Microencapsulation of flavour-enhancing enzymes for acceleration of Cheddar cheese ripening

By

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Centre for Plant and Food Science
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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at any other institution.

Kavya Anjani
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List of Publications

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Runner up, Best poster at the 38th Annual Australian Institute of Food Science & Technology Convention, 10-13th July, 2005, Sydney, Australia.
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<th>Description</th>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSNP</td>
<td><em>Bacillus subtilis</em> neutral proteinase</td>
</tr>
<tr>
<td>EE</td>
<td>Encapsulation efficiency</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>CFE</td>
<td>Cell free extract</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl esters</td>
</tr>
<tr>
<td>FAA</td>
<td>Free amino acids</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein iso-thio-cyanate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared (spectroscopy)</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HTST</td>
<td>High-temperature short-time (pasteurisation)</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>LAPU</td>
<td>Leucine amino peptidase units</td>
</tr>
<tr>
<td>LC-ESI-MS/MS</td>
<td>Liquid chromatography electrospray ionisation tandem mass spectrometry</td>
</tr>
<tr>
<td>LU</td>
<td>Lipase units</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>MFGM</td>
<td>Milk fat globule membrane</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>NSLAB</td>
<td>Non starter lactic acid bacteria</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>RP-UPLC</td>
<td>Reverse phase ultra performance liquid chromatography</td>
</tr>
<tr>
<td>SLAB</td>
<td>Starter lactic acid bacteria</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>SSA</td>
<td>Sulphosalicylic acid</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>TFA</td>
<td>Tri fluoro acetic acid</td>
</tr>
<tr>
<td>TMAH</td>
<td>Tetra methyl ammonium hydroxide</td>
</tr>
</tbody>
</table>
Abstract

Commercial flavour-enhancing enzymes were delivered in an encapsulated form to accelerate Cheddar cheese ripening. Polymers such as alginate, chitosan and κ-Carrageenan were screened to be used as encapsulant material for microencapsulation of the commercial protease enzyme, Flavourzyme®. Alginate was found to be a suitable polymer for Flavourzyme encapsulation using the Inotech® encapsulator while κ-Carrageenan and chitosan were too viscous for extrusion through the encapsulator nozzle. Gelling of alginate-Flavourzyme microcapsules in 0.1M CaCl₂ resulted in poor encapsulation efficiency (ranging 17-18% depending on the alginate concentration). Incorporation of Hi-Maize™ starch or pectin as filler materials into the alginate-Flavourzyme encapsulation matrix to increase encapsulation efficiency by minimising porosity also resulted in poor encapsulation efficiency.

An alternative approach to the modification of the cationic gelling solution, by adding chitosan, significantly increased the encapsulation efficiency to 70-88% and produced mostly spherical capsules with an average diameter of 500μm. Encapsulation efficiency increased with an increase in chitosan concentration from 0.1 to 0.3% (w/v) in the cationic gelling solution of 0.1M CaCl₂. Though gelling of alginate-Flavourzyme microcapsules in gelling solution of 0.1M CaCl₂ containing 0.3% (w/v) chitosan resulted in higher encapsulation efficiency, a chitosan concentration of 0.1% (w/v) was chosen for further work as higher concentrations of chitosan in the gelling solution resulted in aggregation of capsules during formation.

Gelling time of 10 min and alginate concentrations in the range 1.6 to 2.0% (w/v) were found to be optimal encapsulation parameters for Flavourzyme encapsulation
while 2.0% (w/v) solution of trisodium citrate was found to be optimal for *in vitro* release of encapsulated enzymes for measurement of enzyme activity.

Flavourzyme capsules stored frozen or freeze-dried were shelf stable for at least 10 weeks retaining about 80% of the initial enzyme activity as opposed to retention of 25-34% activity in air-dried capsules.

Leakage of encapsulated Flavourzyme prepared from 1.6% (w/v) alginate was slightly higher than those prepared from 1.8 and 2.0% (w/v) alginate in cheese milk. Flavourzyme-alginate capsules prepared from 1.6, 1.8 and 2.0% (w/v) alginate retained over 70% of the initial enzyme activity under simulated cheese-press pressure. Concentration of alginate had no significant effect (p > 0.05) on the retention of encapsulated Flavourzyme when the capsules were pressed for 4h; however when the simulated cheese press duration increased to 8 and 16h the retention of encapsulated Flavourzyme was significantly higher (p < 0.01) in capsules produced from 2.0% (w/v) alginate.

Incorporation of encapsulated enzymes into the milk prior to rennetting resulted in an even distribution of capsules in the cheese matrix compared to aggregation of capsules, when added to milled curd prior to salting. All cheeses; control with no added enzymes and experimental cheeses with free and encapsulated Flavourzyme and/or Palatase showed higher levels of moisture and lower levels of fat compared to standard Cheddar cheese due to the variation in the manufacturing protocol. There was no significant difference (p > 0.05) in fat and final pH between control and experimental cheeses and there was no difference in the numbers of coliforms, *E.coli, Salmonella, Listeria*, coagulase positive staphylococci, *Bacillus cereus*, yeast and moulds in control or experimental cheeses.
Increased and prolonged proteolysis was observed in cheeses with encapsulated Flavourzyme showing increased release of several peptides, also with the formation of new peptides absent in the control cheese with no added enzymes. Accumulation of high molecular weight/hydrophobic peptides was higher in cheeses with free Flavourzyme followed by cheeses with encapsulated Flavourzyme. Concentration of water-soluble peptides increased with the increase in the concentration of encapsulated Flavourzyme in the cheese. Concentration of water-insoluble peptides was higher in control cheese compared to cheeses with encapsulated Flavourzyme even after 180 days ripening. After 30 days of ripening, concentration of most free amino acids was about 3 times greater in cheeses with encapsulated Flavourzyme than in control and about 7 times higher after 90 days ripening. Concentration of total amino acids was consistently higher in cheeses with encapsulated Flavourzyme compared to control.

Cheese grading scores for body, texture and appearance of all cheeses with encapsulated enzymes were lower than control and free enzyme treated cheeses during the entire grading period of about 100 days due to crumbly and pasty texture. Control and cheeses with added Flavourzyme received high overall score for flavour. Flavour score of cheese with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein was higher than all cheeses around 50 days with better overall flavour score until about 94 days ripening with improved flavour and elimination of bitterness. However the flavour of enzyme treated cheeses deteriorated with time and the control cheese scored the highest for flavour.

Though increased concentration of free fatty acids was detected in cheeses treated with encapsulated lipase; Palatase, these cheeses developed rancid, unpleasant, strong lipolytic flavours as early as 55 days ripening.
1 Introduction

Cheese is a popular dairy product in most regions of the world with a growing consumer demand. Consistent growth in cheese consumption has been the result of good dairying and marketing ability of the cheese industry and also due to increased awareness of the nutritional benefits of cheese. Cheese is made from coagulation of milk followed by fermentation, dehydration and salting, though bacterial activity continues throughout the ripening of cheese.

Several groups of cheese have evolved over time with diverse varieties dominating different markets. Cheddar has been the most popular cheese variety in Australia as it accounted for more than 50% of the total cheese manufactured in 2005/2006 and total Cheddar (natural (76.6%) and processed (23.4%)) constituting 71.7% of the total cheese supermarket sales in 2005/2006 (Anon, 2007b, 2007d).

Cheddar cheese is usually ripened around 8°C (Fox, 1988). Flavour development in cheese is the result of biochemical processes occurring during ripening which are proteolysis: breakdown of milk proteins to peptides and amino acids, lipolysis: breakdown of milk fat to fatty acids and glycolysis: hydrolysis of milk sugars mainly lactose and citrate to smaller compounds and catabolism of these products to smaller compounds by cheese microflora. All these processes lead to development of characteristic flavour and texture during ripening (Adda et al., 1982; Forde and Fitzgerald, 2000; McSweeney, 2004). Flavour formation in low-moisture varieties like Cheddar range from 2 months to over 2 years depending on the intensity of flavour sought (McSweeney, 2004).

Long ripening duration in varieties like Cheddar implies additional expenses in maintenance, refrigeration and risk of low yield due to contamination and syneresis...
thus increasing the overall production costs (Cliffe and Law, 1990; Picon et al., 1997). Demand for consistent low-cost high-intensity flavour in cheese has lead to an intensive research on potential means for acceleration of ripening. Several attempts at minimising the ripening duration have been reported and those techniques include elevation of ripening temperature, high pressure processing, use of attenuated starters, addition of adjunct cultures, use of genetically modified starters and addition of exogenous enzymes.

Elevation of ripening temperature was thought to be a straightforward approach towards accelerating cheese ripening but it was limited as ripening at high temperatures led to increased risk of contamination and syneresis leading to textural defects and low yield (Wilkinson, 1993, Fox et al., 1996; Hannon et al., 2005). High pressure treatment of cheeses has been reported for acceleration of ripening however, there are no known reports of successful commercial application. Use of genetically modified starters to produce excess proteolytic enzymes was also limited as it was technically complex and has problems with acceptance by the consumer. Addition of adjunct cultures known to produce proteolytic enzymes similar to the starter and non starter lactic acid bacteria is limited by the availability of strains also with the risk of development of off-flavours. Addition of cultures attenuated by various ways such as heat, lysozyme, and freeze-thaw treatments resulting in earlier lysis and release of proteolytic enzymes during ripening leading to an accelerated flavour development was reported however, this approach was not very successful due to low specificity.

Addition of exogenous enzymes to catalyse the biochemical reactions occurring during cheese ripening was studied by many researchers with the aim of accelerating ripening (Fox and Stepaniak, 1993). Addition of free enzymes (mainly proteolytic enzymes) led to premature and extensive proteolysis with the development of off-
flavours and bitterness in addition to contamination of whey (Law and Kirby, 1987; Wilkinson and Kilcawley, 2005).

Microencapsulation is a well known technique used for protection, isolation and controlled release. However, its application to deliver enzymes into cheese system for acceleration of ripening is a recent technique and is innovative. Encapsulation of enzymes was thought to prevent immediate proteolysis and contamination of whey also aiding in an even distribution of the enzymes in cheese matrix resulting in prolonged and relatively controlled release of the enzymes during ripening.

Several materials such as milk fat, food gums and liposomes were tested for delivering enzymes into the cheese system to accelerate ripening with reports of poor encapsulation efficiency, poor stability of capsules in cheese and very little understanding of the release mechanism of the encapsulated enzymes during ripening (Perols et al., 1997; Picon et al., 1997; Kailasapathy et al., 1998; Laloy et al., 1998; Kheadr et al., 2000, 2003).

Potential of alternative materials and techniques for enzyme microencapsulation for application in cheese has not been well reported. Though a large number of techniques and ingredients are used for microencapsulation, suitability of a technique largely depends on the substance subjected to microencapsulation and its application. In the current study microencapsulated enzymes were delivered into cheese for acceleration of ripening, as per this requirement it was necessary to deliver the enzymes in inert, non-fat capsules that do not alter the composition of cheese, in line with the focus of this study to microencapsulate flavour-enhancing enzymes for controlled release in Cheddar cheese during ripening.

Entrapment of biological material in hydrogels or polymers for the purpose of protection, isolation or controlled release is very popular. Use of the encapsulator for
enzyme encapsulation eliminated the need for solvents and emulsifying agents otherwise used in common encapsulation procedures. Microencapsulation of enzymes in food-grade polymers using the encapsulator was found to be ideal for this study. Suitability of food gums for microencapsulation of flavour-enhancing enzymes was investigated in this study for application into cheese for acceleration of ripening.
1.1 Aim

The aim of this study was to evaluate an accelerated Cheddar cheese ripening process by microencapsulating flavour-enhancing enzymes in food gums and delivering into cheese for controlled release during ripening.

1.2 Objectives

i. Screening of food gums suitable for enzyme encapsulation using the Inotech® encapsulator followed by optimisation of enzyme encapsulation, shelf life study of encapsulated enzymes and morphological characterisation of enzyme capsules

ii. Studies on the stability of the enzyme capsules under simulated conditions of cheese manufacture

iii. Evaluation of enzyme delivery into cheese for maximum retention and an even distribution

iv. Monitoring of cheese ripening by analysis of water-soluble and water-insoluble peptides, free amino acids and free fatty acids

v. Organoleptic evaluation of accelerated ripened Cheddar cheese
2 REVIEW OF LITERATURE
2.1 History and background of cheese

Cheese is one of the oldest, diverse and most widely consumed groups of dairy products with high nutritional value. Cheese is made from coagulation of milk followed by separation of whey and pressing the curd. The pressed curd can be consumed fresh or after a period of ripening depending on the variety of cheese and intensity of the flavour sought.

Cheese is said to have evolved about 8000 years ago in the region around southern Turkey and spread to rest of the world with the civilization (Fox and McSweeney, 2004). Cheese was the only means of milk preservation for centuries and some long-ripened varieties can be considered as long-shelf life products (Adda et al., 1982). Originally cheese was manufactured using milk from all forms of domesticated animals however, in recent times cheese is mostly made using bovine milk. Curdling of milk stored in the bags made of young animal’s stomach lead to the discovery of milk coagulating properties of digestive juices from the stomach tissue. It is now known that rennet is the milk coagulating extract obtained from the fourth stomach of young animals. Shortage of rennet lead to the development of rennet substitutes for cheese manufacture; yet rennet is the principal milk coagulating agent used to date.

Cheese is a popular dairy product globally and several varieties have evolved over time depending on the source of milk, origin, variation in the manufacturing process and maturation. Cheddar is the most popular cheese variety in Australia as it accounted for over 51% of the total cheese manufactured while 71% of the cheese supermarket sales accounted for total Cheddar (natural and processed) in 2005/2006 (Anon, 2007b, 2007d). Natural Cheddar alone accounted for about 54% of the total cheese supermarket sales demonstrating the popularity of this variety in Australia.
2.2 Cheese production

Cheese is inherently, biologically and biochemically unstable in contrast to many other dairy products. cheeses can be produced by acid-coagulation or rennet-coagulation. Most acid-coagulated cheeses (e.g. Cottage) are consumed fresh, soon after manufacture; while most rennet-coagulated cheeses (e.g. Cheddar, Parmesan) are consumed after ripening, the production of these involve two stages; manufacture and ripening (Fox and McSweeney, 2004).

2.2.1 Cheese manufacture

Cheese manufacture is mainly a dehydration process involving concentration of milk fat and casein between 6 and 12 folds depending on the variety and may last for 5-24h. The basic steps involved in cheese manufacture are acidification, coagulation, dehydration, salting and pressing or moulding. Stage of milk supply such as early or late lactation milk also affects the quality of cheese, increased proteolysis was observed in cheeses made from late lactation milk. Increased level of free fatty acids (FFA) was also noted in late lactation milk and hence higher in cheeses manufactured from it (Hickey et al., 2006). In modern production, milk of high microbiological quality, free from antibiotics is standardised for required amount of casein and fat ratio followed by pasteurisation. Microorganisms in milk that survive pasteurisation contribute to the population of non-starter lactic acid bacteria (NSLAB) in cheese. In current dairy processing, pasteurised milk is stored in cool rooms until manufacture. Pasteurised cheese milk is heated to about 30°C depending on the variety being manufactured (Fox and McSweeney, 2004).

Acidification during cheese manufacture is a crucial step for certain varieties and is achieved by encouraging production of lactic acid from the starter culture. For
varieties such as Cheddar that are cooked not more than 40°C *Lactococcus lactis* subsp *cremoris* and/or *Lc. lactis* subsp *lactis* are the most commonly used starter cultures whereas *Streptococcus thermophilus* is used as a starter in high temperature cooked cheeses and other *Lactobacillus* spp are also used as starter lactic acid bacteria (SLAB) in most varieties of cheese. Time required to attain the end pH during cheese manufacture can vary depending on the variety. Calcium chloride is added early during cheese manufacture, it influences acid development, coagulation properties and gel syneresis (Fox and McSweeney, 2004).

Coagulation is one of the most important stages of cheese manufacture. Most fresh cheeses are acid-coagulated while long-ripened cheeses are rennet-coagulated. Rennet is extracted from the stomach of young animals; the principal enzyme in rennet is chymosin, accounting for about 95% of the milk-clotting activity along with small amount of pepsin which hydrolyse milk proteins (mainly caseins). Casein micelles constituting about 80% of bovine milk proteins is composed of several thousand individual molecules of all four major types of caseins (*α_s1*, *α_s2*, *β* and *κ*) with *κ*-casein located on the outer surface of the micelle exerting a stabilising effect on the micelles, preventing them from coagulating. The *κ*-casein constitutes hydrophobic and hydrophilic moieties, and is linked to the micelle by the hydrophobic moiety while the hydrophilic moiety interacts with the solvent stabilising the casein micelle (Dalgleish, 1993). Milk-clotting enzymes such as chymosin (and pepsin) hydrolyse *κ*-casein at the junction of hydrophobic para-*κ*-casein and hydrophilic macropptide moieties, the macrop peptide diffuses into the solution following hydrolysis and the stabilising influence of *κ*-casein is lost leading to coagulation of casein micelles. This marks the initial coagulation of milk after renneting while *β*-casein is untouched by rennet. This leads to a curd formation.
The curd is cut into distinct size depending on the variety. Cutting, stirring, size of the curd and temperature at which it is cut greatly influences the rate of expulsion of whey; determining the loss of milk components in whey and also affecting moisture content and water activity of cheese (Lawrence et al., 2004). The curd is cooked to specific temperature and subjected to processes such as cheddaring, kneading or stretching depending on the variety and the whey is expelled. The curd is either dry salted or brined. Salt concentration in cheese can vary between 0.7 and 6% and can greatly influence moisture, water activity, shelf life, growth and activity of SLAB and NSLAB thus indirectly affecting the protein and fat stability, enzyme activity during ripening and hence the quality of cheese (Laan et al., 1998). The salted, dehydrated pressed curd is (treated with secondary starters for some varieties) moulded, packed and allowed to ripen.

2.2.2 Cheese ripening

Some varieties of cheese are consumed fresh while certain varieties are consumed after ripening, which can range from two weeks (e.g. Mozzarella) to two or more years (e.g. Parmigiano-Reggiano or vintage Cheddar) (McSweeny, 2004). The diversity in the properties of different varieties of cheese is mainly due to the variation in the source of milk, starter cultures, manufacturing processes, ripening time and temperature and secondary cultures. The flavour of freshly produced cheese curd is bland, characteristic cheese flavour develops during ripening due to the production of a wide range of sapid compounds by biochemical pathways (Adda et al., 1982; McSweeny, 2004). A range of biochemical and microbiological events contribute to the flavour and texture development of the cheese during ripening.
2.2.2.1 Microbiology of cheese ripening

Starter lactic acid bacteria are primarily used for acid production and their growth ceases soon after manufacture due to low pH, but continue to contribute towards ripening as they produce proteolytic and lipolytic enzymes responsible for conversion of peptides, amino acids and fatty acids to flavour compounds (Fox and Wallace, 1997) however, most starter enzymes are intracellular and are released only upon cell lysis during ripening.

Non starter lactic acid bacteria (NSLAB) also form a significant portion of the microbial load in most ripened cheese varieties (Beresford and Williams, 2004). The population of NSLAB is greater and diverse in cheeses made from raw milk and their source can range from milk to manufacturing equipment. The number of NSLAB is highest around 3 months of ripening and is stable for rest of the maturation period. Different strains may dominate during different stages of ripening while some persist during the entire ripening period. Lactobacilli are the predominant species of NSLAB in Cheddar cheese during ripening. Non starter lactic acid bacteria like SLAB contribute to ripening through release of intracellular and extracellular enzymes responsible for conversion of peptides, amino acids and fatty acids to flavour compounds (McSweeney et al., 1993).

Secondary starters like *Penicillium roqueforti* in blue cheeses such as Roquefort, Gorgonzola, Stilton and Danish blue; *Penicillium camemberti* in surface-mould ripened cheeses such as Brie and Camembert; Propionic acid bacteria in Swiss type cheeses are also used for development of characteristic flavour and texture. However there is no deliberate addition of secondary starters in Cheddar cheese and hence will not be discussed further.
2.2.2.2 The biochemistry of cheese ripening

In mature cheeses such as Cheddar, development of flavour and texture is largely controlled by complex biochemical reactions occurring during ripening (Forde and Fitzgerald, 2000). These biochemical reactions can be grouped into three major categories;

i. **Proteolysis**: breakdown of casein to peptides and amino acids and their catabolism

ii. **Lipolysis**: breakdown of milk fat to free fatty acids (short, medium and long chain length) and their catabolism and

iii. **Glycolysis**: metabolism of lactose, lactate and citrate by SLAB and NSLAB with the development of flavour compounds (McSweeny, 2004) (Figure 2.1).

Enzymes that catalyse these biochemical reactions during ripening originate from milk, coagulant, SLAB, NSLAB, secondary starters (in certain varieties) and exogenous enzymes (McSweeney, 2004; Wilkinson and Kilcawley, 2005).
Figure 2.1 Overview of biochemistry of cheese ripening (Law, 2001).
2.2.2.2.1 Proteolysis

Proteolysis is the most complex and most important biochemical event occurring during ripening in cheese varieties such as Cheddar (Forde and Fitzgerald, 2000; Upadhyay et al., 2004a). This topic has been extensively reviewed (Fox, 1989; Fox and Law, 1991; Fox and McSweeny, 1996; Sousa et al., 2001; Upadhyay et al., 2004a). Proteolysis is responsible for the change in pH, water activity, and protein structure of cheese thus affecting the texture and flavour (Upadhyay et al., 2004a). During ripening, proteins in cheese curd are hydrolysed to smaller peptides and amino acids. The mixture of peptides and amino acids not only contribute directly to the flavour and mouth feel of the cheese, but also act as substrates for further metabolism by SLAB and NSLAB generating a range of flavour compounds (Law, 2001; Upadhyay et al., 2004a).

Proteolysis in cheese during ripening is catalysed by proteinases and peptidases from milk, coagulant, SLAB, NSLAB, secondary starters and in some cases exogenous enzymes (Fox and McSweeny, 1996; Law, 2001; Sousa et al., 2001 and McSweeny, 2004) (Figure 2.2).
Figure 2.2 Proteolytic agents in cheese during ripening (Sousa et al., 2001).
Rennet is the principal coagulant used in cheese manufacture and consists of chymosin, pepsin and other proteinases. Chymosin is the principal proteinase in most commercial rennets as it accounts for about 95% of the milk-clotting activity of rennet. It hydrolyses at the Phe$_{105}$-Met$_{106}$ bond of $\kappa$-casein leading to destabilisation of casein micelle and coagulation of the milk. Though most of the rennet is lost during whey drainage, a small amount is trapped in the cheese curd. The residual chymosin from the trapped rennet in cheese curd cleaves $\alpha_{s1}$-casein at various sites (McSweeney et al., 1993) influenced by pH and ionic strength (Mulvihill and Fox, 1977). Pepsins are also present in considerable amount in rennet and their activity is similar to chymosin.

The main proteolytic agents from SLAB are proteinases and a range of intracellular peptidases. Lactocepin, a cell envelop associated proteinase, degrades casein to short peptides. The degradation of these peptides to amino acids is catalysed by intracellular peptidases (Christensen et al., 1999 as cited by Upadhyay et al., 2004a). While low pH of curd inhibits further growth of SLAB in cheese, its intracellular enzymes are released into cheese upon lysis. A range of endopeptidases, di- and tripeptidases and aminopeptidases have been studied from LAB; both SLAB and NSLAB and carboxypeptidases in a few strains of lactobacilli have also been studied (Figure 2.3) (El Soda et al., 1987; Upadhyay et al., 2004a).

There is no contribution of secondary starters to the ripening of traditional Cheddar cheese as there is no deliberate addition of secondary cultures during manufacture.
**Endopeptidases (PepO, PepF)**

**Exopeptidases**

Aminopeptidases (PepN, PepA, PepC, PepL)  \(\text{Iminopeptidase (PepI)}\)

Pyrolidonyl carboxylyl peptidase (PCP)

X Prolydipeptidyl aminopeptidase (PepX)  \(\text{Carboxypeptidase}\)

Dipeptidases (PepV, PepD)  \(\text{Prolidase (PepQ)}\)  \(\text{Prolinase (PepR)}\)

Tripeptidase (PepT)

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Figure 2.3 Schematic representation of the action of peptidases found in lactic acid bacteria (Upadhyay et al., 2004a).
2.2.2.2.11 Catabolism of amino acids during cheese ripening

Catabolism of amino acids is a major flavour-forming process during ripening (Yvon and Rijnen, 2001; Ardo, 2006), which can be initiated by decarboxylases, lyases, transaminases, deaminases and dehydratases (Ardo, 2006). The major pathway for degradation of amino acids by LAB is an α-keto acid pathway initiated by transamination catalysed by aminotransferases (Yvon and Rijnen, 2001; Williams et al., 2001). Aminotransferases catalyse amino acid transamination to α-keto acid which is further degraded to a range of compounds (Yvon and Rijnen, 2001; Curtin and McSweeney, 2004) (Figure 2.4, Figure 2.5, Table 2.1). Alternative amino acid catabolism pathways include cleavage of amino acid side chains by amino acid lyases; formation of amines by decarboxylases and ammonia by deaminases and hydrolysis of –OH and –SH groups in amino acids by dehydratases (Figure 2.6) (Smit et al., 2000, Smit, 2005; Ardo, 2006). Aminotransferase activity was detected in all LAB (SLAB and NSLAB) isolates from Cheddar cheese (Williams et al., 2001) indicating the role of LAB and their enzymes in flavour formation during ripening; by catabolism of amino acids.
Figure 2.4 Schematic diagram of amino acid catabolism pathways found in different microorganisms and some chemical reactions (dotted line) occurring in cheese during ripening. HA-DH: hydroxyacid dehydrogenase, α-KADH: α-ketoacid dehydrogenase, α-KADC: α-ketoacid decarboxylase, alDH: aldehyde dehydrogenase, alcoDH: alcohol dehydrogenase, MGL: methionine γ-lyase, CGL: cystathionine γ-lyase, CBL: cystathionine β-lyase, TPL: tyrosine-phenol lyase, TIL: tryptophan-indole lyase (Yvon and Rijnen, 2001; modified by Curtin and McSweeney, 2004).
Figure 2.5 Catabolism of leucine initiated by transaminase, deaminase or decarboxylase action and volatile flavour compounds that may be formed from this amino acid. Similar catabolic pathways operate for the other branched-chain aliphatic amino acids (isoleucine and valine) (McSweeney, 2004).
Figure 2.6 Methionine degradation pathways by starter cultures. PLP: pyridoxal phosphate; TPP thiamine pyrophosphate (Smit et al., 2000).
Table 2.1 Flavour compounds derived from amino acids via α-keto acids by the transaminase pathway. Compounds in italics are formed by non-enzymatic reactions (Adapted from Smit, 2005).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Keto acid</th>
<th>Aldehyde</th>
<th>Organic acid</th>
<th>Alcohol (thiol)</th>
<th>Esters (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile</td>
<td>α-keto-3-methyl-pentanoic acid</td>
<td>2-methylbutanal</td>
<td>2-methylbutyric acid</td>
<td>2-methyl butanol</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>α-ketoisocaproic acid</td>
<td>3-methylbutanal</td>
<td>3-methylbutyric acid</td>
<td>2-methyl propanol</td>
<td>Ethyl-3-methylbutanoate</td>
</tr>
<tr>
<td>Val</td>
<td>α-ketoisovaleric acid</td>
<td>2-methylpropanal</td>
<td>2-methyl propanoic acid</td>
<td>2-methyl propanol</td>
<td>Ethyl isobutanoate</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenyl pyruvate</td>
<td>Phenyl pyruvate</td>
<td>Phenyl acetaldehyde</td>
<td>Phenyl methanol</td>
<td>Ethyl benzoate</td>
</tr>
<tr>
<td>Trp</td>
<td>Indole-3-pyruvate</td>
<td>Indole-3-acetaldehyde</td>
<td>Indole-3-acetic acid</td>
<td>Phenyl ethanol</td>
<td>Phenylethyl acetate</td>
</tr>
<tr>
<td>Met</td>
<td>α-keto methylthio butyrate</td>
<td>Methional</td>
<td>Methylthiobutyric acid</td>
<td>Methionol</td>
<td>Ethyl-3-methylthiopropionate</td>
</tr>
</tbody>
</table>

*Ethyl-3-methylbutanoate, Phenyl acetaldehyde, Ethyl benzoate, Phenylethyl acetate*
2.2.2.2 Lipolysis

Lipolysis during cheese ripening mainly refers to the enzymatic hydrolysis of triglycerides to fatty acids and glycerol, mono-, di- or triglycerides and is essential for flavour formation in varieties such as Blue but in cheeses such as Cheddar due to low level of lipolysis, its influence on flavour has received little attention (McSweeney and Sousa, 2000). Extensive lipolysis is characteristic of mould-ripened (Gripon, 1993; Spinnler and Gripon, 2004) and Italian cheeses (Bosset and Gauch, 1993), and only moderate lipolysis occurs in Cheddar cheese while extensive lipolysis is considered undesirable in Cheddar (McSweeney and Fox, 1993; McSweeney and Sousa, 2000). Lipolysis in cheese influences flavour through formation of free fatty acids (FFA), which act as precursors for formation of several flavour compounds such as lactones in Cheddar cheese. Lipids also act as solvents for many sapid flavour compounds in cheese and also influence the texture of cheese (Figure 2.7) (Adda et al., 1982; McSweeney, 2004).

