and sour taste (Folkenberg and Martens, 2003). However, there are apparently some characteristics of EPS other than ropiness which would govern their effects on yoghurt texture, such as their interactions with other yoghurt components, molecular weight, degree of branching, and chain flexibility (Folkenberg et al., 2006a, Folkenberg et al., 2006b).

The EPS hinder the structural rebuilding of yoghurt after rupturing by shear, a phenomenon often expressed as thixotropy (Rao, 1999) and is also perceived as 'a loss of consistency' after shear (Penna et al., 2001). During shearing in a rheometer or chewing in the mouth, the EPS which are originally located in the void among casein particles are driven out, which leads into aggregation with EPS from other interspace cavities. The formation of large EPS aggregates eventually leads to phase separation. Due to incompatibility of EPS and caseins, re-association of casein particles is obstructed by EPS (Folkenberg et al., 2006a), especially EPS which form attractive bonds with casein micelle (Girard and Schaffer-Lequart, 2007). Therefore, factors inhibiting the attractive interactions between EPS and casein would potentially support better recovery. Some of these EPS characteristics include low molecular weight and weak charge (Girard and Schaffer-Lequart, , 2007). On the other hand, in the absence of EPS, the casein particles are easily reconnected regaining their original state (Folkenberg et al., 2006a) or in the case of brittle yoghurt network, the structure may be irrecoverably broken (Girard and Schaffer-Lequart, 2007).

The EPS influence gelation and microstructure of yoghurt. The presence of EPS in yoghurt base mixture induced depletion, and as a consequence gelation as observed by sharp increase in G' started earlier (Girard and Schaffer-Lequart, 2007). The pressure created during depletion interaction causes casein particles to assemble by hydrophobic forces, giving raise to greater elastic properties. The earlier start of gelation in acid milk is especially pronounced when EPS involved in the interactions have stiff chains in their backbone at neutral pH (Tuinier and de

Kruif, 1999). α (1→4) linkage is stiffer than β (1→2) or β (1→3) linkages. Higher concentration of EPS stimulates more extensive depletion (Girard and Schaffer-Lequart, 2007). However, this effect also depends on the molecular of the EPS with low molecular weight producing a smaller depletion effect due to their poor exclusion from the gap between two casein particles (Tuinier and de Kruif, 1999).

2.8 Rheological determination of yoghurt texture

Although creaminess is an important characteristic of yoghurt, it is difficult to make a simple correlation with physical and rheological measurements. Therefore, most of physical and rheological measurements are focused on the gel strength towards mechanical disturbance and syneresis. Rheological and textural measurements are carried out using small and large deformation techniques. Large deformation is carried out by applying a constant shear rate 0.00185/s until yielding of the gel was reached (Lucey et al., 1997a). Large deformation method can be used to collect data on the viscosity which can further be analysed using rheological model such as the Cox-Merz model. Although this model can be fitted to other several semi solid foods, it poorly fitted the properties of a culture-fermented yoghurt (Yu and Gunasekaran, 2001). Small deformation method consists of oscillatory and rotatory measurements, commonly called as SAOS (small amplitude oscillatory shear), by applying low frequency (0.01-100 Hz) at stress <1 Pa (Yu and Gunasekaran, 2001) and low to medium shear rate (0.05-100/s) (Yu and Gunasekaran, 2001), respectively. Using SAOS, the structure remains relatively intact since the deformation is minimal (Yu and Gunasekaran, 2001). The large deformation technique was applied to GDL-acidified yoghurt to reveal several crucial parameters of the gel strength where irreversible structural disruption started to occur (Lucey et al., 1997a). These parameters were γ_{yield} and σ_{yield}, the shear strain at yielding and apparent yield stress, respectively. Hence, lower values of both parameters indicated more brittle gel. In the reported work,

the values of γ_{yield} showed meaningful trend, but those of σ_{yield} did not (Lucey et al., 1997a).

Several rheological parameters commonly derived from SAOS measurement are storage modulus (G'), loss modulus (G"), loss tangent (tan δ), fracture stress ($\sigma_{fracture}$) and fracture strain ($\gamma_{fracture}$) (Lucey, 2001). Storage modulus indicates a solid-like behavior of a material and relates to the strength and number of bonds to resist to the oscillatory deformation (Lucey, 2001). High values of G' thus reflect a large number of strong bonds within the structure. During storage or ageing of the yoghurt gel, the increase in G' may indicate the enhanced fusion of particles in inter- and intramolecular rearrangement (Lucey et al., 1997d). The plot of G' against frequency or strain can be used to reveal the structural changes as an increasing stress is applied (Hess et al., 1997). In yogurt, its shear thinning behaviour is shown by declining storage modulus as the strain or frequency is increased. The change in the rate of decline is indicative of the change in the amount of energy required to break down the casein network into smaller particles. When the EPS are present within the network, they reduce the rate of structural disruption, which is demonstrated as an inflection (Hess et al., 1997). Loss modulus, G", is an indicator of a liquid-like behaviour. Furthermore, the ratio between these two moduli, G"/G' is also known as the phase shift or tan δ, and reflects the degree of the bond relaxation. High value of tan δ shows tendency towards greater relaxation of bonds (Lucey and Singh, 1997, Lucey et al., 2001). A maximum in tan δ is observed during solubilization of CCP and may be related to loosening in structure (Lucey et al., 2001). Fracture stress, $\sigma_{fracture}$, is a shear stress required to break the gel. It indicates the ability of the network to resist to a break-down, as well as shows the shear rate where the structural rearrangement can take place. The fracture strain, $\gamma_{fracture}$, indicates the strain value at which the gel starts to break, and $\sigma_{fracture}$ indicated susceptibility of gel to break upon the application of strain. The higher the values of both parameters, the more intensive association among particles within the network (Lucey et al., 1997d).

A shear sweep method or rotational rheological test is commonly applied to obtain parameters such as apparent viscosity (η_{app}), consistency index (K), flow

behaviour index (n), and yield (Rao, 1999). Apparent viscosity is viscosity as a function of the shear rate (Rao, 1999). During shearing of yoghurt, apparent viscosity is decreasing, indicative of shear thinning behavior. The inflection observed in the curve shows a change in the shear resistance, thus could be a sign of different structural arrangements being exposed to shearing. The EPS residing in the network cause an increase in the shear resistance after the casein network is first disrupted (Hess et al., 1997). Rotational test can also be used to describe flow behaviour of yoghurt. In most cases, the viscosity trend against shear rate follows the modified Power Law model, commonly known as Ostwald – de Waele model (Rao, 1999):

$$\eta_{app} = K\dot{\gamma}^{(n-1)}$$

This model depicts a shear thinning or shear thickening behaviour, and can cover a wide range of shear rates. However, it cannot demonstrate a Newtonian flow commonly encountered at very low shear rate. Hence, two power law indices are derived, those are K and n. K is consistency index and denotes viscosity at shear rate 1/s (Rao, 1999). Whilst, exponent n is flow behaviour index and indicates the deviation of a material from the Newtonian flow (n = 1). Shear thinning is indicated by $n < 1$, while shear thickening is described by $n > 1$.

Similarly, the two indices can also derived from the Power Law model for shear stress plotted against shear rate (Rao, 1999):

$$\tau = K\dot{\gamma}^{n}$$

Where τ is shear stress (Pa s), $\dot{\gamma}$ is shear rate (1/s), K is consistency index (Pa s^n), and n is the flow behaviour index (dimensionless). The values of both indices in the EPS-containing yoghurt are influenced by the shear rate (Hess et al., 1997). At low shear rates, very low values are observed indicating intensive shear thinning. At higher shear rate, the values of both indices are greater in EPS-containing yoghurt compared to those of non-EPS yoghurt. Therefore, EPS in yoghurt seemingly supported the shear resistance.

As the Power Law model did not always fit well to the data obtained by the dynamic measurement (Hassan et al., 2003b), several other rheological models

have been introduced to describe the flow behaviour of the yoghurt gel. By assuming the presence of yield in yoghurt, the Herschel-Bulkley model was employed and gave a good fit, while other yield-consisting models such as Casson and QRS model did not (Hassan et al., 2003b). However, although Herschel-Bulkley model effectively describe the yield of yoghurt, the overall data was poorly fitted into the model (Yu and Gunasekaran, 2001). The Herschel-Bulkley model is expressed as:

$$\sigma - \sigma_0 = \kappa(\dot{\gamma})^n$$

Where σ is shear stress (Pa s), and σ_0 is yield stress (Pa s), $\dot{\gamma}$ is shear rate (1/s), K_H is consistency index (Pa s^n), and n_H is flow behaviour index. It can be seen that the difference of this model from the Power Law model is in the incorporation of the yield expression. The yield measure in yoghurt can be absent, low or high, depending on the preparation method such as type of culture (Hassan et al., 2003b) or heat treatment (Lucey et al., 1997a). In the EPS-containing yoghurt, the yield is lower, and may be a sign of poor interaction between the EPS and protein components (Hassan et al., 2003b).

The Casson model is expressed as:

$$\sigma^{0.5} = K_{0C} + K_c\dot{\gamma}^{0.5}$$

where σ is shear stress (Pa s), K_{0c} and K_c are intercept and slope, respectively; and $\dot{\gamma}$ is shear rate (1/s). The model exhibits a yield and non-Newtonian flow at shear stress above yield. The Casson model is used to analyse food dispersions, and often applied for chocolate products. The limiting viscosity, a viscosity at high shear rates, can be calculated using plastic viscosity, and follows the equation:

$$\eta_\infty = \eta_{Ca} = (K_c)^2$$

In other work, the QRS was better than other models in describing flow behaviour of stirred yoghurt varying in dry matter content (Skriver et al., 1993). This discrepancy may originate from the variation of preparation or processing method, as well as rheological and analytical methods.

The area between upward and down ward curves of shear stress versus shear rate is called thixotropy area. The value denotes structural recovery after shearing (Rao, 1999). Larger values correlate with slow structural recovery after disruption due to shearing. It may also indicate more elastic gel. On the other hand, low value denotes quick recovery after shear-induced deformation. Thixotropy area is also correlated to ropiness of yoghurt, although only in a cautious and limited application (Folkenberg et al., 2006a), by the following equation:

$$\text{Thixotropy area} = a \cdot \text{Ropiness}^{b}$$

The constants, *a* and *b*, have values of 36.6 and 0.27, respectively.

In most cases, the rheological behaviour of set-type yoghurt was carried out by directly placing the sample on the measuring device with a care to maintain the integrity of the acid created gel. However, the homogeneity of the sample is one of the important factors to be considered especially in the case of non-homogenous growth of an EPS producing culture. Therefore, it appears relevant to have the sample pre-sheared and thoroughly mixed before loading it into the measuring device. Pre-shearing at high shear-rate was commonly applied for material possessing thixotropy, in order to eliminate any residual stress, which may cause less reproducible results (Da Cruz et al., 2002). Similarly, the stirred yoghurt was first stirred several times before the measurements were made (Folkenberg et al., 2005).

The onset of gelation is often studied using rheological methods. There are several definitions on the determination of the start of gelation, apparently depending on the nature of materials being tested. It has been described as a sharp increase in G' (Haque et al., 2001), the appearance of maximum tan δ before sudden decline (Lee and Lucey, 2004a), increase of storage modulus greater than 1 Pa (Lee and Lucey, 2004a), or the cross-over between G' and G" (Tung and Dynes, 1982), tan δ independent of frequency (Winter and Chambon, 1986), the appearance of pronounced complex modulus G* above the instrumental noise (Horne, 1998) or when G' and G" shared the Power Law exponent (Winter, 1987). A very low strain (<0.01%) (Hassan et al., 2003b) or 0.5% (Jaros et al., 2002b) and low frequency (0.1 Hz) (Hassan et al., 2003b) is employed in an

oscillatory measurement applied to measure *in situ* gelation. The low oscillation is maintained to avoid destruction of the weak structure at the beginning of development. The development of dynamic moduli over time is then studied. Typically, the storage modulus or complex modulus (G*) exhibits a slow increase at the beginning of fermentation. An abrupt increase in the moduli starts to take place when pH drops to around 5.5 depending on the type of mixture (Lucey et al., 2001) or cultures (Hassan et al., 2002), and continus until pH 4.5 (Hassan et al., 2002). The gelation starts by solubilization of the CCP, which is indicated by a sudden increase in tan δ to reflect loosening of original casein-casein structure in milk (Lucey et al., 1999). The structure weakening possibly originates from a reduction in electrostatic repulsions among micelles (Lucey, 2002). Consequently, it is followed by association of casein particles to form a firm structure, and is reflected in the increase of G' (Lucey, 2002). The casein aggregation may be driven by several forces such as van der Waals, hydrophobic and κ-casein bridging. Some treatments can affect the start of gelation so that the gelation pH is higher, such as pre-heating of milk (Lucey et al., 1997a) or the presence of EPS (Hassan et al., 2002), as well as higher temperature during acidification (Lucey et al., 1997c). The mechanism of gelation itself, as observed by the increase in G' in time, is not affected by protein concentration of yoghurt mixtures (Anema, 2008).

2.9 Enrichment of yoghurt mix

2.9.1 Calcium supplementation

One of the important reasons for calcium supplementation is that calcium daily intake does not meet the recommended level (Cook and Friday, 2003). Calcium is the major mineral (~60 %) within the bone, thus contributes to bone density, bone size, mass and architecture as well as prevention of bone fracture and osteoporosis (Kessenich, 2007). Milk and dairy products including yoghurt and cheese are considered to be the main source for dietary calcium. The

frequency of milk consumption is positively associated with bone density (Kalkwarf, 2007). The main target group for calcium fortification are children and the elderly. Calcium requirement in the diet during childhood and early adulthood is very high as a consequence of the rapid growth with peak bone mass formation in this period. A failure to meet this requirement can lead to childhood fracture epidemy (Kalkwarf, 2007, Matkovic et al., 2007). Milk consumption and consequently the calcium intake during childhood is important, since it is the consumption during this period that determines the total bone mass and resistance against adolescent rib and osteoporotic fractures (Kalkwarf, 2007). On the other hand, calcium intake during adulthood exhibits a poor correlation with the prevention of osteoporotic fractures. The recommended milk consumption for children age 2-8 years is 2 cups (800 mg) of milk or milk equivalent per day, while for 9 year and older is 3 cups (1300 mg) per day (Kalkwarf, 2007). Calcium supplementation during pre-puberty is more effective than post-puberty to maintain bone mass and density after cessation of supplementation. The elderly are a group with high risk of osteoporosis. There are ~44 million people in the US age of 50 years suffering from this disease (Kerstetter et al., 2007). Therefore, calcium intake for this group is given a great attention, and calcium supplementation is important part of the osteoporosis therapy (Kessenich, 2007). The recommended calcium intake for 50-70 year old women is 1200 to 1500 mg per day with no more than 500 mg Ca consumed at one time. Moreover, high calcium intake also reduces the systolic blood pressure (Sugiyama et al., 2007).

Milk is considered to be a good vehicle for calcium fortification, for its phosphopeptide content enabling better calcium absorption likely due to reduced rate of gastric emptying (Kitts and Kwong, 2004). Even more, calcium bioavailability in yoghurt is higher than in milk, likely due to ionisation of calcium in acidic pH of yoghurt and subsequently improved intestinal absorption (Unal et al., 2005). Another reason to make yoghurt an important candidate for calcium and other minerals fortification is its rocketing popularity, and good acceptance to women, children, teenagers and elderly, especially in the US (Achanta et al., 2007). The consumption per capita in 2003 was 8.2 lbs (Dairy-Facts, 2004). Calcium fortification in fruit yoghurt up to 100 mg Ca per 100 mL

yoghurt increases the yoghurt firmness and has no adverse effect on sensory properties including flavour, colour, body, texture, appearance and overall acceptability (Achanta et al., 2007). Calcium bioavailability is also associated with a high protein intake (Kerstetter et al., 2007). Large population data set showed that women with high protein intake had higher bone mass density. Proteins themselves contribute to 50% of the bone volume. High protein intake (2.1 g/kg) increases intestinal calcium absorption, bone mass density, and reduces kinetic measures of the bone turn-over. Bone calcium loss is lower in people with high protein-containing diet than those with low protein diet. There are several proposed mechanisms on the role of protein in the improvement of calcium bioavailability. First, amino acids in proteins may stimulate secretion of gastric juice via the allosteric activation of the gastric parietal cell calcium-sensing receptor. Another possible mechanism is protein activated insulin-like growth factor 1 (IGF-1) which controls the bone growth (Bonjour et al., 1997). A synergy between calcium and protein has been observed, which is involved in the bone and muscle mass protection. Proteins in sufficient amount are required to attain and maintain muscle mass and strength, and this effect only appeares under high calcium intake (Heaney, 2007). In this regard, calcium fortification in yoghurt may need to be combined with protein fortification such as with whey protein. Despite the well-known potential benefit of calcium supplementation milk products, seemingly there is only limited number of studies in this area focusing on the effects of calcium on the yoghurt texture. Commercial calcium preparation for food supplementation includes calcium carbonate, calcium chloride, calcium phosphate, tribasic calcium phosphate, calcium citrate malate, calcium lactate, calcium gluconate, calcium lactate gluconate and natural milk calcium (Goldscher and Edelstein, 1996). When supplemented in the form of Ca-lactate to fruit yoghurt, the water holding capacity was improved, but the thinning behaviour was reduced, while flow behaviour was higher compared to the control (Singh and Muthukumarappan, 2008). The yoghurt gel strengthening effect of calcium may be due to dissolved calcium facilitating cross-linking between protein molecules. The increase in the degree of cross-linking was not accompanied by changes in

bonding, as shown by insignificant increase in apparent viscosity nor loss tangent (Singh and Muthukumarappan, 2008).

2.9.2 Whey protein supplementation

Whey proteins, a by-product of cheese manufacturing, have been extensively used to improve functional, nutritional, therapeutic, and physiological properties of various food products. It consists of mostly (~50%) β-lactoglobulin, and to a lesser extent (~20%), α-lactalbumin (Chatterton et al., 2006). β-Lactoglobulin is a soluble globular protein with a low molecular weight (~18 kDa). At pH 3-7, it forms a dimer with molecular weight of 36 kDa. α-Lactalbumin is also a globular protein soluble in water, and exhibits a pronounced affinity to metals, especially calcium. The released of some portion of calcium from α-lactalbumin may lead to denaturation, commonly called as molten globule state (Chatterton et al., 2006). Calcium in α-lactalbumin exerts its heat stability with melting temperature around 68 °C, while on the depletion of calcium it is reduced to 43 °C.

Whey proteins are related to the biosynthesis of skeletal muscle, especially during excessive physical activity (Ha and Zemel, 2003) and in elderly (Paddon-Jones et al., 2006). The protein anabolism function of whey proteins may relate to their essential amino acids, especially leucine (Ha and Zemel, 2003). Leucine is a substrate for muscle protein biosynthesis and may take part in signalling among molecules during initiation of the anabolism. The muscle building effect of whey proteins also correlates to a rapid absorption of their amino acids in the gut system. There is an apparent synergy between whey proteins and calcium in the muscle development, with calcium supplementation increasing the optimisation of the skeletal muscle composition. Furthermore, calcium inhibits synthesis of adipose tissue through suppression of parathyroid hormone responsible for fat storage. Whey protein consumption reduces food intake during hypercholesterolemic diet (Jacobucci et al., 2001, Minehira et al., 2000). This effect may originate from the reduction in appetite due to accumulation of amino

acid in the blood or rapid enhancement of protein anabolism and catabolism (Jacobucci et al., 2001). Therefore, the combination of whey proteins and calcium appears to be supportive of the good lean muscle development. In the elderly, whey proteins prevent a progressive loss of muscle mass and functional capacity (Paddon-Jones et al., 2006).

Whey protein supplementation results in a strong hypocholesterolemic effect, and thus may reduce the risk of cardiovascular diseases (Jacobucci et al., 2001, Minehira et al., 2000). The rise in blood and liver high-density lipoprotein is inhibited during a high cholesterol diet. However, the oxidised cholesterol in the blood may impair immune response (Minehira et al., 2000). Whey proteins appear to support the gastrointestinal health by providing lactose for the growth of probiotics (Ha and Zemel, 2003) and β-lactoglobulin prevents the adhesion of pathogens in the gut (Chatterton et al., 2006). Antimicrobial activities of whey proteins have been noted against several pathogens (Gill et al., 2006, Madureira et al., 2007, Yalcin, 2006) including cariogenic species (Warner et al., 2001). Upon hydrolysis, these proteins release a wide range of peptides with manifested bioactivities including angiotension-converting enzyme inhibitory activity (Chatterton et al., 2006). Therefore, the inclusion of these highly valuable dairy ingredients into various food products would certainly improve healthy perception of foods. Moreover, it also gave environmental and economical benefits.

The important functional characteristics of whey proteins are their gelling properties which affect physical quality of various food systems including yoghurt. Heat-induced gelation of whey proteins at least involves two steps. These are unfolding of protein three dimensional structure to expose reactive functional thiol groups, and rearrangement of the unfolding state to form aggregates (van Mil and Roefs, 1993). The unfolding renders whey proteins to become amphiphilic with higher water holding capacity (Morr, 1979). Several factors induce the unfolding including temperature and heating rate, pH, lactose content, β-lactoglobulin content and the presence of other proteins in whey. Following unfolding, the aggregation to form fibril-like structure occurs, with the aggregation rate influenced by the concentration of whey proteins, total solids, or β-lactoglobulin. At high concentration of added whey proteins, denaturation as

indicated by significant increase in elastic modulus (G') starts earlier, and may show higher maximum values of G' (Kavanagh et al., 2000). At neutral pH, gelation of whey proteins is correlated to denaturation of β-lactoglobulin, while denaturation of α-lactalbumin only gives increase in viscosity without apparent gelation (Boye et al., 1995). When pH of the system is far from pI, there is a possible additional step after aggregation, called 'random cross-linking' of aggregated fibrils (Clark et al., 2001). Thus, at the gelation point, the aggregation is not considered complet and would continue to eventually reach a plateau. This plateau could be indicated by G'_{inf}, a 'limiting modulus'. For charged proteins, it may take long time to reach this plateau. The unfolding and aggregation may be irreversible or reversible, depending on the temperature of denaturation. Thermal denaturation at neutral pH and 60 °C (Pelegrine and Gasparetto, 2005) resulted in reversible altered tertiary conformation of whey proteins (Iametti et al., 1996). Irreversible denaturation is induced by heating at around 75-80 °C (Clark et al., 2001), and is more extensive at higher temperatures and low heating rates (De Wit and Klarenbeek, 1984). Higher temperatures enable more extensive protein-protein interactions via hydrophobic non-covalent bonds (Lorenzen and Schrader, 2006). Similarly, the strongest WPI gel is structured at pH 5-6 when large aggregates are formed by non-covalent bonds in addition to disulfide bridges (Lorenzen and Schrader, 2006). In the presence of caseins, the aggregation of whey proteins is affected by casein:whey protein ratio (Beaulieu et al., 1999, Puvanenthiran et al., 2002), where high whey protein proportion increases the denaturation rate (Law and Leaver, 1999) and causes formation of large particles consisting of both micelles covered with individual whey proteins as well as whey protein aggregates (Beaulieu et al., 1999).

The supplementation of whey proteins to yoghurt has been studied extensively and the results showed various effects on the final yoghurt texture, apparently influenced by the preparation methods. Native whey protein supplementation reduced yoghurt gel strength parameters such as storage modulus, and yield stress (Guggisberg et al., 2007), viscosity and firmness (Guggisberg et al., 2007, Oliveira et al., 2001), and created porous structure leading to higher syneresis (Gonzalez-Martinez et al., 2002). However, when

native whey proteins were added at high concentration after fermentation of yoghurt, storage modulus was increased (Patocka et al., 2004). When yoghurt milk base supplemented with whey proteins was heated before acidification, the gel strength was significantly increased by heating time, apparently due to larger micelle size which may have resulted from more extensive cross-linking of whey proteins and caseins (Remeuf et al., 2003). Heating milk mixture containing added whey proteins positively affected gel strength, likely due to denaturation of whey proteins. The positive effect of denatured whey proteins on the gel strength of glucono-δ-lactone (Vasbinder et al., 2003) as well as microbial (Puvanenthiran et al., 2002) acidified yoghurt was emphasized. Interestingly, when whey proteins heated at high temperature were added to milk base after homogenization, the gel strength was impaired, which was opposite to unheated counterpart (Sodini et al., 2006). This may reflect the complex nature of the yoghurt structural development, which possibly involves several factors and their interactions.

2.9.3 Addition of sucrose to yoghurt

Sucrose is commonly added during yoghurt making to improve flavour. Sweetness is one of major sensory determinants that positively affects acceptability of yoghurt (Jaworska et al., 2005). Clearly, yoghurt with optimum level of sweetness was preferred than those oversweetened or undersweetened (Vickers et al., 2001). However, oversweetened yoghurt was accepted better than the undersweetened yoghurt. The preferred degree of sweetness in yoghurt is affected by eating situation. In multi-food lunch, for example, over-sweetened yoghurt is disliked. Sucrose content in yoghurt affects palatability (Perez et al., 1994). The favoured sucrose content in yoghurt is between 5 to 10 %, and its consumption increases food intake. Non-sucrose sweeteners also increase appetite (Bellisle and Perez, 1994), with varying effect among sweeteners (Rogers and Blundell, 1989). The addition of sucrose is also very likely to affect the final texture of yoghurt. There is a lack of information on the effects of sucrose addition on the yoghurt texture. Few conducted studies showed that sucrose

reduces gel elasticity of the GDL-acidified sodium caseinate (Braga and Cunha, 2004). Sucrose also potentially weakened the polysaccharide junction zones and altered flow behaviour of liquid food systems. It may increase or decrease viscosity depending on hydration of hydrocolloid molecules, rheological properties of the hydrocolloid and also the influence of other components in the system (Yanes et al., 2002).

2.10 Milk composition and processing factors

Milk composition has an important influence on yoghurt texture, as shown by seasonal variation in yoghurt gel properties (Tamime and Robinson, 1985). Total solid content in milk suspension positively affected the firmness of yoghurt coagulum in linear way, as shown by the increase in storage modulus and lower loss tangent (Kristo et al., 2003). Acidified milk containing higher levels of total solid started to gel earlier, and exhibited greater maximum G' than that with lower level of total solids. Higher concentration of total solids also prolonged the time to reach end of fermentation, probably due to buffering effect of milk solid. Since yoghurt cultures obtained more nutrition from milk containing higher concentration of total solid, the cell growth and acid production is improved. The fat content in milk also showed similar tendency, where full fat yoghurt (3.5 g/100 g fat) exhibited firmer structure than low fat yoghurt (Xu et al., 2008). Consequently, yoghurt made from skim milk may potentially face the problem of weak texture.

Texture of yoghurt is also affected by several processing factors during, before and after fermentation. Pre-heating (95 °C, 5 min) and homogenization are common pre-treatments to milk (Tamime and Robinson, 1985). During pre-heating, denaturation of whey protein takes place, which starts the interactions among β-lactoglobulin, κ-casein, and α-lactalbumin. As a result, yoghurt gel becomes stronger and more stable. Higher temperature potentially escalates the effect. A 5 °C difference in pre-heating temperature between 80-95 °C for 1 min increased storage modulus of resulted fermented milk coagulum (Xu et al., 2008). At the same time, prolonged heating such as 30 min at 95 °C seemingly resulted

in severely denatured whey proteins, and caused substanstial increase in firmness (Knudsen et al., 2006). Pressure treatment applied to milk such as homogenisation or pressurization may also induce particle interaction and give comparable effects to extensive heat-treatment (Knudsen et al., 2006). During yoghurt fermentation, temperature is usually set between 37-45 °C (Tamime and Robinson, 1985). The effect of temperature on yoghurt texture varies. Kristo et al. (2003) reported that higher temperature within the range increased gelation rate, significantly shortened the onset of gelation, and resulted in higher storage modulus, more poreous structure and higher level of syneresis. Higher fermentation temperature supported the growth of yoghurt cultures, thus reduced fermentation time and also affected polysaccharides production. In contrast, Beal et al. (1999) showed that lower fermentation temperature resulted in more viscous gel.

The type and concentration of yoghurt inoculum influence gelation and final texture of yoghurt, by altering the start and onset of gelation (Jumah et al., 2001). The sudden increase in viscosity, indicating the start of gelation, is delayed at low concentration of yoghurt starter inoculum. The onset of gelation and shear-thinning properties of yoghurt gel depend on the type of cultures. The firmness of yoghurt is positively related to slow gelation. In addition, it is also influenced by the type of culture which may in conjunction with the characteristics of polysaccharides produced during fermentation.

3 Production and Rheological Characterization of Exopolysaccharides Produced by Strains of *Streptococcus thermophilus*

3.1 Introduction

In this experiment, the production of exopolysaccharides (EPS) of two strains of *Streptoccous thermophilus* was studied, and the rheological properties of the EPS-suspension were also examined. EPS is one type of hydrocolloids which possess the potential as emulsifier or stabilizer to affect food texture. The study on factors affecting the production of the EPS is thus useful in optimization of their production. The rheological characterisation of EPS is not only important for the application of the EPS as food additives, but also to study its interaction with milk components in fermented milk which may then determine the final textural characteristics of product. Results of such study can be utilised to control the processing environment during the making of fermented milk.

Considerable attention has been given to bacterial exopolysaccharides (EPS) in foods arising their ability to provide potential health benefits to consumers (De Vuyst and Degeest, 1999, Looijesteijn et al., 2001) and their application as thickening agents in processed products (Cerning, 1990). Although certain bacterial species can produce considerable amounts of EPS, yoghurts prepared with strains *Streptococcus thermophilus* result in less than 0.1% EPS in the final product (Cerning, 1990). Nevertheless, EPS play an important role in the development of yoghurt texture, with the type of EPS exerting a greater effect than their concentration (Vaningelgem et al., 2004b). In general, two strain-dependent types of EPS (Mozzi et al., 2006) have been frequently assessed for

their effects on yoghurt texture, namely 'ropy' and 'capsular'. Certain bacterial strains may even produce a mix of these two types in various proportions (Zisu and Shah, 2003, 2005b). Furthermore, combinations of the two types of EPS producing cultures improved not only the total EPS production but also yoghurt texture (Marshall and Rawson, 1999).

Numerous studies have been conducted in order to enhance our understanding of the factors governing EPS production and the mechanisms by which they affect the yoghurt texture. Most of the EPS produced during yoghurt fermentation are heteropolysaccharides, and their production may be unpredictable due to a plasmid-related instability (Boels et al., 2001). EPS production, thickening properties, molecular mass and structural conformation are greatly affected by environmental factors (Ruas-Madiedo et al., 2005a). In some species, these affected only EPS yield, because their monosaccharide composition remained unchanged (Looijesteijn and Hugenholtz, 1999). Strain selection, temperature, pH and growth stage (Aslim et al., 2005), nitrogen source (Degeest et al., 2002), and metabolizable sugars (Mozzi et al., 2003) are among the factors influencing EPS production. EPS production by some *S. thermophilus* strains was coupled to growth (de Vin et al., 2005). However, in some mesophilic bacterial strains, EPS were mainly produced at sub-optimal growth conditions (Cerning, 1990). Depending on the strain and growth conditions, maximum yield of EPS may be achieved in the exponential (Duenas et al., 2003) or stationary growth phase (Gancel and Novel, 1994). At the end of growth phase, there are some indications that EPS undergoes undesirable enzymatic degradation (Degeest et al., 2002, Pham et al., 2000). The type of carbon source apparently governs the total amount of EPS produced with a clear strain-dependence (Mozzi et al., 2001). For example, *S. thermophilus* LY03 produced more EPS with lactose than with glucose (Degeest and de Vuyst, 2000a). Nitrogen compounds in the growth medium are necessary during formation of sugar nucleotides, essential precursors for EPS assembly. However, the type of nitrogen source appears to be less important in EPS production than is the carbon source (Duenas et al., 2003).

Although the type of EPS has been reported to influence yoghurt texture, and some mechanisms on their interactions with milk proteins has been proposed,

there is a lack of information on the rheological properties of EPS produced by yoghurt cultures. Such information can be useful for predicting the possible interaction of EPS with milk component(s) to influence the texture of yoghurt. More importantly these complex carbohydrates could also be applied in other food systems as thickeners. Therefore, this study aimed to examine EPS production by two strains of *S. thermophilus* which produced capsular-ropy and capsular EPS. We report on the effect of fermentation conditions including temperature, time, and carbon source; and on the rheological properties of extracted EPS.

3.2 Materials and methods

3.2.1 Bacterial cultures

The two strains of *S. thermophilus* examined in the study were kindly provided by Australian Starter Culture Research Centre (Werribee, Australia). *S. thermophilus* ST 1275 produces mainly ropy with a smaller portion of capsular EPS, while ST 285 produces only capsular EPS (Zisu & Shah, 2003, 2005). The frozen (-80 °C) cultures in 300 mL/L glycerol were activated by growing them twice in M17 medium (Amyl Media, Merck Pty Ltd., Kilsyth, Victoria, Australia) at 37 °C for at least 24 hours.

3.2.2 Cell growth and EPS production and isolation

The medium used in these experiments was basal M17 medium supplemented with galactose, glucose or lactose at 20 g/L. The cultures were inoculated into 30 mL of the sterile medium at 1 mL/100 mL in 50 mL Falcon tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA) and incubated at 30, 37 or 42 °C for 24 hours aerobically, during which samples were periodically collected for analysis of all growth parameters and determination of EPS concentration.

In this experiment, M17 was chosen as the medium rather than milk due to its more defined and simpler composition. Presence of very complex compounds in milk such as proteins may lead to difficulty in isolation and purification of EPS from milk. Although EPS produced by the same strain in milk may be different from those produced in M17 media, previous studies (Degeest and de Vuys, 2000a; Mozzi et al. 2006) mentioned that the composition of EPS did not alter significantly by external factors during growth.

Determination of viable cell counts was carried out by serial dilutions in sterile 1 g/L peptone water, and incubation at the corresponding temperature for 24 hours to obtain sufficient cell growth. Cell concentration was additionally determined by optical density at 650 nm. EPS assessed in the rheological studies were extracted from the culture grown in M17 medium by precipitation with ethanol (Van Geel-Schutten et al., 1998). Initially, fermentation was stopped by adding 25 mL/L of trichloroacetic acid (80 g/100 g) into the suspension, and keeping the container in a cold room (4 °C) overnight. This was followed by protein removal by centrifugation at 11000 × *g* and 4 °C for 10 min (Model J2-HS, Beckman, Fullerton, California, USA). The supernatant was collected and mixed with two volumes of ethanol. The mixture was allowed to stand for twelve hours for complete precipitation of EPS. This procedure was repeated twice, after which the EPS was freeze-dried (Dynavac freeze drier, Dynavac Eng. Pty. Ltd., Melbourne, Australia). The concentration of EPS was assessed using the phenol–sulphuric acid method (Dubois et al., 1956). Lactic acid concentration was determined by an HPLC method (Donkor et al., 2005). The method uses dilute 0.01 mol/L H_2SO_4 as a mobile phase with flow rate of 0.6 mL/min, and 300 × 7.8 mm ion-exchange Aminex HPX-87H column (Bio-Rad Laboratories, Australia) coupled to a UV detector (Varian Analytical Instruments, Walnut Creek, CA, USA) at 220 nm. The column temperature was maintained at 65 °C.

