INVESTIGATION OF EXOPOLYSACCHARIDE PRODUCTION BY LACTIC ACID BACTERIA.

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Declaration

This thesis, which is submitted for consideration for the degree of Doctor of Philosophy, is a record of research carried out in the School of Life Sciences, The Robert Gordon University, under the supervision of Dr Eoin Cowie, Dr Colin Henderson and Professor Brian Ratcliffe. It is believed to be original except where due reference has been made and has not been presented for any other higher degree.

Rachael Ann Jones
December 2008

"Two roads diverged in a wood, and I –
I took the one less travelled by,
And that has made all the difference"

"The Road Not Taken"
Robert Frost.

ABSTRACT

This thesis is an investigation into the production of exopolysaccharides (EPS) produced by strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis* ssp. *cremoris*. These are used in the dairy industry for the production of yoghurt and fermented drinks products. For many years EPS producing lactic acid bacteria have been used by the dairy industry as a thickening agent in the production of yoghurt. However, this EPS producing trait is unstable and is readily lost which can cause an alteration in the texture of the final product. It was found that all the strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* produced quantities of EPS that could be used for further analysis. They were found to be in the molecular weight range of 6.6×10^6g /mol to 1.26×10^{11} g/mol and were composed of different quantities of glucose, galactose and rhamnose. Temperature, carbon source and shaking all affected the quantities of EPS produced by all strains of *Lc. lactis* ssp. *cremoris*.

The firmness and viscosity of fermented milks produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* were higher than those produced by strains of *Lc. lactis* ssp. *cremoris* indicating that firmness and viscosity are not solely related to the levels of EPS production. A 40kb plasmid was found in all strains of *Lc. lactis* ssp. *cremoris* that could potentially contain the genes for EPS production. The plasmid could not be removed using elevated temperature or with the addition of acriflavin. Fourier transform infrared spectroscopy (FTIR) showed that it was possible to differentiate different strains based on their spectra and that differences were found in the protein and EPS regions of the spectra. It was also established that the age of culture, whether the growth medium was liquid or solid and the carbon source of the growth media had an effect on the FTIR spectra produced and the ability to differentiate between strains. There is further potential to develop this technique to provide a quick and easy method of identifying strains of lactic acid bacteria and monitor their EPS producing ability.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. GENERAL INTRODUCTION

BIOFILMS

The idea that bacteria prefer to grow on surfaces has been around for about 150 years. In 1847 Antoine van Leeuwenhoek, using a primitive microscope described what he called "animalcules" scraped from the surface of human teeth. Almost 100 years later, in 1934, Claude Zobell examined marine populations by direct microscopy, and concluded that there are bacteria attracted to surfaces and adhere (Costerton 1999).

Biofilms are colonies of bacteria that are attached to a surface that can be enclosed in exopolysaccharide (EPS). Biofilms allow nutrients for the growth of microbial population to be trapped and to prevent the detachment of cells when they are present in systems covered with flowing liquids. Biofilm formation is a dynamic process and current models are primarily based on *Pseudomonas* species (Hall-Stoodley and Stoodley 2002). Formation is depicted as a process that begins when free-floating planktonic bacterial cells attach to a surface. This is then followed by growth into a mature complex biofilm (Figure 1.1). In addition to this bacteria can break off from the biofilm and disperse into the surrounding fluid

Where are biofilms found?

Biofilms may form

1. On moist solid surfaces.
2. On soft tissue inside living organisms.
3. At interfaces between a liquid and the air.

Bacteria form biofilms because the biofilm improves the survival of the bacteria by allowing cells to remain a in a favourable niche and live in close association to one another. This facilitates intercellular communication and exchange of genetic material.

Biofilms can be found in a variety of different environments. They are found on the surface of rocks in rivers, lakes, streams and the sea (Costerton and Lappin-Scott 1995).

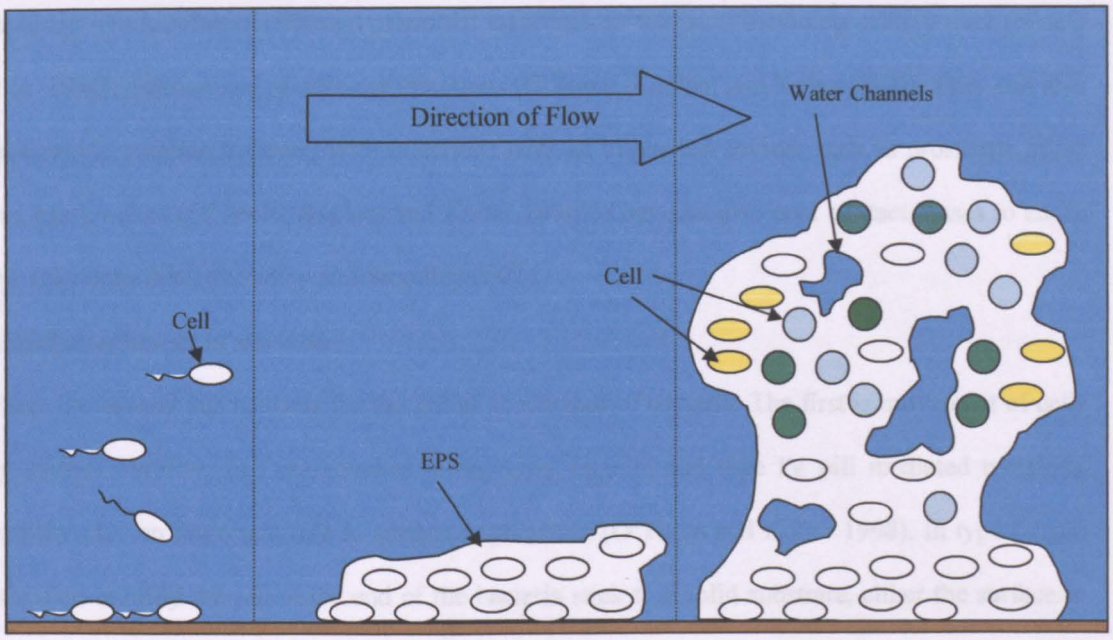


Figure 1.1 Formation of a biofilm.

They act as a food source for small invertebrates which are then eaten by larger predators. Biofilms, therefore, act as an important part of aquatic food chains. Biofilms can also be found growing in hot, acidic volcanic pools in or on glaciers (Costerton 2007).

In industry biofilms can develop on the insides of pipes, which can lead to then becoming blocked or corroded (Hall-Stoodley and Stoodley 2002). Biofilms can also be found in food preparation areas, particularly on floors and counters and this can make cleaning difficult (Costerton 2007). The biofilms could also harbour food poisoning bacteria. Biofilms in cooling water systems can harbour *Legionella* bacteria (Hall-Stoodley and Stoodley 2002).

In sewage treatment plants waste water passes over biofilms grown on filters. These filters can digest organic material present in the water. Drinking water from lakes, springs or rivers can be passed over slow sand filters covered in biofilms to remove certain contaminents (Costerton 2007).

As mentioned earlier biofilms are also present on the teeth of most animals in the form of dental plaque, which is responsible for tooth decay and gum disease (Costerton 2007). They are also

involved in a number of different microbial infections in the body including urinary tract (Nickel et al. 1994), catheter and middle-ear infections (O'Toole, Kaplan, and Kolter 2000). They can also be found in patients suffering with infections from of implanted devices such as prosthetic joints and heart valves (O'Toole, Kaplan, and Kolter 2000). They can also coat contact lenses to cause eye infections (Hall-Stoodley and Stoodley 2002).

Initial attachment of bacteria

There are several mechanisms for the initial attachment of bacteria. The first is movement of cells by surface motility. In *Pseudomonas aeruginosa,* flagella and type IV pili mediated twitching motility play an important role in surface aggregation (O'Toole and Kolter 1998). In type IV pili mediated motility the pili at the end of the bacteria stick to a solid substrate, either the surface to which the bacteria are attached or to other bacteria, and the pilus contracts to pull the bacteria forward, similar to a grappling hook. Movement is typically jerky and is called twitching motility. This style of movement is also seen in a number of other gram negative organisms such as *Escherichia coli* (Jackson et al. 2002) and *Vibrio cholera* (Watnick et al. 2001). However, motility is not essential for the formation of biofilms as *Staphylococcus epidermidis,* a non motile bacteria uses proteins and polysaccharide adhesins for adherence (Rupp et al. 1999).

Another mechanism for the start of biofilm formation is as a result of binary division of attached cells. As the cells divide, new cells spread outwards and upwards to form cell clusters, in a similar way to colony formation (Tolker–Nielson et al. 2000). Surface-associated aggregation can also occur by recruitment of single cells (Tolker–Nielson et al. 2000) or cell clumps (Stoodley, Hall-Stoodley and Lappin-Scott 2000) from the surrounding fluid. However, this only plays a small role in colonization. Lastly, large patches of mature biofilms can be broken off by the flow of fluid over the surface and this can allow colonization of surfaces downstream (Stoodley et al. 1999). The role of the three mechanisms in biofilm formation is dependent on the surface being colonized the conditions of the aqueous environment and the organisms involved.

Genetic regulation of biofilm formation

It has been suggested that biofilm formation is influenced by a number of regulatory pathways. O'Toole et al. (2000) studied the events in biofilm development using a *crc* mutant of *P. aeruginosa* and found that incomplete biofilm formation was due to a defect in type IV pili expression/function. Crc is a carbon metabolism regulator found in all bacteria and its role in influencing biofilm development via type IV pili expression is believed to be due to sensing certain types of nutrition. In a study involving *E. coli*, the effect of a carbon regulator (CsrA) was found to have an effect on biofilm formation (Jackson et al. 2002). In this study, a number of genetic and nutritional conditions were found to effect biofilm formation, suggesting that biofilm development involves a number of factors and is a very complex response. Disruption of *csrA* increased biofilm formation compared with the parent strain and the over expression of CsrA was found to be inhibitory in *E. coli* K12 and in pathogenic isolates. By disrupting *csrA* biofilm formation was improved even when flagella and pili were absent. This implicates *csrA* regulation in a number of biosynthetic processes. Biofilm formation has also been associated with conjugative plasmids. Ghigo (2001) found that certain plasmids induced biofilm formation and suggested that high cell densities in biofilms favour higher rates of transfer of plasmid DNA. Genetic regulation controlling biofilm development appears to be very complex.

Quorum sensing and biofilm structure

The communication of cells is critical in the development and maintenance of a biofilm. In *P. aeruginosa*, one of the most common biofilm formers, the major signalling molecules are compounds called homoserine lactones. As the molecules accumulate, they function as chemotactic agents to recruit nearby *P. aeruginosa* cells, and the biofilm develops. This method of communication is known as quorum sensing. Quorum sensing is a mechanism to ensure that sufficient cell numbers of a given species are present before eliciting a response. Quorum sensing is widespread among gram negative bacteria. Each species synthesizes a specific acetylated homoserine lactone (AHL). The molecule can diffuse outside of the cell but will only build up in large quantities if there are a number of bacterial cells nearby secreting the same AHL. The

specific AHL functions as an inducer that combines with an activator protein and this will cause the transcription of specific genes (Miller and Bassler 2001).

The role of EPS in biofilm formation

Many micro-organisms synthesise polysaccharides that are defined by their location relative to the cell (Cerning 1995). Exopolysaccharides (EPS) are long-chain polysaccharides composed of branched, repeating units of sugars or sugar derivatives. The sugar units present are primarily glucose, galactose and rhamnose, found in different ratios (Welman and Maddox 2003). EPS are secreted into the surroundings during growth and are not attached to the bacterial cell wall. This separates them from structurally similar capsular polysaccharides (CPS) which remain attached to the cell wall. The EPS contribute to the mucoid appearance and sticky texture of bacterial cultures when grown on a solid medium or to the increased viscosity in liquid culture.

Bacterial cells in biofilms are held together primarily by EPS. Polysaccharides are the best-studied component of EPS. However, there is a substantial diversity in EPS produced by different species of bacteria under different growth conditions (Sutherland 2001). This is as a result of difficulties in the separation and extraction of EPS from bacterial cells and the difficulties of analyzing polysaccharides. It has not been established whether the nucleic acids and proteins found in EPS have a structural role or are remnants of cellular debris.

The polysaccharide alginate, produced by mucoid strain of *P. aeruginosa*, is the best-studied component of biofilm EPS. It appears to play an important role in determining biofilm structure. The complexity of mucoid biofilms grown from the cystic fibrosis isolate *P. aeruginosa* FRD1 was suppressed in non mucoid strains, which formed flat, patchy biofilms (Nivens et al. 2001). A structurally complex biofilm was also observed when alginate was overexpressed in a non-mucoid *P. aeruginosa* PAO1 mutant (Hentzer 2001). A glucose and galactose-rich EPS produced by a variant of *V. cholerae* O1 El Tor was necessary for complex biofilm development (Yildiz, Dolganov and Schoolnik 2001). The increased production of EPS in flagellar mutants of *V. cholerae* O139 was linked to reduced intestinal colonization in an infant mouse models (Watnick et al. 2001).

As well as being involved in the formation of biofilms the EPS produced by strains of lactic acid bacteria can be utilized by the dairy industry in the production of fermented yoghurts.

Yoghurt and EPS

Yoghurt and similar fermented milk products are popular food products world-wide and in a number of countries yoghurt is still made using traditional methods. However, in the last 20 years yoghurt consumption has increased in Europe and the United States (Zourari, Accolas and Desmazzeaud 1992). Therefore traditional methods of manufacture using small quantities of milk are no longer sufficient to meet demands in volume and variety of yoghurt required by today's consumers. Yoghurt now has to be manufactured on a much larger scale and use additives, such as pectins and alginates to improve the texture of the final product (Cowie 1993). However, yoghurt manufacturers are also using EPS producing bacteria that can act as natural thickening agents. The use of EPS-producing bacteria in the production of yoghurt is not a new innovation. For many years EPS-producing strains of *Lactococcus lactis* ssp. *cremoris* or *Lc. lactis* ssp. *lactis* were essential for the production of traditional Scandinavian products "Viili" and "Långfil". However, more recently EPS-producing strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* have been used in mainstream yoghurt production as thickening agents. The use of EPS-producing strains has a number of advantages compared with that of non EPS-producing strains. These include the improvement of texture of the product and replacement of artificial thickeners (Cowie 1993).

The EPS-producing trait in lactic acid bacteria has been reported to be unstable and can be readily lost without affecting bacterial growth (Gancel and Novel 1994a, Bouzar, Cerning and Desmazeaud 1996). Quality yoghurt depends not only on flavour and aroma, but also on texture. If texture is dependent on EPS production, then bacterial cultures used in yoghurt production need to have a stable EPS-producing ability. To help in monitoring the phenotype and control the stability of this characteristic, rapid and simple methods for monitoring EPS production are required. A detailed understanding of the production of EPS and its role in affecting the texture of yoghurt is also important.

FERMENTED MILK PRODUCTS.

Fermentation to extend the useful life of milk has been carried out for thousands of years. The first animals to be domesticated are thought to have been goats and sheep in the Middle East about 9000BC (Cowie 1993). Early fermented milks are likely to have come about through the growth of naturally occurring lactic acid bacteria in the milk as a result of the warm climate. Lactic acid bacteria play an important role in the production of fermented dairy products in Northern Europe, Eastern Europe and Asia (Table 1.1).

Table 1.1 Some lactic acid bacteria involved in the production of fermented milk products (adapted from Dubòc and Mollet 2001).

Bacteria	*Fermented milk products*
Lactococci	
Lc. lactis ssp. *lactis*	Cultured buttermilk, kefir
Lc. lactis ssp. *cremoris*	Cultured buttermilk, kefir
Streptococci	
St.thermophilus	Yoghurt, dahi, mozzarella
Leuconostoc	
L.mesenteroides ssp. *mesenteroides*	Kefir, cultured cream
L.mesenteroides ssp. *cremoris*	Kefir, cultured cream
L.mesenteroides ssp. *dextranicum*	Kefir, cultured cream
Lactobacilli	
Lb.delbrueckii ssp. *delbrueckii*	Fermented milk drinks, yoghurt
Lb.delbrueckii ssp. *bulgaricus*	Yoghurt, buttermilk, mozzarella
Lb.delbrueckii ssp. *lactis*	Fermented milk drinks
Lb.helveticus	Kefir, koumiss, mozzarella
Lb.acidophilus	Acidophilus milk, kefir
Lb.paracasei ssp. *paracasei*	Fermented milk drinks
Lb.johnsonii	Probiotic yoghurt fermented milk drinks
Lb.casei	Probiotic yoghurt
Lb.paracasei	Probiotic yoghurt
Lb.reuteri	Probiotic yoghurt
Lb.rhamnosus	Kefir
Lb.plantarum	Kefir
Lb.kefir	Kefir
Lb.kefiranofasciens	Kefir
Lb.fermentum	Kefir

YOGHURT PRODUCTION

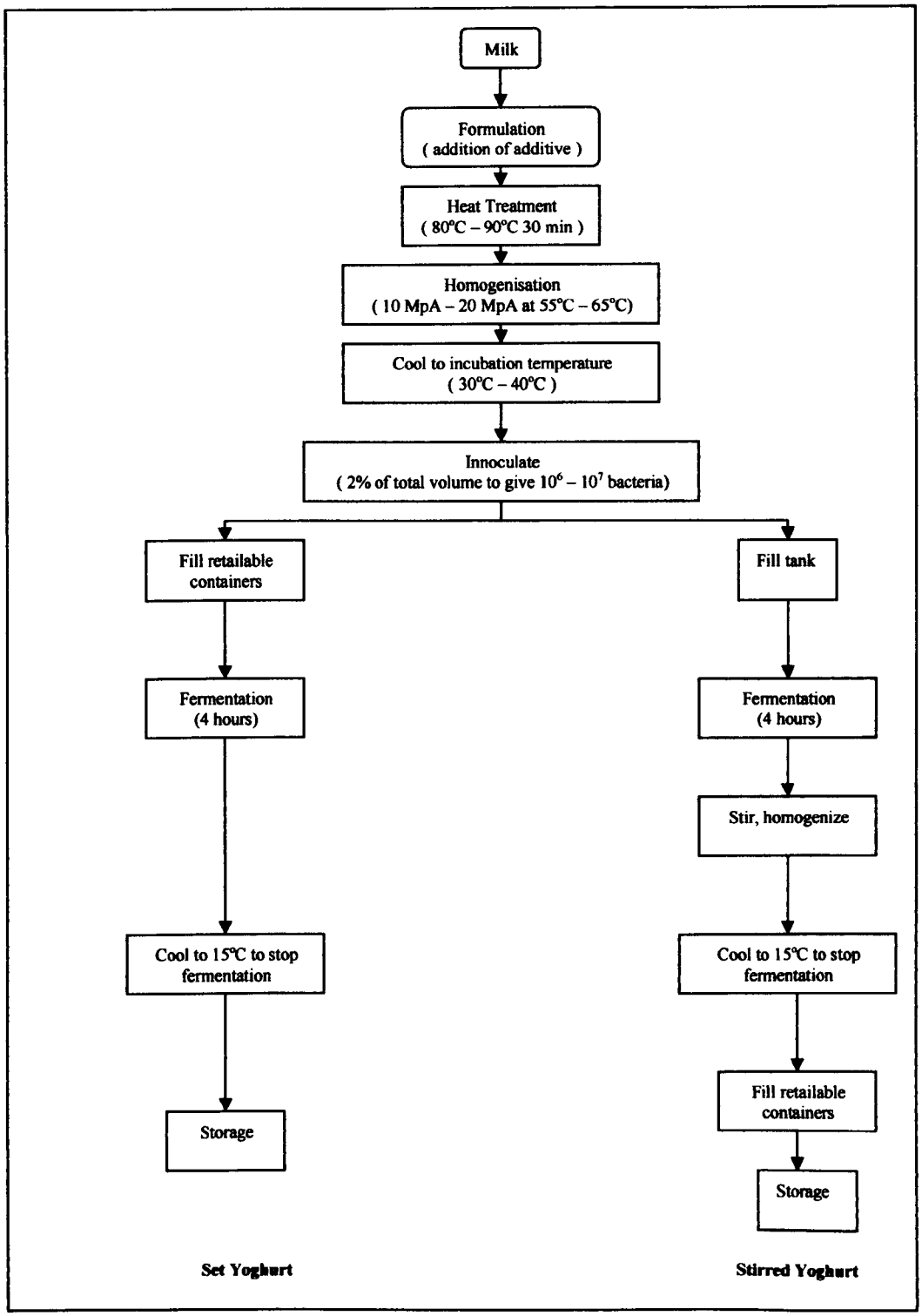


Figure 1.2 Yoghurt Production. Further details for each stage can be found in the text below (adapted from Duboc and Mollet 2001).

The production of yoghurt relies on the formation of a milk gel. This occurs during the fermentation stage. In milk of normal pH, casein micelles are stabilised by hydration, negative charge and steric repulsion (Mulvihill and Grufferty 1995). Caseins make up approximately 80% of the protein in bovine milk, with four main types (α_{s1}-, α_{s2}-, β-, and κ– caseins) in combination with quantities of micellar or colloidal calcium phosphate (CCP) in the form of casein aggregates called casein micelles.

Decreases in the pH, to around pH 5.3, cause changes to occur in casein micelles and make them different from the casein micelles found in normal milk of pH 7 (Lucey and Singh 1998). Bacterial cultures ferment lactose to lactic acid this causes a drop in the pH and casein particles aggregate. As a result charge neutralization occurs leading to the formation of chains and clusters that are linked together to form a three dimensional network (Lucey and Singh 1998). The mechanism of gel formation is still not fully understood but it is known that it takes place during the fermentation stage.

During acidification of milk, many of the physiochemical properties of casein micelles undergo considerable change, especially in the pH range 5.5 to 5.0, including a dissociation of the caseins As the pH of unheated milk is reduced, CCP is dissolved and the caseins are liberated into the liquid phase of the milk (Dalgleish and Law 1988). The extent of liberation of caseins is dependent on temperature; at 30°C a decrease in pH causes virtually no liberation; at 4°C about 40% of the caseins are liberated at pH 5.5 (Dalgleish and Law 1988).

Virtually all milks used in yoghurt manufacture are subjected to an extensive heat treatment. Heating of milk can lead to many changes including denaturation of whey proteins and their interaction with casein micelles. Heat treatment of milk above 70°C causes denaturation of whey proteins, some of which associate with casein micelles, involving κ-casein, via hydrophobic interactions and the formation of intermolecular disulphide bonds (Haque and Kinsella 1988). Moderate heating does not appear to affect the sizes of casein micelles although these treatments cause the whey proteins to denature and bind to micellar κ-casein; more extensive heat treatment causes some degree of micellar aggregation and an increase in particle size (Dalgleish, Pouliot and Paquin 1987).

During acidification of milk most of the CCP in the micelles is solubilized, the charge on individual caseins is altered and the ionic strength of the solution increases. As a result, the forces responsible for the integrity of these 'micelle-like' CCP-depleted casein particles are considerably different from native micelles (Lucey and Singh 1998).

Yoghurt Additives.

Whey separation, the appearance of liquid on the surface of a milk gel, is a common defect in fermented milk products (Lucey and Singh 1998). Whey separation occurs if the gel network is damaged or if the gel undergoes structural rearrangement. Syneresis is the natural shrinkage of a gel and this occurs at the same time as the expulsion of liquid or whey separation.

In yoghurt making, in some countries, it is common practice to supplement the solids content of the milk using skimmed or whole milk powder to enhance the natural texture of the yoghurt. The properties of the yoghurt may also be improved and stabilised by the addition of chemical stabilisers. These stabilisers are added to help to prevent syneresis, and to bind free water (Cerning 1995). Examples of additives include gelatine and the plant polysaccharides, guar gum, carageenan, pectin and starch.

Polysaccharides such as guar gum increase viscosity by forming a network of polysaccharide chains. They give high viscosity at low concentration. Carageenan is well known for its ability to stabilise milk proteins and is often used in the dairy industry as a gelling agent, thickener and a stabiliser. Pectin is another polysaccharide used in yoghurt making and is only required in small amounts. This is useful as too high a concentration of polysaccharide additives can impair flavour. Starch can be used up to 1%, but the concentration of other stabilisers should not exceed 0.5% (Marshall et al. 2001).

In France and the Netherlands, the addition of stabilisers to yoghurt is prohibited (Cerning 1995). Also, the consumer is now demanding low fat, low sugar, and low cost yoghurts with as few additives as possible. Strains of *Lb.delbrueckii* ssp. *bulgaricus, Lc. lactis* ssp. *cremoris* and *St. salivarius* ssp. *thermophilus* all produce polysaccharides that are thought to enhance the natural thickening and stabilising properties of the yoghurt. The use of these EPS-producing starter

cultures may enhance the texture of resultant yoghurts and helps to reduce the incorporation of additives.

Heat Treatment and Homogenisation of Milk

Yoghurt is made from full fat or skimmed milk usually from cows but it can also be made from goat's or sheep's milk. Before the starter culture is added two preliminary steps are required (Figure 1.2). Firstly, the milk is heated at 80 - 90°C for about 30 min. This sterilises the milk and removes microbial competition during fermentation by starter culture organisms. Heating also promotes the interaction of whey proteins and casein (Mottar et al. 1989). It is assumed that the interaction of whey proteins with casein increases the hydrophobicity of the micellar surface, which favours gel formation. This is because the heating process denatures the whey proteins causing β-lactoglobulin to interact with κ-casein and for both to become integrated into the micelles (Dannenberg and Kessler 1988). The heating process increases the yoghurt's viscosity, stabilises the gel and limits syneresis. Secondly, the milk is homogenised at pressure in the range 10-20 MPa and at temperatures in the range 55-65°C (Lucy and Singh 1998). This is to prevent the milk fat from separating out by reducing the average size of the milk fat globules. Homogenisation also improves the product stability, increases the milk viscosity and makes it appear whiter. The milk is then cooled to a temperature of between 30 and 40°C. The temperature should be at the optimum for the starter culture bacteria used.

Fermentation

Once the milk has cooled down, following heat treatment, starter bacteria are added and the fermentation process begins (Figure 1.2). The most common bacteria used as starter cultures for fermentation are *St. salivarius* ssp. *thermophilus* and *Lb delbrueckii* ssp. *bulgaricus*. Mixtures of two cultures of lactic acid bacteria are usually used. These bacteria are added to a level of about 2% of the total volume to give an initial concentration of 10^6- 10^7 cfu/ml composed of roughly equal numbers of both bacteria.

Fermentation is carried out in retail packs to produce set yoghurts or in large fermenters to produce stirred yoghurt. The fermentation process takes about 4 hours. Stirring during production of yoghurt affects the rheology and allows syneresis to occur as the gel network breaks down (Heertje, Visser and Smits 1985; Rawson and Marshall 1997). When fermentation is complete the yoghurt is cooled to 15°C before the addition of fruits and flavours and packaging. It is then cooled to 5°C, a temperature at which it will remain stable for up to three weeks.

THE TEXTURE AND RHEOLOGY OF YOGHURT

The success of a yoghurt product in the market place is dependent on three factors, its taste, texture and rheology. Rheology is defined as the study of the deformation of objects under the influence of applied forces. Foods are subjected to forces during processing such as pressing and grinding and these have an effect on the mechanical properties and texture of food (Lewis 1990). In the chewing process food is subjected to a number of forces. During this process, information is transmitted from sensory receptors in the mouth to specific parts of the brain. Here it is integrated with other incoming information from the nose and tastebuds, as well as information in the memory to give an overall impression of texture (Lewis 1990). Texture is an important determinant of what a consumer sees as a good quality food. If the perceived quality of the food does not match up to the consumers expectations then the consumer is disappointed. Texture of food products can be determined in two ways, either sensory methods involving trained and untrained testers or instrumental methods (Lewis 1990).

Instrumental methods of texture analysis

An understanding of the structure of a material can often lead to a better understanding of its physical properties and textural characteristics. There are three approaches used to analyse texture instrumentally. Fundamental methods measure physical properties of a sample e.g stress-strain relationships and viscoelastic behaviour. Imitative methods attempt to simulate the forces and deformations that affect the food whilst it is being consumed. Empirical methods measure properties of materials that are not well defined and can be used as an indirect measurement of a textural attribute e.g hardness, cohesiveness, adhesiveness, viscosity, sponginess, fracturability and

chewiness. The testing force can be applied in a variety of ways; penetration, shear, compression, extrusion, cutting, flow and mixing.

The use of EPS producing strains in yoghurt manufacture.

EPS producing strains of lactic acid bacteria play a role in the formation of a well textured product. The factors that affect the texture of yoghurt are (Sebastiani and Zegler 1998):

a) The presence of a thickening agent in the liquid phase.
b) The presence of a protein gel primarily composed of casein.
c) The interaction of proteins and polysaccharides.
d) The presence of bacteria with capsular polysaccharide.
e) The binding of water that reduces the amount of free water molecules. This increases the concentration of EPS in the aqueous phase.

The structure of yoghurts made with EPS-producing strains has been visualised using scanning and transmission electron microscopy (Tamime, Kalab and Davies 1984). Yoghurts made with extra casein and skimmed milk showed a high degree of micelle fusion. Increasing the milk protein concentration decreased syneresis and increased gel strength of set yoghurts. It has been suggested (Teggatz and Morris 1990) that the changes to the gel firmness are caused by attachment of ropy strains of bacteria to the protein matrix. These filaments, of protein and bacteria can be visualised by scanning electron microscopy (Teggatz and Morris 1990; Tamime, Kalab and Davies 1984). However, these filaments have not been seen in scanning electron micrographs of mixed cultures (Laws and Marshall 2001) and as a result could be artefacts produced during the preparation of the sample for microscopy.

In formulating mixed cultures for yoghurt manufacture, addition of two EPS producing strains may not have the desired effect for the consumer. Marshall and Rawson (1999) found that combining a non EPS producing strain of *St. thermophilus* with an EPS producing strain of *Lb. delbrueckii* ssp. *bulgaricus* had a greater effect on the viscosity of stirred yoghurt than when combining two EPS producing strains. The hardness of the product was less affected by the EPS content than the viscosity. Viscosity was decreased as a result of fermentation with EPS producing *Lb. delbrueckii* ssp. *bulgaricus* and *St. salivarius* ssp. *thermophilus* strains compared to the non EPS producing

strains. Both EPS producing strains have high molecular mass EPS, but they have different chain lengths and sugar composition (Marshall and Rawson 1999). Yoghurts were made using EPS and non EPS producing cultures as mixed starter cultures (2% cell density of inoculum in equal ratio of rods:cocci). The yield of EPS from combined ropy cultures was not increased. The yield of EPS did increase when EPS producing strains were combined with a non EPS producing partner. Increased EPS production from *Lb. delbrueckii* ssp. *bulgaricus* resulted in higher viscosity and a greater ability to recover viscosity after mechanical testing. The beneficial effects of increased EPS are not always obvious as the EPS from *St.thermophilus* resulted in a greater susceptibility to syneresis. This indicates that more EPS does not necessarily mean a better-textured product.

GENETIC INSTABILITY OF EPS PRODUCTION

The unstable nature of EPS production and variability of yield are well documented problems in the dairy industry (Cerning 1990). Viscosities for a ropy strain of *S. thermophilus* grown under the same conditions varied from 41 to 240 mPa and the amounts of EPS produced varied between 45 and 340 mg L^{-1} (Cerning et al. 1988). The loss of the slime-producing trait can occur after repeated subculture or prolonged incubation, especially at high temperatures (Forsén, Raunio, and Myllymaa 1973). Mutations may cause colony variants, a reduced production or possibly an altered EPS composition (Gancel and Novel 1994b; Yamamoto et al. 1995; Bouzar, Cerning, and Desmazeaud 1996). The producing cells may synthesize enzymes, such as glycohydrolases, that destroy the ropy texture (Pham et al. 2000). The loss of the slime-producing trait from mesophilic lactic acid bacterial strains has been attributed to loss of plasmids (van Kranenburg, Kleerebezem and de Vos 2000).

BIOSYNTHESIS AND REGULATION OF EPS PRODUCTION IN LACTIC ACID BACTERIA.

The biosynthesis of bacterial EPS is complex and involves the action of a large number of gene products. The genes coding for the enzymes and regulatory proteins required for EPS synthesis are

of plasmid origin in the mesophilic lactic acid bacterial strains, e.g., *Lactococcus* and chromosomally based in the thermophilic strains of *Streptococcus* and *Lactobacilli*.

A number of groups have investigated the genetics of EPS production. The investigation of EPS gene clusters in Gram-negative bacteria began over 20 years ago (Sutherland 1985). However, research on EPS genes of Gram-positive bacteria has only recently advanced. Stingele, Neeser, and Mollet (1996) first described the genes directing EPS synthesis for *Streptococcus thermophilus Sfi6*. The 14.5kb gene cluster is composed of 13 genes that are transcribed from a single promoter upstream of the *epsA* gene. This cluster has also been found on a 40kb plasmid in *Lc. lactis* ssp. *cremoris* B40 (van Kranenburg et al. 1997).

The EPS formation in lactic acid bacteria is unstable. For mesophilic lactic acid bacterial strains, the unstable nature of EPS synthesis is consistent with the genes for EPS synthesis being plasmid bound. For the thermophilic lactic acid bacterial strains, it has been proposed that the loss of EPS-producing character is due to deletions and rearrangement resulting from genetic instability (Laws, Gu and Marshall 2001).

Biosynthesis of EPS

The biosynthesis of EPS appears to follow a common mechanism. Sugar units are assembled at the membrane on a lipid carrier anchored into the membrane. After completion of an EPS repeating unit, it is exported and polymerised to form the cell-surface polysaccharides (Sutherland 1998). In addition to EPS specific gene products, the biosynthetic pathway relies on a number of the housekeeping enzymes such as those required for the preparation of sugar nucleotides. Figure 1.3 is a schematic representation of pathways involved in *Lc. lactis* ssp. *cremoris* B40 and *Streptococcus thermophilus Sfi6* EPS biosynthesis. The biosynthetic pathway can be broken down into a number of reaction sequences. The first are those reactions involved with the transport of sugars into the cytoplasm. The second are those involved in the synthesis of sugar-1-phosphates, the third are those involved in the activation and coupling of sugars and the fourth is the process involved in the export of the EPS (Laws, Gu, and Marshall 2001).

Transport of Sugars into the Cytoplasm

There is a highly regulated process that allows the movement of monosaccharides and disaccharides, from the surrounding growth medium into the cytoplasm. The most commonly encountered mechanism is the phosphoenolpyruvate (PEP)-sugar phosphotransferase system (PTS) (Postma, Lengeler and Jacobson 1993). This system contains a group of proteins that are responsible for binding, transmembrane transport and phosphorylation of a variety of sugar substrates and is commonly found in strains of *Lc.lactis*. In addition, a number of other proteins exist that regulate the binding, transmembrane transport and phosphorylation.

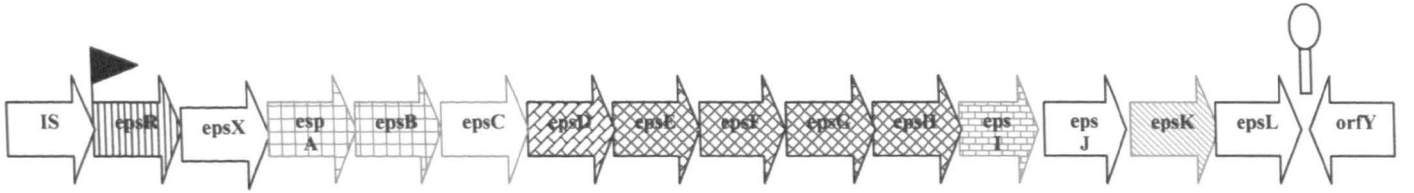
The first group of proteins includes the histidine-containing phosphocarrier protein HPr (enzyme I) and a carbohydrate-specific permease enzyme complex (enzyme II) (Viana et al. 2000). The enzymes act in sequence to provide phosphorylated sugars in the cytoplasm. The sequence is initiated when a phosphate group is transferred from PEP to enzyme I; enzyme I subsequently phosphorylates a histidine residue of HPr to yield HPr (His-P) (Postma, Lengeler and Jacobson 1993). At the same time, a protein of the enzyme II complex binds the carbohydrate (Sliz et al. 1997). The carbohydrate-specific enzymes (II) transport sugars across the membrane and catalyse the transfer of the phosphate group from HPr (His-P) onto the sugar. In the PEP–PTS transport system of *L. lactis*, three Type II enzymes are required for lactose transport: enzymes IIA, IIB and IIC (Wang et al. 2000). Enzymes IIA and IIB are located in the cytoplasm and enzyme IIC acts as a membrane channel.

A second set of proteins is responsible for regulation of nutrient acquisition. The most important of the regulators is the complex formed between the catabolite control protein CcpA and the PTS protein HPr (van den Bogaard et al. 2000).

The histidine-containing phosphocarrier protein HPr has two phosphorylation sites and His (Ser-P) has been shown to regulate sugar transport; CcpA in combination with HPr (Ser-P) binds to the cis-acting DNA sequence termed the catabolite responsive element (cre).

Alternative, non-PEP–PTS transport systems exist for the import of sugars e.g. primary and secondary transport systems. In primary transport systems, transport is directly linked to a chemical or photochemical reaction. There are three classes of primary transport processes.

St. thermophilus Sfi6



Lc.cremoris B40

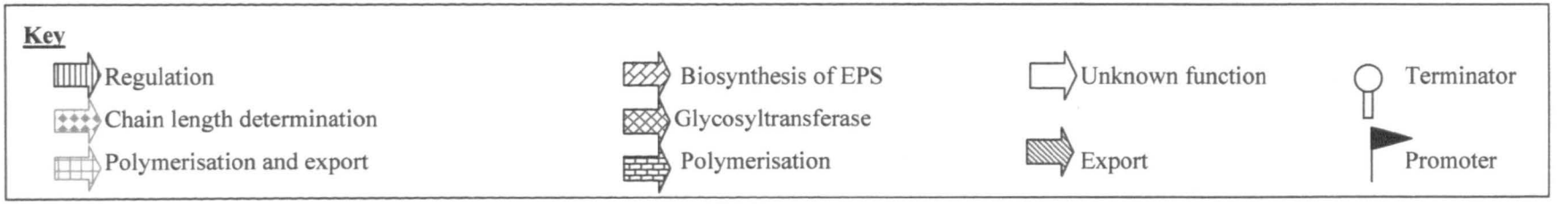


Figure 1.3 Schematic diagram of gene clusters of some strains of lactic acid bacteria (Adapted from Jolly and Stingele 2001).

In class 1 systems, transport is driven by a redox reaction or by light; class 2 transport systems couple movement with ATP hydrolysis or synthesis. Class 3 systems couple sodium transport to a decarboxylation reaction.

Secondary transport systems use pre existing gradients, which are established by the action of primary systems, as driving forces. An example of this is the lactose/H+ symporter (lac Y) of *E.coli*. A primary active transport system pumps H^+ or Na^+ across the membrane establishing an electrochemical ion potential. This is then used for driving the uptake of lactose into the cell against a concentration gradient (Lengeler, Drews and Schlegel 1999).

A number of lactic acid bacteria do not have active PEP–PTS transport system for all the sugars that they are able to internalise. In the absence of a sugar-specific PEP–PTS transport system there is a requirement for active transport.

In *Staphylococcus mutans,* multiple sugars are transported by a primary active transport system (Sutcliffe et al. 1993) and in *St. thermophilus* a secondary transport system is used. This is coded for by the *LacS* gene (Friesen, Knol and Poolman 2000). *LacS* is able to import lactose in symport with protons or, alternatively, *lacS* can function as a lactose–galactose antiport system.

Synthesis of Sugar-1-phosphates

Once inside the cytoplasm, the fate of the carbon feed is determined by the state of phosphorylation of the sugar: sugar-6-phosphates are consumed in catabolic pathways whereas sugar-1-phosphates can participate in polysaccharide synthesis. As the majority of sugars are transported into the cytoplasm by PEP–PTS systems, which generate sugar-6-phosphates, a number of authors have pointed to the possible key role that phosphoglucomutases may play in the phosphorylation process (Laws, Gu, and Marshall 2001).

In the metabolism of maltose, a sugar that is transported via a permease transport protein in *Lc. lactis*, the disaccharide is initially converted into one molecule of glucose and a molecule of β-glucose-1-phosphate. If the β-glucose-1-phosphate is to be used in the catabolic pathway, it must then be converted to glucose-6-phosphate and this reaction is catalysed by β-phosphoglucomutase (Qian et al. 1994).

It has been suggested that α-phosphoglucomutases may play the opposite role in EPS biosynthesis in that they divert glucose-6-phosphate to α-glucose-1-phosphates and EPS biosynthesis (Degeest and de Vuyst, 2000). Ramos et al. (2001) have demonstrated that for the synthesis of EPS in *Lc. lactis* grown on glucose, a proportion of the carbon feed must be converted to glucose-1-phosphate for EPS synthesis.

A number of alternative pathways leading to α-glucose-1-phosphate have been suggested (see bottom right-hand side portion of Fig. 1.4). The mode of synthesis of glucose-1-phosphate is dependent on a number of variables but especially the carbon source on which the culture is grown and the transport system used for sugar import. It has been proposed (Grobben et al. 1996) that for *Lb. bulgaricus* grown on fructose that fructose is imported via a PEP–fructose PTS that specifically yields fructose-1-phosphate. Fructose-1-phosphate is converted, via fructose-1, 6-bisphosphate, to fructose-6-phosphate, to glucose-6-phosphate, and finally to glucose-1-phosphate. A much shorter pathway to glucose-1-phosphate is available for *Lb. bulgaricus* grown on glucose; glucose is internalised via a PEP–glucose PTS yielding glucose-6-phosphate that is transformed directly into glucose-1- phosphate (Grobben et al. 1996).

When *Lc. lactis* is grown on lactose (de Vos and Vaughan 1994), lactose is imported by a lactose-specific phosphotransferase transport system providing internal lactose-6-phosphate. Lactose-6-phosphate is subsequently hydrolysed by a phospho-galactosidase to generate galactose-6-phosphate and glucose. A glucokinase is required for the synthesis of α-glucose-6-phosphate. In contrast, galactose-negative *S. thermophilus* internalises lactose by coupling a lactose permease within an antiport secondary transport system (Poolman 1993). Glucose-6-phosphate is generated using a combination of galactosidase and kinase.

There is also a pathway for the synthesis and subsequent utilisation of galactose-1- phosphate. This uses the enzymes of the Leloir pathway for galactose metabolism. The enzymes of the Leloir pathway convert galactose to α-galactose-1-phosphate (GalK), a-galactose-1-phosphate to UDP-galactose (GalT) and UDP-galactose to UDP-glucose (GalE). For cells grown in galactose the presence of UDP-glucose pyrophosphorylase and a supply of uridine triphosphate would allow the reaction to be considered as catalytic in the formation of UDP-glucose.

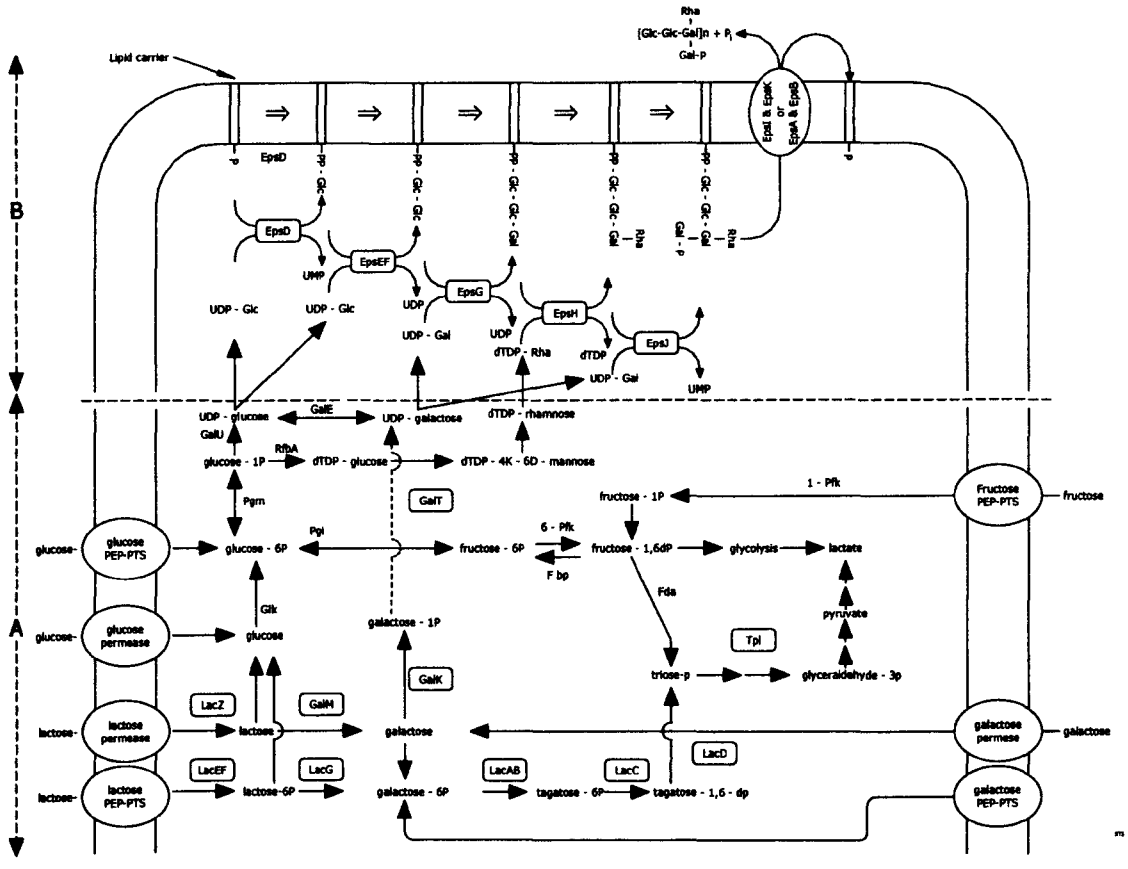


Figure 1.4 (A) Schematic representation of a number of possible pathways for sugar transport and metabolism in LAB. (B) Schematic representation of a possible pathway for EPS biosynthesis in L. lactis NIZO B40 starting from glucose-6-phosphate (the site of polymerisation of the repeat unit has not been established and may occur on either face of the membrane) (adapted from Laws, Gu and Marshall 2001).

The action of the enzymes could supply both UDP-glucose and UDP-galactose without a requirement for a phosphoglucomutase. However, a number of dairy strains of *Streptococcus thermophilus* lack an available galactokinase (Thomas and Crow 1984).

Figure 1.4 shows a schematic representation of some of the pathways involved in EPS synthesis in lactic acid bacteria (Laws, Gu and Marshall 2001). The top half of the diagram shows a possible pathway for EPS biosynthesis in *Lc. lactis* NIZO B40 using glucose-6-phosphate as the starting point. The lower half of the diagram shows the number of possible pathways for sugar transport and metabolism in lactic acid bacteria.

Synthesis of sugar nucleotides and polymerisation

The genes coding for EPS biosynthesis are split into two groups: those required for the synthesis of sugar nucleotides and those that are EPS-specific genes (Laws, Gu and Marshall 2001). The groups of genes are physically separated from each other on the genome. In the case of *Lc.lactis* the EPS biosynthesis genes are extra chromosomal (upper part of Figure 1.4).