Lipolytic enzymes; lipases and esterases can originate from milk, rennet, SLAB, NSLAB, secondary starters and exogenous preparations (Fox and Wallace, 1997).

Milk contains indigenous lipoprotein lipase (LPL) which under optimum conditions can hydrolyse milk fat and release FFA causing rancidity, however, this does not occur as triglycerides in milk are surrounded by milk fat globule membrane (MFGM) and LPL is associated with casein micelles (Collins et al., 2004). High-temperature short-time (HTST) pasteurisation (72°C for 15s) extensively inactivates this enzyme however, it may still contribute to lipolysis during ripening as heating at 78°C for 10s is required for its complete inactivation (Driessen, 1989 as cited by Collins et al., 2004).
Rennet pastes contain lipolytic enzymes however, commercial rennet preparations used in Cheddar cheese manufacture are free from lipolytic enzymes. Hence the principal lipolytic agents in Cheddar cheese originate from LAB (Fox et al., 2000). Lipolytic agents from LAB can hydrolyse a range of FFA, mono-, di- and tri-glyceride substrates (Fox and Wallace, 1997). Though LAB are weakly lipolytic their existence in large numbers over a long period of time can cause substantial amount of lipolysis with liberation of FFA in cheeses without secondary lipolytic starter cultures (Collins et al., 2004). Numerous studies indicate that the lipolytic enzymes of LAB are intracellular; hence lysis of LAB in cheese during ripening causes considerable lipolysis. Cheddar cheese made with highly autolytic starter Lc. lactis subsp cremoris AM2 developed significantly higher levels of caprylate (C8:0), myristate (C14:0), palmitate (C16:0) and stearate (C18:0) during ripening compared to cheese made with less autolytic strain Lc. lactis subsp cremoris HP demonstrating that these lipolytic enzymes of LAB are intracellular as cell free extracts of both strains had similar activities (Collins et al., 2003). Catabolism of FFA is important for formation of flavour compounds; esters impart buttery or fruity aroma, however, thioesters formed by the reaction of short-chain fatty acids with methional impart cheesy aroma to Cheddar cheese (Arora et al., 1995). Important lipolytic flavour compounds such as methyl ketones (Dimos, 1992; Engels et al., 1997), secondary alcohols (Engels et al., 1997) and lactones (Wong et al., 1975) have also been reported in Cheddar cheese.
Figure 2.7 Pathways for the production of flavour compounds from fatty acids during cheese ripening (McSweeney, 2004).
2.2.2.2.3 Glycolysis

During cheese ripening, metabolism of lactose, lactate and citrate are collectively referred to as glycolysis. Lactose is the primary milk sugar, during manufacture of cheeses such as Cheddar; lactose is converted to L-lactic acid by SLAB. The rate and extent of acidification influences the pH, water activity and enzymatic reactions during ripening. Although most of the lactose is lost during whey drainage, small amount still remain in curd (Huffman and Kristoffersen, 1984). This lactose is immediately metabolised to L-lactate by SLAB at low concentration of S/M (salt-in-moisture) and low populations of NSLAB. However, in cheeses with high NSLAB populations such as raw milk cheeses at high S/M, lactose is converted to DL-lactate by NSLAB. High numbers of NSLAB are found to be responsible for racemization of L-lactate in Cheddar and similar cheeses (Crow et al., 2001). Racemization is thought to favour development of Ca-lactate crystals as Ca-DL-lactate is less soluble than Ca-L-lactate (Thomas and Crow, 1983; Dybing et al., 1988). Though racemization does not affect flavour significantly it may result in increased formation of Ca-lactate crystals in cheese resulting in undesirable appearance. Lactate can be oxidised to acetate, formate and ethanol (Figure 2.8) (Fox et al., 2000; McSweeney and Fox, 2004). However, formation of propionate and CO₂ is of importance in Swiss-type cheese for the characteristic eye formation (Piveteau, 1999).

Milk contains 1750mg of citrate per litre, though most of it is lost in whey (Fox et al., 1993), Cheddar cheese curd contains 0.2%-0.5% citrate and is metabolised by a few citrate positive lactococci to diacetyl, acetate, acetoin and CO₂ contributing to the cheese flavour (Figure 2.9) (McSweeney and Fox, 2004).
Figure 2.8 Pathways by which lactate is metabolised in cheese during ripening. (1) racemization, (2) metabolism by Propionibacterium freudereichii in Swiss cheese, (3) oxidative metabolism of lactate, (4) conversion to formate, (5) anaerobic metabolism of lactate to butyrate and H₂, which leads to late gas blowing (McSweeney and Fox, 2004).
Figure 2.9 Pathways for citrate-positive strains of *Lactococcus* and *Leuconostoc* sp (McSweeney and Fox, 2004).
2.2.2.3 Texture

Flavour and texture are the main characteristics responsible for the identification of cheese by consumers. Cheese texture is defined as “the attribute of a cheese resulting from a combination of physical properties including size, shape, number, nature and conformation of the constituent structural elements that are perceived by a combination of the senses of touch (tactile texture), vision (visual texture) and hearing (auditory texture)” (Delahunty and Drake, 2004). Requirements of Cheddar cheese texture vary with the utility, commercial cheeses sold sliced need to be firm whereas as those used as ingredients can be used even when extremely crumbly (Lawrence et al., 2004). Cheese texture is influenced by various factors such as pH, moisture, salt, calcium, fat and protein (Lawrence et al., 2004).

The lactic acid produced by SLAB early in cheese production drops the pH of the curd and at low pH the proteins assume increasingly compact structure resulting in firmer texture of the young cheese. Cheddar cheese in most countries is manufactured with a final pH around 5.1 to 5.3 as compared to 4.9 of the traditional English Cheddar, giving these cheeses a softer texture. The effect of pH on texture can be modified by factors such as moisture, salt and calcium. In the pH range of 5.1 and 5.5, colloidal calcium phosphate and casein are dissociated from sub-micelles and these changes in sub-micelle size and characteristics increases its water binding ability and solubilisation of the micelle by NaCl decreases as the calcium concentration increases (Creamer, 1985). Excessive salting in Cheddar cheese can result in firm-textured drier cheese (Van Slyke and Price, 1952; as cited by Lawrence et al., 2004). Salt concentration is also known to influence enzyme activity and conformation of $\alpha_{s1}$- and $\beta$-casein (Fox and Walley, 1971). In addition enzyme
activity, hydration of protein network and interaction of calcium with casein are also influenced by salt concentration (Delahunty and Drake, 2004).

Protein matrix is responsible for the rigid form of the cheese, thus the increased amount of protein in reduced fat cheese leads to firmer and more elastic texture compared to full fat Cheddar cheese (Lawrence et al., 2004). Breakdown of αs1-casein during ripening considerably softens the texture of the curd. Breakdown of peptide bonds as a result of proteolysis results in formation of water molecules with ionic groups. As ripening progresses and with increase in proteolysis, water available for protein solvation is increasingly bound by new ionic groups leading to firm and easily deformed cheese. Change in texture of the cheese depends on extent of proteolysis which in turn depends on duration of ripening (Lawrence et al., 2004). Increase in fat (Fenelon and Guinee, 2000) and moisture content in cheese results in slightly softer cheese.

2.2.2.4 Flavour

Flavour constitutes the olfactory and taste perception of cheese with aroma being the initial flavour perception. Texture and flavour are considered to be the most important attributes of Cheddar cheese (Jack et al., 1993) and vary considerably between cheese varieties. Raw milk cheeses are intensely flavoured and have atypical flavours (Lawrence et al., 2004). Cheese flavour and texture also vary to a lesser extent within the same batch and even between two regions for the same cheese. Cheddar cheeses are low in flavour at 2 months ripening and have intense flavour at 2 years of ripening. Extent of proteolysis with the release of peptides, amino acids and these acting as substrates for production of a range of compounds contribute to the flavour (McSweeney and Sousa, 2000). Key aroma compounds in
Cheddar cheese are known to originate from amino acids (Urbach, 1995). Bitterness is a very common flavour defect in Cheddar cheese caused by accumulation of low-molecular weight hydrophobic peptides and has been reviewed (Fox, 1989; Habibi-Najafi and Lee, 1996); now several exopeptidases are used to counter bitterness in cheese (Raksakulthai and Haard, 2003).

Low-fat Cheddar cheese lack typical Cheddar flavour (Foda et al., 1974; Olson and Johnson, 1990) indicating the contribution of fat to the Cheddar cheese flavour. Substituting milk fat with vegetable or mineral oil also resulted in atypical flavours with a degree of Cheddar flavour (Foda et al., 1974), suggesting that water-fat interface in cheese is important for flavour development and the flavour components are dissolved and retained in fat (Lawrence et al., 2004). Fatty acids, methyl ketones, keto acids, esters and lactones are the lipolytic flavour compounds in Cheddar cheese derived from milk fat; some may contribute directly to the flavour while some may contribute only to the background flavour (McSweeney and Sousa, 2000).

Starter lactic acid bacteria, NSLAB and secondary starters also contribute to the flavour of cheese by metabolising peptides, amino acids and fatty acids to smaller flavour compounds. Enzymes from cheese microflora are largely responsible for biochemical reactions leading to formation of flavour compounds.

2.3 Acceleration of cheese ripening

Ripening of certain cheese varieties involve complex and often slow biochemical processes (Fox et al., 1993; Fox and McSweeney, 1996; Fox and Wallace, 1997) and represents a significant proportion of the total cost of cheese production (Cliffe and Law, 1990; Picon et al., 1997). Long ripening implies expenses in refrigeration and maintenance with the risk of contamination and low yield, thus increasing the overall
production cost. Any attempt at accelerating cheese ripening is appreciated and is of
economic interest (Cliffe and Law, 1990) in addition to the technological advantage
it offers the cheese makers. Various methods employed to reduce the ripening time
of cheese have been extensively reviewed (Wilkinson, 1993; Law, 2001; Azarnia et
al., 2006). With an understanding of the biochemical processes occurring during
ripening, many researchers have attempted to accelerate cheese ripening by various
ways as summarised below.

### 2.3.1 Elevation of ripening temperature

Most varieties of cheese are ripened at low temperatures, for instance around 6-8°C
for Cheddar (Fox, 1988). At this low temperature the biochemical processes occur at
a very slow rate thus prolonging flavour generation. Increasing the ripening
temperature can increase the rate of biochemical reactions occurring during ripening.
Ripening of cheese at 13-15°C instead of 5-8°C accelerated the ripening time without
any development of off-flavours (El soda, 1993). Folkertsma et al. (1996) reported
accelerated proteolysis and lipolysis with improved flavour scores for Cheddar
cheese ripened at 16°C but suggested ripening at 12°C, as the cheeses ripened at
16°C deteriorated after 6 months. Law (2001) proposed that cheese manufactured
from good quality milk can be ripened at 15°C; while ripening of Cheddar cheese at
12°C can reduce the ripening time by 60-75% without any defects in body. A well
balanced and accelerated flavour development in Cheddar cheese was achieved by
ripening at 20°C or 12°C for 1 or 6 weeks respectively (Hannon et al., 2005). Though
elevation of ripening temperature appear to be a simple technique for acceleration of
cheese ripening, this method has several drawbacks such as risk of microbial
contamination leading to development of off-flavours and unhygienic cheese in
addition to risk of syneresis and fat exudation (Wilkinson, 1993, Fox et al., 1996). Use of this technique is recommended only for cheeses manufactured with high microbiological standards (El soda and Pandian, 1991; El soda, 1993; Wilkinson, 1993).

### 2.3.2 High pressure processing

Subjecting cheeses to high pressure of 50Mpa have shown to accelerate ripening as reviewed by Law (2001). It appears that increase in pressure may stimulate cell lysis; releasing intracellular enzymes leading to acceleration of ripening. La Serena cheeses treated with high pressure (300-400Mpa 10 min at 10°C) on day 2 showed higher levels of hydrophilic peptides and lower levels of hydrophobic peptides, increased levels of free amino acids than control and also showed delayed breakdown of caseins. Cheeses treated at 400Mpa on day 2 had the lowest flavour score while the other high pressure (300Mpa) treated cheeses did not differ from controls in flavour scores (Garde et al., 2007). In a similar study confocal laser scanning microscopy showed differences in the structure of high pressure treated and control cheeses, temperature of pressurisation was suggested as a significant determinant of the overall effect of high pressure treatment (O’Reilly et al., 2003). Increased hydrolysis of β-casein was reported for cheese treated to high pressure on day 15 of ripening, increased levels of free fatty acids were noted compared to control (Juan et al., 2008) suggesting the importance of time of high pressure treatment in acceleration of cheese ripening. However, there are very few reports of application of high pressure to accelerate cheese ripening and its utility is uncertain at this stage.
2.3.3 Attenuated starters

Starters are known to strongly influence flavour and texture development in cheese (Shakeel-Ur-Rehman et al., 1999) and thus are an easy and obvious choice for manipulation for controlled acceleration of ripening (Law, 2001). The main function of starters during cheese manufacture is production of acid at a standard rate, while the enzymes from starters also contribute to ripening. The growth of starters cease immediately after salting and pressing, and the cells lyse and release intracellular and extracellular enzymes contributing to ripening. Increased number of starters can potentially accelerate ripening but also lead to increased acid production and low pH which can be undesirable. Addition of starter bacteria unable to produce significant levels of lactic acid but able to deliver enzymes during cheese ripening can supplement ripening thus resulting in acceleration of ripening. These starters are attenuated by treatments like heating, freezing, spray-drying, freeze-drying or weakened by lysozyme or solvents. Use of attenuated starters to accelerate cheese ripening has been well reviewed by Klein and Lortal (1999) and also by Madkor et al. (2000), Law (2001) and Azarnia et al. (2006).

Heating is the most widely used form of cell attenuation (Klein and Lortal, 1999). Though heat attenuated starters were widely used for acceleration of cheese ripening (Kim et al., 1994; Skeie et al., 1995, 1997; Asensio et al., 1996), only a few studies reported reduction in ripening time (Abdel Baky et al., 1986; Aly, 1990; Kebary et al., 1997). A 90% reduction in acid production also with 60% reduction in cell wall proteinase activity and 10% reduction in amino peptidase activity was noted for *Lactobacillus helveticus* attenuated at 64°C, increasing the temperature to 66°C caused considerable reduction in proteinase and peptidase activities (Castaneda et al., 1990) and development of off-flavours was also reported (Castaneda et al., 1990;
Johnson et al., 1995). Alternative starter attenuation processes used for acceleration of cheese ripening include spray/freeze-drying (Johnson and Etzel, 1995), freezing (Johnson and Etzel, 1995; Verdini et al., 2005), freeze-shock (freeze and rapid thawing) (Spangler et al., 1989; Aly, 1990, El soda et al., 1991, 1992; Johnson et al., 1995; Kebary et al., 1997; Madkor et al., 2000), exposure to lysozyme (Law et al., 1976) and exposure to bacteriocin (Morgan et al., 1995, 1997). Though increased proteolysis, improved flavour score and reduction in bitterness was reported, reduction in actual ripening time was reported only in a few studies also with development of uncharacteristic and off-flavours (Johnson and Etzel, 1995; Johnson et al., 1995). Cheddar cheese manufactured with high pressure attenuated starters *Lc. lactis* ssp *cremoris* HP and *Lc. lactis* ssp *cremoris* 303 had higher levels of amino acids compared to controls (Upadhyay et al., 2007) accelerating secondary proteolysis. Success of this approach towards acceleration of cheese ripening has prompted development of Flavour control FC™ (Chr. Hansen) and Enzobat™ (Medipharm), commercial attenuated starters for enhancement of flavour.

### 2.3.4 Starter selection

The primary function of starters is acid production during cheese manufacture, strain selection offers opportunity to supplement the enzyme system thus potentially accelerating ripening. Muir et al. (1996), Shakeel-Ur-Rehman et al. (1999) and Pripp et al. (1999) suggested use of single strain of *Lactococcus* as the starter for improved cheese quality. Starter lysis ensures the involvement of intracellular enzymes in the gradual process of cheese flavour formation (Meijer et al., 2004); endogenous cell wall associated enzymes, phage encoded enzymes, autolysins, and endolysins can induce this phenomenon (Lortal and Chapot-Chartier, 2005). Premature lysis of
starters can cause early release of intracellular enzymes; hence these starters have the potential to accelerate ripening. Phage-induced lysis of *Lactococcus* in cheese leads to accelerated release of amino acids and reduced bitterness (Crow et al., 1995a, 1995b). Use of fast lysing and heat sensitive stains of *Lactococcus* (Feritag and McKay, 1987), *Lc. lactis* subsp *cremoris* AM2 (Chapot-Chartier et al., 1994), thermophilic *Lactobacillus* spp. and *Streptococcus thermophilus* (Lortal et al., 1997), *Lc. lactis* subsp *cremoris* (Collins et al., 2003) have been shown to increase flavour development in cheese during ripening.

### 2.3.5 Genetically modified starters

Genetically modified starters over expressing genes for proteolytic or lipolytic systems were developed to augment starter enzyme system during ripening. Robust proteinase genes from non-cheese microorganisms were cloned into starters for increased production of proteolytic enzymes for accelerated ripening. 

*Bacillus subtilis* neutral proteinase (BSNP) gene cloned in *Lc. Lactis* UC 317 caused extensive proteolysis in Cheddar cheese within 2 weeks at 8°C; blend of 80:20 unmodified:modified cells were recommended for a more controlled proteolysis (McGarry et al., 1994; as cited by Fox and Tobin, 1999). Proteolysis or texture of Cheddar cheese made from recombinant chymosin from *E. coli* (Green et al., 1985) or *Kluyveromyces lactis* (Bines et al., 1989) did not differ significantly from control cheeses with chymosin from calf rennet. Common peptidases from NSLAB have been expressed in starters for increased proteolytic activity to supplement the contribution of NSLAB to proteolysis during ripening. *Lc. lactis* subsp. *cremoris* MG1363 was genetically modified to over-express the genes pepC, pepN, pepO, pepV or the lysin gene of the bacteriophage ΦvML3 (Guldfeldt et al., 2001). Cheeses
produced with strains over expressing pepN or pepC scored well for flavour with reduced bitterness while cheeses produced with strains over expressing pepO or pepV were indistinguishable from controls and also in the cheeses made from strains with lysin gene were found to be different and it was concluded that lytic property of the recipient strain was important for the flavour development (Guldfeldt et al., 2001). Cheeses made from *Lc. lactis* starters enriched with lactobacilli peptidases led to 3-fold increase in amino acid levels (Courtin et al., 2002). Starter modification for increased sensitivity to lysis has been exploited for early lysis and release of intracellular enzymes during ripening. A three-strain starter system was proposed by Morgan et al. (2002), comprising of a bacteriocin producing strain, a bacteriocin sensitive strain for lysis upon exposure to bacteriocin to release the intracellular enzymes thus accelerating ripening and a bacteriocin insensitive strain for development of acid during manufacture. A *Lactococcus* starter strain PRA270 genetically modified to produce streptokinase, an extracellular protein produced by *Streptococcus uberis* is known to activate plasminogen to plasmin, the active indigenous milk proteinase. Cheddar-type cheeses made with PRA270 showed increased plasmin activity, accelerated hydrolysis of β-casein resulting in increased concentrations of γ-casein during ripening (Upadhyay et al., 2004b). Though genetic modification appears to be a specific approach it is technically complex and is weakly accepted by many consumers.

### 2.3.6 Adjunct cultures

Contribution of NSLAB to cheese ripening is well known and this knowledge has been applied for development of adjunct cultures for acceleration of cheese ripening. Selected strains of NSLAB are deliberately added during cheese manufacture, as
adjunct cultures in the aim of accelerating ripening (McSweeney et al., 1993). Addition of cell free extracts of brevibacteria caused accelerated flavour development in Cheddar cheese (Law, 1987).

Cheese manufactured with adjunct cultures such as are *Brevibacterium linens* (Weimer et al., 1997), NSLAB (Swearingen et al., 2001), *Lactobacillus lactis* (Andersen and Madsen, 2004), probiotic cultures of *Lb. acidophilus*, *Lb casei*, *Lb paracasei* and *Bifidobacterium* spp. (Ong et al., 2006), *Lb. acidophilus* and *Lb. paracasei* subsp. *paracasei* (Bergamini et al., 2006), autolytic or fast lysing strains of *Lb. helveticus* (Hannon et al., 2003; Kenny et al., 2006) NSLAB; *Lb. plantarum* (Tavaria et al., 2006) are reported to have improved flavour development. In addition to use of SLAB and NSLAB as adjunct cultures, yeasts have also been exploited for contribution to cheese ripening; *Geotrichum candidum* (Berger et al., 1999; Martin et al., 1999), *Debryomyces hansenii* and *Yarrowia lipolytica* (Ferreira and Viljoen, 2003; De Wit et al., 2005).

Although increased levels of flavour compounds were detected in cheeses with adjunct cultures, there are no reports of reduction in ripening time or successful commercial application. Few studies have also reported development of off-flavours in cheeses with adjunct cultures.

### 2.3.7 Exogenous enzymes

Cheese ripening is mainly due to the action of enzymes from coagulant, milk, SLAB and NSLAB. Addition of proteolytic and lipolytic enzymes was considered as a more defined and simpler approach to accelerate cheese ripening and has been reviewed by Fox and Stepaniak (1993).
2.3.7.1 Proteolytic enzymes

Early attempts of exogenous enzyme addition to milk to accelerate cheese ripening were not completely successful as bitterness, soft body, brittleness and other defects were associated with increased proteolysis (Sood and Kosikowski, 1979; Law and Wigmore, 1982, 1983; Fernandez-Garcia et al., 1988; Guinee et al., 1991). Flavour intensity, formation of soluble nitrogen, liberation of FAA and FFA were enhanced in Ras cheese made from β-galactosidase treated milk (Farahat et al., 1984).

Addition of cheese slurries to cheese curd have also shown to accelerate ripening in Cheddar (Dulley, 1976; Cliffe and Law, 1990), Swiss (Singh and Kristoffersen, 1971), Ras (Abdel Baky et al., 1982) and Blue (Rabie, 1989) cheeses. Kilcawley et al. (2006) reported development of a novel method to generate enzyme modified cheese (EMC) and Hannon et al. (2006) reported accelerated Cheddar cheese ripening due to addition of EMC powder.

Extracts from crustacean munida was used as coagulant in miniature Cheddar cheese manufacture and complex proteolytic products were observed during ripening compared to cheeses manufactured with chymosin as the coagulant (Rossano et al., 2005).

Hydrolysis of β-casein by plasmin can be augmented by use of plasminogen activators such as urokinase and streptokinase. Exogenous addition of urokinase, a serine proteinase that cleaves plasminogen to plasmin to accelerate cheese ripening resulted in increased proteolysis in Havarti (Bastian et al., 1991), St-Paulin, Swiss (Bastian et al., 1997) and Cheddar cheeses (Barrett et al., 1999). Cheddar cheese manufactured from milk supplemented with semi-purified streptokinase, a plasminogen activator, showed a ~3 fold increase in plasmin activity compared to control with accelerated hydrolysis of β-casein and increased concentrations of γ-
casein. However the levels of FAA were unaffected and increased concentration of hydrophobic peptides were reported (Upadhyay et al., 2004b). Combined action of proteinases and phosphatases are responsible for complete degradation of casein during cheese ripening, hence phosphatases may play a major role in cheese maturation and flavour development (Fox and Stepaniak, 1993). Different peptide pattern was noted while no significant difference in protein or nitrogen content was observed when miniature Cheddar cheeses were manufactured with exogenous preparation of alkaline phosphatase (Shakeel-Ur-Rehman et al., 2006).

Amino acid catabolism during cheese ripening is known to be responsible for formation of flavour compounds, Yvon et al. (1998) and Banks et al. (2001) reported enhanced amino acid catabolism in Cheddar cheese upon exogenous addition of transaminase, α-ketoglutarate. Several commercial enzymes such as Flavourzyme, (Novozymes), Neutrase (Novozymes), Accelase (Danisco) have been recommended by the companies for debittering and accelerated flavour development in cheese and these claims need to be validated.

### 2.3.7.2 Lipolytic enzymes

Contribution of lipolysis to flavour and aroma of Cheddar cheese is not well understood and proteolysis is thought to be the main contributor of flavour compounds in Cheddar cheese. Enzymes from *Aspergillus oryzae* containing both proteolytic and lipolytic fraction, was found to produce Cheddar aroma in cream, and lipase was thought to be the main contributor of the aroma (Arbige, 1986). This was later developed into a commercial enzyme FlavorAge (Chr. Hansen, Inc.) and recommended for acceleration of cheese ripening. Free fatty acid composition of
cheeses treated with this enzyme was similar to control but in higher concentrations (Arbige, 1986). Piccantase (Gist Brocades), Palatase (Novozymes), Capalase (Dairyland) and kid/lamb lipase-esterase (Chr. Hansen) are other commercial lipases recommended for acceleration of cheese ripening. Addition of commercial lipases to milk during cheese manufacture have shown to enhance the FFA profile (Law and Wigmore, 1985) and also has been recommended for acceleration of cheeses such as Tulum (Yilmaz et al., 2005).