The determination of the viable cell counts was carried out by serial dilutions in sterile 0.1 g/100 g peptone water, and incubation at the corresponding temperature for at least 48 hours to obtain sufficient cell growth. The cell concentration was additionally determined by optical density at 650 nm. The EPS quantification was carried out according to a method described previously (Gancel

and Novel, 1994) involving protein digestion using pronase (Sigma Chemical Co.) and dialysis. It was then followed by phenol-sulphuric acid method to quantify glucose (Dubois et al., 1956) with slight modification. In brief, an aliquot sample was heated for 10 min, cooled down and mixed with trichloroacetic acid, as before, for the cell inactivation and protein removal. The EPS was collected as reported previously and further degraded into their sugar units by acid hydrolysis combined with heating. The released reducing sugars were then determined by spectrophotometric/colorimetric method incorporating phenol and concentrated sulphuric acid. The lactic acid concentration was determined following an established HPLC method (Donkor et al., 2005). The method used dilute 0.01 M H_2SO_4 as a mobile phase with flow rate of 0.6 mL/min, and 300 × 7.8 mm ion-exchange Aminex HPX-87H column (Bio-Rad Laboratories, Australia) coupled to Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA, USA). The column temperature was maintained at 65 °C.

3.2.3 Rheological characterization of EPS

Prior to analysis, the freeze-dried crude EPS was dissolved in citric acid-phosphate buffer at pH 3 and also at 6.5 achieving concentrations of 0.1, 0.25, 0.5, 0.75 or 1 g/100 g. Samples were kept in cold (4 °C) storage for 12 hours to allow hydration. Upon full EPS solubilisation, each sample was mixed using Vortex mixer. As much as ~3 mL sample was placed in a cone-plate geometry, using pipette. The gap between two stainless steel geometries was set at 0.3 mm. Any excess of sample was carefully removed using pipette. The sample was then subjected to a controlled shear rate ramp at different temperatures from 0.01 to 100/s in a cone and plate geometry (50 mm diameter, 2°) of a rheometer (Physica MCR 301, Anton Paar, GmbH, Germany). Temperature regulation was achieved with a Viscotherm VT 2 circulating bath and controlled with a Peltier system (Anton Paar) to an accuracy of ± 0.1 °C. The data were analyzed with proprietary software (Rheoplus/32 v2.81, Anton Paar). The flow curves were fitted to rheological models; Ostwald (for overall shear rate curve 0-100/s), and Cross (for

non-Newtonian flow at a shear rate below 10/s). These models are presented by the following equations:

Ostwald model: $$\eta = K\dot{\gamma}^{(n-1)} \quad (1)$$

Cross model: $$\eta = \frac{\eta_0}{1+(\tau\dot{\gamma})^m} \quad (2)$$

with K, n, η, $\dot{\gamma}$ as consistency index (mPa sn), flow behaviour index (dimensionless), apparent viscosity (mPa s) and shear rate (1/s), respectively; η_o presents the zero shear rate viscosity (mPa s), τ relaxation time (s), and m is the limiting slope (dimensionless). The effect of concentration on viscosity was studied by applying the following equation (Speers and Tung, 1986):

$$\eta = a \cdot C^b \quad (3)$$

where a and b are parameters derived from the intercept (mPas g/100g^{-b}) and the slope of a logarithmic plot (dimensionless), respectively. C presents EPS concentration (g/100 g). The influence of temperature on apparent viscosity was also assessed using the Arrhenius model (Macosko, 1994, Speers and Tung, 1986):

$$\eta = A \cdot e^{\frac{\Delta E}{RT}} \quad (4)$$

where η is the apparent viscosity (mPa s) at shear rate of 100/s, A the frequency factor (mPa s), ΔE the activation energy (J/mol), R the gas constant (8.314 J/mol K), and T is the absolute temperature (K).

3.2.4 Statistical analysis

A randomized split plot in time block design was applied to the design of the fermentation experiments. This was set up with four main effects: strain (two

levels), sugar types (three levels), temperatures (three levels), and fermentation time (six levels: 0, 3, 6, 9, 12, 24 hours). This design was replicated twice with at least two sub samplings. Results were analyzed using a General Linear Model procedure (SAS, 1996). The level of significance was set at P=0.05. The best fit correlational analysis for rheological data was carried out using the Rheoplus software (v2.81, Anton Paar).

3.3 Results and discussion

3.3.1 Microbiological and chemical analysis

Viable cell counts were significantly (P<0.5) affected by all the factors: strain, temperature, type of sugar, the fermentation time and their interactions. The capsular strain consistently showed slower growth compared to capsular-ropy culture, regardless the growth conditions. The maximum cell number of the capsular strain was 2.2×10^8 cells/mL achieved in glucose-M17 medium at 42 °C. In contrast, the capsular-ropy culture grew to 1.6×10^{10} cells/mL in lactose-M17 medium at 37 °C (Figure 3.1A, B). Higher temperatures (37 and 42 °C) resulted in greater cell growth than that at 30 °C for both strains and all types of sugar. M17 medium containing glucose or lactose was more growth supportive than that with galactose for both strains and in all temperatures.

The positive effect of higher fermentation temperature, glucose or lactose on the cell growth was also confirmed by other growth parameters such as optical density (OD), pH and lactic acid production (Table 3.1). Statistically, OD readings were only significantly (P<0.05) influenced by the type of sugar and its interactions with other factors. Fermentation time and the type of strain individually had no effect on cell growth as measured by OD, but their interactions did (P<0.05). Sugar type and its interaction with other factors significantly (P<0.05) affected the final pH of the medium. Lactic acid concentration was similarly influenced by the factors examined. The types of sugar substantially (P<0.05) influenced lactate concentration, where glucose produced most lactic acid (~0.80-0.87 $\pm$ 0.02 mg/mL), whereas galactose

produced the lowest levels ~0.55-0.62 ± 0.02 mg/mL. In most cases, from the 8th hour onwards, lactate concentration was either increased or remained unchanged.

The EPS production was only significantly ($P<0.05$) affected by the type of sugar and the fermentation time, and their interactions with other factors. The amount of EPS produced by the two strains was not significantly ($P>0.05$) different. Maximum EPS production by capsular-ropy strain was 441 $\pm$ 36.2 mg/L, produced in galactose-M17 at 42 °C after 12 hours. Similarly, the capsular strain produced 563 $\pm$ 36.2 mg/L, in glucose at 30 °C after 12 hours (Figure 3.2A,B). EPS production of capsular strain at all temperatures was relatively high and the difference among them was negligible. Although not statistically significant ($P\geq0.05$), higher temperature tended to enhance EPS production of capsular-ropy strain in all types of sugar (Figure 3.2A,B). On the other hand, the capsular strain appeared to prefer lower temperature (30 °C) and produced more EPS when grown on glucose or lactose (Figure 3.2A,B). M17 medium containing galactose poorly supported the cell growth, however, the EPS concentration in this medium was relatively high. In medium with lactose at all temperatures, and glucose or galactose at low temperature (30 °C), maximum EPS concentration was reached after 8 hours. After that time it was often slightly reduced towards the end of fermentation. The pattern of EPS production over time by the capsular strain was somewhat similar to that of the capsular-ropy counterpart, except that EPS concentration in galactose medium at low temperature (30 °C) remained stationary after 4 hours. Moreover, EPS concentration in lactose medium at low temperatures (30 and 37 °C) was small, and did not show any considerable change until the end of fermentation.

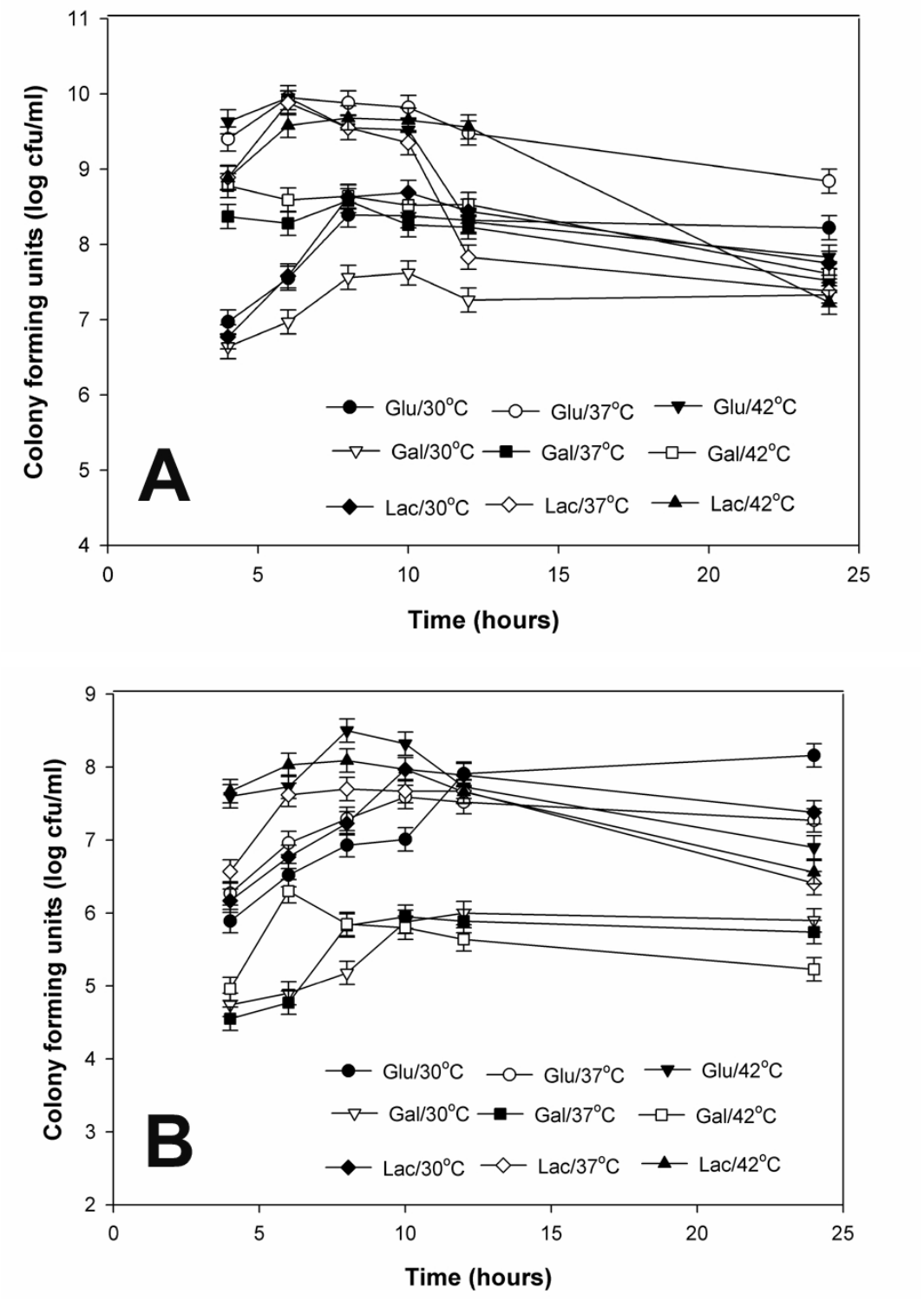


Figure 3.1 The viable cell counts of the capsular-ropy (A) and the capsular (B) strain of *Streptococcus thermophilus* cultivated in a M17 medium supplemented with glucose, galactose or lactose, at 30, 37 or 42 °C, for 24 hours.

Table 3.1 Cell growth parameters of capsular-ropy and capsular strain of *Streptococcus thermophilus* as affected by temperature and sugar source during growth phase

Strain	Temperature, (°C)	Time, (hour)	Types of sugar	pH*	Lactic acid concentration, (g/100 g lactate)*	OD*
Capsular-ropy	30	8	galactose	6.91^{cB}	0.53^{aA}	0.07^{cA}
			lactose	5.99^{aB}	0.67^{bA}	0.86^{bA}
			glucose	6.38^{bB}	0.87^{cA}	0.72^{bA}
		24	galactose	6.88^{cA}	0.54^{aA}	0.09^{aA}
			lactose	5.94^{bB}	0.65^{bB}	0.95^{bA}
			glucose	5.48^{aB}	0.84^{cA}	1.73^{cA}
	37	8	galactose	6.81^{cAB}	0.55^{aAB}	0.15^{aA}
			lactose	5.82^{bB}	0.68^{bA}	1.23^{bB}
			glucose	5.05^{aA}	0.87^{cA}	2.52^{cB}
		24	galactose	6.69^{cA}	0.68^{bC}	0.20^{aA}
			lactose	5.80^{bB}	0.57^{aA}	1.08^{bA}
			glucose	5.01^{aA}	0.88^{cA}	2.08^{cB}
	42	8	galactose	6.62^{bB}	0.58^{aB}	0.28^{aA}
			lactose	5.29^{aA}	0.77^{bB}	1.75^{bC}
			glucose	5.09^{aA}	0.88^{cA}	2.53^{cB}
		24	galactose	6.52^{bA}	0.60^{aB}	0.28^{aA}
			lactose	5.32^{aA}	0.74^{bC}	1.67^{bB}
			glucose	5.09^{aA}	0.88^{cA}	2.78^{cB}
Capsular	30	8	galactose	6.94^{cA}	0.55^{aA}	0.24^{cC}
			lactose	5.93^{bA}	0.65^{bA}	0.94^{aB}
			glucose	5.25^{aA}	0.84^{cB}	2.12^{bB}
		24	galactose	6.84^{cA}	0.56^{aA}	0.27^{cB}
			lactose	5.88^{bA}	0.66^{bA}	1.01^{aB}
			glucose	5.26^{aA}	0.83^{cB}	2.11^{cB}
	37	8	galactose	6.75^{cA}	0.54^{aA}	0.20^{aA}
			lactose	5.89^{bA}	0.64^{bA}	0.57^{bA}
			glucose	5.40^{aA}	0.84^{cB}	1.69^{cB}
		24	galactose	6.59^{cA}	0.57^{aA}	0.14^{aA}
			lactose	5.85^{bA}	0.66^{bA}	0.66^{bB}
			glucose	5.36^{aA}	0.84^{cB}	1.51^{cA}
	42	8	galactose	6.93^{cA}	0.59^{aB}	0.20^{aA}
			lactose	5.96^{bA}	0.66^{bA}	0.57^{bA}
			glucose	5.38^{aA}	0.76^{cA}	1.58^{cA}
		24	galactose	6.85^{cA}	0.57^{aA}	0.16^{aA}
			lactose	6.09^{bB}	0.62^{bA}	0.44^{bA}
			glucose	5.41^{aA}	0.77^{cA}	1.60^{cA}
SEM**				0.10	0.02	0.11

*different small letters in a coloumn denote significant (P>0.05) difference among means of the same culture, temperature and time but different sugar types. The different capital letters depict a difference (P>0.05) among means of the same culture, sugar type and time but different temperature.**SEM, standard error of means, with P=0.05.

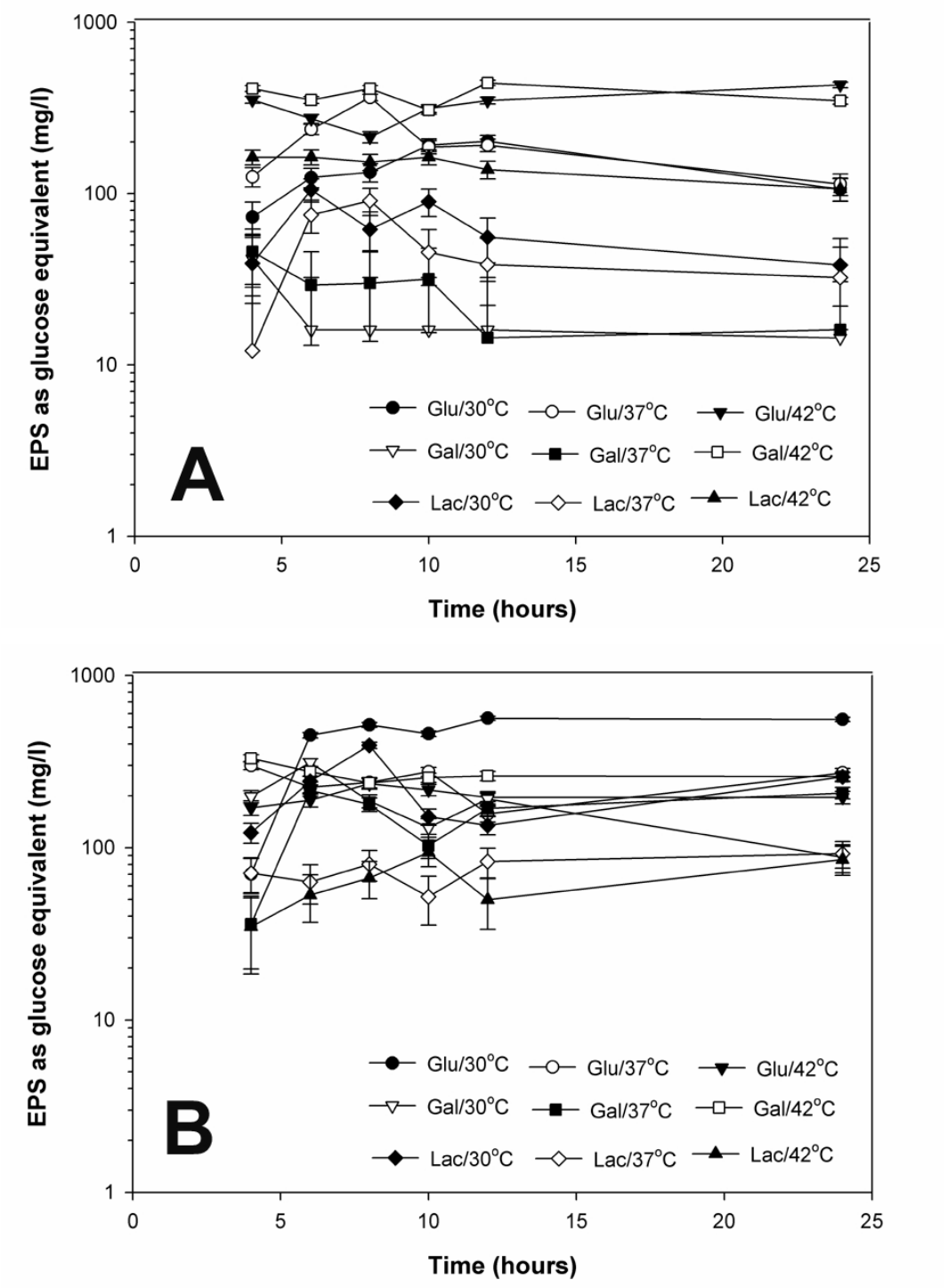


Figure 3.2 The exopolysaccharide production by the capsular-ropy (A) and the capsular (B) strain of *Streptococcus thermophilus* grown in a M17 medium supplemented with glucose, galactose or lactose, at 30, 37 or 42 °C, for 24 hours.

Our results were in agreement with previous work showing comparable cell numbers of *S. thermophilus* grown in lactose- and glucose-containing medium (Degeest and De Vuyst, 2000b). In contrast to these findings, an import system in *S. thermophilus* is lactose-dedicated (van den Bogaard et al., 2000), causing poor growth on glucose (Poolman, 2002b). The growth of *S. thermophilus* strain in the glucose based medium required the activity of phosphoglucomutase which may be repressed in the presence of lactose (Levander and Radstrom, 2001). Galactose did not support the growth of *S. thermophilus* cells (Deegest and de Vuyst, 2000) and its secretion was energetically-favoured (Levander and Radstrom, 2001). Most of the strains were able to utilize only part of excreted galactose moiety (de Vin et al., 2005).

Our experiment showed that both strains produced considerable amounts of EPS in M17 medium (Figure 3.2A, B), even higher than in a recent study (Aslim et al., 2006). Similar to our result, no difference in the EPS production of ropy and non-ropy strains was observed previously (Mozzi et al., 2006). Most of *S. thermophilus* strains own Leloir system for EPS synthesis (Mora et al., 2002). The three sugars examined in our study potentially supported the EPS production, as reported elsewhere (Chervaux et al., 2000), with galactose was frequently related to EPS production (Mozzi et al., 2001). Glucose or glucose moiety of lactose was used for EPS synthesis (Welman et al. 2006). However, when present as a sole carbon source in the medium, glucose poorly supported the EPS production compared to lactose (Deegest and de Vuyst, 2000). Notably in our study, EPS production was only significantly (P<0.05) affected by time. The growth-associated nature of the EPS production of *Streptococcus thermophilus* strains was reported previously, with the EPS was produced mainly during logarithmic phase (Ruas-Madiedo et al., 2005a).

Galactose was the predominant (Goh et al., 2005) and a consistent (Goh et al., 2005, Mozzi et al., 2006) primary unit in the EPS backbone of several *S. thermophilus* strains, although most of them did not utilize galactose (Mora et al., 2002) or metabolized galactose only at the end of growth phase (de Vin et al., 2005). However, several reports found discrepancies between theoretical values and actual galactose concentration in the medium, suggesting a flux to other

metabolites including lactic acid or even assimilation of galactose into EPS in the Gal$^-$ strains of *S. thermophilus* (de Vin et al., 2005). Galactose appeared to contribute to the EPS anabolism rather than the cell growth (Mozzi et al., 2001). The availability of galactose might be initiated by the activation of galactose symporter in the absence of lactose (de Vin et al., 2005) or low lactose/galactose ratio (Poolman, 2002b). Moreover, galactose catabolism was also possible by the activation of some enzymes of Leloir pathway such as galactokinase in lactose-depleted environment (de Vin et al., 2005).

The EPS concentration in the medium was reduced at the end of the growth phase. This could be related to shortage of the available ATP required for EPS polymerization (Welman et al., 2006). Moreover, the cell lysis (Mozzi et al., 2003) could lead to the enzymatic degradation of EPS (Pham et al. 2000) towards the late stages of the growth.

3.3.2 The flow behavior of the crude EPS solutions

The flow curves of the dispersions containing both EPS types at pH 6.5 were fitted to the Ostwald model with high correlation coefficient (between 0.8-0.9). At very low shear rates (below 10/s), a small overshoot as well as thixotropy was observed (figure not shown). In the Ostwald model, consistency index (K) denotes the shear resistance of material, with high value indicates greater resistance. While the other parameter, flow behaviour index n, designates deviation from the Newtonian flow (n = 1) (Rao, 1999). The values of both parameters were affected significantly (P<0.05) by strain, pH, and temperature (Table 3.2). The effect of interaction between strain and pH was only significant (P<0.05) for K value, whilst, the n value was affected significantly by the interaction between strain and temperature. K values were in general low (Table 3.3, 3.4), apparently a sign of a low shear-resistance (Speers and Tung, 1986). Capsular EPS showed higher K values than the capsular-ropy EPS, indicating more shear-resistant nature. Moreover, the flow behaviour indices (n) of the capsular-ropy EPS dispersions were close to that of Newtonian fluid. In contrast, n values of the capsular EPS dispersions were low, with a considerable deviation

Table 3.2 Adjusted mean squares from analysis of variance of the Ostwald parameters of capsular-ropy and capsular exopolysaccharide, at pH 3 and 6.5, different concentrations and temperatures

Source of variation	DF	*p*-value	
		K	n
Strain	1	<0.0001	<0.0001
Concentration	3	0.076	0.211
pH	1	<0.0001	<0.0001
Temperature	3	<0.0001	<0.0001
Strain × concentration	3	0.118	0.389
Strain × pH	1	<0.0001	<0.0001
Strain × temperature	3	0.009	<0.0001
Concentration × pH	3	0.294	0.018
Concentration × temperature	9	0.147	0.001
pH × temperature	3		<0.0001
Error	192		

Table 3.3 Concentration dependency of consistency (K) and flow behaviour (n) index of EPS dispersions at pH 3 and 4, 20, 37 or 42 °C

EPS (g/100 g)	Temperature (°C)	Strain					
		Capsular-ropy			Capsular		
		K (mPa s^n)	n	r^2	K (mPa s^n)	n	r^2
1.00	4	7.5	0.79	0.909	14.8	0.57	0.750
	20	5.3	0.80	0.981	7.9	0.63	0.773
	37	9.9	0.44	0.871	7.6	0.55	0.766
	42	7.7	0.63	0.949	8.1	0.62	0.804
0.75	4	7.8	0.70	0.799	7.3	0.73	0.775
	20	6.5	0.88	0.895	5.7	0.67	0.781
	37	8.7	0.47	0.778	7.6	0.51	0.793
	42	3.8	0.76	0.923	3.3	0.79	0.956
0.50	4	5.8	0.79	0.874	5.6	0.78	0.844
	20	2.8	0.89	0.934	4.1	0.68	0.764
	37	7.6	0.48	0.820	5.6	0.55	0.746
	42	3.6	0.71	0.960	3.4	0.72	0.953
0.25	4	5.1	0.74	0.834	4.2	0.82	0.836
	20	1.9	0.98	0.921	3.0	0.77	0.863
	37	5.0	0.62	0.848	7.5	0.44	0.740
	42	4.6	0.63	0.937	2.5	0.78	0.950

Table 3.4 Concentration dependency of consistency (K) and flow behaviour (n) index of EPS dispersions at pH 6.5 and 4, 20, 37 or 42 °C

EPS (g/100 g)	Temperature (°C)	Strain					
		Capsular-ropy			Capsular		
		K (mPa s^n)	n	r^2	K (mPa s^n)	n	r^2
1.00	4	4.2	0.84	0.929	19.4	0.37	0.867
	20	2.7	0.87	0.971	20.5	0.32	0.948
	37	1.8	0.90	0.969	21.6	0.23	0.732
	42	3.8	0.62	0.801	12.1	0.38	0.759
0.75	4	5.9	0.79	0.868	19.2	0.36	0.746
	20	3.4	0.77	0.932	17.9	0.31	0.945
	37	5.7	0.44	0.796	13.4	0.25	0.817
	42	1.5	0.90	0.987	7.3	0.45	0.725
0.50	4	3.8	0.85	0.923	16.3	0.36	0.415
	20	2.8	0.74	0.898	19.5	0.22	0.818
	37	2.6	0.65	0.786	10.5	0.34	0.916
	42	1.7	0.82	0.975	6.0	0.45	0.753
0.25	4	3.2	0.85	0.898	12.3	0.49	0.660
	20	1.8	0.81	0.848	18.3	0.19	0.915
	37	2.3	0.67	0.784	6.1	0.14	0.895
	42	1.7	0.78	0.975	5.4	0.45	0.694

from the Newtonian flow and thus exhibiting greater pseudoplastic behaviour. In most samples, lower EPS concentrations and higher temperatures had a negative effect on K values, with no apparent trend for n values. However, EPS dispersions at pH 3 did not show any observable difference of Ostwald constants between the two types of EPS. The low pH may diminish the rheological difference between the two.

An increase of the EPS concentration caused concomitant increase of the apparent viscosity. The parameter 'a' was slightly greater for the capsular than capsular-ropy EPS, which denoted the more temperature-sensitive nature of the capsular EPS (Table 3.5). The Arrhenius plot showed that the temperature negatively affected the apparent viscosity of both the colloids (Figure 3.3, 3.4). In the same manner, the activation energy also decreased with the increasing temperature (Table 3.5). The activation energy (ΔE) is the energy required to facilitate the motion of a liquid during flow, and smaller when kinetic energy of the material increases due to rise in temperature (Speers and Tung, 1986). This phenomenon was also shown in our work, with the ΔE values for the capsular-ropy EPS were slightly higher than those of the capsular EPS (Table 3.6). Apparently, the capsular-ropy EPS molecules were slightly more restricted to move than those of the capsular EPS. Activation energy was correlated to cohesion and stickiness of sugar dispersions (Chen, 2007). Nevertheless, the relatively low values of ΔE obtained in the present study were somewhat comparable to earlier reports (Kwon et al., 1996, Speers and Tung, 1986), thus emphasizing a negligible influence of temperature on the apparent viscosity (Figure 3.3, 3.4). pH had no apparent effect on the viscosity at different temperatures. The activation energy at low pH, however, tended to be slightly lower than at higher pH (Table 3.5).

There was a clear difference among the methods employed in this experiment to reveal the effect of temperature on the viscosity of EPS. While the results presented in Tables 3.3 and 3.4 did not reveal clearly the effect of temperature on viscosity, the result in Tables 3.5 and 3.6 showed obvious effect. The K values did not show the decreasing viscosity as the temperature was

Table 3.5 Parameters of apparent viscosity (at 100/s shear rate) as a function of concentration for capsular-ropy and capsular EPS dispersions at temperatures ranging from 4 to 42 °C

Temperature (°C)	Capsular-ropy			Capsular		
	a (mPa s%$^{-b}$)	b	r^2	a (mPa s%$^{-b}$)	b	r^2
			pH 3.0			
4	9.96	0.21	0.889	13.96	0.27	0.929
20	5.23	0.18	0.828	7.42	0.25	0.907
37	3.27	0.17	0.764	5.75	0.27	0.956
42	2.76	0.16	0.703	6.31	0.32	0.958
			pH 6.5			
4	3.00	0.12	0.614	3.07	0.23	0.914
20	1.83	0.10	0.598	1.88	0.21	0.905
37	1.15	0.11	0.577	1.21	0.21	0.911
42	0.91	0.11	0.614	0.99	0.22	0.901

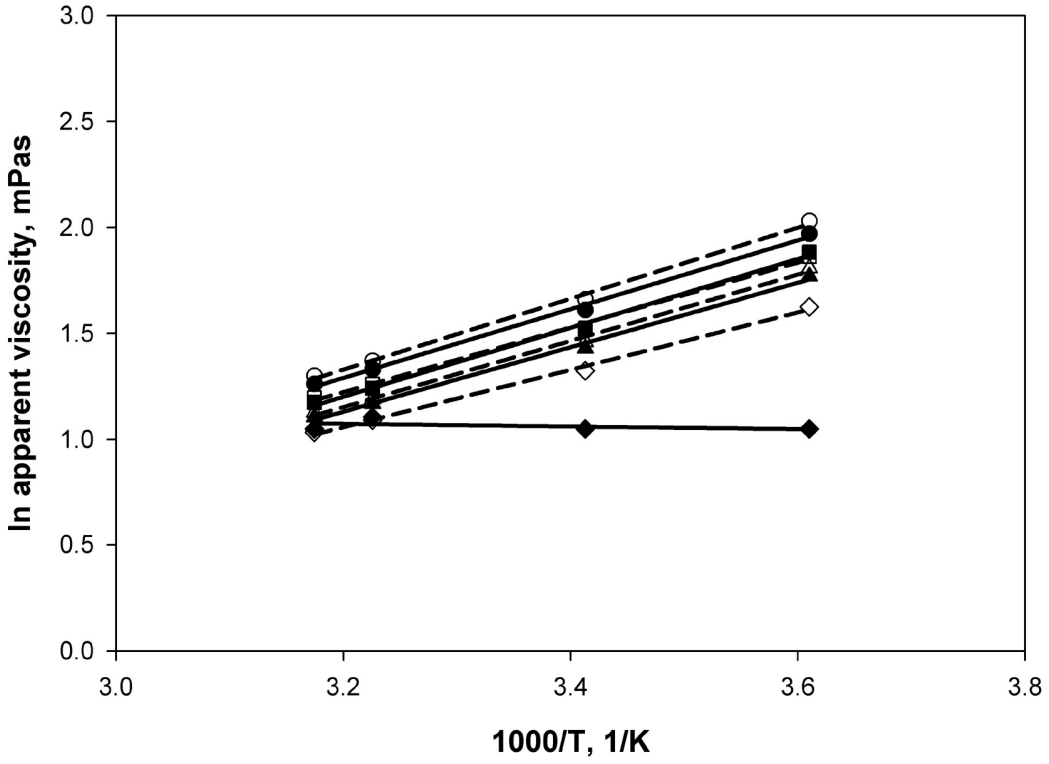


Figure 3.3 Arrhenius plot of temperature from 0 to 42 °C and concentration from 0.25-1 g/100 g (0.25 - ◇; 0.5 - ■; 0.75 - ▽; 1 g/100 g EPS - ●) at pH 3.0 and apparent viscosity of capsular-ropy (solid line) and capsular (dashed line) EPS at shear rate of 100/s.

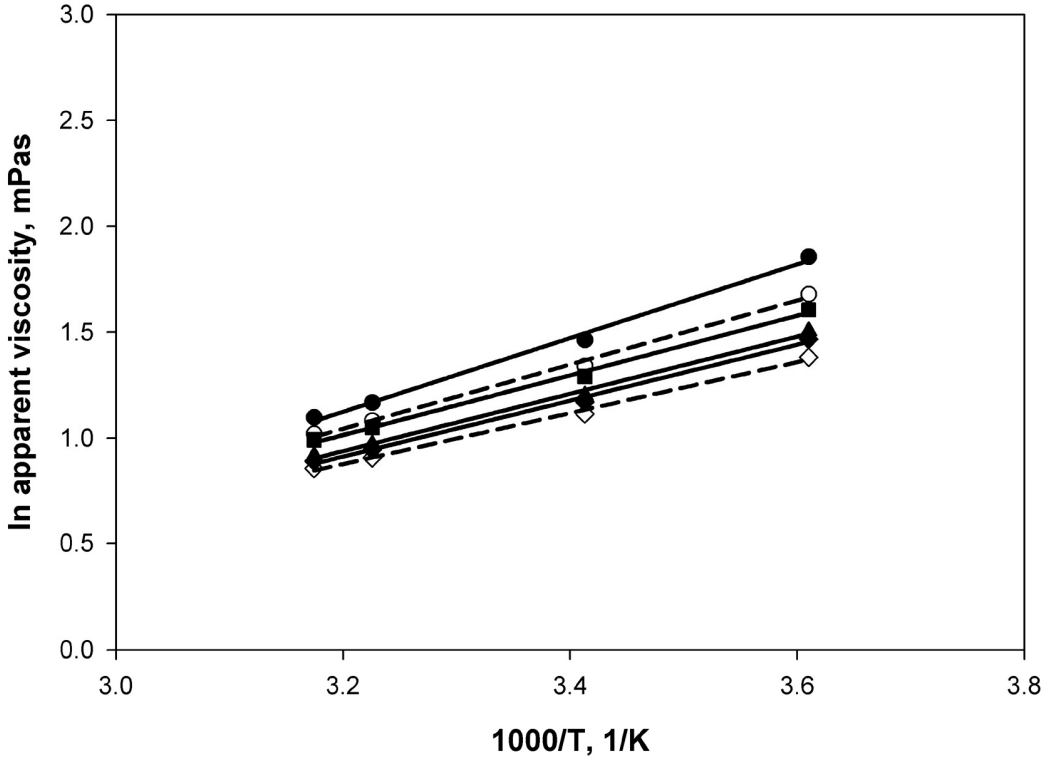


Figure 3.4 Arrhenius plot of temperature from 0 to 42 °C and concentration from 0.25-1 g/100 g (0.25 - ◇; 0.5 - ■; 0.75 - ▽; 1 g/100 g EPS - ●) at pH 6.5 and apparent viscosity of capsular-ropy (solid line) and capsular (dashed line) EPS at shear rate of 100/s.

