

# **ENZYMES FROM YEAST ADJUNCTS IN PROTEOLYSIS DURING CHEDDAR CHEESE RIPENING**

By

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**To my parents  
and Adolf**

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## LIST OF ABBREVIATIONS

<b>AACE</b>	amino-acid-converting-enzyme
<b>ArAAs</b>	aromatic amino acids
<b>BcAAs</b>	branched chain amino acids
<b>BCA</b>	bicinchoninic acid
<b>BSA</b>	bovine serum albumin
<b>DWW</b>	distilled deionized water
<b>CCP</b>	colloidal calcium phosphate
<b>Cd-NR</b>	cadmium ninhydrin
<b>CMP</b>	casein macropeptide
<b>CFU</b>	colony forming units
<b>CN</b>	casein
<b>FAA</b>	free amino acids
<b>FFA</b>	free fatty acid
<b>GDH</b>	glutamate dehydrogenase
<b>GMP</b>	glycomacropeptide
<b>HPLC</b>	High Performance Liquid Chromatography
<b>HTST</b>	high temperature short time
<b>LAB</b>	lactic acid bacteria
<b>NAD<sup>+</sup></b>	nicotinamide adenine dinucleotide
<b>NADP<sup>+</sup></b>	nicotinamide adenine dinucleotide phosphate
<b>NSLAB</b>	non starter lactic acid bacteria
<b>PA</b>	plasminogen activator
<b>PAB</b>	propionic acid bacteria
<b>PAGE</b>	polyacrylamide gel electrophoresis
<b>PAI</b>	plasminogen activator inhibitor

<b>PG</b>	plasminogen
<b>PL</b>	plasmin
<b>Prt</b>	proteinase
<b>SpecPL</b>	Spectrozyme PL
<b>SLAB</b>	starter lactic acid bacteria
<b>TFA</b>	trifluoroacetic acid
<b>TFFA</b>	total free fatty acid
<b>TN</b>	total nitrogen
<b>tPA</b>	tissue plasminogen activator
<b>UHT</b>	ultra high temperature
<b>uPA</b>	urokinase plasminogen activator
<b>WSF</b>	water-soluble fraction
<b>WISN</b>	water-insoluble nitrogen
<b>WSN</b>	water-soluble nitrogen
<b>YM</b>	yeast extract-malt extract broth

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## Chapter 1

### LITERATURE REVIEW

#### 1.1 Introduction

Early man had no concept of microorganisms, enzymes or chemical changes in substrates, but as soon as the milk was removed from the animal, it soured naturally, and was preserved in its unique way, becoming a staple in the diet. A logical variation of sour milk was the development of cheese fermentation. This was due to the use of calf stomachs for storage of fresh milk. The milk soured in the presence of rennet and the curd losing its whey through porosity in the calf stomach became primitive cheeses. Cheeses are well preserved milk foods contributing protein, calories and vitamins to consumers while also providing a diversity of flavours, aromas and textures to the diet (Steinkraus, 1994).

Cheese is the generic name for a group of fermented milk-based food products, produced in a great range of flavours and forms around the world (Fox, 1993). It is generally believed that cheese-making developed around the Tigris and Euphrates rivers in Iraq around 8000 years ago when the first animals (sheep and/or goats) were domesticated (Cogan and Beresford, 2002). In the Middle East and Indian subcontinent, milk was fermented, which provided nutrients in a safely preserved form. These were principally lactic bacterial fermentations, which used the carbohydrate lactose and converted it into glucose, galactose and lactic acid, in so making the milk longer lasting and more palatable (Campbell-Platt, 1994).

Cheese is an excellent dietary source of high-quality protein, vitamins and minerals such as absorbable dietary calcium. Cheddar cheese possesses a pleasing, walnut flavour and a waxy body, which breaks down smoothly when small portions are kneaded between the fingers and contains a minimum of gas holes. The colour of cheddar cheese is variable,

white or yellow, but always uniform. Salting of cheddar cheese is done by the direct addition of coarse salt to milled curd followed by mixing and packing (Smit *et al.*, 2001).

Originally the primary objective of cheese manufacture was to extend the shelf life of milk and conserve its nutritive value. This was achieved by either acid production and/or dehydration. Most rennet-coagulated cheese undergoes a period of ripening which can range from three weeks to two years (Cogan and Beresford, 2002).

Fermented foods are those foods which have been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification of the food. Food fermentation represents one of the oldest known uses of biotechnology. This traditional biotechnology has evolved from 'natural' processes in which nutrient availability and environmental conditions selected particular microorganisms, to the use of specific starter cultures. Fermented foods and beverages represent a significant proportion of all diets world wide, consisting of about one third of the food intake (Campbell-Platt, 1994). Fermented foods are of great importance because they provide and preserve great quantities of nutritious food in a wide diversity of flavours, aromas and textures which enrich the human diet (Steinkraus, 1994).

A major source of variation in the characteristics of cheese resides from the species from which the milk was produced – the cow being the most important (McSweeney and Fox, 1993). The fat of cheese is dispersed as an emulsion but it is not uniformly distributed as in milk. Variations in the clustering of the fat globule in cheese depend on the handling and treatment of the milk, the skill of the cheese maker and the nature of the facilities (Smit *et al.*, 2001).

Protein is the most important element in the manufacturing of cheese. Cheese is a rich source of protein although the amino-acid composition of cheese and milk is not identical as cysteine is concentrated in the whey proteins and therefore lost in the whey. Cheese contains all the essential amino acids and only small variations are reported between different cheeses (Smit *et al.*, 2001).

Throughout manufacture and ripening, cheese production represents a finely orchestrated series of consecutive and concomitant biochemical events which, if synchronized and balanced, lead to products with highly desirable aromas and flavours, but when unbalanced, result in off-flavours and odours (Fox, 1993). At the end of many cheese-making processes, a bland rubbery mass of curd is obtained. It is during ripening that many cheese types develop their characteristic flavours through the gradual breakdown of carbohydrates, fats, and proteins. Several agents are involved in the ripening of cheese, and the impact of the contribution of each of these agents varies according to the type of cheese (El Soda *et al.*, 2000).

Ripening of hard cheeses is a slow and time consuming process, making ripening an expensive process because of capital immobilization, large refrigerated storage facilities, weight losses and spoilage caused by undesirable fermentations. Owing to the cost of ripening, there are obvious economic advantages to be gained by accelerating the process, provided that the final product has the same flavour profile and rheological attributes as conventional cheese (Wilkinson, 1993; Cogan and Beresford, 2002; Singh *et al.*, 2003).

The main methods used for accelerating cheese ripening may be summarized as (Singh *et al.*, 2003)

- elevated ripening temperature
- modified starters
- cheese slurries
- adjunct nonstarter lactic acid bacteria
- exogenous enzymes

The ripening temperature for Cheddar cheese is 6 - 24°C. Because the starter plays a key role in cheese ripening, it might be expected that increasing cell numbers would accelerate ripening, but high cell numbers have been associated with the development of bitterness in Cheddar cheese (Cogan and Beresford, 2002). Elevated ripening temperatures (e.g. 16°C) are the most effective method of accelerating the ripening of

Cheddar cheeses (Cogan and Beresford, 2002; McSweeney, 2004a). The temperature and relative humidity of ripening rooms must be controlled, adding to the cost of cheese ripening (McSweeney, 2004a).

Problems associated with accelerating cheese ripening:

- Elevated temperature tends to distort the balance of microbial and enzyme processes that produce structure forming peptides and flavour compounds
- Over activity of exogenous enzymatic processes distorts the balance of flavour compound composition

## **1.2 Outline of cheese manufacture**

The definition of cheese is a concentrated form of milk obtained by coagulating the casein. This entraps milk fat, lactose, water and serum proteins (albumin, globulins). Most of the water and water soluble constituents are expelled as whey. The seven steps in cheese manufacture are:

- Addition of lactic acid starter bacteria cultures to milk (to produce lactic acid)
- Coagulation of milk followed by cutting the curd
- Cooking at temperatures from 32 – 54°C, which, together with acid production, assists expulsion of whey from the curd
- Separation of whey and curd
- Molding and pressing the curd at low (soft cheese) or relatively high (hard cheese) pressure
- Salting or brining
- Ripening at temperatures of 6 – 24°C to allow characteristic flavour and texture development (Cogan and Beresford, 2002).

Most cheeses are brined after pressing, but Cheddar is an exception; it is dry-salted and milled before being pressed (Cogan and Beresford, 2002). Cooking temperature has the

potential to inhibit the growth of some organisms and plays an important role in controlling the growth of starters and undesirable microorganisms (McSweeney, 2004a).

Cheese manufacture is a highly complex process. The composition of the initial milk is adjusted by centrifugal separation and possibly also ultrafiltration. The milk will then be pasteurised at 72°C/15s to reduce the risk from pathogenic organisms, adjusted to the desired fermentation temperature, and then pumped into a cheese vat. Starter cultures consisting of a carefully selected species of lactic acid bacteria and a coagulant (rennet) are added to the milk and allowed to coagulate. This is by destabilization of the casein micelle (Singh and Bennett, 2002).

Once the coagulum is of sufficient strength, it is cut into small particles and, by a process of controlled heating and fermentation, syneresis or expulsion of moisture and minerals (whey) occurs. Separation of the curd from the whey with filtration follows. In the case of Cheddar, the curd is allowed to fuse together. Salt may then be incorporated into the curd for preservation, as dry granules. The curd is pressed into blocks by either gravity or mechanical compression, and the cheese goes into controlled storage conditions for final fermentation and maturation (Singh and Bennett, 2002).

Cheese manufacture is essentially a microbial fermentation of milk by selected lactic acid bacteria whose major function is to produce lactic acid from lactose, which, in turn, causes the pH of the curd to decrease. The final pH after manufacture ranges from 4.6 – 5.3, depending on the buffering capacity of the curd. Dehydration also occurs during cheese making from an initial value of 88%, in the case of cow's milk, to  $\leq 50\%$ . An enzyme called rennet (coagulant), a protease, is used to coagulate casein, the major milk protein (Cogan and Beresford, 2002).

In milk, the primary soluble proteins are the whey proteins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin. The insoluble proteins are found in large colloidal particles, called casein micelles (Crabbe, 2004). Casein may be considered as spherical, composed of four types of caseins ( $\alpha$ -s<sub>1</sub>,  $\alpha$ -s<sub>2</sub>,  $\beta$ ,  $\kappa$ ).  $\kappa$  – Casein is a calcium-insensitive protein which forms a



protective layer at the surface of the micelle around the calcium-sensitive caseins ( $\alpha$ -s<sub>1</sub>,  $\alpha$ -s<sub>2</sub>,  $\beta$ ,  $\gamma$ ) (Crabbe, 2004). The stabilizing effect arises because  $\kappa$  – casein is divided into a hydrophobic para-  $\kappa$  – casein and a hydrophilic region called  $\kappa$  -casein glycomacropeptide (GMP). It is also negatively charged and repulses other casein micelles, thus ensuring that coagulation does not occur in milk. When rennet is added, only the  $\kappa$  – casein molecule is hydrolyzed to para-  $\kappa$  – casein, and subsequent destabilization of the micelles occurs, which then coagulate and curd formation is obtained (Dalglish, 1993; Dejmeek and Walstra, 2004).

Rennet-catalysed coagulation of milk occurs in two phases: a primary enzymatic phase and a secondary non-enzymatic phase (Berridge, 1942). The primary phase of rennet action involves the formation of small peptides during renneting.  $\kappa$  –Casein is the only milk protein hydrolysed during the primary phase of rennet action (Waugh and Von Hippel, 1956). Only one peptide bond, Phe<sub>105</sub>-Met<sub>106</sub>, is hydrolysed, resulting in the release of the hydrophilic, negatively-charged C-terminal segment of  $\kappa$  – casein.  $\kappa$  – Casein hydrolysis destabilizes the “residual” (para-casein) micelles, which coagulate in the presence of a critical concentration of Ca<sup>2+</sup> at temperatures > ~ 20°C, i.e. the secondary, non-enzymatic phase of rennet coagulation (Schmidt, 1982; McMahon and Brown, 1984; Sheehan *et al.*, 2006). A critical concentration of Ca<sup>2+</sup> and a minimum temperature (~ 20°C) are required for coagulation. Reduction of the colloidal calcium phosphate (CCP) content of the casein micelles prevents coagulation unless the Ca<sup>2+</sup> concentration is increased (Walstra and Jenness, 1984; Fox, 1993).

$\kappa$ -Casein remains soluble at all these calcium concentrations and prevents the precipitation reaction when present with the other casein types, producing a colloidal suspension instead (Horn and Banks, 2004).  $\kappa$  – Casein must first be removed so that other caseins can precipitate if calcium is added.

A rennet gel is quite stable under quiescent conditions, but if it is cut or broken, syneresis occurs rapidly, expelling whey. The rate and extent of syneresis are influenced by milk composition, especially Ca<sup>2+</sup> and casein concentration, pH of the whey, cooking

temperature, rate of stirring of the curd-whey mixture and time. The composition of the finished cheese is to a very large degree determined by the extent of syneresis (Scott, 1986; Fox, 1993).

### **1.3 Ripening of cheese**

Cheese ripening is a very complex biochemical process by which the rubbery or elastic curd is converted into a smooth-bodied and fully flavoured cheese. Flavour and texture are considered as the two main criteria in determining the acceptability of aged cheese. The time required to develop characteristic flavour and texture varies from a few weeks for soft cheeses up to three years for very hard varieties. During this period, cheeses attain their own characteristics through a multitude of chemical, microbiological and biochemical changes whereby protein, fat and residual lactose are broken down to primary products which are further degraded to secondary products (Kheadr *et al.*, 2003).

Cheese ripening is a complex process involving a range of microbiological and biochemical reactions. Microorganisms present in cheese throughout ripening, play a significant role in the ripening process (Cogan and Beresford, 2002).

Cheese manufacture and ripening involves the action of enzymes (from rennet and milk) and selected microorganisms, both directly, while growing, and indirectly, through their enzymes after death and lysis (McSweeney, 2004a). Microbiological changes to cheese during ripening include the death and lysis of starter cells, and the growth of adventitious flora like nonstarter lactic acid bacteria. Cheese texture softens during ripening as a consequence of hydrolysis of the casein micelle during proteolysis, changes to the water-binding ability of the curd and changes in pH (which in turn may cause other changes such as the migration and precipitation of calcium phosphate) (McSweeney, 2004b).

The biochemical changes occurring during ripening may be grouped into primary events that include the metabolism of residual lactose and of lactate and citrate, lipolysis and proteolysis. Following these primary events, secondary biochemical events are very

important for the development of many volatile flavour compounds and include the metabolism of fatty acids and of amino acids (McSweeney, 2004b). A fine equilibrium between primary and secondary products has been shown to be responsible for typical cheese flavour and texture (Kheadr *et al.*, 2003).

Rennet-coagulated cheeses are ripened for a period ranging from two weeks to two or more years, e.g. extra-mature Cheddar cheese, during which the flavour and texture characteristics of the variety develop. Ripening usually involves changes of the microorganisms of the cheese, including death and lysis of starter cells, development of adventitious non-starter microorganisms and, in many cheeses, growth of secondary microorganisms (McSweeney, 2004b).

Cheddar cheese ripening is mainly affected by the rate and extent of proteolysis (Fox, 1989). Enzymes from several sources contribute to proteolysis and the development of cheese texture and flavour during ripening. These enzymes originate from the milk (principally plasmin), the coagulant (rennet), starter, secondary starter and non-starter microorganisms (Fox *et al.*, 1994; Lane *et al.*, 1997).

The unique characteristics of the individual cheeses develop during ripening, and hence the flavour, aroma and texture of the mature cheese, are largely predetermined by the manufacturing process e.g. moisture, NaCl, type of starter and secondary inocula added. During ripening an extremely complex set of biochemical changes occurs through the catalytic action of the following agencies:

- coagulant
- indigenous milk enzymes, especially proteinase and lipase, which are particularly important in cheeses made from raw milk
- starter bacteria and their enzymes which are released after the cells have died and lysed
- secondary microorganisms and their enzymes
- non-starter bacteria

- exogenous enzymes added to accelerate cheese ripening

The secondary microorganisms may arise from the indigenous microorganisms of milk that survive pasteurisation or gain entry to the milk after pasteurisation, e.g. *Lactobacillus*, *Pediococcus*, *Micrococcus*, or they may arise through the use of a secondary starter (Beresford and Williams, 2004). A great deal of research in Cheddar cheese technology is devoted towards the addition of adjunct cultures which may accelerate ripening times or to improve flavour (Wilkinson, 1993).

During the ripening of cheese, three major/primary biochemical events – glycolysis, lipolysis and proteolysis- occur, each of which is involved in flavour formation. The latter is the most important and also the most complex (Cogan and Beresford, 2002).

Glycolysis is the conversion of lactose to lactic acid and is due to the growth of starter bacteria and the lactate produced gives the freshly made cheese its overall acidic taste. They can also produce diacetyl, acetate and acetaldehyde, which are important compounds in flavour formation in fresh cheeses; diacetyl is also an important flavour compound in hard cheeses (Cogan and Beresford, 2002).

Lipolysis results in hydrolysis of the milk fat and production of glycerol and free fatty acids, many of which, particularly the short-chain ones, have strong characteristic flavour. The fatty acids can be further metabolized to methyl ketones and fat also acts as a solvent for many of the flavour compounds produced in the cheese (Cogan and Beresford, 2002).

The sources of proteinase in cheese are milk itself, chymosin (rennet), starter lactic acid bacteria (SLAB), nonstarter lactic acid bacteria (NSLAB), and the secondary microorganisms (micrococci, yeasts and moulds). Milk proteinase is plasmin and is significant in cheese when the chymosin is inactivated during cooking of the cheese (Cogan and Beresford, 2002).

Small peptides produced from hydrolysis of casein, can be broken down into smaller amino acids, peptides, amines, alcohols and sulphur containing compounds, which contribute to flavour formation, by autolysis of the SLAB which releases proteinases and peptidases (Cogan and Beresford, 2002). In the right combination these compounds are responsible for the flavour of various cheeses (de Wit *et al.*, 2005). It is usually the large peptides that provide texture to the cheese while the smaller peptides are used as precursors for flavour formation. For a general overview of the biochemical pathways during cheese ripening refer to fig. 1.1.

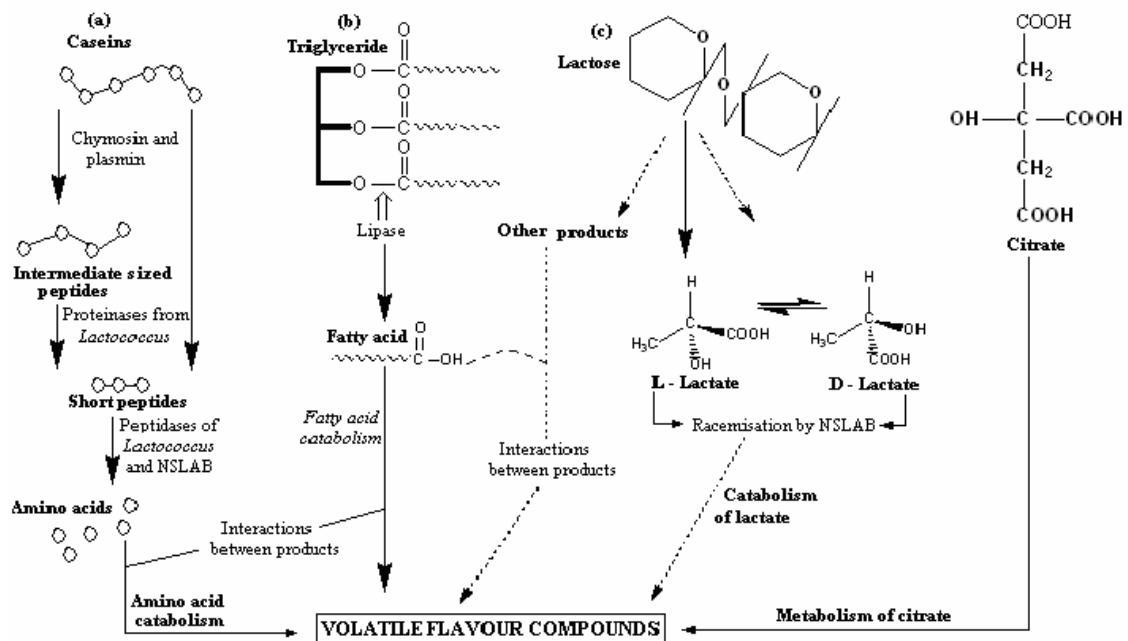


Fig.1.1 General overview of the biochemical pathways in cheese ripening

(McSweeney, 2004a)

#### 1.4 Microorganisms involved in cheese ripening

The microorganisms involved in cheese making and cheese ripening can be divided into two major groups:

- 1) Microorganisms (SLAB) that are added to the cheese milk after being carefully selected
- 2) nonstarter lactic acid bacteria (NSLAB).

Group one can be further subdivided into two groups: the primary and secondary starter (El Soda *et al.*, 2000). Starter lactic acid bacteria are involved in acid production during manufacture and contribute to the ripening process. The secondary microorganisms do not contribute to acid production and consist of (a) nonstarter lactic acid bacteria (NSLAB) and (b) other bacteria, yeasts and/or moulds. The microorganisms and their function in cheese production are summarized in table 1.1.

#### *1.4.1 Types of starter bacteria*

Two types of starter bacteria are used in cheese-making: thermophilic with optimum temperatures of 42°C and mesophilic with optimum temperatures of 30°C (Cogan and Beresford, 2002).

The selection criteria for O-type (a specific starter type) mesophilic starter cultures in Cheddar cheeses are as follows (Cogan *et al.*, 1991):

- Phage resistance at 25, 30 and 37°C
- Acid production at 21 and 30-40°C
- Salt tolerance
- Proteinase and peptidase activity

Starter bacteria are lactic acid bacteria (LAB) and their function is to produce acid during the fermentation process, acidify the cheese milk at an appropriate rate and also contribute to proteolysis during ripening (Fox, 1989; Lane and Fox, 1996). They also provide a suitable environment with respect to redox potential, pH, moisture, and salt contents to allow enzyme activity from the chymosin (rennet) and starter to proceed favorably in the cheese (Cogan and Beresford, 2002).

**Table 1.1** Microorganisms in cheese and their function

<b>Genus or species</b>	<b>Major function</b>
<b>Starter bacteria</b>	
<i>Lactococcus lactis</i>	Acid production; flavour formation during ripening
<i>Leuconostoc</i> spp.	Metabolism of citrate
<i>Lactobacillus delbrueckii</i>	Acid production; flavour formation during ripening
<i>Streptococcus thermophilus</i>	Acid production; flavour formation during ripening
<i>Enterococcus</i> spp.	Flavour formation during ripening
<b>Nonstarter lactic acid bacteria</b>	
<i>Lactobacillus casei</i>	Flavour formation during ripening
<i>Pediococcus</i> spp.	Flavour formation during ripening
<b>Secondary flora</b>	
<i>Corynebacterium</i> spp.	Production of menthanthiol
<i>Brevibacterium linens</i>	Production of menthanthiol
<i>Penicillium camemberti</i>	Proteolysis
<i>Penicillium roqueforti</i>	Proteolysis
Yeasts	Deacidification (conversion of lactate to CO <sub>2</sub> and H <sub>2</sub> O)

(Cogan and Beresford, 2002).

Apart from milk plasmin and rennet, lactic acid bacteria (LAB) are the major source of proteolytic enzymes (proteinases and peptidases) in cheeses (Kunji *et al.*, 1996; Lane and Fox, 1997). They transform caseins into small peptides and free amino acids, which contribute to flavour and serve as aroma precursors (Engels and Visser, 1996; Fox and Wallace, 1997).

The starter bacteria grow rapidly in cheese milk and curd during manufacture, reaching  $10^8$ -  $10^9$  cfu g<sup>-1</sup>, but subsequently decrease to approximately 1% of maximum numbers within 1 month of ripening due to the low curd pH, depletion of lactose and the high salt concentration in the curd. The death and lysis of the starter cells are important as intracellular proteolytic enzymes are released into the cheese matrix where they degrade

oligopeptides (casein derived peptides produced by the coagulant and milk proteinase) to smaller peptides and amino acids (Lane and Fox, 1996). Their enzymes are involved in the conversion of proteins into amino acids and fatty acids from which flavour compounds are produced (Cogan and Beresford, 2002).

The starter bacteria are members of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* (Cogan and Beresford, 2002). Also, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactobacillus helveticus* are regarded as starter bacteria (Beresford and Williams, 2004). See also table 1.1.

The starters are frozen in volumes (200-1000 liter) or freeze-dried which can then be added directly to the cheese milk. Inoculating the milk directly minimizes the risk of contamination and the aroma formers contributing to flavour development are known to be present (Cogan *et al.*, 1991).