The first group of genes consists of those coding for enzymes and proteins required for the synthesis of sugar nucleotides from which the repeat unit is constructed. Sugar nucleotides are needed for the synthesis of a range of polysaccharides and are not specific to EPS biosynthesis. The sugar nucleotides required for the construction of the majority of EPS structures are UDP-glucose, UDP-galactose, and dTDP-rhamnose: the precursors of the repeat unit. The genes coding for the enzymes needed for the synthesis of the sugar nucleotides from glucose-1-phosphate (*galU, galE, rfbA, rfbB, rfbC, and rfbD*) have been identified and cloned from *Lc.lactis* strain MG1363 (Kleerebezem et al. 1999). The first enzyme in the sequence is GalU a UDP-glucose pyrophosphorylase (Figure 1.4). Kleerebezem et al. (1999) reported that intracellular levels of UDP-glucose are determined by the activity of the enzyme GalU. Overexpression of the lactococcal *galU* gene results in much larger UDP-glucose levels in *Lc. lactis*. The production of UDP-Gal is believed to come from the interconversion of UDP-Glc to UDP-Gal, catalysed by the action of GalE. Kleerebezem et al. (1999) showed that a *galE* mutant was not able to synthesise EPS when grown on glucose but EPS was produced when the mutant was grown on galactose. This implies that in the absence of galactose the UDP-galactose required for EPS synthesis is derived from UDP-glucose. The production of dTDP-rhamnose was determined using Gram negative bacteria where rhamnose is found in the O antigens of the lipopolysaccharide (Reeves 1993). Four enzymes, RfbA, RfbB, RfbC, and RfbD, convert a-glucose-1-phosphate to dTDP-glucose then to 4-keto-6-deoxymannose and then to dTDP-rhamnose.

The next stage of biosynthesis uses EPS-specific enzymes. The first EPS production gene clusters were identified for *St.thermophilus* Sfi6 (Stingele, Neeser and Mollet 1996) and *Lc.lactis* NIZO B40 (van Kranenburg et al. 1997). In *St.thermophilus* Sfi6 the gene cluster is 14.5kb in size,

contains 13 genes and is located on the chromosome. In *Lc.lactis* NIZO B40 the EPS gene cluster size is 12kb, contains 14 genes and found on a 40-kb plasmid (Figure 1.3).

All the EPS gene clusters have very similar structures. They have an operon structure with a high coding density. These genes are orientated in one direction and are transcribed as a polycistronic single mRNA (Stingele, Neeser and Mollet 1996). The sequence of genes in these EPS producing strain clusters seems to follow a similar pattern. A central core coding for the glycosyl-transferases has genes at either end that have a strong homology with enzymes used for polymerisation and export. A regulatory domain is present at the start of the gene cluster. Stingele et al. (1999) have demonstrated that this regulatory sequence could be used to stimulate EPS synthesis in a non-EPS producing bacterial strain, *Lc. lactis* MG1363. *In vitro* experiments using [^{14}C]-labelled sugar nucleotides have shown that the monosaccharide repeat unit is assembled on a lipid carrier, attached to the cytoplasmic membrane (van Kranenburg et al. 1997). In O antigen synthesis, the repeat unit construction occurs on the inside surface of the cytoplasmic membrane (see section B Figure 1.4)

For *Lc. lactis* NIZO B40, the first sugar to be attached to the membrane-anchored phosphorylated lipid is glucose. The mode of action of the 'priming' glycosyl- transferase, a UDP-glucose transferase for *Lc. lactis* NIZO B40, requires donation of the sugar and a phosphate group to the lipid. In *Lc. lactis* NIZO B40, the second sugar in the repeat unit is glucose and this is added, with a β-glycosidic link, through the attachment of the anomeric carbon of a-UDP-glucose to the 4-hydroxy group of the lipid-bound glucose. This process requires two EPS gene products, EpsE and EpsF. The final backbone residue, a β (1–4)-linked-galactose, is derived from UDP-galactose and this is catalysed by the product of *epsG*. Van Kranenburg et al. (1997) suggest that the rhamnose and a phosphogalactose, are added to the 2- and 3- positions of the repeat unit in successive glycosyl/phosphoglycosyltransferase catalysed steps, generating the required repeat unit and these are joined by a diphosphate link to the lipid. The catalytic mechanism of the glycosyl-transferases determines whether the sugar is an α- or β-glycoside.

The assembly of the repeat unit on a lipid carrier is a process that is used for the synthesis of excreted polysaccharides, for cell wall peptidoglycans and for cell surface oligosaccharides and

polysaccharides(Garcia-Garibay and Marshall 1991). There is evidence to suggest that the various oligosaccharide and polysaccharide syntheses use the same building blocks (sugar nucleotides) and scaffolding (lipid carrier). The latter may account for the close relationships between rates of EPS synthesis and cell growth that have been observed by a number of authors (Cerning et al. 1992).

Regulation

The *epsA* gene from *St.thermophilus* Sfi6 has been shown to have significant homology with a gene in *Bacillus subtilis* (Stingele, Neeser and Mollet 1996). The gene in *B.subtilis* is involved in the regulation of an autolysin operon where it acts as an attenuator of expression (Lazarevic et al. 1992). A similar regulatory gene seems to be conserved throughout other lactic acid bacteria (Jolly and Stingele 2001). In the case of *Lc. lactis ssp. cremoris* B40 this gene is called *epsR*. It has been suggested that all the homologues from lactic acid bacteria could play a role in the regulation of the *eps* cluster, though there is no current indication as to the nature of regulation of EPS biosynthesis.

Chain Length Determination.

The consistently high molecular mass of the EPS produced by lactic acid bacteria suggests the presence of a mechanism regulating its chain length during polymerisation. This process could be similar to the production of bacterial surface O-antigens (Jolly and Stingele 2001). Most gene clusters in the lactic acid bacteria contain homologues for the O-antigen polymerisation and export proteins Wzx, Wzy and Wzz (*Esherichia coli*) and homologues to ExoP-like (*Sinorhizobium meliloti* formerly *Rhizobium meliloti*) proteins(Jolly and Stingele 2001).

Lc. cremoris B40 EpsA and *St. thermophilus* Sfi6 EpsC (Figure 1.3) share similarity with the product from *wzz* gene in *E.coli* at their N-terminal ends. Wzz proteins are thought to play a role in chain length determination of the O-antigen (Jolly and Stingele 2001). Lactic acid bacteria homologues are thought to possess the same properties and their central domain is thought to be extracellular.

The *Lactococcus epsB* gene contains a conserved nucleotide-binding motif, which is present in the C-terminal part of ExoP (Stingele, Neeser and Mollet 1996; van Kranenburg et al. 1997). The protein Wzc from *E.coli*, homologe of ExoP, is an autophosphorylating protein tyrosine kinase that

is associated with a phosphotyrosine-protein phosphatase, Wzb (Vincent et al. 1999). Wzc is a member of a distinct protein family of prokaryotic membrane-associated protein tyrosine kinases. This protein is believed to be associated with EPS production essential for virulence (Ilan et al. 1999).

Homologues from *St. pneumoniae*, CspC and CspD, have been found to interact with each other and they both are active in capsular polysaccharide biosynthesis. The nucleotide-binding motif is present in CspD, and is required for the functional phosphorylation of CspD (Morona et al. 2000). The interplay of CspC, CspD and ATP is thought to be organised in such a way as to achieve maximum capsular polysaccharide biosynthesis. This is caused by the interaction of CspC and CspD that allows ATP to bind. When CspD autophosphorylates using bound ATP, the tyrosine-phosphorylated CspD dissociates from CspC and reduces capsular polysaccharide production to a low level. The action of a phosphotyrosine-protein phosphatase regenerates CspD allowing it to interact with CspC again. It is thought that the phosphotyrosine-protein phosphatase in *St. pneumoniae* is CspB. Mutants in the *cspB* gene contain CspD in an inactive tyrosine phosphorylated form and capsular polysaccharide production is at a low level. However, when CspD is present in a form that cannot be phosphorylated, CspB is no longer required (Morona et al. 2000).

It is assumed that the ATP-binding sites present in the lactic acid bacteria homologues are also essential for the biosynthesis of EPS. They could be involved in the formation of an EpsC-EpsD complex that initiated EPS polymerisation. However, this may not be the case as the tyrosine-rich region is not found in all homologues. It has also been found that all the clusters contain a gene product that shares 60% - 70% homology with the CspB protein from *St. pneumoniae* (Jolly and Stingele 2001). Another possibility is that enzymes from lactic acid bacteria may use another site for phosphorylation. However the high molecular masses of EPS found in lactic acid bacteria suggest that the regulation of polymerisation is different from the one for O-antigen or capsule biosynthesis.

Polymerization/export

Gonzalez et al. (1998) have studied the genes involved in polymerisation and export of the EPS succinoglycan in *S. meliloti*. They observed that the subunits are constructed on an undecaprenol lipid carrier on the cytoplasmic face of the plasma membrane, which are polymerised in a block fashion. The mechanism by which blocks are polymerised in EPS biosynthesis is not known. In O antigen synthesis, three gene products are required for polymerisation and export (Whitfield and Valvano 1993). There are genes that code for proteins that catalyse the movement of the lipid-bound material from the cytoplasmic face of the membrane to the periplasmic face know as flippase or translocase. There are proteins that catalyses the polymerisation of the blocks (polymerase) and a protein responsible for controlling polymer chain length.

EPS polymerisation and export requires the action of a flippase to translocate the lipid-bound repeat units, a polymerase to catalyse the coupling of repeat units and finally an enzyme to catalyse the detachment of the lipid-bound polymer and that will control chain length. In succinoglycan synthesis, there is strong evidence to suggest that the gene product from *exoQ* is the polymerase (Gonzalez et al. 1998). ExoQ proteins show sequence and topological similarities with the O antigen polymerase gene (Rfc) product (Wzy) (Whitfield and Valvano 1993). All the Wzy-homologues in lactic acid bacteria have similar structures. *Lc. cremoris* B40 EpsK and *St.thermophilus* Sfi6 EpsM share homology with Wzx from *E. coli* and are thought to be involved in the export of bacterial polysaccharides. Wzx is often referred to as flippase. It is thought to translocate the undecaprenyl-linked O-antigen subunits across the membrane so that the carbohydrate is on the periplasmic side of the membrane (Whitfield and Roberts 1999).

Becker, Niehaus and Puhler (1995) have suggested that the ExoP product of *S. meliloti* is involved in chain length determination. In *Lc. lactis* NIZO B40, the genes epsA and epsB show homology with the *S. meliloti* ExoP gene and the gene products are proposed to be involved in chain length determination (one protein may detach the polymer whilst the second may regulate the activity of the first). EpsK and EpsI are homologous to the flippase and polymerases in *Salmonella* or *Shigella* O antigen synthesis (Morona et al. 1994).

INFRARED SPECTROSCOPY

Infrared spectroscopy is a technique that can be used to analyse the composition of a molecule based on the vibrations of the atoms. An infrared spectrum is obtained by an infrared radiation beam being passed through a sample and determining how much of the incident radiation is absorbed. The energy at which a peak in an absorption spectrum appears corresponds to the frequency of a vibration of part of the sample molecule.

The visible part of the electromagnetic spectrum is radiation visible to the human eye. Other types of spectroscopy involve radiation beyond the visible regions of the spectrum. These are: radiowave, microwave, infrared, ultraviolet, X-ray and γ-ray (Figure 1.5).

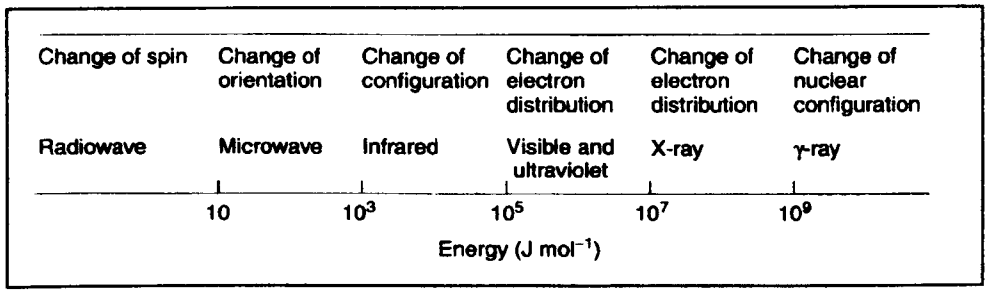


Figure 1.5 The Electromagnetic Spectrum (Stuart. 2008).

Electromagnetic radiation can interact with matter, transferring energy to the molecules involved. The molecules absorb energy, and the energy can make changes occur in the molecules. The changes that occur depend on:

- The chemical involved
- The amount of energy involved

Molecules are continually moving, they rotate and the bonds in the molecules vibrate. The electrons in the molecule have energy and they can move between different energy levels. A molecule has energy associated with different aspects of its behaviour, including: energy associated with translation (the molecule moving around as a whole), rotation (of the molecule as a whole), vibration of the bonds and electrons. These different kinds of energetic activities involve different amounts of energy (Figure 1.6)

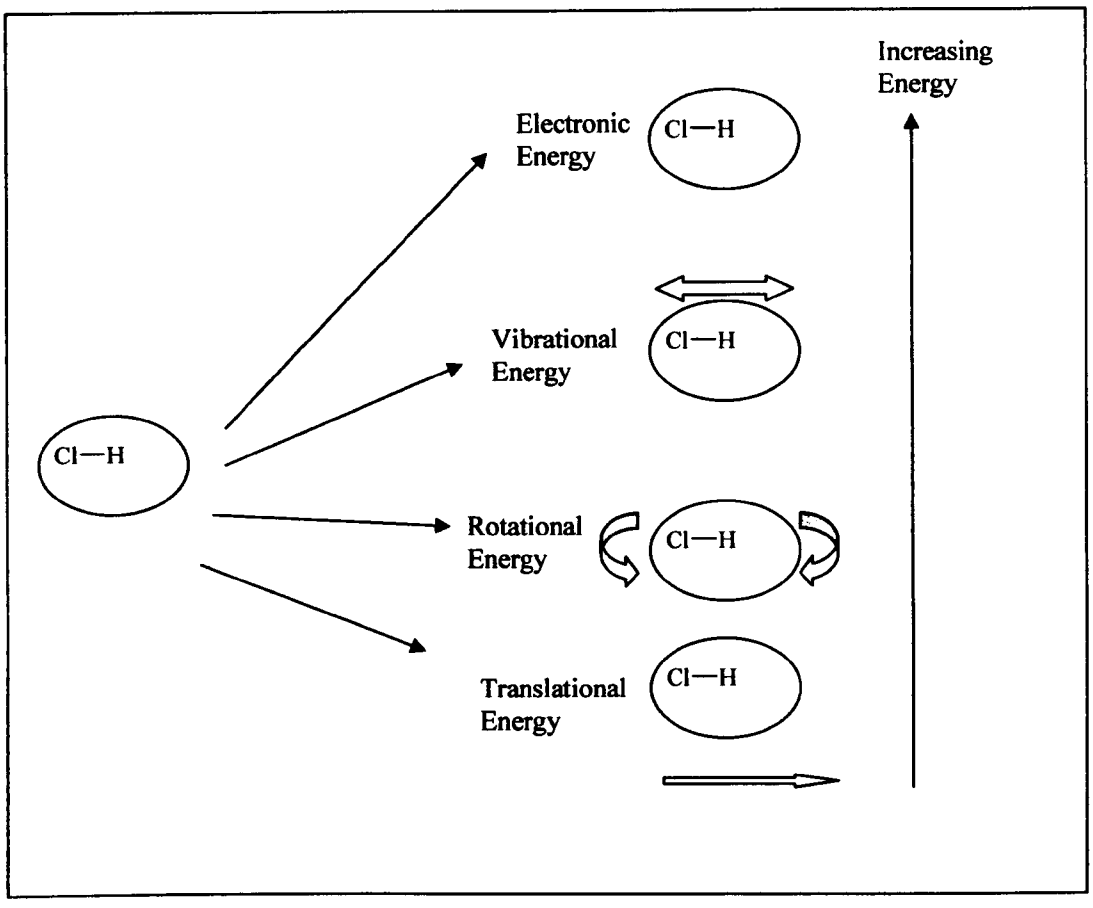


Figure 1.6 Energy associated with different aspects of molecular behaviour.

The energy that causes different types of change within a molecule can be quantified. The gap between energy levels corresponds to the energy of a photon of infrared radiation. Infrared radiation is able to make molecules increase their vibrational energy and move from one vibrational level to a higher one. Rotational energy can be generated by microwaves whereas translational and electronic changes are caused by visible and UV light respectively.

In infrared spectroscopy, substances are exposed to radiation in the frequency range 10^{14}Hz - 10^{13}Hz. For a molecule to absorb infrared it must possess a specific feature such as a dipole (a molecule with a slight positive and negative charge).

Vibrations involve a change in bond length (stretching) or a change in bond angle (bending) (Figure 1.7). Some bonds can stretch in-phase (symmetrical stretching) or out of phase (asymmetric stretching) (Figure 1.8).

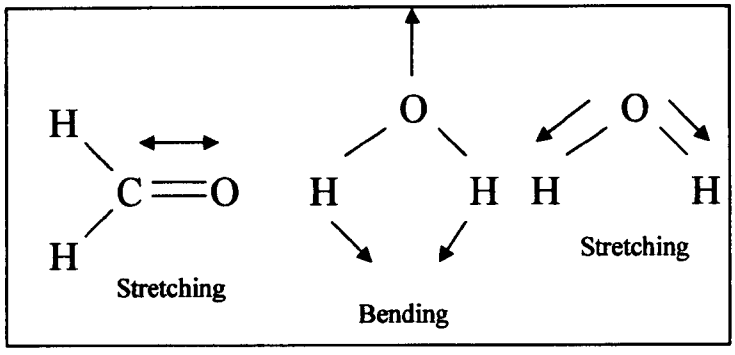


Figure 1.7 Stretching and bending vibrations

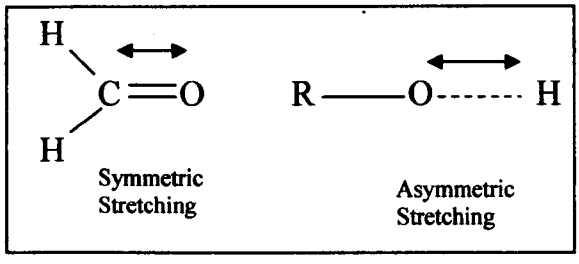


Figure 1.8 Symmetric and asymmetric stretching vibrations

Bending vibrations contribute to infrared spectra (Figure 1.9). Bound hydrogen atoms can move in the same direction or in opposite directions in the plane. In more complex molecules, the analysis becomes simpler since hydrogen atoms may be considered in isolation because they are usually attached to larger, more rigid parts of the molecule. This results in in-plane and out of plane bending vibrations (Figure 1.10).

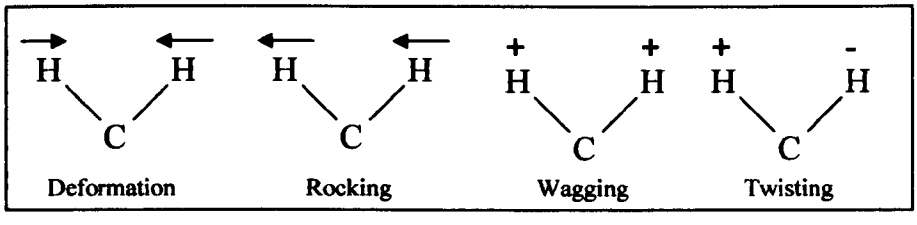


Figure 1.9 Different types of bending vibrations.

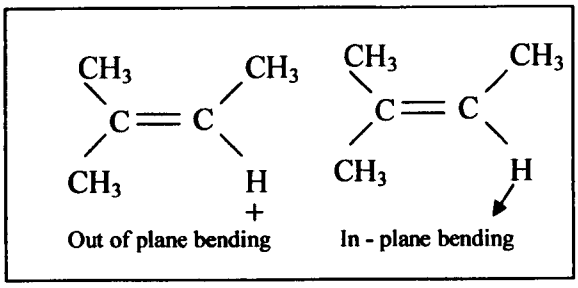


Figure 1.10 Out of plane and in plane bending vibrations.

Fourier Transform Infrared Spectroscopy - FTIR

Fourier transform infrared (FTIR) spectroscopy is based on the idea of the interference of radiation between two beams to yield an interferogram. This is a signal produced as a function of the change of pathlength between the two infrared beams. The interferogram is then subjected to the mathematical method of a Fourier transformation.

The basic components of an FTIR spectrometer are shown in Figure 1.11. The radiation emerging from the source is passed through an interferometer to the sample before reaching a detector. When the signal has been amplified and a filter has eliminated the interference, the data is converted to a digital form by an analogue-digital converter (Stuart 1996). This data is then manipulated using a Fourier transformation calculation to generate precise absorption spectra.

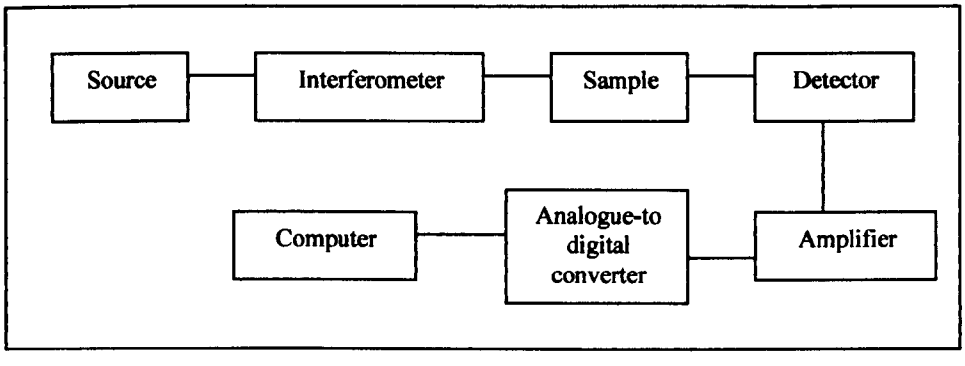


Figure 1.11 The components of an FTIR spectrometer.

The most common interferogram used in FTIR spectrometry is a Michelson interferometer, which consists of two perpendicularly plane mirrors, one of which can travel in a direction perpendicular to the plane (Figure 1.12) A semi-reflecting film, the beamsplitter, bisects the planes of these two mirrors.

If a beam of monochromatic radiation of wavelength λ (cm) is passed into a beamsplitter, 50% of the incident radiation will be reflected to one of the mirrors while 50% will be transmitted to the other mirror. The two beams are reflected from these mirrors, returning to the beamsplitter where they recombine and interfere. Fifty percent of the beam reflected from the fixed mirror is transmitted through the beamsplitter while 50% is reflected back in the direction of the source. The beam which emerges from the interferometer at 90° to the input beam is called the transmitted beam and this is the beam detected in FTIR spectrometry.

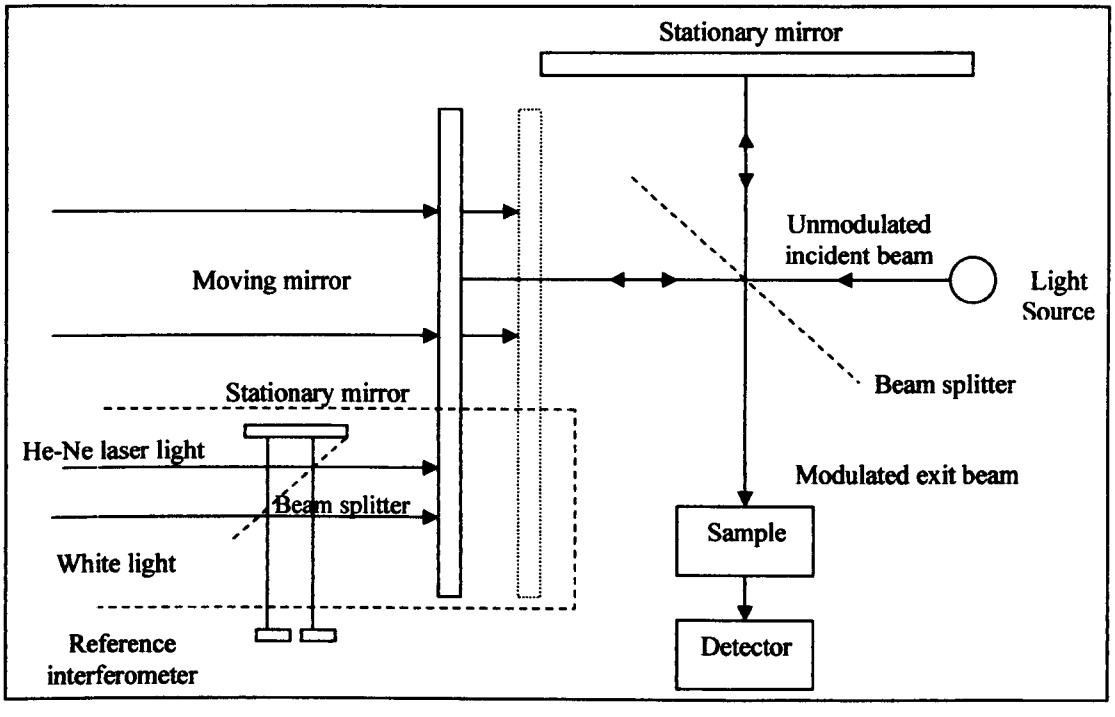


Figure 1.12 A diagram of a Michelson interferometer (adapted from Stuart 2008).

The moving mirror produces an optical path difference between the two arms of the interferometer. The resulting interferogram can be seen below (Figure 1.13).

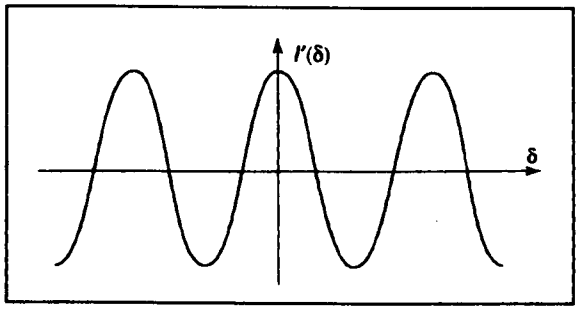


Figure 1.13 An interferogram (Stuart 2008.)

As mentioned earlier once the interferogram has been produced it is subjected to a Fourier transform to produce a spectrum. The spectra can be either an absorption or transmission spectra.

Spectral Analysis

Once an infrared spectrum has been recorded, the next stage is interpretation and this can be simplified by the fact that the bands that appear can be assigned to particular parts of a molecule, producing what are known as group frequencies. These can be found in the mid-infrared region, the near-infra red region and the far-infrared region.

The mid-infrared spectrum (4000 - 400 cm^{-1}) can be divided into four regions and the nature of a group frequency may be determined by the region in which it is located. The regions are generalized as follows: the X-H stretching region (4000 - 2500 cm^{-1}), the triple-bond region (2500 - 2000 cm^{-1}), the double-bond region (2000 - 1500 cm^{-1}) and the fingerprint region (1500 - 600 cm^{-1}) (Stuart 2008).

The fundamental vibrations in the 4000 - 2500 cm^{-1} region are generally due to O-H, C-H and N-H stretching. O-H stretching produces a broad band that occurs in the range 3700 - 3600 cm^{-1}. By comparison, N-H stretching is usually observed between 3400 and 3300 cm^{-1}. This absorption is generally much sharper than O-H stretching and may, therefore, be differentiated. C-H stretching bands from aliphatic compounds occur in the range 3000 - 2850 cm^{-1}. If the C-H bond is adjacent to a double bond or aromatic ring, the C-H stretching wavenumber increases and absorbs between 3100 and 3000 cm^{-1} (Stuart 2008).

Triple-bond stretching absorptions fall in the 2500 - 2000 cm^{-1} region because the high force constants of the bonds. C-C triple bonds absorb between 2300 and 50 cm^{-1}, while the nitrile group (C-N triple bond) occurs between 2300 and 2200 cm^{-1}. These groups may be distinguished since C-C triple bond stretching is normally very weak, while C-N triple bond stretching is of medium intensity. These are the most common absorptions in this region.

The principal bands in the 2000 - 1500 cm^{1} region are due to C=C and C=O stretching. Carbonyl stretching is one of the easiest absorptions to recognize in infrared spectrum. It is usually the most intense band in the spectrum and depending on the type of C=O bond, occurs in the 1830 - 1650 cm^{-1} region. C=C stretching is much weaker and occurs at around

1650 cm^{-1}. C=N stretching also occurs in this region and is usually stronger (Stuart 2008).

It is possible to assign a number of bands in a spectrum to a particular deformation of the molecule, the movement of a group of atoms, or the bending or stretching of a particular bond. Many vibrations do not behave in a particular way and may vary by hundreds of wavenumbers, even for similar molecules. This applies to most bending and skeletal vibrations, which absorb in the 1500 - 650 cm^{-1} for which small steric or electronic effects in the molecule lead to large shifts. A spectrum of a molecule may have a hundred or more absorption bands present but there is no need to assign the vast majority. The spectrum can be regarded as a "fingerprint" of the molecule and so this region is referred to as the fingerprint region.

The absorptions observed in the near-infrared region (13000 - 4000 cm^{-1}) are overtones or combinations of the fundamental stretching bands which occur in the 3000 - 1700 cm^{-1} region. The bands involved are usually due to C-H, N-H or O-H stretching. The resulting bands in the near-infrared are usually weak in intensity and the intensity generally decreases by a factor of 10 from one overtone to the next. The bands in the near-infrared are often overlapped making them less useful than the mid-infrared region for qualitative analysis.

The far-infrared region is defined as the region between 400 and 100 cm^{-1}. This region is more limited than the mid-infrared for spectra-structure correlations, but does provide information regarding the vibrations of molecules containing heavy atoms, molecular skeleton vibrations, molecular torsions and crystal lattice vibrations.

Hydrogen bonds can be found in a variety of different molecules. For example, the biological activity of DNA relies on hydrogen bonding and hydrogen bonding also occurs in water. A hydrogen bond is a weak bond formed between an electronegative atom (e.g O, N, F) and a hydrogen atom attached to another electronegative atom.

Hydrogen bonding has an important effect in infrared spectroscopy. This bonding influences the bond stiffness and so alters the frequency of vibration. There are a number of things that can affect the degree of hydrogen bonding in a compound. These include the solvents used, the concentration of the compound and the temperature.

There are a number of techniques available to users of infrared spectrometers that help with both the qualitative and quantitative interpretation of spectra.

Baseline correction

It is usual in IR spectroscopy to use a baseline joining the points of lowest absorbance on a peak. The absorbance difference between the baseline and the top of the band is then used.

Smoothing

Noise in a spectrum can be diminished by a smoothing process. After a spectrum is smoothed, it becomes similar to the result of an experiment at a lower resolution. The features are blended into each other and the noise decreases. A smoothing function is a convolution between the spectrum and a vector whose points are determined by the degree of smoothing applied.

Difference Spectra

The most straightforward method of analysis for complex spectra is difference spectroscopy. This technique may be carried out by simply subtracting the infrared spectrum of one component of the system from the combined spectrum to leave the spectrum of the other component (Figure 1.14).

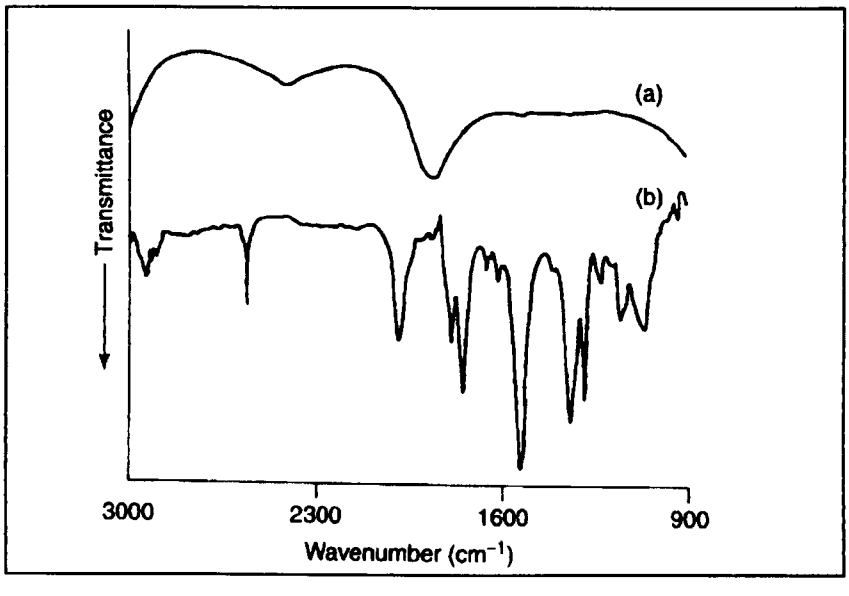


Figure 1.14 Infrared spectra of (a) a 1% (wt/vol) solution of aspirin in water and (b) the same solution after subtraction of the water spectrum (Stuart 2008).

Derivatives

Spectra may also be differentiated. Figure 1.15 shows a single absorption peak its first and second derivative. The benefits of derivative techniques is that resolution is enhanced in the first derivative since changes in the gradient are examined. The second derivative gives a negative peak for each band in the absorption spectrum.

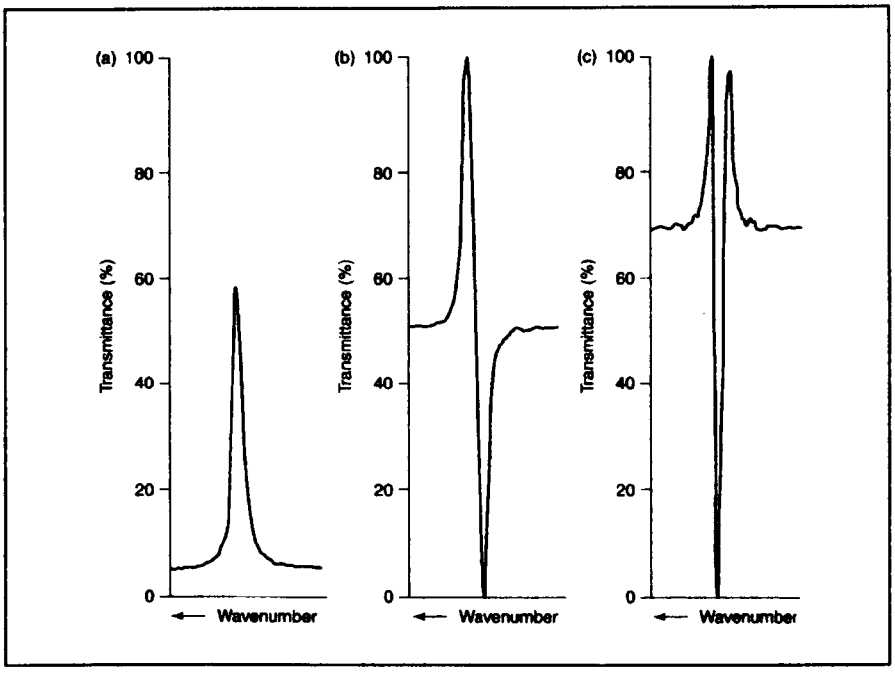


Figure 1.15 Differentiation of spectra: (a) single absorption peak; (b) first derivative (c) second derivative (Stuart 2008).

Figure 1.16 shows how differentiation may be used to resolve and locate peaks in an "envelope". Sharp bands are enhanced and this may allow for the selection of a suitable peak, even when there is a broad band beneath.

Deconvolution

Deconvolution is the process of compensating for the intrinsic line widths of bands in order to resolve overlapping bands. This technique yields spectra that have much narrower bands and is able to distinguish closely spaced features.

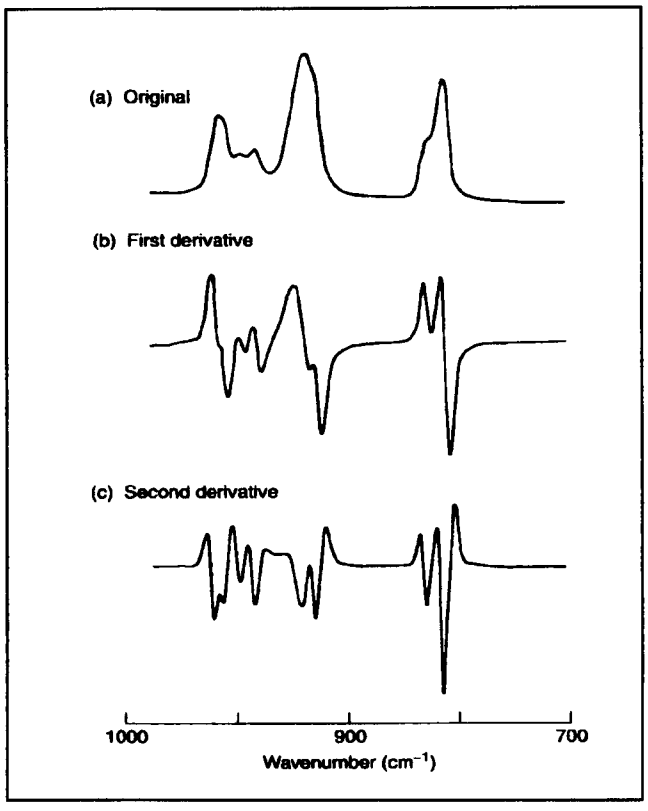


Figure 1.16 Complex absorption band (a, plus corresponding first (b) and second (c) derivatives (Stuart 2008).

Analysis of Biological samples

Infrared spectroscopy has been used to study a variety of biological systems including, lipids, proteins, peptides, nucleic acids, microbial cells and animal and plant tissue.

Lipids

Infrared spectroscopy can provide valuable structural information about lipids, which are important molecular components of membranes. Many lipids contain phosphorus and are classified as phospholipids. Lipids are organized in bilayers of about 40 - 80 Å in thickness where the polar head group points towards the aqueous phase and the hydrophobic tails point towards the tails of a second layer.

The infrared spectra of phospholipids can be divided into the spectral regions that originate from the molecular vibrations of the hydrocarbon tail, the interface region and the head group (Watts and De Pont 1986; Lewis and McElhaney 2002).

The hydrocarbon tail gives rise to acyl chain modes. The most intense vibrations in the infrared spectra of lipid systems are the CH_2 stretching vibrations and these give rise to bands in the 3100 to 2800 cm^{-1} region.

The CH_2 asymmetric and symmetric stretching modes, at 2920 and 2851 cm^{-1}, respectively, are generally the strongest bands in the spectra. The double bond C-H stretching bands due to unsaturated acyl chains are found at 3012 cm^{-1} and the bands due to methylene and methyl groups occur in the 1500 - 1350 cm^{-1} region. At around 1470 cm^{-1}, there are bands due to CH_2 bending and the number and wavenumbers of these bands are dependent on acyl chain packing and conformation. While the asymmetric deformation modes of the CH_3 group are obscured, the symmetric deformation mode appears at 1378 cm^{-1}.

Quantitative infrared analysis can be carried out on blood serum to determine the relative amounts of lipid present (Bhandara et al. 1994). Triglycerides, phospholipids and cholesterol esters are the classes of lipid that occur in blood serum and such compounds occur naturally in concentrations that make them suitable for infrared analysis.

Proteins and Peptides

The infrared spectra of proteins exhibit absorption bands associated with their characteristic amide group (Fabian and Mantele 2000) In-plane modes are due to C=O stretching, C-N stretching, N-H stretching and O-C-N bending, while an out-of-plane mode is due to C-N torsion. The characteristic bands of the amide groups of protein chains are similar to the absorption bands exhibited by secondary amides in general, and are labelled as amide bands. There are nine bands, amide A, amide B and amides I-VII, in order of decreasing wavenumber. Some of the bands are more useful for conformation studies than others and the amide I and amide II bands have been most frequently used for analysis.

Nucleic Acids

The nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), may be studied by using infrared spectroscopy (Taillandier and Liquier 2002). The spectra of nucleic acids may be divided into groups due to the constituent base, sugar and phosphate groups. The bases (thymine, adenine, cytosine, guanine and uracil) give rise to purinic and pyrimidinic vibrations in the 1800 -

1500 cm^{-1} range and these bands are sensitive markers for base pairing and base-stacking effects. Bands in the 1500 - 1250 cm^{-1} region of nucleic acids are due to the vibrational coupling between a base and a sugar, while in the 1250 - 1000 cm^{-1} range sugar-phosphate chain vibrations are observed. These bands provide information about backbone conformations. In the 1000-800 cm^{-1} region, sugar/sugar-phosphate vibrations are observed.

The use of FTIR to analyse bacteria.

Given the complex nature of micro-organisms the FTIR spectra of bacteria show bands predominantly due to the protein component. Although the cells also contain DNA and RNA structures, carbohydrates and lipids, the various cell and membrane proteins form the major part of the cell mass. Figure 1.17 shows parts of the infrared spectra of *Escherichia coli* and a typical protein, ribonuclease A, illustrating their similarity. Bands due to carbohydrates and lipids may also be observed in bacterial infrared spectra to a lesser extent.

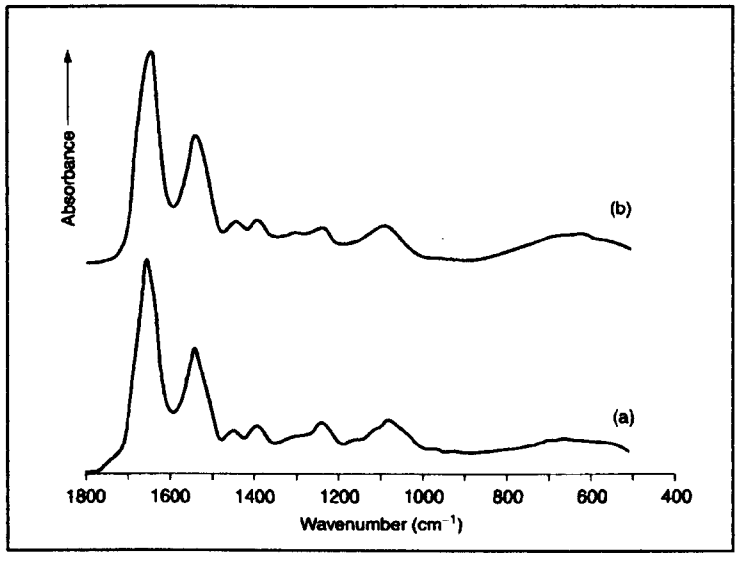


Figure 1.17 Infrared spectra of (a) Escherichia coli and (b) ribonuclease (Stuart 2008).

FTIR spectroscopy provides a rapid method for identifying micro-organisms responsible for infections(Stuart 2008). The technique has been used to monitor the biochemical heterogeneity of microcolonies of *E. coli* (Choo-Smith et al. 2001). Individual spectra were examined and difference spectra calculated. Figure 1.18 illustrates the infrared spectra obtained from the centre and the edge of an *E. coli* colony. From the difference spectra (4) it can be seen that there are

differences in the region near 1230 cm^{-1}, which can be assigned to the phosphate double-bond asymmetric stretching vibration of the phosphodiester, free phosphate and monoester phosphate functional groups. There also differences observed for the protein amide I region (1670 - 1620 cm^{-1}), symmetric stretching vibrations of the COO functional groups (1400 cm^{-1}) and the carbohydrate region (1200 - 900 cm^{-1}).

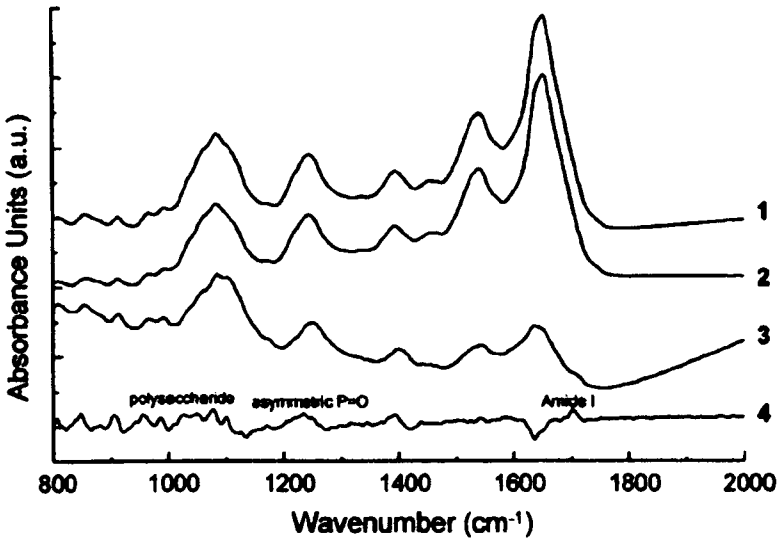


Figure 1.18 Infrared spectra of an Escherichia coli colony measured at (1) the centre and (2) the edge of the colony, the corresponding difference spectrum (3) and as well as the difference spectrum (4) obtained from the first-derivative spectra. (Choo-Smith et al. 2001).

Hierarchical cluster analysis of the spectra of 7 hour old colonies of two *E. coli* strains was carried out and Figure 1.19 shows the resulting dendrogram. This clearly shows the formation of two major clusters corresponding to the different strains.

Studies have shown that differentiating between different genera, species and even strains of bacteria was possible by the calculation of cross-correlation charts. The spectra were taken as bacterial fingerprints. They were compared to each other by computing correlation coefficients (Helm et al. 1991).

FTIR spectroscopy can be used to analyse entire bacterial cells or to compare only certain bacterial components. The absorption characteristics of complete bacterial cells produce a highly specific pattern unique for individual strains. The spectra represent the total chemical composition of cellular components such as proteins, membranes, cell wall and

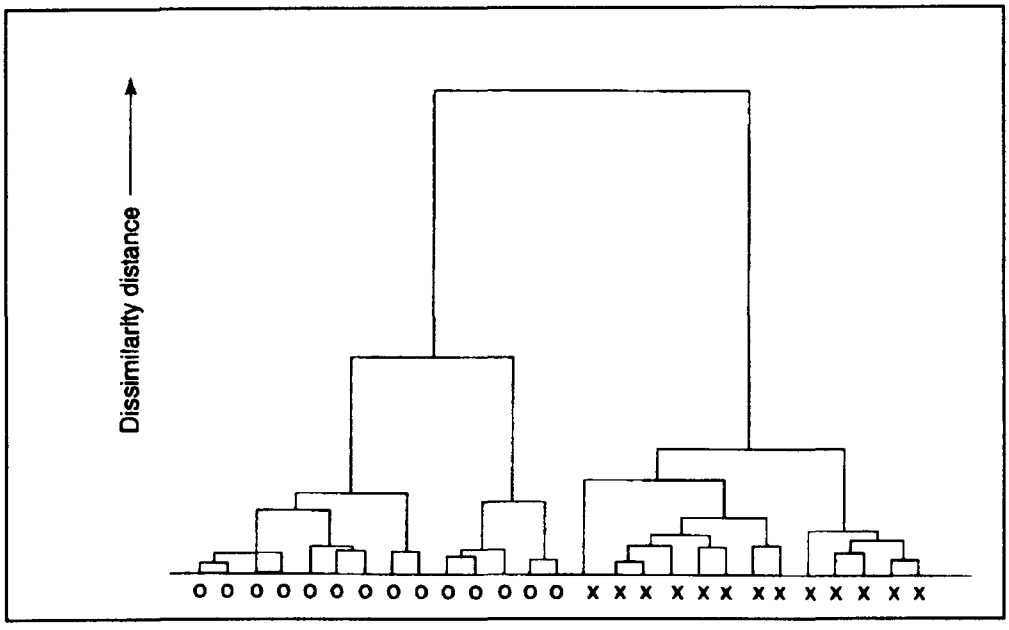


Figure 1.19 Dendrogram from hierarchical clustering analysis of the infrared spectra of 7h colonies of two* Escherichia coli *strains. o and x represent the two strains (Choo-Smith et al. 2001).

nucleic acids of bacterial cells (Helm, Labischinski and Naumann 1991).

A number of studies were been carried out using FTIR to identify different strains and species of bacteria. Helm *et al.* (1991) analysed samples of *Staphylococcus*, *Streptococcus* and *Clostridium* and found it possible to differentiate bacteria based on their FTIR profile. They also noted that FTIR could be used as an easy and safe method for rapid identification of clinical isolates.

Lin, Schraft and Griffiths (1998) undertook a study involving FTIR and the identification of *Bacillus cereus*. The objective of the study was to evaluate the potential of FTIR for rapid identification of *Bacillus cereus* isolates. The study showed that absorbance peaks between 1800 and 1500 cm^{-1} of members of the *B. cereus* group had different shapes and sizes suggesting that FTIR was a suitable technique for identifying *B. cereus*.

Little work has been done on the identification of lactic acid bacteria using FTIR. Lefier, Lamprell and Mazerolles (2000) used FTIR to study the diversity of *Lactococcus* flora during the maturation of Brie produced using 2 different starter cultures. The starter cultures were composed of a mixture of *Lactococcus* strains and the FTIR was used to determine whether 1 strain became dominant during the ripening process.