Table 3.6 Arrhenius-like temperature dependency of apparent viscosity of capsular-ropy and capsular EPS dispersions of different concentrations determined at 100/s shear rate and pH 3 and 6.5

EPS concentration (g/ 100 g)	Capsular-ropy			Capsular		
	ΔE (kJoule/mole)	A (mPa s)	r^2	ΔE (kJoule/mole)	A (mPa s)	r^2
			pH 3.0			
1.00	8.31	0.049	0.998	8.32	0.051	0.998
0.75	8.82	0.037	0.999	8.26	0.048	0.998
0.50	8.77	0.036	0.999	8.83	0.036	0.992
0.25	8.22	0.042	0.998	8.43	0.038	0.996
0.10	7.73	0.052	0.999	9.13	0.027	0.996
			pH 6.5			
1.00	9.82	2.35	0.994	9.52	0.027	0.990
0.75	9.22	2.20	0.992	9.18	0.029	0.992
0.50	9.47	2.26	0.994	9.48	0.024	0.994
0.25	9.52	2.27	0.993	9.11	0.026	0.983
0.10	9.41	2.25	0.992	9.20	0.025	0.988

increased. This was especially observed in capsular-ropy EPS and temperature of 25 and 37 °C. Nevertheless, the effect of temperature was obvious in most of the data between 4 and 42 °C, especially in capsular EPS. It seems that it is due to less suitability of the method used, where K values did not reveal clearly the effect of temperature, or the EPS concentration used was too low.

In contrast, the effect of temperature was obvious from Table 3.5, which showed that temperature negatively correlated to apparent viscosity. Further, the method employing Arrhenius equation (Table 3.6) was able to show that activation energy ΔE of capsular-ropy EPS was higher than that of capsular EPS, suggesting relatively restricted movement of molecules of capsular EPS. The less free movement of capsular-ropy EPS and its temperature-insensitivity can explain more the inconsistency of the K values for capsular-ropy EPS in table 3.3 and 3.4. Thus, it was suggested that the use of parameter 'a' and activation energy ΔE is better than K values in revealing the effect of temperature on the viscosity of these types of EPS.

Polymer characteristics are often studied by assessing flow behaviour at very low shear rate, such as below 10/s (Gorret et al., 2003). Some polysaccharides exhibit non-Newtonian flow, obeying the power law only in the intermediate shear-rate region, but not at low and high shear rates (Rao, 1999). In our work, the data were fitted to the Cross model with a relatively high degree of correlation (Table 3.7). The Cross model is commonly used to describe the flow characteristics of materials possessing first and second Newtonian plateaus at low and high shear rates and the power law flow in between (Macosko, 1994). Zero-shear viscosity (η_o) was affected significantly ($P<0.05$) by strain and pH, but not by concentration ($P>0.05$) (Table 3.7). Any interaction between two factors involving concentration was not significant ($P>0.05$). In acidic environment (pH 3), dispersions of the capsular-ropy EPS showed higher η_o than those of the capsular EPS dispersions (Table 3.8). However, the opposite occurred at pH 6.5. Higher η_o may relate to lower mobility due to water-binding and enlargement of EPS molecules (Ravi and Bhattacharya, 2004), and may indicate more extensive coil overlap (Gorret et al., 2003). Similarly, the values of τ (structural relaxation time) were significantly ($P<0.05$) affected by all factors examined except

Table 3.7 Adjusted mean squares from analysis of variance of the Cross model parameters of capsular-ropy and capsular exopolysaccharide, at pH 3 and 6.5, and different concentrations

Source of variation	DF	*p*-value		
		η_0	τ	m
Strain	1	<0.0001	<0.0001	0.095
Concentration	3	0.1960	0.017	0.044
pH	1	<0.0001	<0.0001	0.019
Strain × concentration	3	0.278	<0.0001	0.010
Strain × pH	1	<0.0001	<0.0001	0.070
Concentration × pH	2	0.168	<0.0001	<0.0001
Error	42			

Table 3.8 The Cross model parameters describing the dependence of the apparent viscosity of capsular-ropy and capsular EPS dispersions on the concentration at pH 3 and 6.5

EPS concentration (g/100 g)	CAPSULAR-ROPY η_0 (mPa s)	τ (s)	m	r^2	CAPSULAR η_0 (mPa s)	τ (s)	m	r^2
				pH 3.0				
1.00	150.82	15.82	0.47	0.920	93.72	9.36	0.61	0.850
0.75	30.49	3.96	0.45	0.785	27.09	5.11	0.74	0.741
0.50	19.59	2.92	0.45	0.766	13.81	1.77	0.94	0.763
0.25	15.91	1.84	0.64	0.724	8.79	0.72	1.04	0.942
0.1	14.36	0.43	1.21	0.731	9.24	1.05	0.97	0.831
				pH 6.5				
1.00	7.80	0.08	2.15	0.981	376.81	8.19	1.88	0.880
0.75	8.01	0.05	2.26	0.935	352.56	14.59	1.10	0.919
0.50	6.96	0.15	1.88	0.878	218.79	15.44	1.65	0.841
0.25	5.88	0.14	1.88	0.878	113.76	19.62	3.49	0.926
0.1	5.45	0.14	1.99	0.875	90.83	27.20	5.92	0.904

concentration. A greater structural relaxation time (τ) was a sign of more extensive entanglement leading to a less mobile EPS chain, and consequently a longer time to develop new entanglement after disruption during shearing (Gorret et al., 2003). Greater values of τ at increasing polymer concentration (Table 3.8) as shown in our work might be ascribed to non-gelling properties (Gorret et al., 2003). However, we observed that higher concentrations of capsular EPS at pH 6.5 exhibited lower τ (Table 3.8), indicating a gelling character under these conditions. The value of m is equal to (1-n) where n is flow behaviour index in the power law model (Ravi and Bhattacharya, 2004). Therefore, the greater the m values, the more the material deviates from the Newtonian behaviour. The m values were not affected (P>0.05) by strain and interaction between strain and pH (Table 3.7). They were affected by concentration, pH, and temperature, and the combinations of two factors among them. The insignificant role of concentration in our study may be due to the low concentrations used. Considerably higher concentration was required to enable examination of viscoelastic properties, eg. 6 g/L in the case of the EPS from *Propionibacterium acidipropionici* (Gorret et al., 2003). Although it was apparent from our work that the two types of EPS exhibited different rheological characteristics, their interaction with milk components during fermentation may be more influential in determining the final texture of yoghurt (Rohm and Kovac, 1994).

3.4 Conclusions

The cell growth and EPS production of the two strains of *S. thermophilus* was affected by fermentation conditions, with capsular strain showed slower growth but similar amount of EPS produced. In general, high temperature, glucose and lactose supported cell growth. The EPS-production in both strains was growth-associated. Galactose was not supportive to cell growth, but EPS production in medium containing this sugar was as much as in other sugars. Thus, both strains were able to utilize galactose supplemented into the medium, seemingly for EPS synthesis. In most cases, maximum EPS production was attained after 4-8 hours. The viscosity of capsular EPS was seemingly more

influenced by temperature. Compared to capsular EPS, capsular-ropy EPS apparently exhibited more resistant to flow and less ability to structural rearrangement after shear-induced disruption. The activation energy of capsular-ropy EPS tended to be higher than that of the capsular EPS. Strain, temperature and pH were significant in determining the flow behaviour of the EPS dispersions.

In the next chapter, the suitability of lactose for both growth and EPS production will be examined in fermented milk product. The effect of processing factors especially incubation temperature and storage will be evaluated. The possible influence of the rheological difference between EPS of the two strains on texture of fermented milk will also be investigated.

4 Effects of Exopolysaccharide-Producing Strains of *Streptococus thermophilus* on Technological and Rheological Properties of Fermented Milk

4.1 Introduction

In the previous chapter, it was shown that capsular-ropy EPS exhibited lower mobility in the aqueous system and was also less sensitive to temperature changes in comparison to capsular EPS. The EPS production itself and the cell growth were positively affected by temperature. These properties will be evaluated in real food system, i.e. fermented milk. It was expected that high incubation temperature would result in higher EPS production as well as improved cell growth. The effect of two different EPS on fermented milk texture, however, may not be easily predicted since it would involve their interactions with other milk components. Studying the texture of fermented milk produced using the strains in different processing conditions would provide better insight on how the two EPS would affect real food system.

Many cultures have appreciated yoghurt as an integral part of everyday diet for centuries. The rich flavour and smooth body have been major contributors to its consumer acceptance, although these attributes nowadays are accompanied with certain health benefits. In early 20th century, Metchnikoff suggested that the longevity of Bulgarian peasants could have been attributed to the consumption of yoghurt. The fermenting cultures used in yoghurt production could minimize detrimental effect of putrefactive bacteria in the gut (Vasiljevic and Shah, 2007). Recent marketing reports indicate that the production of fermented dairy products

is on the rise with yoghurt being the second most popular snack among children in the USA (Sloan, 2006).

In addition to health benefits, another important characteristic of its attractive perception is textural properties such as viscosity (Marshall and Rawson, 1999), smoothness and thickness (Jaworska et al., 2005) and structural resistance to stress (Skriver et al., 1993). Yoghurt cultures commonly used are strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. *L. bulgaricus* may impart specific sensory properties due to 'post-acidification' (Oliveira et al., 2001). Alternatively, yoghurt may be produced by a single strain culture of *S. thermophilus,* which results in a mild flavour. This approach has been accommodated by several regulatory bodies. In Australia, yoghurt is defined as a fermented milk produced by fermentation with lactic acid producing microorganisms (FSANZ, 2003). This particular strain also gives a desirable body to yoghurt due to its production of exopolysaccharides (Hassan et al., 1996a). The presence of EPS in fermented products influences several important sensory properties, including mouth thickness, shininess, clean cut, ropiness and creaminess (Folkenberg et al., 2005). Due to discrepancy in regulations worldwide, the products produced from a yoghurt base and fermented by a single strain of a mixed yoghurt culture or any other lactic acid bacterium are generally termed fermented milks.

The semi solid texture of yoghurt is a result of the development of a three-dimensional protein network during fermentation. The formation of the yoghurt gel is related to pH decrease and culture behavior during fermentation (Hassan et al., 1996b). The development of the elastic gel structure with a solid like behaviour starts at pH around 5.6, caused by structural changes of micelle due to solubilization of colloidal calcium phosphate (Lee and Lucey, 2004b). Further pH reduction causes more complex and extensive interconnection of casein particles, leading to the formation of a continuous protein network and thus governing the structure of yoghurt. Textural properties of yoghurt such as viscosity (Marshall and Rawson, 1999), smoothness and thickness (Jaworska et al., 2005) and structural resistance to stress (Skriver et al., 1993) are important attributes determining its consumer acceptance. In this regard, viscosity of yoghurt appears

to be more influential than flavor (Jaworska et al., 2005) and affected by type of culture used (Guzel-Seydim et al., 2005, Haque et al., 2001), fermentation temperature and storage time (Guzel-Seydim et al., 2005).

The traditional yoghurt production includes the fermentation of milk with a thermophilic mixed cultures consisting of strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. In addition to the pH lowering effect and flavour formation, it plays a major role in the yoghurt texture creation through its *in situ* exopolysaccharides (EPS) production. The EPS produced by yoghurt starter cultures affect the texture of yoghurt and improve sensory characteristics such as mouthfeel, shininess, clean cut, ropiness and creaminess (Folkenberg et al., 2005). However, the final textural characteristics of yoghurt are strongly dependent on structural properties of EPS, such as type (capsular or ropy) (Bouzar et al., 1997), the degree of ropiness (Hassan et al., 1996b), sugar composition (Petry et al. 2000, Petry et al., 2003) and degree of branching (Rinaudo, 2004). The role of the capsular and ropy EPS on the texture of yoghurt has been extensively studied for their distinctly different behaviour in relation to the interaction with milk proteins during yoghurt manufacturing. They differed in their localization within the protein network (Folkenberg et al., 2005, Hassan et al., 2003b) and their effect on the viscosity and consistency of yoghurt (Hassan et al., 2002). Although a great deal of work has been done in this area, the effect of EPS and other processing factors on rheological properties of yoghurt appears to vary greatly, which may suggest a complex nature of the EPS-protein interactions that affect the texture.

The temperature of fermentation may affect the viscosity of yoghurt directly (Lee and Lucey, 2004b) or indirectly via the bacterial EPS production (Ruas-Madiedo et al., 2002). Higher fermentation temperature causes higher gelation pH and subsequently lowers the solubilization of colloidal calcium phosphate leading to a weak structure of the gel. The EPS also production appears temperature dependent (Haque et al., 2001, Lee and Lucey, 2004b), although the viscosity of yoghurt was not always positively correlated to EPS concentration (De Vuyst et al., 2003, Ruas-Madiedo et al., 2002). For example, relatively low EPS concentration produced by *L. lactis* ssp. *cremoris* B40 resulted in the viscosity

similar to that of the strain with highest EPS concentration (Ruas-Madiedo et al., 2002). Also, yoghurt with highest EPS content had the lowest viscosity (Bouzar et al., 1997). Furthermore, the application of EPS-producing yoghurt cultures may decrease the extent of syneresis (Hess et al., 1997, Moreira et al., 2000) likely due to enhanced water-binding ability (Vinarta et al., 2006).

We have recently identified a strain capable of producing both, capsular and ropy EPS, and tested its applicability in the manufacturing of low-fat cheese (Zisu and Shah, 2003, 2005b). *S. thermophilus* ST 285 produces capsular EPS which attached to outer cell wall as assessed using staining and microscopic technique (Zisu and Shah, 2002). On the other hand, when examined using similar technique combined with viscosity measurement, strain of ST 1275 produces capsular EPS, as well as ropy EPS, which is excreted into the medium and consequently increased viscosity of medium (Zisu and Shah, 2003). Our goal in this study was to compare the performance of these two strains, ST 1275 and ST 285, in the manufacturing of fermented milk. More specifically, the effects of fermentation temperature and storage time were assessed in relation to culture growth, EPS production and subsequent *in situ* content and rheological properties of fermented milk.

4.2 Materials and methods

4.2.1 Materials

The starter cultures used in this study were obtained from the Australian Starter Culture Research Centre (Werribee, Australia) and have been characterized for their EPS production by Zisu and Shah (Zisu and Shah, 2002, 2003). *S. thermophilus* ASCC 1275 (ST 1275) produces both capsular and ropy EPS, while *S. thermophilu*s ASCC 285 (ST 285) produces capsular EPS only. Frozen (at -80 °C) glycerol stocks of the cultures were activated by incubating them twice in 30 mL sterile 14 g/100 g skim milk at 37 °C for 24 hours, before using them in the fermented milk manufacturing. Skim milk was prepared by reconstituting corresponding amount of skim milk powder (“Our Milk”, Cowbell,

Metallstrasse, Switzerland) in distilled water and subsequently sterilizing in an autoclave at 121 °C for 15 min.

4.2.2 Experimental design and statistical analysis

All experiments were organized as a randomized full factorial block design with culture, temperature and storage time as main factors. This block structure was replicated twice with at least 2 subsamplings. Results were analyzed as split plot in time measurements using General Linear Model procedure of the SAS System (SAS, 1996). Where appropriate, correlational analysis was employed using Microsoft Excel StatPro^{TM}. The level of significance was preset at $P = 0.05$.

4.2.3 Preparation of fermented milk batches

Skim milk powder was reconstituted to 14 g/100 g with distilled water, pasteurized at 90 °C for 5 min in a water bath and stored in a cold room (4 °C) overnight. The following day, it was pre-warmed in a water bath and inoculated with 1 mL/100 mL of each strain of *S. thermophilus*. Inoculated milk was poured aseptically into sterile 250 mL plastic containers, which were then placed in an incubator at different temperatures: 30, 37 or 42 °C. During fermentation, the pH change of all batches was recorded every 15 min using a pH meter (model 8417; HANNA Instruments, Singapore). The fermentation time, in min, was defined as time required to reach the mandatory pH of 4.5. The acidification rate was inferred from the linear slopes of pH versus time functions and expressed as pH milliunit/min (mU/min).

The fermentation was terminated at pH 4.5 and fermented milk samples were immediately stored in a cold room (4 °C) for 30 days. During this storage period, yoghurt batches were assessed for microbial, chemical and rheological properties at day 1 (approximately 20 h post-fermentation), after 7 days (day 7) and at the end (day 30) of the storage period.

4.2.4 Technological, microbial and chemical properties of fermented milk

The enumeration of *S. thermophilus* strains followed the established procedure reported previously (Donkor et al., 2006). Briefly, 1 mL of a fermented milk sample was resuspended in 0.1 g/100 g peptone water and serially diluted to desired levels. Diluted samples were plated on M17 agar (Merck Pty. Ltd., Kilsyth, Victoria, Australia) and incubated aerobically at 37 °C for 48 h. The results obtained as means of six observations were expressed as log of colony forming units per mL of fermented milk.

The pH change of fermented milk batches during storage was monitored using a pH-meter (HANNA Instruments, Singapore). The titrable acidity of fermented milk was also assessed following AOAC titration method using 0.1 M NaOH (AOAC 33.2.06, 1999). Approximately 10 g of fermented milk was diluted with approximately same volume of distilled water before titration. Titrable acidity was expressed as percentage of lactic acid, determined using the following equation:

$$\text{Lactic acid}, \% = \frac{0.1\,\text{M NaOH (mL)} \cdot 0.009}{\text{Sample (g)}} \times 100$$

The crude EPS was determined following the method previously reported (Rimada and Abraham, 2003) with some modifications (Figure 4.1). The method was selected based on its reported high reliability (Rimada and Abraham, 2003). Approximately 30 g of fermented milk was first centrifuged (Model J2-HS, Beckman, Fullerton, California, USA) at 11000 × *g* at 4 °C for 4 min. The supernatant was collected and combined with two volumes of chilled ethanol and stored at 4 °C overnight. Consequently, the precipitate was collected by centrifugation at 2000 × *g* at 4 °C for 15 min (J2-HS, Beckman). About 10 mL of distilled water was then added to dissolve the EPS-containing precipitate, followed by addition of 250 μL 80 g/100 g trichloroacetic acid to precipitate the remaining proteins. The mixture was stored overnight at 4 °C, centrifuged at 2000 × *g* at 4 °C for 15 min (J2-HS, Beckman) and the supernatant was again collected.

The EPS in the supernatant was finally collected using ethanol precipitation and cold storage as described above. The whole procedure for EPS purification using water and TCA was repeated once more. After that, the EPS was vacuum-dried at 55 °C and weighed. The results were expressed as the amount of crude EPS per kg of fermented milk.

The extent of syneresis during cold storage of fermented milk batches was analysed by a centrifugation method previously reported (Amatayakul et al., 2006) with a slight modification. Inoculated milk samples, prepared following procedure described above, were fermented in centrifugation tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA) and centrifuged (model RT7, Sorvall, DuPont, Newtown, Connecticut, USA) at 70 × *g* at 8 °C for 10 min. The weight of the drained liquid was recorded and related to the initial weight of fermented milk. The degree of syneresis was expressed as a percentage.

4.2.5 Rheological properties of fermented milk

The rheological properties of fermented milk batches were characterized by initially assessing flow behavior subsequently followed by a small amplitude oscillatory measurement combined in one test. The measurements were carried out using a Haake RheoStress rheometer (RS 50, Haake Rheometer, Karlsruhe, Germany) fitted with a cone-and-plate measuring system (35 mm/2° angle). Prior to analysis, all samples were brought to the room temperature (controlled at 20±1 °C) and all determinations were performed at this temperature. The data acquisition was carried out by a RheoWin Pro software package (Version 2.94, Haake). The rheometer was calibrated every 60 days by motor adjustment and two oils with different viscosities as per manufacturer's instructions. The gap width was preset as per the hardware specifications (MCR301, Anton Paar).

The samples were initially gently stirred with a spatula 20 times prior to loading to achieve homogenous mixture. About 10 g sample was loaded into the bottom plate with spoon. After lowering the upper geometry, excess was then removed using spoon. Sample was subjected to a high shearing at 500/s for 60 s to

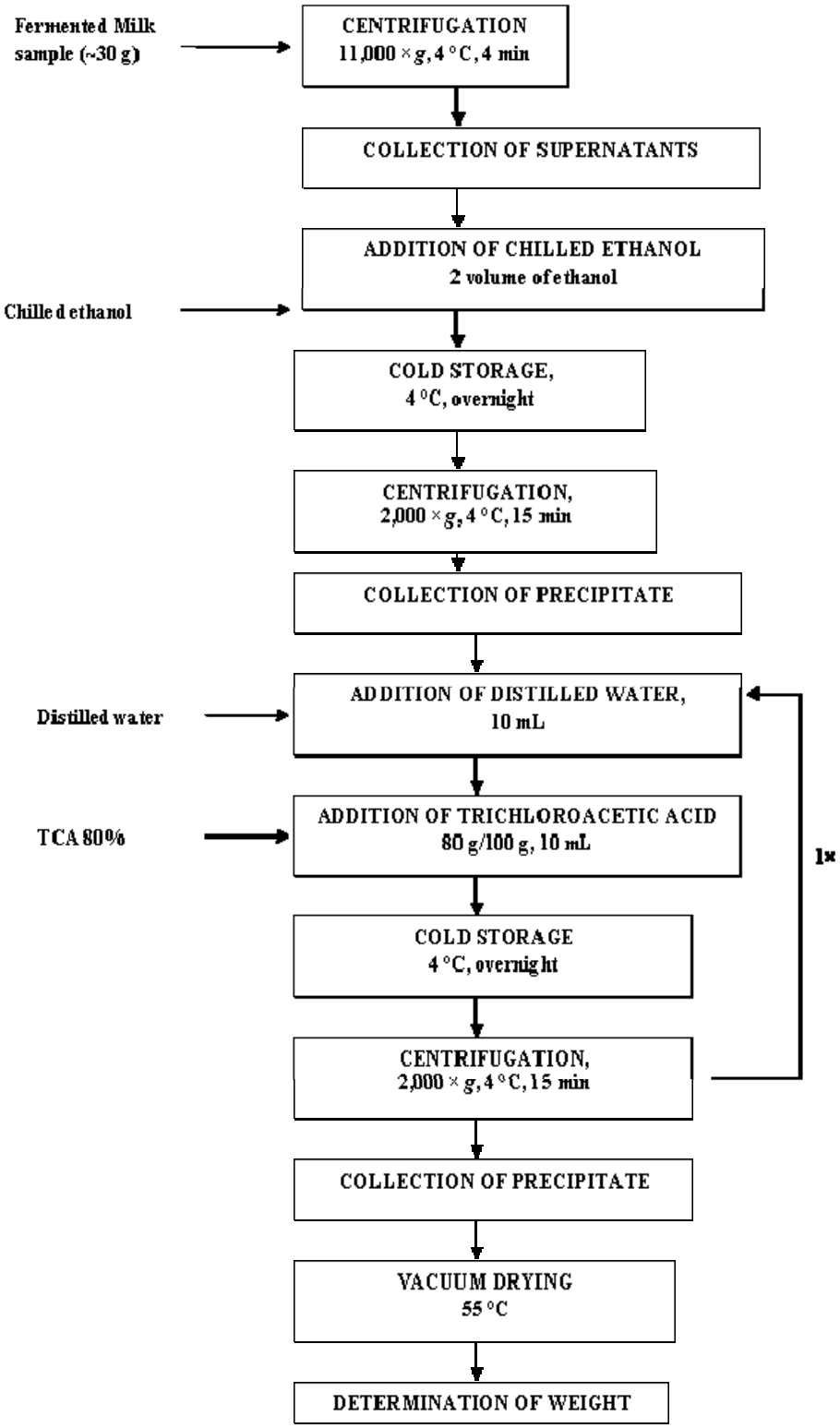


Figure 4.1 Method for determination of EPS concentration

diminish structural differences among samples caused by different treatments. This was followed by 300 s equilibration to allow for a structural rebuilding that would solely be dependent on the composition of the fermented milk. The elimination of gel structure was also necessary prior to measurement of hysteresis-loop-curves to eliminate the effect of probable cross-linking that may have occurred during cold storage (Benezech and Maingonnat, 1994). The flow curves were generated by measuring shear stress as a function of shear rates from 0.1 to 100/s (up and down sweeps). The flow behaviour was described by the Ostwald-de Waele model ($\tau = K\ \dot{\gamma}^n$), where τ presents shear stress (Pa), $\dot{\gamma}$ is shear rate (1/s), while K and n are consistency factor (Pa s^n) and flow behaviour index, respectively. The hysteresis loop area between the upward and downward flow curves (shear rates from 0 to 100/s) was also calculated using the RheoWin Pro software (Version 2.94, Haake).

After ascertaining the flow behaviour, the sample was left to equilibrate again for 300 s and then subjected to the dynamic oscillatory sweep. During these determinations, the frequency was varied between 0.1 and 10 Hz in 14 steps at 5% strain (determined from an amplitude sweep at 1 Hz). Dynamic moduli (G', G") and their ratio, loss tangent (tan δ), were recorded as a function of frequency. All determinations were independently repeated at least three times.

4.3 Results and discussion

4.3.1 Technological, microbial and chemical properties of fermented milk

The technological performances of two EPS producing strains during fermented milk manufacturing were assessed as the function of the fermentation time, acidification rate and titrable acidity. The fermentation temperature had a significant ($P<0.05$) effect on the rate of acidification. In general, the ropy (ST 1275) strain reached the end of fermentation at pH 4.5 faster (Figure 4.2) with a greater acidification rate in comparison to the capsular (ST 285) strain (Table 4.1).

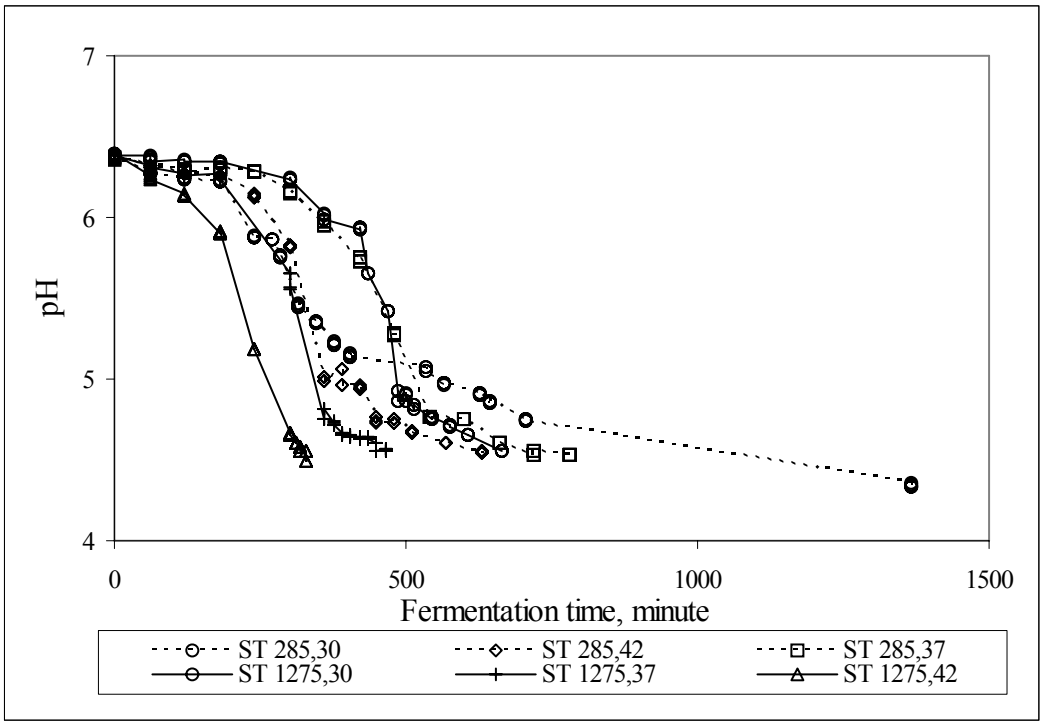
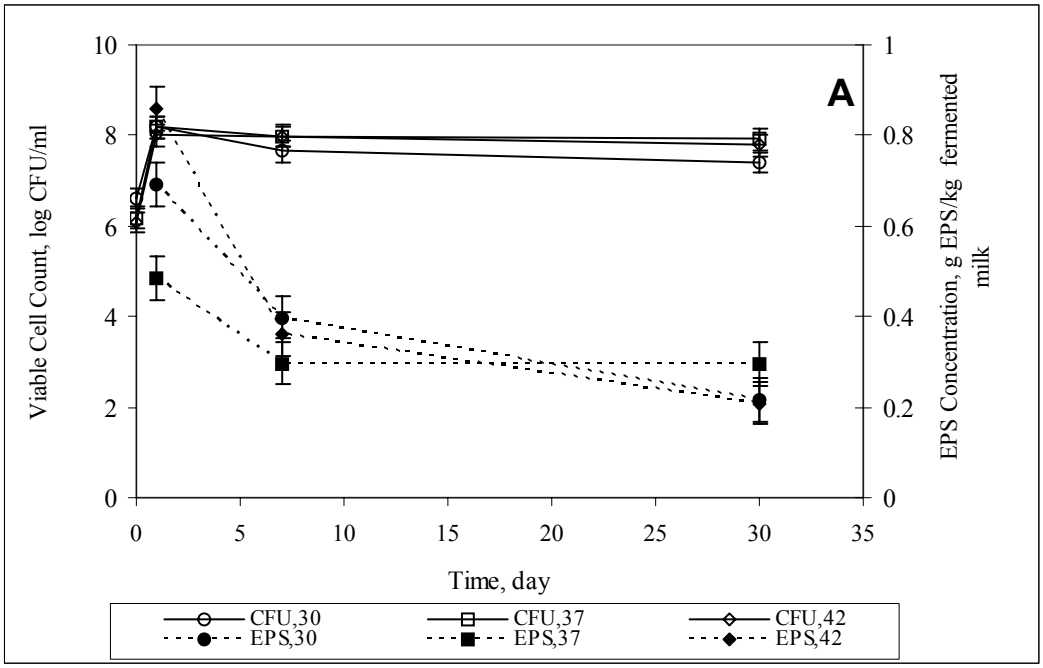


Figure 0.1 Time dependent pH change during the production of fermented milk batches using *Streptococcus thermophilus* ST 1275 (capsular-ropy) or *Streptococcus thermophilus* ST 285 (capsular) at 30, 37 or 42 °C prior to the cold storage. (Legend presented as i.e. ST1275, 30 indicates the strain and fermentation temperature).

Due to significantly (P<0.05) greater acidification rate at 42 °C (6.4 pH mU/min), the ropy strain reached pH of 4.5 in 304.5 min, which was significantly (P<0.05) faster than any other strain/temperature combination (Table 4.1). On the contrary, the longest fermentation time was observed for ST 285 during fermentation at 30 ^{o}C with the lowest acidification of 3 pH mU/min. Interestingly, the similar acidification rate by the same strain was obtained at 42 ^{o}C, indicating a mesophilic nature of this culture which was in contrast to ST 1275 which preferred the higher fermentation temperature.

The initial colony counts of both strains during fermented milk storage were clearly strain- and the fermentation temperature dependent (P<0.05, Figure 4.3). At the end of the fermentation, the fermented milk batches produced with the capsular-ropy strain fermented at 30 and 42 ^{o}C contained significantly (P<0.05) higher number of cells than that fermented at 37 ^{o}C. Furthermore, the number of viable cell of the capsular-ropy strain was also significantly (P<0.05) higher in comparison to that of the capsular strain regardless the fermentation temperature. Interestingly during cold storage, both strains cultivated at 37 ^{o}C experienced an increase in number of viable cells, which was greater (P<0.05) for the capsular strain. The number of the viable cells also decreased slightly (P>0.05) towards the end of the storage. Thes cell growth and EPS production profile of the two strains in fermented milk was in connection with their pattern in M17 medium as reported in Chapter 3.

The titrable acidity expressed as the amount of lactic acid was also strongly (P<0.05) strain and storage time dependent and not significantly (P>0.05) affected by the fermentation temperature. Similar to the growth, titrable acidity in some samples of ropy strain fermented milk tended to be significantly (P<0.05) higher in some samples than in those of the capsular strain (Table 4.1). During the storage, ST 1275 stopped producing acid after the day 7, while ST 285 continued producing acid until end of 30 days, which resulted in subsequent increase in titrable acidity. Similarly, other studies (Haque et al., 2001, Hassan et al., 1995a, Hassan et al., 2001b) observed that ropy strains grew faster than capsular strains during the fermentation of milk, although the exceptions were noted



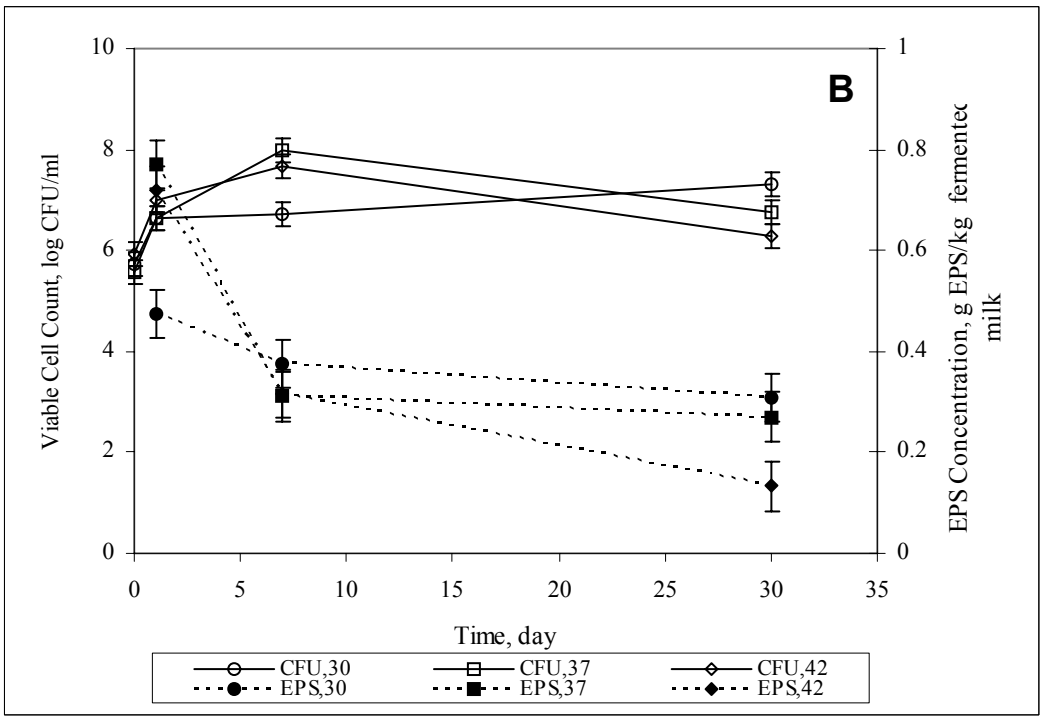


Figure 0.2 The viable cell counts and EPS concentrations in fermented milk batches fermented by *Streptococcus thermophilus* ST 1275 (A) or ST 285 (B) at 30, 37 or 42 °C and stored for 30 days at 4 °C. (The error bars indicate the SEM of 0.24 cfu/mL and 0.05 cfu/mL for the cell count and EPS concentration, respectively, n≥4; legend presented as i.e. CFU,30 indicates the measured characteristic and fermentation temperature).