#### *1.4.2 Growth of starter bacteria during manufacture*

Most of the starter cells are concentrated in the curd. Consequently, there is a higher concentration of lactose within the curd than in the surrounding whey. During the first few hours, acid production will depend on the rate of inoculation, the time of renneting and the rate of cooking the curd (Cogan and Beresford, 2002).

Rates of acid production in Cheddar cheese is relatively rapid and the pH decreases from 6.6, the initial pH of milk, to pH 6.1, the pH at which whey is drained about 3h after the addition of the starter. The more rapid acid production results in a greater rate of whey expulsion, and consequently of lactose, from the curd. During this time, lactate levels in the curd are increasing (Cogan and Beresford, 2002).

Cooking temperatures are also important in controlling the growth of the starters. Mesophilic cultures have optimum temperatures of 30°C and are used in the manufacture of Cheddar cheese. But Cheddar cheese is cooked to 40°C which will reduce the acid



production by the mesophilic starter. This cooking temperature will have no effect on thermophilic organisms, which may be used as adjunct cultures (Cogan and Beresford, 2002).

#### *1.4.3 Growth of starter bacteria during ripening*

Within 24h from addition of the starter, the number of starter cells will have increased to  $10^9$  CFU g<sup>-1</sup> (colony forming units per 1 gram) and most of the lactose will have been transformed to lactate (Cogan and Beresford, 2002).

#### *1.4.4 Autolysis of starter bacteria*

Autolysis of starter bacteria plays an important role in proteolysis because intracellular enzymes, particularly peptidases, are released that will further hydrolyse any peptides which are present. Should the starter reach too high a population or survive too long, flavour defects (bitterness) are produced. Bitter peptides are mainly produced from the hydrophobic regions of the different caseins by chymosin and starter proteinases. It is believed that intracellular peptidases released through autolysis of the starter cells will hydrolyse the bitter peptides to smaller nonbitter peptides and amino acids. Amino acids are the precursors of the compounds required for flavour production. Some amino acids (glycine, alanine) are sweet whereas others (glutamate) may potentiate flavour (Cogan and Beresford, 2002).

Lysis of lactococci in Cheddar cheese has been demonstrated for a large number of lactococcal strains and muraminidase is the major autolytic enzyme in lactococci. The extent of autolysis varied between strains and had a direct impact on the degree of proteolysis in cheese (Beresford and Williams, 2004).

#### 1.4.5 Secondary and adjunct cultures

Adjunct (secondary) cultures may be defined as those added to cheese for purposes other than acid production and essentially serve as an additional source of enzymes (Fox *et al.*, 1998).

Successful NSLAB adjuncts require two important features. The first is that the strains must provide a balance of beneficial ripening reactions in cheese. Adjuncts are selected on the basis of the absence of specific defects. The second consideration is that adjunct strains need to be competitive against the adventitious NSLAB in the Cheddar cheese and remain the dominant NSLAB during ripening to affect their flavour benefits. In many cases, this feature is obtained by selecting NSLAB isolates from good quality Cheddar (Crow *et al.*, 2001).

Studies have shown that lactobacilli, used as adjuncts, can affect flavour development in Cheddar cheese. Most researchers report enhanced levels of proteolysis and enhanced flavour intensity. Thus the selection of the adjunct strain is crucial, because certain strains of *Lactococcus casei* produced high-quality Cheddar, while other strains of these species resulted in cheese with acid and bitter flavour defects (Cogan and Beresford, 2002). Typically, the inclusion of adjunct strains of nonstarter lactobacilli results in improved flavour intensity, increased aroma and accelerated ripening (Beresford and Williams, 2004).

The contribution of NSLAB to the quality of pasteurised milk Cheddar is unclear but the flavour of such cheese can be intensified by adding adjuncts of mesophilic and thermophilic lactobacilli. The effectiveness of the latter may be due to the fact that they die off rapidly and lyse, releasing intracellular enzymes. The use of adjunct lactobacilli, and perhaps other genera, makes it possible to intensify and alter flavour of Cheddar cheese (Fox *et al.*, 1998).

The secondary cultures involved include nonstarter lactic acid bacteria (NSLAB), which grow internally in most cheese varieties, bacteria (*Staphylococcus*, *Micrococcus*, *Corynebacterium*), propionic acid bacteria (PAB), yeasts (*Debaryomyces hansenii*), moulds (*Penicillium camemberti*) and heterofermentative lactobacilli (table 1.1). Most of the secondary cultures grow mainly on the cheese surface. They are either (a) adventitious contaminants or (b) deliberately added (yeasts) (Cogan and Beresford, 2002).

The interior of cheese is a rather hostile environment for the growth of microorganisms: it has a low pH (~5), a relatively high salt content (1-4%) lacks a fermentable carbohydrate, is anaerobic and may contain bacteriocins produced by the starter bacteria (Fox *et al.*, 1998).

Microorganisms other than the starter bacteria are capable of growing in Cheddar cheese (Lane and Fox, 1996). In the cases of cheeses made from raw milk, the main source is likely to have been the cheese milk. This is also the most likely source in cheeses manufactured from pasteurised milk, because some of them withstand pasteurisation to some extent; post pasteurisation contamination may also occur (Cogan and Beresford, 2002).

NSLAB present in cheese are adventitious microorganisms, which originate either from the factory environment or from the milk or is usually present because of post-pasteurisation contamination (Lane and Fox, 1996; Kieronczyk *et al.*, 2003). Lactobacilli are found in cheese in low numbers (<50 CFU g<sup>-1</sup>) at the day of manufacture and become the dominant microorganisms (10<sup>7</sup> to 10<sup>8</sup> CFU g of cheese<sup>-1</sup>) in the mature cheese (Kieronczyk *et al.*, 2003). Possible substrate(s) for NSLAB include lactose, citrate, fatty acids, glycerol, lactate, amino acids, and sugars from glycoproteins (Fox *et al.*, 1998). It is likely that interactions occur between strains of NSLAB and other strains on the cheese. NSLAB are responsible for transforming L-lactate to D-lactate during ripening, but this has no effect on flavour development, but can affect the appearance of the cheese (Cogan and Beresford, 2002). They do not contribute to acid production during cheese

manufacture, but impact on flavour development in the ripening of cheese. NSLAB are believed to add desirable flavour notes and reduce harshness and bitterness associated with some starter cultures in Cheddar cheese.

The presence of NSLAB in commercial cheese is associated with the development of more intense Cheddar flavour in a shorter time (Beresford and Williams, 2004). The dominant non-starter bacteria in Cheddar cheese are facultatively heterofermentative species of lactobacilli (Shakeel-Ur-Rehman *et al.*, 2000). Heterofermentation is the fermentation of a sugar to a mixture of reduced products (Madigan *et al.*, 2003).

### **1.5 Yeast in the dairy industry**

Dairy products offer a special ecological niche that is selective for the occurrence and activity of specific yeasts. Yeasts, however, play an essential role in the preparation of certain fermented dairy products, ripening of certain cheeses and contribute to the final product (Viljoen, 2001). Yeasts can also cause spoilage and produce excessive gas formation, off-flavours, slime formation or discoloration (Fleet, 1990).

Dairy products develop their nutritional and organoleptic qualities as a result of the metabolic activity of a succession of different microorganisms since more than one type of interaction may occur simultaneously (Verachtert and Dawoud, 1990). The yeasts, as part of the interactions, either contribute to the fermentation by supporting the starter cultures (Jakobsen and Narvhus, 1996), inhibiting undesired microorganisms causing quality defects, or adding to the final product by means of desirable biochemical changes such as the production of aromatic compounds, proteolytic and lipolytic activities (Viljoen, 2001).

It was shown that yeasts in cheese are considered as insignificant at the earlier stages of cheese production, but play a significant role in the later stages, being present as natural contaminants in the curd during maturation (Welthagen and Viljoen, 1998; 1999). Yeast

species also play a synergistic role with the utilization of lactic acid which leads to an increase in pH thus favoring the growth of bacteria and contributing to the ripening and de-acidification of the cheese (Viljoen and Greyling, 1995; Viljoen, 2001; Ferreira and Viljoen, 2003).

High numbers of yeasts are frequently observed in cheeses and are believed to make a significant contribution to the maturation process. Yeasts possess the ability to grow under conditions unfavourable to many bacteria in cheese and therefore play a significant role in the spoilage of dairy products as well as the ripening of some cheese varieties (Wyder and Puhan, 1999; Viljoen, 2001; Wyder, 2001). However they are also responsible for desirable biochemical changes in dairy products (Viljoen and Greyling, 1995).

Their occurrence may be attributed to the yeast's ability to grow at low temperatures, the assimilation/fermentation of lactose, the assimilation of organic acids produced by the lactic acid bacteria such as succinic, lactic and citric acid, their proteolytic and lipolytic activities, resistance against high salt concentrations and resistance to cleaning compounds and sanitizers. They can inhibit undesired microorganisms and liberate growth factors (through autolysis or excretion) like B-vitamins, pantothenic acid, niacin, riboflavin and biotin. Yeasts have the ability to tolerate low pH and water activity values (Wyder and Puhan, 1999; Viljoen, 2001; Ferreira and Viljoen, 2003; Laciotti *et al.*, 2005). They are widely dispersed in the dairy environments and appear as natural contaminants in raw milk, air, dairy utensils, brine and smear water (Wyder and Puhan, 1999).

Studies of the addition of certain yeast species as part of the starter culture in the cheese manufacturing processes have been done. Certain yeast species are known for their proteolytic and lipolytic activity as well as their compatibility and stimulating action with the lactic acid starter cultures when co-inoculated. Addition of these yeast species enhanced flavour development and reduced ripening times during cheese maturation (Ferreira and Viljoen, 2003).

Some yeast species associated with Cheddar are: *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Torulasporea delbruekii*, *Kluyveromyces marxianus*, *Trichosporon beigellii* (Viljoen and Greyling, 1995; Wyder and Puhan, 1999). It is still not widely appreciated that yeasts can be an important component of many, if not all, cheese varieties (Ferreira and Viljoen, 2003).

*Debaryomyces hansenii* and *Yarrowia lipolytica* are typical yeast species frequently associated with dairy products. *Debaryomyces hansenii* and *Yarrowia lipolytica* have been regarded as good candidates for ripening agents in cheese fulfilling specific criteria to be regarded as co-starters for cheesemaking (Ferreira and Viljoen, 2003). The two species fulfill a number of criteria to be regarded as co-starters for cheesemaking. They are known for their proteolytic and lipolytic activity as well as their compatibility and stimulating action with the lactic acid starter cultures when co-inoculated. Recent studies indicated that yeasts could be included as part of starter cultures for the manufacturing of cheese, enhancing flavour development during the maturation (Ferreira and Viljoen, 2003; de Wit *et al.*, 2005).

The inclusion of *Debaryomyces hansenii* as part of the starter culture was shown to have a dual role by inhibiting undesired microorganisms and also its proteolytic activity encouraging the survival and growth of LAB (de Wit *et al.*, 2005). When both species were incorporated as part of the starter culture, the cheese had a good strong flavour after a ripening period of four months. The cheese had a clean, slightly sweet, pleasant taste and still retained its good, strong flavour after nine months (Ferreira and Viljoen, 2003). Ferreira and Viljoen, (2003) concluded that *Debaryomyces hansenii* and *Yarrowia lipolytica* grew and competed with other naturally occurring yeasts in the cheese and with the starter bacteria without any inhibition of the starter culture. The species also contributed to the accelerated development of a strong Cheddar flavour, although bitter and fruity flavours were detected when the yeasts were inoculated individually. When both species were incorporated as part of the starter culture, the cheese had a good strong flavour after a ripening period of four months, and after nine months retained its good, strong flavour. The control cheese developed a bitter and impure taste after nine months.

Thus it is proposed that *Debaryomyces hansenii* and *Yarrowia lipolytica* are both incorporated as part of the starter culture for the production of mature Cheddar cheese (de Wit *et al.*, 2005).

## 1.6 Proteolysis in cheese

Proteolysis contributes to the development of cheese texture through hydrolysis of the protein matrix of cheese, a decrease in  $a_w$  through changes to water binding by the new carboxylic and amino groups liberated on hydrolysis of peptide bonds and these groups are ionized at the pH of the cheese and thus bind water. Flavour and off-flavour of cheese also develops through proteolysis by directly producing short peptides and amino acids and indirectly liberating amino acids which act as substrates for a range of catabolic reactions which generate important volatile flavour compounds (Upadhyay *et al.*, 2004).

### 1.6.1 Proteins in milk

About 80% of the milk proteins consist of caseins. Almost all casein is present as casein micelles (de Wit *et al.*, 2005). See also section 1.2.  $\alpha_{S1}$ -CN is hydrolyzed by chymosin at Phe<sub>23</sub> - Phe<sub>24</sub> which results in the production of a small peptide ( $\alpha_{S1}$ -CN f1-23) that is rapidly hydrolyzed by starter proteinases (McSweeney, 2004a).  $\gamma$ -Caseins have been known to correspond to C-terminal portions of  $\beta$ -casein sequence. These are formed by cleavage of  $\beta$ -casein at positions 28/29, 105/106 and 107/108 by the enzyme plasmin. The fragments 29-209, 106-209 and 108-209 constitute the  $\gamma$ -caseins (Walstra and Jenness, 1984).

### 1.6.2 Proteolytic agents in cheese

Proteolysis in cheese during ripening is catalysed by enzymes from:

- a) Coagulant (chymosin, pepsin or fungal acid proteinase)
- b) Milk indigenous proteinases (plasmin)
- c) Starter bacteria

- d) Nonstarter, adventitious microorganisms
- e) Secondary inoculum (in some varieties), e.g. *Penicilium (P.) roqueforti*, *P. camemberti*, *Brevibacterium (Br.) lines*, *Lactobacillus* spp. (a recent development in Cheddar)
- f) Exogenous proteinases and/ or peptidases or attenuated bacterial cells have been investigated recently as a means of accelerating ripening or accentuating flavour (Fox and McSweeney, 1996; Upadhyay *et al.*, 2004).

The progress of proteolysis in most ripened cheeses can be summarized as follows: initial hydrolysis of caseins is catalyzed primarily by residual coagulant, and to a lesser extent, by plasmin and perhaps cathepsin D and other somatic cell proteinases. This result in the formation of large and intermediate-sized peptides which are then subsequently degraded by the coagulant and enzymes from the starter and nonstarter lactic acid bacteria. Small peptides and free amino acids are produced from the action of bacterial proteinases and peptidases (Fox and McSweeney, 1996).

### *1.6.3 Proteolysis during ripening*

Proteolysis is the most important event and the most complex (Cogan and Beresford, 2002; de Wit *et al.*, 2005). Proteolysis is very important for cheese texture by hydrolysing the *para*-casein matrix which gives cheese its structure and by increasing the water-binding capacity of the curd (i.e. to the new  $\alpha$  – carboxylic and  $\alpha$  – amino groups produced on cleavage of peptide bonds). However, the major role of proteolysis in cheese flavour is in the production of amino acids which act as precursors for a range of catabolic reactions which produce many important volatile flavour compounds (McSweeney, 2004a). A high correlation exists between the intensity of Cheddar cheese flavour and the concentration of free amino acids (Fox, 1989). Due to features such as high proteolytic and lipolytic activities, some yeast species play an important role in the formation of aroma precursors such as amino acids, fatty acids and esters (Ferreira and Viljoen, 2003).



The principal amino acids in Cheddar cheese are Glu, Leu, Arg, Lys, Phe, and Ser. Medium and small peptides contribute to a brothy background flavour in many cheese varieties; short, hydrophobic peptides are bitter. Amino acids contribute directly to cheese flavour as some amino acids taste sweet (e.g. Gly, Ser, Thr, Ala, Pro), sour (e.g. His, Glu, Asp), or bitter (e.g. Arg, Met, Val, Leu, Phe) (McSweeney, 2004a).

## **1.7 Action of the principal proteinases during cheese ripening**

The rate, extent and nature of proteolysis during cheese ripening as well as the amount and nature of the degradation products, vary according to the enzyme involved, the type of cheese and the environmental conditions of ripening. Primary proteolysis in cheese may be defined as the degradation of various individual proteins that constitute the cheese matrix, that is, the changes in  $\beta$ -,  $\gamma$ -,  $\alpha$ -caseins, peptides and other minor bands that can be detected by polyacrylamide gel electrophoresis (PAGE) (Grappin *et al.*, 1985).

### *1.7.1 Chymosin/Rennet*

Chymosin has been used as the milk-clotting enzyme for the industrial production of cheese (Crabbe, 2004). Chymosin, the principle enzyme used to coagulate milk, initially hydrolyses the Phe<sub>105</sub>-Met<sub>106</sub> bond of  $\kappa$ -CN, which results in the release of the negatively-charged C-terminal (CMP) fragment, thereby initiating coagulation of milk (Sheehan *et al.*, 2006).

Rennet specifically hydrolyses the Phe<sub>105</sub>-Met<sub>106</sub> bond of the  $\kappa$ -casein. The  $\alpha_{S1}$ -,  $\alpha_{S2}$ -, and  $\beta$ -caseins are not hydrolysed during milk coagulation but may be hydrolysed in cheese during ripening (section 1.6.1). Most of the rennet added to the cheesemilk is removed in the whey, but more or less 6% is retained in the curd and plays a major role in the initial proteolysis of caseins in many cheese varieties (Fox *et al.*, 2004).

The coagulant contributes indirectly to the development of Cheddar cheese flavour by producing large- and medium sized peptides which act as substrates for starter (and possibly non starter) proteinases and peptidases which produce small peptides and free amino acids from these substrates. The liberated amino acids may contribute directly to flavour or serve as precursors of flavour compounds. The coagulant may also produce bitter peptides (Lane *et al.*, 1997).

Chymosin is most stable at pH values between 5.3 and 6.3. Under acidic conditions (pH 3-4), the enzyme loses its activity rapidly, probably caused by auto-degradation. The solubility of chymosin is affected by pH, temperature and ionic strength of the solution (Crabbe, 2004).

#### *1.7.2 Indigenous milk proteinases (Plasmin)*

Plasmin (PL) contributes to proteolysis during ripening of some cheese varieties depending on cooking temperature and pH during ripening. The enzyme is heat resistant and survives UHT treatments (Bastian and Brown, 1996; McSweeney, 2004b). Plasmin could be a valuable enzyme for accelerated ripening and improved flavour development in natural cheeses. Excessive hydrolysis of caseins results in reduced rennet curd-forming properties of milk, smaller micelles and increased surface activity of casein at air-water interfaces (Bastian and Brown, 1996). The presence of plasminogen and plasmin in milk greatly influence milk quality and cheese ripening (Benfeldt *et al.*, 1995).

Plasmin may significantly affect cheese making properties of milk, especially in terms of cheese ripening. It has also been reported that increased proteolysis due to increased plasmin activity in cheese results in improved flavour and overall quality (Somers and Kelly, 2002).

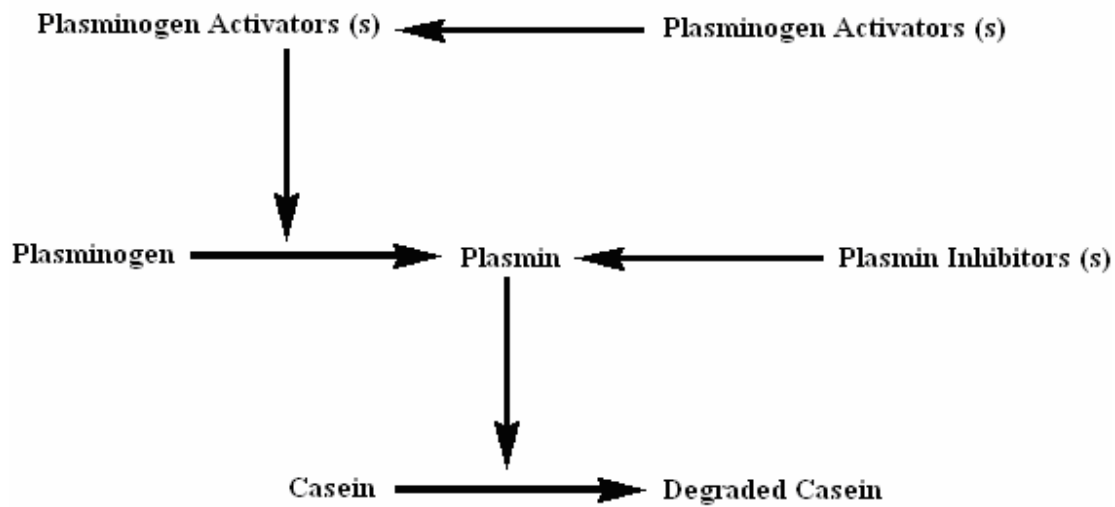
Plasmin exists in milk in inactive and active forms, both of which are associated with casein micelles. Plasminogen activators are also associated with the casein micelles in milk and activate the zymogen (Bastian and Brown, 1996). In cheeses cooked at high

temperatures ( $>50^{\circ}\text{C}$ ), plasmin plays an important role as chymosin is inactivated in these cheeses (Cogan and Beresford, 2002). Plasmin is produced from its inactive zymogen, plasminogen, and its conversion to its active form is mediated by a complex system of urokinase-type (uPA) and tissue- type (tPA) plasminogen activators (PA) (Dalglish, 1993; McSweeney, 2004a).

Plasmin is associated with the casein micelles in milk (Crudden and Kelly, 2003). It hydrolyses  $\alpha_{\text{S1}}$ -casein and, to a lesser extent  $\alpha_{\text{S2}}$ -casein and  $\beta$ -casein (Politis *et al.*, 1993; McSweeney, 2004b). Several studies have shown that  $\kappa$ -casein is resistant to hydrolysis by plasmin (Richardson and Pearce, 1981; Bastian and Brown, 1996; Egito *et al.*, 2003).

The enzyme has an affinity for lysine and arginine residues and preferentially cleaves Lys-X and to a lesser extent Arg-X bonds. Plasmin purified from milk has optimum activity at pH 7.5 and  $37^{\circ}\text{C}$  (Bastian and Brown, 1996; McSweeney, 2004b). There are three plasmin-sensitive bonds in  $\beta$ -casein; Lys<sup>28</sup> – Lys<sup>29</sup>, Lys<sup>105</sup> – His<sup>106</sup>, Lys<sup>107</sup> – Glu<sup>108</sup>. When plasmin hydrolyses these bonds, the following peptides are released:  $\gamma_1$ -caseins ( $\beta$ -CN f29-209),  $\gamma_2$ -caseins (f 106-209),  $\gamma_3$ - (f 108-209), proteose peptone 8-fast (f 1-28), proteose peptone 8-slow (f 29-105 and 29-107) (Bastian and Brown, 1996). Plasmin also rapidly cleaves  $\alpha_{\text{S2}}$ -casein and, more slowly,  $\alpha_{\text{S1}}$ -casein (Barrett *et al.*, 1999).

Addition of urokinase to cheese milk effectively increases plasmin activity by conversion of the inactive precursor plasminogen, to plasmin. Plasminogen activation occurs during curd manufacture and in the first 24 hours of cheese ripening leading to accelerated proteolysis in Cheddar cheese (Barrett *et al.*, 1999). Urokinase is a serine proteinase composed of two disulphide-linked subunits, which cleaves plasminogen at two sites to produce active plasmin (Barrett *et al.*, 1999). Since plasmin associates with the casein micelles, most exogenous plasmin added to milk is retained in the curd (McSweeney, 2004a). For a schematic representation of the plasmin enzyme system in milk, refer to fig. 1.2.



**Fig.1.2** Schematic representation of the plasmin enzyme system in milk  
(Bastian and Brown, 1996).

### 1.8 Aroma, flavour and texture of Cheddar cheese

The sensory perception of a food is a very important product characteristic. Sensory perception is a complex process, which is influenced by factors such as content of flavour compounds, the texture, and appearance of the product, but also several characteristics of the individual consuming the product in a certain environment. Flavour (perception) is defined as the sensation arising from the integration or interplay of signals produced as a consequence of sensing chemical substances by smell, taste and irritation stimuli from food or beverage (Laing and Jinks, 1996). In the mouth only basic differences like sweet, umami (a Japanese word meaning delicious) acid, bitter, salt are sensed by taste-receptor cells, while in the nose different neurons are able to respond to different volatile compounds (Laing and Jinks, 1996; Ninomiya, 2002). The objective of cheese manufacture is to produce a product with the flavour, aroma and texture of the intended variety, free of defects and in the shortest time possible (Fox, 1993). Flavour is the complex sensation that includes taste, aroma and texture (Walstra and Jenness, 1984).

The flavour of cheese originates from microbial, enzymatic and chemical transformations. The breakdown of milk proteins, fat, lactose and citrate during ripening

gives rise to a series of volatile and non-volatile compounds which may contribute to cheese flavour. Proteolysis, by enzymes from milk, rennet and microorganisms, is a major biochemical event. Other factors, such as lipolysis and lactose fermentation, also play an important role (Engels *et al.*, 1997).