Enzymes ranging from proteinases, exopeptidases, plasminogen activators, lipases, β-galactosidases, phosphatase and enzymes involved in amino acid catabolism have been exogenously added to cheese in the aim of increased flavour development, reduction of bitterness and accelerated ripening. Accelerated proteolysis was achieved, often with textural defects or with no significant improvement in flavour profile. Though commercial proteinases have been successful in reducing bitterness, attaining balanced textural and flavour profiles still remain a challenge (Wilkinson and Kilcawley, 2005). Addition of free enzyme to the milk, with rennet or starter culture results in loss of nearly 90% of the enzyme at whey drainage (Law and Kirby, 1987). Addition of enzymes to cheese curd at dry salting stage would raise the problem of uneven distribution of small amount of enzyme in large masses of curd (Law, 2001; Wilkinson and Kilcawley, 2005). Uncontrolled premature and extensive proteolysis with development of bitterness and off-flavours in addition to whey contamination and loss of yield are some of the problems associated with addition of free enzymes to accelerate cheese ripening (Law, 2001).
2.3.7.3 Microencapsulation of enzymes for acceleration of cheese ripening

Encapsulation involves confinement of substance/substances of interest within a membrane or a matrix for isolation (Dziejazk, 1988), protection from environment (Gibbs et al., 1999) or for controlled delivery (Skeie, 1994) of the entrapped material. The technique of microencapsulation has been studied by a number of groups for delivering flavour-enhancing enzymes for acceleration of cheese ripening and is an alternative approach towards accelerating cheese ripening. This method of adding exogenous enzymes is free from the problems associated with addition of free enzymes (Wilkinson and Kilcawley, 2005). Several proteolytic/lipolytic enzymes have been microencapsulated for this purpose and this subject has been reviewed (El Soda et al., 1989; Skeie, 1994; Wilkinson and Kilcawley, 2005).

Early attempt at acceleration of cheese ripening by microencapsulated cell free extracts (CFE) of *Lc. lactis* ssp *diacetylactis* in milk fat was by Magee et al. (1981), cooking cheese at 30°C or 33°C disrupted the capsules causing early release of CFE. More heat stable milk fat capsules with CFE of *Lc. lactis* var. *maltigens* and *Gluconobacter oxydans* were used and higher levels of flavour compounds were reported. These capsules were found to be stable at 42°C for only 60 min.

Hydrogel beads designed to melt at <10°C and hence ideal for release of enzymes during cheese ripening was proposed by Perols et al. (1997) with an encapsulation efficiency of ~50%; however, bead syneresis interfered with the enzyme release. Kailasapathy et al. (1998) used food gums such as agar, alginate, gellan, κ and τ-Carrageenan and κ-Carrageenan-locust bean gum for application of encapsulated enzymes into cheese for acceleration of ripening. Encapsulation efficiencies ranging form 29-57% and capsule retention in curd ranging from 74-91% was reported. Increased proteolysis, lower β-casein and higher amino acid levels were observed in
cheeses treated with enzyme capsules. Flavour of experimental cheeses did not differ from controls. Cheeses made with enzyme capsules scored lower for texture while bitterness was highest in cheeses with κ-Carrageenan capsules (Kailasapathy and Lam, 2005).

Alternative method of enzyme encapsulation in liposomes was developed by Law and King, (1985). Liposomes are phospholipid bilayers that form spherical structures upon dispersion in aqueous solutions and these vesicles can be unilamellar or multilamellar (MLV) depending on the manufacturing method (Walde and Ichikawa, 2001). This method has evolved as the dominant method of enzyme encapsulation to accelerate cheese ripening and many researchers have reported acceleration of proteolysis in cheese during ripening by addition of liposome-encapsulated enzymes. Encapsulation efficiency in small unilamellar vesicles (SUV) was very poor at ~1-3% (Kirby et al., 1987), hence most of the studies have focused on multilamellar vesicles (MLV) and they can be produced in various ways. Encapsulation efficiency of ~30% was achieved with multilamellar vesicles produced by reverse phase evaporation technique (Alkhalaf et al., 1989). Multilamellar vesicles, produced by hydration had encapsulation efficiencies 45-70% (Fresta et al., 1995); Multilamellar vesicles produced by microfluidization had encapsulation efficiencies 11-22% (Lariviere et al., 1991). Multilamellar vesicles produced using dehydration-rehydration technique had encapsulation efficiencies ~35% (Skeie et al., 1995). Enhanced proteolysis, no bitterness and firmer texture were characteristic of cheeses with *Bacillus subtilis* neutral proteinase (BSNP) entrapped in liposomes by dehydration-rehydration with encapsulation efficiency of 54.3% (Picon et al., 1995). Encapsulation efficiencies of proteinases encapsulated in stimulated release liposomes were 14.5% for chymosin, 27.5% for BSNP and 11.7% for cyprosins.
(Picon et al., 1997). Multilamellar vesicles produced from proliposomes had encapsulation efficiencies up to 96% and curd entrapment level of 64% (Dufour et al., 1996). Release of liposome-encapsulated enzymes in cheese is influenced by pH, fat content and temperature. Release of enzymes from liposomes was significantly stimulated by increasing the fat content from 0 to 20% and pH from 4.9 to 5.5; however increasing the ripening temperature had no significant effect on the release of enzymes from liposomes and the principles involved in the release of liposome encapsulated enzymes could not be completely explained (Laloy et al., 1994, 1998). Acceleration of Cheddar cheese ripening with liposome encapsulated bacterial and fungal proteinases was achieved with encapsulation efficiencies of 32% and 33% respectively, improved organoleptic properties and no bitterness was detected (Kheadr et al., 2000). Enzyme mixtures containing bacterial protease and lipase, fungal protease and lipase and Flavourzyme® and lipase encapsulated in liposomes, accelerated Cheddar cheese ripening with higher flavour intensity in short period without texture, rancid or bitter defects (Kheadr et al., 2003).

Majority of the studies involving enzyme encapsulation for acceleration of cheese ripening have used liposomes as the encapsulating material often with low encapsulation efficiency and poor understanding of the release mechanism of the enzymes during ripening.

2.4 Microencapsulation

While liposomes have been the predominant materials used for enzyme encapsulation for acceleration of cheese ripening they have poor encapsulation efficiencies and also are expensive ingredients to be applied on a commercial scale. Though a few researchers have reported use of alternative materials like food gums
and milk fat for enzyme encapsulation for acceleration of cheese ripening, the potential of other materials for this purpose has not been exploited. Currently almost any substance can be encapsulated for isolation, purification, protection or controlled release.

### 2.4.1 Techniques of Microencapsulation

Microencapsulation techniques currently used in the food industry are spray-drying, spray-chilling or spray-cooling, fluidized-bed coating, liposome entrapment, coacervation, inclusion complexation, rotational or centrifugal suspension separation and extrusion as reviewed by Gibbs et al. (1999).

Spray-drying involves homogenisation of the material of interest with the carrier material in 1:4 ratios and the mixture is fed into a dryer through a nozzle as opposed to cooling of the mixture in cool air by spray-chilling/cooling. The latter requires vegetable oil or hydrogenated oil.

In fluidized-bed coating, solid particles are suspended and the coating material is atomised, the amount of coating depends on the length of time the particles are in chamber.

Extrusion mainly involves dispersion of materials into a liquid; the dispersed material solidifies on contact with the liquid trapping the particles within a matrix.

Liposomes consist of one or more layers of phospholipids, hydrophilic portion orients towards aqueous phase while the hydrophobic portion associates with hydrophobic portions of other lipid molecules. Folding of the lipid sheet into a spherical shape forms stable capsules and this folding can be induced by various solvents or aqueous solutions.
Coacervation involves emulsification of the material followed by separation of the liquid phase which is used to coat the core material. This method though efficient can be expensive.

Microencapsulation by inclusion complexation involves entrapment of materials in the hydrophobic core of β-Cyclodextrin with hydrophilic surface.

Centrifugal suspension separation is a relatively new technique and involves mixing of the core and wall materials and then adding to a rotating disk. The core materials then leave the disk with a coating of residual liquid. The microcapsules are then dried or chilled after removal from the disk.

### 2.4.2 Hydrogels

Hydrogels are formed from hydrophilic polymers which may absorb from 10-20% to thousands of times their dry weight in water. They can be chemically stable or degradable. When a polyelectrolyte is combined with a multivalent ion of opposite charge, it may form a hydrogel known as an ionotropic hydrogel, for example calcium alginate. When polyelectrolytes are mixed they may gel or precipitate. Pores may be formed in hydrogels by phase separation during synthesis or may exist as smaller pores in the matrix (Hoffman, 2002). Labelled molecular probes of a range of molecular weights are used to probe the pore size in hydrogels (Dong et al., 1994). Polymers such as alginate, κ-Carrageenan, chitosan form firm hydrogels and could also be potential entrapment materials for enzymes for application in acceleration of cheese ripening.
2.4.3 Alginate

Alginate is a polysaccharide that can form a hydrogel. It is available as the sodium salt of polyuronic acid containing varying proportions of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). An ionic network developed in the presence of Ca\(^{2+}\) or other multivalent cations forms a gel (Betigeri and Neau, 2002). CaCl\(_2\) is the commonly used cation for gelation of alginate. It is widely used in matrix immobilisation of wide range of biological materials ranging from bacterial cells (Champagne et al., 1992; Kailasapathy, 2002), mammalian cells (Koch et al., 2003), enzymes (Blandino et al., 2000), vitamins (Madziva et al., 2006) and also for controlled drug delivery (Sugawara et al., 1994). The wide application of this polymer can be attributed to the mild conditions involved in the preparation and low cost (Fundueanu et al., 1999).

Properties of Ca-alginate hydrogels can be easily modified on treatment with starch (Sultana et al., 2000), pectin (Madziva et al., 2006), xanthan (Elcin, 1995), poly-L-lysine (Strand et al., 2002), polyethylene glycol (Chandy et al., 1999) or chitosan (Sezer and Akbuga, 1999) to suit the application it is being prepared for and is a potential entrapment material for enzymes for application in acceleration of cheese ripening.
Figure 2.10 Structure of alginate. Alginate molecules are linear block copolymers of β-D-mannuronic acid (M) and α-L-guluronic acids (G) with a variation in composition and sequential arrangements (De Vos et al., 2006).
2.4.4 Carrageenan

Carrageen is a food grade, biocompatible high molecular weight polysaccharide extracted from seaweed that forms a hydrogel (Velde et al., 2002). Its primary structure is made up of alternating $\alpha$-(1,3)-D-galactose-4-sulphate and $\beta$-(1,4)-3,6-anhydro-D-galactose residues. $\kappa$-Carrageenan forms a thermoreversible gel on cooling and also in the presence of salts; mainly potassium, which bind specifically to the helices. Molecules undergo a coil-helix transition followed by aggregation of helices and salts reduce electrostatic repulsion between chains thus promoting aggregation leading to gel formation (Williams and Phillips 2000). Sodium salt of $\kappa$-Carrageenan is soluble in cold water and sets into a gel on cooling between 40-60°C depending on the cations present. It is used in the encapsulation of cells (Cassidy et al., 1997), enzymes like $\alpha$-chymotripsin (Belyaeva et al., 2004) and its other applications for immobilisation has been reviewed by Velde et al. (2002).

2.4.5 Chitosan

Chitosan is a polymer of $\beta$ (1→4)-2-amino-2-deoxy-D-glucose obtained from chitin by N-deacetylation. Chitosan is insoluble in aqueous solution at pH 6 except for low molecular weight samples (Terbojevich and Muzzarelli, 2000). Chitosan gels on addition of cross-linking reagent or by ionotropic gelation with certain anionic counterions (Cabral and Kennedy, 1991). It is a non-toxic, biocompatible, antibacterial and biodegradable polyelectrolyte and for this reason has been used for a variety of applications including controlled drug delivery (Borzacchielo et al., 2001). Cross linking of chitosan with other polymers has shown to improve its physical and mechanical properties. Chitosan has several applications including cell
encapsulation (Zielinski and Aebischer, 1994; Chandy et al., 1999), drug delivery (Sezer and Akbuga, 1999) and plasmid delivery (Bozkir and Saka, 2004). It appears to be a suitable material for enzyme encapsulation for acceleration of cheese ripening.

2.4.6 Starch

Starch is the second most naturally abundant polysaccharide of plant origin. It is made up of glucose polymers. Linear polymer of glucose forms the amylose molecule while the branched polymer forms the amylopectin molecule. Starch is made up of insoluble microscopic semi crystalline granules 1 - 100µm in size (Murphy, 2000). It is used with hydrogels for encapsulation of bacteria (Sultana et al., 2000).

2.4.7 Pectin

Pectins are hetero-polysaccharides with at least 65% of galacturonic acid units. The acid group may be free or esterified with methanol. De-esterification yields a range of ‘low methoxyl’ pectins that have greater reactivity towards calcium ions which will cause gelation under suitable conditions of soluble solids and pH (May, 2000). It has been used with alginate for encapsulation of folic acid (Madziva et al., 2006).

2.4.8 Poly-L-lysine

Poly-L-lysine (PLL) is a naturally occurring cationic amino acid polymer (Aynie et al, 1999) and the most widely studied polycation for alginate bead coating (Orive et al., 2006). Addition of PLL is known to strengthen the alginate gel structure (Lim

2.5 Analysis and evaluation of cheese ripening

The demand for constant supply of consistent low-cost high intensity flavour in cheese had led to development of novel ways for monitoring cheese ripening. At first basic analytical techniques were developed to measure proteolysis and lipolysis; the major biochemical processes occurring in cheese during ripening. Proteolysis during cheese ripening was initially monitored by spectroscopy/colorimetry (trinitrobenzene sulphonic acid soluble fraction, trichloroacetic acid soluble nitrogen and Kjeldahl method for determination of total nitrogen) for measurement of amino acids and peptides. Advanced techniques involving electrophoresis (capillary electrophoresis, polyacrylamide gel electrophoresis) and chromatography (reverse phase - high performance liquid chromatography, gas chromatography) are now widely used for characterisation and quantification of peptides, fatty acids and amino acids from cheese (Cliffe et al., 1989; Cliffe and law, 1990; Verdini et al., 2004; Veloso et al., 2004). Moisture and textural changes in cheese during ripening were monitored by measuring the ultrasonic velocity of Mahon cheese (Benedito et al., 2000, 2002). Dupont et al. (2003) used rabbit polyclonal anti-peptide antibodies against the cleavage sites to measure the extent of cleavage of peptide bonds by protease, proposing the use of ELISA with the specific antibodies for assessing proteolysis.
Statistical methods have also been employed to analyse data to predict cheese ripening. Ripening time of Manchego cheese was predicted using partial least squares (PLS) regression (Poveda et al., 2004). Coker et al. (2005) statistically analysed proteolysis data by principal component analysis (PCA) and multiple linear regression to predict ripening of cheese. Advanced spectroscopy and proteomics were employed by Gagnaire et al. (2004) to investigate the release of enzymes in Emmental cheese aqueous phase by using methodology based on size exclusion chromatography, 2D-electrophoresis, MALDI-TOF MS (matrix assisted laser desorption/ionisation – time of flight mass spectrometry) and LC-ESI-MS/MS (liquid chromatography electrospray ionisation tandem mass spectrometry) and identified five functional groups of proteins involved in 1) proteolysis, 2) lipolysis, 3) stress response, 4) DNA and RNA repair of LAB and 5) oxido-reduction. Proteomics is fast evolving as a powerful tool for monitoring cheese ripening, reports of use of proteomics for analysis in cheese science is constantly increasing. Piraino et al. (2007) developed a rapid method using (MALDI-TOF MS) for analysis of peptides from the ripening cheese, the analysis time was reported to be ~ 3 min compared to 100 min for analysis by RP-HPLC. Infrared spectroscopy has also been exploited as a potential tool for measuring cheese composition (Rodriguez-Soana et al., 2006) and texture (Blasquez et al., 2006; Karoui et al., 2007). Applying similar technique, Martin-del-campo et al. (2007) followed proteolysis, lipolysis and glycolysis by Fourier transform infrared (FTIR) spectroscopy to monitor cheese ripening. Analysis of variance (ANOVA) showed significant changes on spectra sets and two different steps of ripening were described by principal component analysis of the spectral data.
Sensory methods are also employed to forecast ripening trend and also for quality control. These methods involve a scoring system with the grant of maximum score based on desirable qualities of the cheese and deduction of score for undesirable characteristics or sensory evaluation by a panel of trained judges (Delahunty and Drake, 2004).

### 2.6 Factors affecting cheese quality

Ripening of cheese is due to the action of coagulant, microorganisms and their enzymes. Every batch of cheese varies even though all the variables such as starters, coagulant and ripening atmosphere are constant. This variability in the final cheese quality can be attributed to several factors and their interaction (Fox and Cogan, 2004). Source and quality of milk, coagulant, NSLAB and ripening temperature are the major factors affecting cheese quality.

Milk quality, stage of lactation and animal nutrition affect cheese quality as pasteur-fed animal milk contain higher levels of carotene and hence impart deeper colour to cheese. Increased proteolysis and higher levels of free fatty acids may be present in late-lactation milk (Hickey et al., 2006) and pathogens from milk of poor microbiological quality can cause off-flavours in cheese (Fox and Cogan, 2004). Acidification may be insufficient if the milk is contaminated with excessive antibiotics or if inactive starters are used thus affecting coagulation, syneresis, flavour and texture. In modern practice standard casein-fat composition is used for consistent cheese quality.
2.7 Summary

Cheese is an important part of the daily diet with high per capita consumption in Australia and most other parts of the world. Most cheeses are consumed after ripening, an important stage of cheese production. It involves complex biochemical processes occurring in cheese curd after manufacture. Cheese ripening can range from a few weeks to over 3 years depending on the variety, thus contributing considerably to the cost of cheese production. Any attempt at minimising the ripening duration has attracted attention. Though researchers have been trying over decades to understand cheese ripening process in order to accelerate ripening, there are several steps during ripening that are not yet well understood, thus limiting the efforts at acceleration of ripening. There are several reports of acceleration of cheese ripening in various varieties using a range of methods but very few reports of successful commercial application possibly due to complexity of the methods and poor results at industrial scale. Addition of exogenous enzymes during cheese manufacture appears to be a specific and simple approach towards accelerating ripening. Extensive proteolysis and bitterness associated with addition of free enzymes can be avoided by addition of microencapsulated enzymes. Though several commercial lipases and proteases are recommended for acceleration of cheese ripening, very few reports have tested these enzymes. Enzymes for cheese ripening have been mostly encapsulated in liposomes, which are expensive ingredients and hence are not feasible for commercial application. Reports of enzyme encapsulation in other economic ingredients such as milk fats and food gums show poor efficiency of the enzyme in addition to poor understanding of the release mechanism of encapsulated enzymes in cheese during ripening. Inert economical food grade polymers such as alginate, chitosan and κ-Carrageenan have been extensively used.
for encapsulation of biological and biochemical materials like bacterial and mammalian cells, tissues, enzymes, vitamins and also for controlled drug delivery. These polymers can be used to microencapsulate enzymes to be delivered into cheese for acceleration of ripening.
3 SCREENING AND SELECTION OF POLYMERS FOR ENZYME ENCAPSULATION
3.1 Abstract

Screening of alginate, chitosan and κ-Carrageenan for microencapsulation of Flavourzyme showed that alginate was a suitable polymer for enzyme encapsulation using the Inotech® encapsulator, as chitosan and κ-Carrageenan were too viscous even at low concentrations to give satisfactory capsules. Encapsulation efficiency of Flavourzyme-alginate gel beads formed in 0.1M CaCl₂ was found to be ~17-18%. Poor encapsulation efficiency was attributed to the high porosity of alginate gel beads and possible diffusion of Flavourzyme during encapsulation. Starch (Hi-Maize™) and pectin were mixed with alginate to minimise the porosity of the gel beads however there was no improvement in the encapsulation efficiency of Flavourzyme. Gelling of the alginate-Flavourzyme beads in the chitosan treated cationic solution of 0.1M CaCl₂ significantly increased (p < 0.05) the encapsulation efficiency to 70-88% depending on the chitosan concentration. Though encapsulation efficiency increased with the increase in chitosan concentration, 0.1% was used as higher concentrations caused clumping of capsules during formation. Optimum gelling time of the capsules in chitosan treated cationic solution was found to be 10 min. Alginate concentration in the range 1.6 to 2.0% (w/v) was found to be suitable for Flavourzyme encapsulation and 2% (w/v) tri-sodium citrate was found to release most of the encapsulated enzymes \textit{in vitro} for measurement of enzyme activity. Coating of the Flavourzyme capsules in 0.15% (w/v) alginate or 0.05% (w/v) poly-L-lysine showed no significant difference (p > 0.05) in enzyme retention compared to those without any coating. Enzyme capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate allowed to gel in 0.1M CaCl₂ containing 0.1% chitosan, stored frozen and freeze-dried were found to be shelf-stable for at least 10 weeks retaining about 80% of the initial enzyme activity unlike capsules stored after air-drying which retained
Enzyme encapsulation

only 25-50% of the initial enzyme activity. Morphological assessment of enzyme capsules gelled in cationic solution containing chitosan revealed mainly spherical capsules with an average diameter of 500μm.
3.2 Introduction

Cheese is a popular dairy product with some varieties requiring extended ripening which is an economical burden (Picon et al., 1997). Though several techniques were developed for acceleration of cheese ripening, not many have been successful at commercial scale. Exogenous addition of enzymes that catalyse the biochemical reactions occurring during cheese ripening appears to be a simple approach but has lead to premature extensive proteolysis in addition to contamination of whey. Enzyme delivery in the microencapsulated form was found to be a solution for the problems associated with the use of free enzymes (Wilkinson and Kilcawley, 2005). Encapsulation in milk fat (Magee et al., 1981; Braun and Olson, 1984), hydrogel (Perols et al. 1997), food gums (Kailasapathy et al. 1998; Kailasapathy and Lam, 2005) and liposomes (Law and King 1985; Picon et al., 1995, 1997; Dufour et al., 1996; Laloy et al., 1998; Kheadr et al., 2000, 2003) have been used for enzyme encapsulation for acceleration of cheese ripening. Liposomes appear to be the popular material for this purpose as a number of studies have reported use of liposome-encapsulated enzymes for acceleration of cheese ripening. Liposomes are expensive to be used on an industrial scale and hence not feasible for commercial acceleration of cheese ripening.

Though there have been significant developments in the encapsulation technology for application in biological science, very few techniques have been studied for delivery of enzymes into cheese for acceleration of ripening. Polysaccharides such as alginate, chitosan and κ-Carrageenan are food-grade polymers that form firm gels with ions of opposite charge. These polymers are inert and inexpensive and have been widely used for encapsulation of biological and biochemical substances ranging from cells, enzymes and drugs to plasmid for the purpose of protection, isolation or
Enzyme encapsulation controlled delivery (Champagne et al., 1992; Sezer and Akbuga, 1999; Blandino et al., 2000; Koch et al., 2003; Bozkir and Saka, 2004). These polymers can be used to encapsulate flavour-enhancing enzymes for acceleration of Cheddar cheese ripening. Food-grade polymers were screened in this study for the encapsulation of flavour-enhancing enzymes. Though several advanced techniques for efficient enzyme encapsulation have been reported, many techniques involve use of strong chemicals, solvents or fats which is undesirable for application in cheese due increased reports of obesity and related illnesses and also the risk of rejection by consumers. Hence the Inotech® encapsulator was chosen for enzyme encapsulation as it requires mild conditions such as only enzyme-polymer mixture and gelling solution for enzyme encapsulation.

Polymers such as alginate, κ-Carrageenan and chitosan were screened for encapsulation of Flavourzyme (flavour-enhancing enzyme) using the Inotech® encapsulator. After selection of a suitable polymer, encapsulation parameters such as concentration of polymer and gelling solution, gelling duration and in vitro release were optimised. This study was aimed at assessing the suitability of selected polymers/hydrogels for microencapsulation of flavour-enhancing enzymes and optimisation of the encapsulation parameters to enhance the encapsulation efficiency and enzyme retention after manufacture and sustained release during ripening.
3.3 Materials and methods

Milli Q water (Millipore, Massachusetts, USA) was used for all preparations unless otherwise mentioned.

3.3.1 Flavourzyme® assay

Flavourzyme® (Novozymes, Sydney, Australia) a fungal protease/peptidase complex from *Aspergillus oryzae*, known to contain both endoprotease and exopeptidase activities and highly recommended for debittering was employed in this study to accelerate Cheddar cheese ripening. This enzyme is standardised in leucine amino peptidase units (LAPU) with declared activity of 500LAPU/g of 500L solution or 1000LAPU/g of 1000L solution. One LAPU is the amount of enzyme which hydrolyses 1 µmol of L-leucine-p-nitroanilide per min. Flavourzyme activity was measured based on the rate of reaction of the enzyme with the substrate L-leucine-p-nitroanilide (Fluka, Sydney, Australia). The assay sensitivity was determined to be in the range 0.02 to 0.1LAPU/mL hence all the samples and standards were diluted in this range prior to assay. All standards and samples were diluted with 0.2mM ZnCl₂ solution (Sigma, Sydney, Australia) prepared with 0.15% brij, (Sigma, Sydney, Australia) and 80µL of this diluted enzyme mixture was incubated with 840µL of assay working solution of 0.1M tris buffer, pH 8.0 (Sigma, Sydney, Australia) for 10 min at 40°C followed by addition of 80µL of reagent; 26mM L-leucine-p-nitroanilide (Fluka, Sydney, Australia) prepared in absolute ethanol (Sigma, Sydney, Australia). The absorbance was read at first and tenth min at 405nm, the change in the absorbance of the samples was compared to that of known standards to estimate the enzyme activity. This Flavourzyme assay is a modified adaptation of the method supplied by Novozymes® Australia Pty Ltd.
3.3.2 Palatase® assay

Palatase® 20000L a lipase from Novozymes (Sydney, Australia) was tested for acceleration of flavour development during Cheddar cheese ripening. It is standardised in lipase units (LU) with declared activity of 20000LU/g of preparation. Standards and samples were diluted with 0.1M sodium phosphate buffer, pH 7.4 which was also used as the assay working solution and 20µL of standard or samples were added to 200µL of phosphate buffer in a 96 well plate and heated to 30°C, followed by addition of 10µL of the reagent p-nitrophenyl butyrate; 25µL dissolved in 10mL 2-propanol (Sigma, Sydney, Australia) and the enzyme kinetics was read at 410nm at 30°C in the Multiskan Spectrum Microplate Spectrophotometer 1500 (Thermo Lab systems, Vantaa, Finland). The Multiskan Spectrum software was used to interpret the data and calculate the Palatase activity in unknown samples using the standard curve.

3.3.3 Selection of polymers for enzyme encapsulation

Flavourzyme (500LAPU), 2mL was diluted in 8mL ZnCl₂-brij solution and added to 90mL filtered Alginate (molecular weight range of 12000 to 80000, viscosity of ~250cP, 2% (25°C) L and composed of approximately 61% mannuronic acid and 39% guluronic acid with a M/G ratio of 1.56 from Sigma, Sydney, Australia) or chitosan (minimum 85% deacetylated from Sigma, Sydney, Australia) or κ-Carrageenan (Fluka, Sydney, Australia) solutions of 1.0, 0.1 and 0.1% (w/v) respectively and extruded through the 300µ nozzle of the Inotech® encapsulator. The droplets from alginate, κ-Carrageenan and chitosan were allowed to gel for 10 min in 0.1M CaCl₂, 0.1M KCl and 0.1M sodium phosphate solutions respectively. The gelling solution was drained and the enzyme microcapsules were washed with water...
and 1g dissolved in 9mL of 2.0% (w/v) tri-sodium citrate and the Flavourzyme activity was measured as described in section 3.3.1 to determine the encapsulation efficiency (EE) which was expressed as:

\[
\text{Encapsulation efficiency} = \frac{\text{Enzyme activity recovered from the capsules}}{\text{Initial enzyme activity}*} \times 100
\]

* (In the polymer-enzyme solution)

**Note:** Enzyme activity recovered from the capsules explained in section 3.3.4

All polymers were prepared in higher concentration so as to yield desired concentration when made up to 100mL from 90mL to compensate for dilution of the polymer upon mixing with diluted enzyme. Enzyme polymer mixture (100mL) and the capsules obtained from this mixture were weighed to estimate the loss of water from the hydrogel beads during formation as a result of diffusion. The difference in the weight of capsules and enzyme-polymer mixture was used to correct the final enzyme activity, as undiluted Flavourzyme was used initially and later enzyme microcapsules were used for the measurement of EE and all further experiments.