Table 4.1 The fermentation time, acidification rate, titratable acidity, syneresis, hysteresis loop area, elastic modulus and loss tangents of fermented milk batches incubated by *Streptococcus thermophilus* ST1275 or ST285 at 30, 37 or 42 °C and stored for 30 days at 4 °C

Culture	Fermentation temperature, (°C)	Fermentation time*, (min)	Acidification rate* (mU/min)	Day	Titratable acidity**, (g/100 g lactate)	Syneresis** (g/100 g)	Hysteresis loop**, (Unit)	G'**, (Pa)
ST 1275	30	645.7^{d}	3.30^{b}	1	0.85^{aA}	6.2^{aA}	168.1^{aA}	23.8^{aA}
				7	1.03^{bA}	4.1^{aA}	135.1^{aA}	41.1^{aA}
				30	1.1^{bA}	5.5^{aA}	245.1^{aA}	105.5^{aA}
	37	445.5^{b}	4.77^{d}	1	0.87^{aA}	12.0^{aA}	215.9^{aA}	116.7^{aA}
				7	1.06^{bA}	9.1^{aB}	634.4^{bB}	116.7^{aA}
				30	1.14^{bA}	15.0^{aA}	905.5^{cB}	229.9^{aA}
	42	304.5^{a}	6.35^{e}	1	0.86^{aA}	21.4^{aB}	385.4^{aA}	108.4^{aA}
				7	0.99^{bA}	14.1^{bB}	458.8^{aB}	117.9^{aA}
				30	1.01^{bB}	13.8^{bB}	553.6^{aA}	365.1^{aA}
ST 285	30	719.5^{f}	3.30^{b}	1	0.82^{aA}	14.8^{aA}	164.9^{aA}	57.6^{aA}
				7	0.89^{aA}	11.9^{aA}	310.6^{aA}	273.5^{aA}
				30	0.95^{bA}	10.9^{aA}	258.5^{aA}	262.2^{aA}
	37	579.8^{c}	3.7^{c}	1	0.81^{aA}	17.2^{aB}	308.2^{aA}	281.1^{aA}
				7	0.93^{bA}	11.7^{bA}	409.1^{aA}	404.6^{aA}
				30	0.97^{bA}	20.5^{aA}	471.1^{aB}	848.5^{bB}
	42	696.3^{e}	3.00^{a}	1	0.82^{aA}	26.6^{aA}	302.1^{aA}	362.7^{aA}
				7	0.84^{aB}	19.2^{bA}	408.6^{aA}	352.9^{aA}
				30	0.97^{bA}	18.9^{bA}	383.9^{aC}	1040.9^{bB}
SEM***		3.7	0.02		0.029	2.1	81.4	149.2

***SEM - pooled standard error of the mean, $P<0.05$. Small letter superscripts in a column denote significant difference ($P<0.05$) among samples (*) fermented at the same temperature for a particular strain (**). Capital letter superscripts in a column indicate significant difference ($P<0.05$) among samples on a same day of storage for a particular strain.

(Hassan et al., 2001b). The slow growth and acidification rate of capsular strains may be attributed to greater energy expenditure on the capsule production or inhibition of metabolic activity due to reduced permeability of capsular material on cell surface (Hassan et al., 1995a). The ability of *S. thermophilus* to produce basic metabolites from urea at low temperatures may also contribute to the slow decrease of pH in addition to slow metabolic performance (Tinson et al., 1982).

Both strains showed similar tendency for the EPS production, which was greatly (P<0.05) affected by the fermentation temperature (Figure 4.3). The maximum quantity of the EPS produced by both strains differed slightly (P> 0.05), with ST 1275 producing 860±47.5 mg EPS/kg fermented milk at 42 °C as opposed to ST 285 which generated 769±47.5 mg EPS/kg fermented milk at 37 °C. More importantly, the EPS concentration in all batches declined substantially (P<0.05) during storage, especially in the first week. Despite different initial EPS concentrations, all yoghurt batches with the exception of one produced with ST 285 at 42 °C, showed no statistical difference (P>0.05) in the final EPS concentration at the end of the storage.

Relatively high fermentation temperature of around 40 °C supported the cell growth and EPS production of thermophilic lactic acid bacteria (Deegest et al., 2002, Petry et al., 2000). Apparently, the EPS production in our study was associated with the culture growth, since both strains produced the maximum EPS at the optimum growth temperatures (Figure 4.3). A significant (P<0.05) reduction of the EPS concentration in all batches during first weeks of storage may indicate the activity of enzymes capable of degrading the EPS (Deegest *et al*., 2002). This mechanism was likely activated due to energy requirements for the cellular maintenance since the EPS may serve as an energy storage in the presence of an excess of nutrients, which may be used later in the cell metabolism (Tolstoguzov, 2003).

The extent of syneresis during the cold storage was also significantly (P<0.05) affected by the strain selection, fermentation temperature and time. For both strains, the fermentation at 42 °C resulted in products with greater (P<0.05) syneresis than those at 30 °C. Prolonged storage affected the extent of syneresis greatly (P<0.05) with a general tendency towards reduction, although the

exceptions might be noticed. This result was somewhat similar to a previous report where the use of a 'more-ropy' strain resulted in less syneresis (Folkenberg *et al.*, 2005) as occurred with ST 1275. On the other hand, the 'less-ropy' strain produced the EPS, which gave greater syneresis, as in the case of the ST 285 yoghurt. Furthermore, higher fermentation temperature may also lead to greater syneresis (Lee and Lucey, 2004b) due to formation of large pores (Lucey et al., 2003, Ruas-Madiedo and Zoon, 2003). In general, our results showed no apparent effect of EPS concentration on the extent of syneresis in fermented milk batches.

4.3.2 Rheological properties

The storage modulus (G') of the fermented milk batches was significantly (P<0.05) affected by the storage time, while strain and fermentation temperature had no apparent effect (P>0.05). Similarly, the more solid-like character was also shown by greater tan δ which was affected by storage time but not affected by the strain or the fermentation temperature (P>0.05). G' increased towards the end of storage (Table 4.1, Figure 4.4), although the difference was rather slight (P>0.05) among fermented milk batches. The highest G' (1040.98 Pa) was obtained for the fermented milk made by the capsular strain at 42 °C on the day-30 of the storage. On the other hand, the lowest elastic modulus of 23.8 Pa was determined for the fermented milk containing capsular-ropy EPS incubated at 30 °C at the beginning of the storage. In general, the milk fermented with ST 285 had slightly (P>0.05) more elastic, solid character as shown by greater storage modulus than that of ST 1275 (Table 4.1, Figure 4.4). For both strains, the low fermentation temperature (30 ^{o}C) resulted in the fermented milk batches with low storage modulus and vice versa.

Low G' of the ropy yoghurt was observed earlier (Hassan et al., 1996a, Hess et al., 1997), while the yoghurt prepared with the addition of a capsular culture had higher elastic properties (Guzel-Seydim et al., 2005). The tendency of yoghurt gel to become more solid in the presence of the capsular EPS than with the ropy EPS may be influenced by a higher degree of cross-linking in capsular EPS (Tolstoguzov, 2003). Concomitant increase of G' with the increase of the

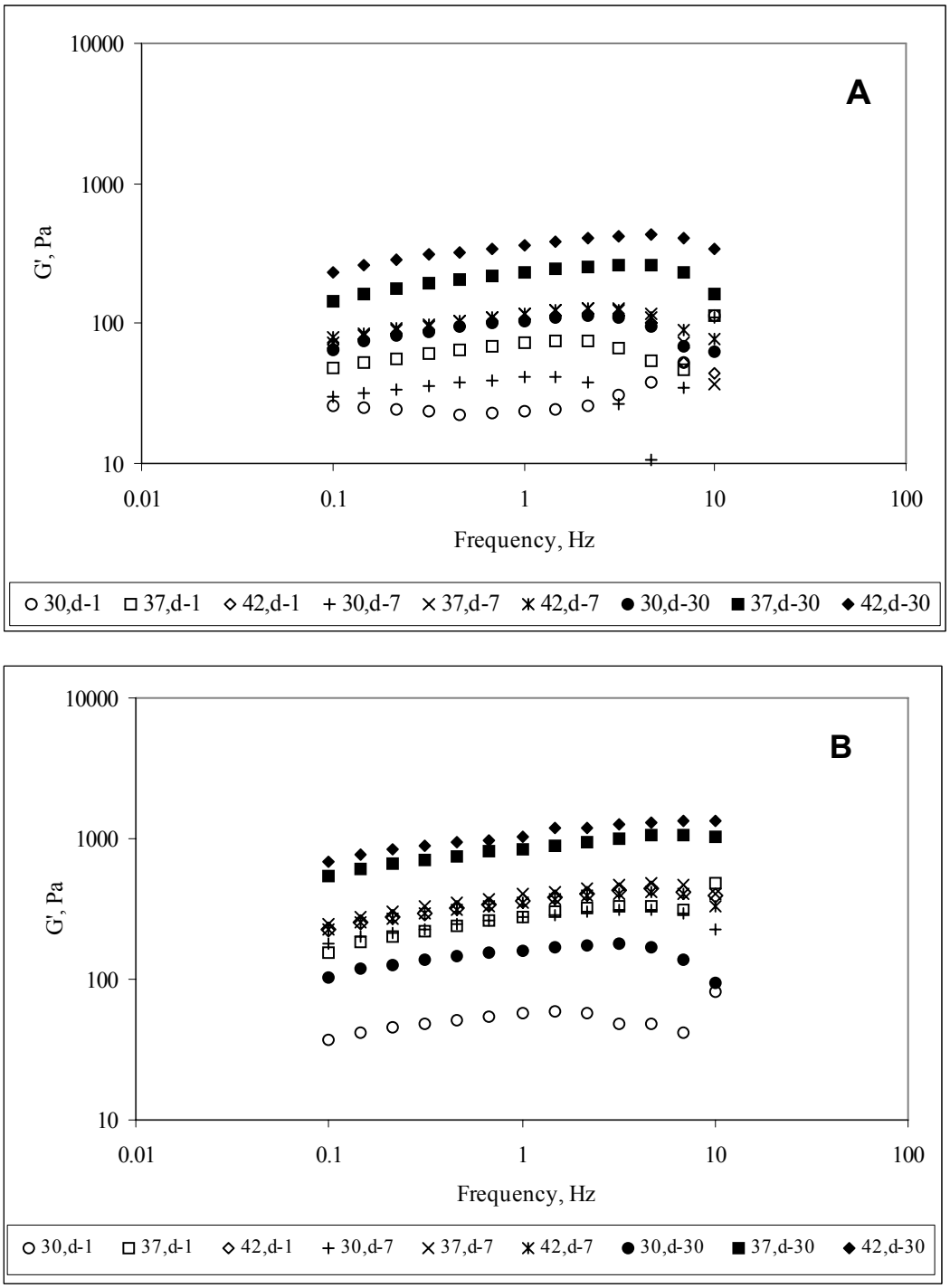


Figure 0.3 Storage modulus (G') of yoghurt batches as a function of oscillatory frequency. Yoghurt samples were produced by *Streptococcus thermophilus* ST 1275 (A) or ST 285 (B) by fermentation at 30, 37 or 42 °C and stored for 30 days at 4 °C. Samples were taken at day 1 (○, □, ◊} for 30, 37 and 42 °C, respectively), day 7 (+, x, * for 30, 37 and 42 °C, respectively), and day 30 (●, ■, ♦ for 30, 37 and 42 °C, respectively), of storage.

fermentation temperature in our work may be attributed to a greater extent of the hydrophobic bonding among caseins during fermentation at higher temperatures (Haque et al., 2001). Our results, however, contradicted several other reports, which found that a higher fermentation temperature produced less firm yoghurt (Kristo et al., 2003, Skriver et al., 1993). This discrepancy may be due to the difference in acidification rate and time required for the development of casein aggregates, which affects gel strength (Horne, 1998, Lucey et al., 1998).

The results obtained by examination of the flow behaviour of the fermented milk batches were fitted to power law function of Ostwald-de Waele model (Table 4.2). The model parameters, consistency index (K) and flow behaviour index (n) showed contrasting dependence. While the consistency index of fermented milk batches was significantly (P<0.05) affected by the storage time, the flow behavior index was strongly (P<0.05) influenced by the strains used in fermentations. The consistency index (K) of fermented milk batches increased considerably (P<0.05) during storage (Table 4.2). The highest K value (6.94 Pa s^n) was determined for the capsular EPS containing fermented milk incubated at 42 °C on the day-7 of storage, while the lowest value (0.84 Pa s^n) was that of capsular-ropy EPS containing milk fermented at 30 °C on the first day (Table 4.2). The use of the capsular ST 285 strain resulted in fermented milk batches with low flow behaviour index values in comparison to those produced with ST 1275, which gave further evidence of strain-dependent nature of fermented milk viscosity (Faber et al., 2001). The flow behaviour of fermented milk batches deviated significantly (P<0.05) from the Newtonian fluids in the presence of the capsular EPS. This was previously attributed to stronger interaction between milk protein and the EPS (Hassan et al., 2001a). However, these interactions appear to be driven by chemical structure of EPS (Folkenberg et al., 2006b), leading to thermodynamically stable composite gels or phase separation in the case of thermodynamic incompatibility (de Kruif and Tuinier, 2001).

The thixotropy hysteresis loops of fermented milk batches were also affected significantly (P<0.05) by storage, while the type of EPS producing strain had no apparent effect (P>0.05) (Table 4.1). The greatest hysteresis for both

Table 4.2 Consistency (K) and flow behaviour (n) index during cold storage of fermented milk batches produced with *Streptococcus thermophilus* ST 1275 or ST 285 by fermentation at 30, 37 or 42 °C and stored at 4 °C for 30 days.

Culture	Temperature, (^{o}C)	Storage time, (day)	K*, (Pa s^{n})	n*, -	R^{2} **
ST 1275	30	1	0.85^{aA}	0.67 ^{aA}	0.9215
		7	0.97^{aA}	0.69 ^{aA}	0.9805
		30	1.56^{aA}	0.61 ^{aA}	0.9547
	37	1	2.94^{aA}	0.53^{aB}	0.9023
		7	3.09^{aA}	0.53 ^{aB}	0.979
		30	5.50^{bB}	0.49 ^{bB}	0.9586
	42	1	2.35^{aA}	0.55 ^{aB}	0.9609
		7	3.21^{aA}	0.49 ^{aB}	0.979
		30	3.12^{aA}	0.51 ^{aB}	0.9439
ST 285	30	1	2.06^{aA}	0.41 ^{aA}	0.9714
		7	3.40^{aA}	0.23 ^{aA}	0.9562
		30	2.87^{aA}	0.40 ^{aA}	0.9654
	37	1	3.78^{aA}	0.38 ^{aA}	0.9644
		7	3.45^{aA}	0.41 ^{aA}	0.9588
		30	6.30^{bB}	0.28^{bB}	0.9909
	42	1	3.05^{aA}	0.40^{abA}	0.9693
		7	6.94^{bB}	0.34 ^{aA}	0.9872
		30	3.22^{aA}	0.45^{bA}	0.9464
SEM***			0.76	0.03	

* Small letter superscripts in a column denote significant difference (P<0.05) among samples fermented at the same temperature for a particular strain. Capital letter superscripts in a column indicate significant difference (P<0.05) among samples on a same day of storage for a particular strain. **Coefficient of regression of power law equation, n≥4; ***SEM - pooled standard error of the mean, P<0.05.

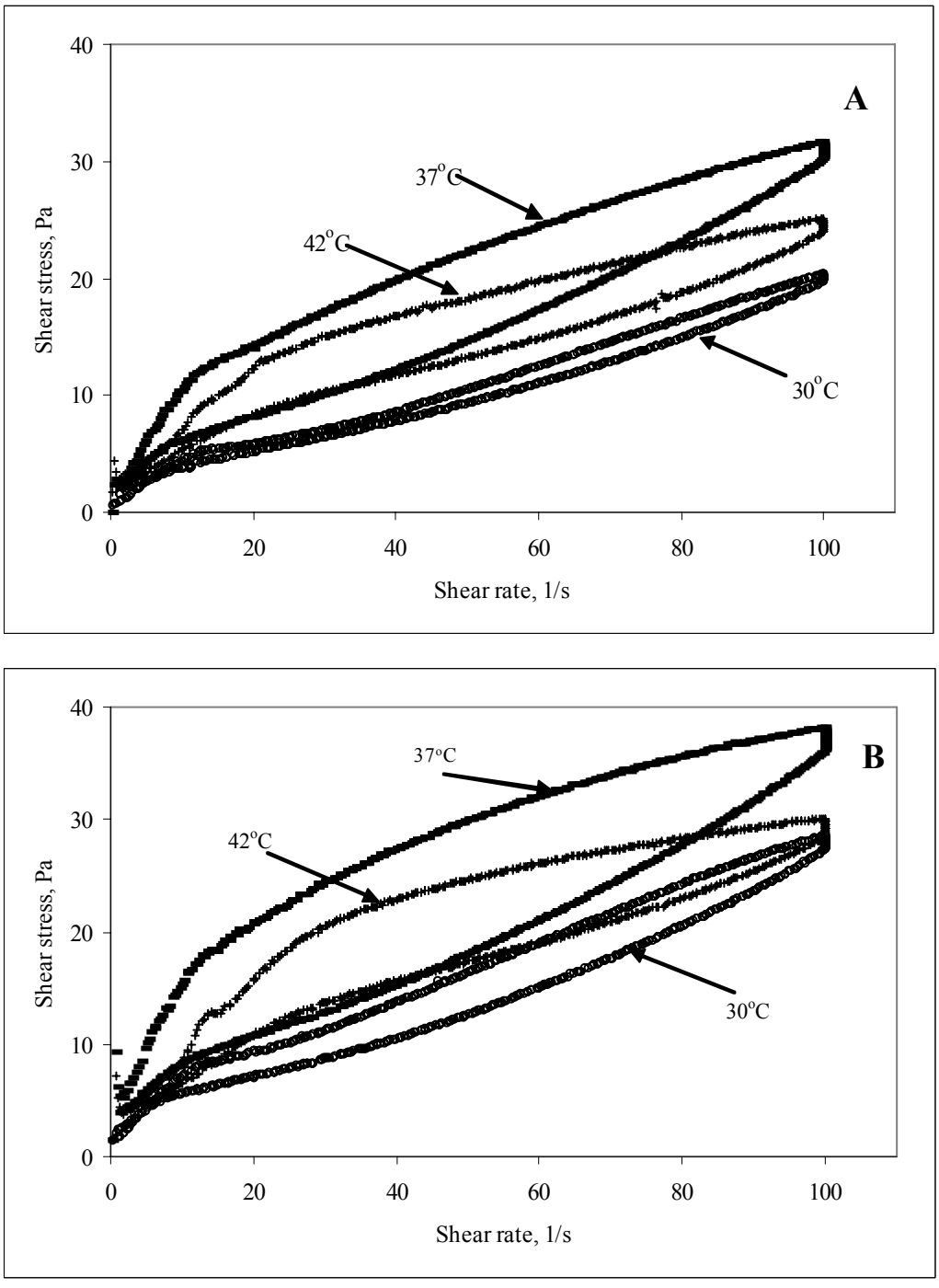


Figure 0.4 The thixotropy loop of fermented milk batches prepared by *Streptococcus thermophilus* ST 1275 at 30, 37 or 42 °C and stored for 1 day (A) or 30 days (B) at 4 °C.

strains was observed for batches prepared at 37 °C at the end of storage. The hysteresis loop, that is frequently noticed during the shear rate sweep of viscoelastic materials, may be assumed as the difference between energies required for the structural breakdown and rebuilding (Gambus et al., 2004). In our study, all fermented milk batches at the end of storage tended to have better structural reversibility (greater hysteresis loop area) from those at the beginning. The thixotropy of fermented milk with capsular-ropy EPS is illustratively shown in Figure 4.5. Although not statistically significant (P>0.05), the yoghurt produced with capsular strain followed similar pattern, but had a smaller hysteresis area.

It appears that the rheological behaviour and syneresis of fermented milk containing different type of EPS have some connection with the behaviour of EPS dispersion as reported in Chapter 3. Some differences between the two EPS were their zero-shear η_o and relaxation time τ, where they reflected a less mobile molecules of capsular-ropy EPS possibly due to higher water-binding capacity or more extensive molecular enlargement (Ravi and Bhattacharya, 2004), as compared to capsular EPS. The similar characteristics were shown in fermented milk gel, where that of fermented milk made using capsular-ropy EPS producer exhibited gel with less syneresis, more flexible, and less susceptible to break-down. Although the milk gel mainly consists of casein and bacterial exopolysaccharides and their interaction determines the texture, the rheological and physicochemical behaviour of EPS seems to give important effect. Prolonged cold storage gave substantial changes in syneresis and textural properties; however the difference between fermented milk made by each of the EPS producer remained. This may again highlighted the essential role of EPS type on the texture of fermented milk, which did not diminish by the effect of structural rearrangement (de Kruif and Tuinier, 2001, Hassan et al., 2003) during storage.

The statistical analysis revealed a weak correlation between the EPS concentration and selected rheological and physical parameters. In both capsular and capsular-ropy EPS fermented milk, the EPS amount was inversely correlated to G' (r = -0.5026, and -0.5597 for capsular-ropy- and capsular fermented milk , respectively), hysteresis loop area (r = -0.3813 for capsular-ropy- and -0.4399 for

capsular fermented milk), and consistency index (r = -0.3493 and -0.2529 for ropy- and capsular fermented milk, respectively), but positively related to the extent of syneresis (r = 0.3617 for ropy- and 0.3480 for capsular fermented milk). The amount of EPS in yoghurt did not have a major impact on selected rheological characteristics confirming findings of several other reports (Bouzar et al., 1997, Folkenberg et al., 2006b, Marshall and Rawson, 1999, Petry et al., 2000b, Skriver et al., 1993). Noteworthy, greater EPS concentration may lead to phase separation due to depletion effect (de Kruif and Tuinier, 2001) and enhanced syneresis. Also, a higher amount of EPS at the beginning of storage in our study may have hindered casein-casein interactions leading to a protein network with lower ability to retain serum (Hassan et al., 1996b). Conversely, reduced concentration of EPS at the end of storage resulted in a more thermodynamically stable system (de Kruif and Tuinier, 2001) with a consequent decline in the degree of syneresis due to better water-holding ability (Hassan et al., 2003b).

4.4 Conclusions

The fermented milk made with a capsular-ropy or capsular strain of *S. thermophilus* at different fermentation temperatures showed different rheological and physical properties at the end of fermentation and during cold storage. The duration of the storage of fermented milk had a more pronounced effect on the EPS concentration, parameters of viscoelastic and flow behaviour, as well as syneresis than the type of culture or the fermentation temperature. Both cultures produced appreciable amounts of the EPS at higher temperature and their production appeared to be related to the culture growth. The weak correlation between the EPS concentration and rheological characteristics of fermented milk was observed. The fermented milk prepared by capsular EPS producing strain had more solid characteristics but greater syneresis in comparison to that prepared by capsular-ropy strain. Different behaviour of two cultures in acidification and rheological properties of fermented milk may need to be considered during processing to control the textural quality of the final product. Although the ropy

strain in this work appears to be more suitable for fermented milk production, the relation between the observed textural characteristics and sensory perception needs yet to be assessed.

Result of this study will be used in the next experiment on the making of calcium-fortified fermented milk prepared by the two strains. Calcium is an important mineral in the diet which is commonly added into several popular products in order to enhance the intake of this mineral. However, since calcium carries positive charges, the presence of calcium in a food system containing charged components in the three-dimensional network such as in fermented milk may influence the textural properties. Theoretically, positive charges of calcium may weaken the fermented milk gel. As uncharged or weakly charged EPS were also present in the system, they may affect the gel texture in positive or negative way. Therefore, it is important to investigate the effect of EPS type on the textural properties of calcium-fortified fermented milk.

5 Rheological Properties of Fermented Milk Produced by a Single Exopolysaccharide Producing *Streptococcus thermophilus* Strain in the Presence of Added Calcium and Sucrose

5.1 Introduction

In the previous chapter, it was revealed that final texture of fermented gel was affected by the type of strain. However the difference was diminished to a certain extent with prolonged storage due to seemingly extensive structural rearrangement and likely EPS degradation. The use of capsular-ropy EPS producing strain in milk fermentation led to less firm, more shear-resistant gel, and lower syneresis. In contrast, milk fermented with capsular EPS producing strain resulted in more brittle gel and higher degree of serum expulsion. In this chapter, the effects of calcium and sucrose additions on the formation of acid gels in the presence of two different types of EPS were examined. Calcium fortification was likely to disrupt the fermented gel, while sucrose or EPS may diminish this effect. These effects have not been assessed previously therefore well defined experimental design would be required to address many confounding factors.

These days, yoghurt is the second most favorite snack among children in the US (Sloan 2006). An important characteristic of its attractive perception is its textural properties such as viscosity (Marshall & Rawson 1999), smoothness and thickness (Jaworska *et al.* 2005) and structural resistance to stress (Skriver *et al.* 1993). Yoghurt cultures commonly used are strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. *L. bulgaricus* may impart specific sensory defects due to 'post-acidification' (Oliveira *et al.* 2001). Legal

specifications in many countries require use of a mixed yoghurt culture, although yoghurt may also be produced by a single strain culture of *S. thermophilus* as a main acid producer and in conjunction with probiotics such in case of ABT culture. This particular strain also gives a desirable body to yoghurt as a result of its production of exopolysaccharides (EPS) (Hassan *et al.* 1996). The presence of EPS in fermented products influences several important sensory properties, including mouth thickness, shininess, clean cut, ropiness and creaminess

Dairy products have been considered a good medium for calcium fortification (Singh & Muthukumarappan 2008) mainly due to presence of various bioactive compounds that improve calcium absorption in the intestine primarily by increasing calcium solubility (Kitts & Kwong 2004). Current market trends show that the milk consumption is declining in the industrialized countries (Perales *et al.*, 2006) leading to inadequate intake of calcium as well as increased incidence of hypovitaminosis D and related diseases (Calvo *et al.* 2004) in some populations. The addition of calcium to dairy products to improve nutrition may also strengthen several structural characteristics; however, this fortification may have detrimental textural consequences when its concentration exceeds a certain level (Matia-Merino *et al.* 2004).

On the other hand, sucrose may improve texture and reduce syneresis of acid induced casein gels (Schorsch *et al.* 2002). Although there is a need for greater understanding of the effects of sucrose addition on the textural properties of acid induced gels, sucrose is more frequently incorporated in fermented milk products including yoghurt for taste improvement. In milk beverages supplemented with hydrocolloids, sucrose may increase or decrease viscosity, depending on the degree of hydration of the hydrocolloid (Yanes *et al.* 2002). It alleviates syneresis by reducing incompatibility between milk proteins and polysaccharides (Schorsch *et al.* 1999b, Choi *et al.* 2004) and affects dominance of the hydrophilic over hydrophobic sites (Belyakova *et al.* 2003). Sucrose addition at a certain concentration enhanced the elastic properties of a GDL-acidified gel (Belyakova *et al.* 2003). Since the addition of sucrose may be beneficial in restoring the structural weakening of calcium-supplemented yoghurt, we aimed to assess the effects of calcium and sucrose additions to fermented milk produced solely by

single, EPS producing *S. thermophilus* strains. Additionally, the role of glucono-δ-lactone (GDL, an artificial acidifier) (Lucey *et al.* 1998) in modulating of the rheological properties of an acid set gel structure was also examined.

5.2 Materials and methods

5.2.1 Materials

Batches of fermented milk were prepared from a yoghurt base (see below) and acidified by addition of one of two EPS-producing strains of *S. thermophilus* ASCC1275 (ST1275) and *S. thermophilus* ASCC285 (ST285) or by GDL. ST1275 produces mixed, capsular and ropy EPS, and ST285 produces capsular EPS (Zisu & Shah 2002, 2003, 2005). Both strains were kindly provided by the Australian Starter Culture Research Center (ASCRC, Werribee, Victoria, Australia). Frozen (at –80 ^{o}C) glycerol stocks of the cultures were activated by incubating them twice in 30 mL sterile 14 g/100 g skim milk at 37 ^{o}C for 24 hours, before using them in manufacturing.

5.2.2 Experimental design and statistical analysis

Experiments were arranged in a randomized full factorial block design with three factors: types of acidulant, calcium and sucrose concentrations. This block structure was replicated twice with at least two sub-samplings. Results were analyzed as a split plot design using a General Linear Model procedure (SAS, 1996) with acidulants as the main plot and calcium and sucrose as subplot. This analysis can be described by following equation:

$$X_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \delta_l + (\alpha\gamma)_{ik} + (\alpha\delta)_{jl} + (\gamma\delta)_{kl} + \varepsilon_{ijkl}$$

Where X corresponds to the actual value and μ the expected value, ε is the error term and α, β, γ and δ denote acidulants, replications, calcium and sucrose supplementation, respectively. Terms in brackets present corresponding

interactions. Correlational analysis was performed whenever necessary. The level of significance was preset at $P = 0.05$.

5.2.3 Preparation of fermented milk batches

Reconstituted skim milk (14 g/100 g) was prepared by dissolving skim milk powder (Murray Goulburn Co-operative Co. Ltd., Brunswick, VIC., Australia) in Milli-Q® water, followed by heat treatment at 90 °C for 5 minutes in a water bath, and cooling to 42 °C. This base was then inoculated with 1 mL/100 mL of each strain of *S. thermophilus* or mixed with 2 g/100 g of GDL (Sigma-Aldrich, Inc., St. Louis, MO, USA). The appropriate amounts of 0.1 M $CaCl_2$ solution and sucrose were then added resulting in calcium and sucrose concentrations of 0, 3, 6 or 9 and 0, 15, 30 or 45 mM, respectively. Selected maximum concentration of added calcium would increase the total calcium concentration in the product by almost 30 g/100 g (Singh & Muthukumarappan 2008). The whole mixture was mixed thoroughly using a magnetic stirrer for ten minutes. After addition of culture or GDL, the milk was poured aseptically into sterile 100 mL plastic containers, and placed in an incubator at 42 °C. The process was terminated when the pH reached 4.5 by transfer into a cold room (4 °C), and kept overnight. The fermentation time depended on the type of acidifier, with the GDL lowering pH to 4.5 within 6 hours. While the rate of acid production from the GDL dissociation was always greater than those produced by the cultures, the final pH of all batches was 4.5. The capsular-ropy and capsular EPS starter culture attained pH 4.5 after 8 (±0.2) and 12 (±0.3) hours, respectively. Following overnight storage, the pH of all batches was checked prior to all rheological measurements and was found to remain stable.

5.2.4 Bacterial growth and EPS concentration in fermented milk

The enumeration of *S. thermophilus* strains followed established procedures (Donkor *et al.* 2007). Briefly, approximately 1 mL of homogenized fermented milk sample was resuspended in 0.1 g/100 g peptone water and serially

diluted to required levels. Such diluted samples were then plated on M17 agar (Merck Pty Ltd., Kilsyth, VIC, Australia) and incubated aerobically at 37 °C for 48 h. The results obtained as means of at least four independent observations were expressed as a log of colony forming units per mL of fermented milk.

The titratable acidity of yoghurt was assessed using the AOAC titration method with 0.1 M NaOH (AOAC 1999). Approximately 10 g of fermented milk was diluted with approximately same volume of distilled water before titration. Titratable acidity was expressed as percentage of lactic acid.

The concentration of crude EPS was determined in fermented samples following established methodology (Rimada & Abraham 2003) with some modifications as reported in Chapter 4. Even capsule producing strains may produce EPS that can be loosely attached to the cell wall (Wicken *et al.* 1983), thus the EPS portion isolated from the medium would be of a greater interest for the study of texture (Folkenberg *et al.* 2006b). The size of samples used for EPS analysis ranged between 30 and 100 g (Folkenberg *et al.* 2006b). Protein precipitation was carried out by addition of 80 g/100 g tricholoroacetic acid (TCA), while EPS was precipitated using chilled ethanol (Rimada & Abraham 2003). First, the sample was centrifuged (Model J2-HS, Beckman, Fullerton, California, USA) at 11000 × *g* at 4 °C for 4 min. The supernatant was collected, combined with two volumes of chilled ethanol and stored at 4 °C overnight. The precipitate was collected by centrifugation at 2000 × *g*, 4 °C for 15 min (model RT7, Sorvall, DuPont, Newtown, Connecticut, USA). About 10 mL of distilled water was then added to the precipitate and mixed before addition of 250 μL 80 g/100 g TCA, and then stored overnight at 4 °C before being centrifuged at 4000 × *g* and 4 °C for 15 min (Sorvall). The precipitate was discarded. This final procedure was repeated twice. To the remaining supernatant was added 10 mL chilled ethanol, which was then stored overnight and then centrifuged at 4000 × *g* and 4 °C for 15 minutes. The precipitate was collected, dried at 55 °C and weighed. The results were expressed as the weight (mg) of crude EPS per kg of yoghurt.

Syneresis is a common defect of yoghurt (IDF 1992). The extent of syneresis during cold storage of all batches was determined by a centrifugation

method (Jaros *et al.* 2002) with slight modification. Batches were prepared by *in situ* fermentation as described above in 50 mL centrifuge tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA) and centrifuged (Sorvall) at 70 × *g* at 8 ^{o}C for 10 min. The weight of the drained liquid was recorded and related to the initial weight of yoghurt with the degree of syneresis expressed as a percentage.

5.2.5 Rheological and physical properties of fermented milk

The rheological properties of all acidified batches were evaluated by controlled stress rheometry and small-deformation oscillatory shear rheology using a Haake RheoStress rheometer (RS 50, Haake Rheometer, Karlsruhe, Germany) fitted with a cone-and-plate measuring system (35 mm/2^{o} angle). The rheometer was calibrated every 60 days by motor adjustment and two oils with different viscosities as per manufacturer's instructions. The gap width was preset as per the hardware specifications (MCR301, Anton Paar). Prior to analysis performed approximately 15 h post-fermentation, all samples were equilibrated to 20±1°C. The pH of all samples was 4.40 ± 0.05. About 10 g sample was loaded into the bottom plate with spoon. Any excess was then removed using spoon. Dynamic oscillatory measurements were carried out over 0.01 to 10 Hz to determine storage (G') and loss modulus (G"), and the ratio between G"/G' (tan δ) was calculated. Strain was maintained constant at 0.5% and was inferred from the linear viscoelastic region determined by amplitude sweep at a constant frequency (1 Hz). The data were analyzed using RheoWin software package (v 2.94).

Textural properties of fermented milk are often assessed on samples acidified with GDL, at a rate controlled by its concentration and temperature (Lucey *et al.* 1998). The difference in acidification rates between GDL- and bacterial-acidified yoghurt may result in substantial discrepancy in the gel properties and final texture (Lucey *et al.* 1998). To avoid this confounding in our study, all samples, after *in situ* thorough mixing and loading, were pre-sheared at a high shear rate of 500/s for 5 s, followed by 300 s rest to allow structural rebuilding, before rheological examination. Pre-shearing at high shear rate was

required to erase the processing history of these semisolid materials. The newly formed structures would clearly be dependent on the medium composition (Da Cruz *et al.* 2002b). Moreover, the pre-shear eliminates residual stress or anisotropy, which consequently, gives reproducible results. These restructured gels in general have mechanical spectra resembling those of the set-type yoghurt with viscoelastic moduli being 8-10 times lower (Jaros & Rohm 2003). Hysteresis loops were generated by measuring shear stress upon increasing shear rate from 0.1 to 100/s in 300 s (upward curve in the rheogram), then holding the rate at 100/s for 5 s and finally decelerating from 100 to 0.1/s in 300 s (downward curve in the rheogram). Data from the upward curve of the shear cycle were fitted to the modified Ostwald-de Waele power law model ($\eta = K\dot{\gamma}^{n-1}$) where η presents apparent viscosity (Pa.s), $\dot{\gamma}$ is shear rate (1/s), while K and n are consistency factor ($Pa.s^n$) and flow behaviour index, respectively. The thixotropy was determined by calculating the area between the upward and downward flow curves of the hysteresis loop. All determinations were repeated at least three times.