At this stage it is sufficient to note that free amino acids such as cysteine, methionine, isoleucine, valine and phenylalanine are intermediates in flavour and aroma production in cheese, and other amino acids such as proline and glutamic acid influence cheese flavour profiles directly (Law, 2001).

Development of strategies for flavour control and manipulation in hard ripened cheeses have become increasingly important in recent years as producers strive to satisfy consumer demands for consistent quality cheeses which have distinctive flavour profiles. Reducing extensive maturation periods provide further challenges to the technologist (Banks *et al.*, 2001).

#### *1.8.1 Texture of Cheddar cheese*

The texture of cheese is determined initially by the composition of the cheese milk, especially by the fat:casein ratio, by the manufacturing operations which control the extent of syneresis and hence the moisture content of the cheese, and the rate of acidification which controls the extent of demineralization of the curd and which in turn has a major influence on the textural parameters of the cheese (Fox, 1993). The texture of the cheese changes during ripening due to proteolysis, the decrease in  $a_w$  due to the liberation of water-binding ionic groups, redistribution of salt, and, in many cases, evaporation of water and to changes in pH due to proteolysis and catabolism of lactic acid (Lawrence *et al.*, 1987).

Undoubtedly, cheese aroma and flavour are influenced by cheese texture, e.g. by consumer perception and release of sapid and aromatic compounds from the cheese mass during mastication (McGugan *et al.*, 1979).

### 1.8.2 Flavour compounds of Cheddar cheese

Lipolysis results in the formation of FFA (free fatty acids), which can be precursors of flavour compounds such as methylketones, alcohols and lactones. FFA are constituents of Cheddar cheese flavour. Milk fat seems to be essential to the development of Cheddar flavour. The background flavour is caused mainly by peptides, amino acids and other products of protein breakdown, whereas hydrogen sulphide, methanethiol (essential to Cheddar cheese aroma), dimethyl sulfide and diacetyl are found in the volatile fraction. Volatile acids appear not to contribute to the aroma, but they may affect the taste of the cheese. A good Cheddar cheese flavour seems to be related to the balance between the FFA and the sulfur compounds (Walstra and Jenness, 1984).

Fatty acids seem to contribute a great deal to the flavour of Cheddar cheese. Low concentrations of fatty acids in cheese indicate a young cheese, one that has not undergone much ripening. At very high concentrations, fatty acids are perceived as off flavours. Yet, when existing at just the right levels, fatty acids contribute to the well balanced, full flavour that is associated with Cheddar cheese (House and Acree, 2002).

In internally bacterially ripened cheese such as Cheddar, there appears to be agreement that the water-insoluble nitrogen (WISN) fraction (consisting mainly of proteins and large peptides) is devoid of flavour and aroma, that the water-soluble nitrogen (WSN), non-volatile fraction (small peptides, amino acids, organic acids) contains most of the compounds responsible for flavour while the aroma is principally in the volatile fraction. There appears to be strong support for the view that products of proteolysis are the principal contributors to cheese flavour (McGugan *et al.*, 1979; Fox, 1993).

Non-volatile WSN fractions contribute to the essential background flavour, while volatile components contribute to more characteristic “cheesy” qualities. As already discussed above, various proteinases and peptidases in cheese hydrolyze caseins to peptides and free amino acids. Small peptides and free amino acids contribute directly to the background flavour of cheese (McGugan *et al.*, 1979).

## 1.9 Catabolism of amino acids

Amino acids are the precursors of various volatile compounds, which have been identified in cheese (Urbach, 1995; Engels *et al.*, 1997). Catabolism of amino acids results in the formation of numerous compounds that may be involved in cheese flavour. Degradative mechanisms potentially include deamination, decarboxylation, desulphuration, oxidation and reduction reactions resulting in the formation of amines, aldehydes, alcohols, indoles, acids, phenolic and sulphur-containing moieties (Hansen *et al.*, 2001; Williams *et al.*, 2001).

Glutamate dehydrogenase (GDH) activity appears to be a key activity in the amino acid catabolism by lactic acid bacteria (LAB) since it produces  $\alpha$ -ketoglutarate, which is essential for amino acid transaminations (Kieronczyk *et al.*, 2003; McSweeney, 2004a). Lactic acid bacteria (LAB) have the enzyme potential to transform amino acids into aroma compounds that contribute to cheese flavour. Amino acid conversion by LAB needs  $\alpha$ -ketoglutarate since this  $\alpha$ -ketoacid is essential for the first step of the conversion. The presence of glutamate dehydrogenase (GDH) activity required for the conversion of glutamate to  $\alpha$ -ketoglutarate is reported to be present in LAB strains commonly used in cheese manufacturing. The ability of LAB to produce aroma compounds from amino acids is closely related to their GDH activity (Tanous *et al.*, 2002). Keto acids are the nitrogen-free analogs of amino acids, and are transaminated to form the respective amino acid in the body (Ödman *et al.*, 2004).

Amino acid transamination is catalysed by aminotransferases which transfer the amino group of an amino acid to an  $\alpha$ -ketoacid,  $\alpha$ -ketoglutarate being the  $\alpha$ -ketoacid acceptor in LAB. The  $\alpha$ -ketoacid acceptor is thus transformed into the corresponding amino acid while the amino acid is deaminated to the corresponding  $\alpha$ -ketoacid (Tanous *et al.*, 2002).

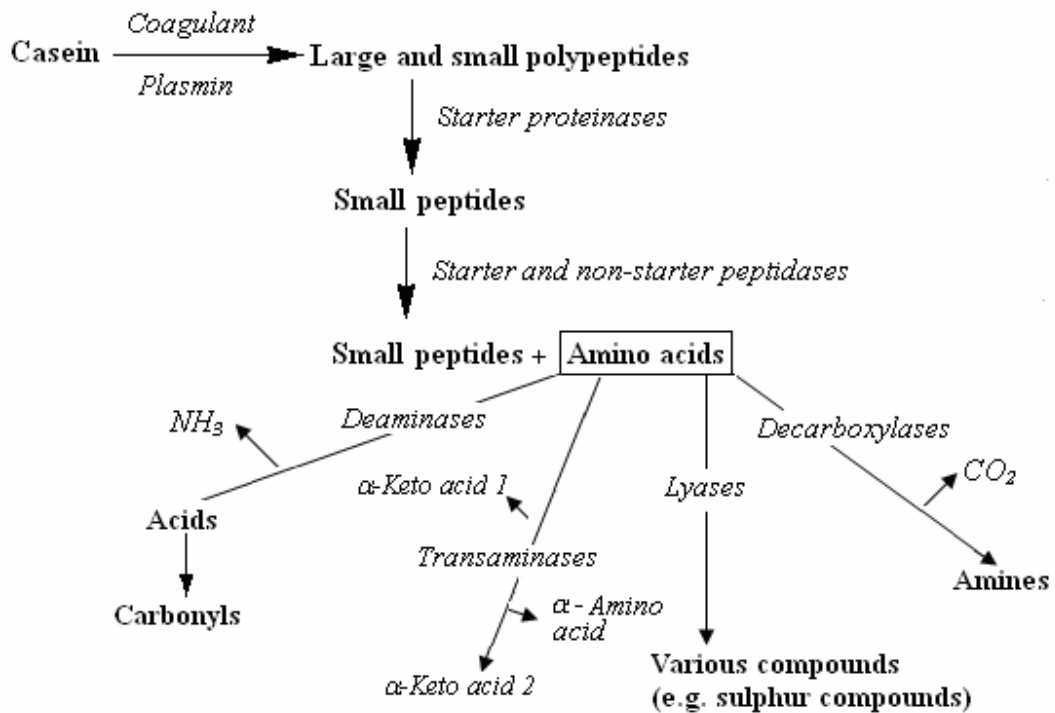
The amino group of glutamate can be transferred to many  $\alpha$ -keto acids in reactions catalyzed by enzymes known as transaminases or aminotransferases. In amino acid biosynthesis, the amino group of glutamate is transferred to various  $\alpha$ -keto acids,

generating the corresponding  $\alpha$ -amino acids. Most of the common amino acids can be formed by transamination. In amino acid catabolism, transamination reactions generate glutamate or aspartate from  $\alpha$ -ketoglutarate (Horton *et al.*, 2002). The transamination reaction is also shown in fig. 1.3.

This was demonstrated in mesophilic lactobacilli such as *Lactobacillus casei*, *Lactobacillus plantarum*; in thermophilic LAB such as *Streptococcus thermophilus*. The  $\alpha$ -ketoacids are then degraded in one or two additional steps into the different aroma compounds, which are aldehydes, alcohols, carboxylic acids and sulphur compounds. Addition of  $\alpha$ -ketoglutarate to Cheddar cheese increased the aroma intensity, aroma creamy aroma and fruity aroma (Tanous *et al.*, 2002). Many of these compounds are odour-active and contribute to the overall flavour of the dairy product (de Wit *et al.*, 2005).

It was postulated that the rate limiting factor in flavour biogenesis was not the release of free amino acids but their subsequent conversion to aroma compounds. The transaminase acceptor  $\alpha$ -ketoglutarate was suggested as the first rate limiting factor in the degradation of amino acids. Addition of the transaminase acceptor  $\alpha$ -ketoglutarate to Cheddar cheese has been shown to enhance amino acid catabolism, and the aroma intensity of a 24 week old Cheddar has been reproduced in a 12 week old Cheddar (Banks *et al.*, 2001). Banks *et al* (2001) supplemented Cheddar cheese curd with  $\alpha$ -ketoglutarate and found better flavour development than in untreated control cheeses.





**Fig.1.3** Summary of proteolysis and amino acid catabolism in cheese during ripening

(McSweeney, 2004a).

GDHs are ubiquitous enzymes that catalyze the reversible oxidative deamination of glutamate to  $\alpha$ -ketoglutarate and ammonium, utilizing  $\text{NAD}^+$ ,  $\text{NADP}^+$  or both as cofactor. It is believed that GDH- $\text{NAD}^+$  is involved in glutamate catabolism, and GDH- $\text{NADP}^+$  has a role in glutamate biosynthesis.

Selecting LAB strains with high GDH activity as a starter or as an adjunct appears to be a specific way to intensify aroma formation in some cheeses (Tanous *et al.*, 2002).

### 1.9.1 Cheese flavour formation by amino acid catabolism

The balance between the formation of peptides and their subsequent degradation into free amino acids is very important, since accumulation of peptides might lead to a bitter off-flavour in cheese (Stadhouders *et al.*, 1983; Smit *et al.*, 1998).

The ability of cultures to degrade bitter tasting-peptides is dependent on the strain used as well as the growth conditions. Following proteolysis, the free amino acids can be converted into volatile flavour components through the action of amino-acid-converting-enzymes (AACEs). Depending on the enzymes present in the cultures used, different flavours can be obtained (Smit *et al.*, 2000).

Enzymes of mesophilic starter lactococci are probably involved in the conversion of amino acids to aroma compounds (Engels and Visser, 1996). Much attention has been paid to the use of (adjunct) starter cultures which would enhance the ripening and flavour formation in cheese, especially the use of thermophilic LAB as adjunct starters to the mesophilic starters. In cheese-types such as Gouda and Cheddar, proteolysis of caseins is essential for sufficient flavour formation. This implies that the amino acids liberated during proteolysis and peptidolysis (phase one and two of cheese ripening) are converted into volatile flavour compounds during the so-called third phase of ripening (Smit *et al.*, 2000).

Amino acids are the precursors of various volatile compounds, which have been identified in cheese (Urbach, 1995; Engels *et al.*, 1997). They can be converted in many different ways by enzymes such as deaminases, decarboxylases, transaminases (aminotransferases) and lyases. Transamination of amino acids results in the formation of  $\alpha$ -keto acids that can be converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation. Many of these compounds are odour-active and contribute to the overall flavour of the dairy product (Hansen *et al.*, 2001). Amino acid conversion to aroma compounds proceeds by two different pathways. The first one is initiated by elimination reactions catalyzed by amino acid lyases, which cleave the side chain of amino acids. This pathway has been observed for aromatic amino acids (ArAAs) and methionine and leads by a single step to phenol, indol and methanethiol, respectively. The second pathway goes through  $\alpha$ -keto acid intermediates initiated by a transamination reaction catalyzed by aminotransferases and has been observed for ArAAs, branched chain amino acids (BcAAs) and methionine. The resulting

$\alpha$ -keto acids are then degraded to aldehydes, alcohols, carboxylic acids, hydroxy acids or methanethiol for methionine via one or two additional steps (Yvon and Rijnen, 2001).

## **1.10 Accelerated ripening**

### *1.10.1 Encapsulated enzymes*

When the objective of cheese production was primarily the conservation of milk constituents, then the more stable the product, i.e., the less change, the better. While storage stability is still important, it is no longer the primary objective of cheese manufacture, a consistently high quality being the target. Since ripening is expensive, acceleration of ripening, especially of low-moisture, slow ripening varieties, is desirable, provided that the proper balance can be maintained (Fox *et al.*, 1996.)

A period of ripening is required for flavour development in most cheese types and can extend up to 1-2 years for some hard cheese varieties. Attempts to shorten the ripening time using a range of ripening systems have had varying degrees of success. Approaches which have been used include the addition of exogenous enzymes or cheese slurries to the curd, use of modified or novel starters or starter adjunct cultures and elevated ripening temperatures (Cogan and Beresford, 2002).

Since the characteristic aroma, flavour and texture of a cheese is the result of the action of numerous enzymes, the use of a single enzyme to accelerate ripening is likely to disturb the flavour component equilibrium and cause flavour defects. Crude cell-free extracts prepared from bacteria, yeasts or molds have been extensively studied. Blends of protease/peptidase, protease/lipase or protease/peptidase/lipase have also been evaluated to accelerate maturation of several types of cheeses. All of these studies were carried out using free proteolytic and lipolytic enzymes added to cheese milk, which may adversely affect flavour and textural criteria of the resulting cheese by prematurely attacking substrates in addition to contaminating cheese whey. The use of microencapsulated

enzymes has been proposed to regulate enzyme/substrate reactions and thereby avoid the drawbacks of free enzymes (Kheadr *et al.*, 2003).

In 1978 only commercially available “off the shelf” food-grade proteinase and lipase enzymes had been added to cheese to try to make it ripen more quickly. These enzymes certainly made the cheese stronger, but their flavour became unbalanced, with a high incidence of bitterness, “meaty notes” and lipolytic rancidity. Also, the cheeses containing proteinases had poor texture, due to the too rapid and excessive breakdown of caseins (Law, 2001).

Proteinases added to break down casein in cheese are needed only in very small amounts because, like all enzymes, they are catalysts, and a small quantity will convert a large amount of substrate. Ripening proteases also quickly remove soluble peptides from caseins. These peptides are lost into the whey when the cheese curd is separated, causing unacceptable losses to cheese yield. Also, the early breakdown of caseins disrupts their orderly structure, prevents proper gel formation, and renders the curds soft and unworkable in the later stages of curd acidification, prior to salting and pressing into cheese. Add to these problems the loss of added enzymes into the whey and it is clear that addition of proteinases directly to the milk is not an option (Law, 2001).

Mammalian serine proteinases participate in physiological processes, some of the best known are digestion and blood clotting. Chymotrypsin-, subtilisin- and trypsin like proteinases are a diverse family of enzymes appearing in animals, bacteria (like *Streptomyces fradiae* and *Bacillus* TA39) and viruses (Katoh *et al.*, 1995; Lesk and Fordham, 1996; Narinx *et al.*, 2003). Plasmin itself is a trypsin-like serine proteinase originating in blood (McSweeney, 2004b)

Enzyme microencapsulation is the obvious solution to the above problem, to protect caseins in the milk and ensure their physical entrapment in the curd gel matrix. Options include fat, starch or gelatine capsules, but none of these has a satisfactory “release” mechanism in cheese (Law, 2001). Enzyme microcapsules physically separate the

enzyme from the substrate in the curd and the enzyme is only released into the curd upon capsule breakdown during ripening (Kailasapathy and Lam, 2005).

Several attempts have been made to reduce the ripening period by addition of enzymes some of which have been reported to halve the normal maturation period of cheese. Direct addition of enzymes to the cheese milk was not successful due to loss of enzymes in the whey, poor enzyme distribution, reduced yield and poor quality cheese. Incorporation of encapsulated enzyme eliminated the problems associated with direct enzyme addition. Enzyme microcapsules physically separate the enzyme from the substrate in the curd and the enzyme is only released during ripening (Kailasapathy and Lam 2005).

#### *1.10.2 Adjuncts*

Adjuncts are microorganisms that do not produce acid, and are only used as an additional source of proteolytic enzymes. They can act as a source of encapsulated enzymes to ensure the controlled release of enzymes. Gradual release of enzymes ensures that good flavour compounds are formed and bitter peptide formation is minimised.

For this study, it is suggested that the yeast cells themselves serve to be a source of encapsulated enzymes. The yeasts contain intracellular enzymes which are slowly released during autolysis into Cheddar cheese during ripening. This should ensure that the enzymes do not degrade the casein proteins so rapidly and cause defects in the Cheddar cheese and it may even play a favorable role in the ripening of the cheese.

A comparative study between a standard Cheddar and yeast-inoculated Cheddar cheeses was done sensorically and chemically (de Wit *et al.*, 2005). *Debaryomyces hansenii* and *Yarrowia lipolytica* contributed to the accelerated development of a strong Cheddar flavour, but bitter and fruity flavours developed when the yeasts were inoculated individually (de Wit *et al.*, 2005; Ferreira and Viljoen, 2003). At six months the cheese inoculated with both yeast cultures had a matured taste that was described as being

comparable to an eight month old commercial Cheddar cheese. At nine months only the cheese inoculated with both yeast cultures, *Debaryomyces hansenii* + *Yarrowia lipolytica*, showed the best potential to ripen to a mature Cheddar while cheeses inoculated with individual yeasts developed off-flavours and bitter tastes (de Wit *et al.*, 2005). This difference was ascribed to differences in hydrolysis and FAA formation.

A general increase in TFFA (total free fatty acids) content was observed during ripening at 150 days in the cheese *Debaryomyces hansenii* + *Yarrowia lipolytica*. Butyric acid (C<sub>4:0</sub>), responsible for rancidity when present in high concentrations, was not detected. Although TFFA may be responsible, in part, for a better Cheddar flavour (de Wit *et al.*, 2005), high concentrations were not detected in these cheeses.

Biochemical explanations of proteolytic methods by the inoculated yeasts were not provided in the study of de Wit *et al* 2005, and would form the basis of the current study.

### **1.11 Aim**

Most studies of yeasts in cheese were carried out on mould-ripened cheeses (cheeses that contain moulds as a feature of the production process). In hard types of cheese (Cheddar cheese) only the microbial ecology has been studied. de Wit *et al* (2005), Ferreira and Viljoen (2003) were the only ones to use yeasts to accelerating the ripening times of Cheddar cheese and explain the chemical changes that took place.

The aim of this project is to:

- Investigate whether yeasts of dairy origin may possess activity of GDH, PG activation and PL
- Investigate whether accelerated ripening of yeast inoculated Cheddar cheese is due to GDH-, PG activation- and PL activity.

This will be carried out by:

- Screening yeasts of dairy origin for GDH -, PG- and PL activity
- Selecting yeasts with GDH-, PG – and PL activity as adjuncts
- Manufacture yeast inoculated Cheddar cheeses
- Determine effect of the yeast adjuncts on the cheese by
  - chemical monitoring
  - sensory analysis

## Chapter 2

### SCREENING OF DAIRY ASSOCIATED YEASTS FOR PROTEASE-, PLASMINOGEN ACTIVATION-, PLASMIN- AND GLUTAMATE DEHYDROGENASE ACTIVITY

#### 2.1 Introduction

Proteolysis in cheese during ripening plays a vital role in the development of texture as well as flavour. Proteolysis contributes to textural changes of the cheese matrix, due to breakdown of the protein network, decrease in  $a_w$  through water binding by liberated carboxyl and amino groups and increase in pH, which facilitates the release of sapid compounds during mastication. It contributes directly to flavour and to off-flavour (e.g. bitterness) of cheese through the formation of peptides and free amino acids as well as liberation of substrates (amino acids) for secondary catabolic changes, i.e. transamination, deamination, decarboxylation, desulphuration, catabolism of aromatic amino acids and reactions of amino acids with other compounds (Sousa *et al.*, 2001).

Proteolysis appears to be rate-limiting in the maturation of most cheese varieties and hence has been the focus of most research on the acceleration of ripening. Acceleration of ripening is most pertinent for low-moisture, slow-ripening varieties and most published work has been on Cheddar cheese (Fox *et al.*, 1996).

During ripening, proteolysis in cheese is catalysed by enzymes from (i) coagulant (e.g. chymosin, pepsin, microbial or plant acid proteinases), (ii) milk (plasmin and perhaps cathepsin D and other somatic cell proteinases), (iii) enzymes from the starter, (iv) non-starter or (v) secondary cultures (e.g. *Propionibacterium* sp.) and (vi) exogenous proteinases or peptidases, or both, used to accelerate ripening (Fig 1.3). The initial hydrolysis of caseins is caused by the coagulant and to a lesser extent by plasmin, which results in the formation of large (water-insoluble) and intermediate-sized (water-soluble) peptides which are degraded by the coagulant and enzymes from the starter and non-



starter lactic acid bacteria of the cheese. The final products of proteolysis are free amino acids (Sousa *et al.*, 2001).

Plasmin (PL) is an alkaline serine proteinase, which has similar activity and characteristics to trypsin. PL enters the milk from the blood via the mammary cell wall lining, where it associates mainly with the casein fraction of the milk (Politis *et al.*, 1993; McSweeney, 2004 b; Choi *et al.*, 2006). PL is very heat stable and can survive heat treatment of dairy foods, such as high-temperature short time (HTST) pasteurisation and ultra high temperature (UHT) treatment. Like PL, PG is heat-stable under HTST pasteurisation conditions (72°C for 15s) and loses approximately 10% activity under these conditions (Choi *et al.*, 2006).

Amino acid catabolism is a major process for flavour formation in cheese. Amino acid transamination is catalysed by aminotransferases which convert an amino acid to the corresponding  $\alpha$ -ketoacid (and in turn transfer the amino group of the amino acid to an acceptor molecule, usually  $\alpha$ -ketoglutarate, producing a new amino acid, usually glutamic acid) (Tanous *et al.*, 2002; Mcsweeney, 2004b).

The aim of the work in this chapter is to screen dairy associated yeast species for PG activation- , PL- and GDH enzyme activities. Certain yeast species will then be chosen, according to the screening results obtained, to be inoculated into model Cheddar cheeses.

## **2.2 Materials and Methods**

Twelve yeasts cultures from the UFS cultivar collection that were isolated from dairy sources (table 2.1) were included in the study and subjected to screening for proteases, PL-activation and GDH activity.

**Table 2.1** Yeast cultures selected for this study

<b>Yeast species</b>	<b>Culture ref. number 1</b>	<b>Culture ref. number 2</b>
<i>Debaryomyces hansenii</i>	UOFS y-0610	CSIR y-953
<i>Yarrowia lipolytica</i>	UOFS y-1138	CBS 599
<i>Saccharomyces cerevisiae</i>	UOFS y-2169 NT	OBS 1171 NT
<i>Rhodotorula glutinis</i>	UOFS y-0519	CBS 20
<i>Candida intermedia</i>	UOFS y-2166	CBS 7153
<i>Rhodotorula mucilaginosa</i>	UOFS y-0478	CBS 316
<i>Rhodotorula minuta</i>	UOFS y-0835	CSIR y-105
<i>Candida rugosa</i>	UOFS y-0537	CSIR y-295
<i>Torulaspora delbrueckii</i>	UOFS y-137	CBS 133
<i>Candida zeylanoides</i>	UOFS y-1295 T	CBS 6519 T
<i>Candida sake</i>	UOFS y-1056	CSIR y-489
<i>Dekkera bruxellensis</i>	UOFS y-0937	CSIR y-506

### 2.2.1 Yeast cultivation

Yeast cultures were plated out (in duplicate) on petri dishes containing YM agar and incubated at 25°C for three days. Yeast extract-malt extract (YM) broth (200ml) was made up in 250ml Erlenmeyer flasks and autoclaved for 20 min at 120°C. The flasks were then inoculated with the yeast species in a laminar flow cabinet and incubated at 25°C for 24h and 48h. At 24 hours cells should be in the exponential growth phase while cells at 48 hours should be in the stationary or early death phase. At 48 hours cells should have autolysed and proteins or enzymes be released.