Curk, Peladan and Hubert (1994) used FTIR in the identification of *Lactobacillus* species. They were interested in identifying different species of *Lactobacillus* found in the beer-making process, with the aim of separating spoilage from non-spoilage organisms. The first part of the study involved a study of time of incubation and incubation temperature. They showed that changes in growth temperature produced slight differences in the spectra. The differences between species were greater than the differences between different cultures of a single strain grown at different temperatures. The time of culture also had a similar effect. The differences in spectra of organisms cultured for 24, 36, 48 and 72 hours were often smaller than spectral differences between different species. Temperature and time of culture did not have as great an effect on the spectra as was originally thought.

Using the information that is already known on how different wavenumbers correspond to different components of bacteria it was decided that this was a technique that could possibly be used to identify different strains of lactic acid bacteria and identify whether they are EPS or non EPS producing strains.

AIMS OF THE STUDY

From the introduction it can be seen that a great deal of interest has been shown in the use of EPS-producing lactic acid bacteria as a source of thickeners in yoghurt, since they would be considered natural products. Following a literature study a list of aims were drawn up to investigate EPS production by strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* and their role in textural forming abilities in yoghurt. These were:

1) Isolation of EPS from different strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*.

The first aim of this study was to isolate quantities EPS from different strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. A number of different media and isolation techniques found in the literature would be investigated. This was to enable the production of large enough quantities of crude EPS that could be used for further analysis.

2) Characterise EPS isolated from different strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*.

In the literature it has been reported that there are a number of differences in the composition of the EPS isolated from different strains of the same bacterial species. The second aim of the study was to analyse the isolated EPS for sugar composition, molecular mass, and the presence of charged groups. All of these factors are thought to play a role in the texture of yoghurts.

3) Identify the factors that affect the quantity of EPS production in strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*.

A number of different factors have an effect on the quantity of EPS produced by different strains of lactic acid bacteria. Because EPS plays a role in the texture of yoghurt it is important to determine which factors can effect EPS production and in what way they exert such and effect

4) Investigate the genetic instability of EPS production by strains of *Lc. lactis* ssp. *cremoris*.

The production of EPS by strains of lactic acid bacteria is controlled by a series of genes. In some strains of *Lc.lactis* ssp. *cremoris* these genes are plasmid encoded. The production of EPS by lactic acid bacteria is linked to the expression of these genes. The inconsistencies in this EPS production could be linked to factors that affect the growth of the bacteria. This could also be linked in some way to the possible loss of the plasmid containing the EPS-producing genes.

5) Investigate the textural properties of EPS when strains of *Lc.lactis* ssp *cremoris* and *Lb.delbrueckii* ssp. *bulgaricus* are grown in milk.

When lactic acid bacteria are grown in milk they produce EPS which interacts with the components of the milk to produce a thick gel which improves the rheology of the final fermented product. The EPS contributes to the texture, mouth-feel and stability of the final yoghurt product. It was decided to investigate the texture of fermented milk produced by different strains of *Lc.lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* and the effect of subculturing on the texture of fermented milk.

6) Investigate the use of Fourier transform infrared spectroscopy (FTIR) as a method for studying strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*.

The methods required to identify EPS producing strains of lactic acid bacteria are time consuming require a number of steps and are often subjective. It was decided to investigate Fourier transform infrared spectroscopy (FTIR). The aims of this part of the study were firstly to develop a workable method that could be used to investigate lactic acid bacteria and then use the technique to identify a possible marker in the spectrum that could be used to identify EPS producing ability. The second aim was to develop a method that could be used to identify EPS producing bacteria quickly and easily. This would be useful in the manufacture of fermented diary products for the identification of new bacteria that could be used in starter cultures as well as the continuous monitoring of EPS production throughout the manufacturing process.

CHAPTER 2. ANALYSIS OF EXOPOLYSACCHARIDES (EPS) ISOLATED FROM STRAINS OF *Lb. delbrueckii* ssp. *bulgaricus* AND *Lc. lactis* ssp. *cremoris*.

INTRODUCTION

The first two aims of the study required the isolation and structural analysis of EPS produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. The structural analysis was to consist of analysis of the composition of the crude EPS in relation to its sugar residues, its molecular size and the possibility of charged fragments. Methods for further purification of isolated EPS were also to be investigated.

THE STRUCTURE OF EPS PRODUCED BY LACTIC ACID BACTERIA

EPS are long chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. In the case of the EPS produced by lactic acid bacteria these sugar units are mainly glucose, galactose and rhamnose, in different ratios (Welman and Maddox 2003). EPS from lactic acid bacteria vary in molecular weight and this is thought to be one of the factors determining the functional properties of EPS and its ability to form a viscous solution (Cerning et al. 1992; Welman and Maddox 2003). However, the physiological and rheological properties of EPS are possibly also related to their three-dimensional structure or conformation. The potential of the polysaccharide molecules to form intermolecular associations may also affect the properties of a solution. Polysaccharide chains in solution undergo changes in shape, from disordered random coils to more ordered conformations (Cerning et al. 1992). The secondary and tertiary structures of a polysaccharide are determined by its primary structure. Therefore, small changes in this primary structure may have a significant effect on the final conformation and properties of the polysaccharide.

The structure of the repeating unit of a heteropolysaccharide, produced by *St. thermophilus*, was first determined by Doco et al. (1990). The structures of other polysaccharides have been determined by a number of different techniques including: acid hydrolysis, methylation analysis,

enzymatic digestion and 1D and 2D ^{1}H-NMR spectroscopy (de Vuyst and Degeest 1999). Their repeating structures range from a disaccharide to a heptasaccharide. They share some common features but the relationship between EPS structure and the textural properties that they confer is unclear.

A study carried out by Marshall, Cowie and Moreton (1995) reported the existence of charged and uncharged EPS in a sample of crude EPS extracted from *Lc. lactis* ssp. *cremoris* LC1. When separated the low molecular weight EPS was found to have a charge and the high molecular weight EPS was uncharged. The presence of charged fractions may indicate that EPS take part in or has an ability to form protein-polysaccharide or polysaccharide-polysaccharide complexes within cultured milk.

SUGAR COMPOSITION OF EXOPOLYSACCHARIDES

The sugar composition of polysaccharides varies depending on the strain of lactic acid bacteria producing them (Table 2.1). The sugar compositions for EPS from a number of species and strains of lactic acid bacteria have been determined in a variety of studies, as shown in Table 2.1, using either gas chromatography or high performance liquid chromatography.

ISOLATION AND PURIFICATION OF EXOPOLYSACCHARIDE

A number of different methods have been reported for the isolation of EPS from lactic acid bacteria. They are all preceded by a centrifugation step to remove bacterial cells and solids, leaving the EPS dissolved in the supernatant. The EPS is then precipitated from the supernatant solution by the addition of a suitable solvent.

Garcia-Garibay and Marshall (1991) precipitated the milk proteins from an overnight culture grown in milk using 80% (w/v) trichloroacetic acid (TCA). Centrifugation was used to remove the precipitated proteins. EPS were then precipitated from supernatant using 3 volumes of absolute ethanol. The EPS ethanol solution was left for 20 minutes and then centrifuged to collect precipitated EPS. The precipitate was redissolved in water at pH 4. The solution was filtered and the polymer precipitated again by the addition of 3 volumes of absolute ethanol.

Table 2.1 Summary of Polysaccharide Sugar Composition.

Reference	*Bacteria*	*Strain*	*Molecular Mass (Da)*	*Sugars*	*Ratio*
van Casteren et al. (1998)	*Lactococcus lactis* ssp *cremoris*	B40	6.8×10^5	rhamnose:galactose:glucose: phosphate	1:1.3:2:1.1
Urashima T et al. (1999)	*St. thermophilus* ssp *filant*		4.5×10^6	D-galactose L-rhamnose	
de Vuyst et al. (1998)	*St. thermophilus*	480		gal:glu	3:1
	St. thermophilus	LY03		gal:glu	4:1
	St. thermophilus	BTC		gal:glu	3:1
	St. thermophilus	Sfi20		gal:glu	3:1
	Lb sake	0-1		rha:glu	2:3
Ariga H *et* al. (1992)	*St. thermophilus*	OR901 S1-poly	$9x10^6$	rha:glu	1:1.47
		S2- poly	$1.1x10^6$	L-rhamnose:D-glucose	1:1.46
van Casteren et al. (2000)	*Lactococcus lactis* ssp *cremoris*	B39		L-rha:D-gal:D-glu	2:3:2
Gruter et al. (1992)	*Lactococcus lactis* ssp *cremoris*	H414		gal:glu	2:1
Gopal and Crow (1993)	*Lactococcus lactis* ssp *cremoris*	E8		rhamnose:galactosamine:glucosamine:galactose:glucose	0.6:0.08:1.1: 0.65:1
		398		rhamnose:galactosamine:glucosamine:galactose:glucose	2.2:0.3:1.2:0.9:1
Nakajima et al. (1992)	*Lactococcus lactis* ssp *cremoris*	SBT 0495		D-glucose, D-galactose, L-rhamnose, phosphate	
Nakajima et al. (1990)	*Lactococcus lactis* ssp *cremoris*	SBT 0495		rhamnose, glucose galactose	1:1.45:1.75
Marshall, Cowie and Moreton (1995)	*Lactococcus lactis* ssp *cremoris*	LC330 Charged	1×10^4	glucose:rhamnose:galactose:glucosamine	6:5:4:1
		Neutral	1×10^6	glucose:galactose:glucosamine	6:3:2
Grobben et al. (1997)	*Lb. delbrueckii* ssp *bulgaricus*	2772	1.7×10^6	galactose:glucose:rhamnose	5:1:1
			4×10^4	galactose:glucose:rhamnose	11:1:0.4
Gruter et al. (1993)	*Lb. delbrueckii* ssp *bulgaricus*	rr		galactose:glucose	2:1
Cerning et al. (1986)	*Lb. bulgaricus*		5×10^5	galactose:glucose:rhamnose	4:1:1
Bubb et al. (1997)	*St. thermophilus*	OR901		D-galactopyranose: L-rhamnopyranose	5:2
Doco et al. (1990)	*St. thermophilus*			D-glucose, D-galactose, N-acetyl-D-galactosamine	1:2:1
Faber et al. (1998)	*St. thermophilus*	Rs	2.6×10^3	D-gal:L-rha	5:2
		Sts	3.7×10^3	D-gal:L-rha	5:2
Lemoine et al.(1997)	*St. thermophilus*	SFi39	$<2 \times 10^6$	D-glucose:D-galactose	1:1
		SFi12	$<2 \times 10^6$	D-galactose:L-rhamnose:D-glucose	3:2:1

Grobben et al. (1995) used an extraction process based on the method of Garcia-Garibay and Marshall (1991). After the second precipitation step, the EPS was centrifuged and resuspended in water at pH 4. The EPS solution was dialysed and lyophilized.

Gancel and Novel (1994a) used cultures grown in a derivative of M17 media and centrifuged to remove bacterial cells, then collected the supernatant. Pronase was added to a final concentration of 250 μg/ml to hydrolyse proteins. Samples were incubated for 1 hour at 37°C and residual polypeptides were removed by precipitation with 10% (w/v) TCA. Supernatants were dialysed and the carbohydrate concentration was measured in the dialysate.

The method used by Kimmel, Roberts and Ziegler (1998) was based on the method of Gancel and Novel (1994a). Cultures were grown in Man, Rogosa and Sharpe (MRS) broth. Following culturing, the media was heated in a water bath to inactivate enzymes that could degrade the EPS. The heat treated medium was then cooled to room temperature and 5% pronase E added to hydrolyse proteins. This mixture was then incubated at 37°C for 1 hour. 80% TCA was added and the media was incubated at 40°C for 30 minutes to precipitate any remaining proteins. The cells and precipitated proteins were removed by centrifugation. The supernatant was dialysed for 48 hours. Carbohydrate concentration was measured in the dialysate.

Cerning et al. (1992) cultured the bacteria in milk. Milk proteins were digested with pronase and the media was then incubated at 30°C for 16 hours. Merthiolate was added during incubation to inhibit bacterial cell growth. After incubation the broth was heated to 100°C for 10 minutes to inactivate enzymes. The supernatant was concentrated by ultrafiltration under gaseous nitrogen, followed by the addition of equal volumes of 100% ethanol to precipitate the EPS. The precipitate was collected by centrifugation and dissolved in distilled water, dialysed against distilled water and freeze dried.

Further purification of extracted polysaccharides can be carried out using a number of different chromatographic methods. Gruter et al. (1993) used a column containing Sephacryl S-500 with mobile phase of NH_4HCO_3 buffer and the eluate was monitored using a refractive index detector. Cerning et al. (1986) used a DEAE Trisacryl column. Bubb et al. (1997) used a column containing DEAE Sephadex A-50 ion exchange resin. Van Castern et al. (2000) used TCA and ethanol then a

column containing Sephacryl S-500 with a sodium acetate buffer and monitored the eluent with a refractive index detector.

All of these published methods have been used to extract EPS produced by lactic acid bacteria from a growth media with varying degrees of success. There seems to be very little data available on the carbohydrate content of the EPS extracted by these methods. It could be inferred that as EPS is a carbohydrate, high carbohydrate content would indicate an EPS extract of high purity. In this study a variety of methods were tested, the aim being to extract the maximum yield of EPS from the lactic acid bacteria so that further structural analysis could be carried out.

METHODS

MICROORGANISMS

This section details the compositions of media and general methods used during the isolation and analysis of EPS produced by lactic acid bacteria. A list of materials, reagents and equipment can be found in Appendix 2.

As mentioned in Aims 1 and 2 most of the lactic acid bacteria used were known EPS and non EPS producing strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* (Table 2.2)

Table 2.2 The strains of bacteria used for this research.

Species	Strain	EPS producing ability
Lactobacillus delbrueckii ssp. *bulgaricus*	NCIMB 702482 (2482)	Yes
Lactobacillus delbrueckii ssp. *bulgaricus*	NCIMB 702483 (2483)	Yes
Lactobacillus delbrueckii ssp. *bulgaricus*	NCIMB 702772 (2772)	Yes
Lactobacillus delbrueckii ssp. *bulgaricus*	NCIMB 702394 (2394)	No
Lactobacillus delbrueckii ssp. *bulgaricus*	Ldb1 (Ldb1)	Yes
Lactococcus lactis ssp. *cremoris*	NCIMB 701934 (1934)	Yes
Lactococcus lactis ssp. *cremoris*	NCIMB 700967 (0967)	Yes
Lactococcus lactis ssp. *cremoris*	NCIMB 700968 (0968)	Yes
Lactococcus lactis ssp. *cremoris*	NCIMB 702006 (2006)	Yes
Lactococcus lactis ssp. *cremoris*	LC1 (LC1)	Yes
Lactococcus lactis ssp. *cremoris*	NIZO B40 (B40)	Yes

Bacterial cultures were supplied as freeze dried cultures. These were reanimated in appropriate media. Cultures were then maintained as frozen stock on plastic beads at -75°C. Prior to inoculation for the experiments, cultures were grown from a bead in the same medium as that of the experiments, under static conditions at 37°C for strains of *Lb. delbrueckii* ssp. *bulgaricus* or

30°C for *Lc. lactis* ssp. *cremoris*. They were then subcultured once to create the culture to be used in the experiments.

MEDIA COMPOSITION

Man, Rogosa and Sharpe (MRS) broth/agar

This medium was used for the growth of *Lb. delbrueckii* ssp. *bulgaricus* and was prepared according to manufacturers instructions.

M17 broth/agar

This medium was used for the growth of *Lc. lactis* ssp. *cremoris* and was prepared according to manufacturers instructions.

Skimmed milk medium

Skimmed milk powder was made up to 10% (w/v) in distilled water. This was then autoclaved at 110°C for 15 minutes. This medium was used for the growth of all strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

MICROBIOLOGICAL EXAMINATION

At regular intervals the cell morphology all of the cultures used were examined microscopically using Gram staining. They were also examined using the capsular stain technique to check that the cultures were still EPS producing.

Gram Stain

Crystal Violet Solution: 20 g of crystal violet was dissolved in 100 ml of ethanol to make a stock solution. 1 g of ammonium oxalate was dissolved in 100 ml of water to make an oxalate stock solution. A working solution was obtained by mixing 1 ml of the crystal violet stock solution with 10 ml of water and 40 ml of the oxalate stock solution. The working solution was stored in a drop bottle.

Safranin Solution: 2.5 g of safranin was dissolved in 100 ml of 95 % ethanol to make a stock solution. A working solution was obtained by diluting one part of the stock solution with five parts of water.

Method

A heat-fixed slide of bacterial culture was prepared. Crystal violet was applied and left for 1 min. This was then washed off with water. 0.5 % wt/vol Iodine was applied and left for 1 min. The specimen was decolorised with 100% ethanol. The slide was then rinsed under running water to remove all traces of the ethanol. The sample was then counterstained with safranine for 30 seconds. The slide was washed with water and air dried. The prepared slides were then observed under x100 oil immersion objective lens.

Capsule Stain

A heat-fixed slide of bacterial culture was prepared. The bacteria were then stained with crystal violet, as prepared for the Gram stain. Indian ink was applied and allowed to dry. The slide was observed using x100 oil immersion objective lens.

ISOLATION OF EXOPOLYSACCHARIDE (EPS).

Extraction of EPS from milk (adapted from Kimmel, Roberts and Ziegler 1998; Cerning et al. 1992).

For analysis of the EPS produced by different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* the bacteria were grown on a larger-scale using skimmed milk. Following the testing of other published methods it was decided to extract the EPS using a combination of two different methods to maximise the yield.

All bacterial strains were grown in 1 litre of 10% (w/v) skimmed milk media for 12 hours until the pH reached a value of 4.0. The cultures were then cooled down to 5°C in the refrigerator. Whilst stirring, 190 ml of 80% (w/v) trichloroacetic acid was added. The mixture was then centrifuged for 30 min at 4000 rpm. The supernatant was dialysed against frequent changes of distilled water for 3 days. Ethanol was added whilst stirring to a concentration of 70% (v/v). This was then refrigerated

for 48 h. The precipitate that formed was recovered using centrifugation (10 min at 3,000 rpm) then the pellet dissolved in 20 ml of distilled water. This was followed by dialysis against distilled water to remove traces of ethanol. Dialysis was carried out twice using 2.5 litres of distilled water each time. The EPS solution was then freeze-dried.

Dialysis

Dialysis tubing (6000 - 8000 MW) was prepared by boiling in a solution of 2% (w/v) sodium hydrogen carbonate containing 1mM EDTA for 10 min, rinsing in deionised water then boiling for a further 10 min in deionised water. Samples were dialysed against frequent changes of distilled water. Dialysis was carried out at 4°C to prevent the growth of any contaminating moulds or bacteria.

Storage of EPS isolates

Freeze-dried EPS were stored in a vacuum dessicator with silica gel as a desiccant. EPS solutions were frozen and stored at -20°C.

ANALYSIS OF CRUDE AND FRACTIONATED EPS

Following the large-scale extraction of EPS from different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus,* the structure of the EPS were analysed using a number of different methods.

Determination of total carbohydrate.

Total carbohydrate in solutions of crude and fractionated EPS or dialysates (1 ml) was measured colourimetrically after the addition of 5% (w/v) phenol solution (1 ml) and concentrated sulphuric acid (5 ml). A stable chromophore formed after 15 min heating in a boiling water bath. The absorbance value, measured at 490 nm, was compared with glucose standard solutions (Dubois et al. 1956). The carbohydrate content of the polysaccharides gives an indication of the total

carbohydrate content and the purity of the polysaccharide. Calibration was carried out using glucose solutions with concentrations ranging from 0 -100 μg/ml.

Calibration graph can be found in Appendix 1.1

Lowry method for protein determination

Total protein content in solutions of crude and fractionated EPS (0.5 ml) was measured colourimetrically. The reagent solution (0.6 ml) (Table 2.3) was added and the mixture was allowed to stand for 10 min, then 0.5 ml of Folin Ciocalteau's reagent was added. A stable chromophore was formed after 30 min at room temperature. The absorbance value, measured at 600 nm, was compared with those obtained for bovine serum albumin standard solutions. The protein content of the EPS was determined to give an indication of possible levels of protein contamination of the EPS. Any protein present was likely to have come from milk proteins or bacterial cellular proteins associated with the EPS.

Calibration graph can be found in Appendix 1.2

Table 2.3 Lowry Reagent Solution

Component	Quantity (g/L)
Copper sulphate	0.02
Potassium sodium tartarate	0.02
Sodium hydroxide	40
Sodium carbonate	20

Analysis of EPS using size exclusion chromatography

Size exclusion chromatography was investigated as a possible method of purification of the EPS. Separation on a small scale was carried out using columns of 400 mm in length and 20 mm in diameter containing Sephacryl S-500-HR. A 5mM phosphate buffer (pH 6.5) was used as an eluent at a flow rate of 0.5 ml/min. Fractions (5 ml) were collected using a fraction collector and 1 ml analysed for carbohydrate content.

Large scale fractionation of the crude EPS was carried out using a column 1 m in length and 50 mm in diameter containing Sephacryl S-500-HR. A 5mM phosphate buffer (pH 6.5) was used as

an eluent at a flow rate of 0.5 ml/min. Fractions (10 ml) were collected using a fraction collector and 1 ml analysed for carbohydrate content. These were plotted against elution volume to obtain a chromatogram. Fractions corresponding to separated polysaccharides were pooled, dialysed then freeze dried. Analysis of total carbohydrate content was carried out on solutions of these fractions.

Analysis of polysaccharides on DEAE Sepharose.

Ion exchange chromatography was used to investigate the possibility of the EPS containing charged and uncharged fragments based on work carried out by Marshall, Cowie and Moreton (1995).

Separation of crude and fractionated EPS samples into charged and uncharged fractions was achieved by passing solutions of EPS through DEAE Sepharose (2 ml) held in small disposable columns (10 ml volume). The solutions were allowed to drain through the bed of resin without the use of a pump. After washing the resin with distilled water (4 x 2 ml) the charged material bound to the resin was washed off using 1 M NaCl (4 x 1 ml). Salt was removed by dialysis. Fractions were collected and analysed for carbohydrate content.

Determination of the sugar composition of prepared EPS.

To compare the composition of EPS produced by different strains of lactic acid bacteria HPLC analysis of the hydrolysed EPS was carried out.

Tubes containing 1 ml of 100 µl/ml (w/v) stock solution of crude or fractionated polysaccharide and 1ml of 2 M trifluroacetic acid (TFA) were flushed with N_2 gas and then sealed. These were heated for 1 h at 120°C in an oil bath to hydrolyse the EPS. The TFA was removed using a stream of N_2 whilst warming the tubes at 70°C. The residue was dissolved in 0.5 ml distilled water and then analysed using HPLC.

The following conditions were used for the HPLC:

Column	REZEX 8U 8% H. Organic acid
Temp	65°C
Eluent	0.005M H_2SO_4
Flow Rate	0.6 ml/min
Detector	Shodex R1 SE-61 Refractive Index

Concentrations of sugars in hydrolysed EPS samples were calculated from calibration curves. The calibration curves were prepared by analysing using 0.1 mM standard solutions of glucose, galactose, rhamnose, arabinose, fructose and glucuronic acid.

Calibration graphs can be found in Appendix 1.4, 1.5, 1.6 & 1.7

Molecular sizing of prepared EPS.

A sample of 1mg/ml (w/v) of crude polysaccharide solution was injected into an HPLC Gel exclusion column. The size of the polysaccharides was estimated by comparing the retention times with that of dextran standards of known molecular weight.

The following HPLC conditions were used:

Column	TSK-Gel GMPW 30 cm x 7.8 mm with guard column
Temp	35°C
Eluent	Distilled water
Flow Rate	0.6 ml/min
Detector	Shodex R1 SE-61 Refractive Index

Calibration graph can be found in Appendix 1.3

RESULTS

ISOLATION OF EPS FROM STRAINS OF *Lc. lactis* ssp. *cremoris* AND *Lb. delbrueckii* ssp. *bulgaricus* CULTURED IN SKIMMED MILK.

EPS were isolated from different strains of *Lc. lactis ssp. cremoris* and *Lb. delbrueckii ssp. bulgaricus* grown in 10% (w/v) skimmed milk. The extraction process was carried out as described in the Methods section.

Table 2.4 Concentration of EPS isolated from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus *grown in 10% skimmed milk.

Strain	*Yield(mg/l)*
Lb. delbrueckii ssp. *bulgaricus* 2482	71
Lb. delbrueckii ssp. *bulgaricus* 2483	91
Lb. delbrueckii ssp. *bulgaricus* 2772	128
Lb. delbrueckii ssp. *bulgaricus* 2394	10
Lb. delbrueckii ssp. *bulgaricus* Ldb1	157
Lc. lactis ssp. *cremoris* 1934	84
Lc. lactis ssp. *cremoris* 0967	144
Lc. lactis ssp. *cremoris* 0968	151
Lc. lactis ssp. *cremoris* 2006	203
Lc. lactis ssp. *cremoris* LC1	67
Lc. lactis ssp. *cremoris* B40	253
Skimmed milk	0

From Table 2.4 it can be seen that the EPS yield varied between the strains of both *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. *Lc. lactis* ssp. *cremoris* B40 produced the largest quantities of EPS of all the *Lc. lactis* ssp. *cremoris* strains (253 mg/l w/v) and strain LC1 produced the least (67 mg/l w/v). *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 produced the largest quantities of EPS of all the *Lb. delbrueckii* ssp. *bulgaricus* strains (157 mg/l w/v). *Lb. delbrueckii* ssp. *bulgaricus* 2394 only produced 10 mg/l (w/v) when grown in milk.

THE ANALYSIS OF CARBOHYDRATE AND PROTEIN CONTENT OF EPS ISOLATED FROM STRAINS OF *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* CULTURED IN SKIMMED MILK.

The extracted EPS were first analysed for total carbohydrate content as a possible indicator of purity. The protein content of the EPS was determined in duplicate using the Lowry method. The presence of protein may be an indication of contamination of the EPS by either proteinaceous material from the growth media or from the bacteria itself. Both sets of analyses were carried out on 100 μg/ml (w/v) solutions of the crude EPS.

Table 2.5 Total carbohydrate content of EPS produced by strains of* Lb. delbrueckii *ssp.* bulgaricus *and* Lc. lactis *ssp.* cremoris *in skimmed milk.

Strain	*Carbohydrate content (μg/100μg dry weight)*		
	1	*2*	*Mean*
Lb. delbrueckii ssp. *bulgaricus* 2482	109	105	107
Lb. delbrueckii ssp. *bulgaricus* 2483	91	90	91
Lb. delbrueckii ssp. *bulgaricus* 2772	76	76	76
Lb. delbrueckii ssp. *bulgaricus* 2394	25	25	25
Lb. delbrueckii ssp. *bulgaricus* Ldb1	107	105	106
Lc. lactis ssp. *cremoris* 1934	9	9	9
Lc. lactis ssp. *cremoris* 0967	9	7	8
Lc. lactis ssp. *cremoris* 0968	13	12	13
Lc. lactis ssp. *cremoris* 2006	18	19	19
Lc. lactis ssp. *cremoris* LC1	36	36	36
Lc. lactis ssp. *cremoris* B40	32	32	32

Table 2.5 shows the variation in total carbohydrate content of the crude EPS obtained from the different strains of *Lb. delbrueckii ssp. bulgaricus* and *Lc. lactis ssp. cremoris* cultured in skimmed milk. *Lb. delbrueckii ssp. bulgaricus* produced EPS of a higher purity than *Lc. lactis ssp. cremoris. Lb. delbrueckii ssp. bulgaricus* 2394 produced EPS that had low purity in comparison to the other strains. Of the strains of *Lc. lactis ssp. cremoris*, the EPS produced by strain LC1 had the highest purity at 36% and 0967 and 1934 had the lowest purity values (8 and 9%). Overall all the EPS produced by strains of *Lc. lactis ssp. cremoris* had low purity values. The carbohydrate content for strains 2482 and Ldb1 are both greater than 100 μg. Values higher than 100 μg / 100 μg dry weight indicate a degree of inaccuracy in the method.

Table 2.6 Total protein content of EPS isolated from strains of* Lb. delbrueckii *ssp.* bulgaricus *and* Lc. lactis *ssp.* cremoris *in skimmed milk as determined using the Lowry method.

Strain	*Protein content (μg/100μg dry weight)*		
	1	*2*	*Mean*
Lb. delbrueckii ssp. *bulgaricus* 2482	16	16	16
Lb. delbrueckii ssp. *bulgaricus* 2483	23	22	23
Lb. delbrueckii ssp. *bulgaricus* 2772	19	17	18
Lb. delbrueckii ssp. *bulgaricus* 2394	0	0	0
Lb. delbrueckii ssp. *bulgaricus* Ldb1	11	10	10
Lc. lactis ssp. *cremoris* 1934	10	9	10
Lc. lactis ssp. *cremoris* 0967	9	8	9
Lc. lactis ssp. *cremoris* 0968	9	10	10
Lc. lactis ssp. *cremoris* 2006	12	12	12
Lc. lactis ssp. *cremoris* LC1	8	8	8
Lc. lactis ssp. *cremoris* B40	13	12	13

From Table 2.6 it can been seen that protein was found within all the EPS samples. The EPS from *Lc. lactis* ssp. *cremoris* B40 contains the most (13%) and LC1 contained the least (8%). In *Lb. delbrueckii* ssp. *bulgaricus* 2483 contained the most (23%) and 2394 the least (0%). Overall EPS from strains of *Lb. delbrueckii* ssp. *bulgaricus* contain more protein than strains of *Lc. lactis* ssp. *cremoris.*

ESTIMATION OF MOLECULAR SIZE OF CRUDE EPS ISOLATED FROM STRAINS OF *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* CULTURED IN SKIMMED MILK.

Studies have shown that EPS produced by strains of lactic acid bacteria vary in molecular weight (Table 2.1). The molecular weight of the isolated crude EPS was estimated by comparison with dextran standards using high performance liquid chromatography (HPLC). Dextran standards were used for comparison because they are readily available in a number of molecular weights. A calibration curve relating retention time to molecular weight was prepared using different molecular weight dextrans (Appendix 1.3). The larger molecular weight molecules eluted from the column first. The analyses were carried out using EPS solutions of 1000 μg/ml.

Table 2.7 Estimated molecular weight of crude EPS (in relation to dextran standards) isolated from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus *cultured in skimmed milk.

Strain	*Peak Number*	*% Area of chromatogram*	*Molecular Weight g/mol*
Lb. delbrueckii ssp. *bulgaricus* 2482	1	12	1.51×10^{11}
	2	88	4.17×10^{7}
Lb. delbrueckii ssp. *bulgaricus* 2483	1	58	3.72×10^{7}
	2	42	8.32×10^{6}
Lb. delbrueckii ssp. *bulgaricus* 2772	1	61	2.34×10^{7}
	2	39	6.61×10^{6}
Lb. delbrueckii ssp. *bulgaricus* 2394	1	100	2.63×10^{7}
Lb. delbrueckii ssp. *bulgaricus* Ldb1	1	41	1.55×10^{7}
	2	59	7.94×10^{6}
Range			**$6.61 \times 10^{6} - 1.51 \times 10^{11}$**
Lc. lactis ssp. *cremoris* 1934			No peaks visible
Lc. lactis ssp. *cremoris* 0967	1	100	1.26×10^{9}
Lc. lactis ssp. *cremoris* 0968	1	100	1.77×10^{8}
Lc. lactis ssp. *cremoris* 2006	1	67	3.16×10^{8}
Lc. lactis ssp. *cremoris* LC1	1	5	6.30×10^{8}
	2	76	6.46×10^{7}
Lc. lactis ssp. *cremoris* B40	1	100	5.01×10^{8}
Range			**$6.46 \times 10^{7} - 1.26 \times 10^{9}$**

Table 2.7 shows the different molecular weights of the EPS produced by *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. The EPS produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* are generally smaller than those produced by strains of *Lc. lactis* ssp. *cremoris*. Strains of *Lb. delbrueckii* ssp. *bulgaricus* produced EPS ranging from 6.61×10^{6} g/mol (2772) to 1.51×10^{11} g/mol (2482). Strains of *Lc. lactis* ssp. *cremoris* produced EPS ranging from 6.46×10^{7} g/mol (LC1) to 1.26×10^{9} g/mol (0967). The EPS produced by *Lc. lactis* ssp. *cremoris* 1934 did not produce any peaks in the HPLC so could not be sized. All EPS from strains of *Lb. delbrueckii* ssp. *bulgaricus* except 2394 produced more than one peak on the HPLC. Of the EPS produced by strains of *Lc. lactis* ssp. *cremoris* only LC1 produced more than one peak. Late peaks corresponding to very low molecular weight oligosaccharides/monosaccharides were obtained for EPS produced by strain LC1. These are not shown in Table 2.7.

AN INVESTIGATION INTO THE PRESENCE OF CHARGED AND UNCHARGED FRAGMENTS OF EPS IN CRUDE EPS PRODUCED BY STRAINS OF *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* CULTURED IN SKIMMED MILK.

The strains used in this study were investigated for their ability to produce charged and uncharged EPS. The presence of charged fractions may indicate that EPS take part in or have an ability to form protein-polysaccharide complexes within cultured milk.

Table 2.8 Charged and uncharged carbohydrate fragments found in EPS isolated from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus *cultured in skimmed milk.

Sample	*Ratio of Fractions(μg/ml:μg/ml)*	
	Charged	*Uncharged*
Lb. delbrueckii ssp. *bulgaricus* 2482	1.0	23.5
Lb. delbrueckii ssp. *bulgaricus* 2483	1.0	24.6
Lb. delbrueckii ssp. *bulgaricus* 2772	1.0	14.4
Lb. delbrueckii ssp. bulgaricus 2394	Insufficient crude EPS for analysis	
Lb. delbrueckii ssp. *bulgaricus* Ldb1	1.0	77.3
Lc. lactis ssp. *cremoris* 1934	1.0	1.1
Lc. lactis ssp. *cremoris* 0967	7.2	1.0
Lc. lactis ssp. *cremoris* 0968	1.0	1.7
Lc. lactis ssp. *cremoris* 2006	1.0	1.3
Lc. lactis ssp. *cremoris* LC1	1.0	4.6
Lc. lactis ssp. *cremoris* B40	1.0	1.0

Table 2.8 shows that the EPS from strains of both *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* contained charged and uncharged carbohydrate fragments. In all EPS, except that from strain 0967 and B40, there was more uncharged than charged EPS and in B40 they were found in equal quantities. In strain 0967, there was more charged than uncharged EPS. In strains of *Lb. delbrueckii* ssp. *bulgaricus* there were much larger proportions of uncharged than charged carbohydrate fragments.

DETERMINATION OF MONOSACCHARIDE COMPOSITION FOUND IN CRUDE EPS FROM STRAINS OF *Lc. lactis* ssp. *cremoris* AND *Lb. delbrueckii* ssp. *bulgaricus* CULTURED IN SKIMMED MILK.

A number of studies have reported on the sugar composition of the EPS produced by strains of lactic acid bacteria (Table 2.1). Monosaccharide composition of the EPS isolated from different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* used in this study is shown in Table 2.9.

Table 2.9 Monosaccharide composition of EPS isolated from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus *cultured in skimmed milk.

Strain	*Ratio of Sugars (μg/ml:μg/ml:μg/ml)*		
	Glucose	*Galactose*	*Rhamnose*
Lb. delbrueckii ssp. *bulgaricus* 2482	1.2	3.7	1.0
Lb. delbrueckii ssp. *bulgaricus* 2483	1.0	3.1	3.1
Lb. delbrueckii ssp. *bulgaricus* 2772	1.2	4.2	1.0
Lb. delbrueckii ssp. *bulgaricus* 2394	Insufficient crude EPS for analysis		
Lb. delbrueckii ssp. *bulgaricus* Ldb1	1.4	4.5	1.0
Lc. lactis ssp. *cremoris* 1934	7.0	12.5	1.0
Lc. lactis ssp. *cremoris* 0967	1.0	6.3	2.5
Lc. lactis ssp. *cremoris* 0968	5.0	7.5	1.0
Lc. lactis ssp. *cremoris* 2006	10.0	6.7	1.0
Lc. lactis ssp. *cremoris* LC1	30.0	12.0	1.0
Lc. lactis ssp. *cremoris* B40	3.0	1.7	1.0

It can be seen from Table 2.9 that the monosaccharide composition of the EPS varies between the strains. However, all the EPS were composed of glucose, galactose and rhamnose. In *Lc. lactis* ssp. *cremoris* strains 1934, 0967, 0968 there is more galactose than glucose. In *Lc. lactis* ssp. *cremoris* strains LC1, B40 and 2006 there is more glucose than galactose. Rhamnose is present in the smallest quantities in the EPS from all strains of *Lc. lactis* ssp. *cremoris* except strain 0967. In the EPS from all strains of *Lb. delbrueckii* ssp. *bulgaricus* there is more galactose than glucose. Rhamnose is the least common sugar except in EPS produced by strain 2483 where it is found in equal quantities to galactose.

FRACTIONATION OF CRUDE EPS ISOLATED FROM *Lc. lactis* ssp. *cremoris* USING SIZE EXCLUSION CHROMATOGRAPHY.

From the results seen in Table 2.5, the purity of the EPS produced by strains of *Lc. lactis ssp. cremoris* grown in milk was low. The potential use of size exclusion chromatography as an additional purification step was investigated. Separation was carried out using Sephacryl S-500-HR as described in the method section.

Figure 2.1 shows the chromatograms of the eluted EPS from the size exclusion column. It was possible to separate all but 2 of the EPS produced by strains of *Lc. lactis* ssp. *cremoris* into two components. From the chromatogram (Fig. 2.1) it can be seen that all the strains of *Lc. lactis* ssp. *cremoris* produced two peaks that could indicate the presence of two polysaccharides. It was concluded that this method could be used for large-scale separation. The results confirmed that each EPS is composed of 2 smaller EPS components as suggested by Marshall, Cowie and Moreton (1995).

To enable further study and characterisation of the EPS corresponding to the two peaks. The crude EPS from *Lc. lactis* ssp. *cremoris* was separated using a larger column (1 m x 50 mm) also packed with Sephacryl S-500-HR. In the large-scale purification, the first peak will henceforth be referred to as fraction A (Volume 35-50 ml) and the second peak will be referred to as fraction B (50 – 80 ml). The contents of all the tubes corresponding to EPS fraction A were combined, dialysed and freeze-dried. The same process was repeated for the tubes corresponding fraction B.

Table 2.10 shows the ratio of the mass of the fractionated EPS produced by *Lc. lactis* ssp. *cremoris* corresponding to fraction A and fraction B. It can be seen that it was possible to separate the crude EPS from all strains, except 1934 and 0967 into two freeze dried fractions. For all the EPS samples except, strain 2006 and B40, a larger mass of freeze dried fraction A than fraction B was obtained. In B40 the two fractions were found in equal quantities. Dried fractionated EPS were subsequently analysed for carbohydrate and protein content as well as sugar composition using the same methods as those used to analyse the crude EPS.

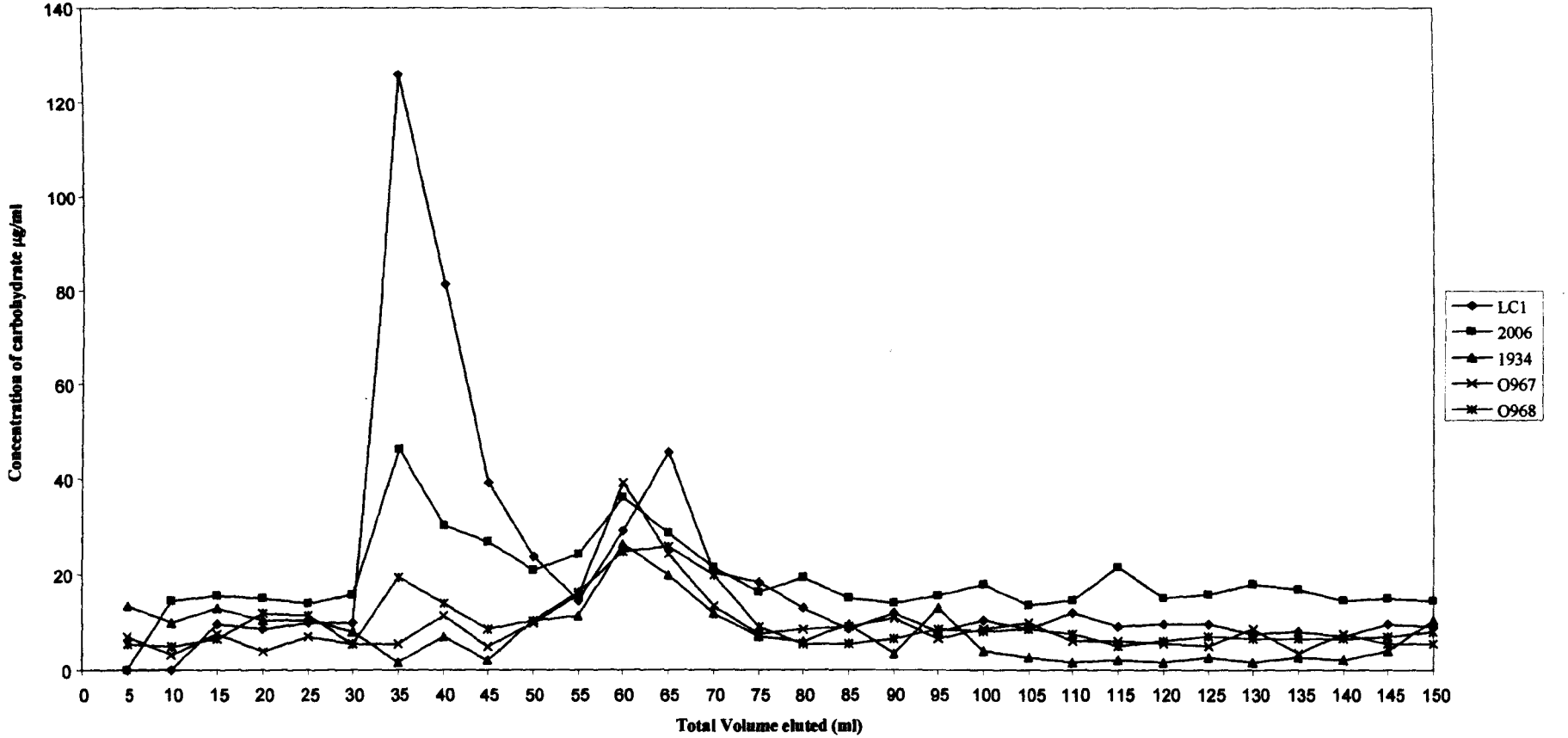


Figure 2.1 Separation of EPS isolated from strains of Lc. lactis *ssp.* cremoris *using a size exclusion column.*

Table 2.10 Fractionation of EPS produced by **Lc. lactis *ssp.* cremoris.**

Strain	*Ratio of mass of fractions (mg:mg)*	
	Fraction A	*Fraction B*
Lc. lactis ssp. *cremoris* 1934	1	0
Lc. lactis ssp. *cremoris* 0967	1	0
Lc. lactis ssp. *cremoris* 0968	3	1
Lc. lactis ssp. *cremoris* 2006	1	2
Lc. lactis ssp. *cremoris* LC1	3	2
Lc. lactis ssp. *cremoris* B40	1	1

THE ANALYSIS OF CARBOHYDRATE AND PROTEIN CONTENT OF FRACTIONATED EPS.

The fractionated EPS were analysed using the phenol sulphuric method, to estimate the carbohydrate content of the polysaccharides. Analysis of the protein content of the EPS was carried out using the Lowry method. Both sets of analyses were carried out on 100 μg/ml solutions of the EPS fractions.

Table 2.11 The carbohydrate content of fractionated EPS.

Strain	*Average carbohydrate content (μg/100μg dry weight)*	
	Fraction A	*Fraction B*
Lc. lactis ssp. *cremoris* 1934	8.9	No fraction B isolated
Lc. lactis ssp. *cremoris* 0967	11.8	No fraction B isolated
Lc. lactis ssp. *cremoris* 0968	18.9	11.2
Lc. lactis ssp. *cremoris* 2006	14.7	4.3
Lc. lactis ssp. *cremoris* LC1	15.8	3.1
Lc. lactis ssp. *cremoris* B40	18.2	4.6

Table 2.11 shows the carbohydrate content of the fractionated EPS based on an average of 2 sets of analysis. The values for all of the strains are very low. Fraction A contained more carbohydrate than fraction B. In the samples extracted from strains 2006, LC1 and B40 the difference in carbohydrate content between fractions A and B is large. Overall these values are also lower than the carbohydrate values for the crude EPS suggesting that gel exclusion chromatography did not improve the purity of the EPS.

Table 2.12 The protein content of fractionated EPS.

Strain	*Average protein content (μg/100μg dry weight)*	
	Fraction A	*Fraction B*
Lc. lactis ssp. *cremoris* 1934	7.9	No fraction B isolated
Lc. lactis ssp. *cremoris* 0967	7.3	No fraction B isolated
Lc. lactis ssp. *cremoris* 0968	5.8	9.5
Lc. lactis ssp. *cremoris* 2006	4.8	2.3
Lc. lactis ssp. *cremoris* LC1	3.8	3.9
Lc. lactis ssp. *cremoris* B40	4.1	4.9

Table 2.12 shows the protein content of the fractionated EPS. From the table it can be seen that the protein content of the fractionated EPS was low in comparison to the values for the crude EPS. In all the strains except B40 and 0968, fraction A contained more protein than fraction B. However, the protein cannot entirely account for the apparent non-carbohydrate content of the EPS fractions.

AN INVESTIGATION INTO PRODUCTION OF CHARGED AND UNCHARGED CARBOHYDRATE FRAGMENTS OF FRACTIONATED EPS.

The fractionated EPS were also analysed to identify the possible presence of charged and uncharged EPS. This analysis was carried out on 100 μg/ml EPS solutions.

Table 2.13 Charged and uncharged carbohydrate fragments found in fractionated EPS.

Sample		*Ratios of Fractions*	
		Charged	*Uncharged*
Lc. lactis ssp. *cremoris* LC1	Fraction A	3.0	1.0
	Fraction B	1.7	1.0
Lc. lactis ssp. *cremoris* 0968	Fraction A	1.4	1.0
	Fraction B	4.0	1.0
Lc. lactis ssp. *cremoris* 2006	Fraction A	1.1	1.0
	Fraction B	1.3	1.0
Lc. lactis ssp. *cremoris* 1934	Fraction A	5.3	1.0
	Fraction B	No fraction B isolated	
Lc. lactis ssp. *cremoris* 0967	Fraction A	2.6	1.0
	Fraction B	No fraction B isolated	
Lc. lactis ssp. *cremoris* B40	Fraction A	1.0	3.7
	Fraction B	3.1	1.0

Table 2.13 shows the ratios of charged and uncharged polysaccharides in the fragments isolated by gel permeation chromatography. It can be seen that in EPS from all strains that there was no consistent pattern in the amount of charged and uncharged fractions extracted. Therefore size exclusion chromatography did not separate the EPS into charged and uncharged polysaccharides.

COMPOSITIONAL ANALYSIS OF FRACTIONATED EPS.

Monosaccharide analysis of the hydrolysed fractionated EPS carried out using HPLC on 1000 μg/ml solutions.

Table 2.14. Monosaccharide analysis of fractionated EPS using HPLC.