5.3 Results and discussion

5.3.1 Bacterial growth and EPS concentration in fermented milk

Acidification is an important factor governing gelation of casein. In GDL-gels, low pH was achieved by slow release of gluconic acid, which was easily controlled by GDL concentration. In culture fermented milk, the increased acidity resulted from organic acid production which was dependant on microbial metabolism. Cell growth of the capsule-forming strain was significantly ($P<0.05$) slower than that of the other strain (Table 5.1). Higher sucrose concentrations impaired cell growth significantly ($P<0.05$) (Table 5.1). A similar effect was observed upon the addition of calcium, with cells experiencing better growth at low concentrations (Table 5.1). However in many cases the capsular-ropy EPS strain appeared unaffected by calcium concentration.

Table 5.1 Microbiological properties and EPS concentration of yoghurt base fortified with sucrose (0, 15 30, 45 mM) and calcium (0, 3, 6, 9 mM) and fermented using *Streptococcus thermophilus* ST 1275 and ST 285 as acidulants at 42 °C

Type of acidulant	Sucrose, (mM)	$CaCl_2$, (mM)	Titratable Acidity*, (g/100 g lactate)	Δ CFU*, (CFU/ml)	Crude EPS*, (mg/kg fermented milk)
ST 1275	0	0	1.09^{aA}	2.385^{aB}	653.5^{bAB}
		3	1.24^{aA}	2.540^{aB}	228.8^{aA}
		6	1.28^{aA}	2.821^{abC}	489.8^{bAB}
		9	1.26^{aA}	3.081^{bB}	614.4^{bB}
	15	0	1.32^{aA}	2.243^{aAB}	190.4^{aA}
		3	1.47^{aAB}	2.339^{aAB}	358.5^{aA}
		6	1.47^{aAB}	2.531^{aB}	336.2^{aAB}
		9	1.46^{aAB}	2.250^{aA}	533.2^{aB}
	30	0	1.62^{aB}	2.218^{aAB}	528.8^{abAB}
		3	1.69^{aB}	2.323^{aAB}	459.7^{aA}
		6	1.66^{aB}	2.322^{aA}	250.1^{aA}
		9	1.71^{aB}	2.159^{aA}	525.5^{abB}
	45	0	1.43^{aB}	2.073^{aA}	352.7^{aA}
		3	1.53^{aA}	2.106^{aA}	282.7^{aA}
		6	1.44^{aB}	2.232^{aA}	433.9^{aAB}
		9	1.49^{aAB}	2.113^{aA}	249.9^{aA}
ST 285	0	0	1.29^{aA}	1.120^{aAB}	231.9^{aA}
		3	1.42^{aA}	1.975^{cC}	409.6^{abA}
		6	1.18^{aA}	1.570^{bC}	347.5^{abA}
		9	1.39^{aA}	1.101^{aB}	516.7^{abA}
	15	0	1.43^{aAB}	1.396^{bB}	740.2^{bCB}
		3	1.66^{aAB}	1.205^{abB}	364.5^{aA}
		6	1.84^{aB}	1.051^{aB}	466.2^{aA}
		9	1.71^{aB}	1.060^{aA}	299.1^{aA}
	30	0	1.55^{aB}	0.904^{aA}	535.5^{abBC}
		3	1.43^{aA}	0.807^{aA}	220.6^{aA}
		6	1.49^{aB}	1.358^{bC}	285.1^{aA}
		9	1.58^{aAB}	0.851^{aA}	516.5^{abA}
	45	0	1.56^{aB}	1.015^{bAB}	480.9^{aB}
		3	1.69^{aB}	0.981^{abAB}	408.4^{aA}
		6	1.71^{aB}	0.719^{aA}	618.5^{aAB}
		9	1.54^{aAB}	0.804^{abA}	415.0^{aA}
SEM**			0.09	0.104	84.4

*Small letter superscripts in a column denote significant difference (P<0.05) among samples at different calcium concentrations, at a particular sucrose concentration for one type of acidulant. Capital letter superscripts in a column indicate significant difference (P<0.05) among samples at different sucrose concentrations, at a particular calcium concentration for one type of acidulant. **SEM - pooled standard error of the mean, P<0.05.

Capsule production requires a great deal of metabolic energy, and polysaccharides may interfere with nutrient absorption (Hassan *et al.* 1995). High concentrations of added sucrose or calcium has been reported to adversely affect cell growth, which may be related to a high osmotic pressure in the medium (Yannick & Laurent 2005). Differing responses between the two strains towards calcium addition could also be caused by the differences in osmotic tolerance.

While sucrose and calcium concentrations affected cell growth, EPS concentration was influenced only by interactions between acidulant type, sucrose and calcium (Table 5.1). A clear trend could not be observed, and EPS concentrations between two strains differed only slightly (P>0.05, 190 to 653±84.4, and 231 to 740±84.4 mg/kg for capsular-ropy and capsular EPS, respectively). The EPS concentration was weakly and positively correlated to cell growth (r=0.45).

5.3.2 Rheological and physical properties of fermented milk

In our study, elastic moduli as the measure of the solid nature of samples among three types of acidified milk were significantly (P<0.05) affected by types of acidulant, calcium concentration and interactions between acidulant type and sucrose concentration (Table 5.2a,b). In general, artificially acidified milk prepared with GDL alone had greater elastic modulus than either fermented milk prepared under the same conditions. An obvious detrimental effect of calcium addition was observed for all the samples (Table 2a,b), although this was significant (P<0.05) only for milk fermented with the capsular EPS strain. Addition of sucrose had an apparent and significant (P<0.05) effect in fermented milk with capsular EPS present in the system. Our observations further support suggestions that the characteristics of the bacterially produced EPS play an important role in governing the properties of acid induced gels.

Food gels are commonly characterized by their viscoelasticity, which may be related to their sensory perception. For example, apparent viscosity measured at 241/s has been reported to be closely related to mouthfeel properties (Folkenberg *et al.* 2006a). Our work showed that G' of the batches fermented with capsular-ropy strain was lower than that of other examined fermented milk

Table 5.2 Rheological and physical properties of yoghurt bases fortified with sucrose (0, 15 30, 45 mM) and calcium (0, 3, 6, 9 mM) and acidified using glucono-δ-lactone at 42 °C

Sucrose, (mM)	$CaCl_2$ (mM)	Loop area*, (unit)	G'*, (Pa)	Consistency Index*, (Pa s)	Flow Behaviour Index*	Syneresis*, (g/100 g)
0	0	732^{aA}	834^{bA}	7.61^{aA}	0.79^{abA}	38.54^{aA}
	3	822^{aA}	659^{abA}	7.91^{aA}	0.85^{abA}	34.41^{aA}
	6	828^{aA}	535^{aA}	6.00^{aA}	0.74^{aA}	36.47^{aA}
	9	650^{aA}	505^{aA}	7.08^{aA}	0.78^{aA}	36.18^{aA}
15	0	747^{abA}	806^{aA}	6.10^{aA}	0.76^{aA}	33.42^{aA}
	3	824^{abA}	687^{aA}	6.62^{aA}	0.77^{aA}	35.26^{aA}
	6	975^{bA}	1267^{bB}	7.22^{aA}	0.78^{aA}	35.84^{aA}
	9	657^{aA}	838^{aAB}	7.61^{aA}	0.80^{aA}	35.99^{aA}
30	0	846^{aA}	879^{bA}	10.04^{abA}	0.83^{aA}	35.50^{abA}
	3	1077^{bA}	829^{abA}	13.21^{bA}	0.88^{aAB}	33.73^{aA}
	6	704^{aA}	656^{abA}	8.05^{aA}	0.81^{aA}	38.61^{abA}
	9	707^{aA}	574^{aA}	7.70^{aA}	0.80^{aA}	39.07^{bB}
45	0	819^{aA}	861^{aA}	8.95^{aA}	0.80^{aA}	35.84^{aA}
	3	792^{aA}	803^{aA}	9.36^{aA}	0.80^{aA}	36.42^{aA}
	6	768^{aA}	636^{aA}	8.87^{aA}	0.83^{aA}	38.95^{aA}
	9	647^{aA}	719^{aA}	6.19^{aA}	0.76^{aA}	36.07^{aA}
SEM**		103.5	103.6	1.67	0.03	1.89

*Capital letter superscripts in a column indicate significant difference (P<0.05) among samples at different sucrose concentrations, at a particular calcium concentration. **SEM - pooled standard error of the mean, P<0.05. Small letter superscripts in a column denote significant difference (P<0.05) among samples at different calcium concentrations, at a particular sucrose concentration.

Table 5.3 Rheological and physical properties of fermented milk batches fortified with calcium (0, 3, 6, 9 mM) and fermented using *Streptococcus thermophilus* ST 1275 and ST 285 at 42 °C, and addition of sucrose (0, 15 30, 45 mM)

Type of Acidulant	Sucrose, (mM)	$CaCl_2$, (mM)	Titratable Acidity*, (g/100 g lactate)	Δ CFU*, (CFU/ml)	EPS*, (mg/kg fermented milk)
ST 1275	0	0	1.09^{aA}	2.38^{aB}	653.5^{bAB}
		3	1.24^{aA}	2.54^{aB}	228.8^{aA}
		6	1.28^{aA}	2.82^{abC}	489.8^{bAB}
		9	1.26^{aA}	3.08^{bB}	614.4^{bB}
	15	0	1.32^{aA}	2.24^{aAB}	190.4^{aA}
		3	1.47^{aAB}	2.34^{aAB}	358.5^{aA}
		6	1.47^{aAB}	2.53^{aB}	336.2^{aAB}
		9	1.46^{aAB}	2.25^{aA}	533.2^{aB}
	30	0	1.62^{aB}	2.22^{aAB}	528.8^{abAB}
		3	1.69^{aB}	2.32^{aAB}	459.7^{aA}
		6	1.66^{aB}	2.32^{aA}	250.1^{aA}
		9	1.71^{aB}	2.16^{aA}	525.5^{abB}
	45	0	1.43^{aB}	2.07^{aA}	352.7^{aA}
		3	1.53^{aA}	2.11^{aA}	282.7^{aA}
		6	1.44^{aB}	2.23^{aA}	433.9^{aAB}
		9	1.49^{aAB}	2.11^{aA}	249.9^{aA}
ST 285	0	0	1.29^{aA}	1.12^{aAB}	231.9^{aA}
		3	1.42^{aA}	1.97^{cC}	409.6^{abA}
		6	1.18^{aA}	1.57^{bC}	347.5^{abA}
		9	1.39^{aA}	1.10^{aB}	516.7^{abA}
	15	0	1.43^{aAB}	1.39^{bB}	740.2^{bCB}
		3	1.66^{aAB}	1.21^{abB}	364.5^{aA}
		6	1.84^{aB}	1.05^{aB}	466.2^{aA}
		9	1.71^{aB}	1.06^{aA}	299.1^{aA}
	30	0	1.55^{aB}	0.90^{aA}	535.5^{abBC}
		3	1.43^{aA}	0.81^{aA}	220.6^{aA}
		6	1.49^{aB}	1.36^{bC}	285.1^{aA}
		9	1.58^{aAB}	0.85^{aA}	516.5^{abA}
	45	0	1.56^{aB}	1.02^{bAB}	480.9^{aB}
		3	1.69^{aB}	0.98^{abAB}	408.4^{aA}
		6	1.71^{aB}	0.72^{aA}	618.5^{aAB}
		9	1.54^{aAB}	0.80^{abA}	415.0^{aA}
SEM**			0.09	0.10	84.4

*Capital letter superscripts in a column indicate significant difference (P<0.05) among samples at different sucrose concentrations, at a particular calcium concentration for one type of acidulant.

**SEM - pooled standard error of the mean, P<0.05. Small letter superscripts in a column denote significant difference (P<0.05) among samples at different calcium concentrations, at a particular sucrose concentration, for one type of acidulant.

batches (Hassan *et al.* 2001) and less responsive to the addition of calcium or sucrose. However, the gel containing capsular EPS showed higher G' which may be due to localization of EPS within the gel pores (Folkenberg *et al.* 2006b).

Consistency index can be defined as the apparent viscosity at shear rate of 1/s (Rao 1999). In our experiment, the consistency index of all samples was influenced significantly ($P<0.05$) by the interaction of acidulant type and sucrose concentration (Tables 5.2 and 5.3). Sucrose addition increased the consistency index, especially in fermented milk containing capsular-ropy EPS. At lower concentrations of sucrose, consistency indices of the three types of acidified milk gels were generally similar. However, at high concentrations of sucrose, GDL acidified milk had a lower ($P<0.05$) consistency index than those of the culture ferments whose values were not significantly different ($P>0.05$). The flow behaviour index shows the extent of deviation from Newtonian behaviour, with values less than 1 indicating shear-thinning properties and susceptibility to structural breakdown upon application of hydrodynamic force (Rao 1999). Our experiments showed that flow behaviour was significantly ($P<0.05$) affected by sucrose concentration as was its interaction with the type of acidulant. Higher sucrose concentrations increased flow behaviour index, especially in fermented milk containing capsular-ropy EPS (Table 5.2 and 5.3).

Shear-thinning may reflect the changes in entanglement occurring in the structure (Macosko 1994), where shear breaks down the aggregates and further reduces their size. In our study, all types of acidified gel showed an immediate and large reduction of apparent viscosity upon shearing. At the beginning of shearing before attaining the plateau, shear stress increased, likely due to dominance of structural rebuilding over structural breakdown. The rate of the structure rebuilding then declined at higher shear rates to reach a plateau (Da Cruz *et al.* 2002a). Fermented milk produced with capsular-ropy EPS producing strain showed higher shear stress at the plateau range in comparison to the other two acid gels. Higher shear stress values in this range have previously been attributed to greater interactions between EPS and caseins (Girard & Schaffer-Lequart 2007). Higher calcium concentrations increased the susceptibility to structural breakdown caused by shear (Figure 5.1A,B, 5.2A,B, 5.3A,B). Sucrose, on the

other hand, apparently increased the shear stress plateau in all types of acid gels and thus appeared to improve rigidity. The effect of sucrose on improving shear-resistance by increasing relaxation time has been reported earlier (Macosko 1994). Similarly, a greater shear stress magnitude before its maximum has been related to a stronger bond between EPS and the casein network (Skriver et al., 1993).

Plots of apparent viscosity as a function of applied shear rate (Figure 5.1A,B, 5.2A,B, 5.3A,B) indicated that all types of acid gels displayed shear-thinning and thixotropic behaviour. Higher calcium concentration increased susceptibility to shear-induced breakdown. In contrast, addition of sucrose increased the resistance of all gels to shearing and prolonged the time required to attain the plateau.

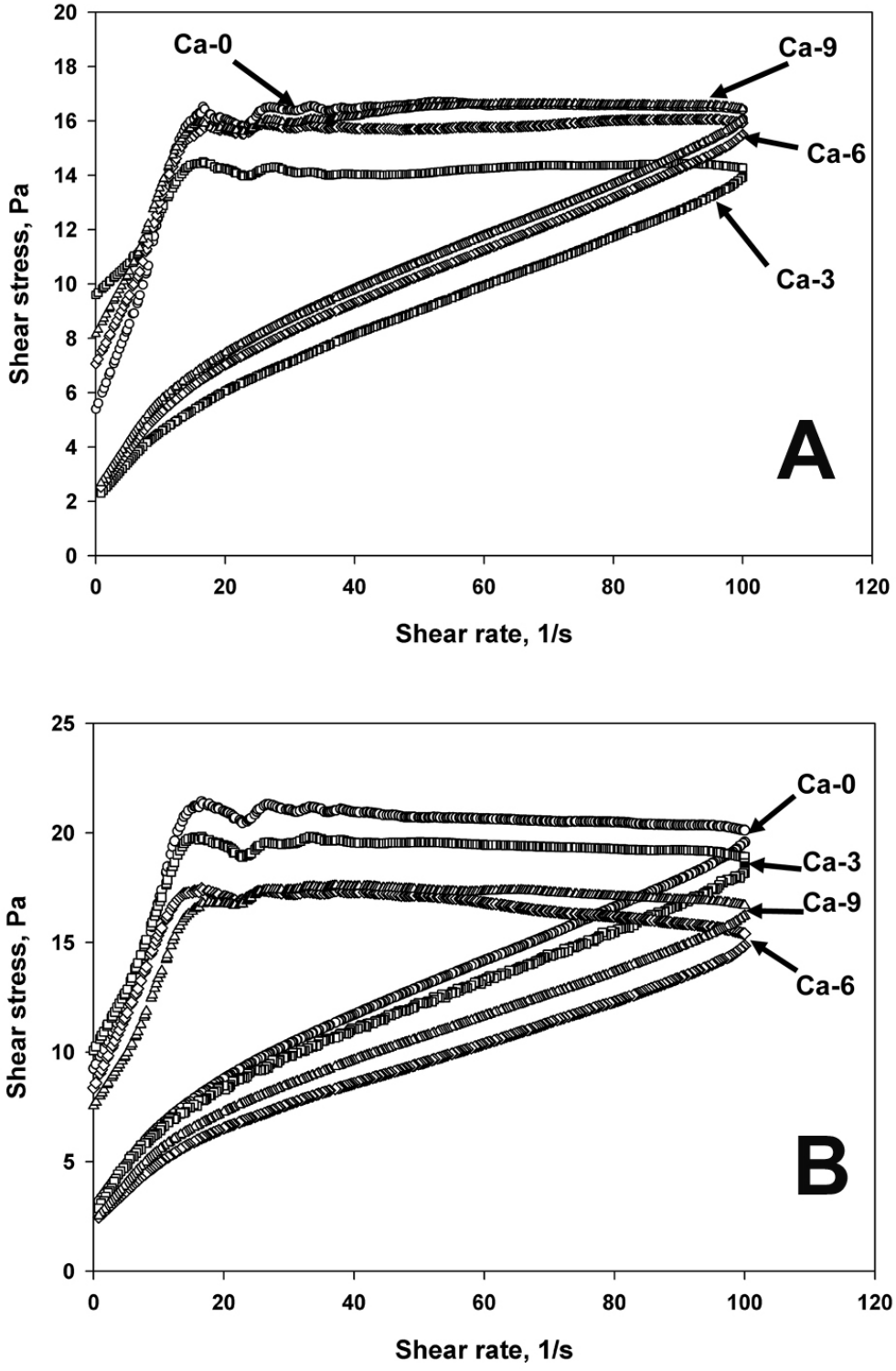


Figure 5.1 The effect of calcium fortification on the flow behaviour of the fermented milk batches prepared using glucono-δ-lactone (GDL) as the acidifier and without (A) or with 45 mM (B) sucrose addition. (The legends depicted by the arrows present the calcium chloride (Ca) concentration of 0, 3, 6 or 9 mM). The data present the average of three independent determinations.

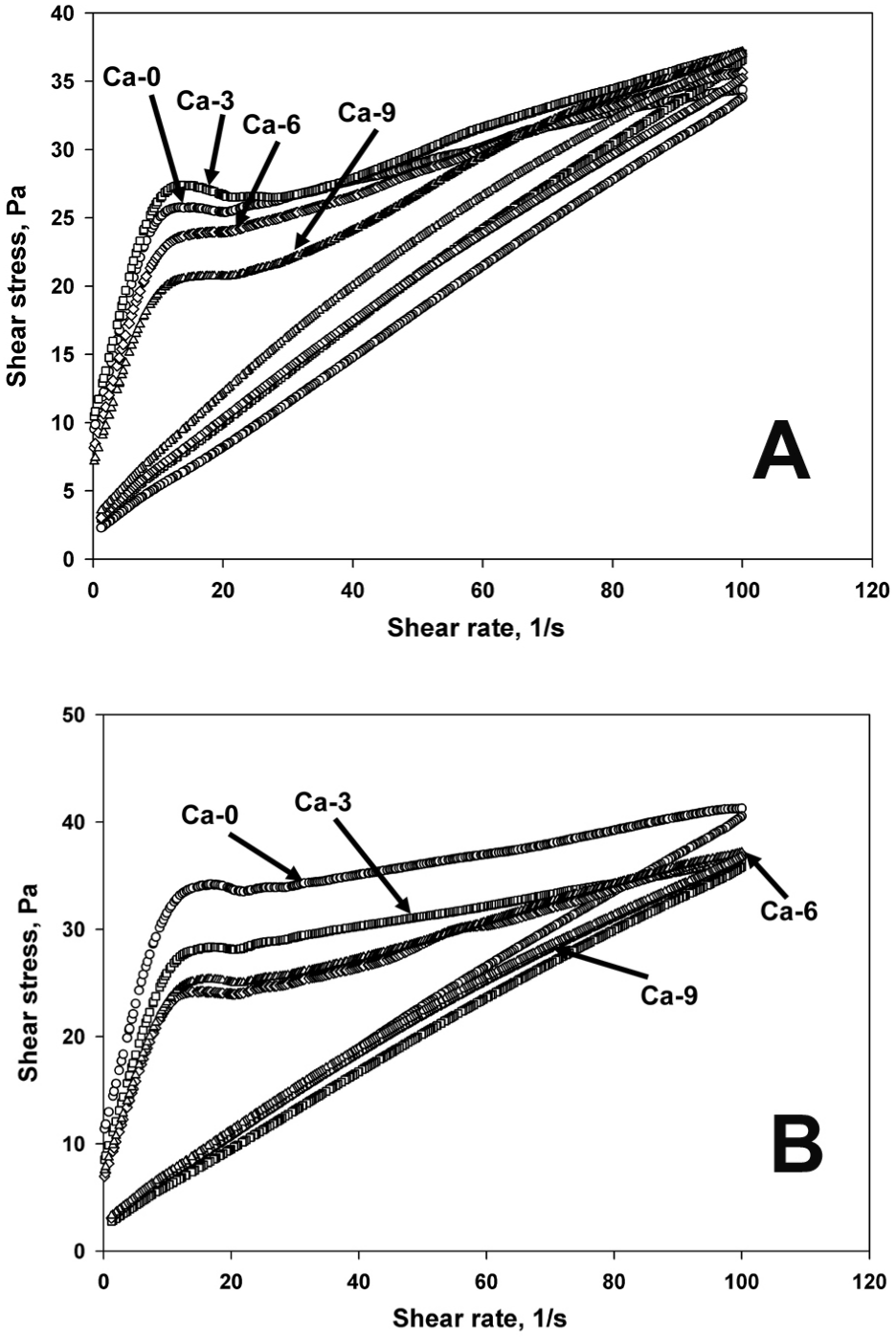


Figure 5.2 The effect of calcium fortification on the flow behaviour of the fermented milk batches prepared using capsular-ropy strain of *Streptococcus thermophilus* and without (A) or with 45 mM (B) sucrose addition. (The legends depicted by the arrows present the calcium chloride (Ca) concentration of 0, 3, 6 or 9 mM). The data present the average of three independent determinations.

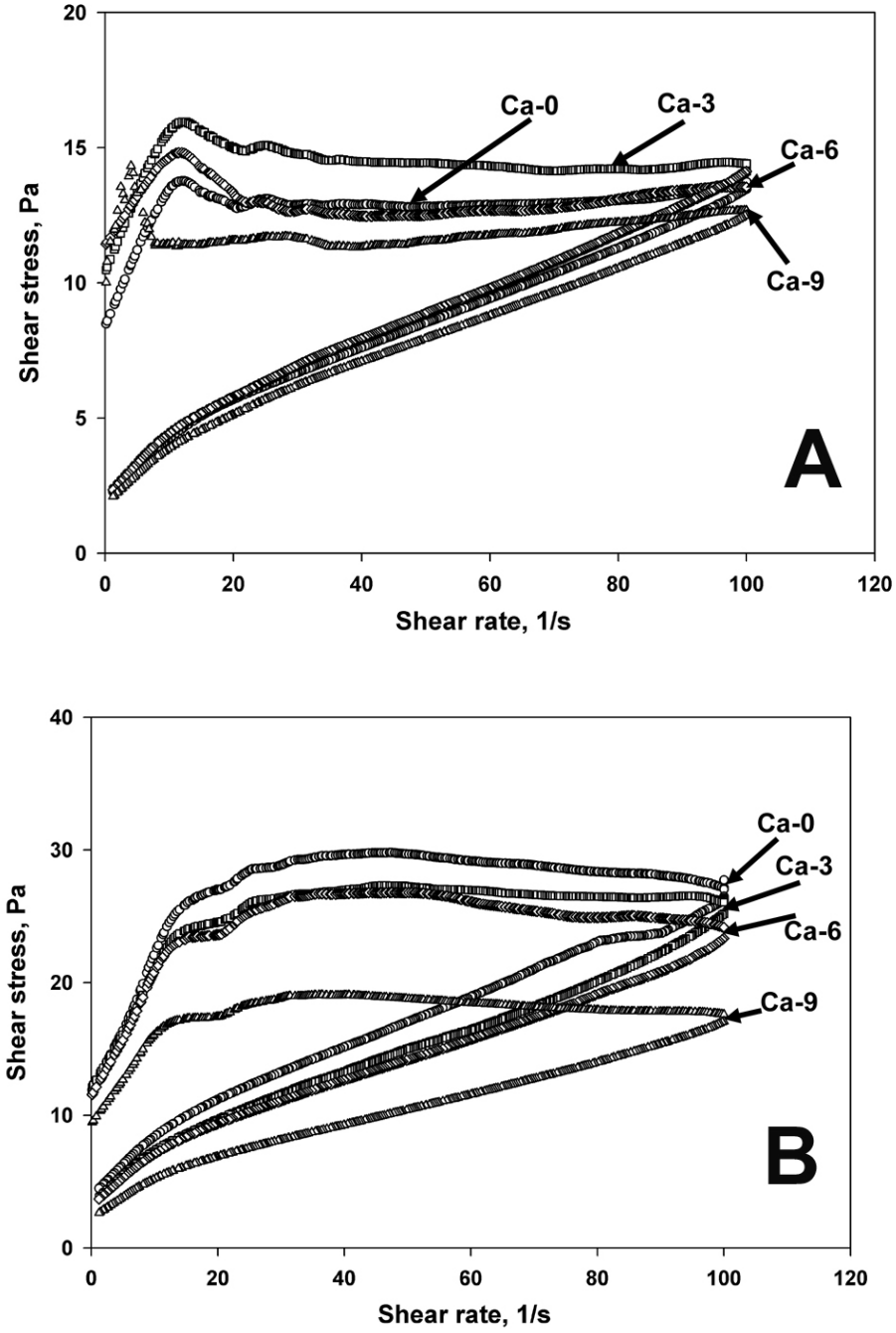


Figure 5.3 The effect of calcium fortification on the flow behaviour of fermented milk batches prepared using capsular strain of *Streptococcus thermophilus* and without (A) or with 45 mM (B) sucrose addition. (The legends depicted by the arrows present the calcium chloride (Ca) concentration of 0, 3, 6 or 9 mM). The data present the average of three independent determinations.

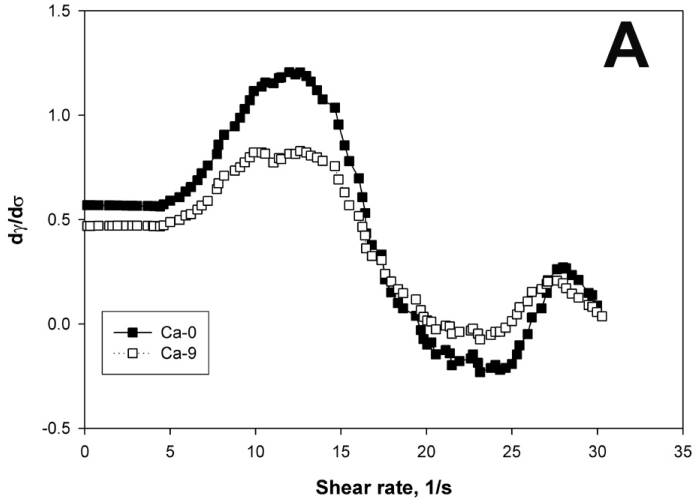
Similarly, a greater shear stress magnitude before its maximum has been related to a stronger bond between EPS and the casein network (Skriver *et al.* 1993).

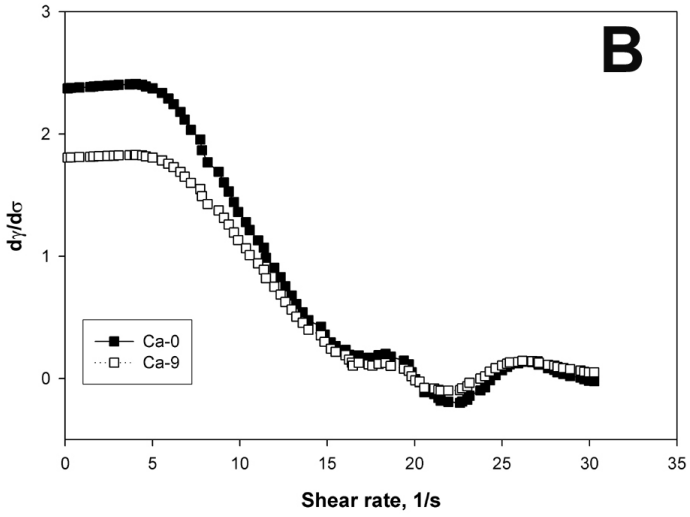
Plots of apparent viscosity as a function of applied shear rate (Figure 5.1A,B, 5.2A,B, 5.3A,B) indicated that all types of acid gels displayed shear-thinning and thixotropic behaviour. In contrast, addition of sucrose increased the resistance of all gels to shearing and prolonged the time required to attain the plateau. In our experiments, hysteresis was also significantly (P<0.05) reduced by calcium concentration in some samples (Table 5.2a,b). Fermented milk containing capsular-ropy EPS appeared to be more sensitive to calcium addition compared to the other batches (Table 5.2 and 5.3) as shown by a greater reduction of the loop area in response to increases in calcium concentration. But the loop area of the GDL-acidified milk appeared unaffected by sucrose addition (Table 5.2 and 5.3).

Fermented milk produced with the EPS strains showed larger loop areas than artificially acidified milk, as has been reported by others (Hassan *et al.* 2003, Folkenberg *et al.* 2006a). Greater hysteresis indicates poorer structural rebuilding after shear-induced breakdown an observation which has been related to incompatibilities between caseins and EPS (Folkenberg *et al.* 2006a). The addition of calcium to fermented milk base appears to have facilitated the structural rebuilding of the acid gel (Figure 5.1, 5.2, 5.3); however, its addition created a more brittle gel in the presence of capsular EPS as indicated by viscosity curves reaching a plateau sooner. This type of gel was poorly resistant to mechanical damage (Folkenberg *et al.* 2006b). Sucrose is believed to form a hydrophilic layer on the casein micelle surface strengthening the association of caseins during acidification (Schorsch *et al.* 1999a) and it may also improve solubility of hydrocolloids (Schorsch *et al.* 1999a). The effect of sucrose we found not to be as evident as that of calcium and probably depended on the type of EPS present in the system.

The second maximum derivative of shear-stress against shear-rate indicates some type of ‘yield’ before shear-induced structural breakdown occurs (Jaros *et al.* 2007). The magnitude of this yield in fermented milk with capsular- ropy EPS was greater than that of other batches (Figure 5.4A,B). The addition of calcium reduced this maximum. Thus, the first derivative of an upward thixotropy curve

plotted against shear rate (Jaros et al., 2007) indicates a more shear-resistant nature of gels containing capsular-ropy EPS than those acidified with GDL or with the capsule-forming ST strain. Plots of apparent viscosity against shear stress described the influence of shear stress when a material starts to flow (Figure 5.5A,B). Thus, the inflection points in the curves show the apparent yield stress (Jaros et al., 2007). Apparent yield stress of fermented milk produced with capsular-ropy EPS strain was higher than that of milk acidified with GDL or fermented by the capsular strain (Figure 5.5A,B). Addition of calcium or sucrose reduced the apparent yield stress of all batches. Fermented milk with capsular-ropy EPS exhibited greater resistance to applied stress, which may be related to a stronger network. Higher values of apparent yield stress have been correlated to higher degree of cross-linking of milk protein in acidified milk (Jaros et al., 2007). Addition of calcium to fermented milk base appeared to have a similar effect to that of sucrose. As the apparent yield stress declined by the addition of either sucrose or calcium, both additives may increase the susceptibility of gels to flow during shearing.





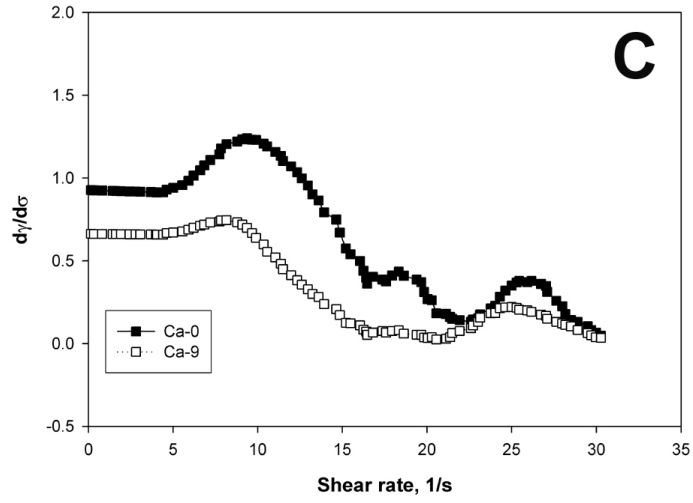


Figure 5.4 The first derivative of the average upward shear stress – shear rate profiles of the fermented milk batches supplemented with 45 mM sucrose and 0 or 45 mM $CaCl_2$ acidified using GDL (A), capsular-ropy strain (B) or capsular strain (C) of *Streptococcus thermophilus*.

calcium reduced this maximum. Thus, the first derivative of an upward thixotropy curve plotted against shear rate (Jaros *et al.* 2007) indicates a more shear-resistant nature of gels containing capsular-ropy EPS than those acidified with GDL or with the capsule-forming ST strain.

Plots of apparent viscosity against shear stress describe the influence of shear stress when a material starts to flow (Figure 5.5A,B). Thus, the inflection points in the curves show the apparent yield stress (Jaros *et al.* 2007). Apparent yield stress of fermented milk produced with capsular-ropy EPS strain was higher than that of milk acidified with GDL or fermented by the capsular strain (Figure 5A,B). Addition of calcium or sucrose reduced the apparent yield stress of all batches. Fermented milk with capsular-ropy EPS exhibited greater resistance to applied stress, which may be related to a stronger network. Higher values of apparent yield stress have been correlated to higher degree of cross-linking of milk protein in acidified milk (Jaros *et al.* 2007). Addition of calcium to fermented milk base appeared to have a similar effect to that of sucrose. As the apparent yield stress declined by the addition of either sucrose or calcium, both additives may increase the susceptibility of gels to flow during shearing.