### 2.2.2 Lysis of yeasts

Yeast lysis was attempted by several methods. Sonification and mechanical breaking with glass beads were not satisfactory, and will therefore not be reported. Osmotic shock proved to be the best lysis method. Yeast cells were centrifuged at 6000 rpm for 7 min in an EBA 12, Hettich centrifuge. Discarding the supernatant, a saturated NaCl solution was added to the yeast cells and left for 30 min. The cells were centrifuged again and the supernatant (containing the NaCl) discarded. Distilled water was added, and left for 30 min. The osmotic shock caused the yeast cells to lyse, which was confirmed by light microscopy.

### 2.2.3 Protein determination

The intracellular protein content of lysed yeasts was determined by the method of Smith *et al*, (1985) by using bicinchoninic acid (BCA) which was supplied by Pierce Biotechnology. A purple reaction product is formed by the interaction of two molecules of BCA with one  $\text{Cu}^{1+}$  ion. The reaction product is water soluble and exhibits strong absorbance at 562nm. Protein standards were prepared with bovine serum albumin (BSA), using  $1\text{mg ml}^{-1}$  BSA ampules and adding 1ml of distilled water to make a stock solution of  $200\mu\text{g ml}^{-1}$ . From there dilutions were made (in duplicate) to make 40, 20, 10, 5, 2.5, 1 and  $0.5\mu\text{g ml}^{-1}$  solutions.

A working reagent (50ml) was also prepared by adding 25ml of reagent A (sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH), 24ml of reagent B (bicinchoninic acid, 4.0%, in water) and 1ml reagent C (4.0% cupric sulfate, pentahydrate in water) in the ratio 25:24:1, reagent A:B:C.

Working reagent (1ml) was added to 1ml protein solution (in test tubes) which was vortexed and incubated in a waterbath at  $60^{\circ}\text{C}$  for 60min to allow colour development. After 60min the tubes were cooled to room temperature and the absorbance measured at 562nm. A standard curve was prepared with the BCA protein standards and was used to determine the protein concentration of the unknown protein samples (fig. 2.1).

## 2.2.4 Determination of enzyme activities in yeasts

### 2.2.4.1 Determination of protease activity

Different substrates selected to specifically detect subtilisin, trypsin or chymotrypsin activity (each 2mM) were used in this assay (table 2.2). Only  $25\mu\text{l}$  of the substrate and  $150\mu\text{l}$  of Tris buffer was used in all of the wells together with  $15\mu\text{l}$  of yeast growth supernatant, or  $15\mu\text{l}$  of broken yeast cells. The blanks only contained distilled water and yeast media without substrate. Tests were done for 24 hour old yeast cultures. This test

method was based upon the method of Feder and Schuck (1970). This assay was employed to determine whether proteases are produced by the yeasts and which type of protease.

**Table 2.2** Substrates for protease assay

<b><u>Substrate</u></b>	<b><u>Enzyme</u></b>
N-Cbz-Gly-Gly-Leu- <i>p</i> -nitroanilide	Subtilisin
L-Leu- <i>p</i> -nitroanilide	Subtilisin
N-Benzoyl-L-Tyr- <i>p</i> -nitroanilide	Chymotrypsin
N-Succinyl-Ala-Ala-Pro-Phe- <i>p</i> -nitroanilide	Chymotrypsin
N-Benzoyl-DL-Arg- <i>p</i> -nitroanilide	Trypsin

#### 2.2.4.2 Determination of plasminogen activation- and plasmin activity

A 2-step reaction involving the activation of PG to PL by uPA (urokinase plasminogen activator), coupled simultaneously with hydrolysis of SpecPL (Spectrozyme PL) as substrate by the resulting PL, was used to assay PA activity. 24h old yeast cultures were used. For the control reaction, 25µl of SpecPL (3.2mM) was reacted with 25µl uPA (1.5 IU/ml), 25µl PG (0.032mg/ml) and 25µl modified Tris buffer (0.05M Tris, 0.1M NaCl, 0.01% Tween 80; pH 7.6) Modified Tris buffer was added to achieve a total volume of 100µl (Rippel *et al.*, 2004). Yeast media and yeast media supernatant were used as controls for analysis of extracellular protease activity and distilled water for intracellular activity. The assays were carried out in 96-well microtiter plates (Costar) that were incubated at 37°C for 60 min. For the standard curve, concentrations of PG ranging from 0 - 25µl were used and it was plotted against the delta absorbance (405-492 nm).

Plates were measured for absorbance at 405, 490, and (405 – 490) nm on an ELISA plate reader (Labsystems iEMS Reader MF). The (405 – 490) nm measurement is a calculated value, whereby the plate reader repeats its measurements at both wavelengths and gives the absorbance value as the difference between the two. The absorbance measurement at 490 nm corrected for any turbidity in the sample (Rippel *et al.*, 2004). Refer to fig. 1.2 for the schematic representation of the plasmin enzymatic system in milk.

#### 2.2.4.3 Preparation for plasmin (PL) activity assay

The preparation was the same as for the assay above, except that this assay contained 25µl plasmin (12.8mU/ml) instead of uPA and PG in all the wells. For the control reaction no PL was added to the assay. Refer to fig. 1.2 for an indication of the enzyme activity in the assay (Rippel *et al.*, 2004).

#### 2.2.4.4 Determination of glutamate dehydrogenase (GDH) activity

For this assay, 48 hour and 24 hour yeast cultures were used. In this assay, the reduced cofactors (NADH<sup>+</sup> or NADPH<sup>+</sup>) produced by oxidative deamination of glutamic acid (Glu) reacts with the iodinitrotetrazolium in the presence of diaphorase to produce a colorimetric product (Kieronczyk *et al.*, 2003).

Assays were carried out in 96 well plates. A reaction mixture consisting of 20µl 50mM potassium phosphate-50mM TEA buffer (pH 9.0) with 1% Triton X-100, 10µl 100mM Glu, 10µl 2mM iodinitrotetrazolium, 10µl 1.76U/ml diaphorase, 10µl 13.8mM NADP<sup>+</sup> or 10µl 17.33mM NAD<sup>+</sup> and 90µl de-ionized water to make a total volume of 150µl (Kieronczyk *et al.*, 2003). Plates were incubated at 37°C and 15µl extracellular (supernatant) or 15µl intracellular (broken yeast cells) phase was added just before absorbance readings were taken at 492nm. Positive controls contained GDH (450mU) and negative controls Tris buffer (30µl). The plates were incubated at 37°C for 60 and 90 min. It was found that NADP<sup>+</sup> caused a very rapid colour change and was judged not suitable for the assay. All further assays were therefore carried out with NAD<sup>+</sup>. Concentrations of GDH ranging from 0 – 20 µl were plotted against absorbance ( $A_{492\text{ nm}}$ ).

#### 2.2.5 Proteolytic activity of yeasts in milk

Fresh milk (i.e., raw milk, unpasteurised, and unhomogenized) was obtained from Dairy Corporation, Bloemfontein, South Africa.

### 2.2.5.1 Milk fractionation

Raw milk was inoculated with twelve yeast species and the samples were treated according to Fajardo-Lira *et al.*, (2000) and centrifuged (4000 g, 25 min, 4°C) to remove the fat from the milk samples. The supernatant was then ultracentrifuged (90,000 g, 1h, 4°C) in Beckman ultracentrifuge tubes to separate water insoluble nitrogen (WISN) and water soluble nitrogen (WSN) fractions of the milk samples. The casein pellets were reconstituted with deionized distilled water (DDW) to the original milk volume. 0.5M sodium citrate buffer (pH 3.2) was added to the whey fraction in a 1:1 (vol/vol) to ensure substrate dissociation from  $\beta$ -lactoglobulin ( $\beta$ -LG) and  $\alpha$ -lactalbumin ( $\alpha$ -LA). Whey samples were centrifuged (4000 g, 20 min) in Amicon Ultra Centrifugal Filter Devices to concentrate the samples to 100 $\mu$ l. Urea-PAGE gel electrophoresis was then done on the WISN as well as the WSN samples to determine whether peptide  $\beta$ -CN (29-?) was released, which would be an indication of PL activity due to the presence of yeasts.

### 2.2.5.2 Analysis by UREA-PAGE

The WISN and WSN samples were analyzed by alkaline urea-polyacrylamide gel electrophoresis (PAGE) using the method of Andrews (1983). Samples were prepared as described by Shalabi and Fox (1987). Staining was by the method of Blakesley and Boezi (1977).

Cheese sample sub-fractions were dispersed in sample buffer, pH 7.6 (Shalabi and Fox 1987) and aliquots of 5-20 $\mu$ l were loaded on the gels consisting of a resolving gel (12.5% T and 4% C, pH 8.8) and stacking gel (12.5% T and 4% C, pH 8.4) according to the method of Andrews (1983). Electrophoresis was done on a Mighty Small miniature slab gel electrophoresis unit, SE 260 (Hoefer Scientific Instruments) using a Hoefer Scientific Instrument, model PS 500X DC Power supply set at 210 volts (constant) (set for 2 gels running simultaneously).

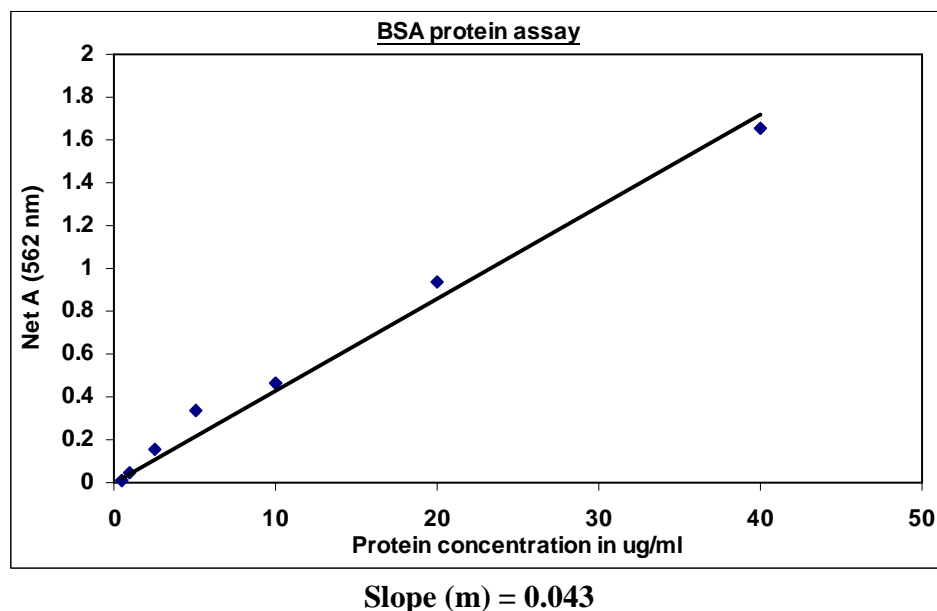
Staining of the peptides bands was done according to the method of direct staining by Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250 (Fluka). No de-staining

was necessary and the gels were stored in distilled water where the protein bands became more intense. Thus, the background was not stained and the protein zones were visualized directly. The electrophoretograms were photographed.

## 2.3 Results

### 2.3.1 Bicinchoninic acid protein assay

The standard curve (fig. 2.1) was used to calculate the intracellular protein content ( $\mu\text{g ml}^{-1}$ ) of lysed yeasts which are shown in table 2.3. Not all of the yeast species tested had high amounts of protein (undiluted). *Rhodotorula glutinis* and *Candida intermedia* had the lowest values. That might be because not all the yeasts had the same growth rate and less cells of these two yeast species were obtained. Differences in protein content could be a reflection of different growth rates, although all yeast were observed to have been in an exponential growth phase at 24h and an early stationary phase at 48h.



**Fig. 2.1** Standard curve for the BCA protein assay with BSA as protein standard.

**Table 2.3** Intracellular content of 24 hour old yeast cultures as determined by BCA assay

<b>Yeast</b>	<b>Protein concentration: undiluted (ug/ml)</b>
<i>Debaryomyces hansenii</i>	170.47 ± 0.01
<i>Yarrowia lipolytica</i>	133.49 ± 0.00
<i>Saccharomyces cerevisiae</i>	97.56 ± 0.08
<i>Rhodotorula glutinis</i>	28.49 ± 0.09
<i>Candida intermedia</i>	48.84 ± 0.06
<i>Rhodotorula mucilaginosa</i>	170.70 ± 0.09
<i>Rhodotorula minuta</i>	165.12 ± 0.01
<i>Candida rugosa</i>	123.60 ± 0.05
<i>Torulaspora delbrueckii</i>	181.98 ± 0.04
<i>Candida zeylanoides</i>	156.40 ± 0.06
<i>Candida sake</i>	162.21 ± 0.14
<i>Dekkera bruxellensis</i>	148.49 ± 0.01

### 2.3.2 Proteolytic activity

In tables 2.4, 2.5, and 2.6 the protease activity in the 12 yeast cultures are shown for the different substrates. The extracellular proteolytic activity was expressed as nmol.min<sup>-1</sup>.ml<sup>-1</sup> enzyme activity in all the tables (tables 2.4, 2.5, 2.6, 2.7, 2.8, 2.9).

**Table 2.4** Subtilisin activity of 24h old yeast cultures for different peptide substrates

	<b>N-Cbz-Gly-Gly-Leu-<i>p</i>-nitroanilide</b>		<b>L-Leu- <i>p</i>-nitroanilide</b>	
	* Extracellular (nmol.min <sup>-1</sup> .ml <sup>-1</sup> )	* Intracellular (nmol.min <sup>-1</sup> .mg protein <sup>-1</sup> )	* Extracellular (nmol.min <sup>-1</sup> .ml <sup>-1</sup> )	* Intracellular (nmol.min <sup>-1</sup> .mg protein <sup>-1</sup> )
<b>Yeast species</b>				
<i>Debaryomyces hansenii</i>	0.360	0.075	0.072	0.086
<i>Yarrowia lipolytica</i>	0.552	0.050	0.192	0.042
<i>Saccharomyces cerevisiae</i>	0.240	0	0.264	0.045
<i>Rhodotorula glutinis</i>	0	0.001	0	0.004
<i>Candida intermedia</i>	0.024	0.181	0.096	0.205
<i>Rhodotorula mucilaginosa</i>	0.048	0.088	0	0.238
<i>Rhodotorula minuta</i>	0.528	0.080	0.168	0
<i>Candida rugosa</i>	0.840	0.156	0.072	0.050
<i>Torulaspora delbrueckii</i>	0.336	0.292	0.072	0.265
<i>Candida zeylanoides</i>	0.504	0.192	0.096	0.105
<i>Candida sake</i>	0.264	0.113	0.120	0.023
<i>Dekkera bruxellensis</i>	0.408	0.031	0.192	0.007

\* Average of duplicates



No parallel can be drawn between the extracellular and intracellular enzyme activity. It is known that proteases may undergo autolysis when released into the medium by secretion or cell lysis (Venter *et al.*, 1999) and because the rate of autolysis differs between enzymes. Further comparisons therefore have to rely on intracellular protease activity.

Subtilisin-like (intracellular) activity ( $>0.1 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ ) was detected in *Candida intermedia*, *Torulaspora delbrueckii* and *Candida zeylanoides* as well as trypsin-like activity (tables 2.4 and 2.6). *Candida rugosa* also displayed higher subtilisin-like (extracellular) activity, but only for substrate N-Cbz-Gly-Gly-Leu-*p*-nitroanilide (table 2.4). *Candida intermedia* and *Torulaspora delbrueckii* displayed high chymotrypsin-like (intracellular) activity, however, *Debaryomyces hansenii* and *Candida rugosa* displayed lower chymotrypsin like activity (table 2.5).

**Table 2.5** Chymotrypsin activity of 24h old yeast cultures for different peptide substrates

	<b><u>N-Benzoyl-L-Tyr- p-nitroanilide</u></b>		<b><u>N-Succinyl-Ala-Ala-Pro-Phe- p-nitroanilide</u></b>	
	<b>* Extracellular</b> ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$ )	<b>* Intracellular</b> ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ )	<b>* Extracellular</b> ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$ )	<b>* Intracellular</b> ( $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ )
<i>Debaryomyces hansenii</i>	0.180	0.143	0	0.106
<i>Yarrowia lipolytica</i>	0.084	0	0.096	0.033
<i>Saccharomyces cerevisiae</i>	0.228	0.039	0.024	0.014
<i>Rhodotorula glutinis</i>	0.012	0.004	0.048	0.003
<i>Candida intermedia</i>	0.516	0.227	0.192	0.204
<i>Rhodotorula mucilaginosa</i>	0.276	0.080	0.072	0.082
<i>Rhodotorula minuta</i>	0.204	0.048	0.096	0
<i>Candida rugosa</i>	0.420	0.129	0.168	0.117
<i>Torulaspora delbrueckii</i>	0.156	0.292	0.048	0.245
<i>Candida zeylanoides</i>	0.636	0.094	0.288	0.092
<i>Candida sake</i>	0	0.110	0.024	0.057
<i>Dekkera bruxellensis</i>	0.396	0.034	0.144	0

\* Average of duplicates

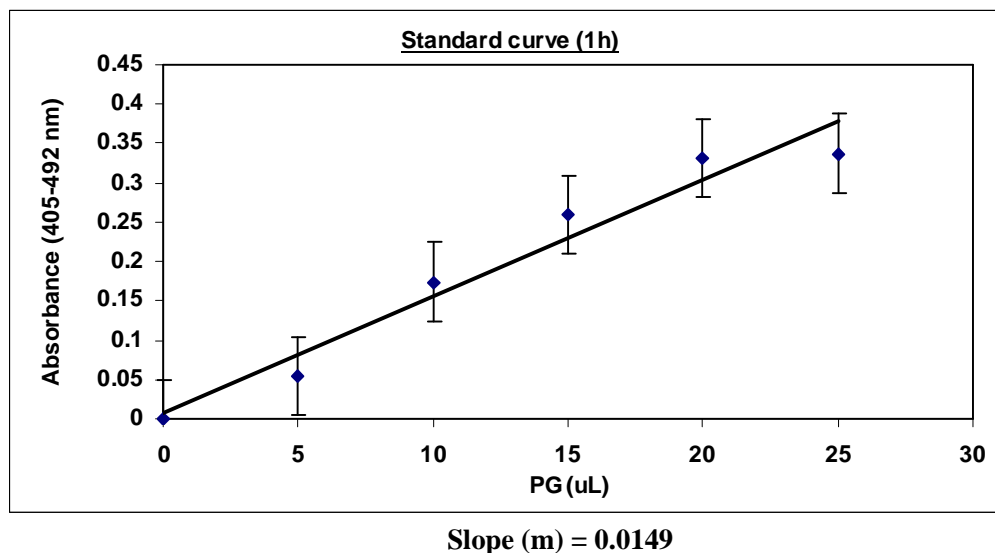
**Table 2.6** Trypsin activity of 24h old yeast cultures for different peptide substrates

<b>Yeast species</b>	<b>N-Benzoyl-DL-Arg-p-nitroanilide</b>	
	<b>* Extracellular</b> (nmol.min <sup>-1</sup> .ml <sup>-1</sup> )	<b>* Intracellular</b> (nmol.min <sup>-1</sup> .mg protein <sup>-1</sup> )
<i>Debaryomyces hansenii</i>	0.144	0.086
<i>Yarrowia lipolytica</i>	0.168	0.020
<i>Saccharomyces cerevisiae</i>	0.240	0.004
<i>Rhodotorula glutinis</i>	0.072	0.012
<i>Candida intermedia</i>	0.216	0.126
<i>Rhodotorula mucilaginosa</i>	0.144	0.086
<i>Rhodotorula minuta</i>	0.144	0
<i>Candida rugosa</i>	0.120	0.069
<i>Torulaspora delbrueckii</i>	0.024	0.290
<i>Candida zeylanoides</i>	0.096	0.107
<i>Candida sake</i>	-0.024	0.026
<i>Dekkera bruxellensis</i>	0.024	0.001

\* Average of duplicates

### 2.3.3 Plasminogen activation activity

The results for the plasminogen activation activity of the 12 yeast cultures after 24 hours are given in table 2.7 and were determined relative to the standard curve in fig. 2.2.



**Fig. 2.2** Standard curve for plasminogen assay at 1h.

**Table 2.7** Plasminogen activation activity of 24h old yeast cultures

<u>Yeast species</u>	<u>PG activation</u>	<u>PG activation</u>
	* <u>Extracellular</u>	<u>Intracellular</u>
	<u>enzyme activity</u>	<u>enzyme activity</u>
	<u>(nmol.min<sup>-1</sup>.ml<sup>-1</sup>)</u>	<u>(nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>)</u>
<i>Debaryomyces hansenii</i>	1.596	0.039 ± 0.001
<i>Yarrowia lipolytica</i>	1.493	0.272 ± 0.001
<i>Saccharomyces cerevisiae</i>	1.184	0.010 ± 0.006
<i>Rhodotorula glutinis</i>	2.419	0.049 ± 0.001
<i>Candida intermedia</i>	1.801	0.029 ± 0.000
<i>Rhodotorula mucilaginosa</i>	2.831	0.078 ± 0.001
<i>Rhodotorula minuta</i>	2.728	0.049 ± 0.001
<i>Candida rugosa</i>	2.007	0.156 ± 0.008
<i>Torulasporea delbrueckii</i>	2.007	0.477 ± 0.007
<i>Candida zeylanoides</i>	2.625	0.126 ± 0.001
<i>Candida sake</i>	2.110	0.019 ± 0.002
<i>Dekkera bruxellensis</i>	2.728	0.097 ± 0.004

\* Average of duplicates

In table 2.7, the only significant enzyme activity noted (>0.1 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>) in the yeast cells (in the intracellular phase) were that of *Yarrowia lipolytica*, *Candida rugosa*, *Candida zeylanoides* and *Torulasporea delbrueckii*. The values obtained shows that the enzymes needed to activate plasminogen (PG) to plasmin (PL) are present.

#### 2.3.4 Plasmin activity

In table 2.8, low intracellular PL activity (>0.2 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>) is noted for *Yarrowia lipolytica* and *Torulasporea delbrueckii*. Other yeasts with low levels of activity are *Candida intermedia* and *Rhodotorula mucilaginosa*. It therefore seems as if these yeasts (*Yarrowia lipolytica* and *Torulasporea delbrueckii*) display plasminogen activation activity as well as plasmin activity. Since *Torulasporea delbrueckii* displayed the highest hydrolysis in this regard, as well as hydrolysis of other peptide bonds, it might be that the plasminogen activation is the result of non-specific proteolysis by several proteases. Confirmation has to be obtained by investigation of hydrolysis of caseins by these yeasts which will be reported in 2.3.6 and 2.3.7.

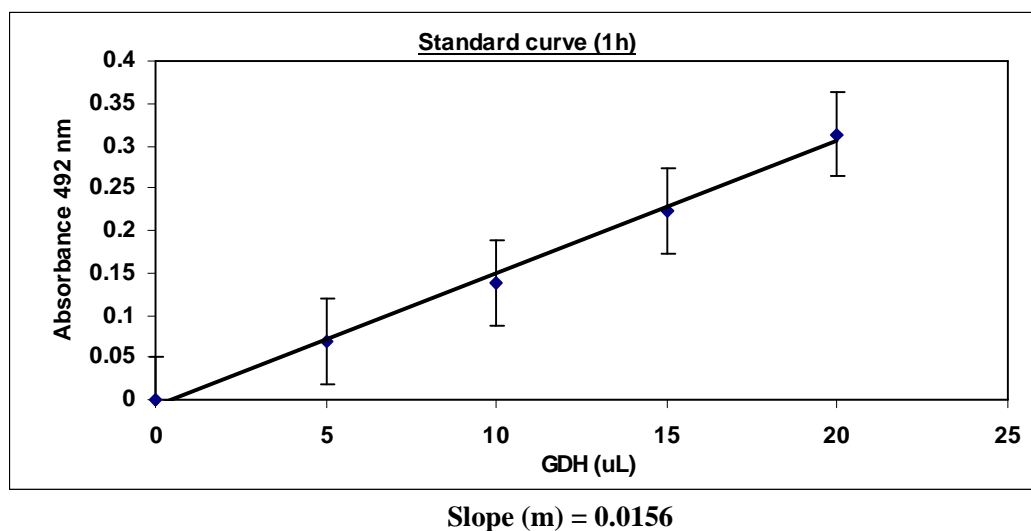
**Table 2.8** Plasmin activity of 24h old yeast cultures

<b>Yeast species</b>	<b>PL</b>	<b>PL</b>
	* <u>Extracellular</u>	<u>Intracellular</u>
	<u>enzyme activity</u> (nmol.min <sup>-1</sup> .ml <sup>-1</sup> )	<u>enzyme activity</u> (nmol.min <sup>-1</sup> .mg protein <sup>-1</sup> )
<i>Debaryomyces hansenii</i>	2.368	0.058 ± 0.004
<i>Yarrowia lipolytica</i>	1.441	0.214 ± 0.004
<i>Saccharomyces cerevisiae</i>	1.029	0.058 ± 0.002
<i>Rhodotorula glutinis</i>	0.618	0.088 ± 0.004
<i>Candida intermedia</i>	0	0.146 ± 0.000
<i>Rhodotorula mucilaginosa</i>	0	0.146 ± 0.001
<i>Rhodotorula minuta</i>	0	0.126 ± 0.007
<i>Candida rugosa</i>	1.544	0.058 ± 0.004
<i>Torulasporea delbrueckii</i>	0.206	0.564 ± 0.001
<i>Candida zeylanoides</i>	0	0.126 ± 0.004
<i>Candida sake</i>	0.823	0 ± 0.002
<i>Dekkera bruxellensis</i>	0.823	0.058 ± 0.008

\* Average of duplicates

### 2.3.5 GDH activity

In table 2.9 the GDH activity for the 12 yeast species at 48 hours are shown. The results given in table 2.9 were determined relative to the standard curve in fig. 2.3.