Sample		*Ratio of Sugars (μg/ml:μg/ml:μg/ml)*		
		Glucose	*Galactose*	*Rhamnose*
Lc. lactis ssp. *cremoris* 1934	Fraction A	1.4	6.0	1.0
	Fraction B		No fraction B isolated	
Lc. lactis ssp. *cremoris* 0967	Fraction A	1.0	3.3	2.2
	Fraction B		No fraction B isolated	
Lc. lactis ssp. *cremoris* 0968	Fraction A	4.0	2.0	1.0
	Fraction B	1.0	2.9	1.4
Lc. lactis ssp. *cremoris* 2006	Fraction A	10.0	6.0	1.0
	Fraction B	1.8	5.0	1.0
Lc. lactis ssp. *cremoris* LC1	Fraction A	6.0	7.0	1.0
	Fraction B	1.0	1.3	6.7
Lc. lactis ssp. *cremoris* B40	Fraction A	10.0	4.0	1.0
	Fraction B	1.3	1.5	1.0

Table 2.14 shows the variation in monosaccharide composition of the different fractions. In fraction A from strains B40, 2006 and 0968 there was more glucose present than galactose and rhamnose. In fraction A of strain 0967, galactose was present in the largest quantities followed by rhamnose then glucose. In fraction A of strains 1934 and LC1 there was more galactose than glucose and rhamnose was present in the smallest quantities. In fraction B of strains B40, 2006 and 0968 galactose was present in the largest quantities followed by glucose then rhamnose. In fraction B of strains LC1 there is more rhamnose than both galactose and glucose. In the EPS from all strains both Fractions A and B have different monosaccharide compositions.

DISCUSSION

Six different strains of *Lc. lactis* ssp. *cremoris* (1934, 0967, 0967, 2006, B40, LC1) and 5 different strains of *Lb. delbrueckii* ssp. *bulgaricus* (2482, 2483, 2772, 2394, Ldb1) were studied. According to the NCIMB catalogue all the strains except *Lb. delbrueckii* ssp. *bulgaricus* 2394 were known to be EPS-producing strains.

As outlined in Chapter 1 the first aim of this study was to isolate as much EPS as possible from all the different strains of both *Lc. lactis* ssp *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. The isolation procedures were undertaken on bacterial cultures grown in reconstituted skimmed milk. This was done using a combination of different methods previously used by Kimmel, Roberts and Ziegler (1998) and Cerning et al. (1992). The objective was to isolate the EPS using as few steps as possible to minimise loss of product and to maintain quality. The different strains of both *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* produced varying quantities of EPS. *Lb. delbrueckii* ssp. *bulgaricus* 2394 produced the least quantity of EPS. This is consistent with this strain not being known as an EPS producer. Strain Ldb1 produced the largest quantities of EPS of the *Lb. delbrueckii* ssp. *bulgaricus* strains. *Lc. lactis* ssp. *cremoris* B40 produced the largest quantities of EPS of the strains of *Lc. lactis* ssp. *cremoris* and strain 1934 the least. These results show that there is variability in the quantities of EPS produced by different strains of lactic acid bacteria when grown in skimmed milk. Variability in the yield of EPS is one of the problems suffered by the dairy industry that could lead to problems in consistency between batches of yoghurt produced by different strains. Strains, Ldb1 and B40 are both strains that are used commercially as starter culture so are likely to have been selected for their EPS producing ability.

The next aim involved the analysis of the isolated crude EPS using a variety of analytical techniques. These included studies into the carbohydrate content, molecular weight and sugar analysis. To increase the purity of the EPS gel exclusion chromatography was used to fractionate the crude EPS.

Overall, crude EPS produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* contained more carbohydrate than strains of *Lc. lactis* ssp. *cremoris*. During the isolation of the EPS it is likely that

not all the impurities were removed as the carbohydrate content of the isolated EPS was in several cases substantially less than 100% for most strains, based on analysis using the phenol sulphuric method for total carbohydrate. The results of this suggest that there is either something remaining associated with the polysaccharide that caused a lower carbohydrate value or that the method of analysis did not give a true value for carbohydrate content. The phenol sulphuric method results are based on a comparison with glucose. EPS are known to contain other sugars which may behave differently during the analysis and this could lead to low purity results.

The EPS produced by strains of *Lb delbrueckii* ssp. *bulgaricus*, except strain 2394, had high carbohydrate content. This is based on the assumption that non-carbohydrate contamination from the medium was present at the same levels in all the crude EPS extracted from skimmed milk. Strain 2394 had a very low yield and low carbohydrate content. The EPS produced by strains of *Lc. lactis* ssp. *cremoris* had very low carbohydrate content. This low purity could also be a result of large quantities of contaminant in relation to the quantity of EPS isolated. Protein analysis showed that EPS from strains *Lb. delbrueckii* ssp. *bulgaricus* contained more protein than the EPS from strains of *Lc. lactis* ssp. *cremoris*. This was therefore the most likely form of contamination of the EPS. In this study skimmed milk powder was used and this would have contained casein. As a result some of the casein from the milk may still be attached to the EPS leading to a large protein content of the EPS. Heertje, Visser and Smits (1985) and Teggatz and Morris (1990) observed that EPS was incorporated within casein micelles. There are obviously interactions between the protein and the polysaccharide and this, together with large size of the EPS molecules, would make it difficult to separate out the protein during the isolation of the EPS. Another possible source of the protein contamination could also be from bacterial intracellular protein that has got caught up in the EPS molecules and could not been removed in the extraction process.

However, protein did not account for all of the non-carbohydrate the contamination found. Other possible forms of contamination could be other cellular components such as parts of the cell wall or DNA and RNA. The EPS molecules themselves are large and this would make is easy for other molecules to get entangled with them during the extraction process. This would also lead to low purity results.

Owing to the lack of available data on purity and purification methods of EPS it is difficult to give definite reasons as to why the purity results are inconclusive. In future it would be useful to carry out a detailed study of purification methods and purity testing methods used in the isolation of EPS produced by lactic acid bacteria.

Once the analysis of purity had been carried out it was then possible to estimate the size of EPS using HPLC (Table 2.7). This showed that EPS produced by strains of *Lc. lactis* ssp. *cremoris* ranged in their molecular weight from 6.46×10^7 to 1.26×10^9g/mol The EPS produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* ranged in molecular weight from $6.46 \times 10^6 - 1.26 \times 10^{11}$g/mol. These sizes were in relation to the dextran standards used for calibration purposes. Separate calibration curves were needed for different molecular species. For strain LC1 there were two peaks that were excluded from the results because they were thought to be too small. These small peaks were probably incomplete polysaccharides that have been damaged in the analysis process. They could also have been media components that had not been removed. Dextrans were found to be relatively heterogeneous in terms of their molecular weight and in retrospect may not have been the best choice of molecular weight standards.

The extra peaks could also be due to more than one EPS being produced by these strains of LAB. Another possibility is that degradation of the EPS took place in the stock solution leading to false low values for the molecular weight. The sizes of the EPS seem to be higher than the published values (Table 2.1). It could well be that the molecular weight of the polysaccharides might have some effect on their functional properties and this could be investigated in a future study.

A study carried out by Marshall, Cowie and Moreton (1995) reported the existence of charged and uncharged EPS in a sample of crude EPS extracted from *Lc. lactis* ssp. *cremoris* LC330. When separated, the low molecular weight EPS was found to have a charge and the high molecular weight EPS was uncharged. In this study all crude polysaccharides extracted were analysed for the presence of charged and uncharged polysaccharides. The crude EPS from both *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* contained both charged and uncharged fragments. In all samples, except strain 0967, there was more uncharged than charged EPS. This seems to agree with what was found by Marshall, Cowie and Moreton (1995).

Sugar analysis of the crude EPS showed that the EPS from all strains contained galactose, glucose and rhamnose in varying ratios. For strains of both *Lb. delbrueckii ssp. bulgaricus* and *Lc. lactis ssp. cremoris* the ratio of sugars was comparable with those found in published work (Table 2.1). It seems that the EPS produced by the different strains of both *Lc. lactis ssp. cremoris* and *Lb. delbrueckii ssp. bulgaricus* had a unique sugar composition and therefore it could be assumed that all the EPS produced by different strains of lactic acid bacteria have their own unique structure. However, this would need to be confirmed using other techniques including NMR-spectroscopy. Laws, Gu and Marshall (2001) reported that when structural analysis of EPS from different strains of lactic acid bacteria was carried out using monomer composition, linkage analysis and NMR analysis the structure could be broken down into a combination of 25 unique structures.

The next stage of the analytical process was to investigate the possibility of purifying the crude EPS using size exclusion low pressure chromatography. It was possible to separate the EPS from all strains, except those from strains 1934 and 0967, into two fractions based on their size. The first fraction extracted was known as fraction A and the second, fraction B. All of the EPS, except those of strain 2006 had more of the larger molecular weight fraction A than frcation B. The carbohydrate and protein content of both fractions for all strains was found to be lower than in the original crude EPS. For all strains, fraction A contained more carbohydrate and protein than fraction B. This could be due to loss of product during the extraction process or a low yield product that contained a large quantity of impurities. These fractions also contained both charged and uncharged polysaccharides. However, considering that between 80- 90% of the fractionated EPS was neither carbohydrate nor protein this suggests that the charges were not related to the EPS and were found on impurities in the EPS. Sugar analysis of the fractions indicated that these EPS were composed of glucose, galactose and rhamnose in varying quantities.

These results show that it is possible to separate the EPS produced by strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* into two fragments and to carry out detailed analysis on their structure. Future work should involve the investigation of the EPS extraction process along with post extraction purification methods. This would allow for better structural analysis of the purified EPS.

CONCLUSION

This particular area of the study showed that it was possible to isolate EPS from strains of *Lb. delbrueckii ssp. bulgaricus* and *Lc. lactis ssp. cremoris*.

More EPS was extracted from strains of *Lb. delbrueckii* ssp. *bulgaricus* than strains of *Lc. lactis* ssp. *cremoris*. EPS from strains of *Lb. delbrueckii* ssp. *bulgaricus* contained more carbohydrate than those from strains of *Lc. lactis* ssp. *cremoris*. Protein analysis of all EPS showed that EPS extracts from strains of *Lb. delbrueckii* ssp. *bulgaricus* contained more protein than those from strains of *Lc. lactis* ssp. *cremoris*.

The EPS from strains of *Lb. delbrueckii* ssp. *bulgaricus* were generally smaller than those of strains of *Lc. lactis* ssp. *cremoris*. EPS from strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* contained both charged and uncharged fragments.

The EPS from strains *Lb. delbrueckii* ssp. *bulgaricus*, LC1, B40 and 2006 contain more galactose than glucose. EPS from strains 1934, 0967 and 0968 contain more glucose than galactose. Rhamnose was found in smallest quantities in all EPS from strains of *Lc. lactis* ssp. *cremoris*, except strain 0967, where it is present in larger quantities than galactose. In the EPS from all strains of *Lb. delbrueckii* ssp. *bulgaricus*, except 2483, there is more galactose than glucose. In strain 2483 rhamnose is found in equal quantities with galactose. In EPS from the other strains rhamnose was found in the smallest quantities.

All EPS, except that of strain 2006, had more fraction A than fraction B when separated using a fractionation column. Fraction A contained more carbohydrate than fraction B. Fractionated EPS also contained less protein than the original crude EPS. From all strains fractions A and B both contain more charged than uncharged EPS. The exception to this was strain B40, fraction A contained more uncharged than charged EPS and fraction B contained more charged than uncharged. The monosaccharide composition of fractionated EPS differed between strains and fractions.

CHAPTER 3. THE PRODUCTION OF EPS BY *Lactococcus lactis* spp. *cremoris*.

INTRODUCTION

Following the isolation and analysis of EPS from different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* the next step was to investigate some of the factors that could affect the quantities of EPS produced e.g. temperature, sugar source and oxygen availability. This section was limited to strains of *Lc. lactis* ssp. *cremoris* as a defined medium that would allow for analysis of EPS production in strains of *Lb. delbrueckii* ssp. *bulgaricus* was not available.

FACTORS AFFECTING PRODUCTION OF EXOPOLYSACCHARIDES IN LACTIC ACID BACTERIA.

EPS production by dairy starter cultures were originally assessed indirectly by visual observations or viscosity measurements. However, in more recent years, methods were developed for quantifying the EPS produced more precisely (Cerning 1995). The quantities of EPS produced in milk by different species and strains vary considerably. The amounts of EPS produced by *Streptococcus thermophilus* spp. *thermophilus* ranges from 50 to 350 mg/l (Garcia-Garibay and Marshall 1991; Cerning 1995). *Lb. delbrueckii* ssp. *bulgaricus* produces between 60 to 150 mg/l (Garcia-Garibay and Marshall 1991; Cerning 1995), and *Lc. lactis* spp. *cremoris* produces between 80 to 600 mg/l (Cerning 1995).

Reconstituted skimmed milk and complex microbial growth media have been used in the study of the production of EPS by lactic acid bacteria. However, quantification of polysaccharide from these media can be difficult due to interference from carbohydrates and polymers contained in media components (Kimmel, Roberts and Ziegler 1998). In semi-defined media, *Lb. delbrueckii* ssp. *bulgaricus* has been shown to produce 354 mg/l polysaccharide (Kimmel, Roberts and Ziegler 1998). When various amino acids were omitted from the semi-defined medium the range in polysaccharide production varied from 32.9 to 55.4 mg/l (Grobben et al. 1998). Kimmel, Roberts

and Ziegler (1998) showed that polysaccharide production could be varied from 28.4 to 504.6 mg/l using MRS broth supplemented with a range of different added components,

Most mucoid bacteria produce EPS under a variety of cultural conditions, but the growth conditions can be optimised to maximise production. Mesophilic strains of lactic acid bacteria grow between 5°C and 40°C but seem to produce maximum amounts of EPS under suboptimal growth conditions. However, in thermophilic strains of lactic acid bacteria, which grow above 40°C, EPS production seems to be growth linked (de Vuyst et al. 1998). In growth associated production, biosynthesis starts simultaneously with growth. It shows an increased rate when the culture is in its exponential phase and reaches a maximum rate when the culture is at the end of exponential growth. This is an indication of primary metabolite growth kinetics (Manca de Nadra et al. 1985; Grobben et al. 1995; de Vuyst et al. 1998). Primary metabolites are produced during the log phase of bacterial growth and are essential for growth. Marshall, Cowie and Moreton (1995) showed that the onset of EPS biosynthesis in a strain of *Lc. lactis* ssp. *cremoris* is observed towards the end of the exponential phase of growth. Other experiments have shown continued EPS production beyond the primary growth stage or only in the stationary phase of growth (Gancel and Novel 1994a; Bouzar, Cerning and Desmazeaud 1996). As EPS is produced during both the initial and later stages of growth, to improve its production, cell growth must also be improved. It has also been found that EPS degradation often takes place after prolonged incubation and this may be due to glycohydrolase activity (Cerning et al. 1986). From the literature available it seems that the factors affecting growth and EPS production seem to vary from strain to strain. Table 3.1 shows some of the factors that have been found to affect EPS production. Owing to the large number of factors that affect EPS production the number to be investigated in this study was reduced, for practical reasons, to temperature, sugar source and oxygen availability, and how the variation of these factors would affect EPS production in strains of *Lc. lactis* ssp. *cremoris*.

Table 3.1 Factors Influencing EPS production in Lactic Acid Bacteria.

Reference	*Bacteria*	*Strain*	*Factors Influencing Growth & EPS production*
Manca de Nadra et al. (1985)	*Lb.bulgaricus*	CRL420	Carbon Source
Cerning (1990)	*Lb.bulgaricus*	CNRZ 416	Media composition
		CNRZ 398	Media composition
	St. thermophilus	CNRZ 404	Media composition
		CNRZ 388	Media composition
Gancel and Novel (1994a)	*St. thermophilus*	S22	Sugar source, temperature, initial pH
Degeest and de Vuyst (2000)	*Lb. sakei*	0-1	Carbon source, temperature
Van den Berg et al. (1995)	*Lb. sakei*	0-1	Temperature, Energy source
Cerning et al. (1994)	*Lb casei*	CG11	Sugar source,
Looijesteijn et al. (2000)	*Lc. cremoris*	B40	Growth rate, Nutrient limitation
Van Niel and Hahn-Hägerdal (1999)	*Lc.lactis*	various	Amino acids, Minerals
	Lc.cremoris	various	Amino acids, Minerals
Torino et al. (2001)	*Lb. helveticus*	ATCC 15807	Environmental pH
Mozzi et al. (1995)	*Lb. acidophilus*	CRL 640	Temperature
Cerning et al. (1992)	*Lc.lactis* ssp.*lactis*	CNRZ 151	Temperature, media composition, sugar source
		CNRZ 156	Temperature, media composition, sugar source
		CNRZ 371	Temperature, media composition, sugar source
	Lc.lactis ssp. cremoris	MLS96	Temperature, media composition, sugar source
		T_5	Temperature, media composition, sugar source
	Lb.casei ssp.casei.	NCIB 4114	Temperature, media composition, sugar source
		CG11	Temperature, media composition, sugar source
Kimmel, Roberts and Ziegler (1998)	*Lb. delbrueckii* ssp. *bulgaricus*	RR	Temperature, pH, media composition
Grobben et al. (1998)	*Lb. delbrueckii* ssp. *bulgaricus*	NCFB 2772	Media composition, Amino acids
Kimmel et al. (1998a)	*Lb. delbrueckii* ssp. *bulgaricus*	RR	Media composition, temperature
Garcia-Garibay and Marshall (1991)	*Lb. delbrueckii* ssp. *bulgaricus*	RR	Temperature, media composition, subculture, growth stimulating factors
Grobben et al. (1995)	*Lb. delbrueckii* ssp. *bulgaricus*	NCFB 2772	Substrate, temperature,
Gassem, Schmidt and Frank (1997)	*Lb. delbrueckii* ssp. *bulgaricus*	RR	Media composition, pH, temperature, incubation time
Mozzi et al. (1996)	*Lb.casei*	CRL87	Temperature, incubation time
Grobben, et al. (1996)	*Lb. delbrueckii* ssp. *bulgaricus*	NCFB 2772	Carbon source,
De Vuyst et al.(1998)	*St. thermophilus*	BTC	Media composition, temperature, pH, O_2 tension, C, N levels
		LY03	Media composition, temperature, pH, O_2 tension, C, N levels
		480	Media composition, temperature, pH, O_2 tension, C, N levels
		Sfi20	Media composition, temperature, pH, O_2 tension, C, N levels
Torino et al. (2001)	*Lb. helveticus*	ATCC 15807	pH

METHODS

MICROORGANISMS

The details of strains of *Lc lactis* ssp. *cremoris* used can be found in the Methods section of Chapter 2. Strain B40 was not included in this section as this work was completed prior to this strain being obtained.

MEDIA COMPOSITION

M17 broth/agar

M17 medium was prepared by dissolving 37.25 g M17 powder in distilled water (950 ml). This was then autoclaved at 121°C for 15 minutes. Lactose (5.0 g) was dissolved in water (50 ml) and also autoclaved at 121°C for 15 minutes. The lactose was added aseptically to the medium after autoclaving.

Milk Indicator Agar (MIA) plates

Agar plates were prepared containing skimmed milk, bromocresol purple and either M17 or MRS agar depending on the species of bacterium used. M17 was used for the growth of *Lc. lactis* ssp. *cremoris.*

Equal volumes of the two solutions were made up (Table 3.2) and autoclaved at 110°C for 15 min and then mixed together before the plates were poured. These plates were designed as part of the study to allow identification of potential EPS producing strains of *Lc. lactis* ssp. *cremoris.*

Cremoris Defined Medium (CDM)

To allow manipulation of the composition of the medium and easier determination of EPS concentration in the medium the bacteria were grown in Cremoris Defined Medium (CDM). CDM was prepared as shown in Table 3.3 and was filter sterilised (Otto, Ten brink and Konings 1983).

Table 3.2 Composition of Cremoris Defined Medium (CDM)

MEDIUM COMPOSITION			
Component	**Amount/Litre**	**Component**	**Amount/Litre**
K_2HPO_4	2.5 g	casamino acids	5.0 g
KH_2PO_4	3.0 g	L-cysteine.HCL	0.25 g
$(NH_4)_3$ citrate	0.6 g	vitamin solution	10 ml
Na acetate.$3H_2O$	1.0 g	metal solution	10 ml
lactose	2.5 g	nucleic acid solution	10 ml
VITAMIN SOLN.			
Component	**Amount/Litre**	**Component**	**Amount/Litre**
pyridoxine HCL	200 mg	vitamin B_{12}	10 mg
nicotinic acid	100 mg	orotic acid	500 mg
thiamine HCL	100 mg	thymidine	500 mg
riboflavin	100 mg	inosine	500 mg
Ca (D+) pantothenate	100 mg	thioctic acid	250 mg
Na-p-aminobenzoate	1.0 g	pyridoxamine. HCL	500 mg
D-biotin	1.0 g	folic acid	100 mg
METAL SOLN.		**NUCLEIC ACID SOLN.**	In 10 ml 0.1N NaOH
Component	**Amount/Litre**	**Component**	**Amount/Litre**
$MgCl_2.6H_2O$	20 g	adenine	10 mg
$CaCl_2.2H_2O$	5.0 g	uracil	10 mg
$FeCl_2.4H_2O$	0.5 g	sodium xanthine	11.52 mg
$ZnSO_4.7H_2O$	0.5 g	guanine	10 mg
$CoCl_2.6H_2O$	0.25 g		

MICROBIOLOGICAL EXAMINATION

For methods regarding microbiological examination see Methods section of Chapter 2.

ISOLATION OF INDIVIDUAL COLONIES OF *Lactococcus cremoris*

Strains were grown in liquid M17 at 30°C. A loopful of culture was then spread onto MIA plates and incubated at 30°C. Individual colonies were selected and transferred onto an individual square of a grid plate of MIA agar. These were incubated at 30°C. The individual colonies were then subcultured in M17 broth and placed onto storage beads.

ANALYSIS OF GROWTH FACTORS EFFECTING EPS PRODUCTION IN STRAINS OF *Lc. lactis* ssp. *cremoris*.

Effect of substrate on EPS production in *Lc. lactis* ssp. *cremoris*.

CDM was prepared using either glucose, fructose, sucrose or lactose as the substrate. Strains of *Lc. lactis* ssp. *cremoris* were grown in the medium at 30°C in an orbital-shaking incubator for 12 hours to pH 4. Optical density readings were taken at 500 nm as an indicator of final levels of growth. Samples of cultures (10 ml) were centrifuged (4000 rpm for 25 min) to remove cells. The supernatant was then dialysed and total carbohydrate content determined (See Methods Chapter 2).

Calibration graph for carbohydrate determination can be found in Appendix 1.7

Effect of incubation temperature on EPS production in *Lc. lactis* ssp. *cremoris*.

Strains of *Lc. lactis* ssp. *cremoris* were grown in CDM containing lactose in orbital-shaking incubators for 12 hours at 25, 30, 35, and 40°C to pH 4. Optical density readings were taken at 500 nm as an indicator of final level of growth. After incubation, 10 ml of cultures were centrifuged (4000 rpm for 25 min) to remove cells. The supernatant was then dialysed and total carbohydrate content determined (See Methods Chapter 2).

Calibration graph for carbohydrate determination can be found in Appendix 1.8

Effect of shaking on EPS production in *Lc. lactis* ssp. *cremoris*.

Strains of *Lc. lactis* ssp. *cremoris* were grown in CDM containing lactose in an orbital-shaking incubator at 30°C and in a static incubator at 30°C for 12 hours to pH 4. Optical density readings were taken at 500 nm as an indicator of final level of growth. After incubation 10 ml of cultures were centrifuged (4000 rpm for 25 min) to remove cells. The supernatant was then dialysed and the total carbohydrate content determined (See Methods Chapter 2).

Calibration graph for carbohydrate determination can be found in Appendix 1.9

EPS production by individual colonies of *Lc. lactis* ssp. *cremoris*.

Beads containing the individual cultures from MIA plates were grown in 10 ml of CDM at 30°C for 12 hours to pH4. After incubation 10 ml were centrifuged (4000 rpm for 25 min) to remove cells. The supernatant was then dialysed and total carbohydrate content determined (See Methods Chapter 2).

RESULTS

EPS PRODUCTION IN STRAINS OF *Lc. lactis* ssp. *cremoris*

Lactose is the naturally occurring sugar in milk and quantities of EPS produced when strains of *Lc. lactis* ssp. *cremoris* were grown in a defined media at 30°C with lactose as the major sugar were measured for comparison with that produced in milk as shown in Table 2.4.

Table 3.3 Average quantities of EPS produced by strains of* Lc. lactis *ssp.* cremoris *in CDM.

Strain	Concentration of EPS (mg/l) from CDM	Concentration of EPS (mg/l) From skimmed milk
1934	2.9	84
0967	2.3	144
0968	4.3	151
2006	25.7	203
LC1	34.8	67

Table 3.3 shows the average quantities of EPS produced by each of the strains of *Lc. lactis* ssp. *cremoris* when grown in CDM or skimmed milk. The information for skimmed milk was taken from Table 2.4. From this it can be seen that the strains differ in the amount of EPS they produce in CDM. It seems that strains 2006 and LC1 produce larger quantities of EPS compared to strains 0967, 0968 and 1934 when grown in CDM. Overall, these results show that there is a difference in quantities of EPS produced between the different strains of *Lc. lactis* ssp. *cremoris* when grown in CDM and that the quantity of EPS produced is substantially less than that produced when the strains are grown in skimmed milk.

THE EFFECT OF TEMPERATURE ON GROWTH AND EPS PRODUCTION IN *Lc. lactis* ssp. *cremoris.*

Following the determination of base line values for the EPS production in CDM, the next stage was to investigate the effect of growth temperature on quantities of EPS produced by strains of *Lc. lactis* ssp. *cremoris*. The organisms were grown in CDM at 25, 30, 35 and 40°C to pH 4. The carbon source in the CDM was lactose. The bacteria were then removed by centrifugation and the CDM dialysed to remove media components. The carbohydrate content of the resultant mix was determined.

Table 3.4 The final absorbencies of strains of* Lc. lactis *ssp.* cremoris *grown for 12 hours in CDM at different temperature as an indication of growth.

Temperature	Absorbence @ 500 nm				
°C	1934	0967	0968	2006	LC1
25	1.21	0.99	1.07	1.09	1.52
30	1.26	1.30	1.37	1.30	1.52
35	1.00	No growth	1.49	1.52	1.30
40	1.11	No growth	1.40	1.51	1.40

Table 3.4 shows the final absorbencies of strains of *Lc. lactis* ssp. *cremoris* when grown in CDM. This is based on an average of two readings. It can be seen that at the end of the incubation the absorbencies were similar indicating that all cultures had reached the same stage of their growth phase. This indicates that any differences in EPS production are not because some of the samples had fewer bacteria than other samples. Strain 0967 did not grow at 35 and 40°C.

Figure 3.1 shows the effect of temperature on EPS production in strains of *Lc. lactis* ssp. *cremoris*. In general lower temperatures stimulated increased EPS production in all strains. Strains LC1 and 2006 both produced increased quantities of EPS at 25 and 30°C. Production in all strains except 0968 was decreased at 35 and 40°C but was still high in comparison to the other strains. Strain 0968 shows maximum production at 25°C and relatively little at 35 and 40°C. Strains 0967 and 1934 showed similar production at 25 and 30°C, the most being produced at 25°C. In strain 0967, no EPS was produced at 35 and 40°C because the bacteria did not grow at these temperatures.

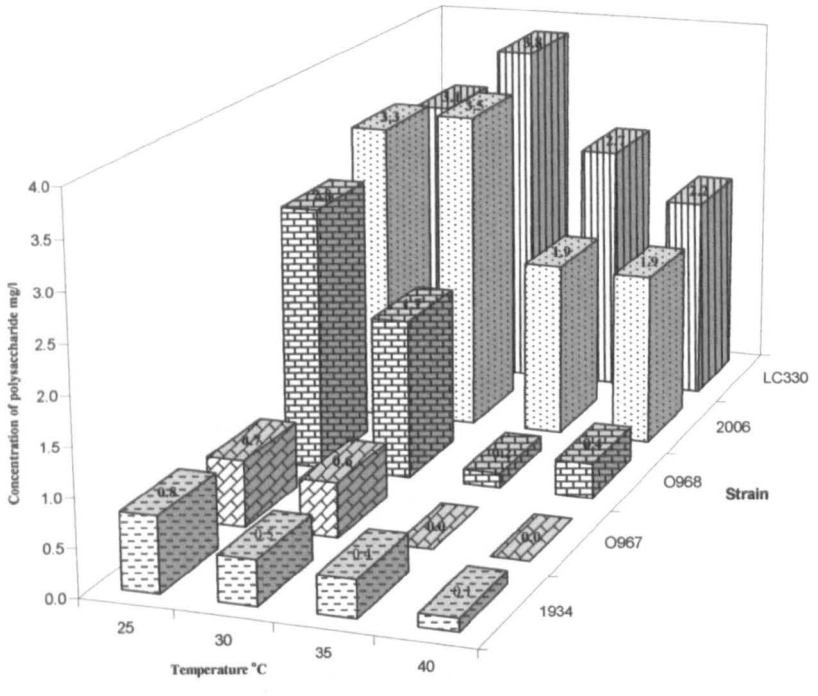


***Figure 3.1 The effect of temperature on the quantity of EPS produced by strains of* Lc. lactis *spp.* cremoris.**

THE EFFECT OF CARBON SOURCE ON GROWTH AND EPS PRODUCTION IN *Lc. lactis* ssp. *cremoris.*

As lactose is the naturally occurring sugar in milk an investigation was carried out into how varying the carbon source in CDM had an effect on the amount of EPS produced by different strains of *Lc. lactis* ssp. *cremoris*.

Table 3.5 The final absorbencies of strains of* Lc. lactis *ssp.* cremoris *grown in CDM for 12 hours at 30°C, with different carbon source as an indication of growth.

	Absorbence @ 500 nm				
	1934	**0967**	**0968**	**2006**	**LC1**
Glucose	1.19	1.53	1.54	1.47	1.48
Lactose	1.40	1.29	1.27	1.23	1.3
Fructose	1.32	1.18	1.16	1.10	1.58
Sucrose	1.44	No growth	No growth	1.35	1.41

Table 3.5 shows an average of two duplicates of absorbency readings taken when strains of *Lc. lactis* ssp *cremoris* were grown in CDM with different carbon sources. This is based on an average of two readings. It can be seen that at the end of the incubation the absorbencies were

similar indicating that all cultures had reached the same stage of their growth phase. This indicates that any differences in EPS production are not because some of the samples had fewer bacteria than other samples. Strains 0967 and 0967 did not grow in CDM containing sucrose so there would not have been any EPS production in these samples.

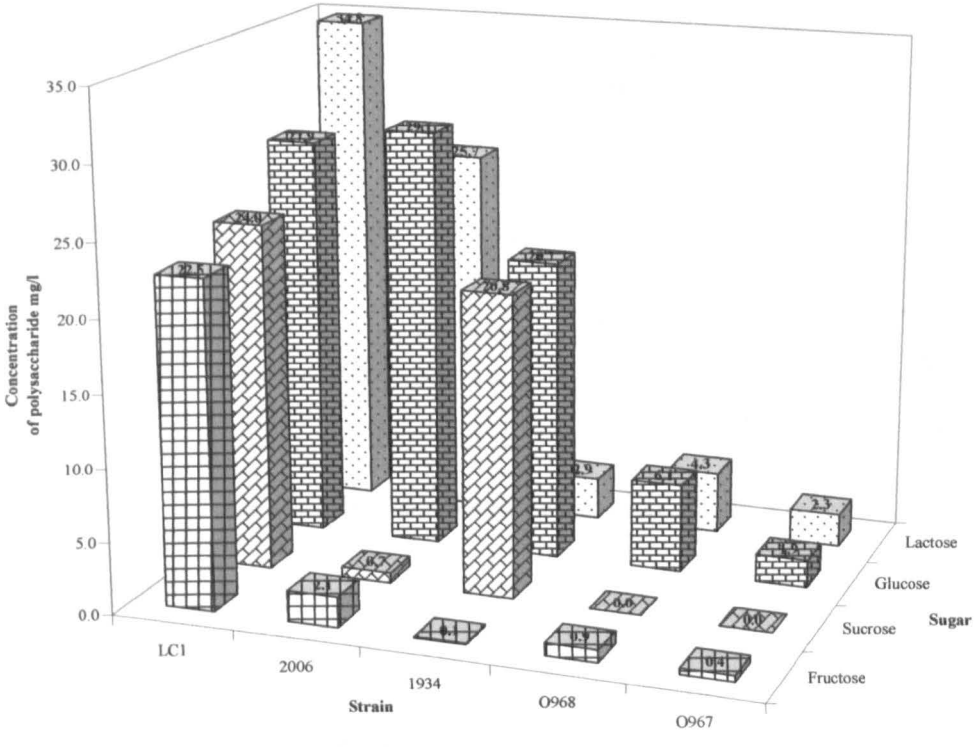


***Figure 3.2 The effect of carbon source on the quantity of EPS produced by strains of* Lc. lactis *ssp.* cremoris.**

Figure 3.2 shows the effect of the carbon source on EPS production in different strains of *Lc. lactis* ssp. *cremoris*. From the graph it can be seen that carbon source influenced EPS production in the different strains. Strain LC1 produced the largest quantities of EPS with all substrates, except glucose where 2006 produced slightly more. LC1 produced most EPS in media containing lactose then, glucose and sucrose with media containing fructose causing least production. For strain 2006, lactose and glucose supported the production of the largest quantities of EPS with sucrose and fructose hardly supporting any EPS production. In strain 1934, glucose and sucrose stimulated the most EPS production. Strains 0967 and 0968 did not grow in media containing sucrose so no EPS

was produced. Fructose, glucose and lactose only supported minimal EPS production in these strains.

THE EFFECT OF SHAKING ON GROWTH AND EPS PRODUCTION IN *Lc. lactis* ssp. *cremoris*.

Following the determination of the effect of sugar source and temperature on the quantities of EPS produced, the next stage was to investigate effects of agitation on the production of EPS in different strains of *Lc. lactis* ssp. *cremoris*.

Table 3.6 The final absorbencies of strains of* Lc. lactis *ssp.* cremoris grown in CDM for 12 hours at 30°C, *grown in static or shaken conditions as an indication of growth.

	Absorbance @ 500 nm				
	1934	**0967**	**0968**	**2006**	**LC1**
Static	1.62	1.67	1.75	1.74	1.87
Shaken	1.27	1.22	1.03	1.26	1.10

From Table 3.6 it can be seen that when the samples were shaken there was reduced bacterial growth.

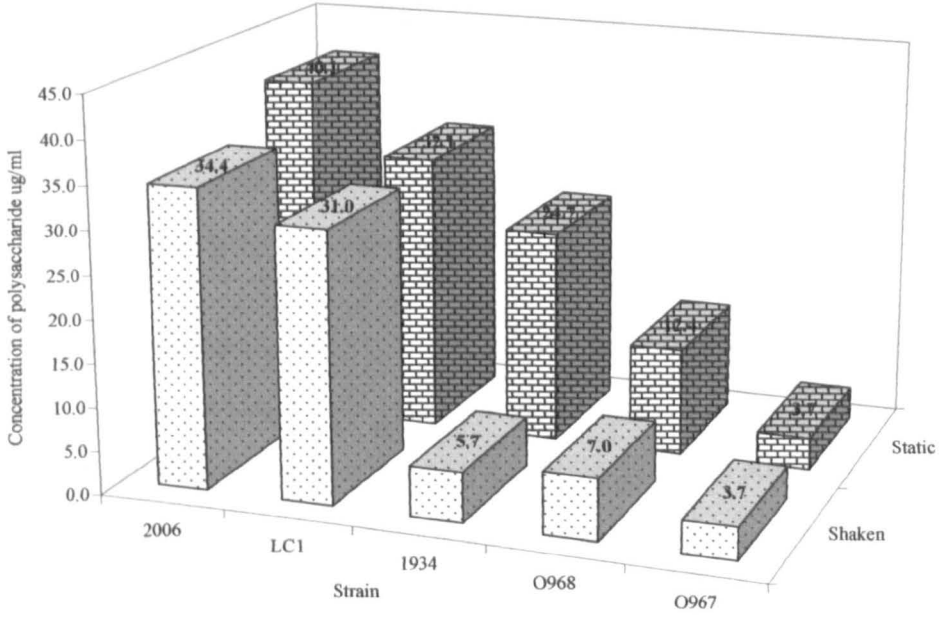


***Figure 3.3. The effect of shaking on EPS production in strains of* Lc. lactis ssp. cremoris.**

Figure 3.3 shows how shaking affected EPS production in strains of *Lc. lactis* ssp. *cremoris*. From this graph it can be seen that shaking seems to have had an effect on EPS production in strains of *Lc. lactis* ssp. *cremoris*. However, when considering the fact that the static samples did not grow as well as the shaken cultures this would suggest that the differences in EPS production were probably due to reduced growth rather than whether the samples were shaken or not. Strains 2006 and LC1 both produced large quantities of EPS under both sets of conditions. In strain 1934, shaking caused a major drop in EPS production. In strain 0968, a small reduction in EPS can be seen when the sample was shaken. EPS production in strain 0967 was relatively unaffected by agitation.

EPS PRODUCTION IN INDIVIDUAL ISOLATES OF *Lc. lactis* ssp. *cremoris*.

The aim of these experiments was to isolate individual colonies of each of the strains and investigate how the quantity of the EPS produced differed within the individual strains of bacteria.

Table 3.7 Production of EPS by individual isolates of* Lc. lactis *ssp.* cremoris *grown in CDM.

	Mean concentration of EPS (mg/l)				
Colony	**1934**	**2006**	**0967**	**0968**	**LC1**
1	10.7	8.3	4.4	0.0	28.8
2	17.9	3.0	10 .0	17.6	47.5
3	12.4	14.3	9.6	7.2	45.8
4	7.7	6.3	11.4	33.9	19.6
5	8.1	9.2	15.5	31.1	34.9
6	8.9	12.7	15.5	9.7	30.2
Mean	8.1 - 17.9	3.0 - 14.3	4.4 - 10.0	0.0 - 33.9	19.6 - 47.5

Figure 3.7 shows the variation in EPS production in individual colonies of the different strains based on the average of two sets of data. For all the strains there is variation in EPS production between colonies. Overall colonies from strain LC1 produced more EPS than colonies from the other strains. Some colonies from strain 0968 also produced larger quantities of EPS than colonies from other strains. Within strain 0968 there was the greatest variation in the amount of EPS produced by the colonies. Colonies from strains 1934, 2006 and 0967 produced similar quantities of EPS. However some of the individual colonies from strains LC1, 0967, 0968 and 1934

produced larger quantities of EPS than that of the original culture from which they were isolated (Table 3.4). The individual colonies of 2006 did not produce more EPS than the original culture.

DISCUSSION

Strains of *Lc. lactis* ssp. *cremoris* are used in the dairy industry in a number of areas due to their ability to produce EPS. This has been supported by the work described in Chapter 2, where it was found that different strains of *Lc. lactis* ssp. *cremoris and Lb. delbrueckii ssp. bulgaricus* produce different quantities of EPS when grown in milk. The next area of study was to investigate how some of the factors that are known to effect EPS production in some strains of lactic acid bacteria (Table 3.1) would effect EPS production in the strains used in this study. A defined medium that supported the growth of all the strains of *Lc. lactis* ssp. *cremoris* was used for detailed studies of EPS production. The components of the defined media could be removed by dialysis leaving only the EPS left in solution.

It was found that growth temperature, carbon source and oxygenation all affected EPS production in strains of *Lc. lactis* ssp. *cremoris*. Strains 0968, 0967 and 1934 had increased EPS production at lower temperatures and, at elevated temperatures EPS production was decreased. The lower temperatures at which the bacteria produced EPS were at the bottom of the growth range for mesophilic bacteria. The EPS production could be produced as a stress response to the unfavourable growth conditions. This links in with the idea that EPS is produced in unfavourable growth conditions as was commented on by de Vuyst et al. 1998. For strains 2006 and LC1, EPS production was high at all temperatures and only slightly decreased at elevated temperatures. This is because EPS is primarily synthesised during the primary stages of growth (Manca de Nadra et al. 1985; Grobben et al. 1995; deVuyst et al. 1998) when the enzymes involved in synthesis are working at their optimum. Above specific temperatures, for that strain, EPS production decreases and this is most likely because enzymes have been denatured.

It was also found that growth substrate had an effect on EPS production. The effect was greatest on strain LC1. Lactose and glucose stimulated the largest quantities of EPS production and sucrose and fructose stimulated low quantities of EPS production. Lactose is found naturally in milk and

this could be the reason as to why it was the most effective carbon source for EPS production. As suggested by Petry et al. (2003) the composition of milk plays and essential role in how well EPS forms textured milk. This is most likely the reason for why lactose is better than sucrose and fructose at stimulating EPS production because it is the sugar found in milk. As seen in Table 2.9 glucose is the main constituent of the EPS produced by all strains of *Lc. lactis* ssp. *cremoris*, except 0967. The bacteria may find it more energy efficient to incorporate glucose directly from the medium into the EPS rather than breakdown the other sugars into glucose for incorporation into the EPS. In future studies it may be useful to study the sugar composition of the EPS of the same bacteria grown in different compositions of media.

Shaking had no effect on EPS production for strains LC1 and 2006 but did have some effect on strains 1934, 0968 and 0967. The presence of oxygen had a negative affect on EPS production. This could be because *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* did not grow well in aerobic conditions (Table 3.6). Manca de Nadra et al. (1985); Grobben et al. (1995) and deVuyst et al. (1998) reported that EPS production took place in the initial stages of growth and therefore EPS is somehow essential for bacterial growth or survival. Any factor that affects the growth of bacteria will also affect EPS production by the bacteria.

When compared with the quantities of EPS produced by the same strains when grown in milk (Table 3.3) it can be seen that all the strains produce less EPS when grown in CDM than when grown in milk. When grown in defined medium *Lc. lactis* ssp. *cremoris* strains LC1 and 2006 produce more EPS than the other strains, 0967, 0968 and 1934. This variability between strains has also been found in a number of other studies (Table 3.1). The environment of the milk is more conducive to EPS production than the defined media which has been developed to provide the minimum growth requirements. This was also confirmed by Petry et al. (2003) who have stated that the composition of milk may modify the physicochemical characteristics of the EPS and this would have an effect on their thickening properties. The differences in the quantities of EPS produced by different colonies within a strain could be one of the primary reasons for the inconsistency problems in the dairy industry. This intra-strain variability suggests that the problem is not only related to the growth of the bacteria but to the level at which the EPS is produced by the

bacteria. This would need to be investigated further by large scale studies on intra-strain variability. The present set of results could not be statistically analysed as there was insufficient data.

The results of this study confirm what has been found previously, see Table 3.1, in that, temperature, growth substrate and oxygen levels, had an effect on EPS production. These factors also have an effect on bacterial growth and each strain has its own optimal levels for these growth factors and these will therefore have some effect on EPS production. However, the experiments on intra-strain variability, suggested that something other than growth is having an effect on the levels of EPS production by the bacteria. This could be something to do with the levels to which the genes related to EPS production are expressed by the bacteria. One of the possible ways of assisting in the optimisation of EPS production by strains used in the dairy industry would be to optimise the growth conditions of the bacteria used. This may help to alleviate some of the problems related to inconsistent texture between batches of yoghurt. However, Petry et al. (2003) and Marshall and Rawson (1999) have suggested that quantity of EPS does not have an effect on the thickening ability of EPS. It could be that the ability of the bacteria to produce EPS, that produces a good texture of yoghurt, is a combination of a number of factors including the growth of the bacteria. As mentioned earlier the optimisation of the growth factors will not solve all of these inconsistency problems other aspects of EPS production needed to be investigated The genetics of EPS production are investigated in the next chapter.

CONCLUSIONS

The amount of EPS produced varies greatly between strains of *Lc. lactis* ssp. *cremoris* when grown in CDM. Strains LC1 and 2006 produce more EPS than the other strains, 0967, 0968 and 1934.

Generally lower temperatures favoured EPS production. Strains LC1 and 2006 produced increased quantities of EPS at 25 and 30°C. Production was decreased at 35 and 40°C but was higher than the other strains. Strain 0968 showed maximum production at 25°C and little at 35 and 40°C. Strains 0967 and 1934 showed similar production at 25 and 30°C, the most being produced at 25°C. In strain 0967, no EPS was produced at 35 and 40°C because the bacteria did not grow.

Strain LC1 produced largest quantities of EPS with all substrates as carbon sources. LC1 produced most EPS in media containing lactose then, glucose and sucrose, with least in media containing fructose. For strain 2006, lactose and glucose support the production of the largest quantities of EPS and sucrose and fructose produced least EPS. In strain 1934 glucose and sucrose produced most EPS production. Strains 0967 and 0968 did not grow in media containing sucrose so no EPS was produced. Fructose, glucose and lactose only supported minimal EPS production. Agitation did not affect the production of EPS for strains LC1, 2006 and 0967 but did reduce production of EPS in strains 1934 and 0968.

EPS production varied between the individual colonies for each strain of *Lc. lactis* ssp. *cremoris*. Colonies from strain LC1 seem to produce more EPS than colonies from the other strains. Some colonies from strain 0968 also produced larger quantities of EPS than colonies from other strains. Colonies from strains 1934, 2006 and 0967 produced similar quantities of EPS. Some of the individual colonies from strains LC1, 0967, 0968 and 1934 produced larger quantities of EPS than that of the parent strain. The individual colonies of 2006 did not produce more EPS than the parent strain.

A number of factors affected the growth of strains of *Lc.lactis* ssp. *cremoris* and these factors had some effect on the quantity of EPS produced.

Chapter 4. THE ROLE OF PLASMIDS IN THE PRODUCTION OF EXOPOLYSACCHARIDES (EPS) PRODUCED BY STRAINS OF *Lactococcus lactis* ssp. *cremoris.*

INTRODUCTION

Loss of EPS producing ability

The EPS secreted by lactic acid bacteria contributes to the texture, mouth-feel and stability of fermented milk products. However it has been found that the ability to produce a thickened milk product is unstable and as a result the texture, in particular the viscosity of the final product can vary between fermentation batches. A number of studies have reported loss of, or reduction in production, or a change in EPS composition between batches (Gancel and Novel, 1994a; Bouzar, Cerning and Desmazeaud 1996). The instability has been observed both in strains containing plasmids encoding genes for EPS production (Cerning 1990) and for strains of *Lb. delbrueckii* ssp. *bulgaricus*, which do not contain EPS producing plasmids (Gancel and Novel, 1994b).

From Chapter 3 it could be seen that the growth of the bacteria was not the only factor that affected the quantity of EPS produced by strains *Lc. lactis* ssp. *cremoris.* Work by van Krannenberg et al. (1997) found that *Lc. lactis* ssp. *cremoris* B40 possessed a plasmid 40 kb in size that contained the genes responsible for EPS production. It was decided to investigate whether the strains of *Lc. lactis* ssp. *cremoris* used in this study also possessed a 40kb plasmid and if so whether the loss of this plasmid would cause them to lose their ability to produce EPS. It was not possible to carry out work on strains of *Lb. delbrueckii* ssp. *bulgaricus* as they rarely contain plasmids (Pouwels and Leer 1993).

Bacterial Plasmids

As mentioned in Chapter 1, the EPS producing genes in certain strains of lactic acid bacteria are believed to be found on plasmids (van Kranenburg et al. 1997, Stingele, Nesser, and Mollet 1996.). Plasmids are sections of circular or linear DNA found in most bacteria they vary in size from a few thousand to hundreds of thousands of base pairs (close to the size of a bacterial chromosome). A cell can harbour two or more different types of plasmid, with hundreds of copies of some plasmid

types and only one or a few copies of other types. The number of a particular plasmid will depend on the genes contained on the plasmid. The higher the copy number the more efficient the plasmid is at replicating itself.

Like chromosomes, plasmids encode for proteins via mRNA molecules and replicate as the cell grows and the replicated copies are usually distributed into each daughter cell when the cell divides. However, unlike chromosomes, plasmids generally do not encode functions essential to bacterial growth. Instead, they provide gene products that can benefit the bacteria under certain circumstances but are not always essential.

Depending on their size, plasmids can encode a few or hundreds of different proteins. Gene products encoded by plasmids include enzymes for the utilization of unusual carbon sources such as toluene (Franklin et al. 1981), resistance to substances such as heavy metals (Schmidt and Schlegel 1989) and antibiotics (Lee and Edlin 1985), synthesis of antibiotics (Neal and Chater 1987), synthesis of toxins (Maeda, Shimada, and Takagi 1978) and proteins that allow the infection of higher organisms (Hooykaas et al. 1985) (Table 4.1).