The extent of syneresis was greatly affected ($P<0.05$) by the type of acidulants and their interaction with sucrose. Generally, the GDL acidified gel had the greatest degree of syneresis, followed by the capsular-EPS and capsular-ropy EPS fermented milks, respectively (Table 2a,b). The effect of calcium addition on the extent of syneresis was not apparent ($P>0.05$). Others have also found that ropy EPS has a greater ability to retain serum, resulting in low syneresis (Folkenberg *et al.* 2006b, Lucey *et al.* 1998). The positive correlation between G' and syneresis seemed to relate to gel compaction causing whey expulsion (Haque *et al.* 2001, Torre *et al.* 2003). The GDL and capsular EPS containing acid gels appeared to be more compact than that containing capsular-ropy EPS. Addition of sucrose reduced syneresis of gels, especially those produced by bacterial cultures. As previously shown, at acidic pH, sucrose increased dehydration of GDL-acidified milk protein, thus improving protein-protein association, but excluding water from the network (Belyakova *et al.* 2003).

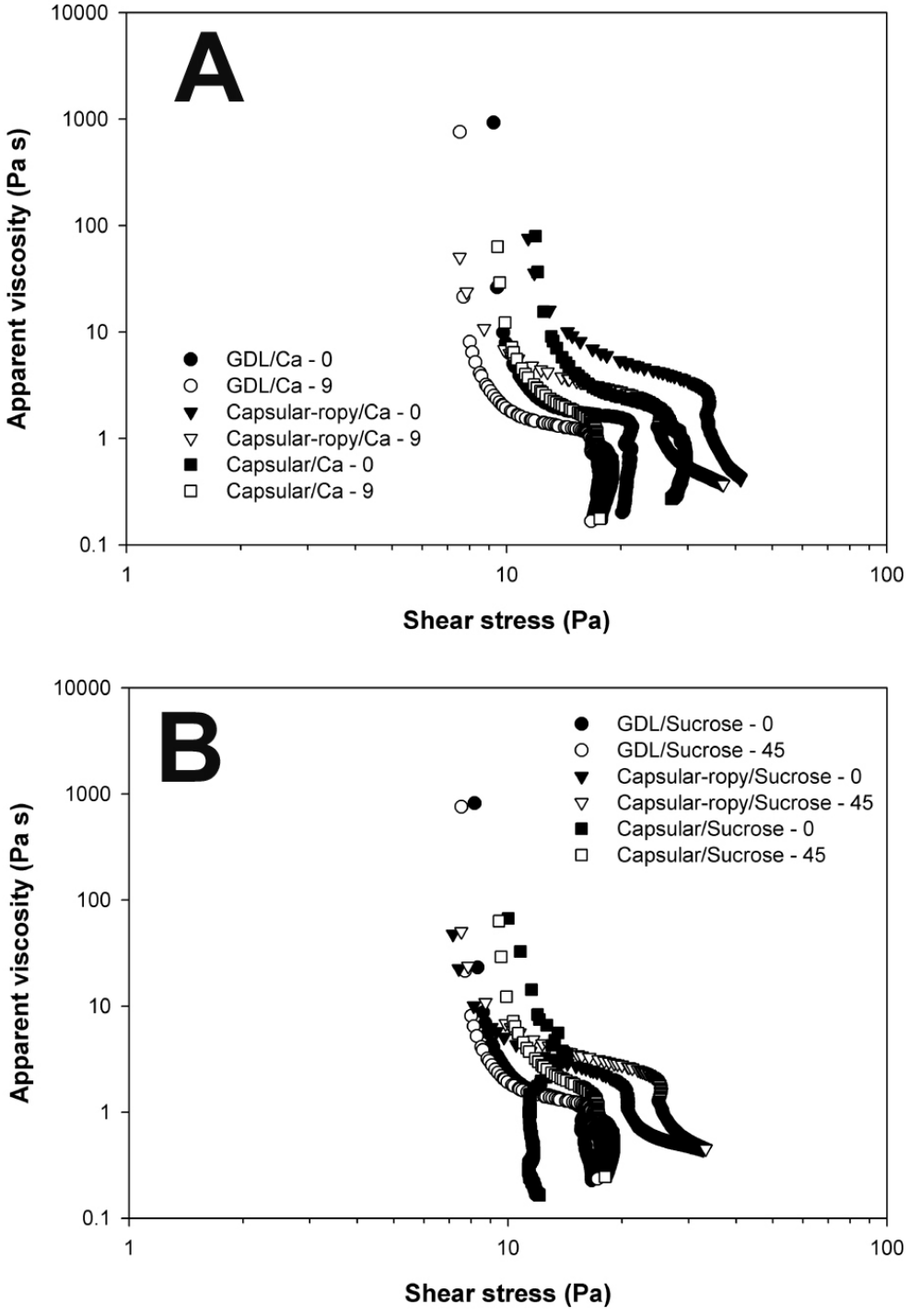


Figure 5.5 Plots of the apparent viscosity versus shear stress for fermented milk batches prepared by direct acidification by GDL or fermentation by capsular-ropy or capsular strains of *Streptococcus thermophilus* and supplemented with (A) 45 mM sucrose and 0 or 9 mM $CaCl_2$ or (B) 9 mM $CaCl_2$ and 0 or 45 mM sucrose.

Similar characteristics as EPS in dispersion was still noticeable in this experiment, where better water binding in capsular-ropy EPS caused less syneresis in the corresponding fermented milk. The tendency towards lower molecular mobility and higher relaxation time of capsular-ropy EPS dispersion also gave shear-resistant characteristics of fermented milk made using the capsular-ropy EPS producer, with or without calcium and sucrose. The addition of calcium seems to magnify the extent of incompatibility between EPS and casein, causing the fermented milk with capsular-ropy EPS became even less solid and that with capsular EPS became more brittle, as compared to fermented milk without calcium. Thus, in calcium-fortified fermented milk, the character of EPS type similar to that shown in dispersion was still evident.

Compared to fermented milk without addition of calcium reported in Chapter 4, calcium-fortified fermented milk showed more compact gel as indicated by more syneresis (around twice higher) combined with higher G' (around ten times higher when sucrose was not present). Calcium added fermented milk contained more lactic acid than normal fermented milk, which may due to pH lowering effect of hydrolysed $CaCl_2$. Shear-resistance of calcium-fortified fermented skim milk was also lower than normal fermented skim milk.

Our results showed that in most cases, fermented milk containing the capsular-ropy EPS produced gel characteristics different from those of milk bases acidified with GDL or fermented by the capsule-forming strain. Although it cannot be concluded from our work that the EPS type was responsible for the textural difference among samples, several previous reports have emphasized the significance of EPS in modifying the properties of gels (Folkenberg *et al*., 2006b, Girard and Schaffer-Lequart, 2007). In our study, EPS concentration at the end of fermentations was weakly correlated with all viscoelastic parameters, although there were some inconsistencies. For example, the concentration of the capsular-ropy EPS was negatively but weakly correlated to all viscoelastic measurements: G' ($r = -0.1738$), consistency index ($r = -0.3246$), and flow behaviour index ($r = -0.2218$). However, the concentration of capsular EPS was positively correlated to the same parameters, with $r = 0.2313$, 0.1624, 0.2374 for G', consistency, and flow behaviour index, respectively.

The correlation between the EPS concentration and syneresis was weakly positive in samples containing capsular-ropy EPS (r = 0.3899) but negative (r = -0.3932) when capsular EPS was present. A higher syneresis of the gel produced with the capsular strain noted by others (Folkenberg *et al*., 2006a) may be associated with a structure-weakening effect of EPS (Lucey *et al.*, 1998a). The observations reported in this work may provide some insight into approaches to modify textural characteristics of different fermented milk products manufactured from the yoghurt base using artificial acidifiers or different EPS-producing cultures with the addition of calcium and sucrose. It may also provide an opportunity for development of a wider range of calcium-fortified dairy based products.

5.4 Conclusion

The textural and physical properties of a yoghurt base acidified by a single, capsular-ropy EPS producing strain of *S. thermophilus* were more profoundly affected by the addition of calcium or sucrose in comparison to that acidified with either GDL or the capsule-forming strain. Added calcium appeared to have detrimental and weakening effects on the properties of acid gels. On the other hand, sucrose addition strengthened and improved certain textural parameters. But calcium and sucrose increased thixotropy and reduced yield stress. The results underline the importance of understanding the role of different types of EPS in the structural formation of acid set gel containing calcium and sucrose.

Result of experiment presented in this chapter highlighted a possible calcium fortification in fermented milk, and that the subsequent gel weakening can be reduced by either type of EPS. Although the response of both EPS type towards addition of calcium and sucrose was similar, the gel of fermented milk prepared with capsular-ropy EPS producer maintained less firm, less syneresis, and more shear-resistant characteristics compared to that fermented with capsular-EPS producer. In the next experiment, the ability of capsular-ropy EPS producing strain to preserve the texture of whey protein-fortified fermented milk is studied.

6 Physico-Chemical and Rheological Properties of Calcium-Fortified Low-Fat Fermented Milk Supplemented with Whey Proteins

6.1 Introduction

It was apparent from the previous chapter that EPS produced by either the *S. themophilus* strain was capable of reducing the deteriorating effect of added calcium and sucrose. In this chapter, the potential of the EPS to attenuate the structure of acid gels was further assessed upon addition of two different types of whey-protein products. Whey proteins used in the experiment were whey protein concentrate (WPC) and whey protein isolate (WPI).

Whey proteins, a by-product of cheese manufacturing, have been extensively used to improve functional, nutritional, therapeutic, and physiological properties of various food products. They influence textural properties of various food products (Christiansen et al., 2006, Davis and Foegeding, 2007, Innocente et al., 2002, Morr et al., 2003, Serdaroglu, 2006, Yildiz-Turp and Serdaroglu, 2008, in press) mainly due to water- as well as protein- binding, emulsifying, and gelling characteristics in various food products. Their functional properties have been used to improve colour, firmness, fracturability and reduce the oil content in deep-fried poultry products (Dogan et al., 2005), to prevent cook loss and improve texture profiles in meat batters (Barbut, 2006), to enhance homogeneity of the crystal size, coldness intensity, and creaminess in ice cream (Ruger et al., 2002), and to create more homogenous size of the air bubbles in whipped-frozen emulsions (Relkin and Sourdet, 2005). The nutritional and health related benefits of whey proteins include provision of essential amino acids in infant formula,

biostatic and antibacterial activity (De Witt, 1998), weight control due to their calcium content (Pilvi et al., 2006), alleviation of stress related ailments (Schaafsma, 2006a) and enhanced satiation during weight loss program (Schaafsma, 2006b). Therefore, the inclusion of these highly valuable dairy ingredients into various food products would certainly improve healthy perception of foods.

The effects of whey protein addition during yoghurt fermentation on the yoghurt texture varies, apparently depending on factors such as whey protein type (Vasbinder et al., 2004), degree of whey protein denaturation (Sodini et al., 2006), point of whey protein addition such as before or after pasteurisation (Schorsch et al., 2001) and casein to whey protein ratio (Puvanenthiran et al., 2002). The interactions between the whey proteins in the form of native whey protein isolate (WPI) and casein led to weakening of the acid gel structure (Patocka et al., 2006). On the other hand, the WPC supplementation to yoghurt improved the yoghurt gel strength (Isleten and Karagul-Yuceer, 2006, Remeuf et al., 2003) especially after whey protein denaturation. The denaturation of whey proteins may also adversely (Sodini et al. 2006) or positively (Isleten and Karagul-Yuceer, 2006) affect syneresis, the expulsion of whey upon prolonged storage.

Calcium is added to milk products not only for nutritional but also for functional purposes. Biologically active phosphopeptides as well as other compounds in milk can improve calcium bioavailability, rendering it as effective means for calcium fortification (Kwit and Kwong, 2004). Moreover, the calcium fortification in milk improved solubility, dialysis, transport and uptake rate of calcium, thus increasing its bioavalability (Perales et al., 2006), as well as enhancing its heat stability (Singh et al., 2007). Therefore, supplementation of both whey protein and calcium (Sanchez-Hidalgo et al., 2000) may synergistically improve nutritional status of certain food products. Although calcium is considered as an inhibitor of Fe and Zn absorption, the adverse effect of Ca fortification may be alleviated by high protein content (Mendoza et al., 2004). Adding low concentrations of $CaCl_2$ increased strength of both WPC and WPI heat-set gels (Lorenzen and Schrader, 2006) due to enhancement of whey protein unfolding and stronger bonding among formed aggregates (Ju and Kilara, 1998,

Lorenzen and Schrader, 2006). Similarly, a gel strengthening may also be caused by the presence of EPS. As EPS are generally 'non-adsorbing' polymers to the protein network, with a weak negative charge (Tuinier et al., 2003), it may perform a 'depletion layer' surrounding every particle when mixed with colloidal protein. This would cause the protein particles to coalesce, causing more rigid structure. The information on the combined effects of the calcium addition and whey protein type on the textural and physico-chemical properties of yoghurt fermented with an EPS-producing culture, is rather limited. Therefore, we aimed with this work to assess the properties of calcium and whey supplemented yoghurt produced with an EPS-producing strain of *Streptococcus thermophilus* using the response surface methodology.

6.2 Materials and methods

6.2.1 Fermented milk cultures

Fermented milk batches were fermented by an EPS-producing strain of *Streptococcus thermophilus* ASCC 1275, which produces mixed EPS (capsular and ropy) (Zisu and Shah, 2003). The strain was obtained from the Australian Starter Culture Research Center (ASCRC, Werribee, Victoria, Australia). Frozen (-80 ^{o}C) glycerol stock of the culture was activated by incubating it twice in 30 mL sterile 14 g/100 g skim milk at 42 °C for 24 hours, before application in the fermented milk manufacturing.

6.2.2 Experimental design and statistical analysis

The experimental design consisted of twelve combinations according to a second order central composite design with two factors at five levels each, and $CaCl_2$, WPC or WPI concentrations as independent variables (Table 6.1). Every combination was at least replicated. The statistical analysis of the data was carried out using the SAS System (SAS, 1996). The full term second order polynomial

response surface models were fitted to each of the response variables, according to the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon$$

Where β_0, β_1, …, β_{22} represented the estimated regression coefficients, with β_0 being the constant term; β_1, β_2 represented the linear effects, β_{11}, β_{22} the quadratic effects; β_{12}, β_{22} the interaction effects; ε was the random error; and X_1, X_2, were the independent coded variables (Myers and Montgomery, 2002). A simple correlational analysis was performed to reveal connection among parameters.

6.2.3 Preparation of fermented milk batches

Low heat-treated skim milk powder (Murray Goulburn Co-operative Co. Ltd., Brunswick, Victoria, Australia) was used alone or in conjugation with whey protein preparations as fermented milk mixes. A portion of skim milk solids was replaced with appropriate amount of whey proteins. The powders were reconstituted in Milli-Q™ water to achieve 14 g/100 g total solid content and subsequently pasteurized at 90 °C for 5 minutes holding time in a water bath. The appropriate amount of Ca was added prior to pasteurization in the form of $CaCl_2$. Fermented milk bases were then cooled to 42 °C and inoculated with 1 mL/100 mL of *S. thermophilus*. Proportions of skim milk solids, whey protein concentrate (WPC 80 instantised, Wynpro, United Milk Tasmania) or whey protein isolate (Alacen 895, Fonterra, Laverton, Victoria, Australia) and added calcium in samples were adjusted according to the central composite design in Table 6.1. After inoculation, the pasteurized fermented milk bases were poured aseptically into sterile 100 mL plastic containers, which were subsequently placed in an incubator preset at 42 °C. The process was terminated when pH reached 4.5 by

Table 6.1 Experimental design and levels of factors in natural and coded values

Factors	Coded factor				
	-2	-1	0	1	2
	Natural values				
Calcium concentration (mM)	0	2.5	5	7.5	10
WPC or WPI concentration (g/100 g)	0	1.25	2.5	3.75	5

Run	Factors	
	Calcium concentration (mM)	WPC or WPI concentration (g/100 g)
1	2.5	1.25
2	7.5	1.25
3	2.5	3.75
4	7.5	3.75
5	0	2.5
6	10	2.5
7	5	0
8	5	5
9	5	2.5
10	5	2.5
11	5	2.5
12	5	2.5

immediate transfer into a cold room (4 °C). After overnight cold storage, randomly selected samples were examined for their rheological properties. The remaining samples were similarly assessed after 21 days.

6.2.4 Microbial and chemical properties of fermented milk

The enumeration of *S. thermophilus* strains followed established procedures reported previously (Donkor et al., 2006) for fresh sample (day-0) and storage samples (day-21). Briefly, approximately 1 g of fermented milk sample weighed precisely was resuspended in 0.1 g/100 g peptone water and serially diluted to required levels. Such diluted samples were then plated on M17 agar (Merck Pty Ltd., Kilsyth, Victoria, Australia) and incubated aerobically at 42 °C for 48 h. The results obtained as means of four independent observations were expressed as a log of colony forming units per g of fermented milk.

The crude EPS was determined following already established methodology with some modifications, which was reported to be highly reliable (Rimada and Abraham, 2006). Approximately 30 g of fermented milk was first centrifuged (Model J2-HS, Beckman, Fullerton, California, USA) at 11000 × *g* at 4 °C for 4 min. The supernatant was collected and combined with two volumes of chilled ethanol and stored at 4 °C overnight. This was followed by centrifugation at 2000 × *g*, 4 °C for 15 min (model RT7, Sorvall, DuPont, Newtown, Connecticut, USA) to enhance the EPS precipitation. Collected EPS-containing precipitate was then dissolved in 10 mL of distilled water, followed by the addition of 250 μL 80 g/100 g trichloroacetic acid for precipitation of the remaining proteins. After storing the mixture overnight at 4 °C, it was centrifuged at 700 × *g*, 4 °C for 15 min (Sorvall) to collect the EPS-containing supernatant. This procedure was repeated twice, where the final precipitate was dried at 55 °C under vacuum and then weighed and expressed as the crude EPS. The extent of syneresis during cold storage of the fermented milk batches was analysed by a centrifugation method previously reported (Jaros et al., 2002b) with slight modifications. For this test, the fermented milk batches were prepared by *in situ* fermentation in 50 mL centrifuge tubes (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA).

Upon termination of fermentation, the tubes were stored in a cold room and centrifuged (Sorvall) at 700 × *g* at 8 °C for 10 min on the following day or after 21 days of cold storage. The weight of the drained liquid was recorded and related to the initial weight of fermented milk with the degree of syneresis expressed as a percentage.

6.2.5 Rheological and physical properties of fermented milk

The rheological properties of the fermented milks were measured using a controlled-stress rheometer (Physica MCR 301, Anton Paar, GmbH, Germany), equipped with a temperature and moisture regulating hood and a cone and plate geometry (CP50-1, 50 mm diameter, 1° angle and 0.49 mm gap). The temperature was regulated by a Viscotherm VT 2 circulating bath and controlled with a Peltier system (Anton Paar). The temperature during all determinations was maintained constant at 5 °C with an accuracy of ± 0.1 °C. The data of all rheological measurements were analyzed with the supporting software Rheoplus/32 v2.81 (Anton Paar). The rheometer was calibrated every 60 days by motor adjustment and two oils with different viscosities as per manufacturer's instructions. The gap width was preset as per the hardware specifications (MCR301, Anton Paar).

Prior to loading, all samples were stirred gently with a spatula to eliminate thixotropy and different concentration (i.e. EPS) effects. About 10 g sample was loaded into the bottom plate with spoon. Any excess was then removed using spatula. The sample was then pre-sheared at a high shear rate of 500/s for 15 s followed by 300 s rest to allow for structural rebuilding. The dynamic oscillatory measurements were carried out over a range of frequencies from 0.1 to 10 Hz to determine elastic properties (storage modulus G') of the samples. The strain was maintained constant at 0.5% and was inferred from the linear viscoelastic region determined by amplitude sweep at a constant frequency (1 Hz). The hysteresis loops were generated by measuring the shear stress upon increasing shear rate from 0.1 to 100/s in 300 s (upward curve in the rheogram), then holding at 100/s for 5 s and finally decelerated from 100 to 0.1/s in 300 s (downward curve in the rheogram). The data from the upward curve of the shear cycle were also fitted to

Ostwald-de Waele power law model ($\tau = K\dot{\gamma}^n$), where τ represents shear stress (Pa), $\dot{\gamma}$ shear rate (1/s), while K and n are consistency factor (Pa s^n) and flow behaviour index, respectively.

The firmness of fermented milk gels was determined using a texture analyzer (TA-XT2plus, Stable Micro System Ltd., Surrey, UK), equipped with 30 kg load cell and 20 mm aluminium cylinder probe (P/20, Stable Micro Systems). The cross-head speed during measurements was set at 1 mm/s with the 50% compression. Every combination was replicated twice with two sub samplings each.

6.3 Results and discussion

6.3.1 Microbial and chemical properties

The concentration of the viable cells in all samples increased as expected during fermentations in a similar fashion between WPC- and WPI-supplemented fermented milk with 1.389-1.510 and 0.913-1.469 log cfu/mL, respectively. Statistically, the cell growth was not significantly (P>0.05) affected by either WPC or WPI concentrations (Table 6.2, 6.3). Interestingly, the calcium addition hindered substantially (P<0.01) the culture growth in the WPI supplemented fermented milk only (Table 6.3).

Table 6.2 Regression coefficients of the second-order polynomial model for the response variables (analysis has been performed using coded units) for WPC

Factors	Regression Coefficient						
	Log G' (mPa)	K ($mPa\ s^n$)	n	Firmness (g)	Syneresis (g/100 g)	EPS production (mg/kg fermented milk)	Cell growth, (Log CFU/g fermented milk)
Day-1							
Constant	2.950^{*}	4.600^{NS}	0.047^{*}	11.567^{*}	22.066^{***}	520.305^{*}	1.057^{*}
Ca	-0.187^{*}	8.272^{NS}	0.003^{**}	-0.313^{NS}	5.879^{**}	-188.230^{NS}	0.009^{NS}
WPC	0.593^{*}	67.441^{*}	0.017^{*}	1.599^{***}	0.175^{NS}	15.664^{*}	-0.205^{NS}
$(Ca)^2$	-0.009^{**}	-0.779^{***}	-0.0003^{*}	0.028^{NS}	-0.436^{**}	15.664^{NS}	0.004^{NS}
$(WPC)^2$	-0.061^{*}	-7.305^{*}	-0.002^{*}	-0.254^{**}	5.218^{NS}	19.723^{**}	0.067^{*}
Ca×WPC	-0.055^{*}	-4.717^{**}	-0.0007^{***}	-0.129^{NS}	0.168^{NS}	8.346^{NS}	-0.016^{NS}
R^2	0.813	0.910	0.919	0.770	0.705	0.7842	0.4355
F	2.652	36.54	41.25	12.03	8.60	13.08	6.48
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	0.0002
Day-21							
Constant	3.513^{*}	0.653^{***}	0.671^{*}	8.652^{*}	29.116^{*}	308.718^{*}	0.303^{NS}
Ca	0.092^{NS}	0.064^{NS}	0.029^{***}	-0.375^{NS}	4.293^{**}	-26.740^{*}	-0.130^{**}
WPC	0.484^{*}	1.115^{*}	-0.027^{NS}	1.244^{***}	-0.881^{NS}	-141.112^{*}	-0.037^{NS}
$(Ca)^2$	-0.005^{NS}	-0.001^{NS}	-0.002^{***}	-0.025^{NS}	-0.333^{**}	4.51^{*}	0.0003^{NS}
$(WPC)^2$	-0.065^{*}	-0.131^{*}	-0.003^{NS}	-0.327^{*}	-0.318^{NS}	24.222^{**}	-0.046^{*}
Ca×WPC	-0.030^{NS}	-0.090^{*}	0.004^{NS}	0.091^{NS}	0.358^{NS}	1.617^{NS}	0.047^{*}
R^2	0.669	0.952	0.853	0.785	0.753	0.979	0.519
F	3.408	71.08	20.88	13.15	10.96	163.96	9.05
Probability of *F*	0.0007	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

* significant difference at $P<0.001$; ** significant difference at $P<0.01$; *** significant different at $P<0.05$.

Table 6.3 Regression coefficients of the second-order polynomial model for the response variables (analysis has been performed using coded units) for WPI

Factors	Regression coefficients					
	Log G' (mPa)	K (mPa s^n)	n	Firmness (g)	Syneresis (g/100 g.)	EPS production (mg/kg fermented milk)	Cell growth (Log CFU/g fermented milk)
Day-1							
Constant	4.008*	0.765**	0.849*	8.906*	14.554^{NS}	211.478*	0.479^{NS}
Ca	-0.082***	-0.101^{NS}	-0.030^{NS}	-0.443^{NS}	9.511**	-15.480***	0.236**
WPI	-0.014^{NS}	0.356**	-0.142*	0.774^{NS}	9.112^{NS}	-76.600*	0.229^{NS}
$(Ca)^2$	-0.011*	0.002^{NS}	0.004*	-0.005^{NS}	-0.895*	2.463*	-0.019*
$(WPI)^2$	-0.029**	-0.061*	0.019*	-0.167**	-0.977^{NS}	8.761*	-0.040***
Ca×WPI	0.048*	-0.006^{NS}	0.002^{NS}	0.0233^{NS}	-0.797^{NS}	4.072***	0.0006^{NS}
R^2	0.895	0.870	0.831	0.832	0.795	0.943	0.428
F	2.626	24.10	17.71	17.88	5.28	62.33	6.29
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	0.0037	<0.0001	0.0002
Day-21							
Constant	4.139*	0.104^{NS}	0.508*	7.014*	45.507*	152.796*	-0.445*
Ca	0.040^{NS}	0.104^{NS}	0.017^{NS}	0.461^{NS}	2.085^{NS}	-2.999^{NS}	0.144*
WPI	0.176^{NS}	0.511*	0.089**	2.24*	-11.031**	-54.484**	0.468*
$(Ca)^2$	-0.011*	-0.011**	-0.006*	-0.073*	0.057^{NS}	0.912^{NS}	-0.009*
$(WPI)^2$	-0.046*	-0.055**	-0.014*	-0.369*	1.663**	4.234***	-0.056*
Ca×WPI	0.008^{NS}	-0.030^{NS}	0.005^{NS}	-0.034^{NS}	0.418^{NS}	4.416***	-0.029
R^2	0.758	0.813	0.935	0.830	0.901	0.896	0.730
F	2.636	15.67		5.28	32.56	31.12	22.6
Probability of *F*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*significant difference at $P<0.001$; **significant difference at $P<0.01$; ***significant different at $P<0.05$.

In fresh fermented milk, WPC and WPI additions significantly ($P<0.05$) affected the EPS production diametrically different way (Table 6.2, 6.3). Whiles the WPC supplementation improved it, EPI addition decreased it. As the EPS production in most cases was growth-coupled (Ruas-Madiedo et al., 2005b), the low EPS concentration in WPI fermented milk seemed to be in accordance with the limited cell growth. The production of EPS by a bacterial culture in a whey-based growth medium was only possible in the presence of hydrolized WPC (Briczinski and Roberts, 2002). In general, the estimated EPS production in the freshly prepared WPC-fermented milk (maximum of 542 mg/kg from combination of 0 /100 g WPC and 4.6 mM Ca) was higher than that produced in the WPI-fermented milk (maximum of 386.8 mg/kg from combination of ~1 g/100 g WPI and ~8.8 mM Ca). Statistically, the calcium addition in both WPC and WPI fermented milk decreased the EPS production, but was significant ($P<0.05$) only in WPI fermented milk (Table 6.2, 6.3). However, the apparent trend according to the model showed that calcium increased EPS production in both types of the supplemented fermented milk (Figure 6.1A, 6.2A). In the WPC fermented milk, increasing the Ca concentration improved the EPS production at any level of WPC. Calcium potentially improved cell growth as well as EPS production under low pH condition (Maccio et al., 2002). Interaction between whey protein and calcium positively affected EPS production in all fresh and storage fermented milk, but was only significant ($P<0.001$) for WPI fermented milk (Table 6.2, 6.3)

The storage time reduced the amount of the EPS in both WPC (estimated maximum of ~333 mg/kg from combination of 1.7 g/100 g WPC and 9.7 mM Ca) and WPI yoghurt (estimated maximum of 202 mg/kg from combination of 0 g/100 g WPI and 5.2 mM Ca) (graph not shown). In this case, the decline in the EPS concentration in the WPI fermented milk was less pronounced than that of the WPC fermented milk. The enzymatic degradation of the EPS beyond stationary phase was a common phenomenon observed (Deegest et al., 2002, Pham et al., 2000). The EPS may be incorporated in the culture metabolism when most of the nutrients in the medium were diminished (Tolstoguzov, 2003). Thus, the limited degradation of the EPS in WPI may be in conjunction with negative cell growth

during storage. Trend in EPS concentration of storage WPC yoghurt was somewhat similar to that of the fresh fermented milk, in which WPC at medium concentrations adversely affected EPS concentration. During storage of WPI fermented milk, this reduction in the EPS concentration occurred in the region of high WPI-low Ca concentration.

6.3.2 Rheological and physical properties

The viscoelastic properties of fermented milk are commonly examined using small amplitude oscillatory measurement as elastic moduli (G') to assess its solid-like character. In general, the estimated log G' of the freshly prepared WPC fermented milk was higher (maximum of ~4.3 log mPa at 3.9 g/100 g WPC and 0.91 mM $CaCl_2$) than that of the fresh WPI fermented milk (maximum of ~3.9 log mPa, at 1.6 g/100 g WPI and 4.2 mM calcium) (Figure 6.1A, 6.2A). As the statistical analysis revealed, the elastic modulus of fermented milks upon the WPC supplementation was greatly (P<0.05) increased by the WPC concentration (Table 6.2), which was in contrast to that of WPI fermented milk (Table 6.3). This effect in the fresh WPC fermented milk was diminished by high Ca concentration. The increasing effect of calcium concentration on G' of the fresh fermented milk was only significant (P<0.01) in WPC fermented milk (Table 6.2, 6.3) especially at its low concentration. In the WPI fermented milk, albeit statistically opposite effect of WPI on G', the G' was improved with the increase in the WPI concentration at high concentrations of Ca.

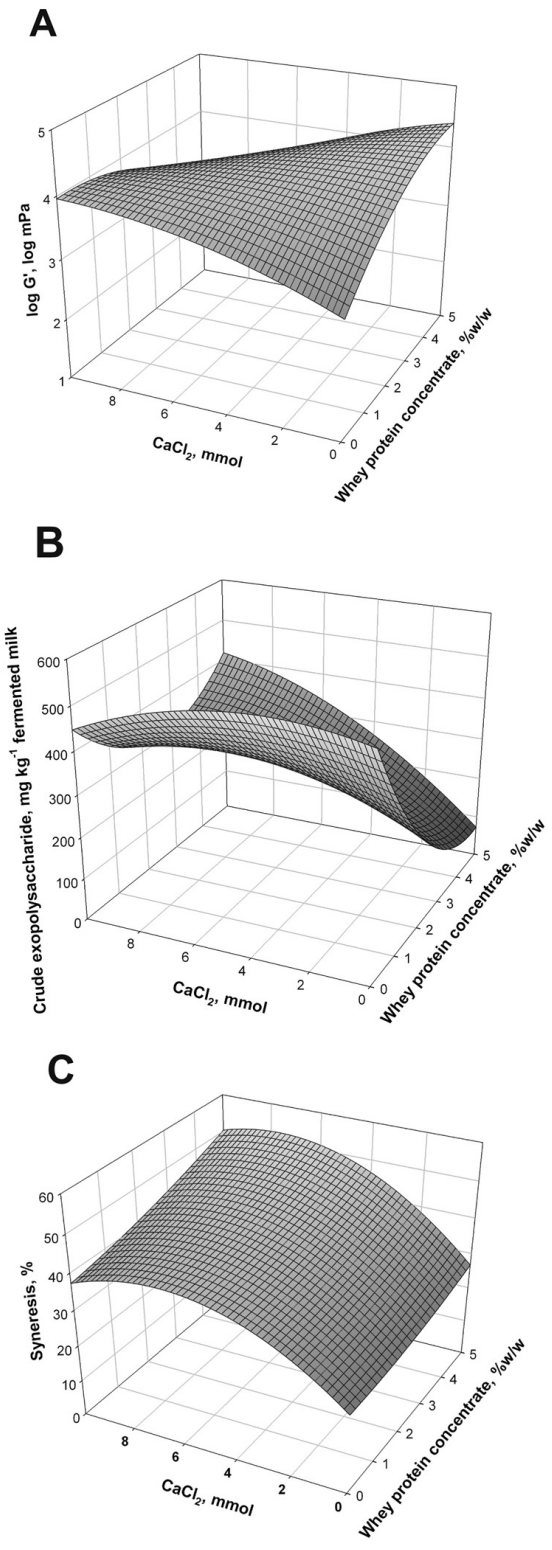


Figure 6.1 Typical estimated responses achieved by the response surface modelling showing the effects of the calcium chloride and whey protein concentrate additions on (A) the gel elastic modulus (G') (B) the EPS production by *Streptococcus thermophilus* and (C) the extent of the syneresis of the fermented milk batches after overnight cold storage.

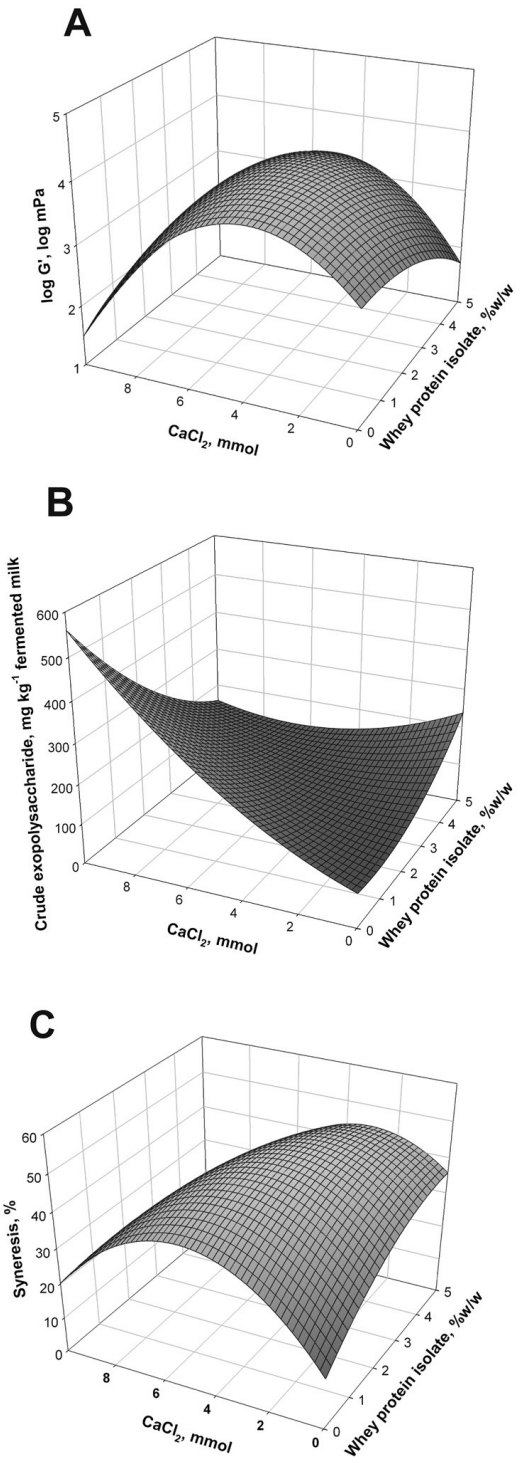


Figure 6.2 Typical estimated responses achieved by the response surface modeling showing the effects of the calcium chloride and whey protein isolate additions on (A) the gel elastic modulus (G') (B) the EPS production by *Streptoccocus thermophilus* and (C) the extent of the syneresis of the fermented milk batches after overnight cold storage.