### 3.3.4 Polymer combinations for improved Flavourzyme retention in capsules

Flavourzyme (500LAPU) 2mL, was mixed with polymer combinations of alginate:starch (Hi-Maize) (Starch Australasia Ltd, Sydney, Australia) in the concentrations 1.0:1.0, 1.5:1.0, 2.0:1.0 and 2.0:1.5 g in 90mL water and alginate:pectin (esterified potassium salt, Sigma, Sydney, Australia) in the concentrations 1.6:0.4 and 1.4:0.6g in 90mL water and the enzyme gel beads were produced by gelling the droplets produced from the encapsulator in the cationic solution of 0.1M CaCl\(_2\) for 10 min. The enzyme microcapsules were harvested after draining the cationic solution and 1g was dissolved in 9mL of 2% (w/v) tri-sodium
citrate and the Flavourzyme activity was measured as described in 3.3.1 after appropriate dilution.

### 3.3.5 Optimisation of polymer concentration

Flavourzyme (500LAPU) 2mL was mixed with 1.4, 1.6, 1.8 and 2.0 % (w/v) alginate and extruded through the 300µm nozzle of the Inotech® encapsulator. The Flavourzyme-alginate droplets were allowed to gel in the cationic solution of 0.1M CaCl$_2$ for 10 min. The enzyme micro capsules were harvested after draining the cationic solution and 1g was dissolved in 9mL of 2.0 % (w/v) tri-sodium citrate and Flavourzyme activity was measured as described in 3.3.1 after appropriate dilutions.
Figure 3.1 Schematic representation of enzyme encapsulation in calcium-alginate by extrusion method (Anjani et al., 2007)
3.3.6 Treatment of cationic solution with chitosan and optimisation

Alginate (2g) - Flavourzyme 500L (2.0mL) (in 100mL), droplets from the encapsulator were allowed to gel in a modified cationic solution of 0.1M CaCl\(_2\) containing 0.1% (w/v) chitosan, a modification of the method reported by Sezer and Akbuga (1999). The pH of the cationic solution containing chitosan was set at 6.2 as it is the optimal pH of Flavourzyme (Flavourzyme data sheet, Novozymes). Chitosan, 0.1g was dissolved in 5mL glacial acetic acid (Ajax chemicals, Sydney, Australia) and the volume was made up to 70mL with Milli Q water and the pH was adjusted to 6.2 using NaOH (1.0M and 0.1M) (Ajax chemicals, Sydney, Australia) followed by addition of 1.1g CaCl\(_2\) (Sigma, Sydney, Australia) dissolved in 10mL Milli Q water and the final volume of the solution was made up to 100mL, this solution was then refrigerated. Gelling solutions of CaCl\(_2\) containing 0.1, 0.2 and 0.3% (w/v) chitosan were studied for maximising EE.

Flavourzyme-alginate gel beads produced from 2mL 500LAPU Flavourzyme and 1.4, 1.6, 1.8 and 2.0% (w/v) alginate were allowed to gel in cationic solution of 0.1M CaCl\(_2\) with 0.1% (w/v) chitosan for 10 min and the Flavourzyme activity was measured in the dissolved beads to optimise the alginate concentration.

3.3.7 Coating of capsules for improved enzyme retention and stability

Flavourzyme-alginate gel beads formed in 0.1M CaCl\(_2\) containing 0.1% (w/v) chitosan was rinsed with Milli Q water and 2g was allowed to stand in 5mL of 0.15% (w/v) alginate or 0.05% (w/v) poly-L-lysine (PLL) solution for 10 min at room temperature with swivelling of the containers every 2 min (Champagne et al., 1992; Quong et al., 1999). After 10 min the capsules were rinsed with Milli Q water and 1g
capsules were dissolved in 9mL 2% (w/v) tri-sodium citrate and the Flavourzyme activity was measured as described in 3.3.1 after appropriate dilution.

3.3.8 Optimisation of gelling duration

The Flavourzyme-alginate droplets from the encapsulator resulting from 2mL 500LAPU Flavourzyme and 1.6, 1.8 and 2.0% alginate with and without PLL coating were allowed to gel in the cationic solution of 0.1M CaCl₂ containing 0.1% (w/v) chitosan for 10, 20 and 40 min to optimise the gelling duration of the capsules for maximum EE. Flavourzyme gel beads (1g) were dissolved in 9mL 2% tri-sodium citrate and Flavourzyme activity was measured as described in 3.3.1.

3.3.9 Optimisation of releasing solution concentration

Tri-sodium citrate in the concentration of 0.5, 1.0 and 2.0% (w/v) was tested for complete in vitro release of encapsulated enzyme. Flavourzyme gel beads (1g) were dissolved in 0.5, 1.0 and 2.0% (w/v) tri-sodium citrate and the Flavourzyme activity was measured as described in 3.3.1 after appropriate dilution. Tri-sodium citrate is used only for in vitro release of encapsulated enzyme for measurement of enzyme activity, however, in cheese; the encapsulated enzymes will be released by diffusion of encapsulated enzymes from the capsules.

3.3.10 Shelf life of encapsulated Flavourzyme

Uncoated and PLL-coated Flavourzyme capsules both in 1g batches were freeze-dried for 16h in an Alpha-1-4 freeze drier with controller LDC-1M (CHRIST® Gefriertrocknungsanlagen, Osterode am Harz, Germany), frozen in a −20°C freezer overnight and air-dried at room temperature (25°C) for 24h. Freeze-dried and air-
dried capsules were stored at 4°C while frozen capsules were stored at –20°C for 10 weeks and the shelf-stability of these capsules was assessed by monitoring the Flavourzyme activity every week by dissolving the capsules in 2% tri-sodium citrate and measuring the activity as described in 3.3.1 after appropriate dilution.

3.3.11 Morphology of enzyme microcapsules

Size of alginate immobilised Flavourzyme gel beads was measured using an optical microscope (Nikon microscope, Labophot-2, Nikon Corporation, Kanagawa, Japan) fitted with a stage ocular of 10X magnification. The average size reported is a mean of 20 randomly selected gel beads.

3.3.12 Statistical analysis

All results are a mean of 6 replicates unless otherwise mentioned and expressed with standard error of mean or standard deviation as mentioned. Data were analysed using one (two) factorial analysis of variance (ANOVA) general linear SPSS® for Windows™ Version 14 (SPSS Inc. 2005). The assumption of normal distribution was checked using P-P plot and homogeneity of variance using Levene’s test of equality of error variances. Treatments were tested against each other using Ryan-Einot-Gabriel-Welsch Q test if the assumption of equality of variance was met or Dunnett’s T-test if the assumption was not met.
3.4 Results

3.4.1 Selection of polymers for enzyme encapsulation

Alginate, κ-Carrageenan and chitosan were studied for encapsulation of Flavourzyme; a flavour-enhancing enzyme, for controlled release of Flavourzyme in Cheddar cheese for acceleration of ripening. The Inotech® encapsulator was selected for enzyme encapsulation as it involved mild conditions for capsule production; hence it was required to pump only relatively low viscosity solutions through the nozzle of the encapsulator as per instructions from the manufacturer in order to allow easy flow of the polymer-enzyme mixture. Chitosan was too viscous and could not be pumped through the nozzle of encapsulator hence was unsuitable for Flavourzyme encapsulation through using the encapsulator. Similarly κ-Carrageenan turned too viscous upon cooling below 65°C and this high temperature was likely to denature the enzyme and hence reduce Flavourzyme activity therefore κ-Carrageenan was also eliminated as a suitable polymer for Flavourzyme encapsulation using this encapsulator. Thus after preliminary screening it was determined that alginate was a suitable polymer for enzyme encapsulation through this encapsulator. Alginate concentrations of 1.4, 1.6, 1.8 and 2.0% (w/v) were considered for Flavourzyme encapsulation with average EE of 16.3, 19.2, 17.4 and 18.6% respectively (Figure 3.2). There was no significant difference (p > 0.05) in the EE on increasing the alginate concentration. Incorporation of starch or pectin into alginate-Flavourzyme mixture prior to encapsulation resulted in the EE ranging from about ~7 to 12% for all combinations of alginate/starch (Figure 3.3) to about ~9% for alginate/pectin (Figure 3.4) tested in this study. Though increase in the alginate and starch concentrations significantly (p < 0.05) improved the EE of Flavourzyme, it was
however lower than that obtained when encapsulated in alginate alone. Hence alginate was used for enzyme encapsulation.
Figure 3.2 Encapsulation efficiency of Flavourzyme immobilised in alginate by gel bead formation in 0.1M CaCl$_2$ and the effect of alginate concentration on encapsulation efficiency. Error bars indicate standard error of mean.
Figure 3.3 Encapsulation efficiency of Flavourzyme immobilised in alginate-starch mixture by gel bead formation in 0.1M CaCl$_2$ and the effect of varying alginate-starch concentration and proportion (in 100mL water) on encapsulation efficiency. Error bars indicate standard error of mean.
Figure 3.4 Encapsulation efficiency of Flavourzyme immobilised in alginate-pectin mixture by gel bead formation in 0.1M CaCl$_2$ and effect of varying concentration and proportion of alginate and pectin (in 100mL water) on encapsulation efficiency. Error bars indicate standard error of mean.
3.4.2 Effect of chitosan concentration in gelling solution on encapsulation efficiency of Flavourzyme

Addition of chitosan to the gelling solution of 0.1M CaCl$_2$ significantly increased the EE of Flavourzyme immobilized in 2 % (w/v) alginate (F$_{2, 6}$ = 19.013, p < 0.001). EE of Flavourzyme capsules formed in cationic solution of 0.1M CaCl$_2$ with 0, 0.1, 0.2 and 0.3% (w/v) chitosan was 18.6$^a$, 71.9$^b$, 75.7$^c$ and 85.0$^d$ % respectively (Figure 3.5). Results are a mean of 5 replicates unless mentioned otherwise and the error bars indicate standard error of mean. Means followed by different superscript letter are significantly different.

3.4.3 Weight of Flavourzyme microcapsules

Since Flavourzyme was supplied in liquid form it was measured in millilitres for all experiments including encapsulation, however; when encapsulated, the weight of Flavourzyme capsules could only be measured in grams before dissolving to measure the enzyme activity. All enzyme capsules were of 1g weight for the ease of dilution and calculations. To adjust for this variation in the measurement, enzyme microcapsules obtained from 100mL enzyme-polymer mixture was weighed and this weight was used to adjust the enzyme activity of the capsules accordingly. For example if 2mL Flavourzyme in 100mL enzyme-polymer mixture yielded 70g capsules then 1mL yields 0.7g, hence enzyme activity in 0.7g was calculated. Weight of Flavourzyme-alginate capsules from 100mL mixture ranged from 58.9, 61.1, 61.5 and 62.9g for 1.4, 1.6, 1.8 and 2.0 % (w/v) alginate respectively while that from 100mL solution of alginate-starch ranged 49-80g and alginate-pectin ~64g. Weight of Flavourzyme capsules formed in the gelling solution containing chitosan was 77.4, 85.5 and 88.0g for 0.1, 0.2 and 0.3% (w/v) chitosan respectively (Table 3.1).
Figure 3.5 Encapsulation efficiency of Flavourzyme immobilised in 2% (w/v) alginate with gel bead formation in cationic solution of 0.1M CaCl$_2$ containing chitosan and the effect of chitosan concentration on encapsulation efficiency (results are a mean of 5 replicates, * data obtained from Figure 3.2, mean of 6 replicates). Error bars indicate standard error of mean.
Table 3.1 Weight of capsules obtained from 100mL polymer-Flavourzyme (90mL:2mL, 8mL Flavourzyme diluent) mixture allowed to gel in cationic solution of 0.1M CaCl₂ with and without chitosan for 10 min. * treatment with varying concentrations

<table>
<thead>
<tr>
<th>Polymer/gelling solution</th>
<th>Concentration (% w/v)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate*/ 0.1M CaCl₂</td>
<td>1.4</td>
<td>58.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>61.1 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td>61.5 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>62.9 ± 0.55</td>
</tr>
<tr>
<td>Alginate-pectin*/ 0.1M CaCl₂</td>
<td>1.4:0.6</td>
<td>63.4 ± 0.66</td>
</tr>
<tr>
<td></td>
<td>1.6:0.4</td>
<td>63.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>1.8:0.2</td>
<td>64.0 ± 0.4</td>
</tr>
<tr>
<td>Alginate-starch*/ 0.1M CaCl₂</td>
<td>1.0:1.0</td>
<td>49.0 ± 0.5ab</td>
</tr>
<tr>
<td></td>
<td>1.5:1.0</td>
<td>51.9 ± 0.05a</td>
</tr>
<tr>
<td></td>
<td>2.0:1.0</td>
<td>60.1 ± 0.46b</td>
</tr>
<tr>
<td></td>
<td>2.0:1.5</td>
<td>80.2 ± 1.1c</td>
</tr>
<tr>
<td>2.0% Alginate/ 0.1M CaCl₂-chitosan*</td>
<td>0.1</td>
<td>77.4 ± 0.7a</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>85.5 ± 0.6b</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>88.0 ± 0.8b</td>
</tr>
</tbody>
</table>

abc Means within the same column followed by different superscript letter are significantly different (p < 0.05).
3.4.4 **Optimisation of polymer concentration**

Varying alginate concentrations were studied for optimisation of Flavourzyme encapsulation. EE of Flavourzyme-alginate gel beads formed in cationic solution of 0.1M CaCl₂ containing 0.1% (w/v) chitosan was 62.4ₐ, 69.7ₐb, 70.2ₐbc and 72.3c for 1.4, 1.6, 1.8 and 2.0% (w/v) alginate respectively (Figure 3.6). Means followed by different superscript letter are significantly different ($F_{3, 8} = 193.754$, $p < 0.01$).

3.4.5 **Effect of coating on Flavourzyme retention in capsules**

Flavourzyme capsules were coated with 0.15% alginate and 0.05% poly-L-lysine for 10 min in an attempt to minimise enzyme loss. Flavourzyme activity in the capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate was recorded before and after coating with alginate and PLL. The enzyme activity ranged from 84.4 to 85.9 LAPU for alginate and 86.1 to 87.0 LAPU for PLL-coated Flavourzyme microcapsules (Figure 3.7). There was no significant difference ($p > 0.05$) in the enzyme activity of capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate or those coated in alginate or PLL.

3.4.6 **Optimisation of gelling duration**

Alginate form gel on contact with cations and is important for efficient entrapment. Properties of alginate microcapsules are influenced by the concentration of cationic solution in which the gel is formed. Hence varying concentrations of chitosan were studied for efficient Flavourzyme encapsulation in uncoated and PLL-coated capsules. Flavourzyme activity decreased with the increase in gelling duration for both uncoated (Figure 3.8a) and PLL-coated capsules (Figure 3.8b). The enzyme retention in uncoated capsules produced from 1.6, 1.8 and 2.0 % (w/v) alginate was not significantly different ($p > 0.05$) but increase in the gelling duration of $10^a$, $20^b$
and 40° min caused significant ($F_{2, 44} = 12.321, p < 0.01$) reduction in enzyme retention.

The enzyme activity of PLL-coated capsules produced from 1.6 % alginate was significantly different ($F_{2, 44} = 33.742, p < 0.01$) from those produced from 1.8 or 2.0% alginate. The enzyme retention in capsules allowed to gel for 10\textsuperscript{a}, 20\textsuperscript{ab} and 40\textsuperscript{b} min was significantly different ($F_{2, 44} = 10.059, p < 0.01$), means followed by different superscript letter are significantly different.
Figure 3.6 Effect of alginate concentration on encapsulation efficiency of Flavourzyme gel beads formed in 0.1M CaCl₂ containing 0.1% (w/v) chitosan. Error bar indicates standard error of mean.
Figure 3.7 Flavourzyme activity in capsules produced from 2% (w/v) alginate allowed to gel in cationic solution of 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan and coated with 0.15% alginate and 0.05% poly-L-lysine for 10 min. Error bars indicate standard error of mean.
Figure 3.8 Effect of gelling duration in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan on Flavourzyme retention in (a) uncoated and (b) poly-L-lysine coated capsules made from 1.6, 1.8 and 2.0% (w/v) alginate. Error bar indicates standard error of mean.
3.4.7 Optimisation of tri-sodium citrate concentration for *in vitro* release of encapsulated enzyme

Activity of encapsulated enzyme can only be measured upon complete release from the capsules. Encapsulated Flavourzyme was released *in vitro* by dissolution of capsules in tri-sodium citrate solution. Complete dissolution of the capsules and subsequent release of entrapped enzyme is greatly influenced by the concentration of tri-sodium citrate solution; hence 0.5, 1.0 and 2.0% (w/v) tri-sodium citrate were studied for complete *in vitro* release of encapsulated Flavourzyme. Flavourzyme release from both uncoated (Figure 3.9a) and PLL-coated capsules (Figure 3.9b) increased with increase in tri-sodium citrate concentration. There was significant interaction ($F_{4, 44} = 892.830$, $p < 0.01$) between the concentration of alginate and concentration of tri-sodium citrate solution hence the differences between buffers were tested for each concentration of alginate separately.

In uncoated capsules produced from 1.6 and 2.0% alginate, the enzyme released from 0.5% tri-sodium citrate was significantly lower (1.6% tri-sodium citrate; $F_{2, 14} = 986.560$, $p < 0.01$) (2.0% tri-sodium citrate; $F_{2, 14} = 10320.078$, $p < 0.01$) than that released from 1.0 and 2.0% tri-sodium citrate but there was no significant difference ($p > 0.05$) in the enzyme released from 1.0 and 2.0% tri-sodium citrate. Where as for the capsules produced from 1.8% alginate the enzyme released from 0.5$^a$, 1.0$^b$ and 2.0$^c$% (w/v) tri-sodium citrate was significantly different ($F_{2, 14} = 4381.913$, $p < 0.01$), means followed by different superscript letter are significantly different.

In PLL-coated capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate, the enzyme released from 0.5% tri-sodium citrate was significantly different (1.6% alginate; $F_{2, 14} = 30.572$, $p < 0.01$), (1.8% alginate; $F_{2, 14} = 23.146$, $p < 0.01$), (2.0% alginate; $F_{2, 14} = 30.288$, $p < 0.01$) from that released from 1.0 and 2.0% tri-sodium citrate while there
was no significant difference (p > 0.05) in the enzyme released from 1.0 and 2.0% tri-sodium citrate.

3.4.8  Shelf life of freeze-dried, air-dried and frozen Flavourzyme microcapsules

Flavourzyme encapsulated in 1.6, 1.8 and 2.0% alginate by gelling in 0.1M CaCl$_2$ solution containing 0.1% chitosan, both uncoated and PLL-coated were stored in three ways; freeze-drying, air-drying and freezing and the effect of these processes and shelf-stability was evaluated by monitoring the activity of encapsulated enzyme over a 10 week storage period. Uncoated capsules from 1.6 (Figure 3.10i), 1.8 (Figure 3.10ii) and 2% alginate (Figure 3.10iii), stored at 4°C after freeze-drying and at -20°C after freezing retained over 80% of the initial enzyme activity while those stored at 4°C after air-drying retained only about 50% of the initial enzyme activity. In PLL-coated capsules from 1.6, 1.8 and 2.0% alginate a similar trend was observed, freeze-dried and frozen capsules retained over 80% of the initial enzyme activity (Figure 3.10iv, v and vi) while air-dried capsules from 1.6 % alginate retained about 34% (Figure 3.10iv), from 1.8% alginate retained about 32% (Figure 3.10v) and from 2.0% alginate retained only about 25% (Figure 3.10vi) of the initial enzyme activity. Loss of enzyme activity appears to occur mainly during the process of air-drying.
3.4.9 Morphology of uncoated and poly-L-lysine coated Flavourzyme microcapsules

Microscopic examination of Flavourzyme capsules showed mainly spherical capsules for both uncoated and PLL-coated capsules while the number of non-spherical capsules was slightly higher in PLL-coated capsules (Table 3.2).
Figure 3.9 Effect of tri-sodium citrate concentration on *in vitro* release of encapsulated Flavourzyme from (a) uncoated and (b) poly-L-lysine coated capsules produced from 1.6, 1.8 and 2.0 % (w/v) alginate and allowed to gel in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan. Error bar indicates standard error of mean.
Figure 3.10 Shelf life of Flavourzyme in uncoated capsules immobilised in 1.6 (i), 1.8 (ii) and 2.0 % (w/v) (iii) alginate by gel bead formation in 0.1M CaCl₂ containing 0.1% (w/v) chitosan, stored by freeze-drying (a), air-drying (b) and freezing (c) over a period of 10 weeks. Error bar indicates standard deviation.
Enzyme encapsulation

(iv) (v) (vi)

Figure 3.10 Shelf life of Flavourzyme in poly-L-lysine coated capsules immobilised in 1.6 (iv), 1.8 (v) and 2.0 % (w/v) (vi) alginate by gel bead formation in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan, stored by freeze-drying (a), air-drying (b) and freezing (c) over a period of 10 weeks. Error bar indicates standard deviation.
Table 3.2 Size and shape of alginate Flavourzyme capsules allowed to gel in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan (mean ± standard deviation, average of 20 replicates)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Size (in µm)</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Alginate concentration (% w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncoated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>410-520</td>
<td>501 ± 32.8</td>
</tr>
<tr>
<td>1.8</td>
<td>500-530</td>
<td>511 ± 11.0</td>
</tr>
<tr>
<td>2.0</td>
<td>510-570</td>
<td>541 ± 19.1</td>
</tr>
<tr>
<td>0.05% PLL coated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>440-500</td>
<td>476 ± 21.1</td>
</tr>
<tr>
<td>1.8</td>
<td>460-550</td>
<td>496 ± 25.9</td>
</tr>
<tr>
<td>2.0</td>
<td>460-620</td>
<td>529 ± 42.5</td>
</tr>
</tbody>
</table>
Enzyme encapsulation

3.5 Discussion

Flavourzyme and Palatase were the two enzymes used in this study for acceleration of Cheddar cheese ripening; all the encapsulation and optimisation studies were performed with Flavourzyme which has similar molecular weight to Palatase (communication from Novozymes). Alginate, chitosan and κ-Carrageenan were studied for encapsulation of Flavourzyme. It was not possible to extrude chitosan through the 300µm nozzle of the encapsulator even at a concentration of 0.1 % (w/v) hence it was eliminated as a suitable biomaterial for enzyme encapsulation. Another food grade polymer, κ-Carrageenan was also studied for Flavourzyme encapsulation as κ-Carrageenan is known to form hard gels compared to weak gels from τ-Carrageenan (Velde et al., 2002). Being a thermo reversible gel it was only possible to extrude κ-Carrageenan through the encapsulator nozzle by maintaining a steady temperature of 70°C as it formed gel easily upon contact with cooler surfaces of the encapsulator even at a low concentration of 0.1 % (w/v). This high temperature for long duration considerably reduces the activity of Flavourzyme (Flavourzyme product sheet, 2004) hence κ-Carrageenan was also eliminated as a suitable polymer for enzyme encapsulation using the Inotech® encapsulator. Alginate was found to be a suitable polymer for enzyme encapsulation using the encapsulator; hence all experiments in the current study were performed on alginate based encapsulation.

Sodium salt of alginic acid from Sigma (Sydney, Australia) and Protanal (Drammen, Norway) were used to encapsulate Flavourzyme using the Inotech® encapsulator followed by capsule gelling in 0.1M CaCl₂, alginate from Sigma showed lower viscosity compared to alginate from Protanal (data not shown) hence alginate from Sigma was used for all further experiments. EE of Flavourzyme encapsulated in alginate ranged from 16 to 18% between 1.4 and 2.0 % (w/v) alginate (Figure 3.2)
with no significant increase (p > 0.05) in EE with increase in alginate concentration. Low EE was due to leakage of Flavourzyme from capsules during encapsulation. Pore size of alginate gels have been studied by various methods. Pore size distribution in 3% alginate gel examined by transmission electron microscope was found to be in the range 100-1000Å with a distribution maxima at 250Å (Smidsrod et al., 1972). Klein et al. (1983) reported pore size of Ca-alginate gels to be about ~7 to ~17nm depending on the gel type. Fundueanu et al. (1999) estimated pores and porous volume of micro particles in Ca-alginate to have a mean diameter of 220µm when obtained by emulsion method and 1.2mm when obtained by dripping method.Alginate gel beads produced using the Inotech® encapsulator have been reported to allow molecules in the weight range of 1000kDa (Inotech® website, 2004). Based on these reports it can be said that pores in alginate matrix is significantly large compared to Flavourzyme molecules with molecular weight ~30kDa (communication from Novozymes) and thus causing excessive leakage and poor EE. Low EE can also be due to Flavourzyme loss as a result of syneresis during gel bead formation. Flavourzyme being a water soluble enzyme was lost by diffusion into the gelling cationic solution during gel bead formation. Water loss from gel beads due to syneresis was found to be 41 to 37% for alginate concentration ranging from 1.4 to 2.0% (Table 3.1) in agreement with the findings of Pommersheim et al., (1994), who reported 34% loss of enzyme by diffusion into solution during the formation of gel beads and Dashevsky (1998) who reported 36% protein loss due to diffusion and 44% water leakage during cross linking of alginate and calcium cations. In the current study, increase in alginate concentration lead to decreased water loss and in turn better retention of enzyme in gel beads.
Increased EE of folic acid on immobilization in a mixture of alginate-pectin (Madziva et al., 2006) and increased cell encapsulation upon incorporation of Hi-Maize starch during Ca-alginate encapsulation (Sultana et al., 2000) have been reported. Similarly in the current study Flavourzyme was immobilised in alginate-starch (Hi-Maize) and alginate-pectin mixtures in varying concentrations and proportions followed by gelling in 0.1M CaCl$_2$ solution. However, EE of Flavourzyme was lower than that obtained when encapsulated in alginate alone, ~7-12% for alginate-starch (Hi-Maize) and ~9% for alginate-pectin (Figure 3.3 & 3.4). Though EE of Flavourzyme significantly increased (p < 0.05) with the increase in alginate and starch (Hi-Maize) concentration, this polymer mixture could not be used due to relatively high viscosity for extrusion through the encapsulator nozzle. Low EE of Flavourzyme in alginate-starch (Hi-Maize) and alginate-pectin may be due to insufficient blocking of the pores in alginate matrix thus causing excessive leakage during encapsulation and gelling. Low EE can also be attributed to possible interference of starch and pectin with the Flavourzyme assay leading to lower activity, in agreement with the report of interaction of lipase with alginate leading to poor EE (Betigeri and Neau, 2002). Based on screening results EE of Flavourzyme was superior on encapsulation in alginate alone, hence alginate was chosen as the preferred polymer.