Table 4.1 Some naturally occurring plasmids and the traits they carry.

Plasmid	Trait	Original Source
ColE1	Bacteriocin which kills *E.coli.*	*Escherichia coli*
Tol	Degradation of toluene and benzoic acid.	*Pseudomonas putida*
Ti	Tumour initiation in plants.	*Agrobacterium tumefaciens*
Sym	Nodulation on roots of legume plants.	*Rhizobium meliloti*
SCP1	Antibiotic methylenomycin biosynthesis.	*Streptomyces coelicolor*

Most plasmids are circular double stranded sealed molecules with no free ends, known as covalently closed circular (CCC) structure. This structure prevents the strands from separating. A CCC plasmid can coil up on itself and this gives rise to a supercoiled structure. If one of the strands gets nicked the plasmid uncoils and becomes what is known as relaxed. Finally if both strands are nicked the plasmid becomes linear (Figure 4.1).

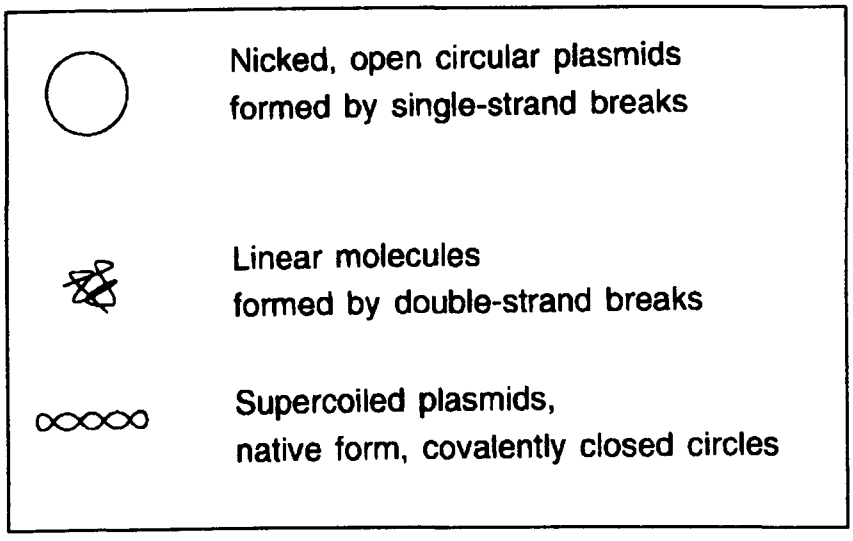


Figure 4.1 Different types of plasmid (Dale and von Schantz 2003).

Separation of plasmids by electrophoresis

Gel electrophoresis is a technique that can be used for the analysis of charged biological molecules such as protein and DNA. When a charged molecule is placed in an electric field, it will migrate towards the electrode with the opposite charge; nucleic acid molecules are negatively charged so they will move towards the positive pole (anode). In a gel, which consists of a complex network of pores, the rate at which a nucleic acid molecule moves will be determined by its ability to penetrate through this network. For linear fragments of double-stranded DNA within a certain size range, this will reflect the size of the molecule. (i.e. the length of the DNA). The larger the DNA the slower it will move through the gel (Dale and von Schantz 2003).

The effective size range that can be separated by a gel is determined by its composition. Agarose gels are used for separating nucleic acid molecules greater than a few hundred base pairs. The agarose concentration can be adjusted to obtain effective separation of larger fragments, or increasing it for small fragments. For even smaller molecules, down to only a few tens of base pairs, polyacrylamide gels would be used (Reed et al. 2003).

Agarose gel electrophoresis can be used for analysing the composition and quality of a nucleic acid sample. In particular, it is useful for determining the size of DNA fragments from a restriction enzyme digest or the products of a PCR reaction. For this purpose it is necessary to

calibrate the gel by running a standard marker containing fragments of known sizes. From the calibration graph the size of unknown fragments can be determined. This is assuming they are linear double-stranded DNA, which restricted DNA and PCR products will be (Reed et al. 2003).

The reason for the non-linearity of the calibration curve with larger DNA molecules is that these molecules move through the gel in a different way. Although these molecules are large, they are also very thin, and they can in effect slither through the gel. It takes some time for them to become lined up, but once they are, then the rate at which they move is independent of their size. So for a particular gel, all molecules above a certain size will have virtually the same mobility. Polyacrylamide gel electrophoresis offers a much sharper size-separation of nucleic acid molecules, down to the separation of fragments that only differ in size by one single base (Dale and von Schantz 2003).

As mentioned earlier, not all DNA molecules are linear. Depending on their form plasmids will behave differently during electrophoresis. All three forms of plasmid will be the same size (in terms of the number of base pairs), but they will move differently in a gel, with the open circular form moving more slowly than either the linear or the supercoiled DNA. The relative mobility of the last two forms is more difficult to predict, and will depend on the size and the conditions of electrophoresis. It is quite normal for a purified plasmid preparation to show two or three bands in a gel; it does not necessarily mean that there is more than one plasmid (Dale and von Schantz 2003).

METHODS

MICROORGANISMS

The details of strains of *Lc lactis* ssp. *cremoris* used can be found in the Methods section of Chapter 2.

MEDIA COMPOSITION

Milk Indicator Agar (MIA) plates

Agar plates were prepared containing skimmed milk, bromocresol purple and M17 agar. Equal volumes of the two solutions were made up (Table 4.2) and autoclaved at 110°C for 15 minutes and then mixed together before the plates were poured. The M17 provide nutrients for growth and the milk is used to encourage EPS production.

Table 4.2 Composition of MIA Agar

Solution A		**Solution B**	
Component	**Quantity (g/l)**	**Component**	**Quantity (g/l)**
M17 Agar	97	Skimmed milk	97
Bromocresol purple	0.1		

Peptonised skimmed milk medium

Peptonised skimmed milk powder was made up to 10% (w/v) in distilled water. This was then autoclaved at 110°C for 15 minutes. This medium was used then aseptically inoculated with acriflavin for loss of plasmid experiments.

TESTING FOR EPS-PRODUCING ABILITY (Toba et al. 1990)

Loopfuls of strains to be tested were spread onto the agar plates. These were then incubated overnight. Touching the colonies on the agar with a wire loop was used to identify EPS-producing ability. If colonies, when touched with a wire loop, came away from the agar plate they were designated as EPS producing. However, if the colonies did not come away form the plate when touched with a wire loop they were designated as non EPS producing.

EXTRACTION OF PLASMIDS AND EPS FROM INDIVIDUAL COLONIES OF *Lc. lactis* ssp. *cremoris*

Initial stock cultures of bacteria were created by placing a bead containing the bacteria into bottles containing 10 ml M17 liquid agar. The bottles were incubated for 12 hours at 30°C. A loopful of the stock culture was then spread onto an MIA plate. The MIA plates were incubated at 30°C for 48 hours. Six colonies for each strain were randomly chosen and half of each colony was inoculated into six separate 10 ml volumes of M17 broth, grown for 12 hours at 30°C, for plasmid analysis. The remainder of each of the colonies was used to inoculate 500 ml volumes of 10% skimmed milk. The skimmed milk was incubated at 30°C for 12 hours and the EPS was extracted using the method described in Chapter 2 (p 49).

PLASMID EXTRACTION

Part of the study was to investigate and try and identify the presence of a 40 kb plasmid that could contain the EPS producing genes in different strains of *Lc. lactis* ssp. *cremoris*. A number of different methods for plasmid isolation were investigated but the one used in the study was that used by Anderson & McKay, 1983.

Plasmid extraction from strains of *Lc. lactis* ssp. *cremoris* (Anderson and McKay 1983).

A 1 ml volume of overnight culture was placed in a 1.5 ml sterile Eppendorf tube and centrifuged for 60 seconds at 15000 rpm. After careful removal of the supernatant the bacterial pellet was resuspended in 379 µl of 6.7% Sucrose-50 mM Tris-1 mM EDTA, pH8 using a vortex mixture. This was then warmed to 37°C. 97 µl of lysozyme (10mg/ml in 25 mM Tris, pH8) was added to the Eppendorf and this was incubated for 5 min at 37°C. Then 49 µl of 0.25 M EDTA-50 mM Tris, pH 8 and 28 µl of sodium dodecyl sulphate (20% w/v in 50 mM Tris-20 mM EDTA pH8) were added and the tube mixed immediately using a vortex mixer. The tube was then incubated for 5 - 10 min at 37°C to complete lysis. 28 µl of 3 M NaOH was added and the tube mixed by inversion for 10 min. Next 50 µl of 2.0 M Tris-hydrochloride, pH7 was added and the tube mixed by

inversion for a further 3 min. 72 µl of 5 M NaCl and 700 µl of phenol saturated with 3% NaCl were added and mixed by inversion. The tube was then centrifuged for 5 min at 5000 rpm.

The upper phase was removed using a sterile pipette tip and placed in a clean Eppendorf tube. This was then mixed, using a vortex mixer with 700 µl of chloroform-isoamylalcohol (24:1). The upper phase was again removed using a sterile pipette tip and placed in a clean Eppendorf tube. 700 µl of isopropanol was added to the tube. This was then incubated at 0°C for 30 min. The tube was then centrifuged at 8000 rpm for 5 min to produce a pellet containing the plasmids. The isopropanol was removed and the pellet resuspended in 20 µl of 10mM Tris-1mM EDTA, pH 7.5. The samples were then analysed by electrophoresis gel.

Agarose gel electrophoresis.

Gels contained 0.6 % agarose with 0.5 µg/ml ethidium bromide. Electrophoresis was performed at 80 V for 5 hours in TAE buffer. Gels were photographed using a Polaroid Gel Camera system.

Induction of loss of EPS producing ability by strains of *Lc. lactis* ssp. *cremoris* using subculturing at elevated temperature.

The experiment was carried out using *Lc. lactis* ssp. *cremoris* 2006 and B40.

Initial stock cultures of the bacteria were created by placing a bead containing the bacteria into bottles containing 10 ml M17 liquid agar. The bottles were incubated for 12 hours at 30°C. A loopful of the stock culture was then used to inoculate 10 ml of 10 % skimmed milk (known as subculture 1) and this was then incubated for 12 hours at 40°C. A loopful of subculture 1 was removed and used to inoculate a fresh 10 ml of 10 % skimmed milk (subculture 2). A loopful of subculture 1 was also spread onto an MIA plate and incubated for 12 hours at 40°C. This process was repeated until 12 subcultures had been carried out. The MIA plates were incubated at 30°C for 48 hours. Fifty colonies for each subculture were tested for stickiness using a wire loop as an indicator of EPS production. If there were any non sticky colonies they were inoculated into 10 ml of M17 broth and incubated at 30°C for 12 hours. These cultures were then analysed for the presence of a 40 kb plasmid.

Loss of EPS-producing ability in *Lc. lactis* ssp. *cremoris* using acriflavin.

Acriflavin was used in this study as it has been shown in previous studies to cause the loss of plasmids (Ishhiwa and Iwata 1980). *Lc. lactis* ssp. *cremoris* 2006 and B40 were grown in 10 ml of 10% (w/v) peptonised skimmed milk (PSM) containing 1 μg/ml acriflavin. The acriflavin was added aseptically to the sterile PSM. Cultures were incubated at 30°C for 24 hours (Ishhiwa and Iwata 1980). After incubation a loopful was transferred into fresh PSM containing acriflavin and reincubated at 30°C for 24 hours. This was repeated for 12 subcultures. A loopful of each subculture was plated onto an MIA plate and incubated for 24 hours at 30°C. Fifty colonies were tested for stickiness and if there were any non sticky colonies they were grown in 10 ml of M17 broth and incubated at 30°C for 12 hours These cultures were analysed for the presence of a 40 kb plasmid.

RESULTS

PLASMID ANALYSIS OF *Lc. lactis* ssp. *cremoris*.

Bacterial cultures of strains of *Lc. lactis* ssp. *cremoris* were analysed for the presence of a 40 kb plasmid that could contain the genes for EPS production. Strains of *Lc. lactis* ssp. *cremoris* were grown in M17 broth. Plasmids were then extracted using the method developed by Anderson and McKay (1983). Samples were then analysed using gel electrophoresis.

Figure 4.2 is a photograph of the gel containing the plasmids from the different strains of *Lc. lactis* ssp. *cremoris*. Lane 1 contained a reference strain of *Lc. lactis* ssp. *cremoris* AC1 containing plasmids of known sizes. Strain B40 (Lane 2) is a strain of *Lc. lactis* ssp. *cremoris* known to contain a 40 kb plasmid that is responsible for EPS production (van Krannenburg et al. 1997). This was used as a reference strain to see if it was possible to identify a 40 kb plasmid in the other strains. From the results, it can be seen that all the strains of *Lc. lactis* ssp. *cremoris* analysed contained a plasmid around the 38kb size and this corresponds to a plasmid of a similar size found in *Lc. lactis* ssp. *cremoris* B40.

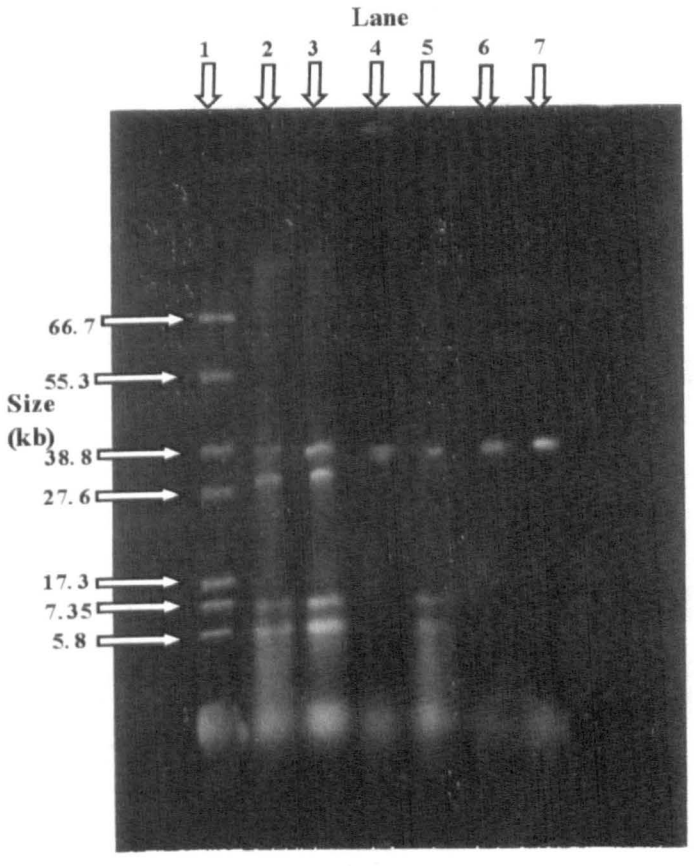


***Figure 4.2 Photo of plasmids found in strains of* Lc. lactis *ssp.* cremoris.**

Lanes: **1.** Plasmid markers from strain *Lc. lactis* ssp. *cremoris* AC1. **2.** *Lc. lactis* ssp. *cremoris* B40. **3.** *Lc. lactis* ssp. *cremoris* 0967. **4.** *Lc. lactis* ssp. *cremoris* 0968. **5.** *Lc. lactis* ssp. *cremoris* LC1. **6.** *Lc. lactis* ssp. *cremoris* 1934. **7.** *Lc. lactis* ssp. *cremoris* 2006.

It can also be seen that strains *Lc. lactis* ssp. *cremoris* B40, 0967 and LC1 contain bands around 7 kb and 6 kb in size. Strains *Lc. lactis* ssp. *cremoris* B40 and 0967 also have a band around 30 kb in size.

Individual colonies of strains of *Lc. lactis* ssp. *cremoris* were isolated using MIA agar. These colonies were analysed for polysaccharide-producing ability and the quantity of EPS was measured using the method described in Chapter 2 (Table 4.3). Some of these colonies produced low quantities of polysaccharides. The plasmid profiles of these individual colonies were determined using strain *Lc. lactis* ssp. *cremoris* AC1 as reference strains to investigate the

presence/absence of a 40 kb plasmid. Figure 4.3 to 4.6 show the plasmid profiles from the individual colonies of the different strains of *Lc. lactis* ssp. *cremoris*. It can be seen that some of the colonies contain a plasmid around 40 kb in size. It can also be noted that there are differences in the plasmid profiles of individual colonies.

Table 4.3 is a comparison of quantities of EPS produced and the presence of a 40 kb plasmid. It can be seen that some of the colonies contain a 40 kb plasmid, whether they produce a large quantity of EPS or not. From this table it can be seen that there does not seem to be a link between the quantity of EPS produced and the presence of a 40 kb plasmid.

***Table 4.3 Comparison of 40 kb plasmid and quantities of EPS produced by individual colonies of strains of* Lc. lactis *ssp.* cremoris.**

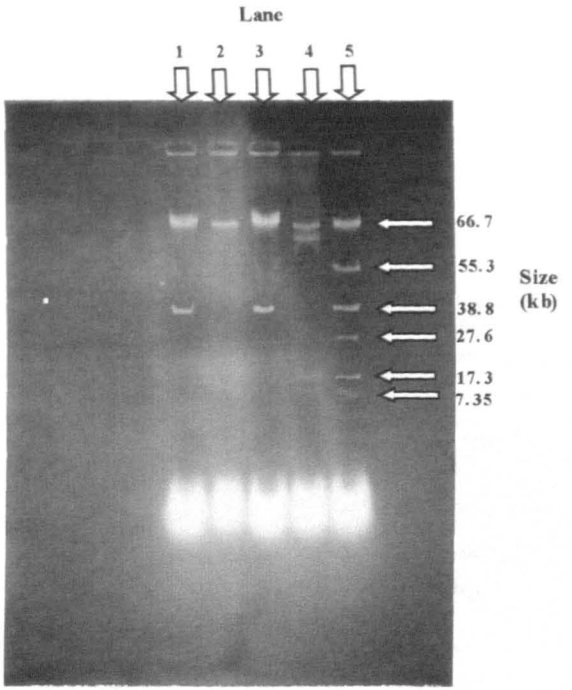
Strain	Colony	Presence of 40 kb plasmid	Average quantity of EPS produced µg/ml
0967	1	Yes	4.4
	2	No	10
	3	Yes	9.6
	4	No	11.4
0968	1	Yes	0
	2	Yes	17.6
	3	Yes	7.2
	4	No	33.9
	5	No	31.1
	6	Yes	9.7
LC1	1	Yes	28.8
	2	Yes	47.5
	3	Yes	45.8
	4	Yes	19.6
	5	No	34.9
1934	1	Yes	10.7
	2	No	17.9
	3	Yes	12.4
	4	No	7.7
	5	No	8.1
	6	Yes	8.9
2006	1	Yes	8.3
	2	No	3
	3	Yes	14.3
	4	Yes	6.3
	5	No	9.2

Use of elevated temperature to investigate loss of EPS producing ability in strains of *Lc. lactis* ssp. *cremoris*.

Table 4.4. The effect of growth at elevated temperature on the ability of strains* Lc. lactis *ssp.* cremoris *B40 and 2006 to produce EPS.

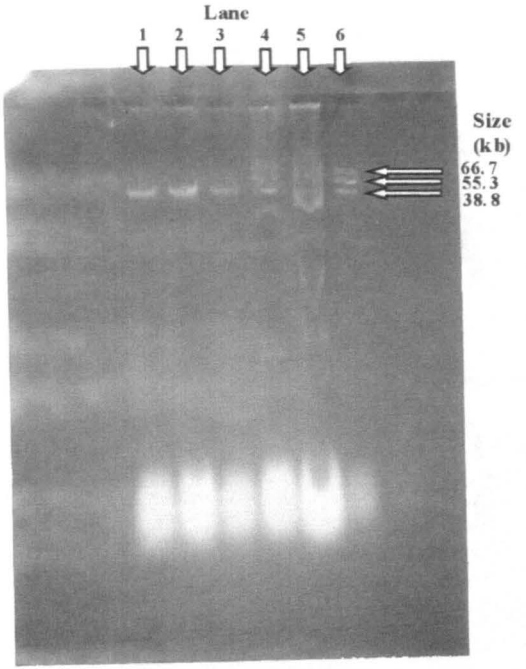
Subculture	No. of colonies that were sticky / 50	
	B40	2006
1	50	50
2	50	50
3	50	50
4	50	50
5	50	50
6	50	50
7	50	50
8	50	50
9	50	50
10	50	50
11	50	50
12	50	50

Table 4.4 shows that subculturing and growing the bacteria at elevated temperatures did not cause the EPS-producing ability to be lost in any of the isolates of either strain 2006 or strain B40 as all 50 colonies tested were all classed as EPS producing.



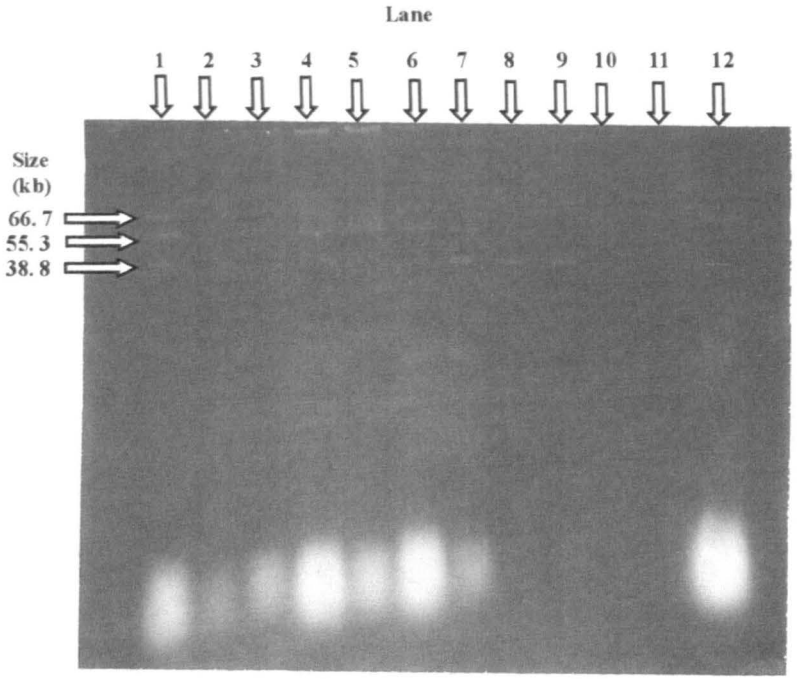
Lane 1-5. *Lc.cremoris* LC1, **6.** Plasmid markers from strain *Lc.cremoris* AC1.

Figure 4.3 Plasmid analysis of individual colonies of strains Lc.lactis* ssp. *cremoris LC1.



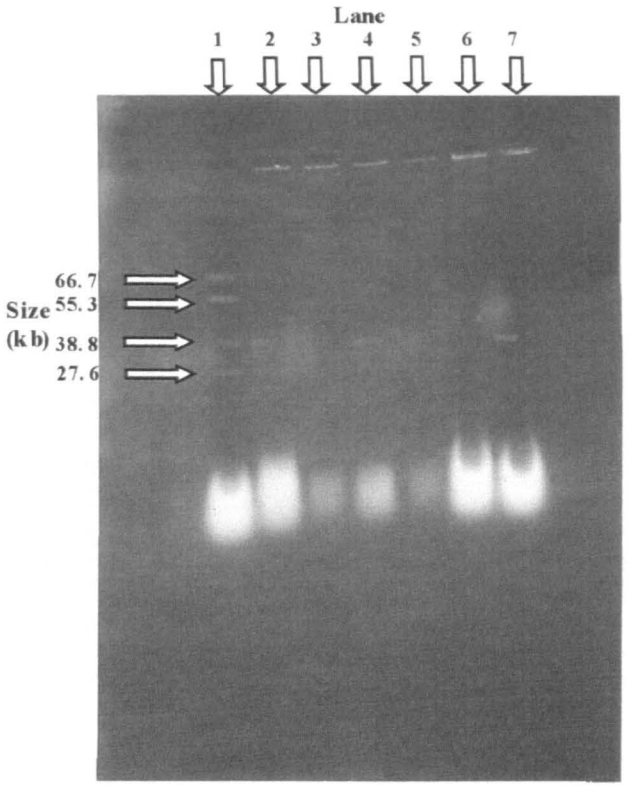
Lane 1-4. *Lc.cremoris* 0967, **5.** Plasmid markers from strain *Lc.cremoris* AC1.

Figure 4.4 Plasmid analysis of individual colonies of strains of of Lc.lactis* ssp. *cremoris 0967.



Lanes: **1.** Plasmid markers from strain *Lc.cremoris* AC1, **2-6**. *Lc.cremoris* 2006, **7-12**. *Lc.cremoris* 0968.

Figure 4.5 Plasmid analysis of individual colonies of strains of Lc.lactis* ssp. *cremoris 2006 and 0968.



Lane 1 Plasmid markers from strain *Lc.cremoris* AC1, **2-7**. *Lc.cremoris* 1934.

Figure 4.6 Plasmid analysis of individual colonies of strains of Lc.lactis* ssp. *cremoris 1934.

The use of acriflavin to stimulate loss of EPS producing ability in strains of *Lc. lactis* ssp. *cremoris*.

Table 4.5. The effect of growth with acriflavin on the ability of strains* Lc. lactis *ssp.* cremoris *B40 and 2006 to produce EPS.

Subculture	No. of colonies that were sticky / 50	
	B40	2006
1	50	50
2	50	50
3	50	50
4	50	50
5	50	50
6	50	50
7	50	50
8	50	50
9	50	50
10	50	50
11	50	50
12	50	50

Table 4.5 shows that subculturing and growth in acriflavin did not cause loss of EPS producing ability in strain B40 or strain 2006 as all 50 colonies tested were all classed as EPS producing.

DISCUSSION

Previous studies have shown that *Lc. lactis* ssp. *cremoris* B40 contained a plasmid 40 kb in size that contained the genes responsible for EPS production (van Kranenburg et al. 1997). An investigation was undertaken to determine the presence or absence of a 40kb plasmid within the strains of *Lc. lactis* ssp. *cremoris* used in this study and to determine whether there is a link between the presence of a 40kb plasmid and the quantity of EPS produce by individual colonies of a strain of *Lc. lactis* ssp. *cremoris*.

It has been reported in a number of studies including that of van Krannenburg et al. (1997) that different strains of lactic acid bacteria contain plasmids that possess the genes responsible for EPS production. Van Krannenburg et al. (1997) reported that in strains of *Lc. lactis* ssp. *cremoris* this plasmid was 40 kb in size and could be observed using gel electrophoresis. Investigations were carried out on all strains of *Lc. lactis* ssp. *cremoris* to see if they contained a 40kb plasmid. This does not however indicate whether this plasmid contains the genes required for EPS production.

For this to be determined the plasmid would need to be removed from the gel and cloned into a strain of *Lc. lactis* ssp. *cremoris* that was a non EPS producer and observe whether EPS production was initiated. All the strains of examined contained a plasmid approximately 38 kb that could possibly contain the EPS producing genes (Figure 4.2). *Lc. lactis* ssp. *cremoris* strains 0967 and B40 contained a plasmid around 30 kb in size and this could be fragments of the the 38 kb plasmid. Some of the colonies for a particular strain did not seem to contain a 40 kb plasmid (Figure 4.3 - Figure 4.6). The most likely explanations for this is are that either the bacteria did not contain the plasmid or that it was lost during the extraction process. In Figures 4.3 to 4.6 it can be seen that were differences in the plasmid profiles of individual colonies of strains of *Lc. lactis* ssp. *cremoris*. There are a number of possible reasons for the differences in the profiles Firstly, they could be due to the different conformations of the plasmids and it is difficult to differentiate between these on a gel because the CCC form will travel much further on the gel than a linear form of the plasmid. The differences could also be due to the fact that they have lost plasmids during the extraction process.

It was observed that individual colonies isolated from different strains of *Lc. lactis* ssp. *cremoris* produced varied quantities of EPS. An investigation was carried out to determine if there was link between the quantities of EPS produced and the presence/ absence of a 40kb plasmid. This involved growing stock culture of bacteria and then spreading a loopful onto MIA plates. Six colonies were randomly selected and grown up in skimmed milk to analyse quantity of EPS produced and also grown in M17 for plasmid analysis. It was found that a large proportion of the individual colonies contained a 40 kb plasmid but the quantity of EPS produced by the individual colonies varied. This suggests that there is considerable variability in the level to which the EPS genes are expressed and this could be linked to the amount of EPS produced (Table 4.3). There was also no link with presence of a plasmid and the quantity of EPS produced. Also, as seen in Chapter 1, EPS production is a complicated process involving a number of different stages which require different carrier proteins and other cellular components. If there is a problem at any of these stages it could lead to the quantity of EPS produced being affected and it could be this that is leading to the variability in the quantity of EPS produced.

The stability of EPS production was investigated by raising the growth temperature and adding acriflavin to the medium to try to remove the plasmid containing the EPS producing genes. The loss of this plasmid could be the result of instability of EPS in yoghurt production. Neither increasing the temperature nor growth in the presence of acriflavin resulted in the removal of the EPS producing ability. This suggests that something else is causing the removal of EPS producing ability. Marshall et al. (2001) have suggested that the instability of EPS production is most likely due to small genetic rearrangements cause by "insertion sequences" or natural transformational exchanges of DNA. This could be why it was not possible to remove EPS producing ability just by growing the bacteria in hostile growth conditions. Something is actively inhibiting or slowing down the bacteria's ability to produce EPS. Also strains of *Lb. delbrueckii* ssp. *bulgaricus* are reported not to contain plasmids so the *eps* genes must be found on the chromosome and they are also reported to lose their ability to produce EPS (Gancel and Novel, 1994b).

This study has shown that the strains of *Lc. lactis* ssp. *cremoris* used in this study contain a 40 kb plasmid but it was not determined as to whether this plasmid contained the genes needed for EPS production. This would need to be carried out in further studies. From this work it suggests that the variability of EPS producing ability could be linked to something other than genetics. Future work could include detailed DNA analysis of the plasmids of the strains of *Lc. lactis* ssp. *cremoris* used in the study to identify and characterise the *eps* genes. Studies could also be undertaken to investigate the possibility of insertion sequences being responsible for the inconsistencies in EPS production within strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

Having undertaken detailed analysis of the production of EPS by the lactic acid bacteria the next stage was to determine the effect of this EPS production on the texture milk products grown using strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

CONCLUSIONS

This chapter described investigations into the possible role of a 40 kb plasmid in the production of EPS by strains of *Lc. lactis* ssp. *cremoris* and whether adverse growth conditions could lead to the loss of EPS producing ability by the bacteria.

Plasmid analysis showed that all strains of *Lc. lactis* ssp. *cremoris* contained a 40 kb plasmid. Individual colonies isolated for their varying EPS producing ability all contained a 40 kb plasmid. It was not possible to remove the EPS producing ability by subculturing at elevated temperature or by subculturing in media containing acriflavin.

Chapter 5.THE EFFECTS OF THE EXOPOLYSACCHARIDES (EPS) PRODUCED BY STRAINS OF *Lactococcus lactis* ssp. *cremoris* AND *Lactobacillus delbrueckii* ssp. *bulgaricus* ON THE TEXTURE OF SKIMMED MILK.

INTRODUCTION

An investigation was undertaken into the texture of milk produced by different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* and the effect of subculturing on the viscosity of the fermented milk.

When strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* are grown in milk EPS produced acts as natural thickener and this has an effect on the texture of the final product. The aim of this section was to investigate how different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* affect texture when grown in skimmed milk. Depending on the type of fermented milk being made the textural properties will differ.

Texture and Yogurts

Three textural characteristics of yoghurts that can be distinguished are viscosity, hardness and elasticity (Rawson and Marshall 1997). Hardness is related to cohesivness in that it is the force that is necessary to attain a given amount of deformation and is an indication of the firmness of the fermented milk gel. Rawson and Marshall (1997) showed that yoghurts made with both EPS and non EPS producing bacterial strains produced hard (firm) yoghurts. This suggests that hardness (firmness) is probably due to the conformation of the milk proteins and the way in which they come together in the formation of the milk gel.

Viscosity is the property of a material to resist deformation and is the "slimy" characteristic of a fermented milk product. The viscosity of the yoghurt can be measured using a viscometer which applies a given shear rate to the sample between a support and a stirring cone. The torque observed results from the resistance of the fluid to movement and allows a measurement of viscosity. An important characteristic of yoghurt is the viscosity profile obtained for different shear rates. In

yoghurt, the viscosity is seen to decrease with increasing shear rates. This means that the more vigorous the agitation, the more fluid the yoghurt (Marshall et al. 2001).

Elasticity is the ability of a material to recover after deformation has occurred and this is linked to a firm body and gum-like fermented product. These properties are important for the appearance and mouthfeel of a product (Sebastiani and Zeleger 1998). Elasticity is measured by applying an oscillatory stress to the sample. Elasticity leads to the conservation of energy whereas viscosity leads to dissipation of energy. The response of a yoghurt to oscillation results from the contribution of the storage modulus G' (elasticity) and the loss modulus G'' (viscosity). Viscous yoghurt will have more elasticity than non-viscous yoghurt Rawson and Marshall (1997).

Bacterial EPS influence the rheology and texture of yoghurts at low concentrations and their thickening power compares favourably with other thickeners (Marshall and Rawson 1999, Rawson and Marshall 1997, Teggatz and Morris 1990). Importantly there is no clear correlation between observed EPS concentration and apparent viscosity of yoghurt.

The structure of EPS has been shown to influence its behaviour in milk. The stiffness of its backbone (van den Berg et al. 1995, Tuinier et al. 2001), its molecular weight and size (Faber et al. 1998), its branching (Tuinier et al. 2000, Tuinier et al. 2001), the nature of its side groups, its monomer composition, and the linkages between its monomers (Tuinier et al.1999, Tuinier et al. 2001; Yang et al. 2000) all effect the texture of fermented milk.

In this study, the two textural characteristics of hardness and viscosity were analysed,

METHODS

MICROORGANISMS.

The details of strains of *Lc.lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* used can be found in the Methods section of Chapter 2.

MEDIA COMPOSITION

The details of media used can be seen in the Methods sections of Chapter 2 and Chapter 3

MICROBIOLOGICAL EXAMINATION

See Methods section of Chapter 2.

TEXTURAL ANALYSIS

Measurment the hardness of bacterial cultures grown in skimmed milk.

Textural analysis of the hardness of skimmed milk cultures was carried out using a Stable Microsystems TA-XT2 Texture Analyser with parameters set as shown in Table 5.1. All strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* were grown in 100 ml of 10% (w/v) skimmed milk. For each strain, 3 cultures were analysed in triplicate. The maximum force was calculated and this is a measure of hardness.

Table 5.1 Parameters for Texture Analyser.

Mode	Measure force in compression
Option	Return to start
Pre Test Speed	1 mm/s
Test Speed	1 mm/s
Post Test Speed	1 mm/s
Distance	40 mm
Trigger Force	5 g
Data Acquisition	200 pps
Probe	Back extrusion cell with 35 mm using 5 kg load cell

Viscosity analysis of bacteria grown in skimmed milk.

All strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* were grown in 100 ml of 10% (w/v) skimmed milk at 30°C (*Lc. lactis* ssp. *cremoris)* or 37°C (*Lb. delbrueckii* ssp. *bulgaricus*) for 12 h. Samples were kept refrigerated until they were analysed. All apparatus was refrigerated so that analysis could be carried out at cooler temperatures. Before analysis each sample was gently stirred, manually with a glass rod, for 1 minute to achieve homogeneity within a sample. Samples were analysed using either a Haake Viscometer using a MVIst spindle or a SV-DIN spindle for milk fermented with *Lc. lactis* ssp. *cremoris* or *Lb. delbrueckii* ssp. *bulgaricus*

respectively. Viscosity readings, in mPa, were taken every 15 seconds for 5 minutes at a rotation speed of 500 rpm^{-1}.

The effect of subculture on the viscosity of *Lb. delbrueckii ssp. bulgaricus* fermented milks.

Lb. delbrueckii ssp. *bulgaricus* Ldb1 and 2772 were grown in 100 ml of 10% (w/v) skimmed milk at 37°C for 12 hours. 10 ml was subcultured into 100 ml of skimmed milk and reincubated. Viscosity analysis was carried out using the method described above. Sub culturing and viscosity analysis was carried out daily for 5 days.

RESULTS

Hardness of milk fermented with strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

The results shown are an average of 3 replicates.

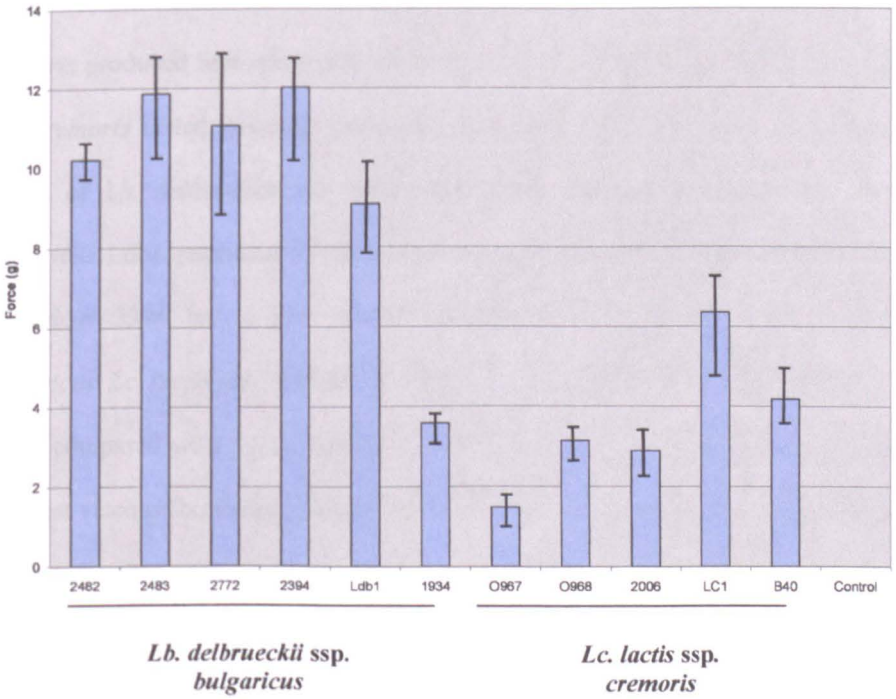


Figure 5.1 Comparison of gel hardness produced when strains of* Lb. delbrueckii *ssp.* bulgaricus *and* Lc. lactis *ssp.* cremoris *were grown in skimmed milk.

Figure 5.1 shows that the gel strength of milk produced by strains of *Lb. delbrueckii* ssp. *bulgaricus* were generally high compared to those of *Lc. lactis* ssp. *cremoris*. The different strains gave similar hardness except for *Lb. delbrueckii* ssp. *bulgaricus* Ldb1, which gave a slightly lower hardness than the others. The hardness of fermented milk prepared using the different strains of *Lc. lactis* ssp. *cremoris* strains were similar and much lower than that of fermented milk prepared using strains of *Lb. delbrueckii* ssp. *bulgaricus*. The results also showed that *Lb. delbrueckii* ssp. *bulgaricus* 2394 produced a hard gel even though it is described as being a non EPS producing strain. The error bars in Figure 5.1 shows that there was also little overlapping of data points between *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* suggesting that differences in gel hardness between the two types of bacteria may be significant. However, for this to be confirmed more data would be needed and a statistical test carried out.

Analysis of viscosity when strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* are grown in 10% (w/v) skimmed milk.

Figure 5.2 shows that all of the strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* produced fermented milk with similar shaped viscosity profiles. The strains of *Lc. lactis* ssp. *cremoris* tested generally produced fermented milks that were less viscous than those of strains of *Lb. delbrueckii* ssp. *bulgaricus* Ldb1, 2483, 2772 and 2482. *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 produced the most viscous milk and milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* 2394 had a low viscosity compared with other strains of *Lb. delbrueckii* ssp. *bulgaricus*. *Lc. lactis* ssp. *cremoris* strain 0967 produced the least viscous milk.

When compared with the gel hardness data (Figure 5.1) it can be seen that strain Ldb1 produced the most viscous fermented milk gel but it did not seem to be a particularly hard gel in comparison to the fermented milk produced using the other strains of *Lb. delbrueckii* ssp. *bulgaricus*. Strain 0967 produced a soft gel and produced the least viscous fermented milk. However, strain 2394 produced the hardest gel but it had the second to lowest viscosity.

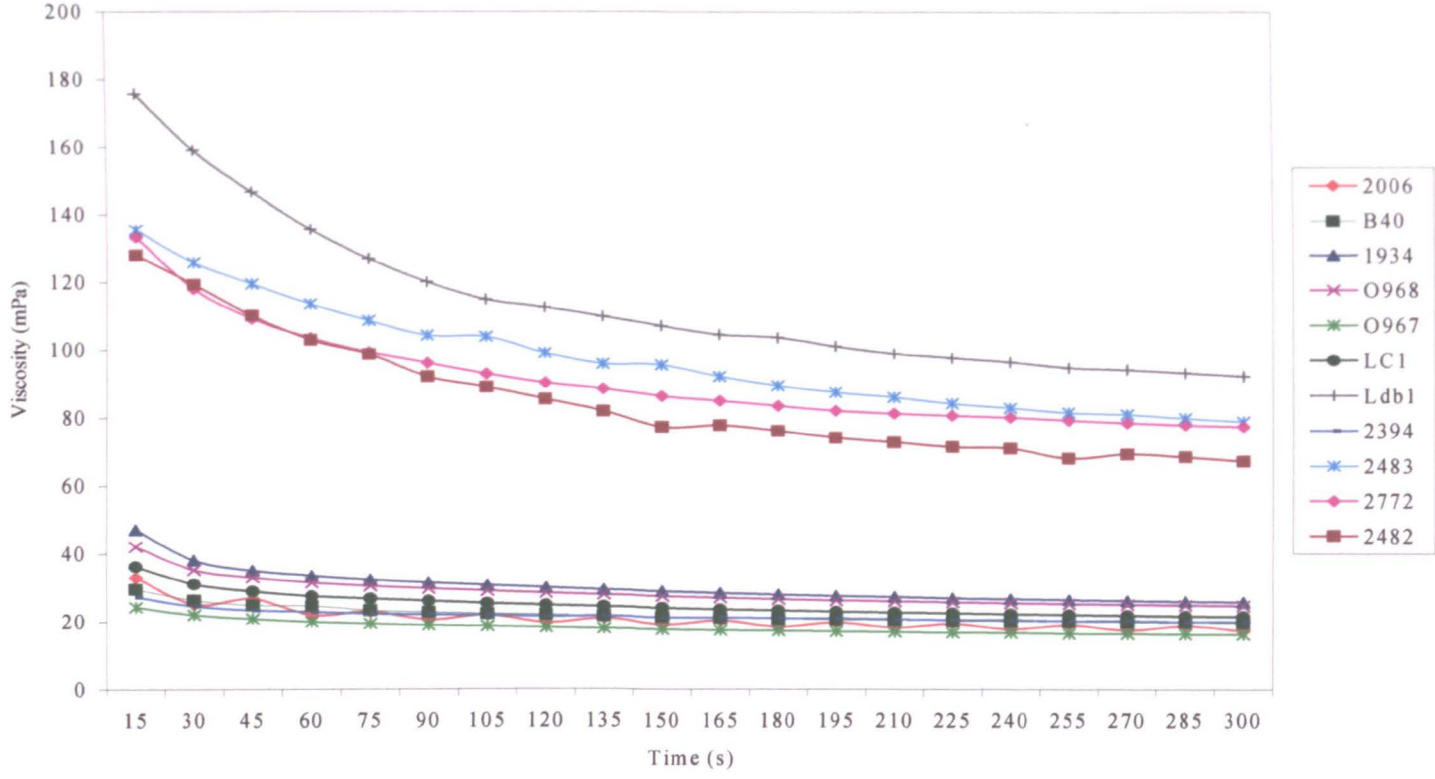


Figure 5.2 Comparison of viscosity profiles produced by strains of* Lc.lactis *ssp.* cremoris *and* Lb.delbrueckii ssp. bulgaricus *when grown in skimmed milk.

The effect of subculturing on the viscosity of strains of *Lb. delbrueckii* ssp. *bulgaricus* grown in 10% skimmed milk.

This section looks at how the viscosity profile of milk fermented with *Lb. delbrueckii* ssp. *bulgaricus* is affected by successive subculturing. The graphs are the average of two repeats. For graphs of the individual experiments see Appendix 1.11 and 1.12.

Figures 5.3 and 5.4 show the effect of subculturing on the viscosity profiles of strain 2772 and strain Ldb1 respectively when grown in 10% skimmed milk. From Figure 5.3 it can be seen that the viscosity of strain 2772 alters between each subculture. After day1 subculture the viscosity drops. On days 3 and 4 the viscosity of the culture increases slightly and on day 5 and 6 there is a sharp increase in the viscosity of the milk. On days 5 and 6 the viscosity increases during the first minute of analysis. It seems that subculturing has a positive effect on the viscosity of strain 2772. From Figure 5.4 it can be seen that the viscosity of strain Ldb1 decreases over time. However, on day 1 the viscosity seems to increase slightly during the first minute of analysis

DISCUSSION

The aim of this part of the study was to determine the texture of the milk gels produced by strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

This initially involved examining and comparing the textural qualities of milk gels produced by the different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. Firstly the milk gels were analysed using a non-destructive method of textural analysis. This involved a probe being lowered into the milk gels to determine the hardness of the gel. This meant that the same samples could subsequently be used for viscosity analysis using a destructive method. Analysis of gel hardness, the ability of the milk gel to resist deformation, of the different strains when grown in milk showed that strains of the same bacteria produced milk gels of similar hardness. The milk gels of *Lb. delbrueckii* ssp. *bulgaricus* strains were harder than those milk gels produced by *Lc. lactis* ssp. *cremoris*. Of the strains of *Lb. delbrueckii* ssp. *bulgaricus* 2394, produced the hardest gel and Ldb1 the softest. *Lb. delbrueckii* ssp. *bulgaricus*, 2394 is considered a non EPS producing strain suggesting that hardness of the gel could be a result of an interaction with milk proteins.

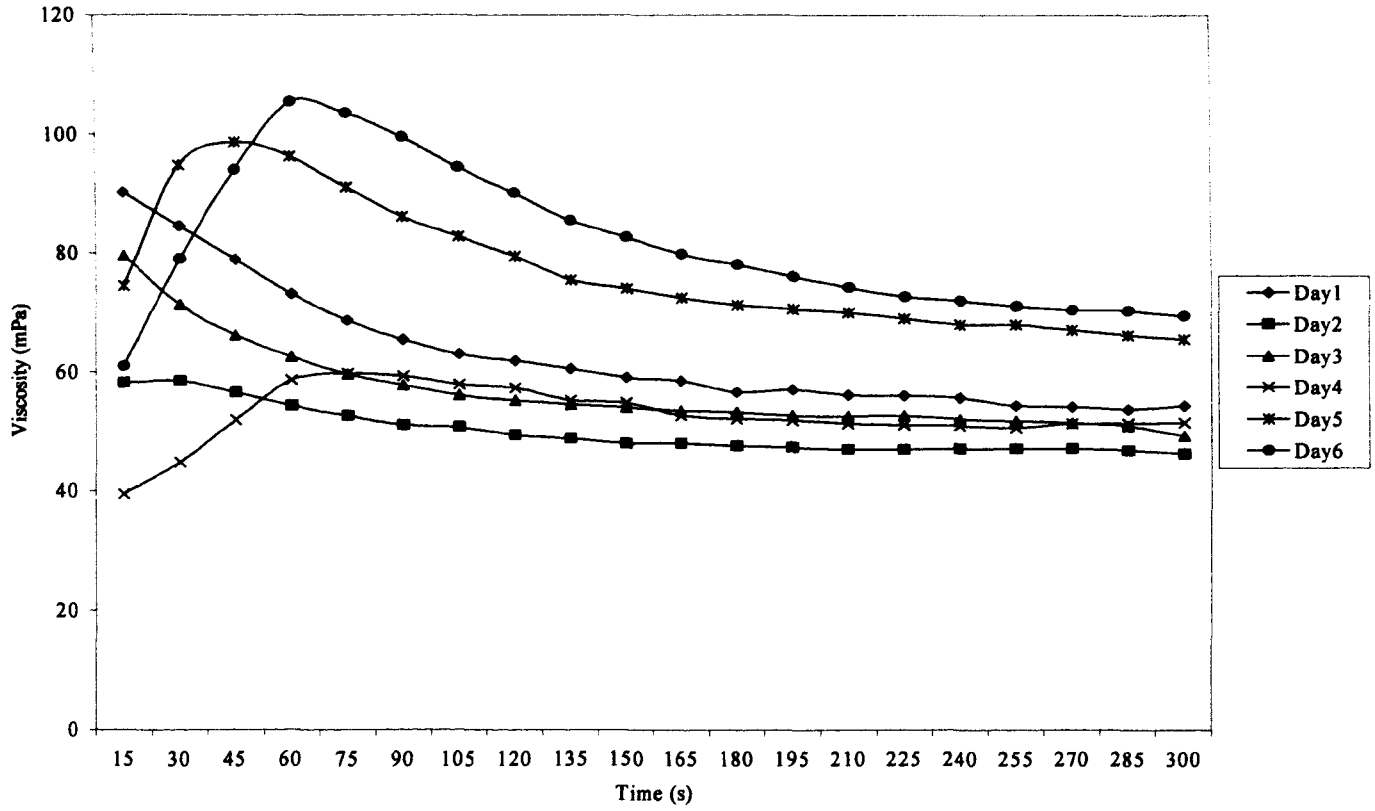


Figure 5.3 The effect of subculturing on the viscosity of* Lb. delbrueckii *ssp.* bulgaricus *2772 when grown in skimmed milk.