Native whey proteins usually disrupt the weak acid gel of yoghurt due to their 'inactive filler' nature (Lucey et al., 1999). However, our model describing WPC addition showed the opposite. Some whey protein denaturation may have likely taken place during heating of fermented milk-bases containing WPC at 90 °C, enabling the formation of a complex between whey proteins and κ-casein, which altered the gel stiffness (Schorsch et al., 2001). Lactic acid was reported to contribute to the increase in the gel stiffness of β-lactoglobulin (Resch et al., 2005), which in our study may have been observed as improvement in G' when WPI concentration was increased. The apparent gel strengthening upon WPC addition may have been also caused by some other essential low molecular weight compounds otherwise absent in WPI due to processing, since the addition of the WPI weakened the acid gel (Schorsch et al., 2001). On the other hand, Ca weakened the WPC gel (Lorenzen and Schrader, 2006), stabilized α-lactalbumin against unfolding and aggregation, thus prevented its denaturation in acidic environment (Pedersen et al., 2006). It also inhibited subsequent complexation between whey proteins with casein, leading to weak gel structure (Schorsch et al., 2001).

After 21 day of storage, the estimated log G' of both WPC (maximum of 4.4 mPa, from combination of 3.5 g/100 g WPC and 0.4 mM Ca) and WPI (maximum of 4.38 mPa, from combination of 2.1 g/100 g WPI and 2.6 mM Ca) fermented milk were higher than those of corresponding fresh fermented milk. Statistically, the WPC concentration substantially (P<0.001) increased G' (Table 6.2), but the WPI concentration had no apparent effect (P>0.05) (Table 6.3). The improvement of G' was more evident in WPI compared to that of WPC fermented milk. WPC contained more calcium, fat and phospholipid than WPI (Lorenzen and Schrader, 2006), that potentially hindered protein-protein interactions during cold storage in the acidic environment (Pedersen et al., 2006). Interaction between whey protein and Ca was only significant (P<0.05) for fresh and storage WPI fermented milk (Table 6.3).

Firmness of the fermented milk gels was the textural characteristic analyzed in this work, while other parameters usually depicted by texture profile analysis were not assessed. Similar to G', fresh WPC fermented milk gel was harder

(maximum firmness of 14 g, at 2.8 g/100 g WPC and 0.04 mM calcium) than that of WPI fermented milk (maximum firmness of 9.8 g, at 2.4 g/100 g WPI and 0.004 mM calcium). The firmness of WPC yoghurt was slightly but significantly altered by types of mixture (Table 6.2). In WPI yoghurt, there was no apparent (P>0.05) effect upon supplementation. The calcium addition had no effect (P>0.05) on firmness of both fresh WPC and WPI fermented milk (Table 6.2, 6.3). In all fresh or storage fermented milk samples, there was no significant interaction (P>0.05) of both whey protein and calcium.

After storage, unlike G', firmness of both fermented milk changed to a lesser extent. While estimated firmness of WPC declined (maximum of 9.8 g from combination of 2.0 g/100 g WPC and 0.1 mM Ca), that of WPI was slightly greater (maximum of 10.9 g resulted from combination of 3.3 g/100 g and 0.3 mM Ca) (graph not shown). The effect of both whey protein additions on gel firmness was significant (P<0.05 and P<0.01 for WPC and WPI yoghurt, respectively). In storage WPC fermented milk, addition of WPC improved firmness, but after ~4 g/100 g WPC, it leveled off. Increasing calcium addition at any WPC concentration decreased firmness. In WPI fermented milk, high values of firmness were achieved by combination of WPI at all concentration and Ca at low concentration (up to ~4 mM). Increasing WPI concentration improved firmness. However, at high WPI concentration, addition of high concentration of Ca decreased it. Although not significant (P>0.05), addition of Ca tended to increase firmness but gradually was reduced at high (~3 g/100 g) WPI concentration.

A close relation between G' and firmness was noted previously (Kealy, 2006). Moreover, a firmer gel of WPC fermented milk may due to higher fat content in WPC. However, the firmness of fermented milk may be better correlated to a yield stress rather than to G'. Both firmness and yield stress are a measure of force to start breaking of gel, while G' was a measurement of gel strength upon oscillation. Nevertheless, in our work, firmness appeared to be less affected by storage in comparison to G'.

Consistency index K is an indicator of the material resistance to deformation (Rao, 1999). Therefore, similar to G' and firmness, it also indicates the strength of

gel, but more specifically related to shear. In our work, similar to G' and firmness, estimated maximum K values of fresh WPC yoghurt were greater (around ten fold) than those of WPI fermented milk. Whey protein concentration was the only factor significantly (P<0.001 to P<0.05 for WPC and WPI, respectively) contributing to the increase in K for both types of fermented milk (Table 6.2, 6.3). In WPC yoghurt, addition of WPC increased K at any Ca concentration. However, increasing WPC concentration as Ca was continuously added gradually reduced K values considerably to reach very low value (close to 0 mPa.s^n) at highest concentration of both Ca and WPC (graph not shown). Similarly, addition of WPI up to ~4 g/100 g also increased K value at any level of Ca, after which K decreased slightly in about similar extent at all Ca concentrations. The effect of Ca was not significant (P>0.05) on either WPC or WPI fermented milk (Table 6.2, 6.3), but its decreasing effect on K of WPI fermented milk was apparent. In WPC yoghurt, Ca addition up to ~6 mM only very slightly increased K, but decreased it afterwards.

During storage, estimated K values of WPC fermented milk were substantially (P<0.001) reduced around 20 times without considerable change in trend, while those of WPI fermented milk were not altered greatly (P>0.05) (Table 6.2, 6.3). This difference in the magnitude of reduction of K values during storage between WPC and WPI fermented milk was somewhat similar to that of firmness. Ca addition had no apparent influence (P>0.05). While the trend in storage WPC did not differ from that of fresh one, Ca addition up to ~6 mM in storage WPI fermented milk increased K, but reduced it afterwards. WPI addition also increased K values but there was a gradual and substantial cut down at high concentration of WPI by increasing Ca concentration. Interaction between whey protein and Ca was only significant for fresh and storage WPC fermented milk (P<0.01 and P<0.001, respectively).

Syneresis or the whey expulsion can result from a weak gel incapable of retaining serum or gel compaction that leads to water expulsion from protein network (Lucey, 2001). The degree of syneresis of the two fresh fermented milks (Figure 6.1C, 6.2C) varied slightly, around 30-50 g/100 g and 20-40 g/100 g for WPC and WPI fermented milk, respectively. In fresh fermented milk, addition of

calcium played a significant (P<0.01) role in enhancing syneresis in both WPC and WPI fermented milk (Table 6.2, 6.3). In WPC fermented milk, addition of either WPC or Ca induced syneresis, although the effect of WPC was lesser than that of Ca. In the WPI fermented milk, the addition of WPI increased syneresis, followed by a steady decline at high concentration of both WPI (~4 g/100 g) and Ca (from ~5 mM).

After the storage, the syneresis of the WPC fermented milk was reduced slightly (around 20-50 g/100 g), while that of the WPI fermented milk increased (up to ~80 g/100 g), especially at high Ca concentrations. Although not statistically significant (P>0.05), WPC intensified syneresis of storage fermented milk at any Ca concentrations with similar trend as that of fresh fermented milk. In the WPI fermented milk, however, increasing both WPI and Ca concentration intensified (P<0.01) syneresis steadily. The influence of interaction between the two factors examined was not significant (P>0.05) in all samples. Relating syneresis to parameters of the gel strength, especially G' and firmness, revealed negative correlations which became more significant (r ranged from -0.483 to –0.8816) after storage. This may indicate that syneresis in both fermented milk was induced by gel-weakening effect, which became more intense during storage.

The correlation between parameters in this study revealed a significant negative correlation between the EPS concentration and all parameters of the gel strength: firmness, G', and K in both types of supplemented fermented milk. In most cases, the storage intensified the extent of these correlations. For example, coefficient of correlation between firmness and EPS concentration in fresh WPC fermented milk was –0.4370, it was then increased to –0.9263 after storage. Similarly, in the case of G', the coefficient was increased from –0.6059 in fresh fermented milk to -0.8852 after storage. Moreover, there was a shift of correlation between EPS concentration and syneresis of both fermented milk, from less significant negative (r of –0.3512 and –0.3087 in WPC and WPI fermented milk, respectively) in fresh fermented milk, became more significant positive (r of 0.4178 and 0.9426 in WPC and WPI fermented milks, respectively) after storage. Probably, although EPS was able to support water holding before storage, in the later stage as it was reduced in quantity, it contributed to the weakening structure

leading to the increase in syneresis, as well as reduce in firmness, G' and *K* of some samples. EPS had been considered to be capable of hindering the development of protein-protein network (Tuinier et al., 2000).

Whey-protein and calcium added fermented milk had very runny characteristics as indicated by very low G', only around one tenth of normal or calcium-fortified fermented milk as reported in Chapter 5. Moreover, the syneresis was also doubled up. This detrimental effect of combined calcium and whey protein added into fermented milk would suggests that this type of fermented milk suits more as drinking rather than spoonable fermented milk. However, it is not impossible to improve the texture by applying several treatments such as heating of whey protein upon addition into milk mixture (Puvanenthiran et al., 2002).

6.4 Conclusions

The supplementation of non-fat set-type EPS-containing fermented milk with calcium and whey protein affected microbial, physical and rheological characteristics. Data derived from small deformation measurement showed trends better than those of large deformation method. The calcium addition tended to weaken the structure, resulted in induced syneresis and lowered storage moduli as well as firmness. The addition of whey protein, on the contrary, tended to strengthen the gel structure as shown by the increase in storage moduli, consistency index *K*, and firmness. The effect of whey protein addition was only significant in WPC fermented milk. The interaction of calcium and whey proteins substantially reduced fermented milk gel strength. Therefore, in WPC fermented milk, low concentration of Ca and high concentration of WPC appeared to improve textural attributes of fermented milk. On the other hand, in WPI supplemented fermented milk, both low concentrations of Ca and whey proteins had resulted in a development of desirable textural characteristics.

This chapter revealed the gel weakening effect of whey protein addition of fermented milk fortified with calcium, whilst EPS concentration was increased. The next chapter deals with the possibility of reducing the weakening effect of whey protein addition on texture of fermented milk prepared by each type of EPS producer.

7 Structural Properties of Fermented Milk as Affected by Culture Type and Casein to Whey Protein Ratio

7.1 Introduction

Previous chapter highlighted the structural disruption of fermented milk gel as a result of the addition of whey protein in combination with calcium. In this chapter an experiment was carried out to rectify the problem by applying heat treatment to whey proteins prior to mixing with the milk base. The effect of whey proteins on the texture formation would be one of the aspects studied in this experiment.

Whey proteins have been incorporated in various food products to provide therapeutic and functional benefits. Their high content of branched amino acids supported the body mass building and reduced body mass loss during physical stress (Schaafsma, 2006a), promoted gut health through protection of intestinal cells, prevented formation of toxic nitrogenous substances by colonic microflora, reduced bowl inflammation and enhanced antimicrobial effects (Schaafsma, 2007). Antimicrobial activities of whey proteins were effective against several pathogens (Gill et al., 2006, Madureira et al., 2007, Yalcin, 2006), including cariogenic species (Warner et al., 2001). Improvement of immune-system and mood control had also been reported (Yalcin, 2006).

In addition to their physiological importance, they also posses other functional characteristics including water holding, interface and gelling behavior that may affect physical properties of various food systems. Whey proteins were used as stabilizing agent in several food systems such as food dressing (Christiansen et al., 2006, Ofstad et al., 2005), low-fat Turkish sausage (Yildiz-Turp and Serdaroglu, 2008) and low-fat meat ball (Serdaroglu, 2006). Whey proteins were incorporated in the development of desirable texture of gluten free

product such as ‘empanadas’ and pie crust (Lorenzo et al., 2008), as well as bread (Gallagher et al., 2003). Their foaming ability was employed to replace egg white (Davis and Foegeding, 2007), and was used in angel cake manufacturing (Morr et al., 2003). In ice cream, a whey protein addition modified melting resistance and stability of air bubbles (Innocente et al., 2002, Zhang and Goff, 2005) without impairing flavour (Innocente et al., 2002).

The supplementation of whey proteins to yoghurt has been studied extensively with varying effects on the final yoghurt texture. Native whey protein supplementation reduced yoghurt gel strength parameters such as storage modulus and yield stress (Guggisberg et al., 2007), viscosity and firmness (Guggisberg et al., 2007, Oliveira et al., 2001), and created porous structure which led to higher syneresis (Gonzalez-Martinez et al., 2002). However, when native whey proteins were added at high concentrations after fermentation of yoghurt, storage moduli were increased (Patocka et al., 2004). When yoghurt milk base supplemented with whey proteins was heated before acidification, the gel strength was significantly increased by heating time, apparently due to larger micelle size which may have resulted from more extensive cross-linking of whey proteins and caseins (Remeuf et al., 2003). Heating milk mixture containing added whey proteins positively affected gel strength, likely due to denaturation of whey protein. The positive effect of denatured whey proteins on gel strength of glucono-δ-lactone (Vasbinder et al., 2003) as well as microbial (Puvanenthiran et al., 2002) acidified yoghurt was emphasized. Interestingly, when whey proteins heated at high temperature were added to milk base after homogenization, the gel strength was impaired, which was opposite to unheated counterpart (Sodini et al., 2006). This may reflect the complex nature of yoghurt structural development, which possibly involves several factors and their interactions.

Heat-induced gelation of whey proteins involves at least two steps, unfolding of protein three dimensional structure to expose reactive functional thiol groups, and rearrangement of the unfolding state to form aggregates (van Mil and Roefs, 1993). The unfolding rendered whey proteins to become amphiphilic with a higher water holding capacity (Morr, 1979). At high concentrations of added whey proteins, denaturation, as indicated by significant increase in elastic

modulus (G'), started earlier and may reach higher maximum values (Kavanagh et al., 2000). Thermal treatment at neutral pH and 60 °C (Pelegrine and Gasparetto, 2005) resulted in reversible altered tertiary conformation (Iametti et al., 1996). Irreversible denaturation was induced by heating at around 75-80 °C (Clark et al., 2001), and was more extensive at higher temperature and low heating rate (De Wit and Klarenbeek, 1984). Higher temperature enabled more extensive protein-protein interaction via hydrophobic non-covalent bonds (Lorenzen and Schrader, 2006). In the presence of casein, the aggregation of whey proteins was affected by caseins to whey proteins ratio (Beaulieu et al., 1999, Puvanenthiran et al., 2002). In this case, high whey protein proportion increased denaturation rate (Law and Leaver, 1999) and caused formation of large particles consisted of both micelles covered with whey protein as well as whey protein aggregates (Beaulieu et al., 1999).

Another factor that governs formation of the yoghurt texture is the presence of exopolysaccharides (EPS) produced *in situ* by yoghurt cultures, especially *Streptococcus thermophilus* strains. The EPS positively contributed to the development of desirable yoghurt characteristics: firmness and low serum expulsion (Folkenberg et al., 2005). In a binary system consisting of an EPS and caseins, the incompatibility may occur leading to a depletion-type interaction (de Kruif, 1999). In this case, higher EPS concentrations and higher molecular weight of the EPS would result in a larger size of aggregated whey proteins and consequently firmer texture. However, in a real yoghurt system, the interaction of the EPS and milk proteins appears more complex. For example, the higher EPS concentration and greater molecular weight detrimentally affected the texture of yoghurt rendering it runny, suggesting the influence of other factors (De Vuyst et al., 2003) such as the location of the EPS within the network (Folkenberg et al., 2005). The effects of whey protein supplementation to EPS-containing yoghurt have not been extensively studied, especially those which involved different EPS types and denaturation state of whey proteins. The aim of this work was to study the effects of incorporating different concentrations of whey protein isolate in its native or heat-treated state on the gelation and final textural characteristics of low-

fat set yoghurt fermented by two types of exopolysaccharide-producing cultures of *Streptococcus thermophilus.*

7.2 Materials and methods

7.2.1 Preparation of mixture

The low heat skim milk powder was obtained from Murray-Goulburn (Victoria, Australia) containing 34-37 g protein/100 g milk which contained 82.59 g of caseins/100 g milk protein. Whey protein isolate (Alacen 895) used was provided by Fonterra (Fonterra, Victoria, Australia) and contained 90-92 g casein /100 g whey proteins as per manufacturer's specification. The fermented milk bases were prepared by reconstituting either skim milk powder (as control) or replacing adequate amounts of skim milk powder with whey protein isolate (WPI) to achieve 14 g/100 g total solid content. The ratio of WPI:casein was adjusted to 3:1 and 1:1, with WPI added in heat-untreated or heat treated form. The heat-treated WPI was prepared by heating WPI 5 g/100 g reconstituted in Milli-Q® water at 80 °C for 10 min followed by immediate cooling by immersing the container in ice to stop heating. After cooling, the mixture was homogenized using microfluidizer (Microfluidics™, Newton, Massachusetts, USA) at ~100 MPa. The microfluidized whey protein dispersion was then mixed with pasteurized (65 °C, 30 min) skim milk suspension in the required ratio giving the final total solid content of 14 g/100 g. The mixtures, including the control sample, were then again homogenized (~100 MPa). In order to produce acid gels, an artificial acidulant in the form of glucono-δ-lactone (GDL) (Sigma-Aldrich, Inc., St. Louis, MO, USA) was used at concentration of 1.3 g/100 g. Fermented milk samples were produced by fermentation using *Streptococcus thermophilus* ST 1275 (capsular-ropy EPS producer) and *S. thermophilus* ST 285 (ropy-EPS producer), provided by the Australian Starter Culture Research Center (ASCRC, Werribee, Victoria, Australia). The frozen (at –80 ^{o}C) glycerol stock of the culture was activated twice by incubating it in 30 mL sterile 14 g skim milk /100 g mixture at 42 °C for 24 hours, before application in the fermented milk

manufacturing. All fermented milk samples were fermented at 42 °C and fermentation was terminated when pH reached 4.5 by placing them in a cold (4 °C) room for 12 hours.

7.2.2 Viable cell count and EPS production

After cooling, the fermented milk samples were stirred to attain homogenous mixture and 1 mL of it was aseptically diluted in 9 mL sterile peptone water, followed by serial dilutions. It was then plated onto M17 agar containing lactose (Merck Pty Ltd., Kilsyth, Victoria, Australia) and incubated at 42 °C for 48 hours aerobically. The EPS production was determined by method previously reported (Rimada and Abraham, 2003) with minor modifications as was used the Chapter 4, 5 and 6. Around 50 g of fermented milk was centrifuged (Model J2-HS, Beckman, Fullerton, California, USA) at ~11,000 × *g*, 4 °C for 10 min and the whey was collected. The whey solution was then mixed with 2 volumes of ethanol and stored overnight at 4 °C. The mixture was centrifuged under conditions reported above and the precipitate was collected and dissolved with 10-20 mL Milli-Q® water. It was then added with 50 μL of concentrated 80 g/100 g TCA solution for every 10 mL mixture. After overnight storage at 4 °C, the mixture was again centrifuged and the supernatant was collected. The whole procedure was repeated once again and the resulting supernatant was collected and dried at 55 °C under vacuum. The amount of EPS was expressed as the crude EPS per kg of fermented milk.

7.2.3 Physical properties of fermented milk gels

The firmness of all acid gels expressed as the force required to achieve 50% strain was determined using a texture analyzer (TA-XT2 plus, Stable Micro Systems Ltd., Surrey, UK), equipped with a 30 kg load cell and 20 mm aluminium cylinder probe. The cross-head speed was set at 1 mm/s and compression was set at 50%. Every determination was at least replicated with two sub samplings each.

Syneresis was determined using method previously established (Jaros et al., 2002a) with slight changes. Undisturbed fermented milk gels were developed in falcon tubes following either the GDL acidification or fermentation protocol. The weight of gels was noted, and then the gels were centrifuged (model RT7, Sorvall, DuPont, Newtown, Connecticut, USA) at ~70 × *g* and 8 °C for 10 min. The whey expelled from the fermented milk was decanted, weighed and related to the initial weight of the sample. The extent of syneresis was expressed as the percentage.

The permeability of the fermented milk gels was determined following an established method (Lee and Lucey, 2004b, Verheul and Roefs, 1998) with slight modifications. In brief, 10 mL of a fermented milk mixture (fermented milk base with GDL or cultures) was placed in a 50 mL falcon tube (Falcon, Blue Max, Becton Dickinson and Company, Franklin Lakes, N.J., USA). A glass tube (inner diameter of 3.7 mm and length of 25 cm, open on both ends) supported with rubber stopper was immersed in the fermented milk mixture. The falcon tube was then closed by a fitting rubber stopper, immersed in a water bath at 42 °C and incubated until pH reached 4.5. After acidification or fermentation, the glass tube containing now the gel inside was removed and the length of the gel was measured. The glass tube was then immersed in 35 mL whey in another falcon tube. By the osmotic pressure, the whey would diffuse up along the length of the gel and fill the surface of the gel. The height of the whey on the gel surface was recorded periodically. Reference tubes were the same type of falcon tubes filled with the whey of the same volume (35 mL), in which a similar glass tube supported with rubber stopper was immersed. The height of whey was measured. Permeability was expressed as permeability coefficient, *B*, as described by Lee & Lucey (Lee and Lucey, 2004b). The temperature of determination was 20 °C, and density of the whey was 1.033 mg/mL. Six tubes for each sample in every subsampling were assessed. Any imperfect gel, which was broken or detached from the glass wall, was excluded. Permeability coefficient was calculated using the following equation:

$$B = -\left[\ln\frac{(h_\infty - h_{t2})}{(h_\infty - h_{t1})}\right]\cdot\frac{\eta\cdot H}{\rho\cdot g\cdot(t_2 - t_1)}$$

Where B is the permeability coefficient (m^2), h_∞ the height of the whey in the reference tube (m), h_{t1} and h_{t2} (m) the height of the serum in the gel tube at time t_1 (s) and and t_2 (s), respectively. η is the viscosity of whey (Pa), ρ density of the whey (kg/mL), g acceleration due to gravity (m/s), and H the length of the gel (m).

7.2.4 The rheological characteristics of set-type fermented milk

The gelation was assessed during acidification using the artificial acidulant (GDL) or two types of the cultures. The GDL was added at 1.3 g/100 g, while suspension of *S. thermophilus* was added as much as 2 mL/10 mL either as capsular-ropy- or capsular-EPS producer. For the GDL-acidified fermented milk, approximately 50 mL of the fermented milk base was thoroughly mixed with the GDL using a vortex mixer, and quickly loaded into the sample chamber of a double-gap type geometry (C-DG 26.7/T200, internal diameter 23.833 mm, external diameter 27.594 mm, Anton Paar) equipped with a solvent trap to prevent the evaporation. A layer of low viscosity silicone oil was placed in the solvent trap ring to prevent evaporation. The structural development was assessed using a controlled rate/controlled strain rheometer (Physica MCR 301, Anton Paar, GmbH, Germany). The rheometer was connected to a circulating water bath (Viscotherm VT 2, Anton Paar), and the temperature was controlled with a Peltier system (Anton Paar) to maintain it constant at 42 °C with an accuracy of ± 0.1 °C. Upon loading, all samples were pre-sheared (500/s, 3 s for GDL fermented milk, and 3 min for bacterial fermented milk) to achieve uniformity in the measuring geometry. The development of G' over time was examined at 0.1 % strain and constant frequency of 1 Hz until pH reached 4.5 or G' showed plateau. The data of all rheological measurements were analyzed with the supporting software Rheoplus/32 v2.81 (Anton Paar). The changes of pH were checked periodically from a simultaneous fermentation batch.

The assessment of the rheological properties of set-type fermented milk after fermentation was carried out in a cone-and-plate geometry (CP50-1, 50 mm

diameter, 1^o angle and 0.02 mm gap) with the CR/CS Anton Paar rheometer. All samples were homogenized by thorough stirring prior to loading. The fermented milk structure was additionally broken by pre-shearing at 500/s for 30 s, followed by the resting period of 300 s to allow for a structural rebuilding. All samples were subjected to a frequency sweep performed in the linear viscoelastic region. The viscoelastic region was determined by applying an amplitude (strain) sweep at 1 Hz. The thixotropy loop experiment was conducted by increasing the shear rate logarithmically from 0.1 to 100/s within 300 s, and then decreasing back to the origin again within 300 s. The data from the upward curve of the shear cycle were fitted to three models: firstly, the Bingham model ($\tau = a + K\dot{\gamma}$), where τ presents shear stress (Pa), $\dot{\gamma}$ shear rate (s^{-1}), while *a* yield stress (Pa s) and *K* consistency coefficient (Pa s^n); secondly, the Herschel-Bulkley model ($\tau = a + K\dot{\gamma}^n$), with *a* yield stress (Pa s), *K* the consistency (Pa s^n) and *n* viscous coefficients (dimensionless), respectively; and thirdly, the power law model ($\tau = K\dot{\gamma}^n$), where *K* and *n* are consistency factor (Pa s^n) and viscous coefficient (dimensionless), respectively.

7.2.5 Statistical analysis

The experiments were arranged according to randomized full factorial block design with two factors: types of acidulant (three levels), and whey protein addition (five levels). This block structure was replicated twice with at least 2 sub-samplings. Results were analyzed using General Linear Model procedure of the SAS System (SAS, 1996), and the level of significance was preset at P = 0.05. The General Linear Model could be described by following equation:

$$X_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$$

Where X corresponds to the actual value and μ expected value, ε is the error term and α, β, denote acidulants (GDL and EPS producing cultures) and whey protein

supplementation, respectively. Terms in brackets present corresponding interactions. The level of significance was preset at $P = 0.05$.

7.3 Result and discussion

7.3.1 Bacterial growth and EPS production

In general, the addition of whey proteins either in heat-untreated or heat-treated form had little or no effect on the growth of both strains of bacteria (results not shown). The EPS concentration in capsular-ropy or capsular fermented milk with the same casein to WPI ratio was in most cases not significantly different ($P>0.05$) (Table 7.1). EPS isolated from fermented milk with capsular-ropy strain was significantly ($P<0.05$) higher than that of capsular strain in some samples with no or low concentration of added whey proteins. The apparent insensitivity of the capsular *S. thermophilus* to the whey protein supplementation may be due to its slow-growth nature. Accordingly, the EPS production by the capsular-ropy strain was more influenced by the media composition compared to that of the capsular *S. thermophilus*. The control fermented milk contained more EPS than those produced by WPI supplementation (Table 7.1). Certain strains of *S. thermophilus* are known to possess a poor proteolytic activity, thus their growth and EPS production may depend on the quality of supplemented proteinaceous nitrogen (Lucas et al., 2004). The strains used in our study produced the EPS in a growth-dependent manner.

Table 7.1 Physical and chemical properties of fermented milk supplemented with either heat-untreated or heat-treated whey protein isolate and acidified using GDL or capsular-ropy or capsular EPS producing strains of *Streptococcus thermophilus* at 42 °C.

Fermented milk samples[*]	Syneresis[**], (g/100 g)	Permeability[**], ($\times 10^{-11}$ m^2)	Firmness[**], (g)	EPS concentration[**], (mg/kg fermented milk)
		GDL		
Control (Skim Milk)	22.95^{bA}	23.2^{bB}	69.9^{aA}	ND
3:1, NWPI	32.93^{bA}	36.0^{cB}	52.4^{aA}	ND
1:1, NWPI	34.82^{bA}	43.8^{dB}	47.4^{aA}	ND
3:1, DWPI	4.35^{aA}	3.58^{aA}	277.8^{bA}	ND
1:1, DWPI	5.88^{aA}	0^{aA}	524.3^{cA}	ND
		Capsular-ropy		
Control (Skim Milk)	21.05^{abA}	13.1^{bA}	26.3^{aA}	223.9^{aB}
3:1, NWPI	29.65^{bA}	15.2^{bA}	18.1^{aA}	142.5^{bB}
1:1, NWPI	41.66^{bA}	65.0^{cC}	17.9^{aA}	104.1^{aA}
3:1, DWPI	18.12^{aB}	3.71^{aA}	217.2^{bA}	50.2^{aA}
1:1, DWPI	9.105^{aA}	0^{aA}	1009.0^{cB}	90.4^{aA}
		Capsular		
Control (Skim Milk)	32.54^{bA}	23.9^{cB}	29.7^{aA}	128.6^{bA}
3:1, NWPI	33.24^{bA}	15.0^{bA}	24.2^{aA}	62.2^{abA}
1:1, NWPI	34.25^{bA}	30.7^{cA}	20.7^{aA}	99.7^{abA}
3:1, DWPI	17.07^{aB}	3.48^{aA}	305.7^{bA}	81.3^{abA}
1:1, DWPI	16.57^{aA}	0^{aA}	1028.5^{cB}	53.4^{aA}
SEM[***]	3.00	1.9	41.7	17.4

[*]Numbers denote casein and whey protein ratio, NWPI stands for heat-untreated whey protein isolate, and DWPI stands for heat-treated whey protein isolate Means present the average of at least 4 determinations (n=4); [**]Small letter superscripts in a column denote significant difference (P<0.05) among samples at different whey protein supplementation, for a particular type of acidulant; Capital letter superscripts in a coloumn denote significant difference (P<0.05) among samples at the same whey protein supplementation, for different acidulant. [***]SEM - pooled standard error of the mean, P<0.05.

7.3.2 Gelling properties of acid induced gels in the presence of EPS and heat-untreated or heat-treated WPI

In the GDL-acidified fermented milk, the WPI supplementation enhanced both maximum G', it shortened the time of onset of gelation, and was positively related to whey protein concentration (Figure 7.1). The fermented milk samples with the heat-treated WPI exhibited higher G' than those of the heat-untreated whey proteins. Similarly, in the capsular-ropy fermented milk, whey proteins also induced earlier start of the gelation (Figure 7.2). However, the low whey protein supplementation (casein:whey protein ratio of 3:1) reduced maximum G' in comparison to the control fermented milk. A typical sharp increase of G' was observed during gelation in all capsular-ropy fermented milk samples, as opposed to those with heat-untreated whey proteins with a rather gradual increase of the storage modulus. This may indicate a slower textural development or a weaker structure during gelation. On the other hand, in the fermented milk acidified with the capsular strain, the whey protein supplementation increased the maximum G' in comparison to control, regardless the concentration and whey protein state (Figure 7.3). The higher concentration of added whey proteins, heat-untreated or heat-treated, appeared to delay the gelation. The results apparently indicated that while the gelation of the GDL-acidified fermented milk was positively correlated to the concentration of added whey proteins regardless their state, those fermented by EPS-producing *S. thermophilus* strains were not. This may reflect that the EPS interfered with the gelation in the whey protein supplemented fermented milks. Moreover, the gelation behaviour among fermented milk samples made by these two EPS producers also differed inferring likely the effect of the EPS nature on the formation of the acid gel (Bouzar et al., 1997).

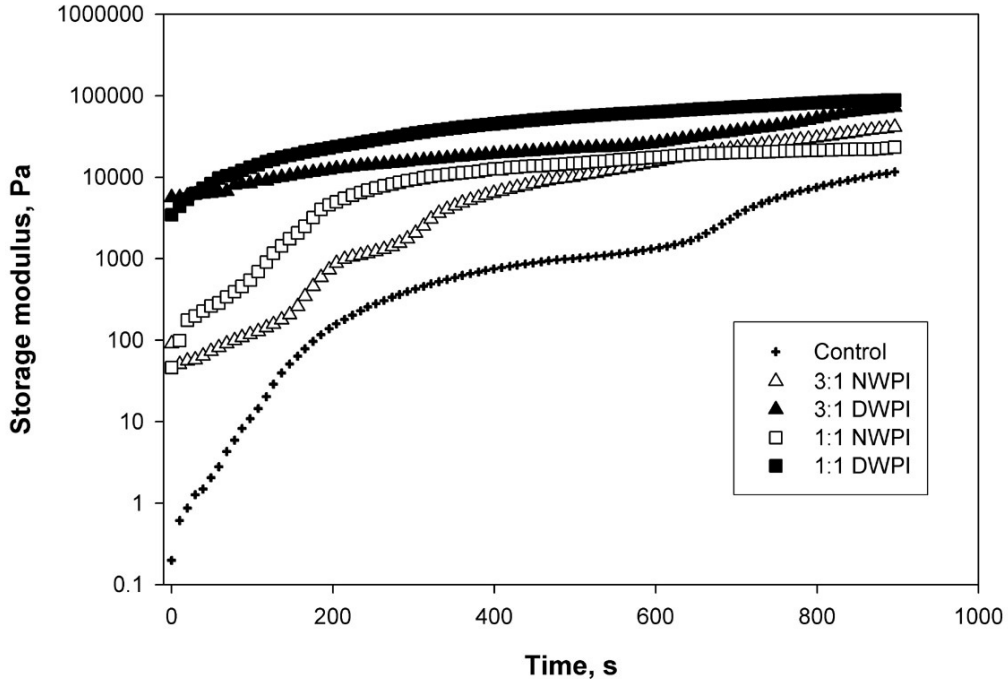


Figure 7.1 Changes in elastic moduli (G') during gelation of fermented milk acidified with glucono-δ-lactone for control mixture (+), and those containing casein:whey protein ratio of 3:1 (Δ for heat-untreated, and ▲for heat-treated whey protein), and casein: heat-untreated whey protein ratio of 1:1 (□ for heat-untreated and ■ for heat-treated whey protein), fermented at 42 °C.

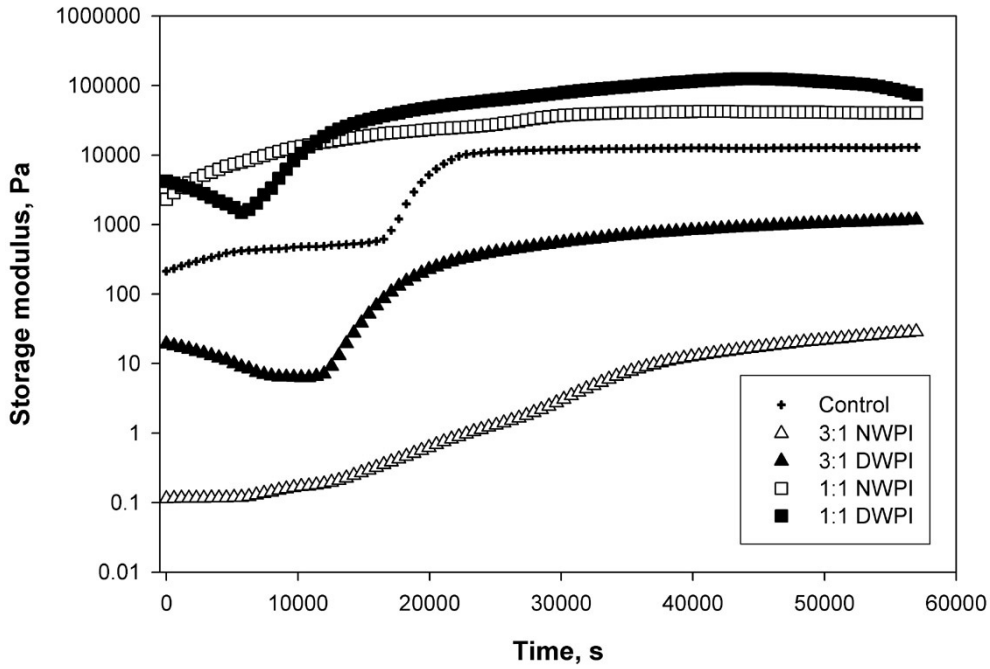


Figure 7.2 Changes in elastic moduli (G') during gelation of fermented milk acidified with capsular-ropy strain of *Streptococcus thermophilus* for control mixture (+), and those containing casein:whey protein ratio of 3:1 (Δ for heat-untreated, and ▲for heat-treated whey protein), and casein: heat-untreated whey protein ratio of 1:1 (□ for heat-untreated and ■ heat-treated whey protein) fermented at 42 °C.