Flavourzyme capsules with low EE was not an efficient means of enzyme incorporation into cheese. Cationic solution was modified in an attempt to enhance EE. Sezer and Akbuga (1999) reported increased drug loading capacity of alginate capsules on addition of 0.3 % chitosan to the gelling solution. In the current study addition of chitosan to cationic solution of 0.1M CaCl$_2$ significantly increased (p < 0.05) the EE to 72.21, 76.28 and 84.0% for 0.1, 0.2 and 0.3% chitosan respectively.
(Figure 3.5) for Flavourzyme immobilized in 2% alginate and gelling for 10 min. Chitosan reacts with alginate as a cation with an anion, similar to CaCl$_2$. As chitosan is a larger molecule compared to calcium ions, it is more efficient in blocking the pores in alginate matrix when combined with CaCl$_2$ compared to CaCl$_2$ alone. Binding of chitosan to alginate is said to increase with decrease in degree of n-acetylation and addition of calcium ions (Gaserod et al., 1998). Chitosan, minimum 85% deacetylated in 0.1M CaCl$_2$ solution was used in this study, enhanced EE of Flavourzyme upon treatment of cationic solution of 0.1M CaCl$_2$ can be attributed to increased binding of chitosan to alginate network thus blocking the pores. Therefore by treating the capsules with chitosan it was possible to reduce the pore size and consequently minimising leakage. Dashevsky (1998) reported weight loss of capsules due to shrinkage and syneresis during cross linking of guluronate with calcium cations, water loss decreased with increase in alginate concentration. Similarly in the current study, capsule weight increased with increase in alginate concentration from 1.4 to 2.0%. Water loss of ~37% was recorded for capsules produced from 2% alginate when gel bead was formed in the cationic solution of 0.1M CaCl$_2$ while water loss of 23, 15 and 12 % was recorded for capsules produced from 2% alginate with gel bead formation in cationic solution of 0.1M CaCl$_2$ containing 0.1, 0.2 and 0.3% chitosan respectively (Table 3.1). These results show that gelling of capsules in 0.1M CaCl$_2$ solution containing chitosan can minimise bead shrinkage and water loss inturn leading to enhanced enzyme retention. Though there was a significant (p < 0.001) yet small increase in the EE of Flavourzyme with the increase in chitosan concentration, 0.1% (w/v) was chosen to be optimal as 0.2 and 0.3% (w/v) chitosan in gelling solution were too viscous for further application.
causing clumping or surface aggregation of capsules during formation, which would obstruct even distribution of capsules in the cheese matrix.

The next step in improving Flavourzyme encapsulation was selection of polymers for efficient robust process. Alginate solutions of concentrations 1.4, 1.6, 1.8 and 2.0\% (w/v) were studied by immobilising Flavourzyme and gelling for 10 min in 0.1M CaCl$_2$ containing 0.1\% (w/v) chitosan. There was a significant increase (p < 0.05) in EE of Flavourzyme on increasing the concentration of alginate from 1.4 to 1.6 \%, however no significant increase (p > 0.05) in EE was observed upon increasing alginate concentration beyond 1.6\% (Figure 3.6), unlike the reports of Blandino et al. (2000), who reported reduced glucose oxidase leakage on increasing the alginate concentration. This disagreement in results may be due to the difference in the nature of the enzymes and gelling solution employed in the two studies and also possibly due to the constant size of the capsules produced from the same nozzle resulting in similar surface area in capsules from different concentration and hence similar enzyme retention capacity of the capsules. Consequently 1.6, 1.8 and 2.0\% alginate were used for further study.

Chitosan and PLL are the most widely used poly-cations for capsule production. Coating of alginate capsules with polycations like PLL has shown to improve mechanical stability of capsules (Lim and Moss, 1981; Liu and Krishnan, 1999, Ouyang et al., 2004). Gugerli et al. (2002) reported < 150 kDa permeability of capsules produced from 1.5\% alginate coated with 0.05\% (w/v) PLL (30-70kDa molecular weight, from Sigma) suggesting that PLL-coating of alginate capsules can minimise the porosity. Coating of calcium alginate capsules with chitosan-alginate polyelectrolyte complex has been shown to reduce permeability (Peniche et al., 2004). As the capsules used in the current study are formed in chitosan treated
cationic solution, alginate was considered as a suitable polymer for coating in addition to PLL and both were used for coating of Flavourzyme capsules in an attempt to minimise enzyme leakage and to improve mechanical stability of capsules. However, initial Flavourzyme activity in capsules decreased from ~ 90 to 85 and 87 LAPU after 10 min of 0.15% alginate and 0.05% poly-L-lysine coating respectively (Figure 3.7) indicating continued leakage of Flavourzyme from the capsules. Thus there was no further improvement in capsule permeability upon coating. Poly-L-lysine coated and uncoated capsules were used in further experiments to test stability, as clumping was observed with alginate-coated capsules. Aggregation of alginate-coated capsules could be due to interaction of residual chitosan on the surface of the capsules with alginate, and this may cause uneven distribution of capsules in a cheese matrix.

Duration of capsule gelling was optimised to obtain efficient enzyme micro-capsules. Enzyme recovery significantly (p < 0.001) decreased with increase in gelling duration of uncoated capsules from 10, 20 and 40 min (Figure 3.8a) whereas in PLL-coated capsules (Figure 3.8b), the enzyme retention in capsules allowed to gel for 10 min was significantly higher (p < 0.001) than those allowed to gel for 40 min while a gelling time of 20 min had no significant effect (p > 0.05) on enzyme retention compared to 10 and 40 min gelling duration. Decrease in enzyme retention in the capsules upon increasing the gelling time may be due to continued diffusion/leakage of Flavourzyme into the gelling solution.

In PLL-coated capsules (Figure 3.8b), as the gelling progressed from 10 to 40 min, the recovery of Flavourzyme activity of capsules produced from 1.8 and 2 % alginate was significantly higher (p < 0.001) than those produced from 1.6 % alginate suggesting better interaction of PLL with alginate at higher concentrations of alginate.
and thus resulting in efficient blocking of pores and better retention of encapsulated enzyme.

Complete *in vitro* release of encapsulated enzyme is essential for the measurement of enzyme activity. Tri-sodium citrate was used for dissolution of capsules for *in vitro* release of encapsulated enzyme from uncoated and PLL-coated capsules.

In uncoated capsules (Figure 3.9a) produced from 1.6 and 2% alginate, the release of the encapsulated enzyme significantly increased ($p < 0.001$) with the increase in tri-sodium citrate concentration from 0.5 to 1.0% however there was no significant increase ($p > 0.05$) in enzyme release on increasing the tri-sodium citrate concentration beyond 1%. whereas for the capsules produced from 1.8% alginate, the release of encapsulated enzyme significantly increased ($p < 0.001$) with increase in tri-sodium citrate concentration from 0.5 to 1.0 to 2.0% indicating that higher concentrations of buffer is required for complete release of the encapsulated enzyme from capsules made from higher concentrations of alginate. However, this trend doesn’t continue beyond the alginate concentration of 1.8%, probably due to relatively high concentration of alginate for dissolution by tri-sodium citrate.

In PLL-coated capsules (Figure 3.9b) produced from 1.6% alginate, there is no significant difference ($p > 0.05$) in the release of encapsulated enzyme on increasing the tri-sodium citrate concentration from 0.5 to 1.0% while an increase in tri-sodium citrate concentration from 0.5 to 2.0% lead to a significant increase ($p < 0.001$) in the release of encapsulated enzyme. Whereas for capsules produced from 1.8 and 2% alginate there was no significant increase ($p > 0.05$) in the release of encapsulated enzyme on increasing the tri-sodium citrate concentration beyond 1.0%. These results suggest that at higher concentration of alginate, relatively lower concentration
of tri-sodium citrate is sufficient for release of encapsulated enzymes from capsules coated in PLL unlike uncoated capsules.

Uncoated and PLL-coated Flavourzyme capsules produced from 1.6, 1.8 and 2.0% alginate were stored at 4°C after freeze-drying and air-drying and at -20°C after freezing and the shelf-stability of the stored capsules was monitored over 10 weeks by measuring the enzyme activity every week. In both uncoated and PLL-coated capsules from all concentration of alginate stored frozen or freeze-dried retained over 80% of the initial enzyme activity at a steady rate over a 10-week period (Figure 3.10). Uncoated capsules stored after air-drying retained only about 50% of the initial enzyme activity (Figure 3.10i, ii and iii) with most of the enzyme activity loss occurring during the process of air-drying. A similar trend was observed in PLL-coated air-dried capsules where only 25-34% of the initial enzyme activity was retained over a 10-week storage period with majority of enzyme activity loss occurring during air-drying (Figure 3.10iv, v and vi). It can be concluded that Flavourzyme is highly sensitive to air-drying at room temperature and may lead to irreversible loss of enzyme activity. In air-dried capsules loss of enzyme activity increases with increase in alginate concentration, though more pronounced in PLL-coated capsules this trend was also observed in uncoated capsules. This could be due to the high surface tension during air-drying and also possibly due to the hydrophilic nature of alginate as also reported by Betigeri and Neau (2002), who observed an increase in leaching of lipase with increase in alginate concentration and hypothesised that it could be due to the hydrophilic nature of the polymer.
3.6 Conclusion

Alginate was found to be a suitable polymer for enzyme encapsulation using the encapsulator. Enzyme encapsulation in alginate alone and also in polymer mixtures of alginate-starch and alginate-pectin followed by gelling in 0.1M CaCl₂ resulted in poor encapsulation efficiency. Flavourzyme immobilization in alginate by gel bead formation in cationic solution of 0.1M CaCl₂ containing 0.1% (w/v) chitosan for 10 min is an efficient and novel means of enzyme encapsulation for acceleration of cheese ripening. Alginate concentration ranging from 1.6 to 2.0% (w/v) is suitable for Flavourzyme encapsulation. There was no enhancement in Flavourzyme retention in capsules coated with alginate or poly-L-lysine. Flavourzyme microcapsules are shelf stable for at least 10 weeks when stored after freezing or freeze-drying hence suitable for large scale applications.
4 APPLICATION OF ENCAPSULATED ENZYMES IN
CHEDDAR CHEESE MANUFACTURE
4.1 Abstract

Flavourzyme-alginate hydrogel beads produced from 1.6, 1.8 and 2.0% (w/v) alginate allowed to gel in chitosan treated CaCl₂ solution were evaluated for stability under simulated conditions of cheese manufacture prior to application in cheese for acceleration of ripening. Flavourzyme leakage from capsules was tested in milk at pH 5.0 & 6.0 and at temperatures 32 & 38°C for 10 min. At 38°C, Flavourzyme retention in capsules was found to be significantly lower (p < 0.001) in capsules produced from 1.6% alginate compared to those from 1.8 or 2.0% and significantly lower (p < 0.001) at pH 6.0 compared to pH 5.0. While at 32°C, the concentration of alginate had no effect on enzyme retention in the capsules. Flavourzyme capsules tested under simulated cheese press showed considerable mechanical stability by retaining over 70% of the encapsulated enzyme under a pressure of 0.79Ncm⁻² for 16h. Leakage of encapsulated Flavourzyme increased with the duration of simulated cheese press from 4 to 16h and was highest in capsules produced from 1.6% alginate. Addition of enzyme capsules to milk prior to renneting resulted in an even distribution of the capsules compared to the aggregation of capsules, when added to milled curd prior to salting. Cheddar cheese manufactured with alginate capsules containing low, medium and high load of Flavourzyme and medium load of Palatase and control cheese with no added enzymes showed higher levels of moisture and lower levels of fat compared to standard Cheddar cheese. Cheeses (control and experimental) produced for grading were tested for microbiological quality. Addition of free or alginate-encapsulated Flavourzyme or Palatase had no effect on the pathogen levels in cheese as there was no difference in numbers of coliforms, *E.coli*, *Salmonella*, *Listeria*, coagulase positive staphylococci, *Bacillus cereus*, yeast and moulds in control or experimental cheeses.
4.2 Introduction

Cheese is one of the most widely consumed dairy products with Cheddar being the most popular variety in Australia as natural Cheddar alone accounted for more than 54% of the total cheese supermarket sales in 2005/2006 (Anon, 2007d). Cheddar is a semi-hard variety that is consumed after a period of ripening ranging from few months to three years implying increased production costs due to refrigeration, maintenance and low yield due to contamination and syneresis. Accelerated ripening can potentially reduce the production costs and any attempt at reducing the ripening time and yet maintaining the quality of cheese is appreciated and has attracted attention of many researchers (Cliffe and Law, 1990).

Though several techniques such as elevation of ripening temperature (Folkertsma et al., 1996; Hannon et al., 2005), high pressure processing (O’Reilly et al., 2003; Garde et al., 2007), genetic modification of starters (Upadhyay et al., 2004b) and addition of attenuated starters (Upadhyay et al., 2007) or adjunct cultures (Ong et al., 2007a, 2007b) have resulted in increased proteolysis or lipolysis and enhanced flavour formation, not many have been successful at accelerating cheese ripening at a commercial scale. Exogenous addition of enzymes that catalyse the biochemical reactions occurring during ripening appears to be a simple approach and a range of enzymes have been tested for this purpose. Though exogenous addition of enzymes caused increased proteolysis, enhanced flavour formation, reduction/elimination of bitterness; premature extensive proteolysis, bitterness and development of off-flavours were also reported by some groups in addition to whey contamination. Addition of exogenous enzymes to milk during cheese manufacture is inefficient as ~90% of enzyme added to milk is lost at whey drainage (Law and Kirby, 1987) in addition to premature extensive proteolysis and development of off-flavours (Skeie,
Addition of enzymes at dry-salting during cheese manufacture can also be disadvantageous owing to the possibility of uneven distribution of the small amount of enzymes in the large blocks of curd (Wilkinson and Kilcawley, 2005).

Enzyme encapsulation was the suitable alternative for delivery of enzymes into cheese; it was first reported by Magee et al. (1981) who observed increased flavour compounds in cheese due to addition of cell free extracts encapsulated in milk fat, these capsules disrupted at 35°C. Later developments included capsules able to withstand higher temperature (Braun and Olson, 1984) and capsules from alternative materials such as hydrogels (Perols et al., 1997), food gums (Kailasapathy et al., 1998) and liposomes (Law and King, 1985; Kirby et al., 1987; Skeie et al., 1995; Picon et al., 1995, 1997; Laloy et al., 1998; Kheadr et al., 2000, 2003). Through intense research by several groups, liposomes were developed for efficient enzyme encapsulation (El Soda et al., 1989; Skeie, 1994; Dufour et al., 1996) for delivery into cheese for acceleration of ripening. However, liposomes are expensive ingredients and there are no known reports of commercial application of liposome-encapsulated enzymes for acceleration of cheese ripening.

Though most studies report accelerated proteolysis, very few report enhancement and acceleration of flavour development during ripening. Kheadr et al., (2000) recommended addition of enzymes encapsulated in liposomes obtained from Pro-lipo for acceleration of cheese ripening with the load of bacterial proteinase up to $1 \times 10^5$ AU/g cheese curd and fungal proteinase up to $1 \times 10^6$ AU/g cheese curd for acceleration of flavour development in Cheddar cheese without any flavour or textural defects. An increase in moisture content of cheeses due to the addition of liposome (Kheadr et al., 2000) or food gum (Kailasapathy and Lam, 2005) encapsulated enzymes have been reported.
Addition of Flavourzyme an enzyme complex with endoprotease and exopeptidase activity with Palatase a fungal lipase, both individually encapsulated in liposomes during cheese manufacture lead to increased and balanced flavour development without any bitterness, off flavours or rancidity (Kheadr et al., 2003).

Earlier studies on enzyme encapsulation for acceleration of cheese ripening involved addition of encapsulated enzymes to milk during cheese manufacture. Picon et al. (1997) reported 64-75% retention of liposome encapsulated enzymes in cheese when added to milk during manufacture while Kailasapathy and Lam (2005) reported over 90% retention of κ-Carrageenan and gellan encapsulated enzymes in cheese when added to milk. Addition of neutral protease from Aspergillus oryzae to the curd at the milling stage during Cheddar cheese manufacture resulted in increased proteolysis (Fedrick et al., 1986). Kheadr et al. (2000, 2003) also reported addition of liposome encapsulated enzymes to milk before cheese manufacture for acceleration of ripening. Stage of addition of folic acid capsules into Cheddar cheese was evaluated by Madziva et al. (2006). Addition of alginate-pectin encapsulated folic acid to milk early during cheese manufacture resulted in even distribution while addition to milled curd and injection into pressed cheese block lead to poor distribution with capsule clusters.

Technique for microencapsulating flavour enhancing enzymes efficiently developed earlier in section 3.0 was used to immobilise Flavourzyme and Palatase; flavour-enhancing enzymes by Novozymes. This study was designed to test the suitability of the enzyme microcapsules developed for application in Cheddar cheese for acceleration of ripening.
4.3 Materials and methods

Flavourzyme (Novozymes, Sydney, Australia) 2mL was dissolved in 8mL ZnCl$_2$ (Sigma, Sydney, Australia) solution prepared with 0.15% (v/v) brij (Sigma, Sydney, Australia) and mixed with 90mL alginate (Sigma, Sydney, Australia) solution to give a final Flavourzyme concentration of 2% (v/v) and alginate concentration of 1.8% (w/v). This mixture was extruded through a 300µm nozzle of the Inotech® encapsulator IE 50R (Inotech, Dottikon, Switzerland) and the droplets were allowed to gel in 0.1M CaCl$_2$ (Sigma, Sydney, Australia) containing 0.1% (w/v) chitosan (Sigma, Sydney, Australia) for 10 min. After gelling the enzyme microcapsules were washed with Milli Q water (Massachusetts, USA) and used for experiments in this section unless mentioned otherwise.

4.3.1 Leakage of encapsulated Flavourzyme in milk

Flavourzyme capsules (5g) from 1.6, 1.8 and 2.0% (w/v) alginate were suspended in 50mL milk at pH 5.0 and pH 6.0 and both held at 32°C and 38°C for 10 min in a water bath to assess the loss of encapsulated Flavourzyme in milk during cheese manufacture. Milk pH was adjusted using lactic acid (Sigma, Sydney, Australia). The capsules were harvested from milk and washed with Milli Q water before 1g was dissolved in 2.0% (w/v) tri-sodium citrate (Sigma, Sydney, Australia) and Flavourzyme activity was measured as described in section 3.3.1. Initial and final Flavourzyme activity was measured.
4.3.2 Stability of encapsulated Flavourzyme under simulated cheese press conditions

Flavourzyme capsules (30g) produced from 1.6, 1.8 and 2.0% alginate were subjected to simulated cheese press pressure of 0.79Ncm$^{-2}$ (a force similar to that applied to cheese blocks during cheese press) for 4, 8 and 16h using the Texture analyser TA-XT2 (Stable Micro Systems, Surrey, UK) to test the enzyme leakage during simulated cheese press. As the capsules were compressed, the leaked enzyme escaped into the space above the syringe plunger through the serrated edges allowing the liquid while retaining the capsules. The leaked enzyme was separated from the capsules by simply siphoning out the liquid above, and the capsules were collected and 1g was dissolved in tri-sodium citrate to release and measure the activity of Flavourzyme retained in the capsules. Flavourzyme activity before and after treating to the simulated cheese press was measured.
Figure 4.1 Application of simulated cheese press pressure on Flavourzyme capsules
4.3.3 Cheddar cheese manufacture with microencapsulated Flavourzyme and Palatase

Cheddar cheese was manufactured in the Armfield cheese vat (Ringhood, England) from 10L milk; 9L pasteurised skim milk combined with 1L fresh cream (35% milk fat, unhomogenised) from the supermarket. Skim milk, 1L was combined with 1L fresh cream and heated to 45-47°C in a water bath with constant stirring and blended with a “House and Home” HHMM4 Multimixer (Sydney, Australia) for ~3 min and mixed with 8L skim milk to yield ~3.5% fat calculated using the Pearson square method. This 10L milk was heated to 31°C with constant stirring in the cheese vat followed by addition of CaCl₂ (50%; Cheeselinks, Victoria, Australia), 2mL diluted to 20mL with sterile Milli Q water. Starter culture Delvo-Tec LL 50C direct set, deep frozen cultures of *Lc. lactis* ssp *cremoris* and/or *Lc. lactis* ssp *lactis* (DSM Food specialities, Sydney, Australia) was added at the rate of 1.85 units/1000L or 0.0185 units/10L without prior activation. Calf rennet 290 IU/mL (Cheeselinks, Victoria, Australia), 2.5mL diluted to 25mL in sterile Milli Q water was added 10 min after starter addition and the stirring was stopped after a min and the vat was left undisturbed for 40 min. Curd was cut and left undisturbed for 10 min followed by heating to 38°C over 55 min with gentle stirring until the pH reached 6.40 to 6.45, followed by drainage of whey. The whey was filtered using a cheese cloth to collect cheese curd and any capsules lost in the whey and added back to the vat. The curd was banked up against the walls of the vat for 20 min allowed to settle and cut into large blocks and turned every 20 min while maintaining the temperature of the curd. Once the pH dropped to 5.40 to 5.45 the curd was cut into 1cm cubes and mixed well with 25g salt and allowed to stand for 10min. followed by hooping and pressing
overnight under 8kg weights. After about 16h of pressing the curd was sliced and vacuum packed and allowed to ripen at 9°C.

Enzyme capsules were added either to the milk before rennetting or to the milled curd before salting to determine the right stage of capsule addition.

Nine batches of Cheddar cheese were manufactured in triplicates. Low (0.5 LAPU/g milk protein), medium (0.75 LAPU/g milk protein) and high (1.0 LAPU/g milk protein) levels of Flavourzyme with Palatase (0.5LU/g milk fat) were added to milk before rennetting, in free and encapsulated form, these included;

1) Cheese with no added enzymes-control, C
2) Cheese with added free Flavourzyme (medium), D
3) Cheese with added encapsulated Flavourzyme (low), F50
4) Cheese with added encapsulated Flavourzyme (medium), F75
5) Cheese with added encapsulated Flavourzyme (high), F100
6) Cheese with added encapsulated Flavourzyme (low) and encapsulated Palatase, PF50
7) Cheese with added encapsulated Flavourzyme (medium) and encapsulated Palatase, PF75
8) Cheese with added encapsulated Flavourzyme (high) and encapsulated Palatase, PF100
9) Cheese with added encapsulated Palatase, P

A, D, F75, PF75 and PF100 were again manufactured in duplicates at Dairy Farmers Innovation center, Sydney for the purpose of grading.
Figure 4.2 Flow process chart of Cheddar cheese manufacture (adapted from Madziva, 2006)
4.3.4 Effect of stage of capsule addition during cheese manufacture on the distribution of enzyme capsules in cheese matrix

Flavourzyme, 2mL was mixed with alginate-starch solution to give a final concentration of 1.8:1.0 (alginate:starch) and gel beads were produced by extruding from 300µm nozzle of the encapsulator into 0.1M CaCl₂ solution containing 0.1% chitosan and allowed to gel for 10 min. Flavourzyme capsules were incorporated into cheese at two stages of manufacture:

1) Flavourzyme capsules (25g) were added to 10L milk prior to rennetting and the cheese block was cut into 50g cubes and vacuum packed.

2) Flavourzyme capsules (25g) were added to milled curd prior to salting and the cheese block was cut into 50g cubes and vacuum packed.

The distribution of the capsules in Cheddar cheese was established by staining the starch containing capsules with iodine. Olympus-SZH-ILK fitted with a digital camera (Olympus Optical Co., Tokyo, Japan) and a Fibreoptic Illuminator-15001 (Fibreoptic Lightguides, Melbourne, Australia) was used to visualise the stained capsules. The number of capsules counted were represented as the number of particles and the area occupied by stained capsules on the surface of cheese slice was established in terms of pixels using the software Image Pro Plus and the ratio of number of pixels to the number of particles was also used to estimate the distribution of capsules in addition to visual observation.

4.3.5 Analysis of Cheddar cheese

Control and experimental Cheddar cheeses manufactured were subjected to following chemical analysis.
4.3.5.1 Moisture

Moisture in cheese was determined by the oven drying method (AOAC 1990, Method 926.08). A clean dry metallic container was filled 1/3rd with acid-washed sand and weighed \((m_0)\). About 5g grated cheese was mixed with the sand and weighed \((m_1)\) again and the mixture was moistened and heated on a hot plate and transferred to an oven at 104°C for drying for about 20h. The container was cooled in a desiccator and weighed \((m_2)\). Percentage moisture was calculated using the following equation

\[
\text{Moisture (\%) = \frac{m_1 - m_2}{m_2 - m_0} \times 100}
\]

4.3.5.2 Fat

Fat in cheese was determined by Babcock method (AOAC 1995, Method 989.04 and 989.10). Hot water (9mL) was added to 9g grated cheese followed by 0.5mL Zephiran and mixed well. Concentrated \(H_2SO_4\) was slowly added drop wise with constant mixing until all the lumps were dissolved and the solution turned into a deep brown/purple colour which was transferred into a Babcock flask and centrifuged for 5 min followed by addition of hot water till the neck of the flask and 2 min centrifugation. Again hot water was added in order to read fat in the graduated section of the flask and centrifuged for another min and the percentage of fat was read from the fat corresponding to the graduations on the Babcock flask.

4.3.5.3 pH

Cheese block was pierced at least 1cm deep with a pointed pH probe and the pH was read using the Inolab pH meter (Weilheim, Germany).
4.3.5.4 Microbiological status of cheese samples

Cheese samples C, D, F75, PF75 and PF100 were manufactured in duplicates for grading and were tested by Silliker Microtech (Sydney, Australia) for following pathogens as per the corresponding Australian Standard\textsuperscript{TM} (Anon, 2007a) or AOAC methods;

Coagulase positive Staphylococci - AS 1766.2.4 - 1994

Coliforms AS 1766 - 1992

\textit{E. coli} AS 1766 - 1992

\textit{Salmonella} AOAC 996.08

\textit{Listeria} AOAC 999.06

\textit{Bacillus cereus} AS 1766.2.6 - 1991

Yeast and moulds AS 1766.2.2 - 1997

4.3.5.5 Statistical analysis

All data presented are a mean of 6 replicates unless otherwise mentioned and expressed with standard error of mean or standard deviation as mentioned. Data were statistically analysed by SPSS\textsuperscript{®} for Windows\textsuperscript{TM} Version 14 (SPSS Inc. 2005) for one (two) factorial, general linear analysis of variance (ANOVA). The assumption of normal distribution was checked using P-P plot and homogeneity of variance using Levene’s test of equality of error variances. Treatments were tested against each other using Ryan-Einot-Gabriel-Welsch Q test if the assumption of equality of variance was met or Dunnett’s T-test if the assumption was not met. Standard deviation was calculated using Microsoft Excel.
4.4 Results

4.4.1 Stability of encapsulated Flavourzyme

Flavourzyme capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate by gelling in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan were assessed for stability in milk by measuring the leakage of encapsulated Flavourzyme from capsules suspended in acidified milk at pH 5.0 and 6.0 at 32°C (Figure 4.3a) and 38°C (Figure 4.3b), conditions similar to that during certain stages of Cheddar cheese manufacture. Flavourzyme activity in capsules was measured before and after treatment in acidified milk and the difference in the activity is the estimated loss of activity due to leakage.

At 38°C, the retention of Flavourzyme activity in capsules produced from 1.8 and 2.0% alginate was significantly higher ($F_{2, 44} = 27.378$, $p < 0.01$) than that retained in capsules produced from 1.6% alginate. Initial activity of Flavourzyme in the capsules prior to treating to acidified milk was significantly higher ($F_{2, 44} = 210.397$, $p < 0.01$) than those treated to pH 5 which in turn was significantly higher than those treated to pH 6 (Figure 4.3b).

At 32°C, there was no significant difference ($p > 0.05$) in the enzyme retention of capsules produced from 1.6, 1.8 and 2.0% alginate. There was also no significant difference ($p > 0.05$) in the enzyme retention in capsules treated to pH 5 or 6, however the retention of Flavourzyme activity in capsules treated to acidified milk was significantly lower ($F_{2, 44} = 7.386$, $p < 0.01$) than the initial activity before subjecting to acidified milk (Figure 4.3a).