**Fig. 2.3** Graph of GDH enzyme activity for the different yeast species at 1h.

**Table 2.9** Glutamate dehydrogenase activity of 48h old yeast cultures

<b>Yeast species</b>	<b>GDH</b>	<b>GDH</b>
	* <u>Extracellular</u>	<u>Intracellular</u>
	<u>enzyme activity</u>	<u>enzyme activity</u>
	(nmol.min <sup>-1</sup> .ml <sup>-1</sup> )	(nmol.min <sup>-1</sup> .mg protein <sup>-1</sup> )
<i>Debaryomyces hansenii</i>	2.661	9.326 ± 0.004
<i>Yarrowia lipolytica</i>	0.242	2.716 ± 0.001
<i>Saccharomyces cerevisiae</i>	0	3.895 ± 0.001
<i>Rhodotorula glutinis</i>	0	5.404 ± 0.000
<i>Candida intermedia</i>	5.080	14.785 ± 0.006
<i>Rhodotorula mucilaginosa</i>	0.726	5.129 ± 0.000
<i>Rhodotorula minuta</i>	5.805	11.438 ± 0.011
<i>Candida rugosa</i>	5.322	14.072 ± 0.002
<i>Torulaspora delbrueckii</i>	0	11.493 ± 0.004
<i>Candida zeylanoides</i>	35.800	21.039 ± 0.008
<i>Candida sake</i>	12.337	9.107 ± 0.008
<i>Dekkera bruxellensis</i>	2.661	9.518 ± 0.003

\* Average of duplicates

The results in table 2.9 show that all the yeasts displayed intracellular GDH activity, with the highest levels (>9 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>) detected in *Debaryomyces hansenii*, *Candida intermedia*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspora delbrueckii*, *Candida zeylanoides*, *Candida sake* and *Dekkera bruxellensis*. GDH enzyme activity may be ranked from highest to lowest: *Candida zeylanoides*> *Candida intermedia*> *Candida rugosa*> *Torulaspora delbrueckii*> *Rhodotorula minuta*> *Dekkera bruxellensis*> *Debaryomyces hansenii*> *Candida sake*> *Rhodotorula mucilaginosa* > *Rhodotorula glutinis* > *Saccharomyces cerevisiae*> *Yarrowia lipolytica*.

High levels of extracellular GDH activity was observed for *Candida zeylanoides* which could best be ascribed to cells lysed before preparation of extracts at 48 hour growth. Thus the conclusion could be made that the yeast cells contain GDH enzyme activity that could play a role in the flavour formation in Cheddar cheeses.

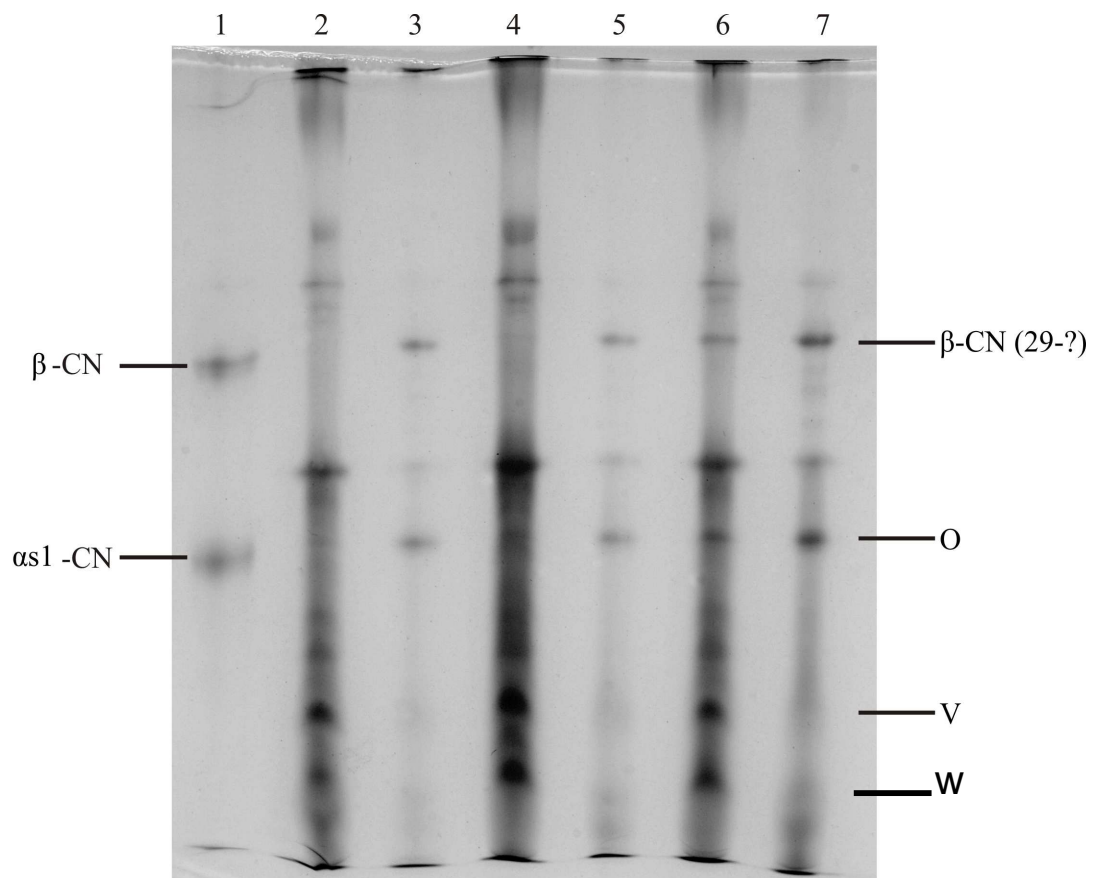
### 2.3.6 UREA-PAGE of the WISN fraction of fresh milk

The electrophoretograms of the WISN fraction of the milk are not shown because no significant differences were noticed at this early stage of proteolysis

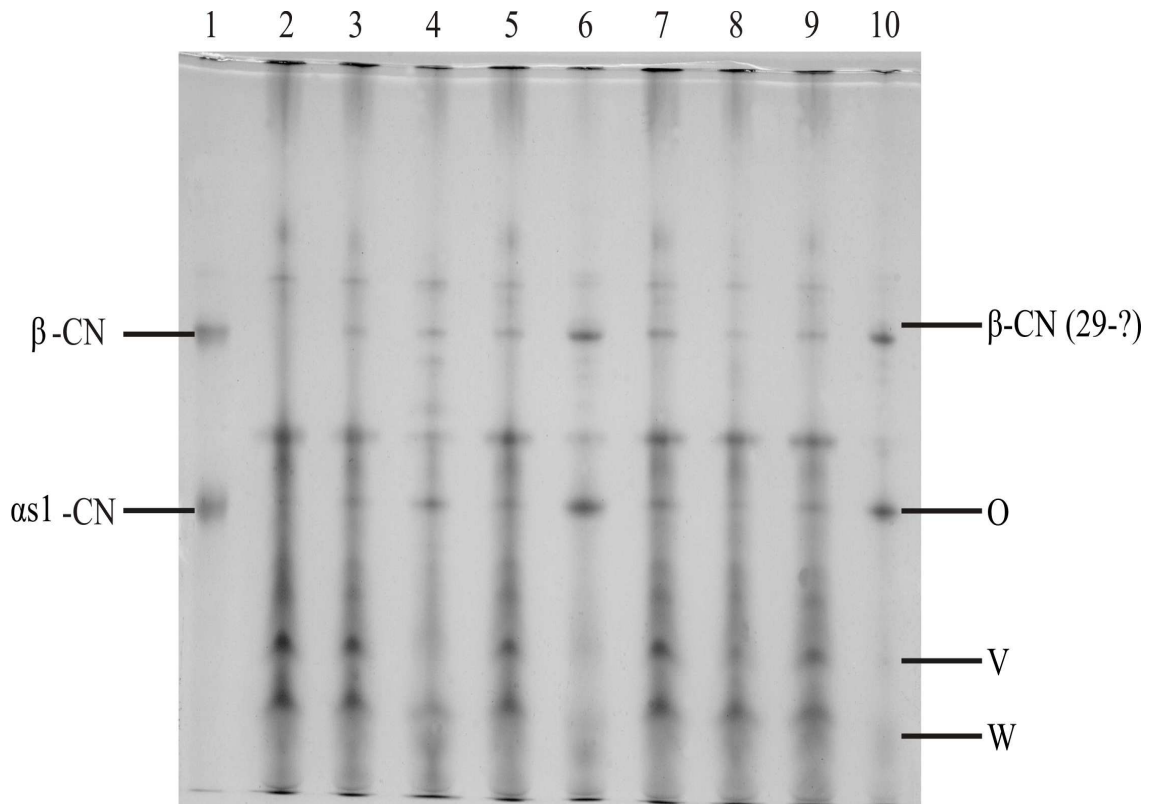
### 2.3.7 UREA -PAGE of the WSN fraction of fresh milk

Changes in proteolysis are shown in the electrophoretograms (fig. 2.4 - 2.7). Of note is the degradation of  $\beta$ -CN and  $\alpha$ -CN into fractions  $\beta$ -CN (29-?) and O respectively.

The formation of peptide  $\beta$ -CN (29-?) is due to plasmin activity and is formed after one day of incubation of the milk sample as well as all yeast inoculated samples (fig. 2.4 – 2.7). Peptides  $\beta$ -CN (29-?) and O are formed at day 0, the same day of inoculation, by *Torulaspora delbrueckii*, *Yarrowia lipolytica*, *Rhodotorula mucilaginosa* and *Candida zeylanoides* (fig. 2.4, 2.5, 2.6 and 2.7) which is an indication of increased PL activity. This could be ascribed to a contribution of PL by the yeasts, or by their PG-activation of milk PG to PL. Yeasts *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Candida intermedia*, *Rhodotorula minuta*, *Candida rugosa*, and *Candida sake* (fig. 2.4 – 2.7) at day one also showed formation of the peptides, but to a lesser extent.

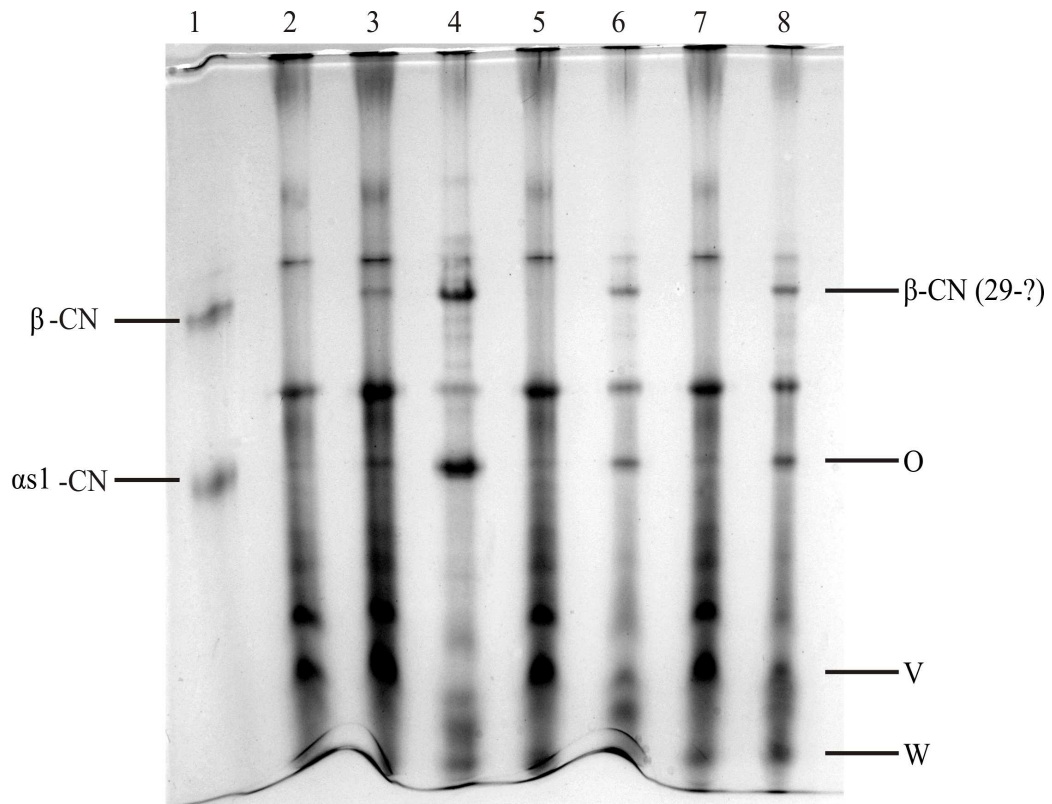


**Fig. 2.4** Urea-PAGE of the WSN fraction of milk containing yeast species. Lane 1: marker, Na-caseinate. Lane 2: milk control day 0. Lane 3: milk control day 1. Lane 4: *Debaryomyces hansenii* day 0. Lane 5: *Debaryomyces hansenii* day 1. Lane 6: *Torulaspora delbrueckii* day 0. Lane 7: *Torulaspora delbrueckii* day 1.

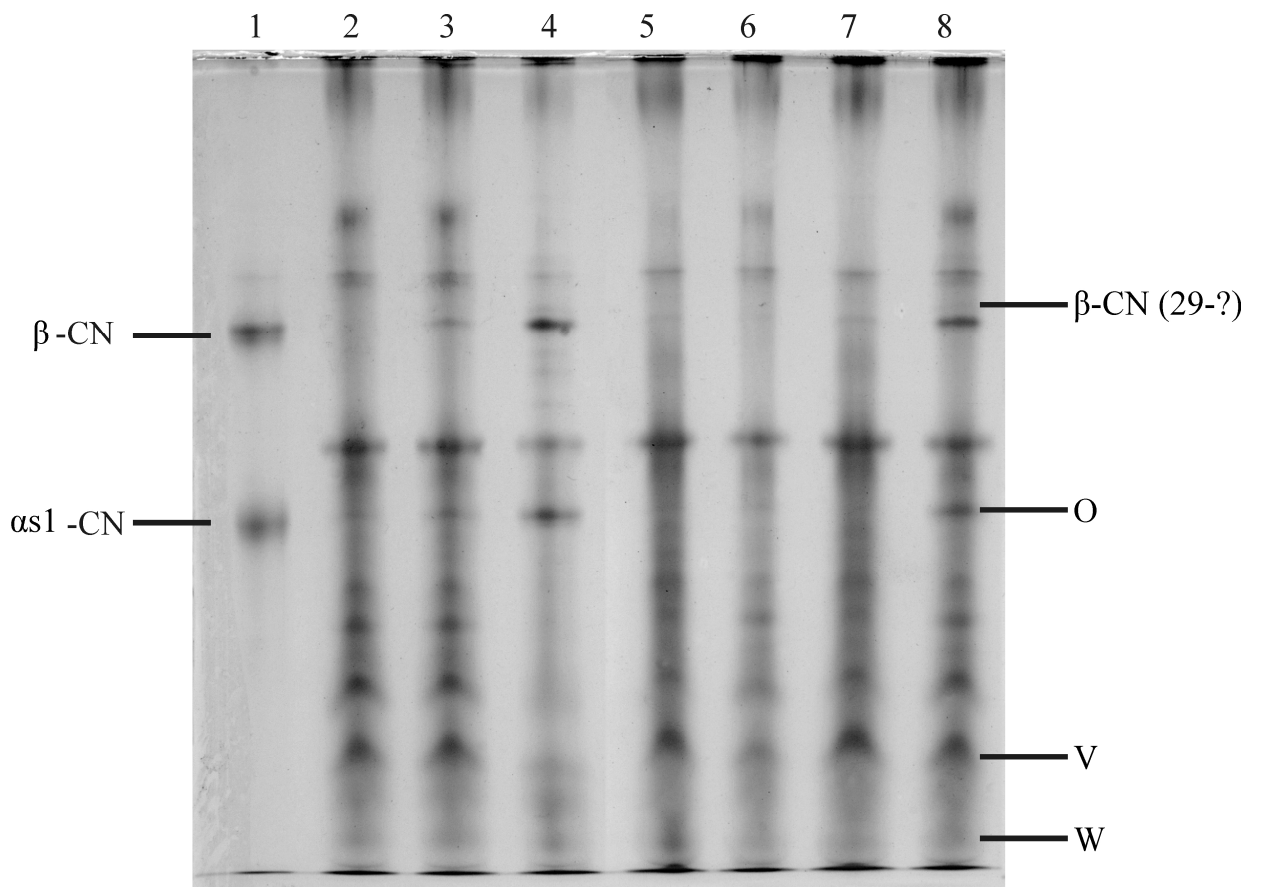


**Fig. 2.5** Urea-PAGE of the WSN fraction of milk containing yeast species. Lane 1: marker, Na-caseinate. Lane 2: milk control day 0. Lane 3: *Saccharomyces cerevisiae* day 0. Lane 4: *Saccharomyces cerevisiae* day 1. Lane 5: *Yarrowia lipolytica* day 0. Lane 6: *Yarrowia lipolytica* day 1. Lane 7: *Rhodotorula glutinis* day 0. Lane 8: *Rhodotorula glutinis* day 1. Lane 9: *Candida intermedia* day 0. Lane 10: *Candida intermedia* day 1.





**Fig. 2.6** Urea-PAGE of the WSN fraction of milk containing yeast species. Lane 1: marker, Na-caseinate. Lane 2: milk control day 0. Lane 3: *Rhodotorula mucilaginosa* day 0. Lane 4: *Rhodotorula mucilaginosa* day 1. Lane 5: *Rhodotorula minuta* day 0. Lane 6: *Rhodotorula minuta* day 1. Lane 7: *Candida rugosa* day 0. Lane 8: *Candida rugosa* day 1.



**Fig. 2.7** Urea-PAGE of the WSN fraction of milk containing yeast species. Lane 1: marker, Na-caseinate. Lane 2: milk control day 0. Lane 3: *Candida zeylanoides* day 0. Lane 4: *Candida zeylanoides* day 1. Lane 5: *Dekkera bruxellensis* day 0. Lane 6: *Dekkera bruxellensis* day 1. Lane 7: *Candida sake* day 0. Lane 8: *Candida sake* day 1.

## 2.4 Discussion

Chymotrypsin-, subtilisin- and trypsin-like proteases is nothing new in yeasts (Katoh *et al.*, 1995; Lesk and Fordham, 1996; Narinx *et al.*, 2003). The results presented here added a few yeast species to the list. Subtilisin like activity was detected in *Candida intermedia*, *Torulaspora delbrueckii*, as well as trypsin like activity (table 2.6). *Candida rugosa* also displayed subtilisin like extracellular activity, but only for substrate N-Cbz-Gly-Gly-Leu-p-nitroanilide (table 2.4). *Candida intermedia* and *Torulaspora delbrueckii* displayed high chymotrypsin like (intracellular) activity, however, *Debaryomyces hansenii* and *Candida rugosa* displayed lower chymotrypsin like activity (table 2.5).

Yeast species that displayed intracellular (although low) PG activation activity above  $0.100 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$  were *Yarrowia lipolytica*, *Candida rugosa* and *Torulaspora delbrueckii*, *Candida zeylanoides*, and intracellular PL above  $0.100 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$  activity were *Yarrowia lipolytica*, *Candida intermedia*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, *Torulaspora delbrueckii* and *Candida zeylanoides*. *Rhodotorula mucilaginosa* displays low levels of PL activity but does not display PG activation activity. *Yarrowia lipolytica*, *Torulaspora delbrueckii* and *Candida zeylanoides* are the only ones to have both of the enzyme activities (tables 2.7 and 2.8).

It is interesting to note that *Torulaspora delbrueckii* is the only one of the yeast species examined that displays PG activation activity, PL activity (more than *Yarrowia lipolytica*), protease activity (in small amounts) and GDH activity (more than *Debaryomyces hansenii*). It even displays the highest levels of all these enzymes. It also displays subtilisin, trypsin and chymotrypsin activity. Taking this into account, the question arises whether the observed PG activation and PL activities are not due to non-specific proteolysis due to the presence of several types of proteases. To specifically investigate the effect of these enzymes on caseins, proteolysis was carried out on milk and the results evaluated by UREA-PAGE.

The UREA-PAGE gels of WISN were inconclusive as proteolysis was not very advanced within the short time and the electrophoretograms were therefore not shown. Evidence of proteolysis could only be detected by the WSN. In fig. 2.4, the milk control at day 1 showed evidence of proteolysis of caseins, which may be ascribed to the action of indigenous plasmin, of which the formation of the peptides  $\beta$ -CN 29-? and O is an indication (Rippel *et al*, 2000; Fadjaro-Lira *et al*, 2004). The milk samples that were inoculated with yeasts also showed plasmin type hydrolysis, but at different degrees. The proteolysis by yeasts *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Rhodotorula glutinis* did not differ much from the control, however, *Torulaspora delbrueckii*, *Yarrowia lipolytica*, *Candida intermedia*, *Rhodotorula mucilaginosa* and *Candida sake* showed the development of peptide  $\beta$ -CN (29-?) and fraction O at day 0 (the same day as inoculation) and a much higher content of this peptide, as evidenced by a relatively darker band on the electrophoretograms. These results would therefore confirm that the PG activation observed by the dedicated assays, are not due to non-specific hydrolysis.

Almost all the yeast species display GDH activity (between 2.5 and 21 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>). *Debaryomyces hansenii*, *Candida intermedia*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspora delbrueckii*, *Candida zeylanoides*, *Candida sake* and *Dekkera bruxellensis*, expressed high levels of GDH activity of above 12 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>. This GDH activity seems very low; however, it is in the same order of magnitude as observed for *Lactobacillus* strains by Kieronczyk *et al* (2004). These authors showed that higher GDH activity may be observed, but that it depends on the type of amino acid that is used as substrate. The highest activity was monitored for aspartate and the lowest for isoleucine.

de Wit *et al* (2005) have shown that a cheese inoculated with *Debaryomyces hansenii* and *Yarrowia lipolytica* developed Cheddar flavour at an accelerated rate (Ferreira and Viljoen, 2003). A chemical explanation was offered as the proteolysis of yeast inoculated cheeses having progressed differently. However, a biochemical explanation could not be provided at that stage. It is postulated that PG activation and GDH activity could have

played a role in the cheese ripening differently as observed by de Wit *et al* (2005), which was the aim of the present study.

Although de Wit *et al* (2005) have not investigated the products formed during amino acid catabolism, the sensory observations showed that yeast inoculated Cheddar cheeses obtained the Cheddar cheese flavour at an earlier ripening time than the standard Cheddar cheeses. It was attributed to higher peptide and amino acid content, which may have a direct influence on cheese flavour or the precursors of flavour components. Ferreira and Viljoen, 2003 have indicated that the accelerated formation of the desired Cheddar flavour may be attributed to the enhanced breakdown of proteins by the yeasts, like those formed by the amino acid transferase (Horton *et al.*, 2002).

de Wit *et al* (2005) have observed that Cheddar cheeses inoculated with *Yarrowia lipolytica* and *Debaryomyces hansenii* resulted in accelerated flavour development, however, separate inoculation of these two yeasts did not result in sensorically acceptable products. The current study shows that *Yarrowia lipolytica* displays PG activation- and PL activity, while *Debaryomyces hansenii* displays high levels of GDH activity. It seems as if the explanation to the accelerated ripening of the yeast inoculated Cheddar cheese lies in the activity of both enzymes, which are contributed by the separate yeasts. An exchange of these yeasts with similar enzyme producers should therefore lead to similar results regarding accelerated flavour development, which is to be attempted in the current study.