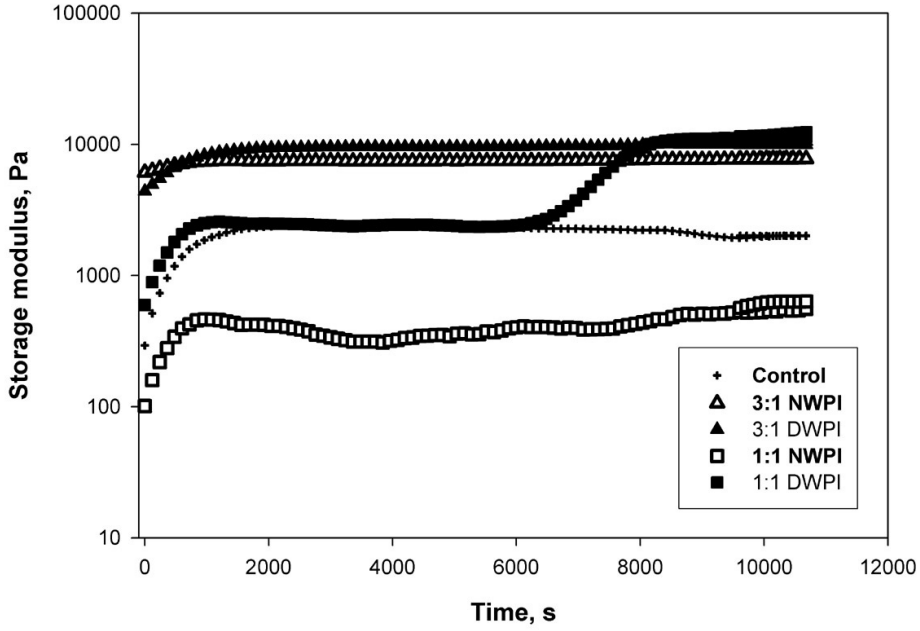


Figure 7.3 Changes in elastic moduli (G') during gelation of fermented milk acidified with capsular strain of *Streptococcus thermophilus* for control mixture (+), and those containing casein:whey protein ratio of 3:1 (Δ for heat-untreated, and ▲for heat-treated whey protein), and casein: heat-untreated whey protein ratio of 1:1 (□ for heat-untreated and ■ for heat-treated whey protein) fermented at 42 °C.

A partial unfolding of whey proteins may occur during heating at 80 °C and pH 7, but the gelation may not occur due to electric repulsion among denatured protein globules (Fitzsimons et al., 2007). The rate of heat denaturation of whey proteins increased at lower proportion of casein to whey protein (Law and Leaver, 1999). Nevertheless, the denaturation could increase viscosity (Hollar et al., 1995). At high pH, dimeric β-lactoglobulin may be activated by exposing the thiol groups (Iametti et al., 1996). Upon acidification during the yoghurt manufacturing, the activated whey proteins may interact with dissolved κ-casein, by partially or fully covering the casein aggregates (Guyomarc'h et al., 2003, Vasbinder et al., 2003) to increase further network strength (Guyomarc'h et al., 2003, Knudsen et al., 2006). In a binary model system consisting of the EPS and whey proteins, the increase in viscosity was directly correlated to the EPS concentration which interacted with aggregated whey proteins (de Kruif, 1999). On the other hand, in a yoghurt system, a minimum concentration of native whey proteins improved the gel strength, while further supplementation had detrimental effects (Patocka et al., 2004). This minimum concentration of added native whey proteins required to improve yoghurt gel structure was apparently shown by the capsular-ropy yoghurt in our study and even masked the gelation point. As the results showed (Figure 7.2), apparently important interactions between types of exopolysaccharide and native or heat-treated whey protein may exist, thus further studies are needed to reveal the mechanism of these interactions.

7.3.3 Physical properties of fermented milk samples

7.3.3.1 Firmness

Type of acidifier had a negligible effect ($P>0.05$) on gel firmness, but the ratio of casein to heat-treated WPI was a significant ($P<0.05$) factor. The fermented milk gel samples produced by the culture fermentation appeared slightly but not significantly ($P>0.05$) firmer than the GDL fermented milk (Table

7.1), whereas the addition of native whey proteins reduced firmness slightly (P>0.05) in all fermented milks (Table 7.1). In contrast, firmness was significantly (P<0.05) increased upon addition of heat-treated WPI in all fermented milks produced by the three types of acidulants with an apparent concentration-dependency. Among the three types of fermented milk with the low proportion of added heat-treated whey proteins, the firmness was not significantly different (P>0.05). However, upon addition of the greater concentration of heat-treated whey proteins, the firmness of the GDL fermented milk (524±41.714g) was lower (P<0.05) and only half as firm as the two culture-fermented fermented milks (1009 and 1028±41.714g for capsular-ropy and capsular fermented milk, respectively). Thus, the influence of EPS during supplementation with denatured whey protein appears to be important determinant of firmness. A heat treatment as low as 68 °C applied to whey protein isolate induces the activation of the thiol groups through unfolding of the native protein structure (Alting et al., 2003). Thiol groups are sensitive to pH, and during acidification, they may form covalent bonds to further cause inter-particle cross-linking (Vasbinder et al., 2003). As a result, a hard gel may be formed that depends upon the initial whey protein concentration.

7.3.3.2 Syneresis

Syneresis in fermented milk occurs as a result of an open structure allowing the flow of serum out from the acid-induced protein network (Puvanenthiran et al., 2002). In this study there was generally no significant difference (P>0.05) in syneresis with the different type of fermented milk mixtures (Table 7.1). Syneresis was significantly (P<0.05) reduced in the fermented milk samples with heat-treated WPI. This is consistent with a previous report, which also noted the increase in the firmness of the gel (Lucey et al., 1997b). The denatured whey protein supplementation caused a development of a more compact protein network, leading to construction of smaller pores which inhibited serum release (Puvanenthiran et al., 2002). In general, the syneresis was insignificantly (P>0.05) affected by addition of native whey proteins, but greatly (P<0.05)

decreased by heat-treated whey protein supplementation (Table 7.1). The effect was not significantly (P>0.05) concentration dependent. Substantially great reduction in syneresis of heat-treated WPI supplemented GDL fermented milk may indicate the more sensitive nature of GDL network to addition of denatured whey protein. On the other hand, apparently there was an interaction between EPS and heat-treated WPI to slightly hinder reduction in syneresis of bacterial fermented milk.

7.3.3.3 Permeability coefficient

The permeability of the fermented milk gels was closely and positively related with the pore size within the gel network (Alting et al., 2003). Among control fermented milk, the permeability of the GDL and capsular fermented milks was not different (P>0.05), but the permeability of both were significantly (P<0.05) higher that that of the capsular-ropy fermented milk (Table 7.1). Upon addition of the lower concentration of native WPI, the permeability of both bacterial fermented milks was the same (P>0.05), but were significantly (P<0.05) lower than that of GDL fermented milk. However, increasing the concentration of native WPI caused significantly (P<0.05) higher permeability in both capsular-ropy and GDL, when compared to that of capsular fermented milk. The permeability of all fermented milk was intensified by the addition of native whey proteins and significant (P<0.05) at casein:WPI of all ratio for the GDL fermented milk. In fermented milk containing capsular-ropy strain, the increasing permeability was demonstrated by mixture of 1:1 native casein:WPI (Table 7.1). Whilst, in capsular fermented milk, native WPI addition at the lower casein:whey protein ratio reduced permeability significantly (P<0.05). At higher ratio, it did not alter permeability (P>0.05). Apparently, the changes in permeability during supplementation with native WPI were too small to cause the changes in syneresis.

In contrast to native WPI, heat-treated WPI reduced permeability significantly (P<0.05), which was practically non-existent at 1:1 casein:WPI ratio. The addition of the denatured whey proteins during yoghurt manufacturing results

not only in the formation of larger aggregates (Puvanenthiran et al., 2002), but also greater bonding between these protein segments (Alting et al., 2003, Puvanenthiran et al., 2002). As a result, yoghurt gel structure was more dense and possessed smaller pores (Puvanenthiran et al., 2002) with consequently low permeability and serum expulsion (Alting et al., 2003, Puvanenthiran et al., 2002). Moreover, soluble denatured whey proteins residing in the pores are also capable of performing physical barrier for serum flow (Puvanenthiran et al., 2002).

7.3.4 Rheological properties of fermented milk with the EPS and native and heat-treated whey proteins

7.3.4.1 Elastic properties of fermented milk gels

The elastic properties of the fermented milk samples expressed as the storage moduli were affected by the acidulant type and their interactions with whey proteins ($P<0.05$) (Table 7.2). In the control and native whey protein supplemented fermented milk, the storage moduli were significantly ($P<0.05$) and consistently lower in the culture fermented fermented milk than in the GDL fermented milk. However, this was reversed upon supplementation by the higher concentration (casein:whey protein ratio of 1:1) of heat treated whey proteins. Higher ($P<0.05$) values of storage moduli were also observed at a lower concentration of heat-treated whey proteins for the capsular fermented milk, when compared to that of GDL fermented milk. The addition of native whey proteins appeared to be more detrimental to the structure of the culture produced fermented milk than that of the GDL with even increased G' (Table 7.2). The reduction of the gel strength as a result of the native whey protein supplementation was previously observed (Oliveira et al., 2001, Sodini et al., 2002) and may be due to certain properties of whey proteins as 'inactive filler' (Guggisberg et al., 2007) which poorly support the formation of the acid induced gel network. Our result was different from earlier work (Patocka et al., 2004) in which the native whey proteins at high concentrations were able to reverse a decline in the storage modulus. This disparity may originate in two different approaches. In our study, a

portion of milk proteins was replaced by whey proteins at the constant total solid level, while in the other study (Patocka *et al*., 2004) the whey protein supplementation also increased the total solid content, which could have also affected the elastic properties.

In contrast to the native whey protein addition, the applied heat treatment resulted in whey proteins that subsequently increased G' in all types of fermented milk in the concentration-dependent manner. The apparent rise of the storage modulus was less pronounced in the GDL fermented milk than in the culture-produced fermented milk. This could likely indicate a possible interaction between the released EPS and heat-treated whey proteins. The greater G' values upon addition of denatured whey proteins were considered to be due to their interactions with casein (Vasbinder et al., 2003). Previously, the EPS exhibited a depletion interaction with denatured whey proteins (de Kruif and Tuinier, 1999), as well as casein (Tuinier et al., 1999) and induced the aggregation of both proteins. Furthermore, partially denatured whey proteins were able to interact with soluble κ-casein aggregates in the acidic environment (Vasbinder et al., 2003), whilst aggregates of denatured whey proteins were also present in the surrounding within the network (Puvanenthiran et al., 2002), both magnified the gel strength.

Table 7.2 Rheological properties of the fermented milk supplemented with either native or heat-treated whey protein isolate and acidified using GDL or capsular-ropy or capsular EPS producing strains of *Streptococcus thermophilus* at 42 °C.

Fermented milk sample*	G', (Pa)	Herschel-Bulkley coefficients			Bingham coefficients		Ostwald Coefficients		Thixotropy, (Pa/s)
		K, (Pa s^n)	n	Yield, (Pa s)	Yield, (Pa s)	b	K (Pa s^n)	n	
GDL									
Control (Skim Milk)	5096.6^{bB}	0.06^{aA}	0.07^{aA}	0.86^{aA}	0.06^{aA}	0.05^{aA}	0.19^{aA}	0.59^{cA}	9.2^{aA}
3:1, NWPI	8067.5^{bB}	0.41^{aA}	0.19^{aA}	0.76^{aB}	0.47^{aA}	0.07^{aA}	0.97^{aA}	0.23^{bA}	22.2^{aA}
1:1, NWPI	5920.0^{bB}	1.65^{aA}	0.35^{aA}	0.71^{aA}	1.79^{aA}	0.11^{aA}	2.63^{aA}	0.22^{bA}	34.8^{aA}
3:1, DWPI	1835.0^{aA}	69.34^{abA}	2.78^{aA}	0.99^{aA}	71.09^{abA}	1.22^{bAB}	104.74^{bA}	-0.22^{aA}	1478.4^{aA}
1:1, DWPI	6760.0^{bA}	99.36^{bA}	7.73^{aB}	1.83^{aA}	105.71^{bA}	0.49^{aA}	119.72^{bA}	0.05^{aA}	5580.2^{bA}
Capsular-ropy									
Control (Skim Milk)	149.7^{bA}	9.08^{aA}	5.29^{abA}	0.60^{aA}	11.25^{aA}	1.08^{bA}	19.36^{aA}	0.24^{bB}	363.8^{aA}
3:1, NWPI	33.4^{bA}	2.52^{aA}	2.53^{abA}	0.52^{aA}	3.54^{aA}	0.37^{aA}	13.47^{aA}	0.23^{bA}	97.6^{aA}
1:1, NWPI	24.5^{bA}	0.89^{aA}	1.81^{abA}	0.41^{aA}	1.43^{aA}	0.17^{aA}	3.14^{aA}	0.26^{bA}	111.9^{aA}
3:1, DWPI	549.0^{aA}	26.34^{aA}	8.550^{bA}	0.52^{aA}	28.49^{aA}	1.07^{bA}	38.88^{aA}	0.15^{aA}	2375.2^{aA}
1:1, DWPI	11627.5^{bB}	593.76^{bB}	-0.73^{aA}	1.42^{bA}	600.62^{bB}	-4.22^{aA}	625.16^{bB}	0.02^{aA}	39919.9^{bC}
Capsular									
Control (Skim Milk)	574.75^{aA}	21.36^{aA}	3.24^{aA}	0.92^{aA}	22.79^{aA}	1.13^{bA}	31.69^{aA}	0.15^{bC}	1182.9^{aA}
3:1, NWPI	132.55^{aA}	4.89^{aA}	1.85^{aA}	0.64^{aA}	5.87^{aA}	0.47^{aA}	8.96^{aA}	0.22^{bA}	389.5^{aA}
1:1, NWPI	74.27^{aA}	2.27^{aA}	1.59^{aA}	0.58^{aA}	2.86^{aA}	0.33^{aA}	5.41^{aA}	0.25^{bA}	287.5^{aA}
3:1, DWPI	7320.00^{bB}	194.22^{bB}	5.81^{bA}	0.93^{aA}	196.39^{abA}	2.51^{cB}	224.54^{bB}	0.05^{aA}	5368.2^{aA}
1:1, DWPI	14850.00^{cC}	593.76^{cB}	-0.73^{aA}	1.42^{aA}	600.61^{bB}	-4.22^{aA}	625.16^{cB}	0.02^{aA}	18017.9^{bB}
SEM**	662.6	23.07	2.01	0.31	23.30	0.34	25.83	0.04	1409.2

* Numbers denote casein and whey protein ratio, NWPI stands for native whey protein isolate, and DWPI stands for heat-treated whey protein isolate **SEM - pooled standard error of the mean, $P<0.05$. Means present the average of at least 4 determinations (n=4); Small letter superscripts in a column denote significant difference ($P<0.05$) among samples at different whey protein supplementation, for a particular type of acidulant; Capital letter superscripts in a coloumn denote significant difference ($P<0.05$) among samples at the same whey protein supplementation, for different acidulant.

7.3.4.2 *Flow behavior of fermented milk samples*

In our study, several models commonly used in the study of rheology of semisolid foods, namely Ostwald, Herschel-Bulkley, and Bingham, were used to ascertain flow behavior of the fermented milk samples after manufacturing (Rao, 1999). The three models describe the flow behaviour of a material possessing pronounced shear-thinning properties at lower shear rates. Both Herschel-Bulkley and Bingham models show yield stress of a material, above which the material may exhibit the Newtonian (Bingham) or either shear-thinning or shear-thickening (Herschel-Bulkley) flow. The consistency index derived from the Ostwald and Herschel-Bulkley models were apparently similar (Table 7.2). However, the Bingham model produced much lower values with no apparent trend, and even showed rheologically meaningless negative values at high concentrations of added heat treated WPI. This may indicate a poor suitability of the Bingham model compared to the other two models employed here. All models indicated higher consistency coefficient of the EPS containing fermented milk as previously reported (Hassan et al., 2003b) than this of the non-EPS fermented milk, although the difference was slight ($P>0.05$). Among similar types of mixture, the difference of consistency index in all models between the GDL and bacterial fermented milks was only significant ($P<0.05$) upon supplementation of heat-treated WPI either at all concentrations (Table 7.2). During supplementation of low concentration of heat-treated whey proteins, consistency index derived from the Herschel-Bulkley or Ostwald model showed higher ($P<0.05$) values for the bacterial than that of the GDL fermented milk. The EPS apparently improved the shear resistance of fermented milk. The addition of the native whey proteins to the fermented milk fermented with the cultures had slightly lower ($P>0.05$) consistency index, reversely related to the WPI concentration, in comparison to that of the GDL sample. On the other hand, the heat treated whey proteins consistently and significantly ($P<0.05$) increased consistency index in all types of fermented milks, although to a lesser extent in the GDL fermented milk. This

observation may further underline a probable interaction of the EPS and heat-treated WP, which consequently improved the strength of the fermented milk gel.

The estimation of the viscous (flow behavior) coefficient by two models (Herschel-Bulkley and Ostwald) delivered different results (Table 7.2). Viscous coefficient derived from the Herschel-Bulkley model was higher for the GDL than bacterial fermented milk with supplementation of higher concentration of heated whey proteins. On the other hand, those of control bacterial fermented milk derived from Ostwald model were higher (P<0.05) than that of the corresponding GDL fermented milk. As the concentration of added whey proteins increased, the Herschel-Bulkley model estimated a decline of n values for native, but increasing values for the heat-treated WPI. However, this model appeared to be unsuitable for the samples containing higher concentrations of heat-treated WPI. On the other hand, the Ostwald model provided more consistent estimation for all the samples with the transposed relation of the n values with the WPI concentration. Similarly to Herschel-Bulkley estimation, the viscous coefficients were lower for the heat treated than the control and native WPI fermented milk. This model underlined shear-thinning characteristics of all samples, with the n values less than 1 (Rao, 1999). The fermented milk supplemented with heat-treated WPI produced a greater deviation from the Newtonian flow, than those of the native WPI fermented milk. Lower viscous coefficient for the EPS-containing fermented milk was observed previously (Hassan et al. 2003b).

The yield stress of all fermented milk samples was estimated using the Herschel-Bulkley and Bingham models with varying results. Using the Herschel-Bulkley model, yield stress was relatively low in all types of fermented milk, ranging from 0.521 to 1.837$\pm$0.309 Pa with no obvious statistical difference (P>0.05) or trend. In contrast, the yield stress estimated by the Bingham model ranged from 0.64 to 600.62$\pm$23.30 Pa with an apparent trend. Therefore, the Bingham model appeared to estimate the yield stress of all samples better than the Herschel-Bulkley model. Upon addition of heat-treated whey proteins, the Bingham model showed significantly higher (P<0.05) yields of bacterial fermented milk than GDL fermented milk. Using Herschel-Bulkley model, however, only GDL fermented milk added with lower concentration of heat

untreated whey protein showed significant (P<0.05) higher yield than those of analogous bacterial fermented milk. The GDL fermented milk had insignificantly (P>0.05) lower yield compared to the culture produced fermented milk with a similar casein:whey protein ration (Table 7.2). Similar to other gel strength parameters studied (G', consistency coefficient, and firmness), the yield was reduced upon supplementation by native whey proteins in the EPS fermented milk, but not in the GDL fermented milk. In contrast, the addition of the heat-treated whey proteins increased significantly (P<0.05) yield in all types of fermented milk. A similar result was reported previously (Puvanenthiran et al., 2002) with denatured whey proteins increasing the yield stress of fermented milk due to a greater aggregate size and more extensive bonding within the gel structure.

The thixotropy area correlates to the structural redevelopment after shear, with a lower value indicating quicker structural rebuilding (Rao, 1999). In general, the capsular fermented milk exhibited larger thixotropy area compared to the analogous fermented milk treated with other acidulants (Table 7.2), which may indicate a more solid nature of the capsular fermented milk. Upon addition of either form of the whey proteins, the viscoelastic behaviour of the GDL fermented milk differed from that of the bacterial fermented milk, with consistently larger values of the thixotropy area. The low addition of native whey proteins to fermented milk caused reduction in the thixotropy area, with subsequent increase at the high concentration. Therefore, higher concentrations of native whey proteins may improve the structural rebuilding after shear in fermented milk. On the other hand, heat-treated whey proteins increased (P<0.05) the thixotropy area in all types of fermented milk in a concentration dependent manner. Smaller thixotropy may reflect quicker recovery after application of shear (Rao, 1999), showing that the material is either more solid or more elastic. In our study, the EPS-containing fermented milk had slower recovery than the GDL fermented milk. However, lower thixotropy of a material may also indicate its brittle character (Lucey et al., 1998), with higher thixotropy better reflecting an ability of the material to regain original texture after shear albeit longer recovery time, as shown by the EPS-containing fermented milk in our study. The rupture of texture,

phase separation and syneresis after stirring of non-EPS fermented milk had been observed previously, while the EPS fermented milk was able to regain its homogeneity (Hassan et al. 2003b).

Bacterial fermented milk showed significantly higher (P<0.05) thixotropy than that of the GDL fermented milk, only when supplemented with higher concentration of heat-treated whey proteins (Table 7.2). In general, although mainly not statistically significant (P>0.05), the capsular fermented milk exhibited larger thixotropy area compared to the analogous fermented milk treated with other acidulants, which may indicate a more solid nature of the capsular fermented milk. The thixotropy observations were consistent to other gel strength parameters (G' and consistency coefficient), showing that the native whey protein supplementation increased solid characteristics of the GDL fermented milk with the opposite tendency in the EPS containing fermented milk. Non-reactive, 'inactive filler' (Guggisberg et al., 2007) nature of the native whey proteins supplemented to milk mixture in bacterial fermented milk may be the cause for lowering the thixotropy, and connected to a weakening of its structure (Lucas et al., 2004, Oliveira et al., 2001, Puvanenthiran et al., 2002). On the other hand, heat treated whey proteins upon addition to the fermented milk increased the strength of casein aggregates via disulfide-bridges linkage to κ-casein, as well as induced β-lactoglobulin inter-particle association to give more rubbery-elastic characteristics (Vasbinder et al., 2003).

Power law, Bingham, Herschel-Bulkley, and Casson models were commonly used to describe flow behaviour of semi-solid foods (Murat and Kokini, 1986). In our work, the values of K were well described by Ostwald and Herschel-Bulkley models, n values by Herschel-Bulkley, and yield stress by Bingham model. However, in overall, apparently the data fit Herschel-Bulkley model better than the other two models, to show shear-thinning material with small yield. Herschel-Bulkley model was also the best model to be applied in the design of flow process of ketchup, mustard, apple sauce, and tomato paste (Murat and Kokini, 1986).

Heat-treatment of whey protein added into fermented milk base at low concentration in this experiment seems to restore the texture which was severely

damaged by addition of heat-untreated whey protein, calcium alone or in combination with heat-untreated whey proteins. However, at higher concentration of heat-treated whey protein added into the milk, the texture became too hard and compact without any pore, causing lost of semi-solid characteristics of this fermented milk.

7.4 Conclusions

The GDL and EPS-containing fermented milk showed different gelation, physical and rheological behaviour as a result of whey protein supplementation. Native or heat-treated whey protein supplementation produced gels with different properties. Gel strength parameters: G', yield, firmness in EPS-containing fermented milks were increased upon the addition of heat-treated whey protein in much greater extent compared to non-EPS fermented milk. Capsular and GDL fermented milk exhibited higher *K* (from Herschel-Bulkley and Ostwald models) and yield (from Bingham model) than the values from capsular-ropy fermented milk. *K* values were increased slightly by addition of native whey protein in GDL fermented milk, but were decreased in EPS-containing fermented milk. Addition of heated WPI increased *K* values in all fermented milk. Storage moduli of non-EPS fermented milk were relatively not influenced by WPI supplementation, in contrast to those of EPS-containing fermented milk. Non-EPS- and EPS-containing fermented milk showed opposite response on thixotropy upon addition of native WPI. Syneresis and permeability of all types of fermented milk were lowered by addition of heat-treated whey protein, while firmness was increased. Herschel-Bulkley model seemed to be the best model to describe flow properties of fermented milk.

In conclusion, further study is needed to reveal the mechanism of apparent interaction between EPS and whey protein during manufacturing of WPI-supplemented fermented milk. Therefore, during production of WPI supplemented fermented milk, the type of EPS produced by the starter cultures and the whey proteins state are apparently important factors to be considered in order to obtain desirable textural characteristics of fermented milk.

8 Summary of Results and Future Research Directions

8.1 Summary of Results

The *Streptococcus thermophilus* strains used in this study (ST 1275 and ST 285) produced exopolysaccharides (EPS) in varying concentrations. They were also capable of utilising galactose present in the medium probably recruiting intracellular processes to incorporate this sugar into newly formed exopolysaccharides. This characteristic may be beneficial to the economy of carbon utilisation of the cells. In M17 medium, the carbon source influenced both growth and EPS production of these two strains. While glucose and lactose supported their cell growth, galactose restricted it. On the other hand, the EPS concentration in galactose-containing M17 medium was comparable to those produced in the presence of glucose and lactose. Higher fermentation temperature favoured the cell growth with apparent growth-associated EPS production.

The capsular EPS exhibited different rheological characteristics in comparison to capsular ropy EPS. The apparent viscosity of capsular EPS dispersions declined to a greater extent by the increase in temperature, compared to that of capsular-ropy. Shear-resistance as indicated by K (consistency index) was positively affected by concentration, and the values in capsular EPS dispersions were higher than those of capsular-ropy. The viscosity at zero shear rate (η_0) and relaxation time (τ) between the two types of EPS were different and influenced by pH. In acidic environment, η_0 and τ of capsular-ropy EPS were higher, which may indicate lower molecular mobility due to higher water-binding, or more extensive entanglement, more extensive coil overlap, or size enlargement of the EPS molecules. Longer time was required by capsular-ropy EPS to rebuild its initial structure after disruption during shearing. In accordance to this, the parameter 'a' in Arrhenius plot showed less sensitive nature of capsular-ropy type of EPS. Less sensitivity may also relate to less mobility of the molecules.

Although not apparent, activation energy (ΔE) of capsular-ropy EPS tended to be lower than that of capsular EPS, further indicating more restricted molecular movement in the capsular ropy EPS. Both EPS showed low sensitivity to temperature, which was comparable to other bacterial hydrocolloid studied by Speers and Tung (1986). While temperature influence on the rheological properties of the two EPS was not shown clearly, the difference of zero-shear viscosity (η_o) between the two EPS was palpable. This rheological difference between the two types of EPS shown by Cross model parameters also emphasized the suitability of this method in the study.

When produced in fermented milk system, two types of EPS caused rheological and physical differences in the product, which were a reflection of the rheological properties of the EPS. Fermented milk made by capsular-ropy strain exhibited greater water-binding capacity, with a less brittle gel and larger thixotropy. Although the final texture of fermented milk gel was basically a network arrangement between milk protein and exopolysaccharide (Hassan et al., 2001a), the original character of EPS appeared to be manifested in the final fermented milk texture. For instance, capsular-ropy EPS in dispersion under acidic environment exhibited higher zero-shear η_o and relaxation time τ which was related to lower mobility. This lower mobility could be associated with its better ability to bind water. This character was displayed in fermented milk system containing capsular-ropy EPS, where the syneresis was lower than in that containing capsular EPS. Larger thixotropy of fermented milk with capsular-ropy EPS may also relate to lower mobility character of capsular-ropy EPS. Furthermore, longer relaxation time of capsular-ropy EPS is in accordance with less brittle gel of the fermented milk containing the EPS. This explanation somewhat added more information to previous report mentioning characteristic of attachment and location of EPS in yoghurt gel (Hassan et al., 2001a) in which ropy EPS resided in the pores while capsular EPS was close to the surface of casein network. The current work showed that higher water holding capacity, more flexible or less brittle and larger thixotropy of fermented milk containing capsular-ropy EPS.

During storage, certain structural changes appeared to have taken place, resulting in a more elastic gel regardless the types of EPS. There was a considerable reduction of the amount of EPS during first week of cold storage, but the textural properties as determined by rheological assessment were not greatly changed. This could have been likely caused by various interactions among fermented milk components that were more influential over the EPS disappearance to govern structural changes during gel ageing. Hindering effect of EPS on structural development of gel (Hassan et al., 1996b) may play role in this case, so that by reducing the EPS concentration during storage the protein-protein interaction is improved. Nevertheless, the difference characteristics between fermented milk prepared by each type of EPS were somewhat similar to their behaviour in dispersion.

Such phenomenon was also shown in calcium-fortified fermented milk, where the texture of fermented milk with capsular producer remained more solid-like with more syneresis than that of capsular-ropy cultures, despite there was some influence of added calcium. In the texture of calcium-fortified fermented milk, texture of non-EPS fermented milk was not affected to a great extent by the additive as compared to EPS-containing fermented milk. This gave the impression that there was possible interaction of EPS and calcium. However, it was more likely that calcium affected casein-casein network rather than EPS, since electrostatic interaction between calcium and uncharged or weakly-charged EPS was very unlikely to occur. It was not possible for us to comment on the charge of the EPS in this work, since it was not one of the objectives but should be carried out in the future. Furthermore the current work highlighted importance of the depletion flocculation due to presence of EPS and their interaction with milk proteins as reported in some studies (de Kruif and Tuinier, 2001). As it appeared in the present study charge screening may also play an important role since addition of calcium had a detrimental effect on the textural properties. Calcium fortification in EPS-containing fermented milk should be carried out with precaution, since it may reduce the bioavailability of calcium, by physically trapping the mineral (Bosscher et al., 2003). In contrast to the the gel strengthening effect of added calcium on fruit yoghurt (Singh and

Muthukumarappan, 2008), calcium addition in our work weakened the texture. This quite opposite observation was likely due to presence of pectin in fruit yoghurt and was not included in our work. This was also another indicator that the EPS examined in the present work were not chared at pH of fermented milk and made no or had adverse effect on the textural properties of acid gels.

Combined effect of calcium and heat-untreated whey protein addition to fermented milk impaired the texture even more, and made it more suitable for drinking yoghurt. This deleterious effect of whey protein supplementation on fermented milk gel had made it 'inactive filler' (Guggisberg et al. 2007). Therefore, the information from our experiment would be useful in order to yield a right combination of calcium and whey proteins to produce a gell with less syneresis, high thixotropy and low G'. From the current study, it appeared that whey proteins interfered with caseins rather than EPS.

Upon addition of heat-treated whey proteins, the texture of fermented milk can be improved and altered by adjusting the proportion between whey protein and casein. Addition of low proportion of heat-treated whey protein and casein resulted in EPS-containing fermented milk that differ in some textural characteristics, but their original EPS characteristics dissimilarity remained, i.e. those fermented with capsular-ropy producer was less solid and less syneresis than their corresponding fermented milk made using capsular producer. However, addition of high proportion of whey protein and casein diminished the difference. This, again, suggested the lack of interaction between EPS and whey protein either with or without heat treatment. The more severe effect of added heat-treated whey protein noticeable in non-EPS fermented milk texture probably due to the less stable system in EPS-containing fermented milk (de Kruif and Tuinier, 2001). Mixture of casein and heat-treated whey protein at high concentration had performed hard gel at the beginning of fermentation, thus eliminated the effect of other components such as EPS and fermentation on the final texture of the product. Heat treated whey proteins may form inter-particle association or more extensive disulfide linkage (Vasbinder et al., 3003), resulted in a very hard and rubbery gel.

As the overall conclusion, the production of fermented milk with additional functional properties was possible through the use of low-fat skim milk, employing EPS-producing strains, and supplementation with calcium or/and whey protein concentrate. The two EPS used in the study showed an important role in the development of fermented milk texture albeit the small quantity present in the fermented milk. Moreover, the rheological behaviour of the two EPS in fermented milk exhibited consistent distinctive characteristics. Since polymer-polymer interaction is beyond the focus of this study, we cannot provide any detail explanation on the nature of interaction between EPS and milk proteins. However, the practical implication of the interaction was consistently shown through various experiments that texture of fermented milk made using capsular-ropy EPS was less solid, less syneresis, and more shear-resistant compared to that made using capsular EPS.

8.2 Future research directions

Based on the findings of this work, several areas have resurfaced and require a closer attention in order to properly assess and understand the complex interactions of EPS-containing fermented milk in their natural or supplemented form. The following points address possible directions that could be taken in resolution of these complex systems:

1. the improvement of metabolic activity of selected cultures - the strains used in this study experienced very slow-growth, therefore the supplementation of the fermented milk mix with relevant growth factors should be examined to shorten the fermentation time; however, the special attention should be taken to maintain or improve the production of exopolysaccharides. Alternatively, a fast growing EPS producing strain should be additionally assessed.
2. the use of mixed culture for the improvement of EPS characteristics produced in fermented milk - two examined strains released EPS, which gave distinctly different textural characteristics. One strain produced EPS which gave stronger solid character and smaller thixotropy but

brittle gel with large whey expulsion of fermented milk. On the other hand, the type of EPS produced by the other strain gave ropy fermented milk with greater shear resistance, less syneresis, but weaker solid character. Thus combining the two strains in a right proportion may better improve the texture of fermented milk.

3. the nature of various interactions - in this study, it was demonstrated the changes in the textural properties of EPS-containing fermented milk as affected by various supplementations. However, controlling the texture creation is an important element in order to attain desirable textural characteristics. To achieve this goal, a thorough study on the understanding of mechanisms among fermented milk constituents would be essential. As shown in several chapters, the EPS appeared to be involved in the interaction with milk protein network as well as supplements, all of which resulted in a substantial effect on fermented milk textural properties.
4. sensory studies - the acceptance of fermented milk, including new type of fermented milk containing supplements, is greatly governed by its creamy characteristics. Therefore, any manufacturing or modification of fermented milk should aim at creating creamy appearance and mouthfeel. A sensory study or studies that would assess the effects of supplementation on the consumer acceptance would be necessary. It would also be important to correlate these sensory characteristics to rheological and physical examinations for practical purpose. Despite its importance, this area has not been widely studied. Since human perception of the sensory quality of fermented milk may vary among groups of people or cultures, the study on this particular topic can be expected to be broad.
5. physiological importance - the efficacy of nutrient supplementation was affected by several factors, including the intake or proportion of mixed supplements. The effect of calcium on bone strengthening may be affected by the concentration of whey proteins added into the fermented milk mix. Similarly, the effect of whey protein on preventing muscular

degeneration may also be influenced by calcium concentration being added into the fermented milk. Some fermented milk strains, exopolysaccharides, or bioactive peptides released by digestion of whey proteins may have potential immunomodulatory properties. The extent of the health benefits of supplemented EPS-containing fermented milk thus needs to be explored.

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