Mechanical stability of Flavourzyme capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate by gelling in 0.1M CaCl$_2$ containing 0.1% (w/v) chitosan was estimated by subjecting the capsules to a pressure similar to that applied to the cheese curd before
packaging. The duration of simulated press tested were 4h as in commercial application (5h high pressure press), 8h and 16h similar to the traditional manufacturing process where the cheese is pressed overnight for at least 16h. The difference in enzyme activity in the capsules before and after subjecting to simulated cheese press pressure was used to estimate the leakage of encapsulated enzyme under simulated cheese press pressure (Figure 4.4). There was significant interaction ($F_{4,45} = 14.197, p < 0.01$) between the concentration of alginate and the duration of cheese press hence the differences between the concentrations of alginate was tested for individual simulated cheese press duration separately. There was no significant difference ($p > 0.05$) in the leakage of Flavourzyme from capsules produced from 1.6, 1.8 and 2% alginate when the capsules were pressed for 4h however, when pressed for 8h the leakage of Flavourzyme from capsules produced from 2% alginate was significantly lower than ($F_{2,15} = 47.866, p < 0.01$) those produced from 1.8 and 1.6% alginate. After 16h simulated cheese press, the leakage of encapsulated Flavourzyme produced from 2% alginate capsules was significantly lower than those produced by 1.8% alginate, which in turn was significantly lower than those produced by 1.6% alginate. Thus at 16h simulated cheese press the leakage of encapsulated Flavourzyme was significantly lower ($F_{2,15} = 197.292, p < 0.01$) in the order of increasing alginate concentration; $2^a < 1.8^b < 1.6^c \%$, values with different superscript letter are significantly different.

### 4.4.2 Determination of stage of capsule addition during cheese manufacture

Starch was incorporated into Flavourzyme microcapsules during encapsulation with 1.8% (w/v) alginate and the capsules were added at two stages of manufacture to determine the ideal stage for addition of enzyme capsules. Distribution of stained
capsules in cheese was observed for both the methods of capsule addition; before renneting (Figure 4.5a) and before salting (Figure 4.5b). Stained capsules appeared as dark objects and were counted using the software Image pro plus. The area occupied by dark objects (capsules) was recorded as the number of pixels for a fixed size of cheese slice. The ratio of number of pixels to the number of particles was also used to establish the pattern of distribution between the treatments (Table 4.1).

### 4.4.3 Composition of cheese

All cheeses were tested for moisture, fat and final pH and there was no significant difference (p > 0.05) in the fat and final pH of control or experimental cheeses. However, the moisture content of control cheeses C was only significantly lower (p < 0.05) than cheeses D with free Flavourzyme, F75 with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein and PF50 with encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein and encapsulated Palatase at a concentration of 0.5 LU/g milk fat (Table 4.2).

### 4.4.4 Microbiological status of cheese

Cheese samples manufactured for grading were tested for microbial pathogens as the pathogen level above the regulatory limits are not permitted for sale or preparation of food for sale (Standard 1.6.1, Australian Standard™). All the pathogens were below the regulatory limits of the Australian standard for microbiological limits in commercial cheese. Though the yeast and mould count was high, there was no difference in the counts of control or experimental cheeses (Table 4.3).
Figure 4.3 Retention of Flavourzyme activity in capsules produced from 1.6, 1.8 and 2.0% (W/V) alginate and gelling in 0.1M CaCl₂ containing 0.1% (w/v) chitosan suspended in milk at pH 5.0 and 6.0 at 32°C (a) and 38°C (b) for 10 min. The Flavourzyme activity in the capsules prior to suspension in acidified milk is labelled initial and the difference in the activity initially and at different pH and temperature treatments is equivalent to the loss of activity as a result of leakage of encapsulated Flavourzyme in milk. Error bars indicate standard error of mean.
Figure 4.4 Retention of Flavourzyme activity in the capsules produced from 1.6, 1.8 and 2.0% (w/v) alginate and gelling in 0.1M CaCl₂ containing 0.1% (w/v) chitosan subjected to simulated cheese press pressure in a texture analyser for 4, 8 and 16h. The Flavourzyme activity was measured before and after subjecting to the simulated cheese press pressure and the difference in the initial and final Flavourzyme activity is presented as the retained activity after simulated cheese press. Error bars indicate standard error of mean.

Note: The initial enzyme activity is different for capsules from different concentrations of alginate.
Figure 4.5 Distribution of stained Flavourzyme capsules in Cheddar cheese, (a) capsule addition to milk prior to renneting and (b) capsule addition to curd prior to salting
Table 4.1 Capsule distribution represented by ratio of number of pixels to number of particles, * mean of 30 fields ± standard deviation

<table>
<thead>
<tr>
<th>Stage of capsule addition</th>
<th>Ratio of number of pixels to number of particles*</th>
</tr>
</thead>
<tbody>
<tr>
<td>To milk prior to renneting</td>
<td>45.6 ± 10.96</td>
</tr>
<tr>
<td>To curd prior to salting</td>
<td>104.5 ± 101.67</td>
</tr>
</tbody>
</table>

**Note:** Aggregated capsules will be counted as smaller number of particles with higher pixels corresponding to the area occupied hence more aggregated the capsules are, greater is the pixels to particle ratio.
Table 4.2 Fat, moisture and final pH of cheeses with and without flavour-enhancing enzymes * mean of 3 samples ± standard error of mean

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fat*</th>
<th>Moisture*</th>
<th>Final pH*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>29.9 ± 0.26</td>
<td>35.86 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.36 ± 0.005</td>
</tr>
<tr>
<td>D</td>
<td>29.9 ± 0.23</td>
<td>39.13 ± 0.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.35 ± 0.005</td>
</tr>
<tr>
<td>F50</td>
<td>29.95 ± 0.12</td>
<td>37.33 ± 0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.35 ± 0.021</td>
</tr>
<tr>
<td>F75</td>
<td>30.16 ± 0.14</td>
<td>37.96 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.35 ± 0.008</td>
</tr>
<tr>
<td>F100</td>
<td>29.9 ± 0.11</td>
<td>37.3 ± 0.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.35 ± 0.020</td>
</tr>
<tr>
<td>PF50</td>
<td>30.23 ± 0.12</td>
<td>37.83 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.34 ± 0.017</td>
</tr>
<tr>
<td>PF75</td>
<td>30.16 ± 0.06</td>
<td>37.0 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.32 ± 0.006</td>
</tr>
<tr>
<td>PF100</td>
<td>30.06 ± 0.13</td>
<td>37.1 ± 0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.35 ± 0.012</td>
</tr>
<tr>
<td>P</td>
<td>29.8 ± 0.05</td>
<td>37.26 ± 0.31&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.36 ± 0.008</td>
</tr>
</tbody>
</table>

<sup>abc</sup> means followed by different superscript letter are significantly different (p < 0.05)

**Note:** Cheese composition measured on the day of packing.
Table 4.3 Pathogens tested in control and experimental cheeses produced for grading (acceptable microbiological level in a cheese sample (AS), other dairy products (M) as per the Australian Standard 1.6.1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Yeast and mould</th>
<th>Coagulase positive staph per g</th>
<th>Coliform per g</th>
<th>E. coli per g</th>
<th>Salmonella per 25 g</th>
<th>Listeria per 25 g</th>
<th>Bacillus cereus CFU per g</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(1)</td>
<td>160,000 yeast (est)</td>
<td>Not detected</td>
<td>4</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>C(2)</td>
<td>140,000 yeast (est)</td>
<td>Not detected</td>
<td>4</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>D(1)</td>
<td>10,000 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>D(2)</td>
<td>46,000 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>F75(1)</td>
<td>320,000 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>F75(2)</td>
<td>160,000 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>PF75(1)</td>
<td>28,000 yeast (est)</td>
<td>Not detected</td>
<td>3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>PF75(2)</td>
<td>8,900 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>PF100(1)</td>
<td>9,100 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>PF100(2)</td>
<td>21,000 yeast (est)</td>
<td>Not detected</td>
<td>&lt;3</td>
<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
<td>&lt;100</td>
</tr>
<tr>
<td>AS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
4.5 Discussion

Flavourzyme capsules were subjected to pH 5 and 6 at temperatures 32°C and 38°C to assess the leakage of encapsulated Flavourzyme during cheese manufacture. The difference in the initial and final Flavourzyme activity in the capsules suspended in acidified milk was used to calculate the loss of enzyme activity as a result of leakage. At 32°C (Figure 4.3a), the activity of Flavourzyme in capsules treated to acidified milk was significantly lower ($F_{2,44} = 7.386$, $p < 0.01$) than the initial activity before subjecting to acidified milk however the variation in alginate concentration or milk pH had no significant effect ($p > 0.05$) on the enzyme retention of capsules. However at 38°C (Figure 4.3b), leakage of encapsulated Flavourzyme significantly increased ($p < 0.01$) with decrease in the alginate concentration and increase in the milk pH from 5 to 6. These results indicate that during early stages of Cheddar cheese manufacture, when the temperature is ~32°C and the pH ~6.6, concentration of alginate or the pH of the milk has no effect on the leakage of encapsulated Flavourzyme, but as the cheese manufacture progresses towards lower pH and higher temperature, leakage of encapsulated enzymes is higher in capsules produced from lower concentration of alginate. Improved retention of Flavourzyme activity in capsules produced from 2% alginate suggests better cross linking of the polymer and hence better retention of encapsulated enzymes at higher concentrations of alginate. Based on these results, highly porous or compact matrix for capsules can be designed for desired release of encapsulated enzymes.

Flavourzyme activity in capsules subjected to simulated cheese press pressure was measured before and after cheese press and the leakage of encapsulated Flavourzyme increased with the duration of cheese press (Figure 4.4). Alginate concentration had no significant effect ($p > 0.05$) on leakage of encapsulated Flavourzyme when
pressed for 4h, however, when the cheese press duration was prolonged, the leakage of encapsulated Flavourzyme increased significantly (p < 0.01) with the decrease in alginate concentration indicating continued leakage under simulated cheese press pressure. These results suggest that capsules produced from higher concentrations of alginate are less porous.

Porosity of the alginate capsules is essential for sustained release of encapsulated enzymes in cheese during ripening and at the same time the capsules are not excessively porous as over 70% of the encapsulated enzyme activity was retained in the capsules even after 16h of pressing indicating that the capsules are elastic and fairly stable under simulated cheese press conditions and are suitable for sustained and prolonged release of encapsulated enzymes in cheese. By altering the concentration of alginate in the capsules and the duration of cheese press it is possible to regulate the initial release of encapsulated enzymes, and these results show that these capsules are suitable for controlled and sustained release of encapsulated enzymes in cheese.

Microscopic observation of stained capsules on the surface of cheese slice shows that capsule addition to milk leads to even distribution in the cheese matrix (Figure 4.5a) which can be attributed to the stirring of capsules in a liquid medium such as milk leading to separation of capsules and thus even distribution in curd. Aggregation of capsules was observed when added to curd before salting (Figure 4.5b). As the enzyme microcapsules were added as a large aggregate, it was difficult to attain even distribution when mixing with cheese curd. Aggregation of enzyme microcapsules in cheese imply localised release of encapsulated enzymes leading to pockets of fast ripened zones (“hot spots”) in the cheese blocks which can be undesirable. These results are in agreement with the findings of Madziva et al. (2006) who reported even
distribution of alginate-pectin encapsulated folic acid in Cheddar cheese when added to milk compared to aggregation of capsules when added to milled curd or injected into the pressed cheese block.

Starch incorporated Flavourzyme capsules were produced from 1.8% alginate and from earlier tests it was found that these capsules range from 500 to 530µm and only the particles in this size range were programmed to be counted by the software Image Pro plus. Due to a small variation in the capsule size, the area occupied by capsules will also vary only to a small extent if distributed evenly. But if the capsules are aggregated together, then will be counted as just one particle but the number of pixels will be higher. The ratio of pixels to particles was calculated to be 45.6 ± 10.96 and 104.5 ± 101.67 for capsule addition to milk and curd respectively (Table 4.1) showing higher value for pixels when added to curd, when same amount of capsules was added in both treatments. The high value of standard deviation, 101.67 obtained for capsule addition to curd show large variation in distribution (dense or sparse) of capsules in cheese. The results for distribution estimated by calculating the ratio of number of pixels to particles support the visual assessment of capsule distribution. These results are in agreement with the proposition of Wilkinson and Kilcawley (2005) that addition of enzymes at dry salting stage would be disadvantageous due to uneven distribution of the small amount of enzyme among large blocks of curd. The whey drained was filtered and the capsules lost in the whey were collected and added back into the curd thus improving the retention of capsules in the curd.

Composition of the control and experimental cheeses were compared in terms of fat, moisture, pH and microbiological level as these are the most important indicators of cheese quality early in ripening. Moisture, fat and pH of the cheese samples were
analysed on the day of vacuum packaging (the day following cheese manufacture), while microbiological level was assessed prior to cheese grading.

High moisture content in all cheeses can be attributed to the variation in the Cheddar cheese manufacture protocol. High moisture in all experimental cheeses can be attributed to addition of Flavourzyme during cheese manufacture, probably interfering with the curd syneresis leading to increased moisture retention in curd during manufacture or the increased breakdown of proteins in cheese decrease protein solvation and increase the levels of moisture in cheeses manufactured with proteolytic enzymes; Flavourzyme in the current study. Higher moisture could also be due to excess moisture from the enzyme capsules. Alginate capsules are hydrogels with bound and free water, which may have diffused into the cheese upon pressing and also due to breakdown of hydrogel beads in cheese during ripening. Hydrogels beads may have absorbed water during cheese manufacture and hence may be responsible for increased moisture in cheese. These results agree with the reports of increased moisture levels in cheeses with liposome (Kheadr et al, 2000) or food gum (Kailasapathy and Lam, 2005) encapsulated enzymes. The moisture level in all cheeses was higher than the standard commercial Cheddar which is 34.5% (Anon, 2007c) hence these cheeses may be called as Cheddar-type cheeses. Moisture content of control cheeses was significantly lower (p < 0.05) than cheeses D with free Flavourzyme, cheese PF75 with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein, and PF50 with encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein and encapsulated Palatase at a concentration of 0.5 LU/g milk fat (Table 4.2). Variation in the moisture content among different cheese can be attributed to the variation in cheese manufacturing process.
The cheese milk was standardised for 3.5% fat, which in theory must yield 35% fat in Cheddar cheese but the fat content in all cheeses was 29.8 to 30.2% (Table 4.2), lower than the standard Cheddar cheese fat content of 33.3% (Anon, 2007c). Higher moisture in cheese would be expected to cause relative reduction in other cheese components such as fat content. There was no significant difference (p > 0.05) in the fat content of control or experimental cheese. Similarly no significant difference (p > 0.05) in final pH was noted for experimental or control cheese (Table 4.2). This indicates that addition of free or encapsulated enzymes during cheese manufacture had no effect on the total fat content or final pH of the cheese.

Cheeses manufactured for grading were tested for pathogens to assess the microbiological quality of cheese. Cheeses with pathogen levels above the regulatory limits are considered unsafe and hence are not permitted for sale or in preparation of food for sale (Australian Standard 1.6.1). There was no difference in the numbers of coliforms, *E.coli, Salmonella, Listeria*, coagulase positive staphylococci, *Bacillus cereus*, yeast and moulds in control or experimental cheeses; hence addition of free or encapsulated enzymes had no effect on the pathogen numbers. All cheeses had pathogen numbers under the regulatory limits except for yeast and mould count (Table 4.3). High levels of yeast and mould in both control and experimental cheeses could be due to contamination of the vat or cheese making equipment. As there is no difference in the yeast and mould count of control or experimental cheeses; Flavourzyme, Palatase or the encapsulation materials such as alginate and chitosan cannot be the sources of contamination. These results show that both Flavourzyme and Palatase are of good microbiological quality and can be used for commercial application.
4.6 Conclusion

Enzyme capsules produced from alginate by gelling in chitosan treated cationic solution are stable in acidified milk and also under simulated cheese press pressure. The Flavourzyme capsules are capable of prolonged and sustained release of encapsulated enzymes during cheese press and also during ripening. By altering the concentration of alginate and the duration of cheese press, it is possible to regulate initial release of encapsulated enzymes into cheese which is advantageous in controlled release of the encapsulated enzymes. Addition of capsules to milk during cheese manufacture is found to yield even distribution, and addition of encapsulated enzymes do not affect the fat content or pH of the cheese, hence are suitable for incorporation into Cheddar cheese for acceleration of ripening.
5 MONITORING AND EVALUATION OF CHEESE RIPENING
5.1 Abstract

Ripening of cheeses manufactured with and without free/encapsulated enzymes was monitored by analysing peptides, amino acids and free fatty acids. Analysis of water-soluble peptides from control and experimental cheeses showed increased and prolonged proteolysis in cheeses incorporated with encapsulated Flavourzyme while rapid proteolysis was observed in cheese with free Flavourzyme. Consequently an increased accumulation of hydrophobic and/or high molecular weight peptides was observed in cheeses with free Flavourzyme followed by encapsulated Flavourzyme and control cheese with no added enzymes. Increase in the concentration of water-soluble peptides was noted with the increase in the concentration of encapsulated Flavourzyme in cheese. Peptide analysis also showed higher concentrations of water-insoluble peptides from 60 to 180 days in control cheese than in cheeses with encapsulated Flavourzyme. Free amino acid analysis showed that the concentration of most amino acids was over 3 times greater in cheese PF75 with encapsulated Flavourzyme than in control cheese C after 30 days of ripening and over 7 times greater after 90 days ripening. Total amino acid concentration increased with the ripening duration and showed rapid increase after 90 days of ripening. Profiles of fatty acid methyl esters showed increased number and concentration of fatty acids in cheeses incorporated with encapsulated Palatase than control cheese with no added Palatase. During grading, cheeses incorporated with free Flavourzyme scored high initially about 30 days ripening while cheese with encapsulated Flavourzyme scored high about 56 and 86 days ripening with a good overall flavour score till about 86 days. Though the flavour of control cheese was scored lower initially, compared to experimental cheese, the flavour improved as the ripening progressed and these cheeses received high overall flavour score. The overall score of experimental
Monitoring & evaluation of cheese ripening

Cheeses with encapsulated Palatase scored lowest for flavour with rancid and strong unpleasant lipolytic flavour.
5.2 Introduction

In long-ripened cheeses a range of biochemical processes leads to development of characteristic flavour, aroma and texture over time and proteolysis, lipolysis and glycolysis are the main biochemical processes occurring in cheese during ripening (McSweeney, 2004). Glycolysis is mainly due to the action of cheese microflora but is greatly influenced by the chemical composition of the cheese. Proteolysis refers to the hydrolysis of milk proteins to peptides and amino acids and their conversion to smaller aroma and flavour compounds while lipolysis refers to the hydrolysis of milk fat to fatty acids and glycerol and their conversion to smaller flavour and aroma compounds (McSweeney, 2004). It is possible to achieve accelerated cheese ripening with an understanding of the biochemical processes occurring in cheese during ripening and this subject has been extensively reviewed (Wilkinson, 1993, Law, 2001; Azarnia et al., 2006).

Exogenous addition of enzymes is a simple and specific approach for accelerating the rate of biochemical reactions occurring during ripening thus accelerating the formation of flavour compounds and reduction of ripening duration. Proteolysis is the main biochemical event occurring in Cheddar cheese (McSweeney, 2004) and can be accelerated by addition of proteolytic enzymes specially exopeptidases known to reduce bitterness as it is one of the major flavour defects in Cheddar cheese. Though lipolytic flavours are not dominant in Cheddar cheese, lipolysis of milk fat contributes to the overall flavour in mature Cheddar (Fox and Stepaniak, 1993). Encapsulated enzymes can minimise the problems associated with addition of free enzymes.

Several techniques have been developed for the assessment and characterisation of cheese ripening by qualitative and quantitative determination of chemical compounds...
formed during cheese ripening. Chromatography is one of the most widely used methods; RP-HPLC is commonly used for analysis of peptides (Aguilar, 2004) and amino acids (Cohen, 2001) while GC is used for fatty acid analysis (Metcalfe and Wang, 1981) although HPLC can also be used for this purpose. Cheese grading is also an important tool for evaluation of ripening. It involves judging cheeses against specified defects on standardised scorecards and is widely used for sensory evaluation in the cheese industry (Delahunty and Drake, 2004).

In the current study, Flavourzyme with exopeptidase and endoprotease activity in combination with Palatase, (a lipase) were efficiently encapsulated in alginate and incorporated into Cheddar-type cheese for the evaluation of acceleration of ripening. Monitoring of proteolysis and lipolysis by HPLC and GC respectively in addition to grading was used to evaluate cheese ripening.
5.3 Materials and methods

Milli Q (Millipore, Massachusetts, USA) water was used for all water-based preparations in this study unless otherwise mentioned.

5.3.1 Monitoring of proteolysis in cheese

Proteolytic pattern in experimental and control cheeses was established by analysing the water-soluble and water-insoluble peptides and free amino acids by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and total amino acids by spectrophotometer.

5.3.1.1 Extraction of water-soluble peptides

All cheeses; control and experimental, were grated and 10g was homogenised with 30g water followed by pH adjustment to 4.4.-4.6. This homogenate was held at 40°C for 1h before centrifuging for 30 min at 5°C and 4800rpm. After centrifugation, fat layer was removed and the supernatant was diluted to 100ml with water and filtered through a No 42 Whatman filter paper. This filtrate was stored at -20°C until RP-HPLC analysis (Verdini et al, 2004).

5.3.1.2 Analysis of water-soluble peptides

Water-soluble extract was filtered through a 0.2µm filter (Sartorius, Melbourne, Australia) and 100µL was injected into Shimadzu LA20 Prominence HPLC system with CBM 20A system controller (Shimadzu Corporation, Kyoto, Japan) fitted with an Alltech Altima C18 reverse phase column of length 250mm and 4.6mm internal diameter (Deerfield, USA). Detection was at 220nm using the SPD M20A Photo Diode Array detector and the gradient elution was with solvent A (0.1% trifluoro
acetic acid (TFA) in water) and solvent B (0.1% TFA in acetonitrile) with a flow rate of 1.0mL/min.

Gradient: Initially 0% B, isocratic step at 0% B for 5 min then linear step to 50% B in 30 min, isocratic step at 50% B for 5 min (Verdini et al., 2004).

5.3.1.3 Extraction of water-insoluble peptides

The precipitate obtained after centrifugation of grated cheese (section 5.3.1.1) was dissolved in 7M urea (Sigma, Sydney, Australia) and dialysed against distilled water for 48h using a MWCO 6000-8000 membrane then freeze-dried in an Alpha-1-4 freeze drier with controller LDC-1M (CHRIST® Gefriertrocknungsanlagen, Osterode am Harz, Germany) and stored at -20°C until RP-HPLC analysis (Verdini et al., 2004).

5.3.1.4 Analysis of water-insoluble peptides

Freeze dried water-insoluble extract (20mg) obtained in section 5.3.1.3 was dissolved in 0.01M imidazole (Fluka, Sydney, Australia) pH 7.0, 0.01M dithioerytritol (Sigma, Sydney, Australia) and 6.6M urea (Sigma, Sydney, Australia), total volume 1.5mL and filtered through a 200µm filter (Sartorius, Melbourne, Australia) and 100µL of this solution was injected into Shimadzu LA20 Prominence HPLC system with CBM 20A system controller fitted with an Alltech Altima C{18} reverse phase column of length 250mm and 4.6mm internal diameter. Detection was at 220nm using the SPD M20A Photo Diode Array detector and the gradient elution was with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile) with a flow rate of 1.0mL/min.
Gradient: Initial composition 0% B, isocratic step at 0%B for 5 min, linear step to 25% B in 5 min, linear step to 35% B in 30 min, linear step to 50% B in 10 min, isocratic step at 50% B for 10 min (Verdini et al., 2004).

5.3.1.5 Extraction of amino acids

To 10mL of the water-soluble extract of cheese obtained in section 5.3.1.1, 2mL 15% sulphosalicylic acid (SSA) (Sigma, Sydney, Australia) was added and centrifuged for 30 min at 20°C and 4800rpm. Supernatant was collected and the pH was adjusted to 4.0. This SSA-soluble extract was stored at -20°C until reverse phase ultra performance liquid chromatography (RP-UPLC) analysis (Verdini et al., 2002).

5.3.1.6 Derivatization of free amino acids and analysis by RP-UPLC

Sulphosalicylic acid-soluble extract obtained in section 5.3.1.5 was analysed for free amino acids at the Australian Proteomic Analysis Facility (APAF, Macquarie University, Sydney, Australia). Free amino acids were analysed using ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, USA) with Waters Empower Pro control and analysis software. Waters AccQTag Ultra column (BEH C$_{18}$, 2.1 X 100mm, 1.7µm) was used for all separations. Free amino acid concentrations were determined using pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) followed by separation of the derivatives and quantification by RP-UPLC (Cohen and Michaud, 1993; Cohen, 2001). 5µL of the sample was dried in a Savant Speed Vac centrifugal concentrator, model SC210A (Thermo Savant, Holbrook, NY, USA) with 10µL of internal standard (α amino butyric acid – AABA). The dried sample was redissolved in 80µL
Monitoring & evaluation of cheese ripening

of borate buffer followed by 20µL derivatizing reagent (Waters AccQTag Ultra reagent). The reaction mixture was incubated at 50°C for 10 min in a heating block. Derivatised amino acid samples were transferred to the auto-sampler. The column and auto-sampler temperatures were maintained at 60°C and 20°C respectively. The sample injection volume and flow rate were 1.0µL and 0.7mL/min respectively. The mobile phase A consisted of AccQTag Ultra eluent A concentrate (Waters) diluted 1 in 20 with milli Q water. The mobile phase B consisted of AccQTag eluent B (Waters). The wavelength of the UV detection was set at 260nm. Analysis time was 10.2 min per sample. Each sample was analysed in duplicate and the results averaged.

5.3.1.7 Determination of total amino acids by spectrophotometer

Water-soluble extract of cheese (100µL) was diluted to 1mL and 2mL of Cadmium-ninhydrin reagent was added and this mixture was incubated at 84°C for 5 min, cooled and the absorbance was read at 507nm in a Helios Gamma spectrophotometer (Thermo electron corporation, England) (Folkertsma and Fox, 1992).

5.3.1.8 Preparation of cadmium-ninhydrin reagent for the determination of total amino acid concentration by spectrophotometer

Ninhydrin (0.8g, Aldrich, Sigma, Sydney, Australia) was dissolved in 80mL absolute ethanol (Aldrich, Sigma, Sydney, Australia) and to this 10mL glacial acetic acid (Sigma-Aldrich, Sydney, Australia) was added followed by 1g cadmium chloride (Aldrich, Sigma, Sydney, Australia) dissolved in 1mL water (Folkertsma and Fox, 1992).
5.3.2 Monitoring of lipolysis in cheese

Free fatty acids from cheese was extracted and derivatized to fatty acid methyl esters (FAME) according to the method of Metcalfe and Wang (1981) modified by Martin-Hernandez et al. (1988) and Chavarri et al. (1997) and analysed by gas chromatograph (Poveda et al., 1999).

5.3.2.1 Extraction and esterification of fatty acids from cheese for Gas Chromatography analysis

To 10g grated cheese, 5mL water, 0.5mL 5.5M H₂SO₄ (adjust pH 0.7-1.3) and 15mL diethyl ether (Sigma-Aldrich) was added and centrifuged at 3000 rpm for 5 min at 0°C. Upper organic layer was transferred to a screw capped bottle containing 1g anhydrous sodium sulphate (Sigma-Aldrich). After 5 min, 3mL was transferred to a screw capped vial and 0.2mL 20% Tetra methyl ammonium hydroxide (TMAH) in methanol (Aldrich, Sigma, Sydney, Australia) was added followed by shaking for 1-2 min. Upper layer with FAMEs from glycerides was injected into a Shimadzu Class 10 Gas Chromatograph. Short/medium chain free fatty acids settled as TMAH soaps were extracted and neutralized to pH 9.0 with thimol blue indicator and 2M methanol-HCl before injecting into a Shimadzu Class 10 GC (Poveda et al., 1999).