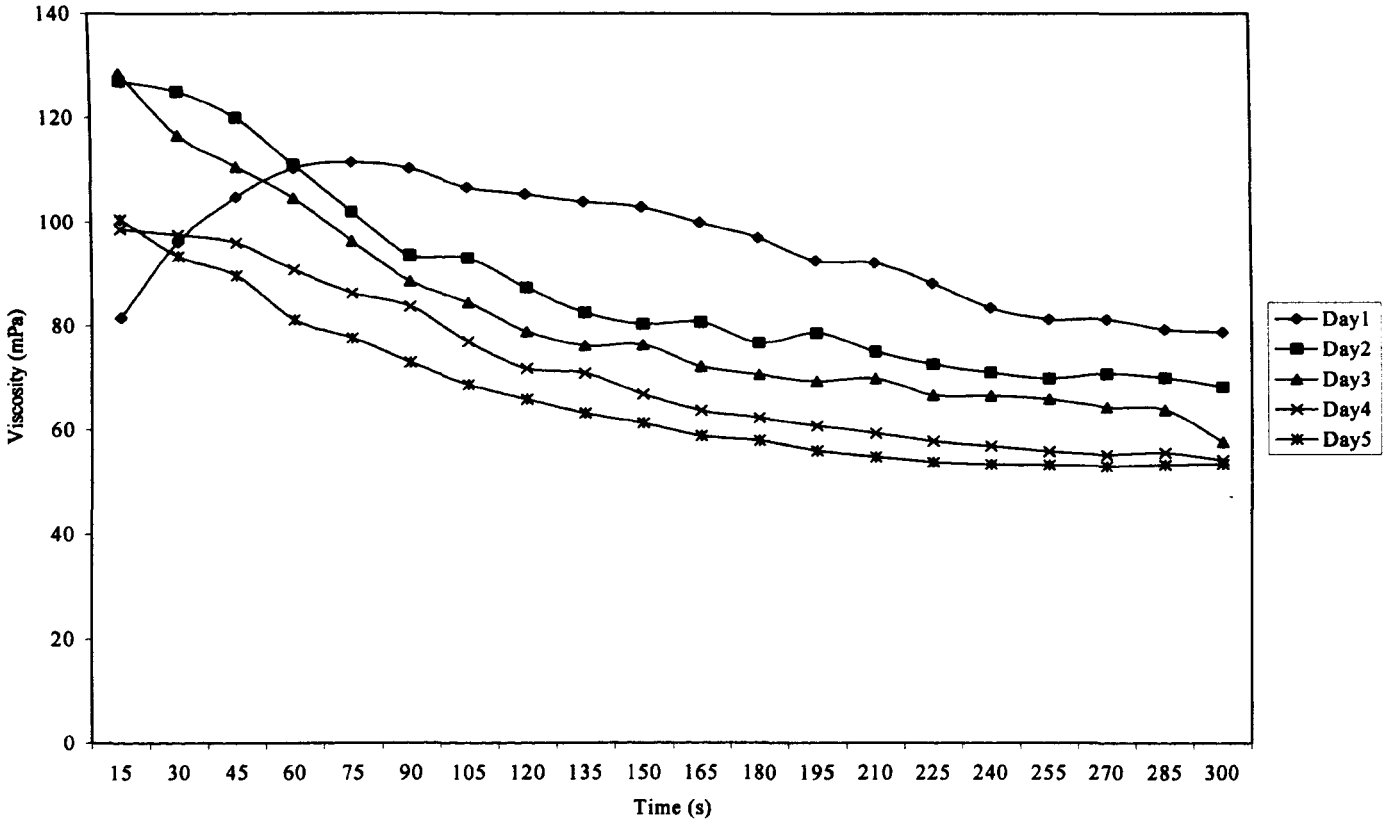


Figure 5.4 The effect of subculturing on the viscosity of* Lb. delbrueckii ssp. bulgaricus *Ldb1 when grown in skimmed milk.

Rawson and Marshall. (1997) reported that yoghurts made with ropy bacterial strains were the hardest (firmest) yoghurts. However, yoghurts made with non-ropy strains also produced hard yoghurts. This indicates that hardness (firmness) is probably due to the interaction of the milk proteins with EPS, which could explain the findings with strains 2394. Ayala-Hernandez et al. (2009) found that milk fermented with a high protein content (between 6 and 8% protein) had significantly higher viscosity and ropiness than those containing a lower protein content.

The next stage was to determine the viscosity of the milk gels. This gives an indication of the slimy thickening characteristic of the milk gels. The results of these experiments showed that all strains of both *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* produced similar shaped viscosity profiles. Strains of *Lc. lactis* ssp. *cremoris* produced milk gels that had lower viscosity profiles than strains of *Lb. delbrueckii* ssp. *bulgaricus*. All strains of *Lb. delbrueckii* ssp. *bulgaricus*, except 2394, produced milk gels that were fairly viscous.

When looking at viscosity on its own, strains of *Lb. delbrueckii* ssp. *bulgaricus* also produced a more viscous gel than *Lc. lactis* ssp. *cremoris* but *Lb. delbrueckii* ssp. *bulgaricus* 2394 (non EPS producer) did not produce a particularly viscous gel suggesting that EPS is necessary for increasing the viscosity. However, strains of *Lc. lactis* ssp. *cremoris* did not produce particular viscous gels and they tended to produce more EPS (Table 2.4) than strains of *Lb. delbrueckii* ssp. *bulgaricus* This leads to the possibility that there may be an optimum amount of EPS needed to produce a viscous gel.

When comparing the viscosity and hardness results with the yield (Table 2.4) and molecular weight (Table 2.7) (Appendix 1.13) of EPS isolated from the different strains when grown in milk it can be seen that strains of *Lc. lactis* ssp. *cremoris* produced large quantities of a high molecular weight EPS but soft and not very viscous gels. On the other hand strains of *Lb. delbrueckii* ssp. *bulgaricus* produced smaller quantities of low molecular weight EPS but hard and quite viscous gels. The exception to this was *Lb. delbrueckii* ssp. *bulgaricus* 2482. This is further evidence to suggest that it is not just the quantity of EPS that affects the final texture of the fermented milk. The molecular weight of EPS may also play a role in viscosity. As mentioned earlier, in yoghurt manufacture the desired textural properties of the final yoghurt would determine the selection of

bacteria used. Set yoghurts would need to be hard and non-viscous and stirred yoghurts would need to be soft and viscous and this is probably why more than one strain is used during yoghurt manufacture to give a better balance of viscosity and hardness. During the process of yoghurt manufacture there are often inconsistencies in the texture of the final product between batches. The next stage of the investigation was to determine whether continuous subculturing reduced the viscosity of fermented milk in comparison with that produced using the initial innoculum. Continuous subculturing of *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 showed that overall there was a rapid drop in the viscosity of subsequent batches. However, for measurements on day 1 the viscosity of the milk gel did seem to increase in the first minute of analysis indicating that during the first minute of analysis the fermented milk actually became thicker, more viscous, than it was at the start of the analysis. When the fermented milk was stirred before being measured by the viscometer it appeared to have quite a glutinous texture and when the viscometer was started some of the fermented milk could have got caught around the spindle. This would give the fermented milk a slightly more viscous texture until the gel had been broken up.

When subculturing *Lb. delbrueckii* ssp. *bulgaricus* 2772 after the day 1 the viscosity dropped. On days 3 and 4 the viscosity of the culture increased slightly and on day 5 and 6 there was a sharp increase in the viscosity of the milk. As with *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 on days 5 and 6 the viscosity also increased during the first minute of analysis. A possible reason for the increase in the whole viscosity profile on days 5 and 6 could be due to the interaction between milk proteins and the EPS making the gel thicker and more viscous. There is more interaction between the milk proteins and the EPS. As mentioned earlier the quantity of EPS produced is highly variable and it could be that between subcultures the amount of EPS varied and it could be this that affected the viscosity of the milk. In future studies it would be desirable to remove a sample of the fermented milk and measures the quantity of EPS produced and determine whether this correlated to any changes in viscosity over time.

Another factor that could affect the viscosity and hardness of the fermented milks is EPS degradation by bacterial glycohydrolase enzymes (Pharm et al. 2000). Petry et al. (2003) suggested that the EPS may be degraded in the stationary phase of growth. It could be that if yoghurts are

incubated for too long during the incubation process the EPS may start to be degraded and the yoghurts would start to lose their viscosity. This could be investigated further using bacteria that have had genes for glycohydrolase enzymes removed.

As mentioned earlier continuous monitoring of EPS producing ability is required to try and ensure textural consistency between batches. At present the only method for testing for EPS producing ability is to grow the colonies on agar plates and then see if the colonies have a slimy consistency when touched with a sterile loop (Toba et al. 1990). This method is very subjective and a more accurate method for determining EPS producing ability by lactic acid bacteria isolates needs to be developed. Ideally such a method could also be used for identifying new strains of lactic acid bacteria with EPS producing ability.

CONCLUSIONS

Non destructive texture analysis allows samples to be analysed for more than one parameter.

The firmness and viscosity of fermented milk produced using strains of *Lc. lactis* ssp. *cremoris* is not related solely to the level of EPS production and it could be linked to the molecular weight or structure of the EPS.

The firmness and viscosity of fermented milks produced using strains of *Lb. delbrueckii* ssp. *bulgaricus* were higher than those produced using strains of *Lc. lactis* ssp. *cremoris*.

Strains of *Lb. delbrueckii* ssp. *bulgaricus*, when grown in skimmed milk gave rise to hard viscous gels.

Strains of *Lc. lactis* ssp. *cremoris*, when grown in skimmed milk gave rise to soft and not very viscous gels.

Loss of viscosity on subculturing can occur in fermented milks of strain *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 but the viscosity seemed to increase in strain 2772 on subculture.

Chapter 6. ANALYSIS OF STRAINS OF *Lactococcus lactis* ssp. *cremoris* AND *Lactobacillus delbrueckii* ssp. *bulgaricus* AND THEIR CRUDE EXOPOLYSACCHARIDES (EPS) USING FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR).

INTRODUCTION

This section deals with the use of Fourier Transform infrared spectroscopy as a technique for analysing *Lactococcus lactis* ssp. *cremoris* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and their crude exopolysaccharides.

As mentioned earlier there are many methods that can be used to identify different species of bacteria. These can range from simple biochemical tests such as the Gram stain, oxidase test and the ability to ferment different sugars (Becker, Weiss and Holzapfel 2009) to genotypic characterisation including PCR and plasmid profiling (Dale and von Schantz 2003).

As well as identifying the bacterial species it is also desirable to be able to differentiate between EPS and non-EPS producing ability. At present the only routine method available for testing for EPS producing ability is to grow the organism on agar plates and then determine whether the colonies have a slimy consistency when touched with a sterile loop (Toba et al. 1990). This method is very subjective and inaccurate and a more reliable method is required. Fourier transform infrared spectroscopy (FTIR) was investigated with the aim of developing methods that could be used to identify lactic acid producing bacteria and to continuously monitor EPS production by strains of bacteria. This would allow the dairy industry to identify EPS producing bacteria and then routinely check their EPS producing ability.

METHODS

MICROORGANISMS.

The microorganisms used can be found listed in Table 2.2.

MEDIA COMPOSITION

See the Methods section of Chapter 2 for composition of MRS for *Lb. delbrueckii* ssp. *bulgaricus* and M17 for *Lc. lactis* ssp. *cremoris*.

FTIR ANALYSIS OF BACTERIAL CULTURES

Analysis of agar plate cultures of bacteria by FTIR.

1 bacterial colony was removed from a spread plate using a sterile loop. This loopful was then resuspended in 80 µl distilled water, using a vortex mixer.

Aliquots of 35 µl of the cell suspension were transferred to a ZnSe optical plate and evacuated to dryness under moderate vacuum. This produced transparent films suitable for absorbance/transmission measurements using FTIR spectroscopy. Spectra were recorded between 4000 and 500 wavenumbers, cm^{-1}, using a Bruker IFS-28B FTIR spectrometer.

Analysis of liquid cultures of bacteria by FTIR.

Cultures were grown in 10 ml of liquid media for 12hr at 30°C (M17) or 37°C (MRS) depending on the bacteria used.

2 ml of culture was concentrated by centrifugation at 5000 rpm for 3 minutes then washed in 2 ml of Ringers solution three times before being resuspended in 80 µl distilled water, with 35 µl aliquots treated as above.

Effect of growth substrate on FTIR profile of *Lc. lactis* ssp. *cremoris*.

10 ml samples of cremoris defined media were prepared using glucose, fructose, sucrose and lactose as the growth substrate (Chapter 2). Strains 0968, 1934, LC1, 0967, 2006 of *Lc. lactis* ssp.

cremoris were grown overnight at 30°C and prepared for FTIR analysis using the protocol described above for liquid cultures.

Effect of age of culture on FTIR profile of *Lc. lactis* ssp. *cremoris*.

Strains 2006, 1934 and LC1 of *Lc. lactis* ssp. *cremoris* were grown on M17 agar plates. After incubation overnight at 30°C samples were removed and analysed by FTIR. The plate cultures were then reincubated at 30°C overnight and a sample removed for FTIR analysis. This procedure was repeated a further 3 times over a total of five days. Samples were prepared for FTIR analysis using method for agar plate cultures.

Analysis of crude polysaccharide by FTIR

1000μg/ml bacterial polysaccharide stock solutions (35 μl) were transferred to a ZnSe optical plate and evacuated to dryness under moderate vacuum. Spectra were recorded between 4000 and 500 wavenumbers, cm^{-1}, using a Bruker IFS-28B FT-IR spectrometer.

Analysis of FTIR Data.

For data-processing the software package OPUS Version 2.2 (Bruker, Karlsruhe, Germany) was used. The instrumental parameters were set up according to the advice of the FTIR workgroup of Robert Koch Institut, Berlin Germany (Helm *et al.* 1991).

Each spectrum resulted from the addition and averaging of 64 interferograms. Spectral resolution was 6 wavenumber cm^{-1}. Smoothed first derivatives of the original spectra were calculated using a Savitzky-Golay algorithm (Savitzky and Golay 1964). Spectra used for cluster analysis were compared using a selection of three spectral windows; 1200-900, 900-700 and 1500-1200 wavenumbers cm^{-1}. The weighting accorded to each window was 1. Data were used for cluster analysis using the Ward algorithm (Ward 1963).

RESULTS

PRODUCTION OF SPECTRA FROM STRAINS OF *Lactobacillus delbrueckii* ssp. *bulgaricus* AND *Lactococcus lactis ssp. cremoris* USING FTIR.

Strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* were grown on agar plates. Absorption spectra were produced using the FTIR. Average 1st derivative spectra and standard deviations were then calculated based on a number of spectra for each strain using the specialised software. Figures 6.1 to Figure 6.10 shows absorption spectra for the bacterial strains used in this experiment Figure 6.11 and Figure 6.12 show the average 1st derivative spectra with + and – standard deviations for strain LC1 of *Lc. lactis* ssp. *cremoris* and strain 2394 of *Lb. delbrueckii* ssp. *bulgaricus* respectively.

Absorption spectra are a direct representation of the different bonds found within the molecule. However, it is difficult to make comparisons based on cellular components from these spectra. First derivative spectra are used because it is easier to separate out the different components and observe smaller differences between spectra.

To assist in spectral interpretation the following features can be observed at these wavelengths.

Wavenumber cm^{-1}	*Feature Observed*	*Molecular Vibrations*
1000 -1300	Sugars, EPS	Vibrations between C-H and C-O.
1210 - 1500	DNA	Vibrational coupling between the base and sugar.
1400 - 1800	Protein	Vibrations in the amide group.
1500 - 1800	Nucleic Acids	Vibrations between the sugar-phosphate chain.
2800 - 3100	Lipid	CH_2 stretching vibrations.

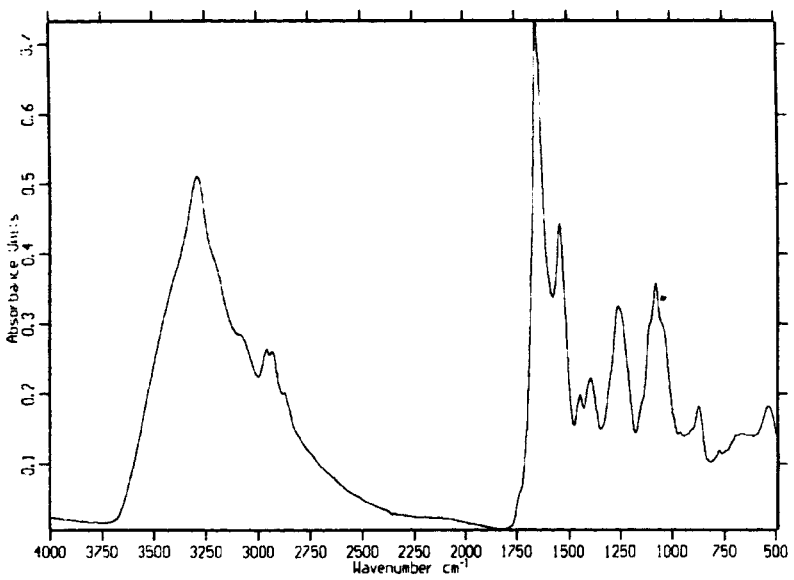


Figure 6.1 Absorption spectra for strain 2482 of **Lb. delbrueckii** *ssp.* **bulgaricus.**

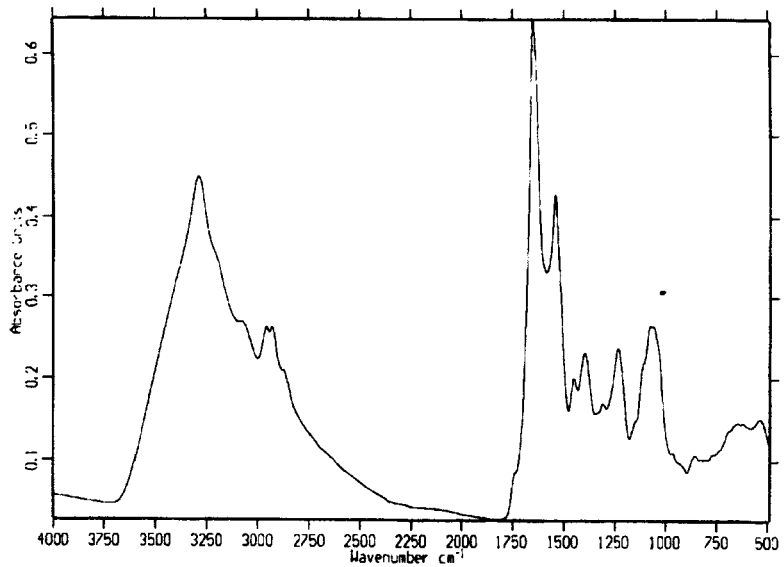


Figure 6.2 Absorption spectra for strain 2483 of **Lb. delbrueckii** *ssp.* **bulgaricus.**

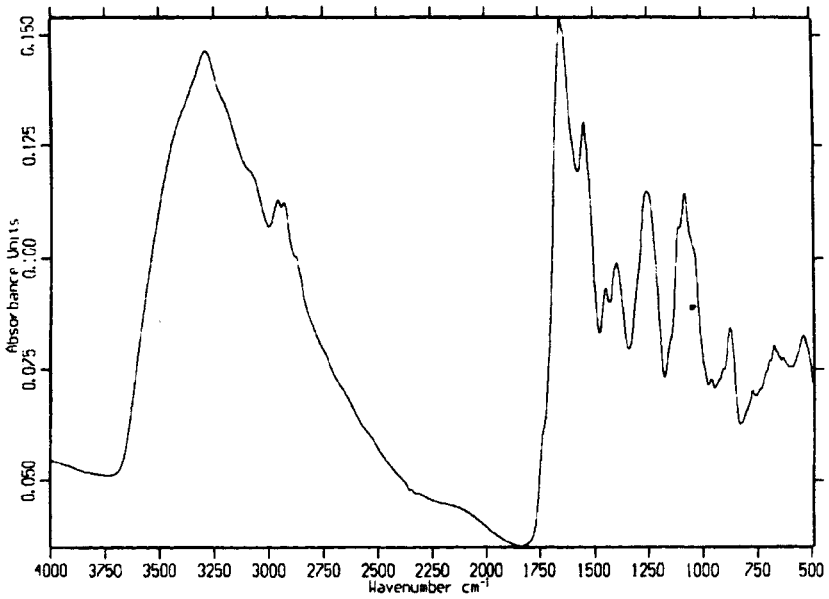


Figure 6.3 Absorption spectra for strain 2772 of **Lb. delbrueckii** *ssp.* **bulgaricus.**

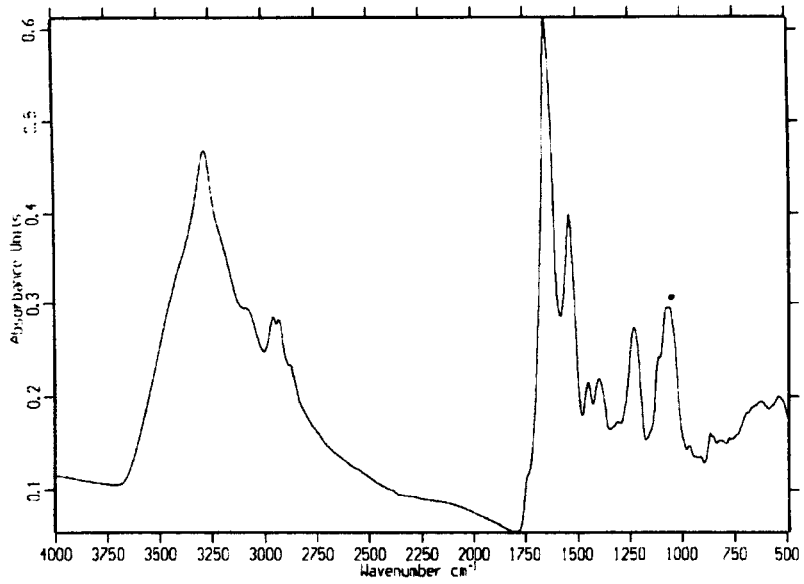


Figure 6.4 Absorption spectra for strain 2394 of **Lb. delbrueckii** *ssp.* **bulgaricus.**

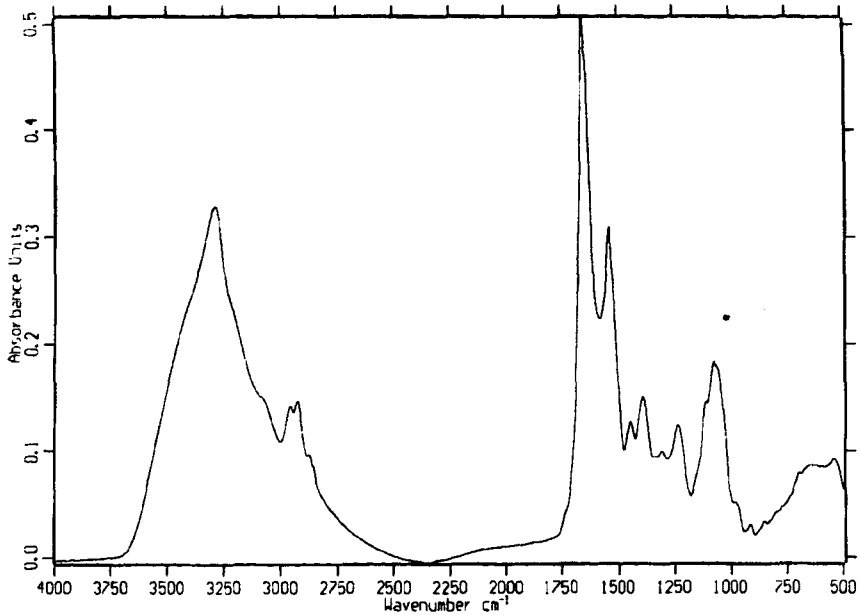


Figure 6.5 Absorption spectra for strain Ldb1 of **Lb. delbrueckii** *ssp.* **bulgaricus.**

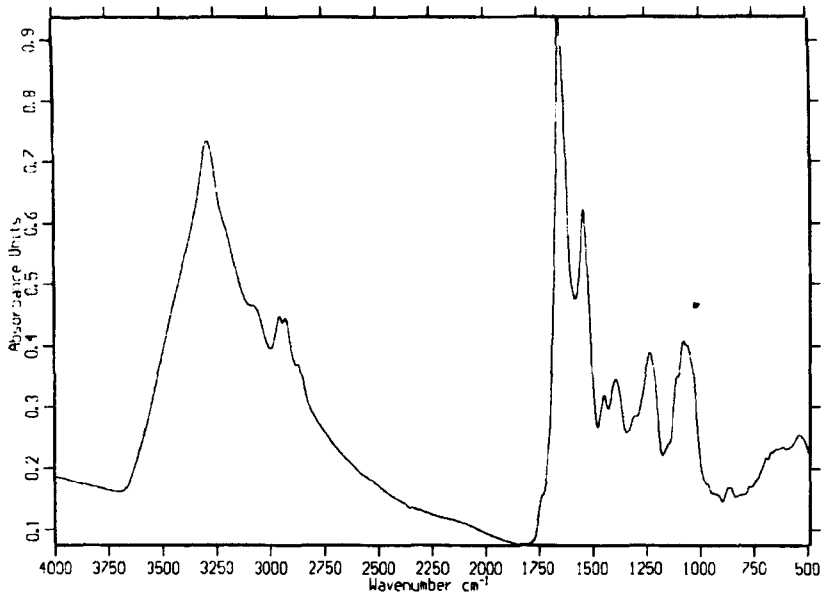


Figure 6.6 Absorption spectra for strain 1934 of **Lc.lactis ssp. cremoris**

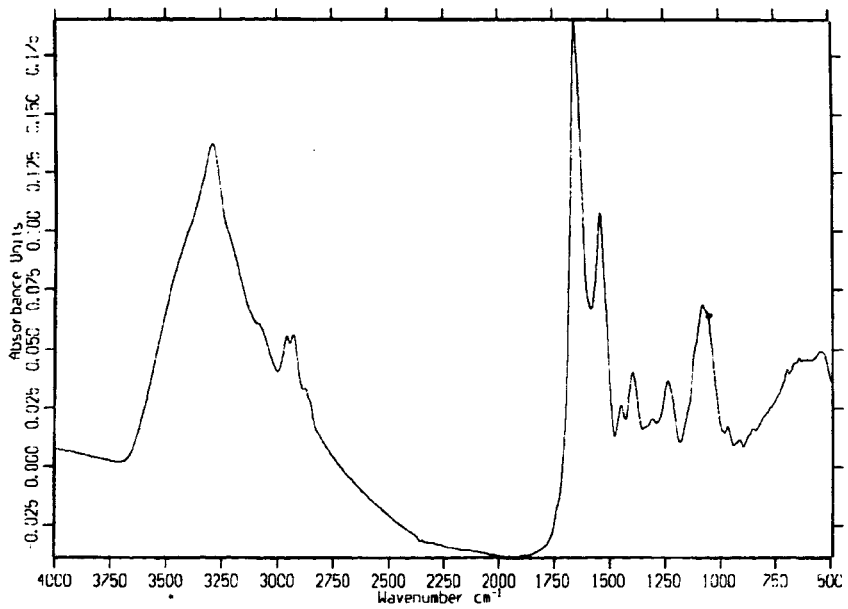


Figure 6.7 Absorption spectra for strain 0967 of **Lc.lactis** *ssp.* **cremoris.**

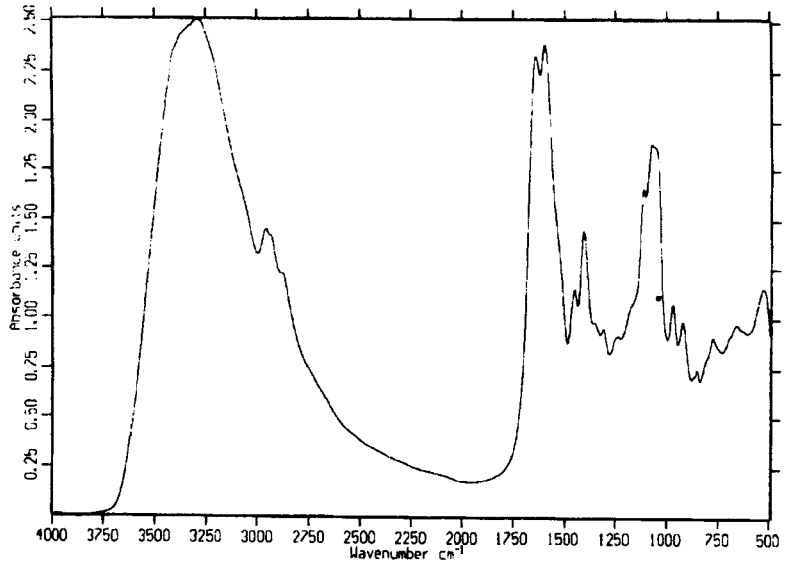


Figure 6.8 Absorption spectra for strain 0968 of **Lc.lactis** ***ssp.*** **cremoris.**

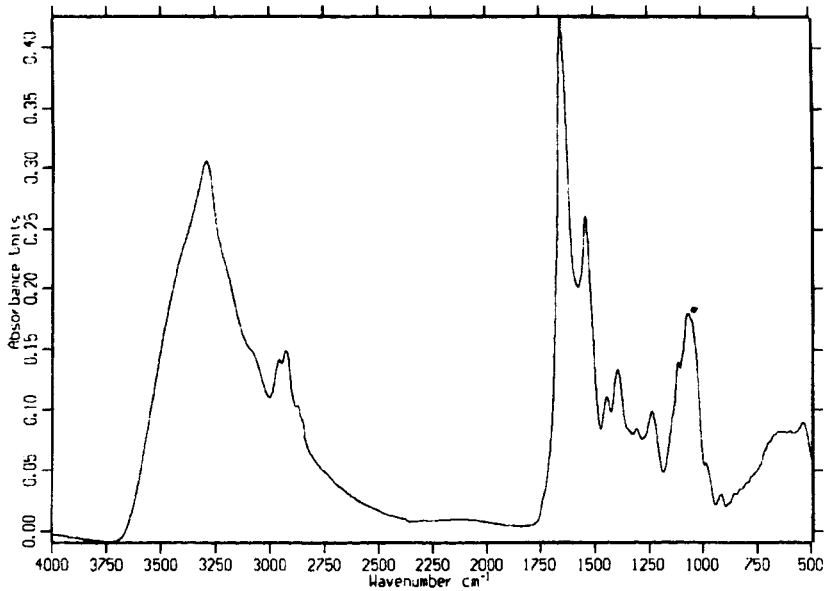


Figure 6.9 Absorption spectra for strain 2006 of **Lc.lactis** *ssp.* **cremoris.**

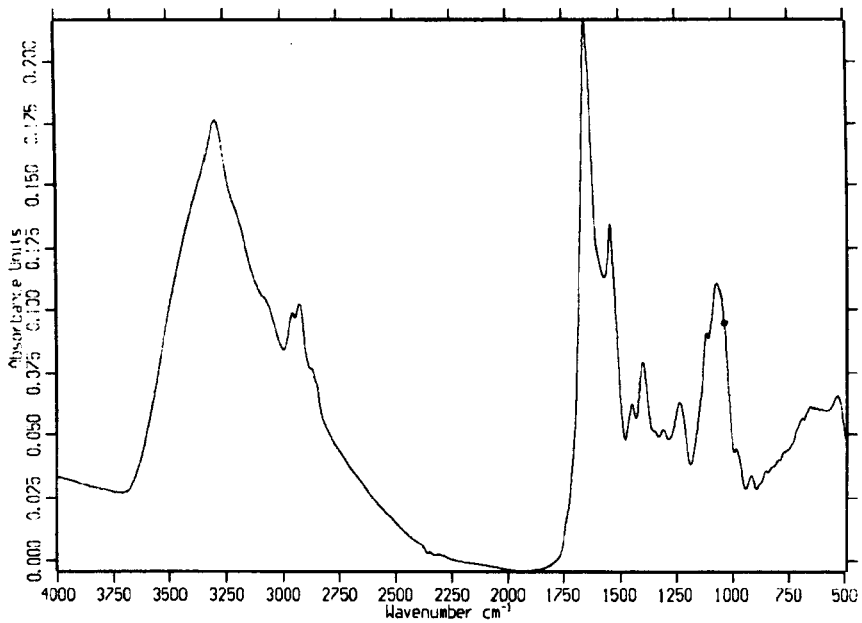


***Figure 6.10 Absorption spectra for strain LC1 of* Lc.lactis *ssp.* cremoris.**

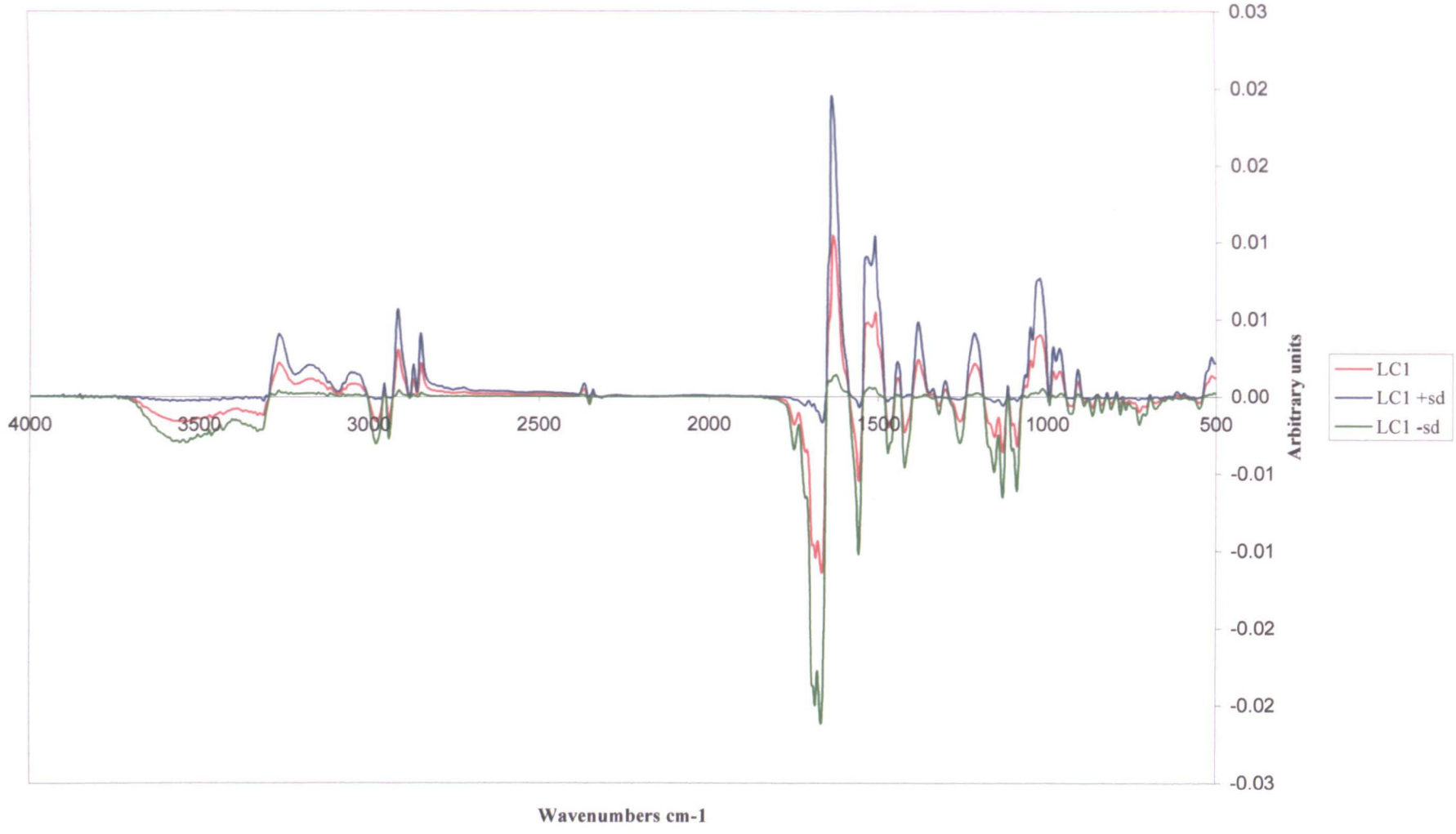


Figure 6.11 Average 1st derivative spectra for strain LC1 of **Lc.lactis** *ssp.* **cremoris.**

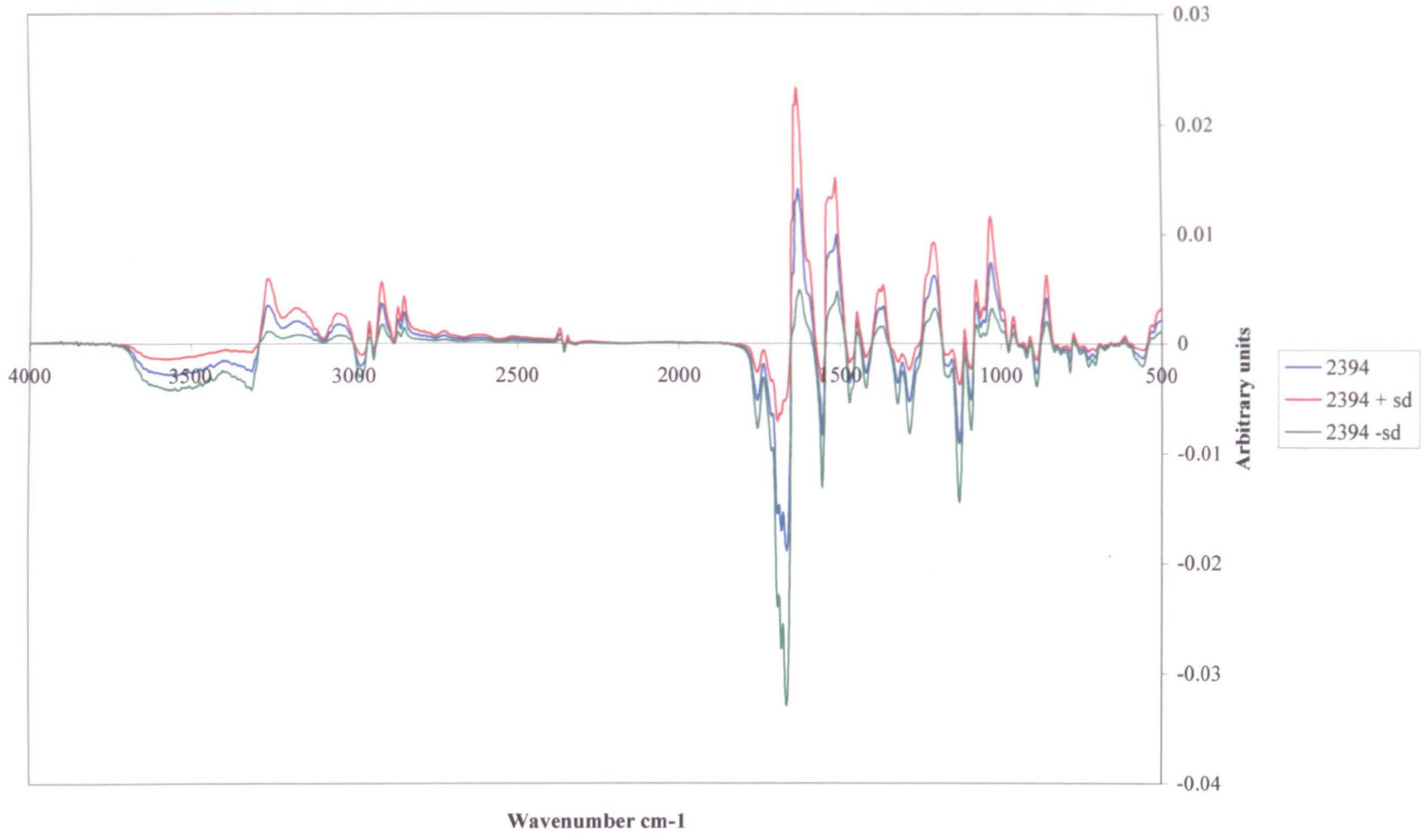


 Figure 6.12 Average 1st derivative spectra for strain 2394 of **Lb.delbrueckii ssp. bulgaricus.**

COMPARISON OF SPECTRA FROM *Lactobacillus delbrueckii ssp. bulgaricus* AND *Lactococcus lactis* ssp. *cremoris* USING THE FTIR.

A comparison was carried out on first derivative spectra produced by strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* using FTIR. First derivatives were based on absorption spectra produced from samples derived from agar plate cultures. The 1st derivative spectra were calculated using a Savitzky-Golay algorithm (Savitzky and Golay 1964). Average 1st derivative spectra were then calculated based on a number of spectra for each strain.

Figure 6.13 is a cluster analysis using the Ward algorithm (Ward 1963) of the first derivatives of spectra from different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. From this it can be seen that it is possible to separate different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. These differences are significant because the heterogeneity of the spectra is greater than one. According to published literature (Lefier, Lamprell and Mazerolles 2000) differences of heterogeneity greater than one are significant.

Figure 6.14 shows where the spectral differences occur between strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*. Specifically differences were found between a number of wavelengths, between 850 and 900 wavelength cm^{-1} (Unknown region) (D), 990 and 1200 wavelength cm^{-1} (Sugars and EPS region) (C), 1250 and 1300 wavelength cm^{-1} (DNA) (B) and 1720 and 1795 wavelength cm^{-1} (Nucleic acids) (A).

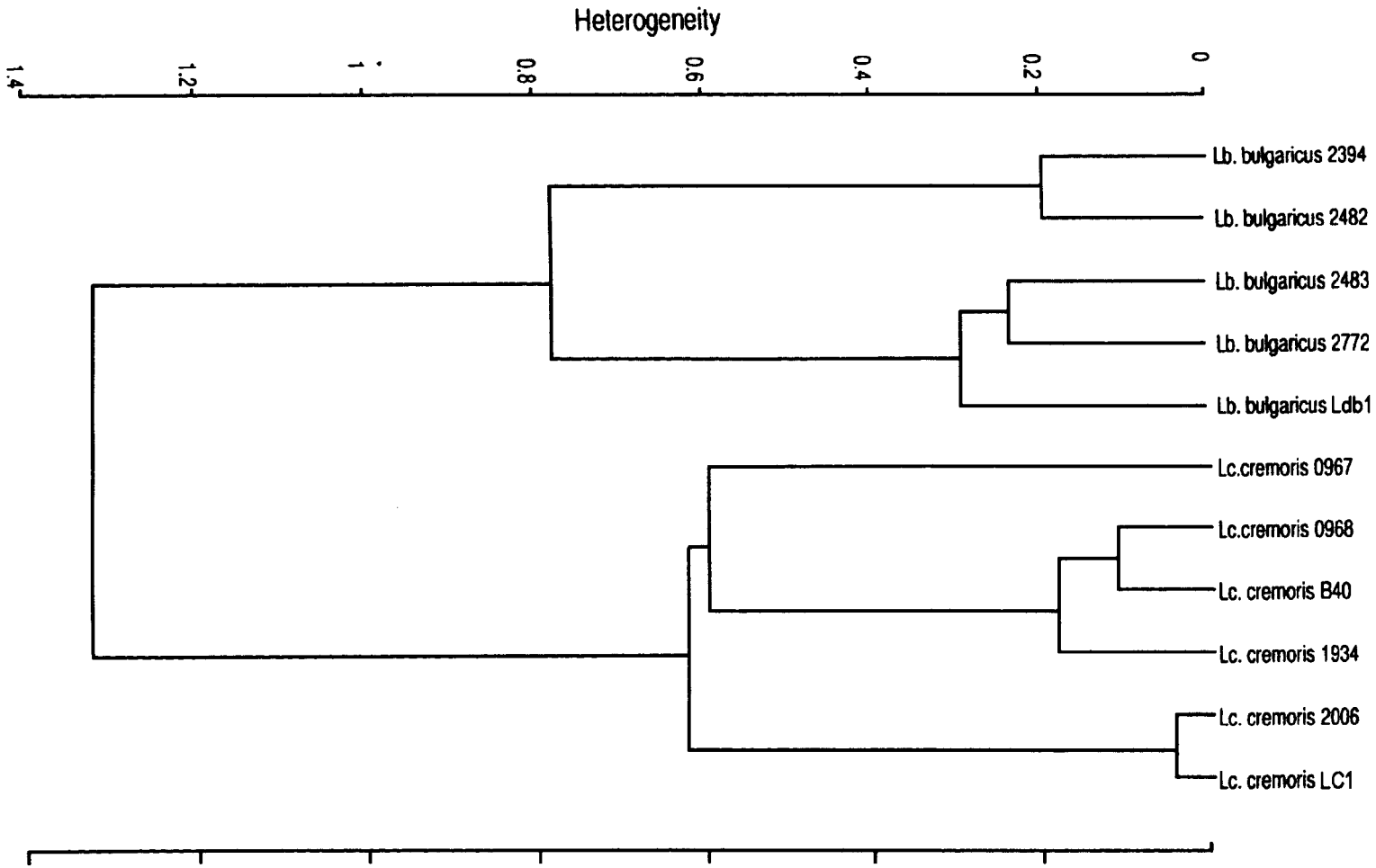


***Figure 6.13 Cluster analyses of spectra from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus.**

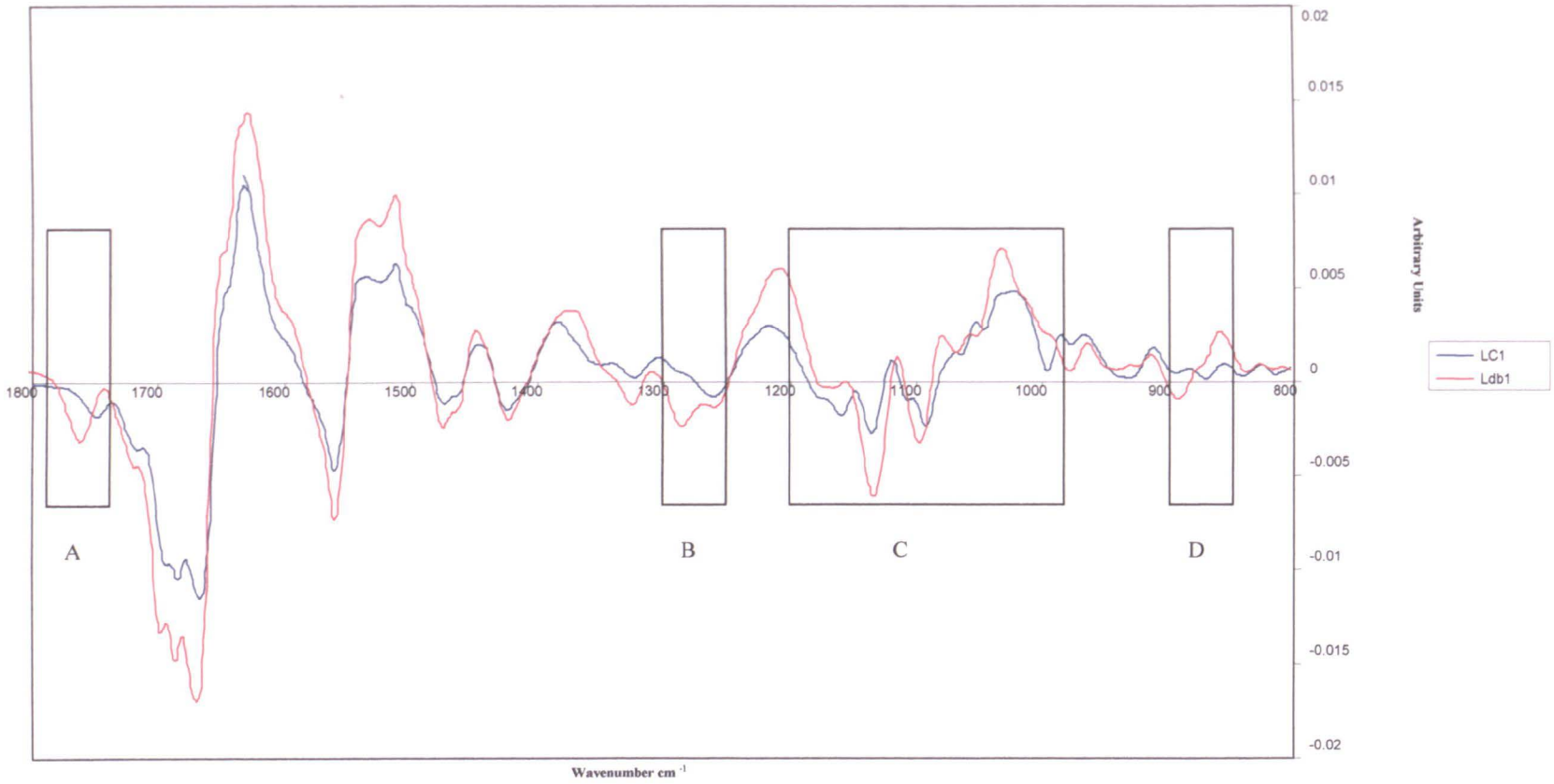


Figure 6.14. Spectra show the differences between strains of **Lc.lactis** *ssp.* **cremoris** *and* **Lb.delbrueckii** *ssp.* **bulgaricus.**

Figure 6.15 shows where the spectral differences can be found within strains of *Lb. delbrueckii* ssp. *bulgaricus*. Figure 6.16 shows where the spectral differences can be found within strains of *Lc. lactis* ssp. *cremoris*.