Yeasts with PG activation- and PL activity such as *Yarrowia lipolytica* would be *Torulaspora delbrueckii*, *Candida rugosa* and *Candida zeylanoides*. Yeasts with GDH activity such as *Debaryomyces hansenii* would be *Candida intermedia*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspora delbrueckii*, *Candida zeylanoides*, *Candida sake* and *Dekkera bruxellensis*. Since *Torulaspora delbrueckii* displays activity of both enzymes, its contribution to accelerated flavour development should be evaluated as single adjunct to LAB. Because, *Candida rugosa*, *Candida zeylanoides*, *Candida intermedia* and *Candida sake* are known as pathogenic organisms, and are therefore not

considered for further use in cheese processing. Is it really the PL and GDH enzyme activities that help to improve ripening times of cheeses? To help answer the question, the adjuncts yeasts selected were *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Torulasporea delbrueckii* and *Dekkera bruxellensis*. *Debaryomyces hansenii* and *Yarrowia lipolytica* were chosen to act as a control for the study that was done by de Wit *et al.*

Taking the enzyme activities into account, it is suggested that yeast inoculated Cheddar cheeses with the following combinations be manufactured: *Debaryomyces hansenii* + *Yarrowia lipolytica*, *Yarrowia lipolytica* + *Torulasporea delbrueckii*, *Dekkera bruxellensis* + *Yarrowia lipolytica*. Cheddar cheese with individual inoculation: Standard (inoculated with LAB as control), *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Torulasporea delbrueckii* and *Dekkera bruxellensis*. The manufacture, sensory analysis and chemical analysis of these will be reported in the following chapters.

## **2.5 Conclusion**

Chymotrypsin-, subtilisin- and trypsin-like proteases, PG activation activity, PL activity and GDH activity were detected in some of the yeast species like *Debaryomyces hansenii*, *Yarrowia lipolytica* and *Torulasporea delbrueckii*. *Torulasporea delbrueckii* showed to possess the highest enzyme activity of all the yeasts. To test what the effect of the enzyme activities will be on Cheddar cheese, four yeast species (*Debaryomyces hansenii*, *Yarrowia lipolytica*, *Torulasporea delbrueckii* and *Dekkera bruxellensis*) were chosen and inoculated with the LAB into miniature Cheddar cheeses. The outcome will be discussed in chapter three and chapter four.

## Chapter 3

### MANUFACTURE AND SENSORY EVALUATION OF MODEL CHEESES WITH AND WITHOUT YEAST ADJUNCTS

#### 3.1 Introduction

Many aspects of the biochemical events involved in cheese production and ageing are not yet completely known. The impact of processing variables such as cheesemilk, temperature profile during cheese-making and starter or adjunct cultures on the quality of the resulting cheese are usually assessed by means of cheese-making experiments (Milesi *et al.*, 2007). However, cheese-making trials and assessment of the performance of enzymes, starters or adjuncts during cheese ripening is expensive and time consuming (Shakeel-Ur-Rehman *et al.*, 1998; Milesi *et al.*, 2007). Reproducibility may also be difficult to obtain at pilot plant scale, and contamination with nonstarter microorganisms as well as sub-lethal phage infections can change ripening patterns leading to misinterpretations of the results (Milesi *et al.*, 2007). Thus, a model system in which their role can be assessed rapidly appears desirable, provided that the conditions in cheese can be replicated closely (Shakeel-Ur-Rehman *et al.*, 1998).

Bacteria usually cause fermentation of milk and are therefore considered to be of major importance during the making of cheese. Yeasts, however, possess the ability to grow under conditions unfavourable to many bacteria and play a significant role in the spoilage of dairy products as well as the ripening of some cheese varieties (Viljoen and Greyling, 1995).

Yeasts frequently occur in dairy products. Yeasts are involved in the ripening process of cheese and partake in microbial interactions and contribute to texture changes and the biosynthesis of aromatic compounds like volatile acids and carbonyl compounds (Ferreira and Viljoen, 2003). Yeasts in cheese are considered as insignificant at the earlier stages of cheese production, but play a role in later stages, being present as natural contaminants

in the curd during maturation (Welthagen and Viljoen, 1999). Traditionally, secondary/adjunct starters are not used in Cheddar-type cheeses but, the development and application of such cultures are among the promising approaches toward accelerating ripening (Fox *et al.*, 1996).

Flavour developments in fermented dairy products is a complex and, in the case of cheese ripening, more specifically PG activation – and PL activity, slow process involving chemical and biochemical conversions of milk components (van Kranenburg *et al.*, 2002). Flavour compounds are formed by various processes, that is, the conversion of lactose and citrate (glycolysis and pyruvate metabolism), fat (lipolysis) and caseins (proteolysis).

In chapter 2 it was found that certain yeast species displayed enzyme activities which could play a role during cheese ripening. The aim of this chapter is to manufacture model cheeses with the following yeast species: *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Yarrowia lipolytica* + *Debaryomyces hansenii*, *Dekkera bruxellensis*, *Dekkera bruxellensis* + *Yarrowia lipolytica*, *Torulaspora delbrueckii* + *Yarrowia lipolytica* and *Torulaspora delbrueckii* strains were inoculated in association with the starter lactic acid bacteria. The different yeasts were used as adjunct starter cultures and ripening agents of the cheese, some were used in combination and others alone. All the combinations had *Yarrowia lipolytica* because of its remarkable lipolytic and proteolytic activities (Welthagen and Viljoen, 1998, 1999; Ferreira and Viljoen 2003; de Wit *et al* 2005). *Torulaspora delbrueckii* was used because it was shown to display GDH- , PG activation - and PL enzyme activity. *Debaryomyces hansenii* and *Dekkera bruxellensis* was chosen because of their almost similar GDH enzyme activity. The aim was also to establish whether the yeast species added to the model cheeses could affect the sensory aspects.



## 3.2 Materials and methods

### 3.2.1 Selection of yeast species

According to the results obtained in chapter 2, as well as results of de Wit *et al* (2005), four yeast species were selected to be inoculated into model cheeses, *Debaryomyces hansenii* and *Dekkera bruxellensis* for GDH activity, *Yarrowia lipolytica* for PG activation/PL activity, and, *Torulaspota delbrueckii* for displaying both these enzyme activities. Although other yeasts such as the *Candida* species displayed similar enzyme activities, they were not included due to their pathogenic character.

Eight Cheddar cheeses were manufactured induplicate. The basic cheese making process was carried out with the addition of LAB (obtained from Dairybelle, Bloemfontein) and the following yeasts were added as adjunct starter cultures:

1. Standard without any adjunct culture inoculation (S)
2. *Debaryomyces hansenii* + *Yarrowia lipolytica* (D+Y)
3. *Debaryomyces hansenii* (D)
4. *Yarrowia lipolytica* (Y)
5. *Torulaspota delbrueckii* + *Yarrowia lipolytica* (T+Y)
6. *Torulaspota delbrueckii* (T)
7. *Dekkera bruxellensis* + *Yarrowia lipolytica* (Db+Y)
8. *Dekkera bruxellensis* (Db)

### 3.2.2 Microbiological analysis

To ensure that yeasts are present in the cheeses, the method described in Ferreira and Viljoen, (2003) was used. This part was done as a separate study and is not reported here, because the current project focused on the chemical changes that occur during ripening. However, the yeasts in 3.2.1 have been found in the miniature Cheddar cheeses.

### *3.2.3 Preparation of yeast inoculums*

The four yeast species chosen were all grown for 48h on YM media and a small amount was inoculated into 10ml of UHT milk. After 24h, a pre-inoculum was made by taking 0.5ml of the inoculated milk samples, inoculating in another 10ml of UHT milk, and left for another 24h. All the samples were incubated at room temperature. To determine the CFU ml<sup>-1</sup> of yeast species to be inoculated into the model cheeses, plate counts have been done.

For the plate counts, dilutions (10<sup>-1</sup> to 10<sup>-5</sup>) of the inoculated milk samples was made in peptone buffer and spread plated onto YM media. After three days the yeast colonies were counted and the concentration (CFU ml<sup>-1</sup>) of each yeast species to be inoculated into model cheeses was determined.

After determining the CFU ml<sup>-1</sup>, a calculated amount of each yeast species was inoculated into 200ml of pasteurised milk as described in 3.2.4 to obtain yeast growth of not more than 10<sup>4</sup> CFU for the 200ml.

### *3.2.4 Protocol for the manufacturing of model cheeses*

The starter culture, FD-DVS pHageControl R-700 Culture Series (CHR Hansen), was supplied by Dairybelle in Bloemfontein, South Africa. The starter culture was divided in amounts of 0.3605g and was frozen until used for cheese manufacture.

Miniature Cheddar cheeses were prepared as described by (Shakeel-Ur-Rehman *et al.*, 1998), but some changes were made to the protocol for use in this project. During the cheese manufacturing process, six wide-mouth plastic Beckman centrifuge bottles, here named cheese bottles, were filled with 200ml raw milk and one wide-mouth plastic centrifuge bottle, here named milk bottle, was filled with 250ml raw milk. Raw milk was obtained from Dairy Corporation, Bloemfontein, South Africa. The milk in the bottles was pasteurised at 63°C for 30 min, followed by cooling to 31°C, and maintained at this

temperature in a water bath. Starter (CHR Hansen), 0.3605g (calculated from the required amount needed for bulk inoculation), was added to the milk bottle containing 250ml of milk and stirred with a glass rod until the starter had dissolved. After stirring, 1ml of the starter-milk-mixture was added to the six cheese bottles containing 200ml of pasteurised milk and stirred. The six cheese bottles were inoculated with the yeast species or combinations thereof together with the starter bacteria (see also 3.2.1.1). The calculated volume of each yeast species to be added, was as follows: *Debaryomyces hansenii* (1.67µl), *Yarrowia lipolytica* (0.83 µl), *Torulaspora delbrueckii* (250µl) and *Dekkera bruxellensis* (10.64 µl). The milk was left to stand for 30 min at 31°C.

CaCl<sub>2</sub> (132µl of a 1mol.L<sup>-1</sup> solution for 200ml of milk) and rennet (43,5µl for 200ml of milk) was then added. The milk was kept for 40-50 min at 31°C until a firm coagulum formed. The coagulum in the cheese bottles was cut manually in 1cm blocks. The curd particles in the whey were heated for 2 min and then stirred slowly for 10 min using glass rods. The temperature was then increased to 38°C over 30 min and the curds/whey mixture stirred continuously at 38°C until the pH reached 6.2. Curd and whey, in the centrifuge bottles, were then centrifuged at room temperature for 60 min at 1700 g in a Beckman, Model J 2-21 centrifuge. These conditions were chosen to achieve a moisture level of 38-40 % in the miniature cheeses. The whey was drained and the curd held in the cheese bottles in a water bath at 38°C. Cheeses were inverted in the bottles and held until the pH decreased to 5.2-5.3 and then centrifuged at 1700 g for 20 min, with the top of the cheeses during the first centrifugation now on the bottom (to give a smooth surface on both sides). After further whey drainage, the cheeses were brine salted (50ml 20 % NaCl, 0.05 % CaCl<sub>2</sub>·2H<sub>2</sub>O) for 30 min at room temperature in the cheese bottles. After salting, the cheeses were removed from the bottles, wiped with tissue paper, vacuum packed and ripened at 8°C. All cheeses were manufactured in duplicate.

### 3.2.5 Sampling methods

Samples of the duplicate cheeses were taken during the manufacturing and ripening of the cheese at the following processing stages:

1. pasteurised milk
2. starter culture
3. whey
4. curd
5. brine
6. cheese at day 0
7. cheese at 2 months
8. cheese at 3 months
9. cheese at 4 months

Liquid samples were taken by aseptically pouring the liquid into a sterile McCartney bottle, and solid cheese samples by aseptically cutting and transferring into sterile bags. The eight cheeses in duplicate were kept under controlled conditions at 8°C and samples taken after processing were frozen. 10 g Samples were taken in duplicate and microbiologically analyzed. See also Ferreira and Viljoen (2003).

### *3.2.6 Moisture determination*

Sea sand, 20g, and a glass rod were placed in an aluminium dish and heated at 105°C for 1h according to the IDF method 4 (1958) to determine the dry matter in cheese and processed cheese. It was then cooled in a desiccator and weighed. About 3g of grated cheese was weighed into the dish and the contents mixed with the glass rod and dried in an oven at 105°C and left over night. The dish was then cooled in a desiccator and weighed again.

### *3.2.7 pH determination*

The pH of cheese samples was determined with a pointed glass electrode and a Hanna instruments 8521 pH meter.

### *3.2.8 Determination of enzyme activity in cheese*

Cheese (1g) was mixed either with 2 ml of Tris buffer (for PG activation and PL assay) or 2 ml of GDH buffer (for GDH assay) in a stomacher. The samples were frozen. The assays for external enzymes were carried out as described in chapter 2, sections 2.2.4.2, 2.2.4.3 and 2.2.4.4.

### *3.2.9 Sensory analysis*

The eight experimental cheeses were sensorically evaluated by a panel of experts in cheese manufacture and sensory evaluation. See Addendum 1 (page 142) for an example of the evaluation form. The sensory quality of the cheeses was evaluated based on a standard protocol including curd openness, texture, taste, flavour and maturity. The cheeses were evaluated after three, six and nine months of ripening.

All the yeasts used in this study have proteolytic activity which converts casein to peptides of different molecular weight and free amino acids which may have direct influence on cheese flavour or be precursors of flavour components (Suzzi *et al.*, 2001).

## **3.3 Results**

### *3.3.1 Microbiological analysis*

Microbial analysis was carried out to determine the growth and survival of bacteria and yeasts. It was carried out as a separate study and is not reported here. Only the major findings are reported as they have an influence on the results of this study. Enumeration of yeasts and lactic acid bacteria present during the processing and maturation stages of the seven cheese treatments showed both enhancement and inhibition in the survival of the lactic acid bacteria compared to the traditional mature Cheddar cheese produced as a control under similar conditions on the cheeses inoculated with (Db), (Db+Y), (Y) and (T). The lactic acid bacteria counts increased to values above 8 log units, although a

decline in numbers was observed after 2 months of maturation. The cheeses inoculated with (D), (D+Y) and (T) had counts of lactic acid bacteria which were lower than those of the control cheese, also with them a decline in counts was observed after 2 months.

In all the cheeses except the one inoculated with (T+Y), a rapid increase in yeast numbers during processing was observed, followed by a decline towards the end of the ripening period. On the cheese that was inoculated with (T+Y), the yeast counts after addition of the starter culture grew at a constant rate throughout the processing and ripening process, with counts never exceeding 4 log units.

This coincides with the findings of Ferreira and Viljoen (2003), who found that when (D) was used as a sole co-inoculum, the yeast numbers decreased gradually during the ripening period to a minimum value of  $4.25 \times 10^2$  CFU g<sup>-1</sup> after 4 months of ripening. When they used (Y) as a sole co-inoculum, yeast numbers also gradually decrease and ceased to survive after 4 months of cheese ripening. Finally, when they incorporated both yeasts as part of the starter culture, the combined yeast count initially decreased during the manufacturing process and they observed a substantial increase (>2-3 log units) compared to the cheeses which they inoculated with individual yeasts.

### 3.3.2 Moisture determination

The ideal amount of moisture that should be present in the cheese is 38-40% (Shakeel-Ur-Rehman *et al.*, 1998). Too much moisture in cheese could affect the pH and acidify the cheese so that the overall texture and cheese taste is influenced. This could lead to the formation of bitter flavours in the cheese. Some of the cheeses had moisture values above 38-40%, being (S) and the combinations of (D+Y) and (T+Y) (table 3.1). This may suggest that the cheeses should be centrifuged a third time to ensure the necessary moisture removal. Moisture determination was done once and no standard deviation analysis was performed on the cheeses.

**Table 3.1** Moisture content of 9 month old standard and yeast inoculated Cheddar cheeses

<u>Organism</u>	<u>% Moisture</u>
Standard	45.87
<i>Debaryomyces hansenii</i> and <i>Yarrowia lipolytica</i>	46.38
<i>Debaryomyces hansenii</i>	40.98
<i>Yarrowia lipolytica</i>	42.24
<i>Torulaspora delbrueckii</i> and <i>Yarrowia lipolytica</i>	44.37
<i>Torulaspora delbrueckii</i>	42.05
<i>Dekkera bruxellensis</i> and <i>Yarrowia lipolytica</i>	38.48
<i>Dekkera bruxellensis</i>	40.06

### 3.3.3 pH determination

The initial pH of the pasteurised milk ranged between 6.6 - 6.7 in the cheeses. The pH began to decrease after the addition of starter lactic acid bacteria, producing organic acids. In table 3.2 the pH is reported as determined over the ripening period of nine months. The pH of the cheeses tended to be uniform with only slight variations such as (T+Y) at eight months. This could be because too much moisture is present in some of the model cheeses that may have adversely affected the pH (table 3.2).

**Table 3.2** pH of 4, 5, 6, 8 and 9 month old standard and yeast inoculated Cheddar cheeses

<u>Cheese description</u>	<u>Months</u>				
	<b>4</b>	<b>5</b>	<b>6</b>	<b>8</b>	<b>9</b>
Standard	5.35	5.38	5.26	5.42	5.37
<i>Debaryomyces hansenii</i> and <i>Yarrowia lipolytica</i>	5.34	5.31	5.34	5.25	5.28
<i>Debaryomyces hansenii</i>	5.34	5.11	5.27	5.21	5.26
<i>Yarrowia lipolytica</i>	5.33	5.31	5.24	5.28	5.20
<i>Torulaspora delbrueckii</i> and <i>Yarrowia lipolytica</i>	5.10	5.11	5.09	4.86	5.28
<i>Torulaspora delbrueckii</i>	5.33	5.14	5.23	5.12	5.30
<i>Dekkera bruxellensis</i> and <i>Yarrowia lipolytica</i>	5.33	5.22	5.25	5.20	5.32
<i>Dekkera bruxellensis</i>	5.27	5.35	5.28	5.30	5.35

### 3.3.4 Enzyme activity in cheese

The levels of PG activation activity, PL activity and GDH activity that were detected were so low that no significant differences between any of the cheeses were obvious and also not over ripening time. It seems as if the method employed to detect these enzyme activities may not be sensitive enough. Results are therefore not shown and not further interpreted.

### 3.3.5 Sensory analysis

After three months ripening time, the cheeses inoculated with (D+Y), (D), (T), (Db+Y) and (Db) displayed acceptable sensory development (table 3.3).

**Table 3.3** Sensory evaluation of 3 month old standard and yeast inoculated Cheddar cheeses

<b><u>Number</u></b>	<b><u>Cheese description</u></b>	<b><u>Texture and taste</u></b>		<b><u>Discussion</u></b>
<b>1</b>	Standard	Soft, clean creamy	Good development	Very bitter after taste
<b>2*</b>	<i>Debaryomyces hansenii</i> and <i>Yarrowia lipolytica</i>	Soft, creamy	Good development	Off flavour, bitter
<b>3*</b>	<i>Debaryomyces hansenii</i>	Sturdy	Clean taste	A little bitter
<b>4</b>	<i>Yarrowia lipolytica</i>	Soft, clean taste	Mealy	Better Cheddar taste development, very bitter/rancid
<b>5</b>	<i>Torulaspora delbrueckii</i> and <i>Yarrowia lipolytica</i>	Sturdy, little creamy	Little mealy	Very bitter, rancid
<b>6*</b>	<i>Torulaspora delbrueckii</i>	Sturdy	Clean, good cheddar taste	A little bitter
<b>7*</b>	<i>Dekkera bruxellensis</i> and <i>Yarrowia lipolytica</i>	Sturdy	Clean, good cheddar taste	A little bitter Almost same as 4
<b>8*</b>	<i>Dekkera bruxellensis</i>	Sturdy	Off flavour, creamy	A little bitter Relative good cheddar taste

\* Good overall taste



At six months of ripening, only (D+Y) and (Db+Y) displayed acceptable sensory qualities (table 3.4) and at nine months (S), (D+Y), (Db+Y) and (Db) (table 3.5). Cheeses inoculated with (Y) and (T) as separate inoculants in all the cases were not evaluated positively at any stage.

**Table 3.4** Sensory evaluation of 6 month old standard and yeast inoculated Cheddar cheeses

<b><u>Number</u></b>	<b><u>Cheese description</u></b>	<b><u>Texture and taste</u></b>		<b><u>Discussion</u></b>
1	Standard	Unclean taste	Soft texture	Very bitter
2*	<i>Debaryomyces hansenii</i> and <i>Yarrowia lipolytica</i>	Cheddar taste, slight unclean taste	Soft texture	Slightly bitter
3	<i>Debaryomyces hansenii</i>	Slight Cheddar and unclean taste	Soft texture	Slightly bitter
4	<i>Yarrowia lipolytica</i>	Slight Cheddar, rancid taste	Soft texture	Very bitter, hides the cheese taste
5	<i>Torulaspora delbrueckii</i> and <i>Yarrowia lipolytica</i>	Unclean taste	Soft texture	Very bitter, hides the cheese taste
6	<i>Torulaspora delbrueckii</i>	Slight Cheddar and unclean taste	Soft texture	Bitter
7*	<i>Dekkera bruxellensis</i> and <i>Yarrowia lipolytica</i>	Cheddar and clean taste	Sturdy texture	Slightly bitter
8	<i>Dekkera bruxellensis</i>	Cheddar taste	Soft texture	Slightly bitter

\* Good overall taste

**Table 3.5** Sensory evaluation of 9 month old standard and yeast inoculated Cheddar cheeses

<b><u>Number</u></b>	<b><u>Cheese description</u></b>	<b><u>Texture and taste</u></b>		<b><u>Discussion</u></b>
1*	Standard	Slight clean cheddar after taste	Firm texture	Bitter
2*	<i>Debaryomyces hansenii</i> and <i>Yarrowia lipolytica</i>	Clean cheddar after taste	Firm texture	Slightly bitter
3	<i>Debaryomyces hansenii</i>	Clean after taste, slight cheddar taste	Firm texture	Slightly bitter, Sour
4	<i>Yarrowia lipolytica</i>	Slight bitter after taste	Soft-firm texture	Bitter
5	<i>Torulaspora delbrueckii</i> and <i>Yarrowia lipolytica</i>	Cheddar taste, slight clean after taste	Firm texture	Bitter
6	<i>Torulaspora delbrueckii</i>	Cheddar taste, slight yeasty after taste	Firm texture	Bitter
7*	<i>Dekkera bruxellensis</i> and <i>Yarrowia lipolytica</i>	Good cheddar taste, slight yeasty after taste, creamy	Firm texture	Slightly bitter
8*	<i>Dekkera bruxellensis</i>	Good clean cheddar taste	Firm texture	Slightly bitter, Sour

\*Good overall taste

Overall, the sensory evaluators observed that the cheeses inoculated with combinations of (D+Y) and (Db+Y) displayed a faster development of a Cheddar cheese flavour than the standard cheese, at least as early as six months of ripening. Separate inoculation of yeasts showed undesired flavour development right through the ripening period, with the exception of the cheese inoculated only with (Db) and starter lactic acid bacteria.

These results seem to repeat those of Ferreira and Viljoen (2003) that Cheddar cheeses inoculated with (D+Y) resulted in the development of a strong Cheddar cheese taste at four months of ripening compared to a control cheese. The observed accelerated Cheddar flavour development was attributed to the enhanced controlled breakdown of proteins by the yeasts (de Wit *et al.*, 2005).

### **3.4 Discussion**

Microbiological tests done on the first four months of ripening found that after two months of ripening, a decline in the numbers of the LAB were found in the cheeses inoculated with (D), (D+Y) and (T).

Therefore the yeasts might have started to overshadow the growth of the starter LAB and they could not work in harmony to produce good flavours in the Cheddar cheese. Thus, for future studies, it is recommended that the amount of the different yeast species to be inoculated into model cheeses, be calculated to ensure good aroma development in Cheddar cheese.

Some of the cheeses like (T+Y) and (T) had bitter, rancid off-flavours and soft texture, while others like (D+Y) had a firmer texture. It is possible that the moisture ratio was incorrect (Thage *et al.*, 2005) (see section 3.3.2, table 3.1) and could have contributed to proteolytic activities.

Too much moisture in Cheddar cheese is not ideal, since it might adversely affect the pH of the cheese. Reduced-fat, semi hard cheese with high moisture content often fails to produce a balanced intense flavour (Thage *et al.*, 2005). Some of the cheeses had moisture levels in the range of 38-40% as suggested by Shakeel-Ur-Rehman *et al* (1998), give or take 2%. The cheeses with (D+Y) and (T+Y) had higher moisture values than the others. Even though (D+Y) had high moisture and pH values (table 3.1 and 3.2), they still developed good flavours during the ripening process. It could therefore be deduced that it

is the enzyme activity of (T+Y) and (T) that result in bitter flavours and not the moisture or pH. However, proof will be provided in the next chapter.

Over the ripening period of nine months, the combinations of (D+Y) and (Db+Y) were the only cheeses to show good development of the Cheddar/cheese taste and development of a firm texture. Even if these cheeses did produce some off flavours, they still showed the best potential of developing a Cheddar taste.