5.3.2.2 Analysis of fatty acid methyl esters by GC

Fatty acid methyl esters from cheese (1µl) was injected into a Shimadzu Class 10 Gas Chromatograph with a flame ionization detector and an Omegawax™ 250 fused silica capillary column of the dimension 30mX0.25mm and 0.25µm film thickness (Supelco, Sydney, Australia). Hydrogen was the carrier gas while hydrogen-nitrogen
was the make up gas and hydrogen-air was the detector gas (Martin-Hernandez et al., 1988, Chavarri et al., 1997 and Poveda et al., 1999).

Time program for separation of FAME by GC:

<table>
<thead>
<tr>
<th>Component</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injector</td>
<td>280°C</td>
</tr>
<tr>
<td>Column</td>
<td>60°C for 3 min</td>
</tr>
<tr>
<td></td>
<td>8°C/min till 230°C and hold for 8 min</td>
</tr>
<tr>
<td>Detector</td>
<td>280°C</td>
</tr>
<tr>
<td>Split ratio</td>
<td>1:80</td>
</tr>
<tr>
<td>Hydrogen flow rate</td>
<td>1.39mL/min</td>
</tr>
<tr>
<td>Linear velocity</td>
<td>38.8cm/sec</td>
</tr>
<tr>
<td>Stop time</td>
<td>38 min</td>
</tr>
</tbody>
</table>

### 5.3.3 Cheese grading

Experimental and control cheeses were graded by a master cheese grader at Dairy Farmers Innovation centre (Sydney, Australia). Cheeses were allowed to warm to 15-22°C before grading for a final score of 100 points; 50 points for flavour, 30 points for body and texture and 20 points for colour and condition. Score was deducted for following defects in each category. Small cubes of cheese was used for grading and expectorated after evaluation and the palate was cleansed with water after each sample.
Table 5.1 Defects in Cheddar cheese considered while grading

<table>
<thead>
<tr>
<th>Flavour and aroma</th>
<th>Body and texture</th>
<th>Colour and condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bitter</td>
<td>Pasty</td>
<td>Uneven</td>
</tr>
<tr>
<td>Stale</td>
<td>Dry / crumbly</td>
<td>Mottled</td>
</tr>
<tr>
<td>Sour / high acid</td>
<td>Mealy</td>
<td>Surface creasing</td>
</tr>
<tr>
<td>Salty</td>
<td>Greasy</td>
<td>Seamy</td>
</tr>
<tr>
<td>Flat / lacks flavour</td>
<td>Gas openness</td>
<td>Free fat</td>
</tr>
<tr>
<td>Fermented</td>
<td>Mechanical openness</td>
<td>Misshapen</td>
</tr>
<tr>
<td>Rancid / lipolytic</td>
<td>Slits</td>
<td>Free moisture / serum</td>
</tr>
<tr>
<td>Chemical / metallic</td>
<td>Short</td>
<td>Lactate</td>
</tr>
<tr>
<td>Weedy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feedy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cowy / barny</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouldy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scorched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harsh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncharacteristic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brothy / umami</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4 Results

5.4.1 Assessment of proteolysis

In Cheddar and related cheese varieties proteolysis is an important factor responsible for development of characteristic flavour and texture (Forde and Fitzgerald, 2000; Upadhyay et al., 2004a). In the current study Flavourzyme, a protease peptidase complex was encapsulated and added during Cheddar-type cheese manufacture and its effect on proteolysis was assessed by monitoring water-soluble and water-insoluble peptides, free amino acids and total amino acids formed during ripening by HPLC. All cheeses were vacuum packed and allowed to ripen at 9°C for 270 days and sampled at intervals of 30 days.

5.4.1.1 Peptide profile: water-soluble and water-insoluble

Comparison of HPLC chromatograms of water-soluble peptides of three cheeses; control C with no added enzyme, cheese D with free Flavourzyme at a concentration of 0.75 LAPU/g milk protein and cheese F75 with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein showed higher area for peptides represented by peaks 1, 4, 10, 11, 13, 14, 18, 19, 21 and 22 in cheeses D and F75 compared to C in addition to formation of new peptides represented by peaks 3, 5, 6, 8, 9, 12, 16, 17, 20 and 23 in cheeses D and F75 which were absent in cheese C. At 30 days ripening, peptides represented by peaks 2, 7 and 15 were of similar concentration in all cheeses; C, D and F75 (Figure 5.1). At this stage of ripening, peptides 1, 2, 5, 8, 10, 11, 18 and 19 are found in relatively higher concentrations in cheese D compared to cheese F75 (Figure 5.1).

As the ripening progressed to 90 days, the concentration of peptides represented by peaks 1, 2, 6, 11, 12, 14, 19 and 20 was higher in cheeses D and F75 compared to C,
while peptides marked by peaks 4, 10 and 15 were at higher concentration in cheese C compared to D and F75. Peptides marked by peaks 3, 5, 7, 8, 9, 16, 17, 18, and 21 were found only in cheeses D and F75 but absent in C while peptide 13 was found only in cheese C and absent in experimental cheeses D and F75 (Figure 5.2). Comparison of water-soluble peptide profile of cheeses D and F75 shows that the peptides represented by peaks 1, 5 and 12 are at higher concentration in cheese F75 compared to D while peptides 4, 10, 14, 16, 18, 19, 20 and 21 are at higher concentration in cheese D compared to F75 while all other peptides appear to be of relatively similar concentration in cheese D and F75 after 90 days ripening (Figure 5.2).

After 180 days ripening, peptides represented by peaks 1, 2, 3, 4, 10, 11, 13, 14, 18, 19 and 20 are in higher concentration in cheeses D and F75 compared to C while peptides represented by peaks 6, 9 and 12 are in higher concentration in cheese C compared to D and F75. Peptides represented by peaks 5, 8, 15, 16 and 17 are found only in cheeses with free and encapsulated Flavourzyme (D and F75) and absent in C while peptide 7 is found only in control cheese C while absent in cheeses D and F75. Comparison of water-soluble peptides of cheeses D and F75 show that the peptides 1 and 14 are at higher concentration in cheese F75 compared to D while peptides 17, 18, 19 and 20 are at higher concentration in cheese D compared to F75 while all other peptides are at relatively similar concentrations after 180 days ripening (Figure 5.3).

Effect of variation in Flavourzyme activity on proteolysis in cheese was assessed by monitoring water-soluble peptides in cheeses with increasing level of encapsulated Flavourzyme activity.

F50 – with encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein
F75 – with encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein

F100 – with encapsulated Flavourzyme at a concentration of 1.0 LAPU/g milk protein

After 60 days ripening, the concentration of peptides represented by peaks 2, 4 and 5 was highest in cheese F100 followed by F75 and F50 while the concentration of peptides 1 and 3 was highest in cheese F100 but at similar concentration in cheeses F50 and F75. All other peptides were of relatively similar concentration in all three cheeses (Figure 5.4).

Water-insoluble peptide profile of control cheese C with no added enzymes was compared with that of cheese F50 with encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein. Area of the peaks from cheese F50 are relatively smaller compared to cheese C and the comparison of the water-insoluble peptide profile with α- and κ-casein indicates that the water insoluble peptides in cheeses C and F50 are mainly α- and κ-caseins, however β-casein could not be identified due to overlapping of the peaks (Figure 5.5).
Figure 5.1 Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 30 days of ripening.
Figure 5.2 Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 90 days of ripening
Figure 5.3 Chromatograms of the water-soluble peptides from control cheese (C), cheese with free Flavourzyme (D) and cheese with encapsulated Flavourzyme (F75) extracted after 180 days of ripening.
Figure 5.4 Chromatograms of the water-soluble peptides from cheeses manufactured with encapsulated Flavourzyme at concentrations of 0.5 (F50), 0.75 (F75) and 1.0 LAPU/g milk protein (F100) after 60 days of ripening.
Figure 5.5 Chromatograms of the water-insoluble peptides from cheese F50 with encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein, cheese C with no added enzymes extracted after 180 days of ripening, α-casein and κ-casein standards at concentration of 15mg/mL.
5.4.1.2 Free and total amino acid composition

Amino acids are largely responsible for the characteristic flavour of cheese (McSweeney and Sousa, 2000; Yvon and Rijnen, 2001), some may contribute positively while an imbalance in amino acid composition may lead to off-flavours (Habibi-Najafi and Lee, 1996). Comparison of 20 standard amino acid composition of control cheese C and cheese F75 with encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein at 30 days ripening showed that the concentration of all amino acids in F75 was higher than in cheese C; concentrations of serine (Ser), glutamine (Gln), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) in cheese F75 were about 3 times that in cheese C while arginine (Arg) was detected only in cheese F75 (Figure 5.6a). After 90 days ripening, concentration of all amino acids from cheese F75 was ~7-8 times greater than that found in cheese C; asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) from cheese F75 were over 10 times that in cheese C while arginine (Arg) and cystine (Cys) were detected only in cheese F75 (Figure 5.6b).

Total amino acid concentration in all cheeses increased with the ripening duration and was highest in cheese F100, with highest level of encapsulated Flavourzyme at a concentration of 1.0 LAPU/g milk protein. Rapid increase in total amino acid concentration was noted after 90 days of ripening (Figure 5.7).
Figure 5.6 Composition of free amino acids from control cheese C with no added enzymes and cheese F75 with encapsulated Flavourzyme. Free amino acid composition in cheeses C and F75 after (a) 30 days ripening (b) 90 days ripening.
Figure 5.7 Total amino acid concentration measured by spectrophotometer in the following cheeses; C, D, F50, F75, F100, PF50, PF75, PF100 & P
C – control with no added enzymes
D – free Flavourzyme at a concentration of 0.75 LAPU/g milk protein
F50 – encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein
F75 – encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein
F100 - encapsulated Flavourzyme at a concentration of 1.0 LAPU/g milk protein
PF50 - encapsulated Flavourzyme at a concentration of 0.5 LAPU/g milk protein + encapsulated Palatase at a concentration of 0.5 LU/g milk fat
PF75 - encapsulated Flavourzyme at a concentration of 0.75 LAPU/g milk protein + encapsulated Palatase at a concentration of 0.5 LU/g milk fat
PF100 - encapsulated Flavourzyme at a concentration of 1.0 LAPU/g milk protein + encapsulated Palatase at a concentration of 0.5 LU/g milk fat
P - encapsulated Palatase at a concentration of 0.5 LU/g milk fat
5.4.2 Assessment of lipolysis

Lipolysis is also known to contribute to Cheddar cheese flavour (Fox and Stepaniak, 1993; Arora et al., 1995) though at a subtle level (Collins et al., 2004). Contribution of Palatase, a lipase to Cheddar-like flavour was assessed by incorporation of encapsulated Palatase at a concentration of 0.5LU/g milk fat with and without encapsulated Flavourzyme during cheese manufacture and ripened at 9°C for 270 days. All cheeses were sampled at 30 day intervals and the evolution of free fatty acids (FFAs) was studied by esterification to fatty acid methyl esters (FAME) using TMAH in methanol followed by neutralising the THMA soaps and monitoring by gas chromatograph (GC).

5.4.2.1 Fatty acid methyl ester profile of Cheddar-type cheese

Fatty acids from the following cheeses were extracted and esterified to fatty acid methyl esters (FAME) using tetra methyl ammonium hydroxide (TMAH).

C – control with no added enzymes
F75 – with encapsulated Flavourzyme
PF75 – with individually encapsulated Flavourzyme and Palatase
P – with encapsulated Palatase

Comparison of FAME profile of cheeses C and P extracted after 90 days ripening shows that the number and concentration of FAME is greater in cheese P than C (Figure 5.8). Similarly comparison of FAME of cheese F75 and PF75 shows that after 90 days ripening the number and concentration of FAME from cheese PF75 is greater than cheese F75 (Figure 5.9). Comparison of FAME of cheeses PF75 and P show similar concentrations and numbers of FAME in both cheeses, though a few more new FAMEs appear to be present in cheese PF75 (Figure 5.10). Comparison of
standard FAME with that of cheese P suggests that most of the free fatty acids extracted from the TMAH soaps fraction of cheese P appear to be in the chain length $C_{11}$ to $C_{23}$ (Figure 5.11). However it was not possible to identify all the compounds due to the lack of appropriate standards.
Figure 5.8 Profile of fatty acid methyl esters of control cheese C (KAA90B, with no added enzymes) and cheese P (KAI90B, with encapsulated Palatase) extracted after 90 days of ripening
Figure 5.9 Profile of fatty acid methyl esters of cheeses with encapsulated Flavourzyme (F75; KAD90B) and with encapsulated Flavourzyme and Palatase (PF75; KAG90B) extracted after 90 days of ripening
Figure 5.10 Profile of fatty acid methyl esters of cheeses; PF75 with encapsulated Flavourzyme and Palatase (KAG90B P) and with encapsulated Palatase (KAI90B) extracted after 90 days of ripening
Figure 5.11 Profile of Standard fatty acid methyl esters (KAFAME7) and FAME from cheese P with encapsulated Palatase (KAI90B) extracted after 90 days of ripening.
5.4.3 Grading of control and accelerated ripened Cheddar-type cheeses

Cheese grading is an essential tool for characterising flavour and for maintenance of consistent quality (Delahunty and Drake, 2004) and involves judgement of cheese on the basis of whether it has properties characteristic of its variety (Lawrence et al., 2004). Following cheeses were graded by judging against specified defects and awarding scores based on flavour, body and texture, colour and condition and a collective final score.

C - control with no added enzymes
D - with free Flavourzyme at a concentration of 0.75LAPU/g milk protein
F75 - with encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein
PF75 - with individually encapsulated Flavourzyme (0.75LAPU/g milk protein) and Palatase (0.5LU/g milk fat)
PF100 - with individually encapsulated Flavourzyme (1.0 LAPU/g milk protein) and Palatase (0.5LU/g milk fat)

Comparison of colour and condition scores of cheeses C, D and F75 showed that cheese C consistently scored better than D and F75, while score of D was similar to C from 50 to 72 days ripening and that of F75 improved around 56 days of ripening but later was scored low due to mechanical holes and mottled surface (Figure 5.12a). Colour and condition scores of cheese F75 and PF75 were similar for the entire period of grading as the appearance was similar (Figure 5.12b). In comparison the appearance of cheese PF100 was similar to PF75 initially but was scored lower later during ripening due to mottled surface and accumulation of free serum (Figure 5.12c).

Comparison of body and texture scores of cheeses C, D and F75 shows that the body and texture of cheese C improved with the ripening duration and was constant during
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the whole grading period, while the overall body and texture score of D and F75 was lower than C due to crumbly and pasty texture (Figure 5.13a) while that of cheeses F75 and PF75 were similar until about 56 days ripening when the body and texture score of F75 increased while that of PF75 decreased due to pastier and crumbly texture with accumulation of excessive serum compared to F75 (Figure 5.13b). Body and texture score of cheese PF100 was lower than PF75 for the most part of the ripening time due to extremely pasty and crumbly texture in addition to pale surface (Figure 5.13c). Enzyme beads in the cheese were not detected during grading by the master cheese grader.

Grading of cheeses C, D and F75 based on flavour showed that initially at about 30 days ripening, cheese D scored higher for flavour compared to C and F75 as cheeses C and F75 were scored lower due to lack of flavour (Figure 5.14a). After about 56 days ripening, flavour score of F75 was highest due to development of savoury umami flavour, followed by D and C. The Flavour of F75 scored lowest about 71 days ripening due to development of bitterness and again improved after 86days ripening while slight unclean flavour was noted in cheese F75 after about 90 days ripening hence downgrading cheese F75 after 90 days (Figure 5.14a). Flavour score of cheese PF75 was lower than F75 for the whole ripening duration due to strong unclean lipolytic flavour (Figure 5.14b) and it was also lower than PF100 for most of the ripening duration due to strong unpleasant pungent flavour compared to the unclean savoury umami flavour with after taste in cheese PF100 (Figure 5.14c).

Final grading score is an aggregate of the scores for flavour, body and texture, colour and condition. Comparison of final scores of cheeses C, D and F75 shows that control cheese C scored consistently during the entire period of ripening while cheese D scored higher initially, the overall score was lower than C (Figure 5.15a).
The overall final score of F75 was constantly lower than C and D (Figure 5.15a) and higher than PF75 (Figure 5.15b) for most of the ripening duration while that of PF100 was higher than PF75 (Figure 5.15c).
Figure 5.12 Comparison of grading score of cheeses (a) C, D and F75, (b) F75 and PF75, (c) PF75 and PF100 based on colour and condition on a scale of 1-20. C – no added enzymes, D - free Flavourzyme at a concentration of 0.75LAPU/g milk protein, F75 – encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein, PF75 – encapsulated Flavourzyme at 0.75LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat and PF100 - encapsulated Flavourzyme at 1.0LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat.
Figure 5.13 Comparison of grading score of cheeses (a) C, D and F75, (b) F75 and PF75, (c) PF75 and PF100 based on body and texture on a scale of 1-30. C – no added enzymes, D - free Flavourzyme at a concentration of 0.75LAPU/g milk protein, F75 – encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein, PF75 – encapsulated Flavourzyme at 0.75LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat and PF100 - encapsulated Flavourzyme at 1.0LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat.
Figure 5.14 Comparison of grading score of cheeses (a) C, D and F75, (b) F75 and PF75, (c) PF75 and PF100 based on flavour on a scale of 1-50. C – no added enzymes, D - free Flavourzyme at a concentration of 0.75LAPU/g milk protein, F75 – encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein, PF75 – encapsulated Flavourzyme at 0.75LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat and PF100 - encapsulated Flavourzyme at 1.0LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat.
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Figure 5.15 Comparison of the final grading score of cheeses (a) C, D and F75, (b) F75 and PF75, (c) PF75 and PF100 on a scale of 1-100. C – no added enzymes, D - free Flavourzyme at a concentration of 0.75LAPU/g milk protein, F75 – encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein, PF75 – encapsulated Flavourzyme at 0.75LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat and PF100 - encapsulated Flavourzyme at 1.0LAPU/g milk protein and encapsulated Palatase at 0.5LU/g milk fat.
5.5 Discussion

The comparison of ripening shows clear differences between cheeses with and without added enzymes and also between free and encapsulated enzymes. Comparison of water-soluble peptide profile of cheeses C, D and F75 after 30 days ripening show that the concentration of peptides in cheeses D and F75 are higher compared to cheese C also with development of a larger number of peptides in cheeses D and F75 but absent in C (Figure 5.1), this can be attributed to the action of Flavourzyme on caseins leading to faster development of peptides, which would take longer when occurring naturally as seen in cheese C. These results are similar to the results reported by Bergamini et al. (2006) who found increased concentration of several peptides by visual matching of chromatograms of water-soluble extract of a semi-hard cheese with probiotic lyophilised culture compared to control cheese. Higher concentration of peptides represented by peaks 1, 4, 10, 11, 13, 14, 18, 19, 21 and 22 in cheeses D and F75 compared to C (Figure 5.1) may possibly be due to faster proteolysis in cheeses D and F75 with free and encapsulated Flavourzyme respectively. These results are in agreement with reports of increased size-exclusion peak areas of astringent fractions of Cheddar cheeses with liposome-encapsulated Flavourzyme added at concentrations of 0.5, 0.75 and 1.0 LAPU/g milk protein at 30 days ripening (Kheadr et al., 2003). In the current study, presence of peptides 3, 5, 6, 8, 9, 12, 16, 17, 20 and 23 in cheeses D and F75 but absence in C (Figure 5.1) suggests that these peptides may be the products of hydrolysis of caseins by Flavourzyme in these cheeses. Higher concentration of the peptides 1, 2, 5, 8, 10, 11, 18 and 19 in cheese D compared to F75 (Figure 5.1) suggests that at 30 days ripening, these peptides may be the products of rapid hydrolysis of caseins by free...
Flavourzyme in cheese D but due to delayed release of encapsulated Flavourzyme in cheese F75, these peptides appear to be at a lower concentration.

After 90 days ripening, most of the free Flavourzyme added to milk during manufacture of cheese D is likely to be lost during whey drainage, but the presence of peptides 1, 2, 6, 11, 12, 14, 19 and 20 at higher concentrations in D and F75 compared to C (Figure 5.2) suggests that these peptides are possibly the result of immediate hydrolysis of cheese proteins and resistance to further hydrolysis by Flavourzyme and hence continue to be present in higher concentrations even after 90 days ripening in cheese D with free Flavourzyme. These results are in agreement with the reports of higher concentration of size-exclusion peak areas of astringent fractions of Cheddar cheeses with liposome-encapsulated Flavourzyme added at concentrations of 0.5, 0.75 and 1.0 LAPU/g milk protein after 90 days ripening (Kheard et al., 2003). In the current study, presence of peptides marked by peaks 4, 10 and 15 at higher concentrations in cheese C compared to D and F75 (Figure 5.2) suggests that these peptides are sensitive to the action of Flavourzyme and hydrolysed by free and encapsulated Flavourzyme in cheese D and F75 and thus found in relatively lower concentrations in these cheeses. Presence of peptides marked by peaks 3, 5, 7, 8, 9, 16, 17, 18, and 21 only in cheeses D and F75 but absence in C (Figure 5.2) indicates that these peptides may be the products of hydrolysis of caseins by Flavourzyme, which is absent in C. Presence of the peptide represented by peak 13 only in cheese C and absence in cheeses D and F75 (Figure 5.2) suggests that this peptide may be highly susceptible to the action of Flavourzyme and may be hydrolysed in cheeses D and F75 hence found only in C. Concentration of peptides represented by peaks 1, 5 and 12 at higher concentration in cheese F75 compared to D (Figure 5.2) suggests that these peptides are products of
sustained action of Flavourzyme released from the capsules in cheese F75 whereas in cheese D, most of the free Flavourzyme would be lost in whey as it is water soluble. Higher concentration of peptides 4, 10, 14, 16, 18, 19, 20 and 21 in cheese D compared to F75 (Figure 5.2) suggests that these peptides may be the products of early hydrolysis by Flavourzyme may possess the site Phe\textsubscript{105}-Met\textsubscript{106} or other cleavage sites for Flavourzyme hence appear to be present in lower concentration in cheese F75.

Presence of peptides 1, 2, 3, 4, 10, 11, 13, 14, 18, 19 and 20 at higher concentration in cheeses D and F75 compared to C (Figure 5.3) is indicative of the prolonged effect of Flavourzyme on cheese proteolysis. Higher concentration of peptides 6, 9 and 12 in cheese C compared to D and F75 and presence of peptide 7 in C but absent in D and F75 suggests that these peptides are sensitive to the action of Flavourzyme and may possess the Flavourzyme cleavage site Phe\textsubscript{105}-Met\textsubscript{106}. Presence of peptides 5, 8, 15, 16 and 17 only in cheese D and F75 but absence in C (Figure 5.3) indicates that these peptides are products of the action of Flavourzyme on cheese proteins. Highest concentration of peptides 1 and 14 in cheese F75 after 180 days ripening (Figure 5.3) suggests that these peptides are constantly released due to the prolonged action of encapsulated Flavourzyme on cheese proteins and tend to accumulate during ripening. Presence of peptides 17, 18, 19 and 20 in cheese D at higher concentration compared to F75 suggests that these peptides are produced as early as the day of manufacture and accumulated due to the diminishing effect of free Flavourzyme, (expected to be lost during whey drainage) in cheese D even after 180 days ripening (Figure 5.3).

Reverse-phase high-performance liquid chromatography (RP-HPLC) involves separation of molecules based on hydrophobicity; solutes are eluted in the order of
increasing molecular hydrophobicity (Cliffe and Law, 1990; Aguilar, 2004). Peptides 20, 21, 22 and 23 (Figure 5.1) appear to be relatively hydrophobic or high molecular weight compared to other peptides as they were eluted after other peptides. These peptides start to accumulate early during ripening and are at high concentration in cheese with free and encapsulated Flavourzyme compared to control C with no added enzyme and these peptides continue to accumulate during ripening and do not appear to be sensitive to the action of Flavourzyme even after 180 days ripening (peaks 17, 18, 19 and 20, Figure 5.3). In a similar study, Ardo et al. (2007) reported that large hydrophobic peptides that elute late in RP-HPLC in a narrow group (83-89 min) and accumulate in the semi-hard cheese Herrgard during ripening and suggested that these peptides are products of early hydrolysis of β-casein by plasmin. In the current study a similar trend in accumulation of hydrophobic or high molecular weight peptides was observed may be the products of β-casein hydrolysis and accumulate during ripening. However in the current study, the retention time of these peaks was found to be 37-40 min and the variation in the retention time of the peaks reported in the current study and that reported by Ardo et al. (2007) may be due to the variation in the type of cheese, extraction, purification and RP-HPLC analysis.

Cheeses F50, F75 and F100 with the increasing levels of encapsulated Flavourzyme at concentrations of 0.5, 0.75 and 1.0 LAPU/g milk protein respectively were analysed for water-soluble peptides to determine the suitable enzyme load for optimal proteolysis. Although most peptides appear to be of similar concentration at 60 days ripening, peptides 1, 2, 3, 4 and 5 were detected at highest concentration in cheese F100 compared to F50 and F75 indicating greater extent of proteolysis in F100 with higher concentration of Flavourzyme. Concentration of peptides 4 and 5
increased with the increase in Flavourzyme concentration (Figure 5.4), indicating varying levels of enzyme activity.

Water-insoluble peptides in cheese mainly consist of caseins, after 180 days of ripening, concentration of water-insoluble peptides from cheese C was found to be higher than those from cheese F50 with encapsulated Flavourzyme (Figure 5.5) suggesting rapid break down of caseins in cheese F50 by encapsulated Flavourzyme resulting in lower concentrations of water-insoluble peptides. Comparison of the chromatograms of α-casein and κ-casein standard, shows that these caseins are one of the major components of water-insoluble peptides from cheeses C and F50 (Figure 5.5).

Amino acids are produced as a result of peptide hydrolysis and are known to contribute to the flavour of Cheddar cheese directly and also indirectly by acting as precursors for the production of flavour compounds (Yvon and Rijnen, 2001). From the water-soluble and water-insoluble peptide profiles it was found that the cheeses with free and encapsulated Flavourzyme showed increased proteolysis compared to control cheese; with no added enzymes. Free amino acid composition of control cheese C and cheese F75; with encapsulated Flavourzyme was compared at 30 and 90 days ripening to assess the exopeptidase activity of Flavourzyme. At both ripening times, levels of all amino acids were at elevated levels in cheese F75 compared to cheese C. The concentration of most free amino acids from cheese F75 were found to be ~1-6 times greater compared to cheese C after 30 days ripening (Figure 5.6a) and ~7-30 times greater after 90 days ripening (Figure 5.6b).