Within both *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*, there are differences between strains. For *Lb. delbrueckii* ssp. *bulgaricus*, strains 2483, 2772 and Ldb1 are similar but they are also slightly different from strains 2482 and 2394. However, these differences are not significant as differences less than 1 are considered not significant (Lefier, Lamprell and Mazerolles 2000). For strains of *Lc. lactis* ssp. *cremoris* strain 0967 is different from the other 5 strains. Strain 0968, B40 and 1934 seem to group together and are different from the others. Strain 2006 and LC1 are also grouped together and are different from the other strains. These differences are also not significant.

When the individual spectra for strains of *Lb. delbrueckii* ssp. *bulgaricus* were observed a number of differences could be found between 1600 and 850 wavenumbers cm^{-1}. For strains 2483 and 2772 the differences from the other strains could be found at 1630 wavenumbers cm^{-1} (Protein region). For strains 2394 and 2482 there were differences from the other strains at 1220 wavenumbers cm^{-1} (EPS region). There were differences between all the 5 strains of *Lb. delbrueckii* ssp. *bulgaricus* between 1250 and 1300 wavenumbers cm^{-1} (EPS region), 850 and 1000 wavenumbers cm^{-1} (Unknown).

When the individual spectra for strains of *Lc. lactis* ssp. *cremoris* were observed the differences were found between 1125cm^{-1} to 1075cm^{-1}. Strain 0967 was different from the other strains between 1150cm^{-1} and 1175cm^{-1} (EPS region). There is also an extra peak at 1430cm^{-1} (Protein region). Strains LC1 and 2006 were different from the other strains between 1075cm^{-1} and 1050cm^{-1}, 950cm^{-1} and 1000cm^{-1} (EPS region).

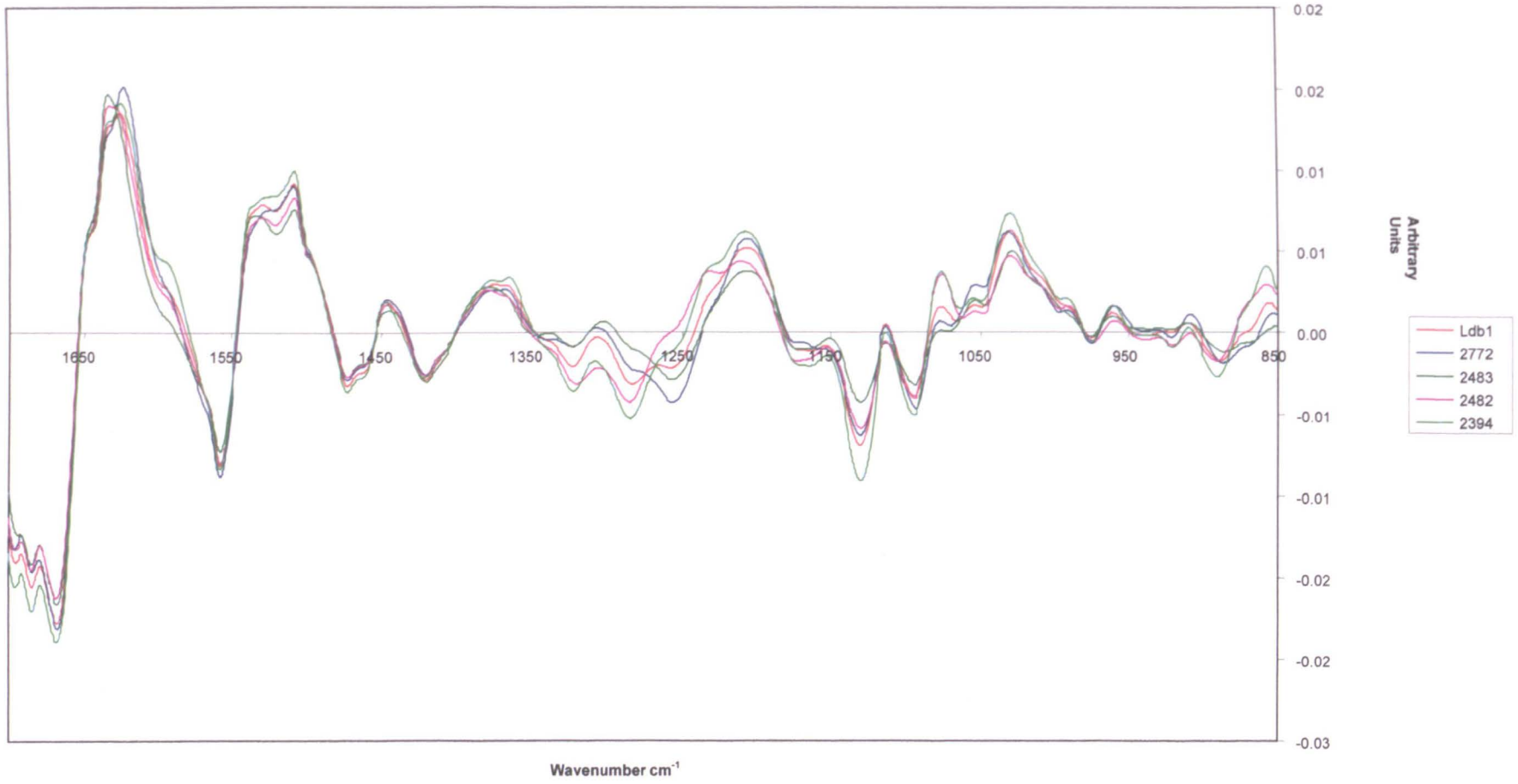


Figure 6.15 Spectra of strains of **Lb.delbrueckii ssp. bulgaricus** *between 800 and 1800 wavenumber* cm^{-1}.

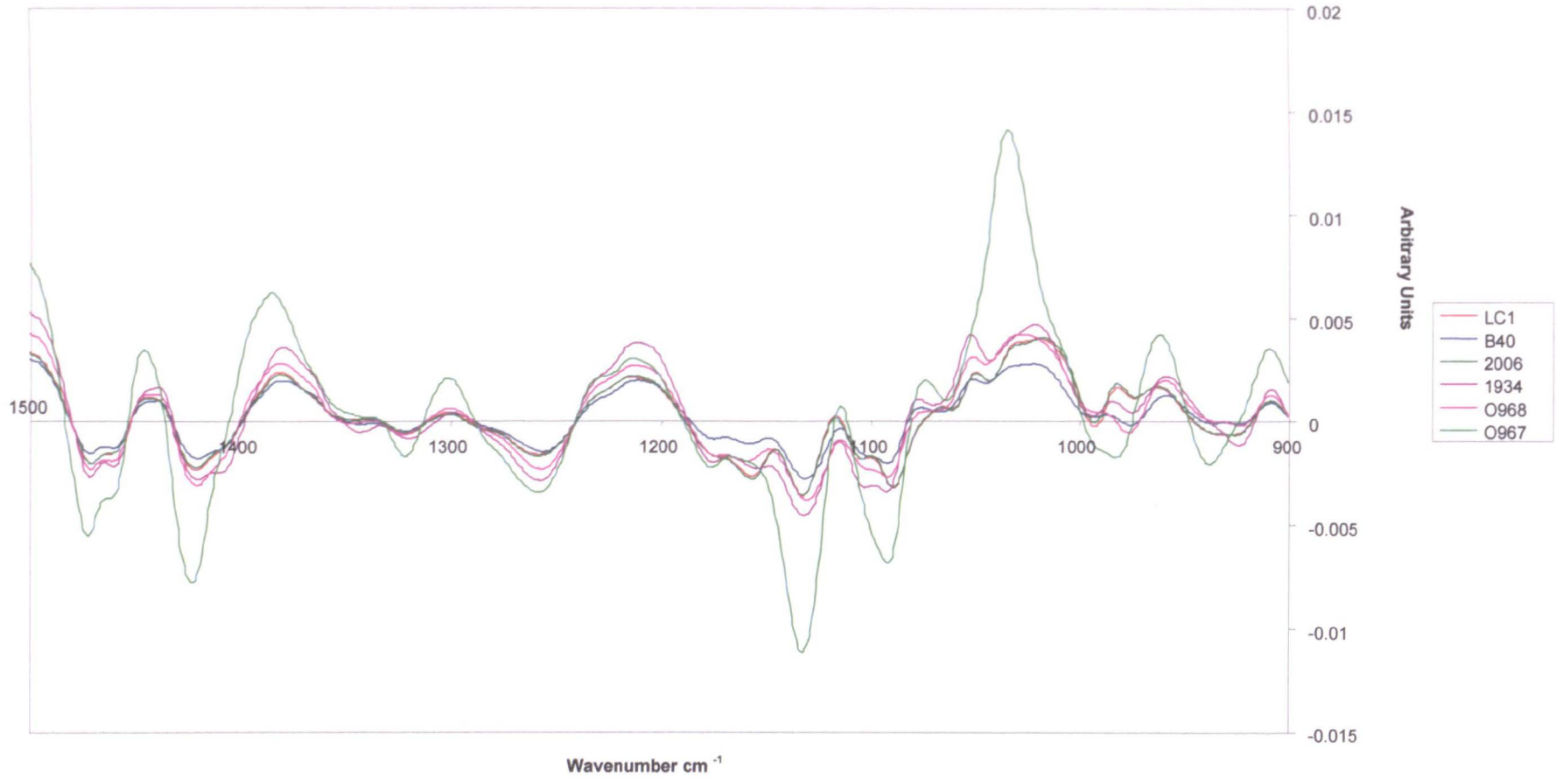


Figure 6.16 Spectra of strains of Lc.lactis ssp. cremoris between 900 and 1500 wavenumber cm^{-1}.

COMPARISON OF SPECTRA OF THE ISOLATED EPS PRODUCED BY STRAINS OF *Lc. lactis ssp. cremoris* AND *Lb. delbrueckii ssp. bulgaricus.*

Following analysis of bacterial samples using FTIR the next step was to analyse the EPS produced by different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* when grown in milk. Spectra of the EPS isolated from strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* were produced using FTIR. First derivative spectra were then calculated. These spectra were analysed using cluster analysis with the 1st derivative spectra of the bacterial strains.

Figure 6.17 shows cluster analysis of the EPS and of strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. From this, it can be seen that the spectra of the EPS of *Lb. delbrueckii* ssp. *bulgaricus* are significantly different from the spectra of the EPS spectra of *Lc. lactis* ssp. *cremoris*. Within the EPS spectra of strains of *Lc. lactis* ssp. *cremoris* there were also significant differences from the strains of *Lb. delbrueckii* ssp. *bulgaricus*. This can also be seen for the bacterial strains (Figure 6.13). However, the differences between strains within *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* were not significant.

On examination of the EPS spectra, it was not possible to directly relate this to the bacterial spectra as in the case of poly-β-hydroxybutyrate, which produces a unique peak in the FTIR spectra (Nichols et al. 1985). The samples also contained protein, cell wall constituents, and nucleic acids that were not removed during the extraction process. The sugar composition of the EPS extracted from the bacterial strains used in this study varies and this will also in turn affect the spectra produced.

However, differences could be seen between the EPS spectra of strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* (Figure 6.18). These differences are found between 700 and 1070 wavelength cm^{-1} (EPS region), at 1700 wavelength cm^{-1} (Nucleic Acids) and between 1400 and 1200 wavelength cm^{-1} (DNA/ Protein region).

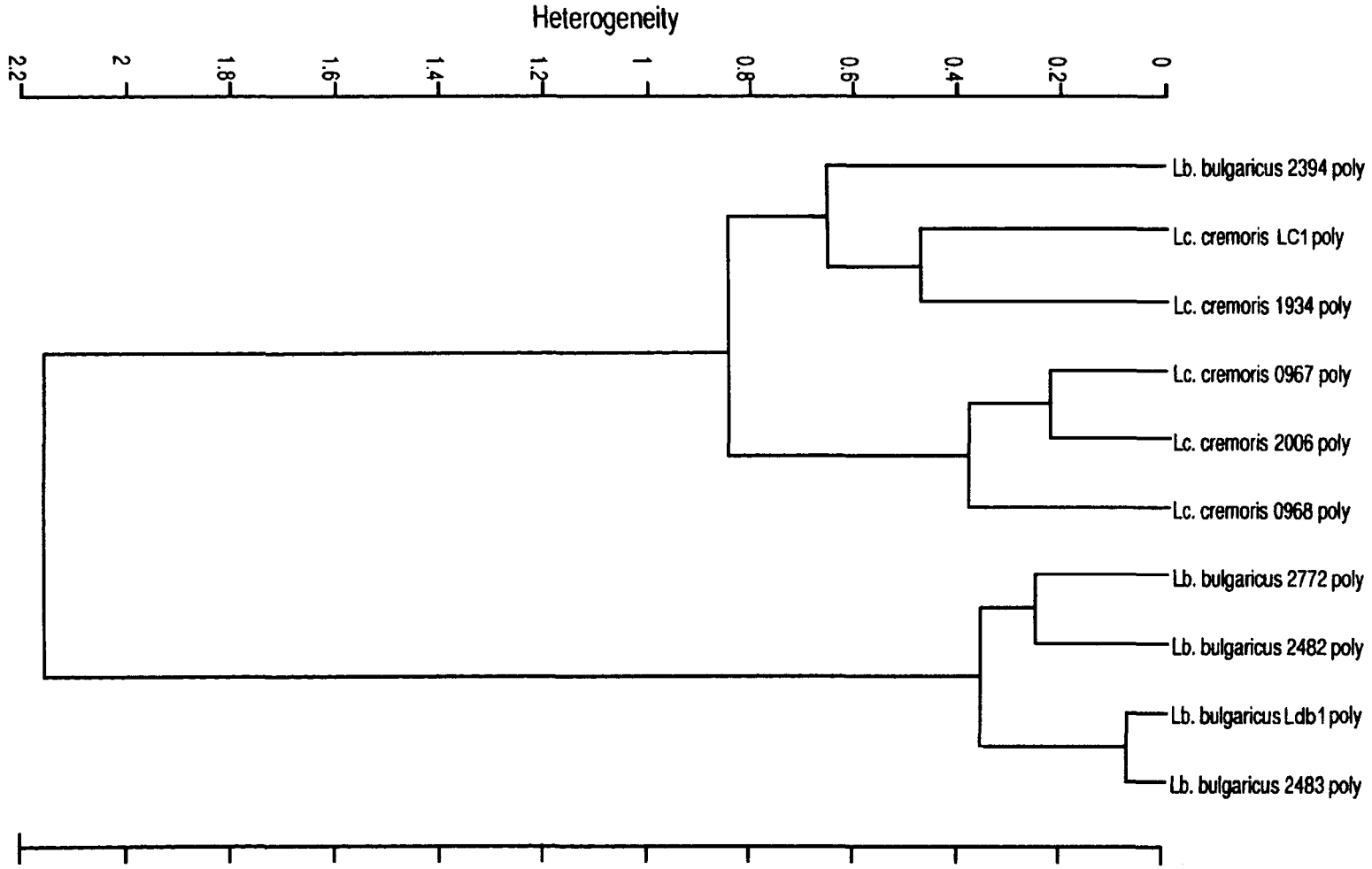


***Figure 6.17 Cluster analyses of spectra of crude polysaccharides from strains of* Lc. lactis *ssp.* cremoris *and* Lb. delbrueckii *ssp.* bulgaricus.**

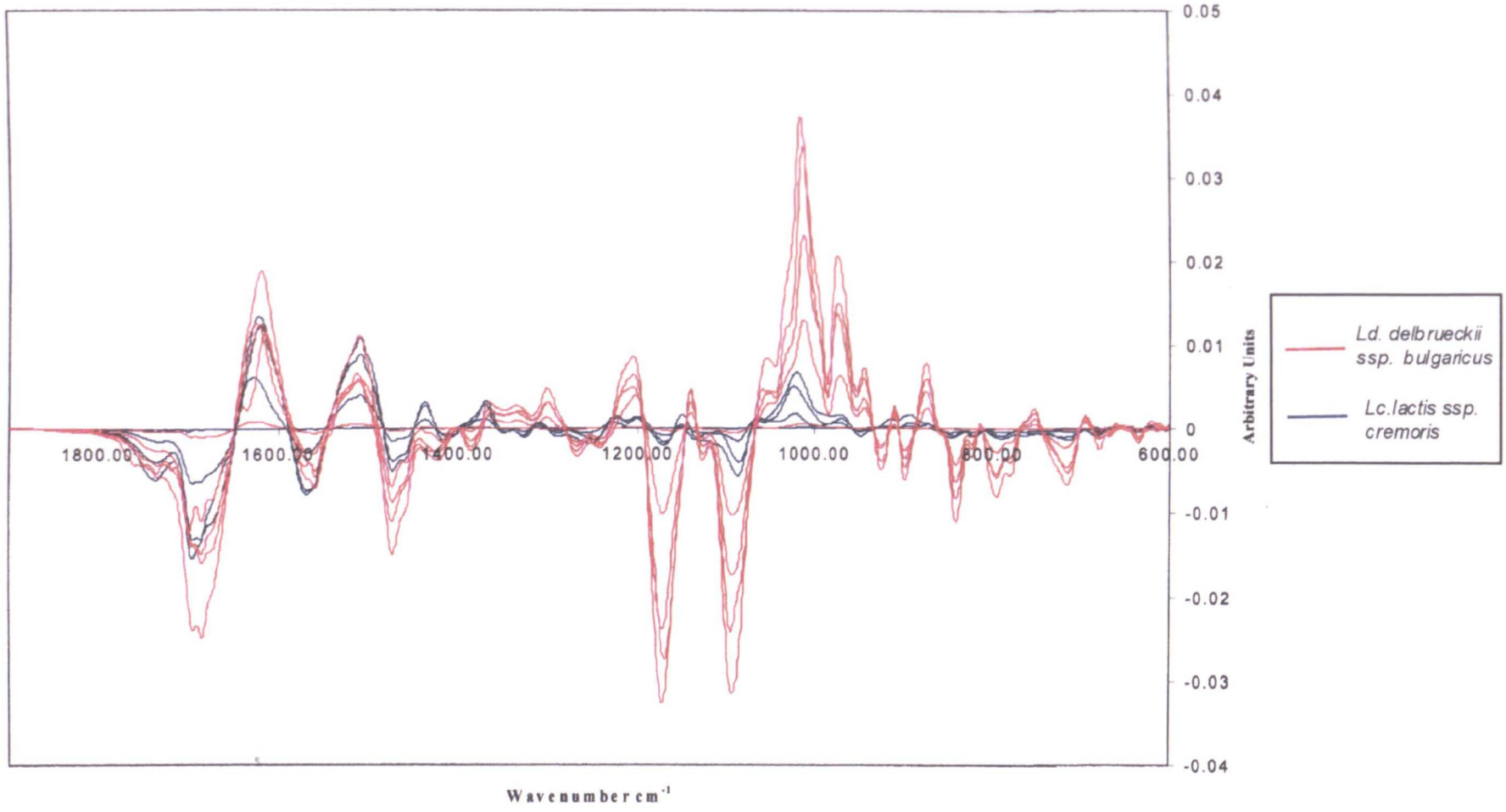


Figure 6.18. Spectral differences between polysaccharides in strains of **Lc. lactis** *ssp.* **cremoris** *and* **Lb. delbrueckii** *ssp.* **bulgaricus.**

THE EFFECT OF AGE OF CULTURE ON THE SPECTRA OF STRAINS OF *Lc. lactis* ssp. *cremoris*.

The aim of this study was to generate spectra from cultures of different ages and compare them. Strains 2006, LC1 and 1934 of *Lc. lactis* ssp. *cremoris* were grown on M17 agar plates overnight. Samples were taken for analysis by FTIR. The agar plates were then reincubated overnight and another sample taken for analysis. This process was repeated for another 2 days. The data was used for cluster analysis using the Ward algorithm (Ward 1963).

Figure 6.19 shows a dendrogram of the different strains and ages of culture. From this, it can be seen that in most strains there was a distinctive change in the spectra with time. Strain 1934 spectra were distinct from strains LC1 and 2006.

Within strain 1934 the largest change took place between 24 and 48 hours incubation, that is, between day 1 and day 2. After this the differences in the spectra were much smaller. Between days 1 – 5, the spectra become less distinct from one another. There is also a slight difference between the spectra from days 2 and 3 and 5. Strains 2006 and LC1 clustered together after 24 hours incubation (day 1). After 24 hours incubation there were large changes in the spectra. Days 2, 3, 4, and 5 are very different from day 1. There are also some spectral differences after day 4. After day 1, it is difficult to differentiate between the spectra of LC1 and 2006. The differences between the spectra were also greater than 1 so the spectra of days 2-5 are significantly different from those obtained on day 1. The spectra of 2006 and LC1 are also significantly different from the spectra obtained for strain 1934.

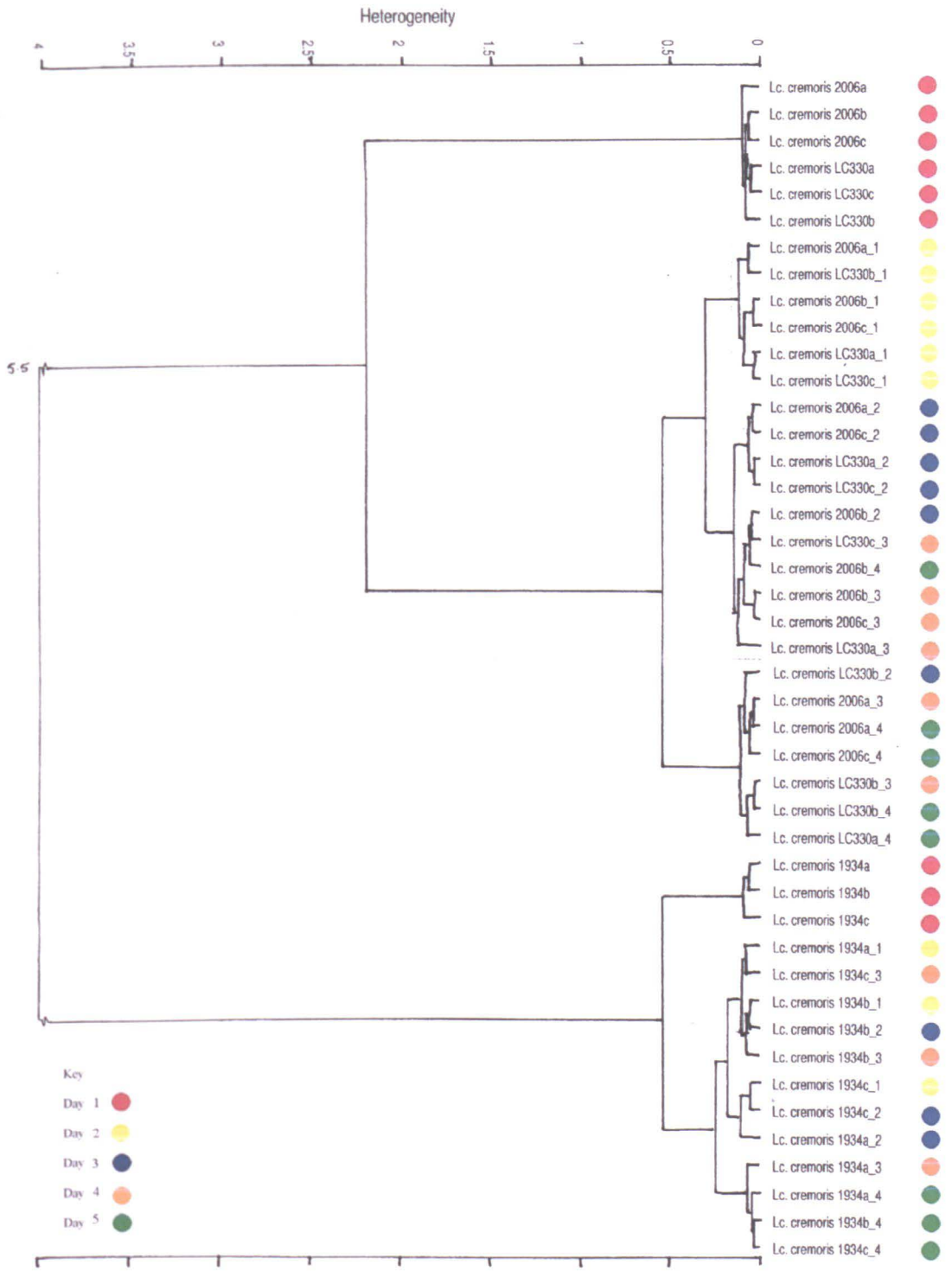


***Figure 6.19 Cluster analyses of spectra of different ages of culture from strains 2006, LC1 and 1934 of* Lc. lactis *ssp.* cremoris.**

THE EFFECT OF SOLID PHASE AND LIQUID PHASE CULTURES ON THE FTIR PROFILE OF STRAINS OF *Lc. lactis* ssp. *cremoris*.

The aim of this part of the study was to see if the nature of growth within a liquid or solid medium had an effect on the FTIR profile. All the strains of *Lc. lactis* ssp. *cremoris* were grown on solid M17 agar plates and in liquid M17 broth overnight. Samples were taken and analysed using the FTIR. The data was used for cluster analysis using the Ward algorithm (Ward 1963).

Figure 6.20 shows cluster analyses of spectra from solid phase and liquid phase cultures of *Lc. lactis* ssp. *cremoris*. From the dendrogram it can be seen that agar cultures produce spectra significantly different from liquid cultures. It can also be seen that spectra of strain 1934 are significantly different from the other strains of *Lc. lactis* ssp. *cremoris* studied.

THE EFFECT OF SUBSTRATE ON THE FTIR PROFILE OF STRAINS OF *Lc. lactis* ssp. *cremoris*.

The aim of this study was to investigate the effect of different substrates on the FTIR profiles of different strains of *Lc. lactis* ssp. *cremoris*. The strains were grown in 10 ml Cremoris Defined Media containing 0.25% of, fructose, glucose, sucrose or lactose. After 24 hours incubation samples were taken for analysis using the FTIR. The data was used for cluster analysis using the Ward algorithm (Ward 1963).

Figure 6.21shows the differences in spectra when strains of *Lc. lactis* ssp. *cremoris* are grown with different carbon sources. From the dendrogram it can be seen that carbon substrate does seem to have an effect on the spectra of different strains of *Lc. lactis* ssp. *cremoris*. Spectra of strain 1934 grown in sucrose seem to be significantly different from spectra of the same strain grown in lactose, glucose and fructose. Spectra of strain 0968 grown in sucrose are significantly different from spectra of the same strain grown in lactose and glucose. Spectra of strain 0967 grown in fructose are different from spectra of the same strain grown in glucose and lactose. Some of the spectra of strains grown in glucose and lactose are significantly different from other strains of the same bacteria grown in glucose and lactose. For strain 2006, spectra of the bacteria grown in

fructose are significantly different from spectra of the same strain grown in lactose, glucose and sucrose. Once again, some of the individual spectra of strains grown in glucose, sucrose and lactose are significantly different from spectra of other strains grown in glucose, sucrose and lactose. For strain LC1 spectra of bacteria grown in glucose are different from spectra of the same strain grown in sucrose and lactose. Some spectra of strains grown in glucose are significantly different from the spectra of other strains also grown in glucose.

DISCUSSION

As mentioned in the general introduction Fourier Transform infrared spectroscopy (FTIR) is a technique that has been used to investigate the structure of bacteria and has been used to a limited extent in the identification of bacteria (Stuart 2008). These bacteria were taken from specimens where it was difficult to culture the bacteria on a large scale. The advantage of this technique as a method of bacterial identification is that FTIR is a very easy analysis to perform. There is very little pre-preparation of the sample required which could lead to external contamination or DNA damage during sample preparations. FTIR has yet to be used on a large scale for bacterial identification. The aim of this section of the study was to develop a method for using FTIR to identify different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. Following this, the next step was to try and separate out EPS and non-EPS producing strains by identifying a unique peak on the spectra for EPS similar to the peak found for poly-β-hydroxybutyrate.

Once the method had been developed it was necessary to determine any factors that may interfere with the identification. The ultimate goal was to develop a method that could eventually be used in the dairy industry to quickly and efficiently identify whether a particular strain of lactic acid bacteria produced EPS and whether an EPS producer continued to do so.

Using FTIR it was possible to generate absorption spectra for all strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. First derivatives of the spectra were calculated and analysed using the Ward algorithm which showed that spectra of strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* were significantly different.

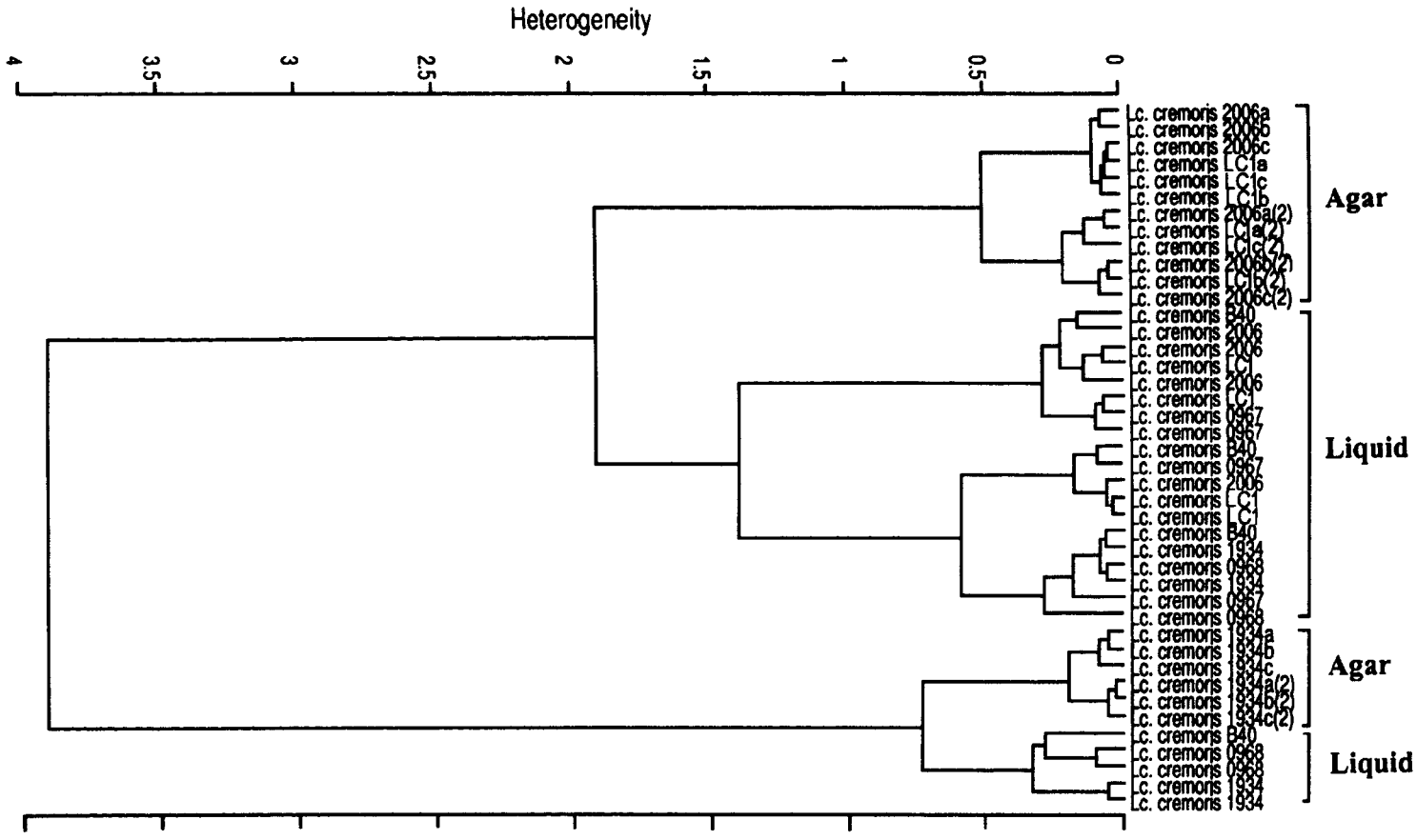


***Figure 6.20 Cluster analyses of spectra of different phases of media on strains of* Lc. lactis *ssp.* cremoris.**

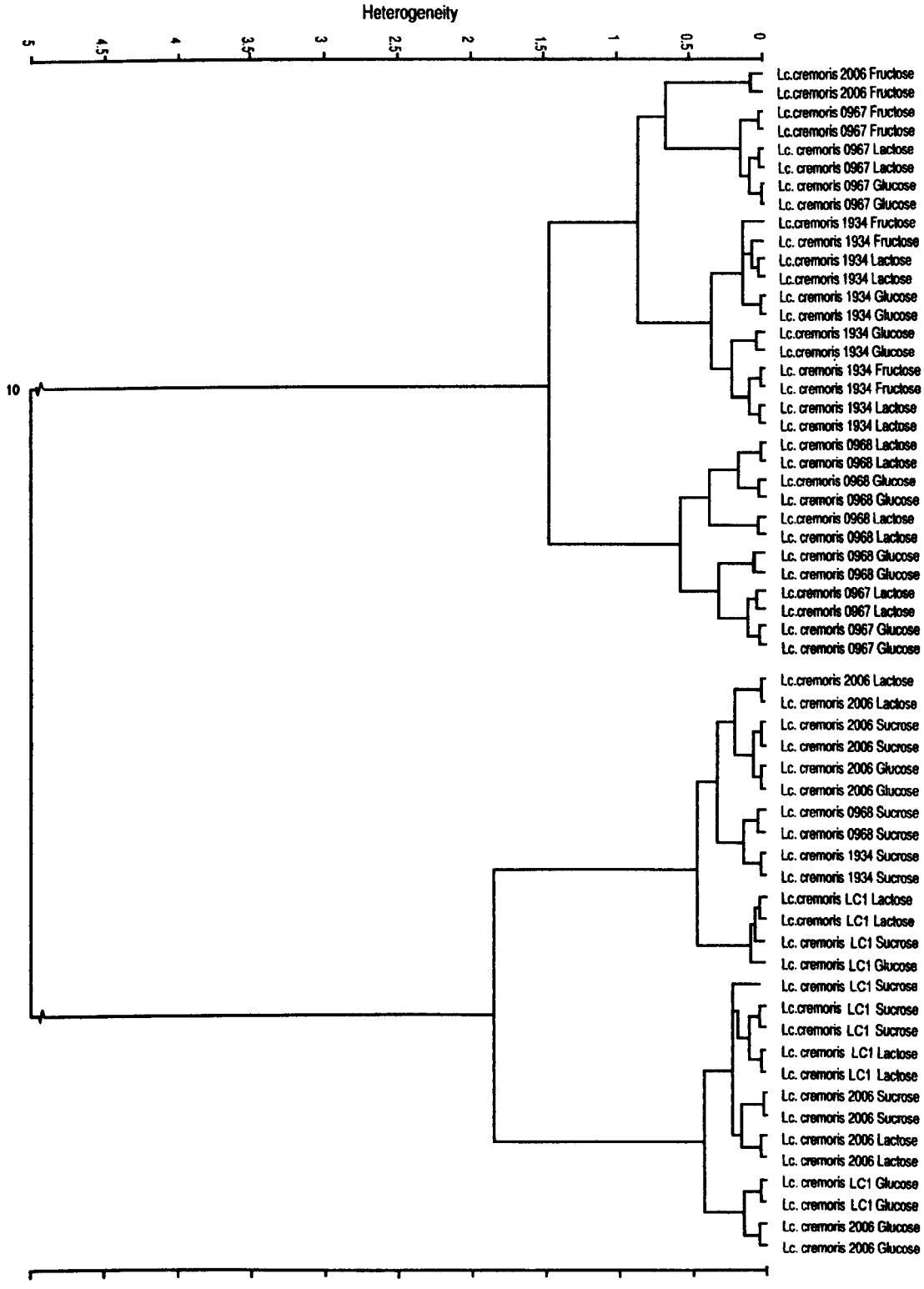


Figure 6.21 Cluster analyses of spectra from strains of* Lc. lactis *ssp.* cremoris *grown in media containing different substrates.

Any differences generated that are greater than 1 is considered to be a significant difference (Lefier, Lamprell and Mazerolles 2000). Therefore it is possible to use FTIR as a method for separating strains of *Lc. lactis* ssp. *cremoris* from strains of *Lb. delbrueckii* ssp. *bulgaricus*. The Ward analysis showed that it was possible to differentiate between the different strains within *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. However, the differences were not significant. This however does not rule out the possibility of using this technique to identify strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. Analysis could further be improved by the creation of a library of spectra of different strains of lactic acid bacteria for comparative purposes. On further examination of the spectra the differences between *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* can be found in the lipid region (wavenumber 2800-3100cm^{-1}) and the EPS region (wavenumber 1000-1300cm^{-1}). The differences in the lipid region are due to differences in the composition of the cell wall between *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. The differences in the EPS region show that the structure of the EPS differs between the two strains. There are also differences found in the region that corresponds to vibrations between the base and ribose sugar in the DNA. This would be expected as the different species are likely to have different quantities of the individual bases.

The differences in the polysaccharide region between strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* are important in terms of meeting the aims of the study. This is where it was anticipated that differences would be found. Structural studies of the extracted EPS from different strains of lactic acid bacteria (Table 2.1) show variation in the sugar composition of the EPS. Results reported in chapter 2 show that the structural composition of the EPS varies between strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. The differences in this region as seen in the FTIR spectra could be related to the differences in the sugar composition of the polysaccharides and this would give rise to differences in the spectra. This work also shows that as well as differences in the polysaccharide composition between *Lc lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* there are also differences in composition of the polysaccharides between strains. This agrees with the results of chemical analysis described in chapter 2. With further work it may well be possible to use this technique to produce unique profiles for the EPS

produced by different strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. FTIR could then be used to identify these strains based on the EPS they produce. It could also be used to identify whether the strains are EPS producing strains or not.

The next stage was to study isolated EPS to try and produce a distinct profile for the individual EPS. Analysis of the EPS spectra showed they were significantly different from the spectra of the bacteria. This was the situation for both strains of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii ssp. bulgaricus*. This is not unexpected, as the EPS should not contain all of the same components that are found in the bacteria. Within the EPS spectra, there were significant differences between the spectra of *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* but not between strains within *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. The spectral differences between the EPS of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* occurred in the EPS (wavenumber 1000 –1300 cm^{-1}), nucleic acid/DNA (wavenumber 1500 - 1800 cm^{-1}, wavenumber 1210 - 1290 cm^{-1}) and protein (wavenumber 1400 – 1800 cm^{-1}) regions of the spectra. The differences in the EPS region were not unexpected as the structure of the EPS differs between different strains of lactic acid bacteria. However, the differences in the other regions are probably contaminants in the sample, such as proteins, cell wall constituents and bacterial DNA acids. This is a further indication that the EPS was not completely pure and needed further purification (See Chapter 2).

This impurity of the EPS means that at present it is not possible to make direct comparisons between the spectra of the EPS and the bacterial spectra. The EPS do not produce a distinct unique peak that can be used to indicate whether the bacterium is an EPS producer or not. The inability to produce distinct peaks could also be due to the differences in the sugar composition of the EPS that should produce different spectra. If the crude EPS can be purified this may help improve the analysis using FTIR and this is something that could be undertaken as future work.

Other experiments showed that the age of the bacterial culture, the substrate composition of the media and the phase of the media also have a significant effect on the ability to separate out the bacterial strains using the FTIR.

Many authors including Cerning et al (1992); Grobben et al (1998); Kimmel and Roberts (1998) and Garcia-Garibay and Marshall (1991) (Table 3.1) have shown that carbon source has an effect on the quantity of EPS production from strains of lactic acid bacteria and this was also confirmed by our own work. Figure 3.2 shows that changing the carbon source does have an effect on the quantity of EPS produced by strains of *Lc. lactis* ssp. *cremoris*. As we saw in Chapter 2 it was difficult to achieve 100% purity when EPS was extracted from milk. This could also apply to strains that were grown in chemically defined media where the carbon source is changed. The extracted EPS could have contained components of the media trapped within its structure and this would have lead to different FTIR spectra being produced as a result of chemical differences. Another explanation is that the bacterium incorporates only the available sugars into the EPS that it produces and this would lead to the production of different FTIR spectra, unique to the carbon source. This is only conjecture and this would need further investigation as to whether the structure of the EPS is altered by the carbon source utilized for growth.

Lactic acid bacteria grown on agar plates are found in isolated colonies and have a sticky consistency as a result of EPS production. The EPS remains attached to the bacteria unlike bacteria grown in liquid media where the EPS is excreted into the liquid media. The differences in the states of the bacteria in the media could lead to the production of unique compounds which would lead to differentiation on the FTIR spectra.

As bacteria age they will produce different enzymes and chemicals for cellular function and as a result this would lead to different peaks being produced on the FTIR spectra that would lead to the differentiation of the bacteria due to age.

In this study it was possible differentiate between different bacterial strains according to the age of the culture and the growth conditions. This means that to make sure experiments using the FTIR are comparable bacterial culture should all be treated in the same way. This was also found by Curk, Peladan and Hubert (1994) who found that growth temperature had an effect on separating out FTIR spectra. FTIR is a useful technique for identifying differences in bacteria due to growth conditions.

The work carried out in this study has shown that FTIR has great potential as an identification tool and could also be useful in other areas in the study of lactic acid bacteria. This study has given us an insight to a number of possible uses and with further work the technique could be refined to produce useful data. Other work that could be carried out in the future could be a detailed structural analysis using FTIR of the EPS produced by the strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* used in this study. Another possible study could be the use of the FTIR to find specific differences between EPS producing and reduced EPS producing colonies of the same strain. If this was possible it may help to identify differences in the bacteria that could cause the variability in the texture of batches of fermented milk.

CONCLUSIONS

Using the FTIR it was possible to generate absorption spectra for all strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. It was also possible to separate different strains within both *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*.

On examination of the spectra, it was found that within *Lb. delbrueckii* ssp. *bulgaricus* differences between strains were found in the protein and EPS regions of the spectra. Within strains of *Lc. lactis* ssp. *cremoris* the differences were found in the protein and EPS regions of the spectra. The differences between *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* were found in the DNA and polysaccharide regions of the spectra.

The spectra of EPS for strains of *Lb. delbrueckii* ssp. *bulgaricus* EPS were all fairly similar, as were the EPS spectra for strains of *Lc. lactis* ssp. *cremoris*. There were however some distinct differences between the spectra of the EPS of *Lb. delbrueckii* ssp. *bulgaricus* and the spectra of EPS of *Lc. lactis* ssp. *cremoris*.

It was not possible to directly relate the polysaccharide spectra to those of the bacteria. Direct comparison of the polysaccharide spectra for both *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* showed that spectral differences between the strains were found in the polysaccharide region. Differences in nucleic acid, DNA and protein regions were most likely contaminants.

The spectra of strains of *Lc. lactis* ssp. *cremoris* were shown to change with time. The greatest differences were shown between cultures grown for 24 and 48 hours respectively. After 48 hours, the cultures could not be reliably separated by cluster analysis. It was easier to discriminate between strains in young cultures incubated up to 24 hours.

The nature of the growth medium was also shown to have an effect on the spectra. There were significant differences between agar and liquid cultures. Growth substrate also has an effect on the spectra and therefore the ability to differentiate between different strains.

Chapter 7. OVERALL DISCUSSION

The ability of some strains of lactic acid bacteria to produce an exopolysaccharide (EPS) is of great interest to the dairy industry. EPS can provide yoghurt, particularly low fat yoghurt, with improved textural properties by thickening the final product without the addition of thickening agents. The problem is that this characteristic appears to be unstable and the resultant loss of textural properties in the final product can have major financial implications. In order to ensure consistency of texture it is therefore important to have a good understanding of how EPS is produced by lactic acid bacteria and the textural role of EPS within yoghurts.

The first part of the study (Chapters 2 – 5) involved gaining a better understanding of EPS production by strains of *Lc.lactis* ssp. *cremoris* and strains of *Lb. delbrueckii* ssp. *bulgaricus*. The second section of the study (Chapter 6) involved evaluating the use of the technique known as Fourier Transform infrared spectroscopy (FTIR) as a method of identifying EPS producing bacteria and its use in the analysis of the ability of bacteria to produce EPS.

7.1 Isolation and characterisation of EPS from different strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*.

The initial aim of the study was to maximize the production of EPS from different strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*. This was to enable the production of large enough quantities of crude EPS that could be used for further analysis. Once the crude EPS from the different strains had been isolated the next task was to analyse the structural compositions of each individual EPS. Using methods published by Kimmel, Roberts & Ziegler (1998) and Cerning et al. (1992) it was possible to isolate enough EPS from all 5 strains of *Lb. delbrueckii* ssp. *bulgaricus* and all 6 strains of *Lc. lactis* ssp. *cremoris* for further analysis. It was also observed that *Lb.delbrueckii* ssp. *bulgaricus* 2394 produced some EPS even though it was not considered to be an EPS producing strain. The strain *Lc.*

lactis ssp. *cremoris* B40 and strain *Lb. delbrueckii* ssp. *bulgaricus* Ldb1, both used in the dairy industry, produced the largest quantities of EPS (Table 2.4). The variability of quantities of EPS produced by strains of lactic acid bacteria has been shown in other studies (Petry et al 2003; Marshall and Rawson 1999; Laws and Marshall 2001).