At three months ripening, the cheeses with (T+Y) and (T) developed a sturdy texture and even a good Cheddar taste (table 3.3). But, at six and nine months ripening time, these cheeses developed bitter compounds and unclean tastes (tables 3.4 and 3.5). *Torulaspota delbrueckii* and *Yarrowia lipolytica* were the yeast species that had the highest amount of PL/PG activation activity (see chapter 2, tables 2.7 and 2.8). The reason might be that proteolysis started out very fast so that bitter compounds formed during the ripening period. It is also possible that the high activity of subtilisin, trypsin and chymotrypsin of *Torulaspota delbrueckii* may contribute to the release of bitter peptides.

To relate the observed sensory aspects to a chemical explanation, the dynamic proteolysis had to be studied. HPLC (High Performance Liquid Chromatography) and UREA-PAGE was carried out on the WSN and WISN fractions of the cheeses which is reported in chapter 4.

## Chapter 4

### PROTEOLYSIS DURING CHEESE RIPENING

#### 4.1 Introduction

Primary proteolysis in cheese may be defined as those changes in  $\beta$ -,  $\gamma$ -,  $\alpha_s$ -caseins, peptides, and other minor proteins that are detected by PAGE (Rank *et al.*, 1985). Primary proteolysis leads to the formation of large water-insoluble peptides and smaller water-soluble peptides (Mooney *et al.*, 1998). Secondary proteolysis products could include those peptides, proteins and amino acids soluble in the aqueous phase of cheese and are extractable as the WSN fraction. The WSN fraction is a complex mixture of large, medium, and small peptides and amino acids. These components result from the action of milk clotting enzymes, milk proteases, starter lactic acid bacteria and contaminating microorganisms (Rank *et al.*, 1985).

The primary role of rennet is to coagulate cheesemilk, but it also plays a major role in proteolysis during maturation and is the principal proteolytic agent responsible for the level of proteolysis detected by gel electrophoresis (Grappin *et al.*, 1985) and for most of the nitrogen soluble in water or in pH 4.6 buffers (O'Keeffe *et al.*, 1976). The coagulant contributes indirectly to the development of Cheddar cheese flavour by producing large- and medium-sized peptides, which act as substrates for starter (and non-starter) proteinases and peptidases which produce small peptides and free amino acids (FAA), from these substrates (Lane *et al.*, 1997). These peptides and amino acids may contribute directly to flavour or serve as precursors of flavour compounds (Lane and Fox, 1997; Lane *et al.*, 1997).

The balance between the formation of peptides and their subsequent degradation into free amino acids is very important since accumulation of peptides might lead to a bitter off-flavour in cheese. Various bitter-tasting peptides have been identified and especially these peptides should be degraded rapidly in order to prevent bitterness (Smit *et al.*,

1998). Five peptides (rich in proline) responsible for bitterness in Cheddar cheese were isolated, namely  $\alpha_{S1}$ -CN (f1-7), (f1-13), (f11-14),  $\alpha_{S2}$ -CN (f191-197) and  $\beta$  (f8-16) (Sousa *et al.*, 2001).

Nitrogen solubility under defined conditions is probably the most widely used method to investigate proteolysis (Kuchroo and Fox, 1982). Several criteria, including nitrogen solubility in a range of solvents and rheological measurements may be used as indices of proteolysis, with electrophoresis being the most useful and widely used method especially for young cheeses (Shalabi and Fox, 1987). Fractionation with water is an efficient procedure for separating the larger peptides in cheese from the smaller ones (Christensen *et al.*, 1991).

In gel electrophoresis the component proteins and peptides are separated either on the basis of their net charge or by molecular weight and visualized by staining and a variety of protein specific dyes. Gel electrophoretic studies showed that the water-soluble fraction contains whey protein, low molecular weight peptides from hydrolysis of casein and free amino acids (Shalabi and Fox, 1987). Cheddar cheese flavour has been located to the water-soluble fraction. The fraction non-soluble in water contains caseins and high molecular weight peptides from the casein hydrolysis and can be analysed by UREA-PAGE. Electrophoretograms of the insoluble fraction are very similar to those of unfractionated cheese and give little information (Shalabi and Fox, 1987).

In chapter 2 certain yeast species that might play a role in accelerated cheese ripening, were selected to be inoculated into model cheeses, chapter 3, with the starter lactic acid bacteria. In chapter 3 sensory evaluations was carried out on the cheeses. The aim of this chapter is to determine the chemical changes in these cheeses in order to explain whether PG activation-, PL- and GDH activity plays the important role in cheese ripening as was set out in the aim of this study (chapter 1) and in the sensory observations (chapter 3).

## 4.2 Materials and Methods

### 4.2.1 Cheeses

Eight Cheddar cheeses were manufactured in duplicate as was described in chapter 3, section 3.2.1 and 3.2.4. They were:

1. Standard without any yeast adjuncts (S)
2. *Debaryomyces hansenii* + *Yarrowia lipolytica* (D+Y)
3. *Debaryomyces hansenii* (D)
4. *Yarrowia lipolytica* (Y)
5. *Torulaspora delbrueckii* + *Yarrowia lipolytica* (T+Y)
6. *Torulaspora delbrueckii* (T)
7. *Dekkera bruxellensis* + *Yarrowia lipolytica* (Db+Y)
8. *Dekkera bruxellensis* (Db)

All the chemical analysis were carried out on the duplicate cheeses.

### 4.2.2 Primary proteolysis

#### 4.2.2.1 Extraction of water-soluble nitrogen (WSN) and water-insoluble nitrogen (WISN) in cheese

Extraction of the WSN was done according to the method of Kuchroo and Fox (1982), where the water-soluble fraction was obtained by homogenizing the cheese with water at a ratio of 1:2 (10g grated cheese + 20ml distilled water) with and Ultra-Turrax T25 (Janke & Kunkel IKA-Labortechnik) at 9500 ( $\text{min}^{-1}$ ) for 1min. The homogenate was then heated to 40°C for 60min and centrifuged (Beckman Model J2-21) at 3000g (5000 rpm) for 30min. This resulted in three distinct fractions; the fat (which was discarded) and the water-soluble and -insoluble fractions. The water-soluble fraction (supernatant) was filtered through glass wool (B. Owen Jones LTD. and McDonald Adams). The procedure

was repeated on the precipitate for complete extraction. Approximately 70% of the soluble N was extracted in the first cycle and a further 20% in the second cycle so that approximately 90% of the WSN was extracted.

Both the WSN and WISN fractions were freeze-dried and frozen at -20°C until analyzed (not longer than three months).

#### *4.2.2.2 Nitrogen (N) determination*

The nitrogen in cheese, total nitrogen (TN) and WSN was determined by means of thermal combustion on a Leco Protein/Nitrogen FP-528 Determinator and the WSN expressed as a percentage of TN (Leco Corporation, 2001).

#### *4.2.2.3 Cd-ninhydrin (CdNR) analysis*

Analysis of the free amino acids was done according to the method of Folkertsma and Fox (1992). A sample of 20µl of WSN was diluted to 1ml in distilled water. To this was added 2ml Cd-ninhydrin reagent (0.8g ninhydrin dissolved in a mixture of 80ml 99.5% ethanol and 10ml acetic acid, followed by the addition of 1g CdCl<sub>2</sub>, dissolved in 1ml distilled water). The mixture was heated at 84°C for 5 min, cooled and the absorbance determined by a Genesys 10 Vis Thermo Spectronic spectrophotometer at 507nm. A blank (reagent without WSN) was prepared. Analysis was performed in duplicate. The results were expressed as mg leucine.g<sup>-1</sup> using a standard curve. A standard curve was prepared by diluting a stock solution of 10mM of leucine in distilled water to a range of 0.5-10mM with distilled water (Lane and Fox, 1997).

#### *4.2.2.4 Analysis by UREA-PAGE*

UREA-PAGE was carried out as described in chapter 2, section 2.2.5.2.



#### 4.2.2.5 Statistical analysis

Differences between the standard and yeast inoculated Cheddar cheeses were determined by analysis of variance (ANOVA) (NCSS, 2004). The Tukey-Kramer multiple comparison test was used to identify differences between treatment means (NCSS, 2004).

#### 4.2.3 Secondary proteolysis

RP- HPLC has been used increasingly to characterize peptides in casein hydrolysates (Bicon, 1983). HPLC patterns becomes more complex as the cheese matures and are useful indices of cheese maturity, and to a lesser extent of its quality and therefore may potentially be useful in the objective assessment of cheese quality (Smith and Nakai, 1990; Fox, 1993; O'Shea *et al.*, 1996).

The aim was to analyze the WSN fraction of the cheese samples to determine if any changes occurred in the peptide peak development during ripening that might help to explain why some cheese sets developed bitter compounds.

##### 4.2.3.1 Sampling methods for RP- HPLC

Sampling of cheeses was discussed in section 4.2.2. Sample preparation was done according to the method of Law *et al.* (1992). Lyophilized samples of the WSN fraction were dissolved in deionized water (DDW) (Milli-Q water) (4mg ml<sup>-1</sup>), and filtered through a 0.45µm Cameo (Separations) pre-filter. The filtrate was stored at -20°C until analysed (not longer than three months).

##### 4.2.3.2 Peptide analysis by RP-HPLC

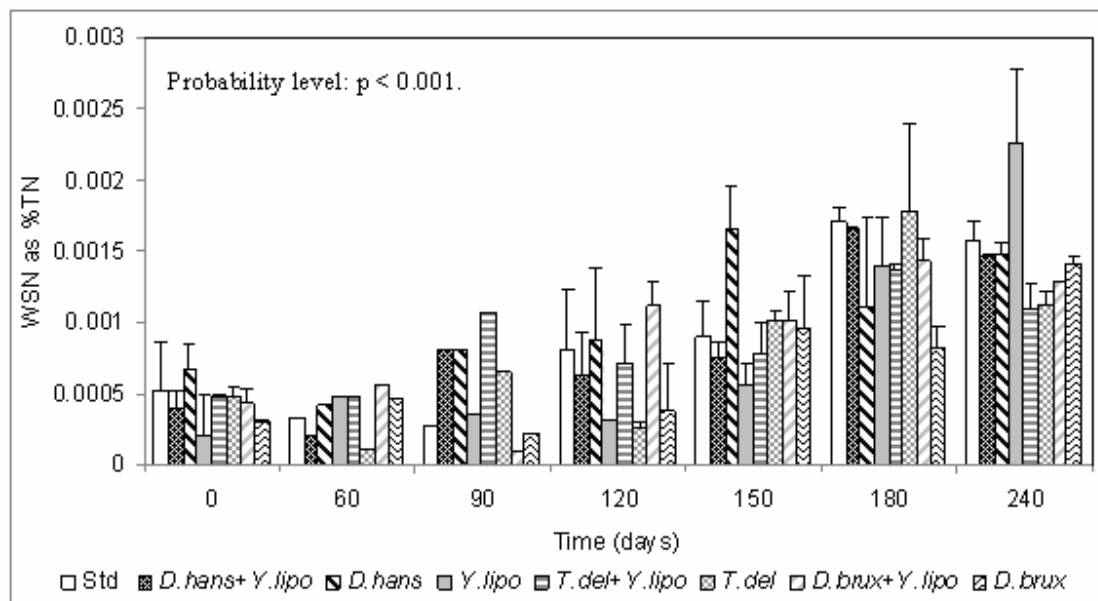
HPLC was carried out on a Thermo Finnigan Surveyor system with a PDA detector, operated at 230 nm. Samples were chromatographed on a 5µm Jupiter (Separations) RP18 250mm x 4,6mm reversed-phase column preceded by a Jour-Guard RP/C18 - 5µm

guard column (Separations) at ambient temperature. A linear gradient of 0.15% TFA, (ion-pairing agent) in DDW (eluant A) to 0.1% TFA (Merck) in 80:20(v/v) acetonitrile (Ultrafine Limited) in DDW(eluant B) was increased to 70% B over 70min at 1ml/min flow rate. Solvents were degassed under vacuum before use.

## **4.3 Results**

### *4.3.1 Nitrogen (N) analysis*

In fig. 4.1 the results of the nitrogen analysis are given. WSN as % of TN only gives information about the extent of proteolysis. Proteolysis was analysed by fractionating the WSN as well as WISN, and study them by PAGE (section 4.3.3) and HPLC (section 4.3.4). There is a tendency that the WSN as %TN increases during ripening from approximately 0.0003 to 0.0023 % (fig. 4.1). It seems as if optimum development of the WSN fractions was obtained at 180 - 240 days. These results of an increase in WSN as % of TN are in agreement with those of Lane and Fox (1996) and de Wit *et al* (2005). No significant statistical difference ( $p < 0.001$ ) was observed between the cheeses at different ripening times.



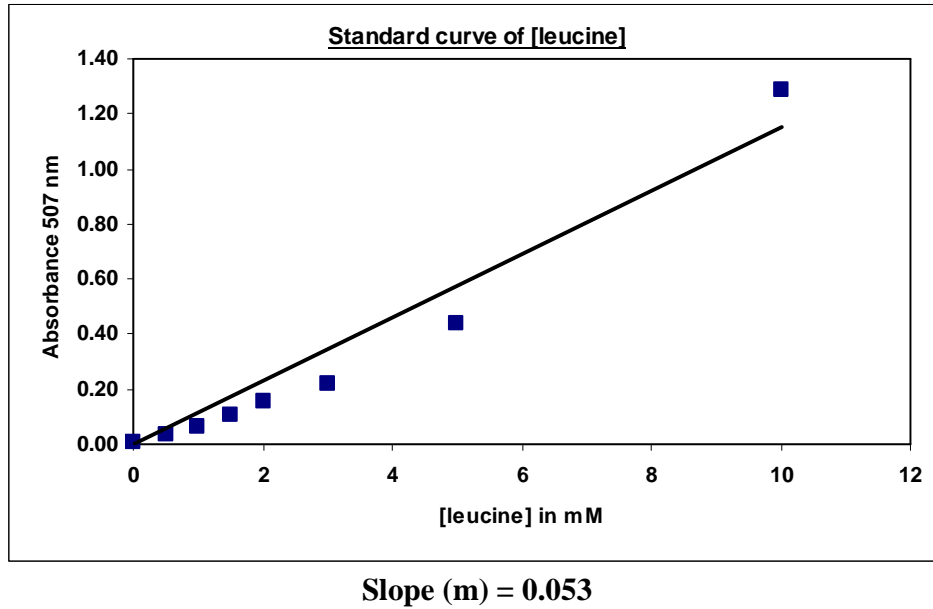
**Fig. 4.1** Changes in WSN as % TN during ripening

#### 4.3.2 Cd-ninhydrin analysis

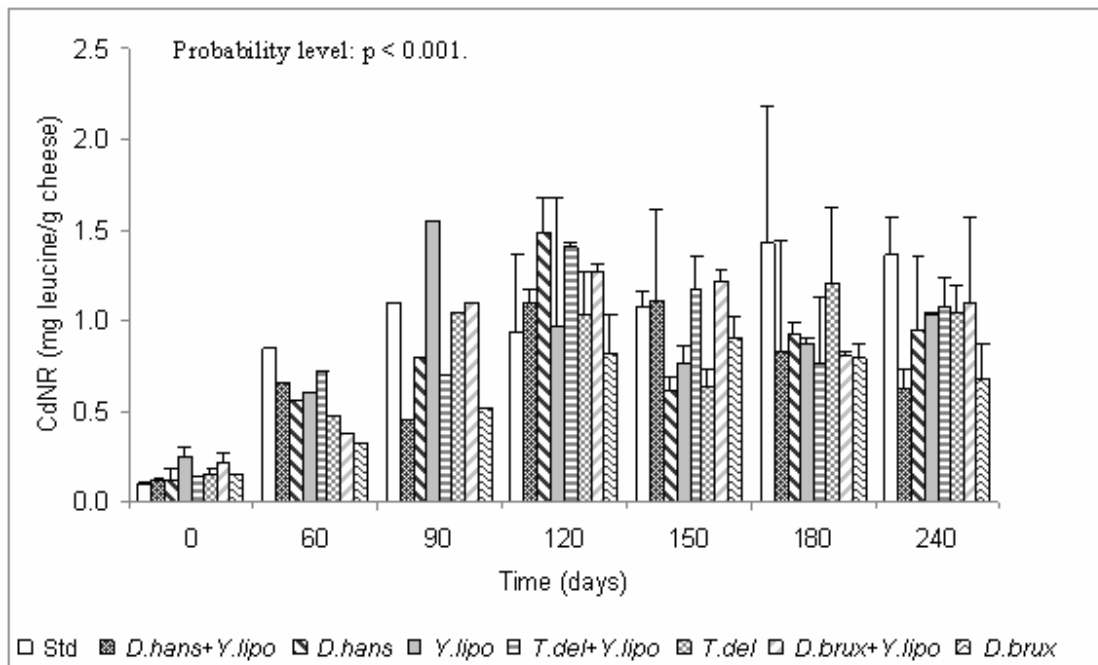
Plasmin action does not result in the production of free amino acids but is responsible for production of intermediate-sized peptides (Farkye and Fox, 1992), which are precursors for production of free amino acids by enzymes of starter bacteria (Upadhyay *et al.*, 2006). Lane and Fox, (1996) also indicated that the proteinase of starter bacteria is important for the production of small peptides and the accumulation of amino acids in cheese during ripening and that the coagulant contributes little to the liberation of free amino acids. Starter and non-starter proteinases and peptidases are mainly responsible for the production of amino acids (Shakeel-Ur-Rehman *et al.*, 2003).

Fig. 4.2 is a graph of leucine standards. The FAA increases over ripening time (fig. 4.3) from an approximate minimum value of 0.1 mg leucine g cheese<sup>-1</sup> at day 0 to an approximate maximum value of 1.5 mg leucine g cheese<sup>-1</sup> at day 120 which agrees with the results of Lane and Fox, (1996) and de Wit *et al.* (2005).

Statistically, no significant difference ( $p < 0.001$ ) was observed between the cheeses at different ripening times.



**Fig. 4.2** Standard curve for determination of free amino acids (as mg leucine g cheese<sup>-1</sup>).



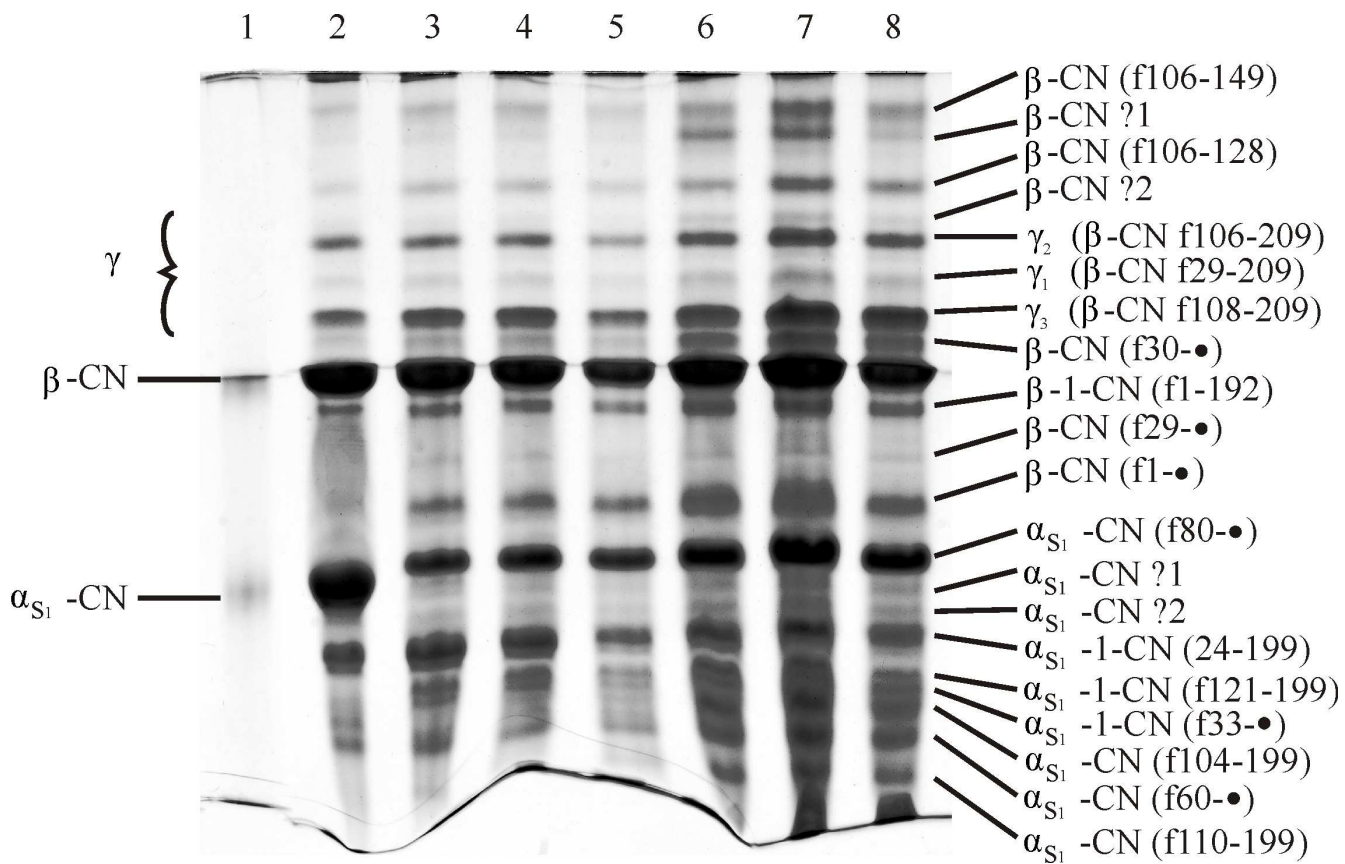
**Fig. 4.3** Changes in free amino acids (CdNR) (as mg leucine g cheese<sup>-1</sup>) over an eight month ripening period for the eight cheeses.

### 4.3.3 UREA-PAGE

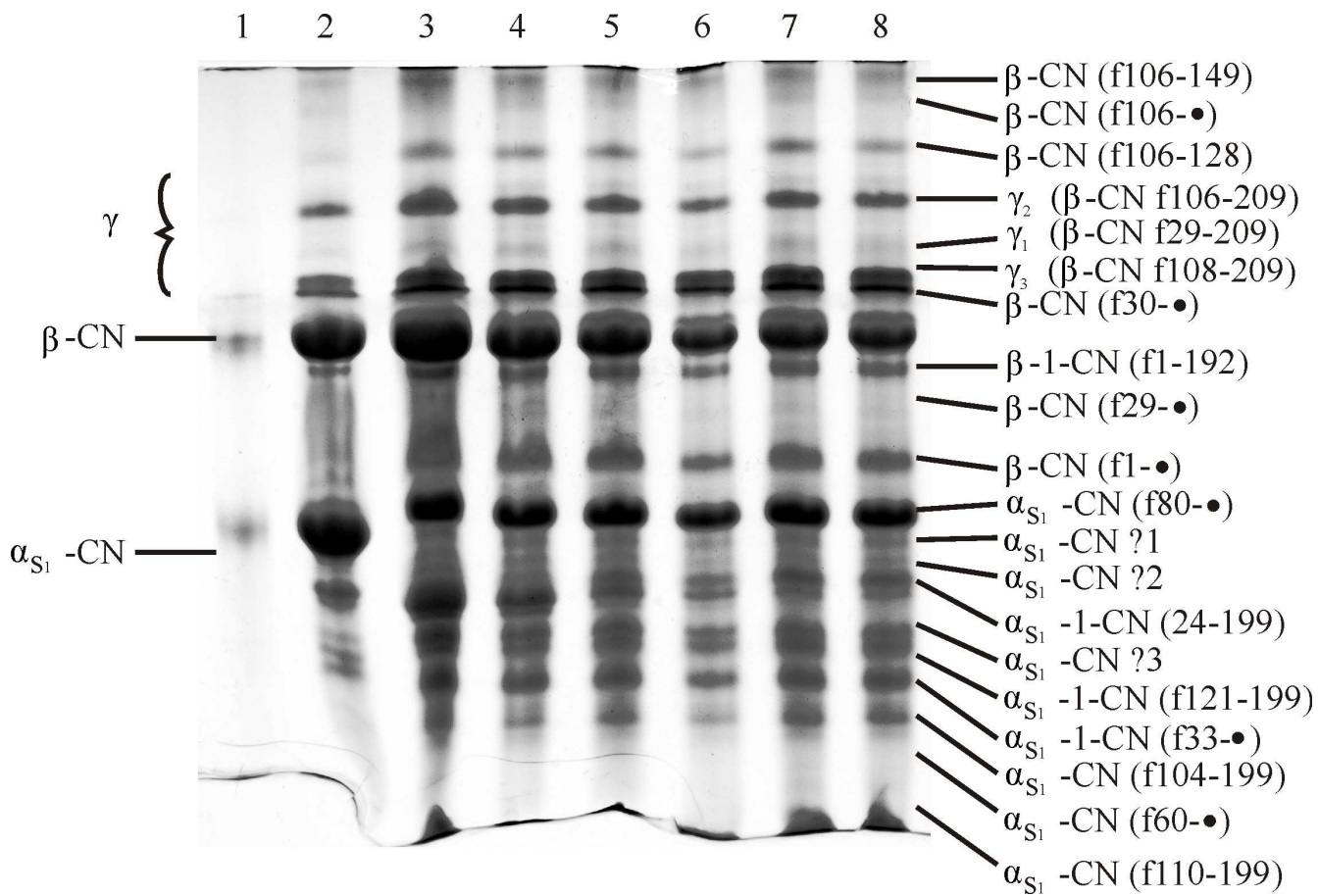
#### 4.3.3.1 Water-insoluble nitrogen peptides (WISN)

Primary proteolysis leads to the formation of large water-insoluble peptides and small water-soluble peptides (Mooney *et al.*, 1998). Mooney *et al.* (1998) also fully or partially identified the WISN fractions of mature Cheddar cheese. The principal water-insoluble peptides are produced either from  $\alpha_{S1}$ -CN by chymosin or from  $\beta$ -CN by plasmin (McSweeney *et al.*, 1994; Mooney *et al.*, 1998).  $\gamma$ -caseins accumulate in all the cheeses and para- $\kappa$ -casein appears to be resistant to proteolysis and no peptides produced from it have been identified (Fox and McSweeney, 1996).