Higher concentration of amino acids in cheese F75 suggests extensive proteolysis due to the action of Flavourzyme. Presence of most amino acids at very high concentration after 90 days of ripening suggests that a large number of amino acids
are released around that time, as the amino acids released earlier would be catabolised to smaller compounds by the cheese microflora, but the presence of most amino acids at very high concentration after around 90 days ripening also suggests that these amino acids may be released in high concentrations at this stage of ripening possibly as a result of low numbers of SLAB and NSLAB reported to be present in Cheddar cheese at this stage of ripening (Fox and McSweeney, 2004). The lower microbial activity would result in less catabolism and thus accumulation of amino acids in the cheese. Release of large number of amino acids especially after 90 days ripening may also be due to the time involved in break down of peptides and subsequent release of amino acids. This observation was also supported by the measurement of total amino acids; the concentration of which increased rapidly after 90 days ripening (Figure 5.7) and was highest in cheese F100 with the highest level of encapsulated Flavourzyme at a concentration of 1.0 LAPU/g milk protein. After 30 days ripening, serine (Ser), glutamine (Gln), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) were about 3 times greater in cheese F75 than in cheese C, while arginine (Arg) was detected only in cheese F75 (Figure 5.6a). Most of these amino acids are hydrophilic and the release of these hydrophilic amino acids indicates the tendency of hydrophilic cleavage of Flavourzyme. Similarly after 90 days ripening, asparagine (Asn), serine (Ser), glutamine (Gln), glycine (Gly), glutamic acid (Glu), threonine (Thr), methionine (Met), valine (Val), isoleucine (Ile) and leucine (Leu) were over 10 times that in cheese C, while arginine (Arg) and cystine (Cys) were detected only in cheese F75 (Figure 5.6b). Most amino acids in C were 10-30% lower compared to F75; these results indicate the exopeptidase activity of Flavourzyme. At 90 days ripening, Leu, Phe and Lys are the most abundant amino
acids in cheese C, where as in cheese F75, Leu, Phe and Glu are the most abundant amino acids. These results agree with Aston and Creamer (1986), McSweeney and Fox (1993) and De Wit et al. (2005) who reported that Leu, Phe and Glu are the most abundant FAA in mature Cheddar cheese. Hence release of free amino acids in cheese F75 appears to be similar to standard Cheddar cheese.

While some amino acids in cheese C decreased marginally after 90 days ripening, some were found at similar concentration. In contrast most amino acids in F75 increased 3-6 folds after 90 days ripening suggesting sustained release of encapsulated Flavourzyme and its effect on peptide hydrolysis. De Wit et al. (2005) also reported a significant increase in free amino acid content of yeast-inoculated Cheddar cheese from 60-120 days.

Lipolysis in Cheddar cheese is known to contribute to the overall flavour only to a small extent and there are very few reports of studies on the effect of lipolysis on Cheddar cheese flavour (Collins et al., 2004). Palatase, a lipolytic enzyme was encapsulated and incorporated into Cheddar-type cheese to study its effect on lipolysis and the overall flavour of Cheddar-type cheeses. Free fatty acids from the 4 cheeses; control cheese with no added enzymes (C), cheese with encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein (F75), cheese with individually encapsulated Flavourzyme (0.75LAPU/gmilk protein) and Palatase (0.5LU/g milk fat) (PF75) and the cheese with encapsulated Palatase at a concentration of 0.5LU/g milk fat (P) was extracted and esterified to fatty acid methyl esters (FAME) and analysed by GC.

Comparison of FAME profile of cheese C and P extracted after 90 days ripening shows smaller number and lower concentration of fatty acids in control cheese C compared to cheese P with encapsulated Palatase (Figure 5.8). Increased numbers
and concentration of free fatty acids in cheese P can be attributed to the hydrolysis of cheese fat by encapsulated Palatase. Similarly, higher number and concentration of FAME was observed in cheese PF75 compared to F75 (Figure 5.9), demonstrating that the presence of a large number of free fatty acids in cheese PF75 is possibly due to the action of encapsulated Palatase which was absent in cheese F75. These results suggest that encapsulated Palatase, like Flavourzyme is efficiently released during Cheddar-type cheese ripening. Cheeses PF75 and P were manufactured with identical load of encapsulated Palatase and the free fatty acids from these cheeses are expected to be identical. FAME profile of cheeses PF75 and P extracted after 90 days ripening are similar (Figure 5.10) suggesting that the release and activity of encapsulated Palatase was not affected by the presence of encapsulated Flavourzyme. Individual encapsulation of Palatase and Flavourzyme in alginate appears to be an efficient means for isolation of Palatase, which may also be a target for hydrolysis by Flavourzyme. Comparison of FAME profile of cheese P with standard indicate that most of the FAME in cheese P are in the chain length range of C\textsubscript{11} and C\textsubscript{23} (Figure 5.11) suggesting that most of the free fatty acids extracted after 90 days ripening were of intermediate length. These results agree with the findings of Yilmaz et al. (2005) who reported increase in concentration of long chain free fatty acids in Tulum cheese made with microbial lipase with palmitic (C\textsubscript{16:0}), oleic (C\textsubscript{18:1}) and myristic (C\textsubscript{14:0}) found to be the most abundant FFA and Fernandez-Garcia et al. (1988) who reported increased levels of (C\textsubscript{16:0}), (C\textsubscript{18:0}) and (C\textsubscript{14:0}) in addition to C\textsubscript{18:1} in Spanish hard cheese manufactured with Palatase.

Cheeses C with no added enzymes, D with free Flavourzyme at a concentration of 0.75LAPU/g milk protein, F75 with encapsulated Flavourzyme at a concentration of 0.75LAPU/g milk protein, PF75 with individually encapsulated Flavourzyme
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(0.75 LAPU/g milk protein) and Palatase (0.5 LU/g milk fat) and PF100 with individually encapsulated Flavourzyme (1.0 LAPU/g milk protein) and Palatase (0.5 LU/g milk fat) were graded by a master cheese grader during ripening. These cheeses were judged for flavour, colour, condition, body and texture against specified defects (Table 5.1) for about 100 days. Grading scores based on colour and condition show that cheeses F75, PF75 and PF100 with encapsulated enzymes scored lower than C and D, but after about 65 days ripening cheese D also scored lower than the control cheese C for colour and condition (Figure 5.12a, b and c) due to mottled surface and accumulation of serum which may be caused by excessive proteolysis. Increased proteolysis leading to extensive breakdown of casein network in cheese is known to decrease the water available for protein solvation resulting in easily deformed cheese (Lawrence et al., 2004). Similarly in the current study, extensive proteolysis may be the cause for liquid expulsion as result of reduced water-binding capacity of the protein network.

Grading of cheeses based on body and texture showed that cheese D initially scored better than C but deteriorated thereafter due to crumbly texture, while the body and texture score of cheese F75 was consistently lower than D and C during the entire grading period due to crumbly and pasty texture (Figure 5.13a). Texture of cheese C improved as the ripening progressed but that of D and F75 improved only till about 86 days ripening and deteriorated thereafter (Figure 5.13a). Body and texture of only cheese C improved with ripening while that of other cheeses deteriorated with time. Crumbly and pasty texture of cheeses D, F75, PF75 and PF100 can be attributed to higher levels of proteolytic enzymes leading to rapid and extensive breakdown of protein network which in turn lowers the water-binding ability of cheese thus resulting in expulsion of water leading accumulation of serum and a pasty and
crumbly texture (Lawrence et al., 2004). Though the body and texture of cheese PF75 was similar to F75 initially, but declined as the ripening progressed (Figure 5.13b). Poor texture of PF75 compared to F75 may be due to extensive lipolysis in PF75 due to the presence of encapsulated Palatase, which was absent in F75. These results suggest that fat is also an important factor affecting cheese texture and extensive lipolysis like proteolysis may lead to texture defects in cheese. Poor texture of cheese PF100 compared to PF75 (Figure 5.13c) suggests extensive protein breakdown in PF100 due to higher level of encapsulated Flavourzyme. These results agree with the reports of brittle texture in Cheddar cheeses with liposome (Kheadr et al., 2000) and food gum (Kailasapathy and Lam, 2005) encapsulated Flavourzyme.

All control cheeses and those with Flavourzyme scored high for pleasant, high intensity acceptable flavour and those with Palatase scored low for unpleasant unacceptable flavour. Flavour scores of cheeses varied with the ripening time, with moderate variation in flavour score was observed for cheese C, more variation was observed in D and F75 (Figure 5.14a). This variation in flavour profile may be due to the formation of new peptides and amino acids some of which may contribute to a pleasant flavour while some to an unpleasant flavour (Raksakulthai and Haard, 2003). Higher flavour score of cheese D at 30 days ripening indicate immediate proteolysis in cheese D resulting from free Flavourzyme leading to rapid development of flavour compared to slower proteolysis in cheese F75 with delayed release of encapsulated Flavourzyme. These results also suggest that the leakage of encapsulated Flavourzyme during cheese press is not responsible for immediate proteolysis as observed in cheese F75.

Cheese F75 scored highest for flavour around 56 days ripening due to development of savoury umami flavour but later scored lower than C and D due to mild bitterness.
and later scored higher due to elimination of bitterness and formation of salty savoury flavour which may be due to the presence of certain peptides and/or amino acids which are absent in C as evidenced by the development of higher number of peptides in cheeses D and F75 (Figure 5.1, 5.2, 5.3 and 5.6) and increased levels of free amino acids in cheese F75 compared to C (Figure 5.6a and b). Better flavour score of cheese F75 may also be due to the formation of the sulphur containing amino acid cysteine in F75 but absent in C and presence of methionine; another sulphur containing amino acid in high concentration in cheese F75. These amino acids are known to impart savoury flavour to cheese and may be released as a result of increased level of proteolysis in F75 as result of sustained release of encapsulated Flavourzyme. Cheeses D and F75 scored better than C for flavour after about 50 days ripening due to elimination of bitterness which was persistent in cheese C with the development of savoury umami flavour. Elimination of bitterness in D and F75 with free and encapsulated Flavourzyme respectively, as early as 50 days ripening suggests that Flavourzyme can be used for debittering cheese. In a similar study bitter and astringent peptides accumulated in experimental cheeses with liposome-encapsulated bacterial and fungal proteinases but were no longer detected after 3 months ripening (Kheadr et al., 2000). However in the current study the flavour of cheese F75 deteriorated after about 86 days ripening with development of bitterness and unclean flavour which did not improve during next grading hence cheese grading was not continued after about 100 days ripening. Arginine was found only in cheese F75 and is thought to be responsible for bitterness (Barcina et al., 1995). Izco et al. (2000) reported arginine concentration of 0.1-3.1mg/g cheese in Ossau-Iraty cheese while Zaki and Salem (1992) reported arginine concentration in the range 2.2-9.3mg/g Edam cheese treated with Neutrase and the bitterness in these cheeses were
attributed to higher concentrations of arginine. Similarly in the current study, higher levels of arginine in the range 0.55 and 0.65mg/g cheese was noted after 30 and 90 days ripening respectively. Development of mild bitterness in cheese F75 after 50 days ripening may be due to increased concentration of arginine at 90 days ripening. Concentration of the amino acid isoleucine in cheese F75 was about 30 times greater than C after 90 days ripening, the hydrophobicity value of Ile is high (Habibi-Najafi and Lee, 1996) and hydrophobic amino acids are known to impart bitterness in cheese (Cliffe and Law, 1990). High concentration of Ile in F75 may also be responsible for the poor flavour developing with longer aging. The concentration of many branched chain amino acids such as threonine, glutamine, glutamate and leucine was higher in cheese F75, these amino acids are also known to impart bitter taste (Raksakulthai and Haard, 2003) and this could also be the reason for development of mild bitterness in cheese F75 after 86 days ripening. Some amino acids such as phenylalanine and some branched amino acids in excess can cause unclean flavour defects in Cheddar cheese (Dunn and Lindsay, 1985). In the current study, Phe, an aromatic amino acid and Glu a branched amino acid were found to be the most abundant FAA after 90 days ripening (Figure 5.6b) and may also be responsible for the unclean flavour of cheese F75 after 86 days ripening.

Though the level of Flavourzyme in cheeses F75 and PF75 were identical, the overall flavour score of F75 was higher than PF75 (Figure 5.14b) as PF75 was downgraded due to development of strong unclean lipolytic flavours possibly due to liberation of free fatty acids released as result of hydrolysis of fat in cheese by encapsulated Palatase. Poor score of PF75 compared to F75 suggests that strong lipolytic flavours are unacceptable in Cheddar-type cheeses. Initially cheese PF100 scored higher than all cheeses for flavour (Figure 5.14c) suggesting that around 27 days ripening, only a
small amount of encapsulated Palatase and Flavourzyme would have been released but sufficient to enhance the flavour. Higher flavour score of PF100 at 26 days ripening suggests that mild lipolysis may contribute positively to the overall Cheddar cheese flavour. However the flavour score of cheese PF100 deteriorated with the progress in the ripening time due to the development of unpleasant unclean flavour. Strong lipolytic flavours are a result of production of large number of FFAs and their catabolism to smaller compounds and these can contribute to flavour either positively or lead to rancid defect depending on the concentration and perception threshold (Molimard and Spinnler, 1996).

Though cheese PF100 had higher level of encapsulated Flavourzyme and similar level of encapsulated Palatase compared to PF75, the overall flavour score of PF75 was lower than PF100 due to strong unpleasant pungent flavour compared to the unclean savoury umami flavour with after taste in cheese PF100 (Figure 5.14c). Though lipolysis is undesirable in young Cheddar cheese, extra mature Cheddar shows considerable lipolysis and the lipolytic flavour impact is balanced by the products of proteolysis (Fox and Stepaniak, 1993). Similarly in the current study, better flavour of PF100 compared to PF75 may be due to masking of strong unpleasant lipolytic flavours in PF100 by generation of more proteolytic compounds as a result of higher rate of proteolysis from higher levels of Flavourzyme.

In a similar study by Kheadr et al. (2003), addition of individually encapsulated Flavourzyme and Palatase in liposomes added to milk during Cheddar cheese manufacture resulted in development of well balanced flavour. It was concluded that addition of Flavourzyme at a concentration of 0.75LAPU/g milk protein and Palatase at a concentration of 0.2 LU/g milk fat was responsible for development of well balanced flavour without textural defects or bitterness. In the current study although
addition of alginate encapsulated Flavourzyme at 0.75LAPU/g milk protein (cheese F75) resulted in increased proteolysis and faster development of flavour as early as 26 days ripening, extensive proteolysis and off-flavours were also observed. The variation in the proteolysis resulting from the same level of Flavourzyme in similar cheeses suggests that the enzymes encapsulated in alginate are released completely compared to those encapsulated in liposomes. In a similar study, Cheddar cheese manufactured with Flavourzyme encapsulated in food gums such as κ-Carrageenan and gellan resulted in bitterness (Kailasapathy et al., 1998) while in the current study, control Cheddar-type cheeses were found to be bitterer than the cheeses with alginate encapsulated Flavourzyme until about 71 days ripening and the development of mild bitterness in cheese F75 with encapsulated Flavourzyme may be due to accumulation of hydrophobic peptides or amino acids as a result of extensive proteolysis. These variations in findings of the current study and that of Kailasapathy et al. (1998) and Kheadr et al. (2003) suggests that alginate is an efficient alternative material for encapsulation of Flavourzyme and Palatase leading to sustained release of encapsulated enzymes. However, development of textural and flavour defects in cheeses with encapsulated and free Flavourzyme suggests that the level of enzyme added was very high. The optimum temperature range of Flavourzyme is 30-50°C which is well above the ripening conditions of Cheddar cheese and it was assumed that the activity of encapsulated Flavourzyme in the cheese would be reduced during ripening due to low temperature, however extensive proteolysis in all the cheeses with free and encapsulated Flavourzyme suggest that the activity of Flavourzyme is not drastically reduced even at Cheddar cheese ripening conditions and the encapsulated enzymes are efficiently released.
5.6 Conclusion

Incorporation of Flavourzyme into Cheddar-type cheese resulted in increased proteolysis. Immediate proteolysis was observed in cheese with free Flavourzyme with the development of flavour as early as 26 days ripening while control cheese at this stage of ripening lacked flavour with pronounced bitterness. Bitterness in cheeses with encapsulated Flavourzyme was eliminated as early as 56 days ripening but persisted in control cheeses with no enzymes. Development of uncharacteristic yet pleasant savoury umami flavour was also observed in cheeses with encapsulated Flavourzyme and this flavour was dominant until about 86 days ripening. However the level of enzymes studied was very high as extensive proteolysis lead to flavour and textural defects in all cheeses with Flavourzyme and unpleasant lipolytic flavours noted in cheeses with encapsulated Palatase. Release and activity of encapsulated Flavourzyme and Palatase was not affected by cheese ripening conditions and individual encapsulation of these two enzymes in alginate is suitable for combined application. Incorporation of encapsulated Flavourzyme in moderation with trace amounts of encapsulated Palatase can potentially lead to enhanced flavour development in Cheddar cheese.
6 CONCLUDING SUMMARY
A range of food grade polymer hydrogels were screened and the most suitable polymer for microencapsulation of Flavourzyme was selected and the encapsulation parameters were optimized for maximum encapsulation efficiency and stability. Flavourzyme microcapsules were tested under simulated conditions of Cheddar cheese manufacture. Flavourzyme and Palatase microcapsules were added to the milk during the manufacture of Cheddar-type cheese for even distribution. Cheese ripening was assessed by analysis of water-soluble peptides, water-insoluble peptides, amino acids and fatty acids and thus monitoring proteolysis and lipolysis. Flavour and texture of accelerated ripened cheese was evaluated by grading.

Cheddar is a widely consumed cheese variety requiring long ripening for the development of mature flavour (Wilkinson and Kilcawley, 2005). Extended ripening of this variety is a financial burden for the manufacturer due to increased costs of refrigeration and maintenance in addition to loss of yield due to contamination and syneresis, thus increasing the overall cost (Cliffe and Law, 1990; Picon et al., 1997; Wilkinson and Kilcawley, 2005). Biochemical reactions occurring in cheese during ripening, leading to the breakdown of proteins, fat and sugars with the formation of smaller compounds are responsible for the development of flavour and texture characteristic of mature cheese (Forde and Fitzgerald, 2000; McSweeny, 2004). As Cheddar is ripened between 8-12°C, the rate of these biochemical reactions is low thus extending the ripening duration (Fox et al., 1993; Fox and McSweeny, 1996; Fox and Wallace, 1997). Though several techniques have been developed to accelerate cheese ripening, development of balanced flavour still remains a challenge (Wilkinson and Kilcawley, 2005). Exogenous addition of enzymes can be used to accelerate cheese ripening.
Microencapsulation of enzymes can minimise the problems associated with addition of free enzymes (Wilkinson and Kilcawley, 2005).

In the current study alginate, chitosan and κ-Carrageenan were studied for microencapsulation of Flavourzyme using the Inotech® encapsulator. Owing to the high viscosity, chitosan and κ-Carrageenan were eliminated as suitable polymers for enzyme encapsulation and alginate was selected for enzyme encapsulation. Gelling of alginate-Flavourzyme mixture in 0.1M CaCl₂ resulted in poor encapsulation efficiency possibly due to high porosity of the capsules and diffusion of Flavourzyme from capsules during formation (Pommersheim et al. 1994; Dashevsky, 1998). Incorporation of starch or pectin into alginate-enzyme matrix as filler material did not improve enzyme retention in capsules unlike reports by Madziva et al. (2006) and Sultana et al. (2000) who reported increased folic acid and cell encapsulation upon addition of pectin and starch to alginate matrix respectively. Low encapsulation efficiency of Flavourzyme in alginate-starch and alginate-pectin could be due to insufficient blocking of the pores in alginate matrix or interference of added starch and pectin with the Flavourzyme assay.

Flavourzyme-alginate gelling in 0.1M CaCl₂ resulted in poor encapsulation efficiency due to increased porosity or diffusion of Flavourzyme from capsules. Treatment of cationic gelling solution with chitosan resulted in an improved encapsulation efficiency. This could be due to blocking of pores in alginate matrix by possible interaction of chitosan with alginate similar to the interaction of CaCl₂ with alginate; gelling of capsules in chitosan treated gelling solution can minimize bead shrinkage and water loss thus minimizing diffusion and leading to enhanced enzyme retention. Though 0.3% (w/v) chitosan in the gelling solution resulted in higher encapsulation efficiency, 0.1% (w/v) was chosen for further work as higher
Concluding summary

Concentrations of chitosan in gelling solution resulted in aggregation of capsules during formation.

There was no enhancement in Flavourzyme retention in capsules coated with alginate or poly-L-lysine, hence uncoated capsules were used for further experiments also poly-L-lysine is an expensive ingredient and hence not suitable for commercial application.

Frozen or freeze-dried Flavourzyme capsules are suitable for industrial application as they retain about 80% of the enzyme activity over 10 weeks when stored appropriately.

Retention of Flavourzyme activity was marginally better (p < 0.01) in capsules produced from 2.0% alginate. As there was no significant difference (p > 0.05) in the enzyme retention of capsules produced from 1.6, 1.8 or 2.0% (w/v) alginate, any of these concentrations of alginate are suitable for delivery of Flavourzyme into cheese. Retention of over 70% of the initial enzyme activity even after 16h simulated cheese press (section 4.4.1), indicates only about 30% of the enzyme was released during cheese press and the rest released in cheese during ripening possibly as a result of breakdown of alginate matrix or through diffusion of the encapsulated enzymes from capsules. There is also the possibility that the surface layer of the beads may show a greater binding of Ca$^{2+}$ and chitosan with alginate and this may form a barrier to the flow of enzymes. Hence the capsules are mechanically stable and are capable of sustained release of encapsulated enzyme thus preventing immature extensive proteolysis. Leakage of encapsulated enzyme increased with the decrease in alginate concentration when subjected to simulated cheese press over 8h indicating that the enzyme microcapsules produced from alginate can be designed
for suitable application in a wide variety of cheeses with varying cheese press duration.

Retention of Flavourzyme activity was marginally better when added to milk at pH 5.0, but similar pH is achieved only after milling, addition of capsules at this stage can be disadvantageous due to the resultant uneven distribution of capsules in cheese. Though the leakage of encapsulated Flavourzyme is higher at milk pH it is recommended to add capsules to milk for even distribution.

Increased moisture, yield, and lower fat content compared to standard Cheddar cheese can be attributed to the variation in cheese manufacture and can be eliminated with the adoption of appropriate manufacturing protocol. There was no difference in fat, moisture, final pH and pathogen levels between control or experimental cheeses, thus addition of encapsulated Flavourzyme does not alter cheese composition and hence suitable for commercial application.

Addition of encapsulated Flavourzyme and Palatase lead to accelerated proteolysis and lipolysis in Cheddar-type cheeses as evidenced by the profiles of water-soluble and water-insoluble peptides, amino acids and fatty acid methyl esters development in these cheeses during ripening. Cheeses with encapsulated Flavourzyme showed reduced bitterness as early as 56 days ripening while bitterness persisted in control cheeses with no added enzymes. Cheese F75 with medium level of encapsulated Flavourzyme scored highest at about 56 and 86 days ripening due to the development of uncharacteristic yet pleasant acceptable savoury umami flavour. Cheese D with free Flavourzyme scored highest initially about 30 days ripening due to immediate and increased proteolysis, but scored lower as the ripening progressed due to development of unpleasant flavour as a result of extensive proteolysis. Flavour of cheeses with encapsulated Flavourzyme deteriorated after about 86 days
ripening possibly due to formation of hydrophobic peptides or amino acids as a result of extensive proteolysis. Texture of all experimental cheeses was crumbly and pasty with excessive serum also due to extensive proteolysis. These flavour and texture defects can be controlled by addition of lower levels of enzymes in the range 0.2LAPU/g milk protein. Hydrophobic or high molecular weight peptides can cause bitterness and accumulation of these peptides can be minimised by addition of much lower levels of enzymes as these are robust enzymes, active even at low ripening temperatures.

The salt content in all cheeses were found to be lower due to increased moisture. Cheeses manufactured with encapsulated Flavourzyme gave salty taste after about 56 days of ripening indicating that the salty taste was due to certain amino acids or peptides. This can be a very useful application in salty cheeses such as Haloumi. Though cheese is consumed in moderation increased salt/NaCl concentration can have undesirable effects on health such as hypertension and osteoporosis (Fox and Cogan. 2004). Use of encapsulated Flavourzyme to develop salty taste can be advantageous for eliminating unwanted excessive salt from cheese which is an essential part of daily diet for many individuals.

Growth in the demand for diversified distinctive food-flavours has lead to increased interest in varied cheese products. Addition of encapsulated Flavourzyme led to development of uncharacteristic Cheddar yet acceptable flavours in addition to accelerated ripening. Encapsulated Flavourzyme can thus be used for development of new flavours in cheeses or cheese products offering a competitive edge commercially. Rancid or lipolytic flavours resulting from addition of encapsulated Palatase were unsuitable in young Cheddar cheese (Fox and Stepaniak, 1993) but mild lipolysis is acceptable in vintage Cheddar cheese (Fox and Stepaniak, 1993;
Collins et al., 2004). Highest levels of lipolysis are observed in mould ripened cheeses (Gripon, 1993; Spinnler and Gripon, 2004) and strong lipolytic flavours are characteristic of many Italian varieties of cheese (Bosset and Gauch, 1993), and hence alginate encapsulated Palatase can be applicable for flavour development in these varieties. It can be concluded that alginate encapsulated Flavourzyme or Palatase can be applied for acceleration of cheese ripening and also for production of low-cost high-intensity cheese flavours for various applications.
7 FUTURE DIRECTIONS
In the current study, alginate-Flavourzyme capsules were allowed to gel in chitosan treated CaCl$_2$ and found to be considerably stable under simulated cheese press pressure, retaining over 70% of the initial enzyme activity. Though prolonged proteolysis was observed in cheeses with encapsulated Flavourzyme, extensive proteolysis leading to formation of unacceptable off-flavours was also a concern. This may be due to immediate breakdown of capsules in cheese matrix. However in the current study it was not possible to study the breakdown of the enzyme microcapsules and understand the release mechanism of encapsulated enzymes in cheese. Though accelerated proteolysis and lipolysis leading to rapid development of flavour in cheese has been reported, mechanism of release of encapsulated enzymes is yet to be understood. In the current study Cheddar-type cheese was manufactured with Fluorescein-isothiocyanate (FITC) labelled Flavourzyme in order to track encapsulated Flavourzyme in cheese, to understand the release mechanism of encapsulated enzymes in cheese matrix during ripening. However, this approach was not successful as the surface of cheese also fluoresced with capsules and it was not possible to differentiate capsules from cheese. Even though FITC-labelled Flavourzyme was treated to resist further binding, fluorescence of the entire surface of cheese suggests that the FITC was bound to cheese proteins also thus causing fluorescence of cheese upon observation at the recommended wavelength ($\lambda_{\text{max}}$). Development of probes/markers to track the encapsulated enzymes in cheese to understand the release mechanisms of encapsulated enzymes is a challenge and is strongly recommended for successful direction of accelerated ripening.

Accelerated ripening due to addition of encapsulated enzymes resulted in development of new peptides absent in ripened control cheese with no added enzymes; these peptides may confer positive or negative effect to the flavour and
Future directions

texture. It is advantageous to isolate and characterise these peptides using amino acid analyser and sequencers to better understand the effects of the encapsulated enzymes on cheese.

Formation of new peptides and amino acids and increased concentration of amino acids can potentially alter cheese composition thus altering the nutrients and in turn leading to change in the microbial composition of cheese. Altered microflora can potentially modify the final flavour and texture of cheese. It is recommended to monitor the SLAB and NSLAB in cheese during ripening.

In the current study, Flavourzyme and Palatase were delivered into Cheddar-type cheese by encapsulating in 1.8% alginate and gelling in 0.1M CaCl$_2$ containing 0.1% chitosan for 10 min. It is known that by altering the polymer concentrations and encapsulation parameters it is possible to obtain various kinds of capsules such as highly porous or very compact and durable capsules. It is recommended to incorporate enzymes encapsulated in various capsules aimed at early, intermediate and delayed release of encapsulated enzymes in cheese for continuous yet moderate proteolysis.

In the current study only 3 polymers; alginate, chitosan and κ-Carrageenan were screened, author recommends screening of more polymers such as pectin, xanthan, agar for enzyme encapsulation for targeted and controlled release of enzymes in cheese.
8 REFERENCES


References

http://www.foodstandards.gov.au/_srcfiles/Micro_limits_edit0702.pdf#search=%22standard%201.6.1%22

ANON (2007b) Australian cheese production by type. Dairy Australia. 


References