In the literature (see Chapter 2) it has been reported that there are a number of structural differences between EPS produced by different strains of the same bacteria (Table 2.1). Differences in sugar composition, molecular mass, and the possibility of the polysaccharide having a charge were all investigated as these are thought to play a role in the ability of the EPS to form a viscous solution (Marshall, Cowie and Moreton 1995). This would therefore have an important role as to whether the bacteria can produce yoghurt with the desired texture. The characterisation would give an indication of the similarities and differences between EPS from different strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*.

The analysis of carbohydrate content showed that in general strains of *Lb.delbrueckii* ssp. *bulgaricus* produced EPS with higher carbohydrate content than strains of *Lc.lactis* ssp. *cremoris* (Table 2.5). It was also noted that of the samples tested strains *Lb.delbrueckii* ssp. *bulgaricus* Ldb1 and *Lc.lactis* ssp. *cremoris* LC1, used in the dairy industry, produced the EPS containing the highest carbohydrate content. There is a lack of data available in the literature on the carbohydrate content of EPS isolated from strains of lactic acid bacteria making it very difficult to identify reasons for the variability in EPS content. All the EPS samples tested from strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris* contained some protein (Table 2.6). This may have been due to the technique used to isolate EPS, but protein does not seem to account for all of the possible contamination of the EPS. Both the methods used for isolation of the EPS and the method used to analyse the carbohydrate content need improving. FTIR analysis of crude EPS showed the EPS also contained cell wall components, proteins and nucleic acids (Figure 6.18). This could account

for the variability in the carbohydrate and protein values determined. The variation in FTIR spectra for the crude EPS was also due to the differing sugar composition of the EPS.

Determining the molecular weight of the EPS isolated from the different strains of *Lc.lactis* ssp. *cremoris* and *Lb.delbrueckii* ssp *bulgaricus* showed that there seemed to be variability in molecular weight between the different strains (Table 2.7). It was observed that all the EPS had a large molecular weight and this was similar to the observations of Grobben et al (1997), Marshall, Cowie and Moreton (1995) and van Casteren et al (1998) who also noted that strains of *Lc.lactis* ssp. *cremoris* and *Lb.delbrueckii* ssp *bulgaricus* had large molecular weight EPS. It could also be noted that the results for the molecular weight of strains *Lc.lactis* ssp. *cremoris* B40, *Lc.lactis* ssp. *cremoris* LC1 and *Lb.delbrueckii* ssp *bulgaricus* 2772 were slightly larger than the published data for those strains (Van Casteren et al. 1998, Marshall, Cowie and Moreton 1995 and Grobben et al. 1997). These differences were most likely due to the resolving power of the column used to determine the molecular weight of these strains. However, it is still possible to show that there was some variability in the molecular weight of different strains. It has yet to be investigated but there could also be a link between the molecular weight of the EPS and their textural forming abilities. When looking at the textural data gathered for this study (Chapter 5) it could be observed that the strains of *Lc.lactis* ssp. *bulgaricus*, which had the smaller molecular weight EPS produced harder and more viscous gels than strains of *Lb.delbrueckii* ssp *cremoris*.

When analysis of the composition of the different strains of *Lb. delbrueckii* ssp *bulgaricus* and *Lc. lactis* ssp. *cremoris* was undertaken it was determined that there was a definite variation in the sugar composition of the EPS produced by the different strains (Table 2.9). Grobben et al. (1997) observed that the ratio of glucose:galactose:rhamnose in *Lb.delbrueckii* ssp *bulgaricus* was very similar to that observed in our own study. This was also found when comparing the observed ratio of glucose:galactose:rhamnose ratio with that observed by Marshall et al. (1995).

It was also noted that the extracted EPS contained charged and uncharged fragments (Table 2.8). This was also noted by Marshall, Cowie and Moreton (1995) who found that *Lc.lactis* ssp. *cremoris* LC1 produced two EPS with different molecular weights. One EPS was charged and one was not.

Post extraction purification of the EPS produced by different strains of *Lc.lactis* ssp. *cremoris* using HPLC did seem to remove some of the impurities without having any major effect on the sugar composition of the EPS (Table 2.14). However, the process did not seem to remove much of the protein in the EPS (Table 2.12).

From this section of the study it can be seen that each of the EPS produced by strains of *Lc.lactis* ssp. *cremoris* and strains of *Lb.delbrueckii* ssp *bulgaricus* have a unique composition. It could be that these differences are what give the EPS its unique textural forming abilities and it makes some strains of lactic acid better than others for use in the in the yoghurt manufacturing process.

7.2 Identification of the factors that affect the quantity of EPS production in strains of *Lb.delbrueckii* ssp. *bulgaricus* and *Lc.lactis* ssp. *cremoris*.

It has been shown in various studies that there are a number of different factors which affect the quantities of EPS produced by strains of lactic acid bacteria (Table 3.1). Due to the significance of EPS in the role of texture it is important to determine what factors affected EPS production. These could then be controlled in the yoghurt manufacturing process to optimize EPS production to create the ideal textured yoghurt. For each of the strains of *Lc.lactis* ssp. *cremoris* some of the factors that affected EPS production were investigated. Only *Lc.lactis* ssp. *cremoris* was investigated in this part of the study as it required the use of a specifically defined medium to allow for quantitative analysis of EPS in the media and a suitable defined medium has yet to be developed for *Lb.delbrueckii* ssp. *bulgaricus*.

It was determined that the growth substrate of the media (Figure 3.2), temperature (Figure 3.1) and agitation (Figure 3.3) all had an effect on the growth of all the strains and as a result

EPS production. EPS is produced in the primary stage of growth when the enzymes involved in synthesis are working at their optimum rate (Manca de Nadra et al. 1985; Grobben et al. 1995; deVuyst et al. 1998) and therefore EPS production is going to be linked to the growth of the bacteria. Other than the factors that were investigated in this study Table 3.1 shows that there seems to be a number of other factors that influence the growth of strains of lactic acid bacteria such as, pH, subculturing and amino acid composition of the media. Therefore, in the yoghurt manufacturing industry it would be necessary to find the optimal growth conditions for the starter culture to be used so as to optimise EPS production.

It was also noted that EPS production was less in defined media than when the strains were grown in milk (Table 3.4). This may be because the defined media is only providing the minimal growth requirements so they will not be the optimum for EPS production. A study of the EPS production of individual colonies of strains of *Lc.lactis* ssp. *cremoris* showed that there was quite a large variability between colonies within strains. This could be due to the level at which the genes for EPS production are expressed in different bacteria and this could be one of the reasons for the variability of batch quality within the dairy industry as has also been suggested by Petry et al. (2003) and Marshall and Rawson (1999). As well as the quantity of EPS produced there are a number of other factors that could be linked to the ability of a strain to produce good textured yoghurt. These are the growth of the bacteria, the structure of the EPS and the level to which the genes of EPS production are expressed.

7.3 Investigation into the genetic instability of EPS production by strains of *Lc.lactis* ssp. *cremoris*.

The production of EPS by strains of lactic acid bacteria is controlled by a series of genes. In strains of *Lc.lactis ssp. cremoris* these genes are usually found on a plasmid. The inconsistencies in EPS production are linked to factors that affect the growth of the bacteria as well as the structure of the isolated EPS. However, they could also be linked in some way to

the interruption of the genes involved in EPS production or the loss of a plasmid containing the genes.

Work by van Krannenberg et al. (1997) found that *Lc.lactis ssp. cremoris* B40 contained a plasmid 40kb in size that possessed the genes responsible for the production of EPS. It was assumed that this plasmid could was to be found in all strains of *Lc.lactis ssp. cremoris*. This bacterial strain was used as a reference strain to see if a 40 kb plasmid could be found in other strains of *Lc. lactis ssp. cremoris*. Further analysis would have been needed for conformation but this was not possible in this study. It was not possible to investigate strains of *Lb. delbrueckii* ssp. *bulgaricus* as they rarely contain plasmids (Pouwels and Leer 1993). It was found that all the strains of *Lc.lactis ssp. cremoris* tested contained a 40kb plasmid. When the same study was carried out on the individual colonies most of them also contained a 40kb plasmid. In order to determine whether or not this plasmid contained genes responsible for EPS production attempts were made to remove it from the strains of *Lc. lactis ssp. cremoris* under investigation. Neither subculturing at elevated temperature nor the use of the chemical acriflavin was found to be successful in its removal. This could be because the plasmid also contains genes necessary for bacterial survival.

The variability in quantity of EPS produced by the different colonies within a strain suggests that the level to which these EPS producing genes are expressed varies. Further work needs to be carried out to investigate levels of expression and the possible effect of insertion sequences on the loss of EPS production.

7.4 Investigation of the textural properties of EPS when strains of *Lc.lactis* ssp *cremoris* and *Lb.delbrueckii* ssp. *bulgaricus* are grown in milk.

When lactic acid bacteria are grown in milk they produce EPS which interacts with the milk to produce a thick gel which improves the rheology of the final fermented product. The EPS contributes to the texture, mouth-feel and stability of the final product. The texture of milk produced by different strains of *Lc.lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*

and the effect of sub culturing and elevated temperature on the loss of this EPS producing ability were investigated. It was noted that strains of *Lb. delbrueckii* ssp. *bulgaricus* produced harder and more viscous gels than strains of *Lc.lactis* ssp. *cremoris* but the strains of *Lb. delbrueckii* ssp. *bulgaricus* produced smaller molecular weight (Table 2.7), and smaller quantities of EPS (Table 2.4) than strains of *Lc.lactis* ssp. *cremoris*. This further enhances the idea that it is not just the quantity of EPS produced that affects the texture of the final product. When strain *Lb. delbrueckii* ssp. *bulgaricus* Ldb1 was sub cultured over time it was noted that there was some decrease in the viscosity of the final product. With strain *Lb. delbrueckii* ssp. *bulgaricus* 2772 there seemed to be variability between subcultures on different days. This is an indication that a fresh starter culture should be used in the yoghurt manufacturing industry. The loss of viscosity could also have been due to the fact that over time as yet unidentified and uncharacterised bacterial enzymes had degraded the EPS. The changes in viscosity over time could have been as a result of different quantities of EPS being produced on each subculture and this would need further investigation.

7.5 Investigation of the use of Fourier Transform infra-red spectroscopy (FTIR) as a method for studying strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris*.

Within the strains of lactic acid bacteria used in the manufacture of yoghurt identification of individual strains as well as their ability to produce EPS is necessary. At present the only method that can be used to identify EPS and non EPS producing strains is to grow them on agar plates. They are then touched with a sterile toothpick and those that produce strands are classed as EPS producers (Toba et al. 1990). This method is very subjective and not very accurate. The final aim of the study was to use the technique of Fourier Transform infra red spectroscopy to characterise different strains of *Lc.lactis ssp. cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. It was also used to determine if EPS produced a unique peak within the spectra. This technique could then be used to monitor EPS production during the manufacture of yoghurts.

A workable method for studying the bacteria was created and subsequently used to separate strains *Lc. lactis ssp. cremoris* and *Lb. delbrueckii* ssp. *bulgaricus*. When the spectra for the strains were studied it was found that differences between *Lc.lactis ssp. cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* could be found in the regions of the spectra that correspond to the vibrations in the DNA and polysaccharides (Figure 6.14). When spectra for strains of *Lc.lactis ssp. cremoris* were studied it was noted that differences occurred in the regions corresponding to polysaccharide and protein (Figure 6.16). The differences in the polysaccharide region are probably due to differences in the sugar and structural composition of the EPS (Chapter 2). Differences between strains of *Lb. delbrueckii* ssp. *bulgaricus* were also found in the regions that correspond to polysaccharide and protein (Figure 6.15). The protein observed was probably that left behind from the extraction process. It was not possible to directly relate the polysaccharide spectra to those of the bacteria. Direct comparison of the polysaccharide spectra for both *Lb. delbrueckii ssp. bulgaricus* and *Lc.lactis ssp. cremoris* showed that spectral differences between the strains were found in the polysaccharide, nucleic acid, DNA and protein regions (Figure 6.18). Due to the fact that it was not possible to identify a unique peak to represent the EPS, it was not possible to link this to the quantities of EPS produced by the different strains of lactic acid bacteria. It was also noted that factors such as the age of the culture and the growth medium also had an effect on the quality of the spectra produced and the ability to separate strains. If this technique was to be used commercially to study lactic acid bacteria the cultures need to be fresh and all grown in the same media.

The work carried out in this study has shown that FTIR has great potential as an identification tool and could also be useful in other areas in the study of lactic acid bacteria. Other work that could be carried out in the future could be a detailed structural analysis of the EPS produced by the strains of *Lb.delbrueckii ssp. bulgaricus* and *Lc.lactis ssp. cremoris* used in this study. Another possible use for FTIR could be to identify specific differences between EPS producing colonies and those which have partially lost their EPS producing ability within the

same strain. If this was possible it may help to identify differences in the bacteria that could cause the variability in the texture of the batches when grown in milk.

7.6 Summary

This study has given us new information on the structure and the production of EPS by strains of *Lb.delbrueckii ssp. bulgaricus* and *Lc.lactis ssp. cremoris*. There are a number of possible reasons as to why the dairy industry suffers with inconsistencies in yoghurt manufacture as a result of EPS production. The degree of viscosity and hardness does not solely depend on the amount of EPS produced because there was no obvious correlation between the EPS produced and the viscosity and hardness of the fermented milk. There is also a possible link between the size and structure of the EPS and its interaction with the components of the milk to produce the desired textured of the final product. It was also observed that there was variability of EPS production between colonies within single strain of bacteria. This suggests that the regulation of the genes of EPS production varies within a strain and this is something that needs to be investigated further. The results of this study suggest that loss or reduction in EPS production is not necessarily explained by the loss of a plasmid. There was a problem with the purity of the isolated EPS and subsequent attempts to purify yielded minimal success. There has been very little published on the purity of isolated EPS or even methods for purifying EPS. This is obviously an area where work could be undertaken and this may help in understanding more about the structure and functionality of the EPS. The use of FTIR was novel and it allowed differentiation between strains of *Lb.delbrueckii ssp. bulgaricus* and *Lc.lactis ssp. cremoris*. There is further potential to develop this technique to provide a quick and easy method of identifying strains of lactic acid bacteria and monitor their EPS producing ability. Further analysis of FTIR spectra could also give us a better understanding of the structure and composition of EPS from different strains of lactic acid bacteria.

7.7 Conclusions

- It was possible to isolate exopolysaccharide from all strains of *Lb.delbrueckii ssp. bulgaricus* and *Lc.lactis ssp. cremoris* used in this study.
- *Lb.delbrueckii* ssp. *bulgaricus* 2394 produced some EPS even though it was not considered to be an EPS producing strain.
- Strains of *Lb.delbrueckii* ssp. *bulgaricus* produced EPS with higher carbohydrate content than strains of *Lc.lactis* ssp. *cremoris.*
- Strains of *Lc.lactis* ssp. *cremoris* and *Lb.delbrueckii* ssp *bulgaricus* all produced large molecular weight EPS.
- There was a variation in the sugar composition of the EPS produced by the different strains of *Lb.delbrueckii* ssp *bulgaricus* and *Lc.lactis* ssp. *cremoris.*
- Strains of *Lb.delbrueckii* ssp *bulgaricus* and *Lc.lactis* ssp. *cremoris* have a unique structural composition.
- The growth substrate of the media, temperature and agitation all had an effect on the growth of all the strains of *Lc.lactis* ssp. *cremoris* and as a result EPS production.
- All strains of *Lc.lactis* ssp. *cremoris* contained a 40kb plasmid which could have contained the genes for EPS production. It was not possible to remove this plasmid by growth in a media containing acriflavin or growth at an elevated temperature.
- Quantity and size of EPS seems to have an effect on the hardness and viscosity of fermented milk as strains of *Lb. delbrueckii* ssp. *bulgaricus* produced smaller molecular weight and smaller quantities of EPS than strains of *Lc.lactis* ssp. *cremoris* but strains of *Lb. delbrueckii* ssp. *bulgaricus* produced harder and more viscous gels than strains of *Lc.lactis* ssp. *cremoris*
- During sub culture, strains of *Lb. delbrueckii* ssp. *bulgaricus* Ldb1seemed to loose viscosity over time but strain 2772 seemed to increase in viscosity over time.
- Using the FTIR it was possible to generate absorption spectra for all strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris.*

- It was possible to separate different strains within both *Lc. lactis* ssp. *cremoris* and *Lb. delbrueckii* ssp. *bulgaricus* using FTIR.
- Within strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* differences were found in the protein and EPS regions of the FTIR spectra.
- The differences between *Lb. delbrueckii* ssp. *bulgaricus* and *Lc. lactis* ssp. *cremoris* were found in the DNA and polysaccharide regions of the spectra.
- It was not possible to directly relate the FTIR polysaccharide spectra to those of the bacteria.
- The FTIR spectra of strains of *Lc. lactis* ssp. *cremoris* were shown to change with time.
- The nature of the growth medium was also shown to have an effect on the spectra and the ability to differentiate between the strains.

Chapter 8. REFERENCES

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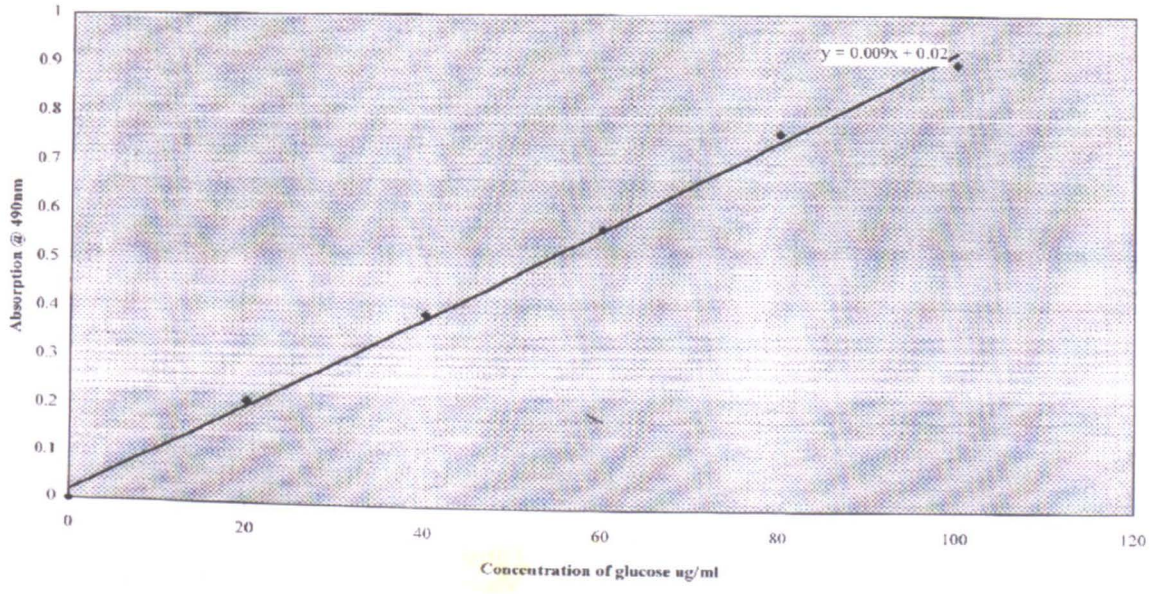
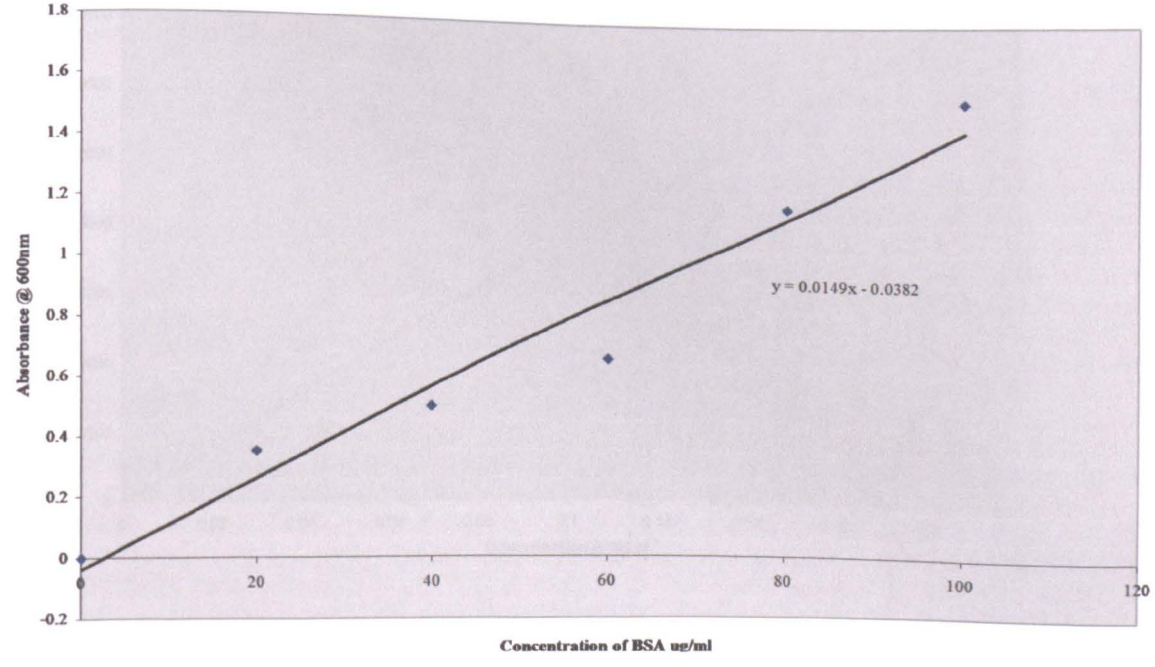
APPENDIX 1 – Calibration Graphs

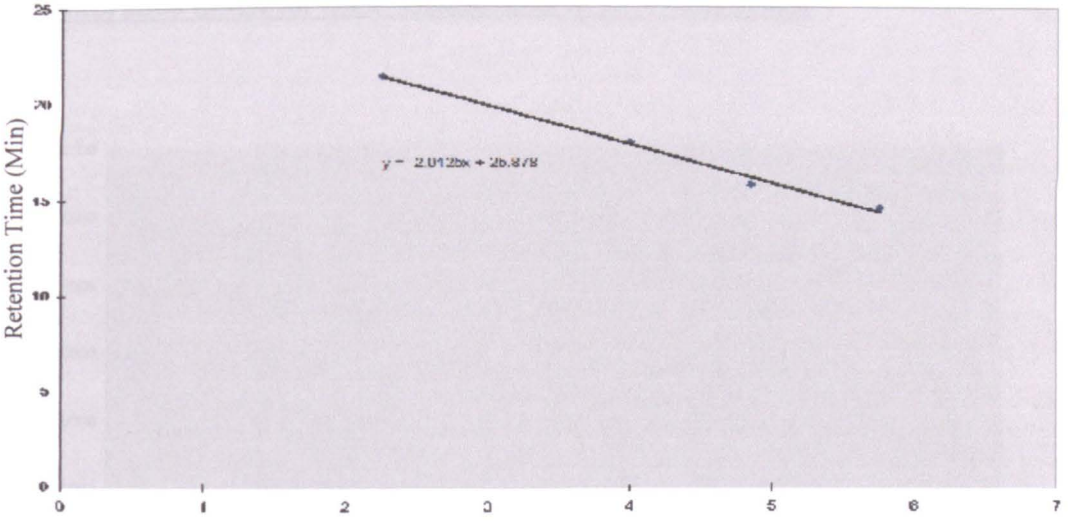
1.1 Calibration graph for total carbohydrate analysis of EPS when bacteria were grown skimmed milk



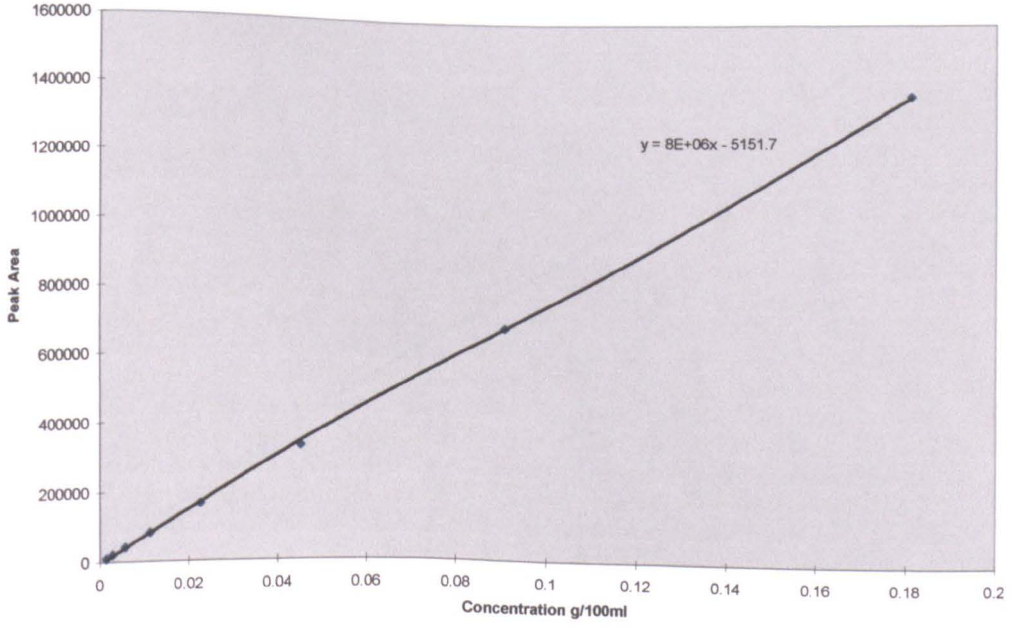
1.2 Calibration graph for protein analysis of EPS when bacteria were grown skimmed milk



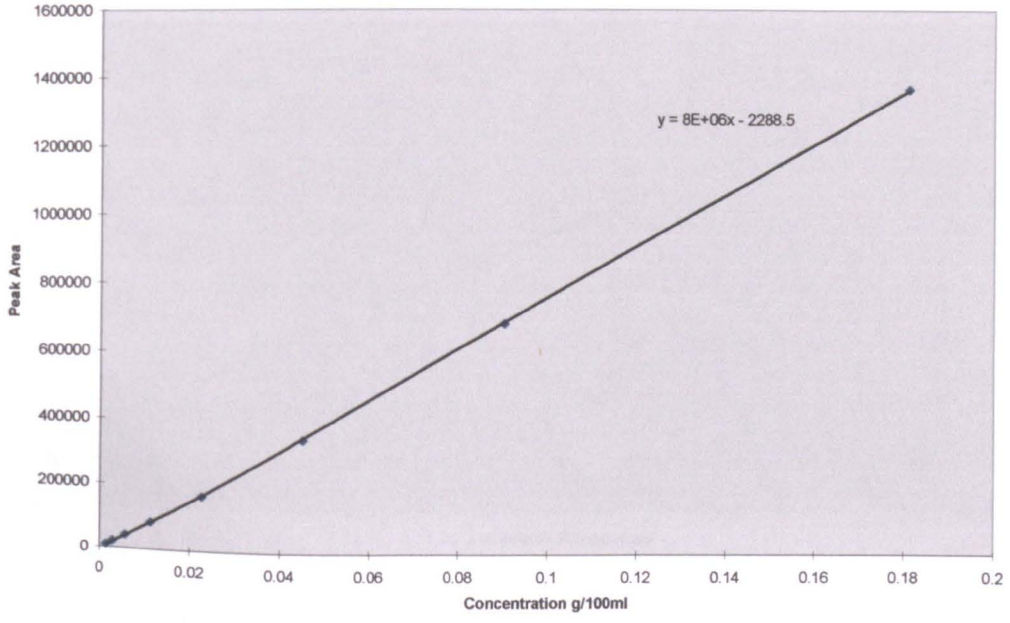
1.3 Calibration graph for sizing of EPS – using Dextran standards



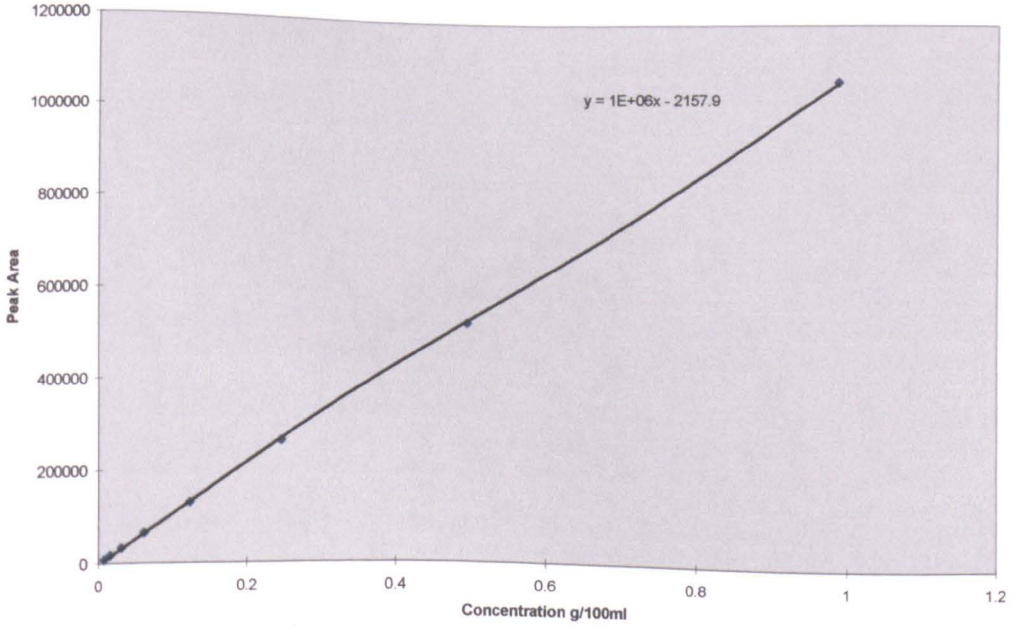
1.4 Calibration graph for sugar composition of EPS – Glucose



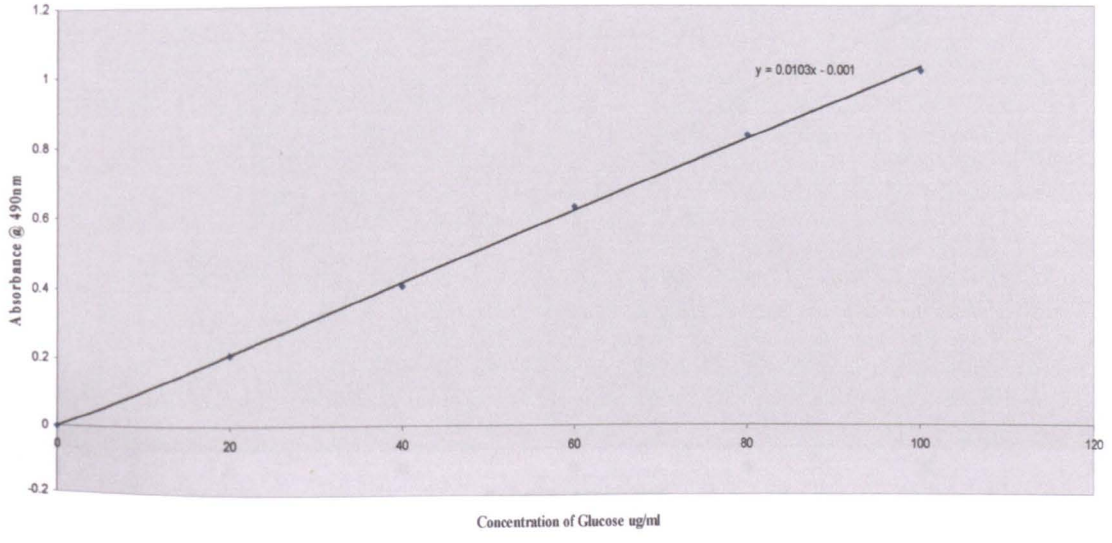
1.5 Calibration graph for sugar composition of EPS – Galactose



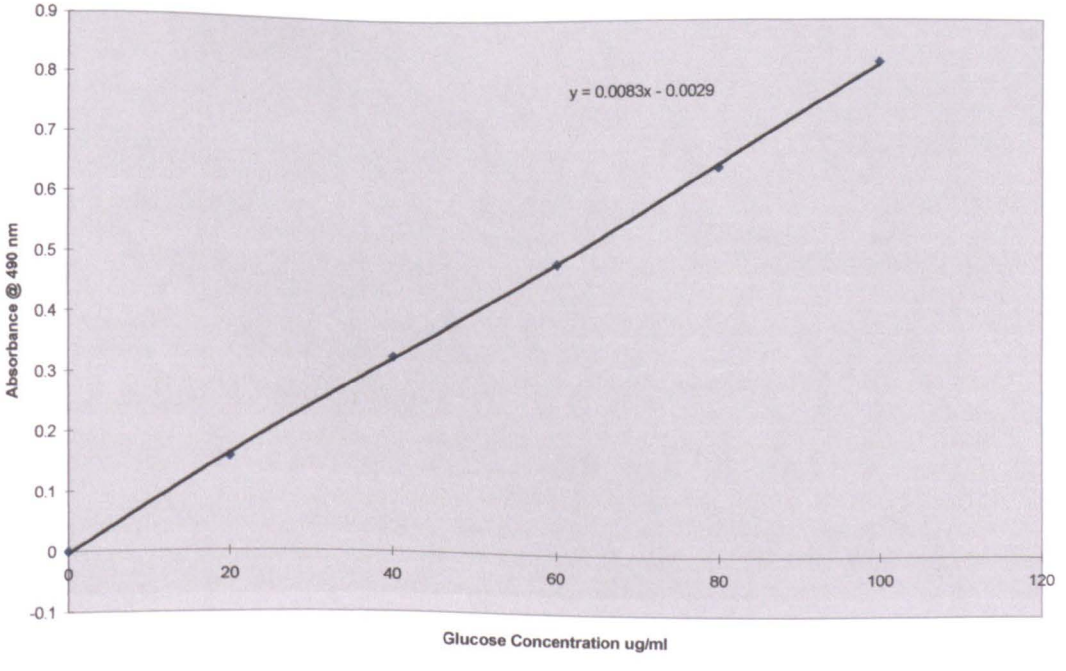
1.6 Calibration graph for sugar composition of EPS – Rhamnose



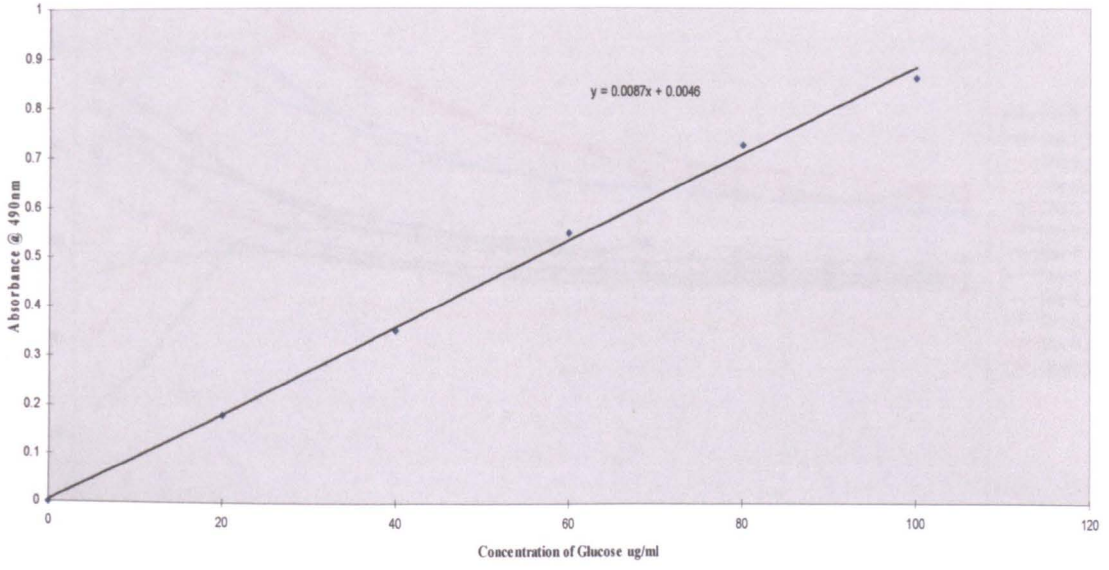
1.7 Calibration Graph total carbohydrate analysis of EPS when bacteria were grown with different carbon sources



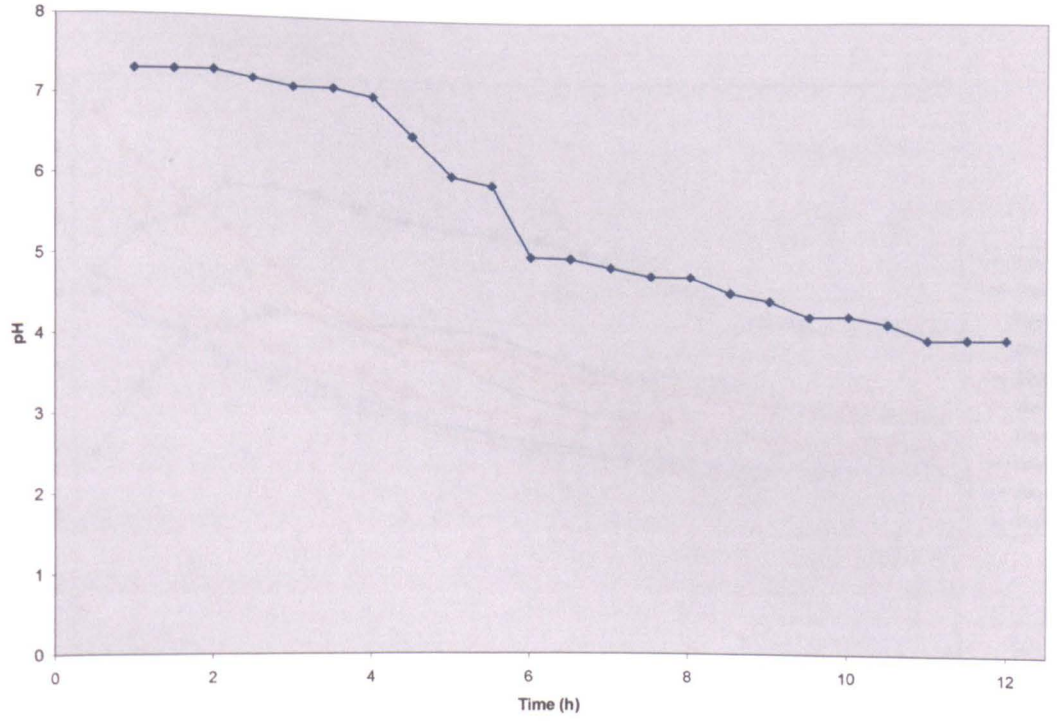
1.8 Calibration graph for total carbohydrate analysis of EPS when bacteria were grown at different temperatures



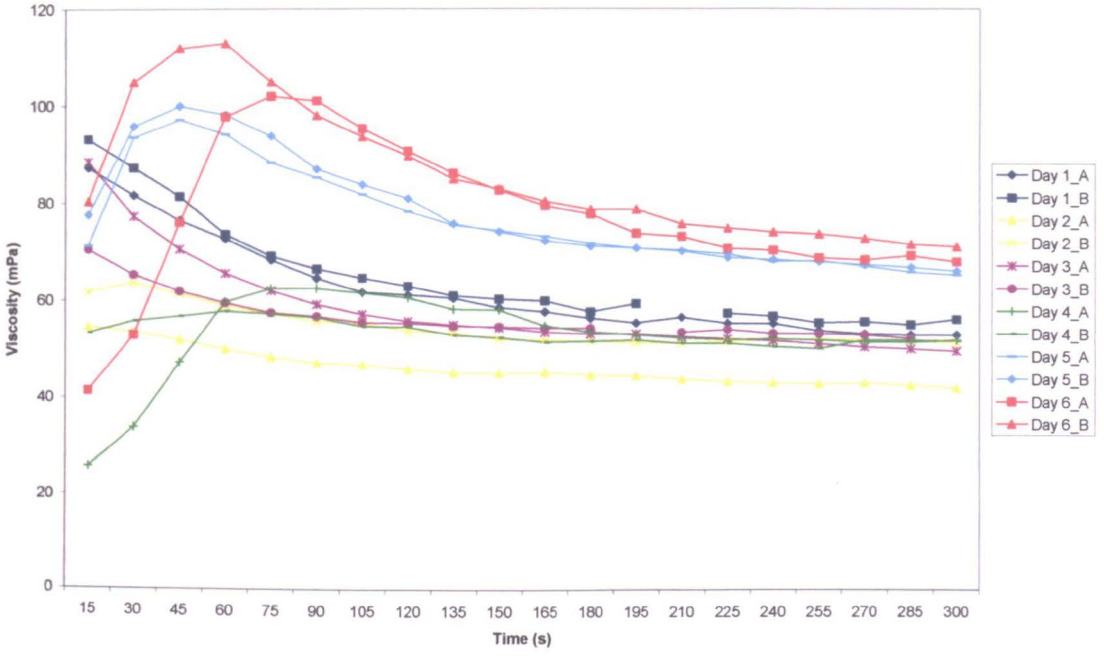
1.9 Calibration graph for total carbohydrate analysis of EPS when bacteria were grown in shaken or unshaken conditions



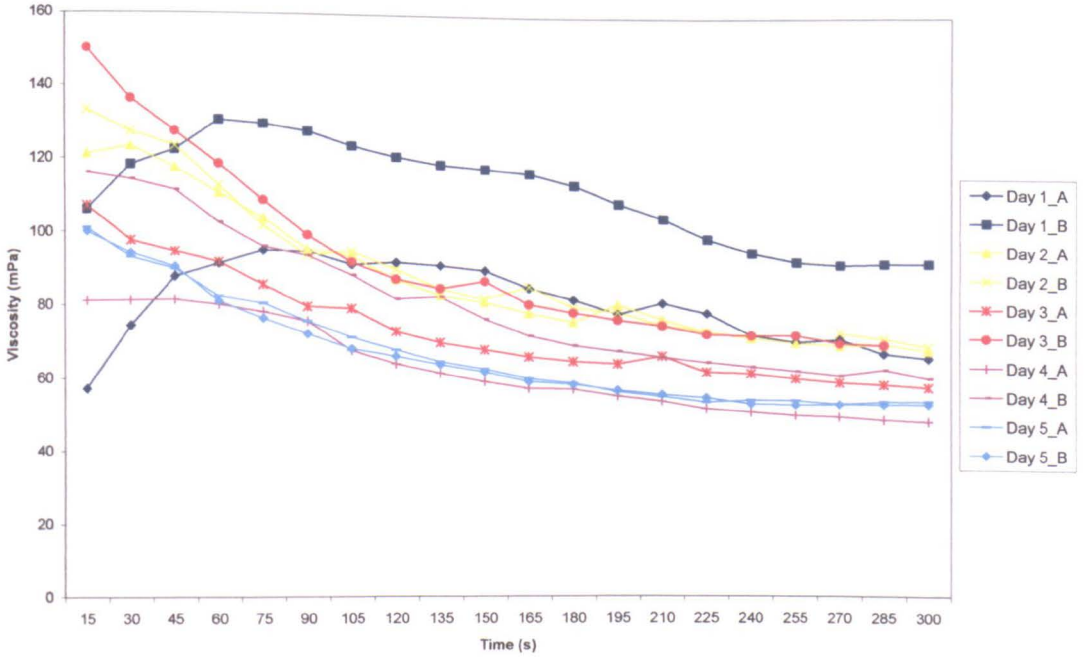
1.10 Graph showing the changes in pH during the growth of *Lactococcus cremoris* 2772 when grown in skimmed milk.



1.11 Graph showing the change in viscosity over time of milk fermented with *Lactococcus cremoris* 2772.



1.12 Graph showing the change in viscosity over time of milk fermented with *Lactobacillus bulgaricus Ldb1*.



APPENDIX 2 – Suppliers

1. Growth Media

M17 broth and agar	Oxoid Ltd (Basingstoke, Hampshire)
Milk agar	Oxoid Ltd
MRS broth and agar	Oxoid Ltd
Peptonised milk powder	Oxoid Ltd
Skimmed milk powder	Oxoid Ltd

2. Chemicals and Media Components

Acriflavin	Sigma
Adenine	Sigma
Agarose	Sigma
Ammonium citrate (tribasic)	Sigma
Bovine Serum Albumin	Sigma
Bromocresol purple	Fisher Scientific (Loughborough)
Calcium (D+) pantothenate	Sigma
Calcium Chloride	Sigma
Casaminoacids	Sigma
Chloroform	BDH Chemicals Ltd (Poole. UK)
Colbalt Chloride	Sigma
Copper Sulphate	Sigma
D-biotin	Sigma
DEAE sepharose	Sigma
Dextran Standards	Sigma
di potassium hydrogen phosphate	BDH
Dialysis Tubing	Fisher Scientific
EDTA	Sigma
Ethanol	Hayman Ltd (Witham Essex)
Ethidium Bromide	Sigma
Ferrous Chloride (tetrahydrate)	Sigma
Folic acid	Sigma
Folin Ciocalteau's Reagent	BDH
Fructose	Fisher Scientific
Glucose	Fisher Scientific
Guanine	Sigma
Inosine	Sigma
Isoamyl alcohol	Fisher Scientific
Lactose	Fisher Scientific
L-cysteine hydrochloride	Sigma
Lysozyme	Sigma
Magnisium Chloride ($6H_2O$)	Fisher Scientific
Nicotinic acid	Sigma
Orotic acid	Sigma
Phenol	Fisher Scientific
Potassium hydrogen orthophosphate	BDH
Potassium Phosphate	Sigma
Potassium Sodium Tartrate	BDH

Propan-1-ol	BDH
Pyroidoamine. HCL	Sigma
Pyroxidine HCl	Sigma
Riboflavin	Sigma
Ruthenium Red	Sigma Chemicals
Sephacryl S-500-HR	Sigma
Sodium acetate (trihydrate)	BDH
Sodium Carbonate	BDH
Sodium Chloride	Fisher Scientific
Sodium dodecyl sulphate	Sigma
Sodium Hydrogen Carbonate	Fisher Scientific
Sodium Hydroxide	Fisher Scientific
Sodium Hydroxide	Fisher Scientific
Sodium Pyruvate	BDH.
Sodium Xanthine	Sigma
Sodium-p-aminobenzoate	Sigma
Sucrose	Fisher Scientific
Sucrose	Fisher Scientific
Sulphuric acid	Fisher Scientific
Thiamine hydrochloride	Sigma
Thioctic acid	Sigma
Thymidine	Sigma
Tricholoroacetic acid	Sigma
Trifluoroacetic acid	Sigma
Tris-Hydrochloride	Sigma
Trizma	Sigma
Uracil	Sigma
Vitamin B_{12}	Sigma
Zinc Sulphate	BDH

3. Equipment

Multispeed Refrigerated Centrifuge, PK121R	ALC International (Italy)
Horizon 20-25 Horizontal Gel Electrophoresis Tank	Gibco (Paisley, Scotland)
Fraction collector & Pump Redifrac Pump P-1	Pharmacia (Sweden)
FTIR system IFS 28/B	Brucker (Germany)
Haake Viscometer 500 with temperature controller	Fisons (Loughborough, UK)
Lowpressure Gel columns	Pharmacia (Sweden)
Powerpack Model 200/2.0	Biorad (USA)
Peristaltic Pump for Tank 101U/R	Watson Marlow (Cornwall, UK)
REZEX 8U 8%H.Organic acid column 30 cm x 7.8 mm	Phenomenex (Cheshire, UK)
Shaking Incubator	Mickie Lab Engineering(Surrey UK)
UV-visible spectrophotometer M330	CamSpec (Sawston, Cambridge)
Texture analyser TA-XT2	Stable Microsystems (Surrey UK)
TSK-Gel GMPW column 7.8 mm x 30cm	TosoHass (USA)
Water bath Y28	Grant Instruments (Royston, Hearts)