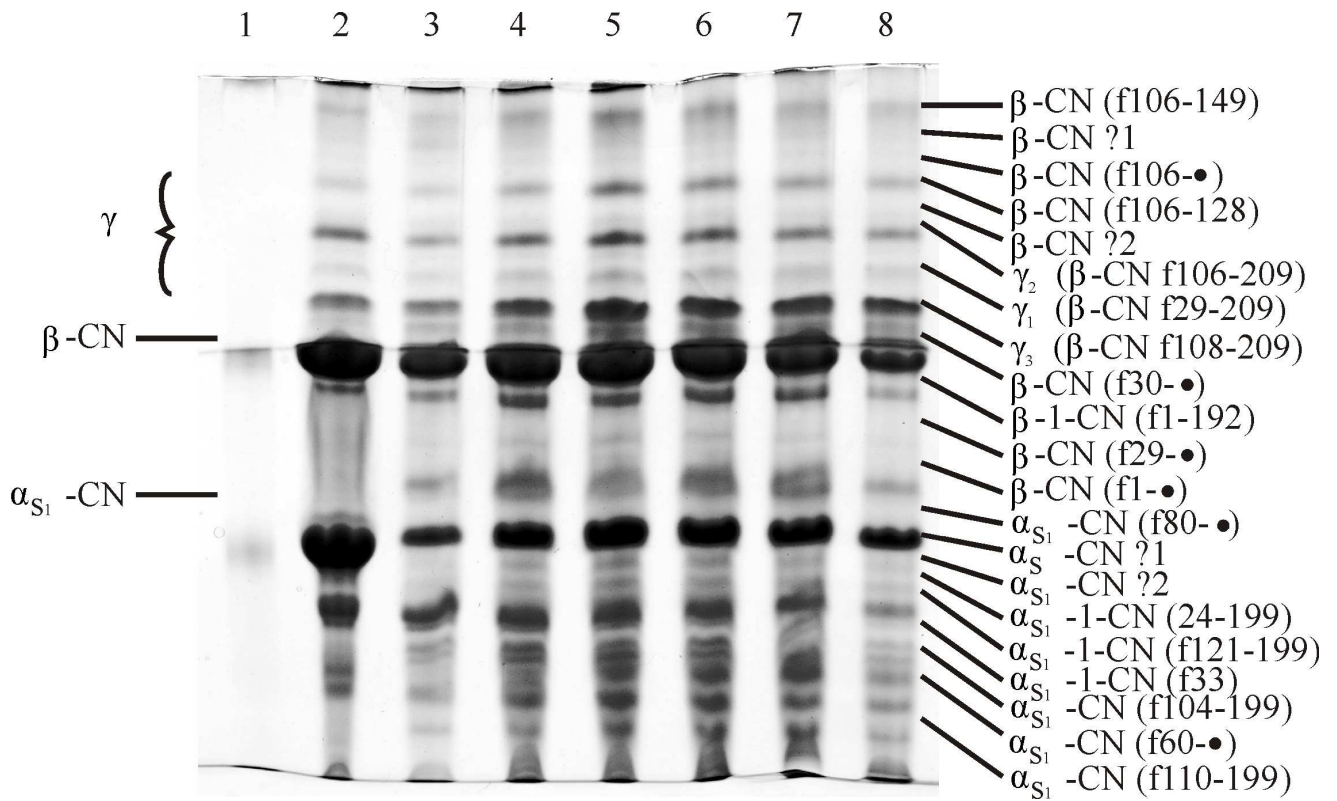
With UREA-PAGE gels (fig. 4.4 – 4.11) of the WISN fraction, the  $\beta$ -CN and  $\alpha_{S1}$ -CN are hydrolyzed to peptides. The WISN fraction of the standard cheese (fig. 4.4) shows that high amounts of the  $\beta$ -CN<sup>1</sup> fraction as well as  $\beta$ -CN (f30- •) develop at a later stage of ripening (day 150-240). The cheeses inoculated with yeasts did not display such a strong development of  $\beta$ -CN<sup>1</sup> and  $\beta$ -CN (f30- •) fractions so clearly seen with the standard cheese. This could mean that caseins in the yeast inoculated cheeses were hydrolyzed differently compared to the standard cheese. The  $\beta$ -CN derived peptides f29-209 and  $\beta$ -CN f29-•, which are formed due to PL hydrolysis at Lys<sup>28</sup> – Lys<sup>29</sup>, for all the cheeses are very light, but development started early on and the intensity increased (fig. 4.4 – 4.11). No other differences could be observed.



**Fig. 4.4** Urea-PAGE of the WISN fraction of standard Cheddar cheese. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

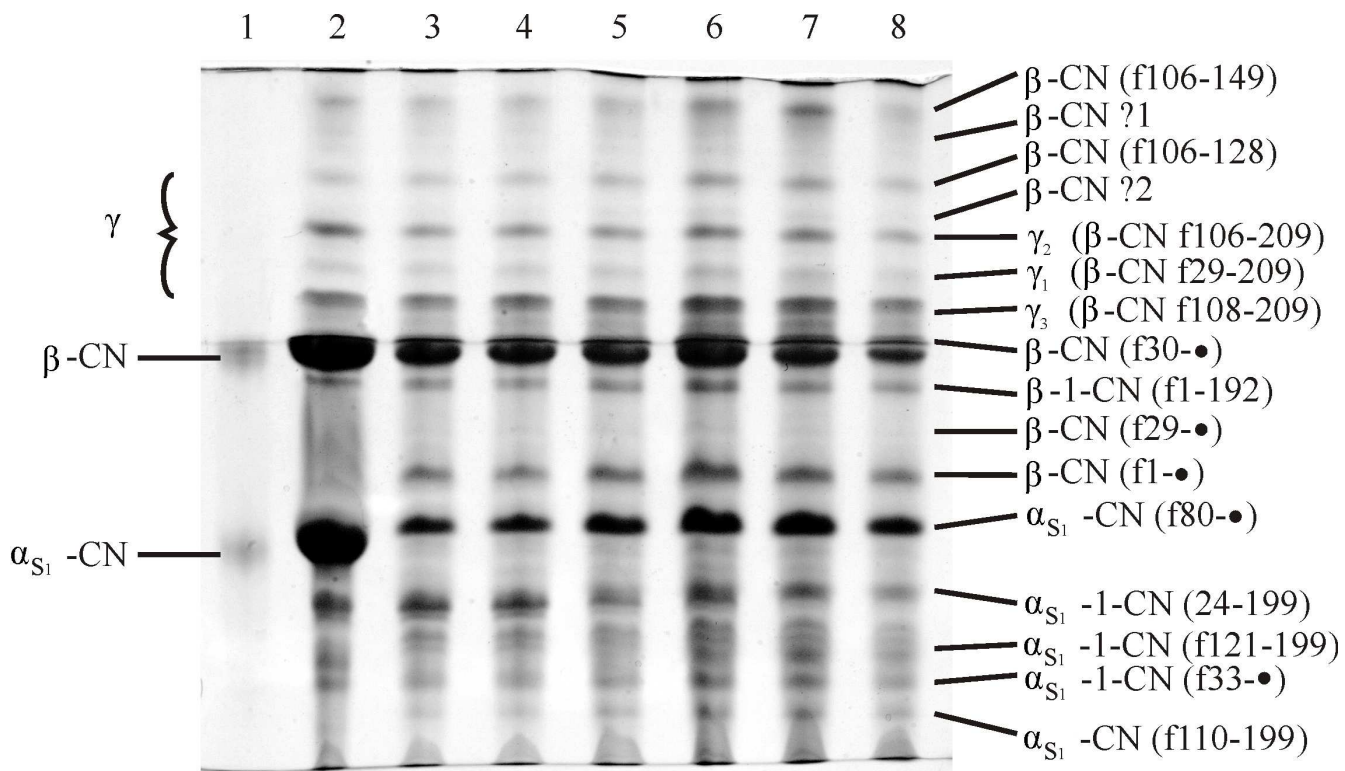


**Fig. 4.5** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Debaryomyces hansenii* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

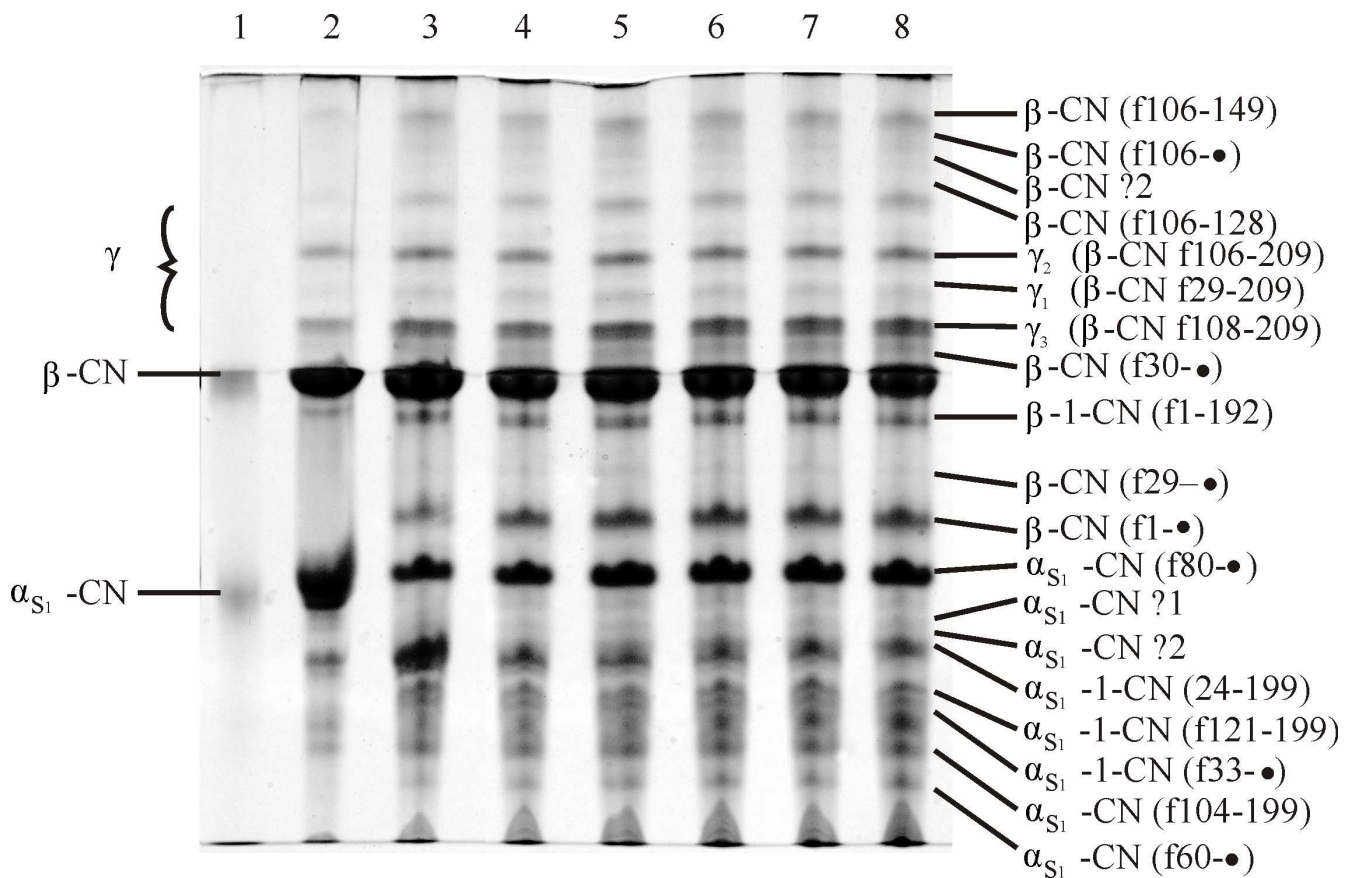


**Fig. 4.6** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Debaryomyces hansenii*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

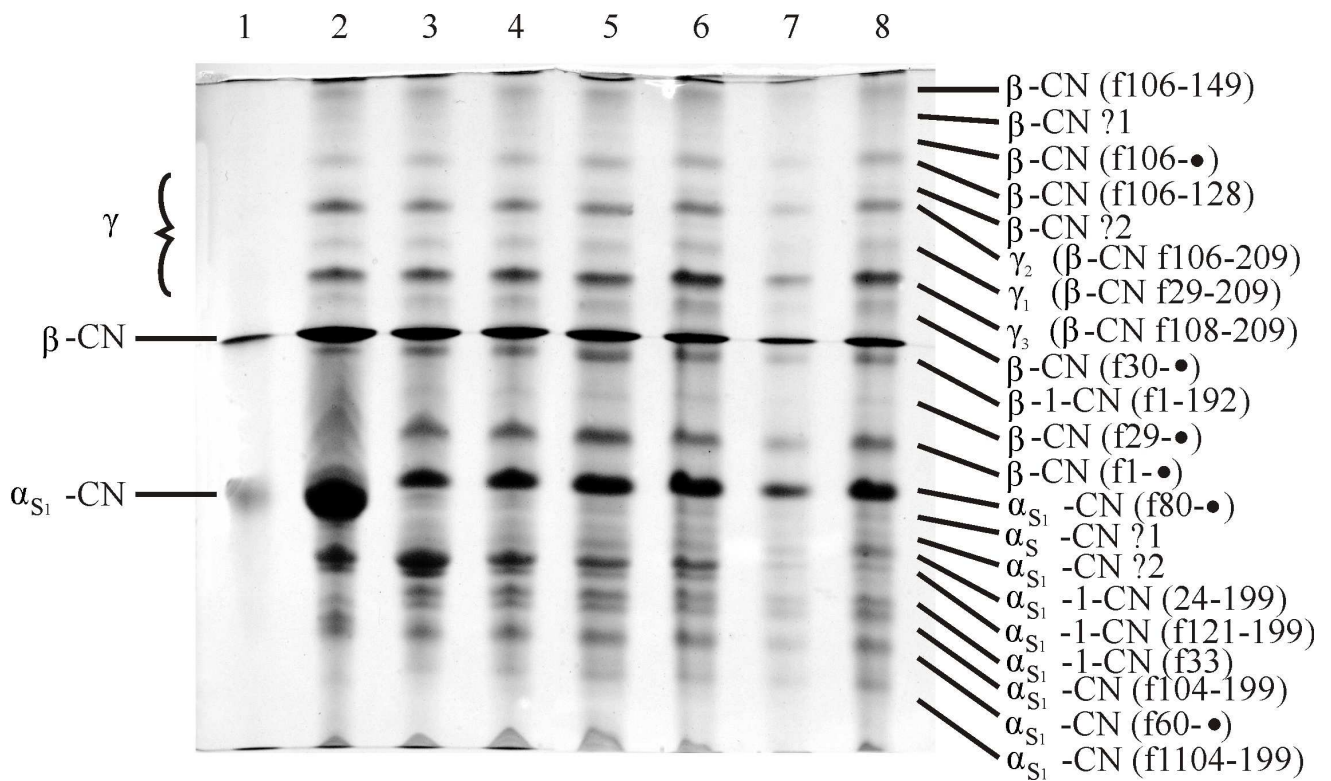




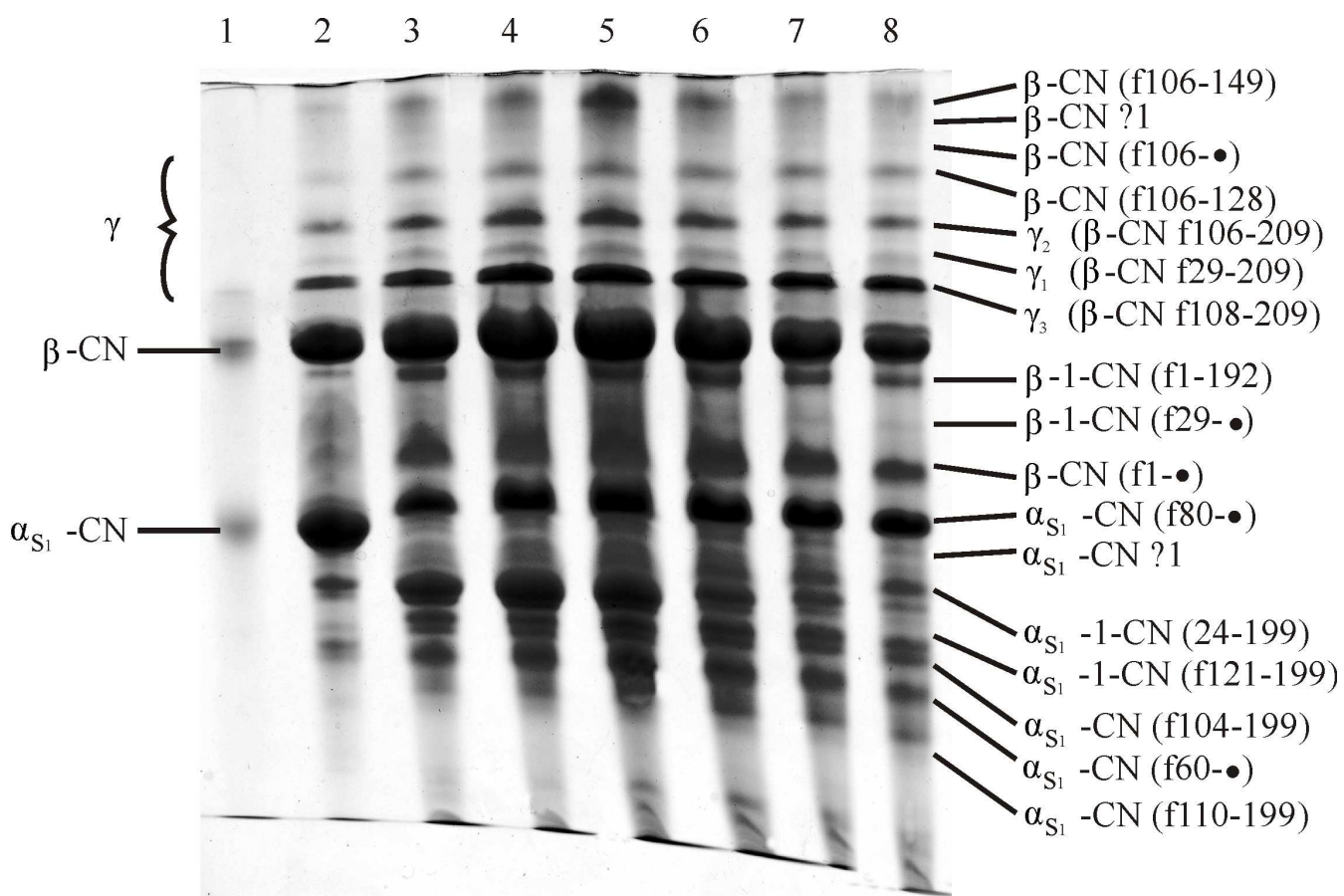
**Fig. 4.7** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



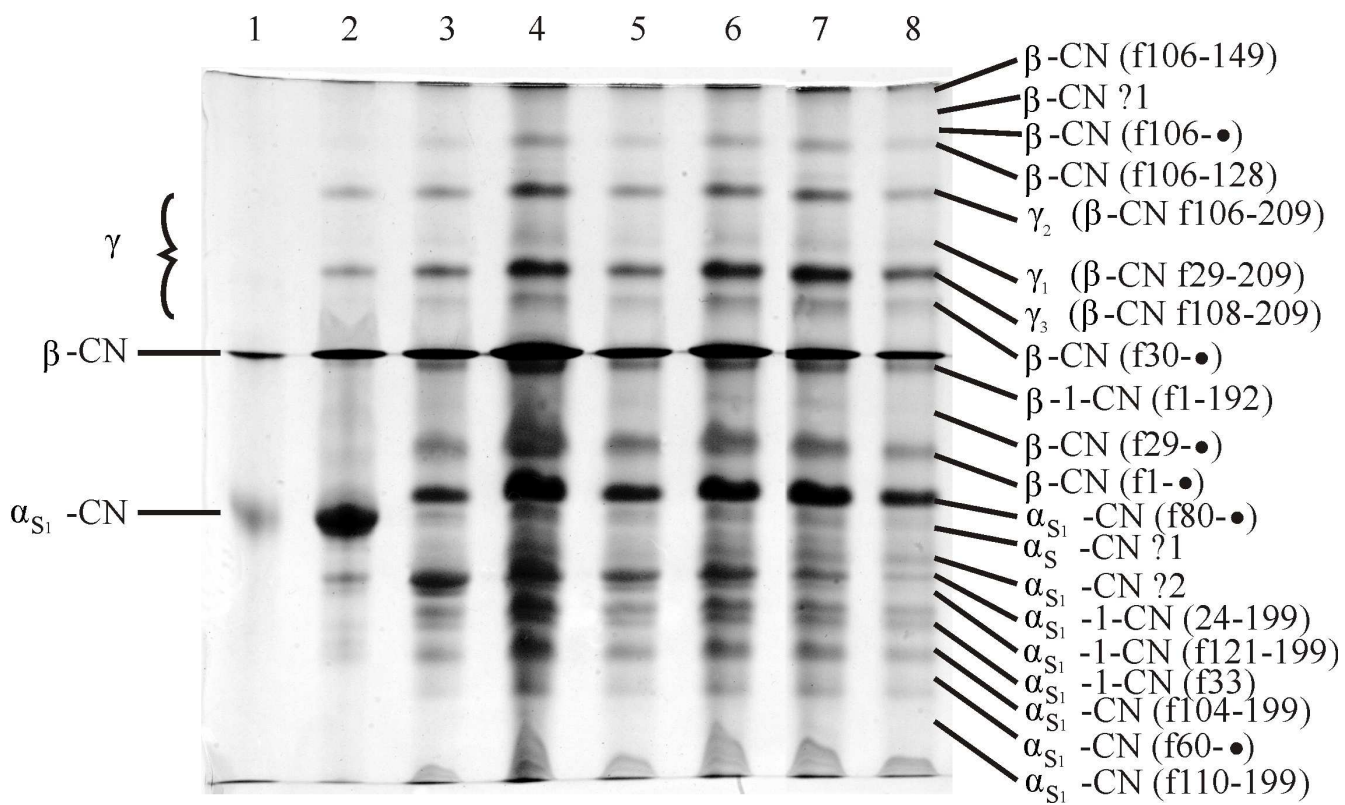
**Fig. 4.8** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Torulaspora delbrueckii* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.9** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Torulaspora delbrueckii*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.10** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Dekkera bruxellensis* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.11** Urea-PAGE of the WISN fraction of Cheddar cheese inoculated with *Dekkera bruxellensis*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

#### 4.3.3.2 Water soluble nitrogen peptides (WSN)

Many of the peptides in the water-soluble fraction have been isolated and identified (Singh *et al.*, 1995, 1997; Mooney *et al.*, 1998). Most of the peptides from the WSF (water-soluble fraction) of Cheddar cheese are derived from the N-terminal half of  $\beta$ -casein (especially from residues 53 to 91) with a smaller number from the N-terminal half of  $\alpha_{S1}$ -casein (Singh *et al.*, 1997; Sousa *et al.*, 2001).

In all the cheese samples (fig. 4.12 – 4.15, 4.17 – 4.19)  $\beta$ -CN (58-?) fraction is displayed as a light band. However, in cheese (T+Y) the  $\beta$ -CN (58-?) fraction is visible as a dark and sharp band (fig. 4.16). The intensity of the fractions  $\beta$ -CN (1-?)\* and  $\beta$ -CN (1-?) decreases during ripening of 240 days in all the cheeses (fig. 4.12 – 4.19). This is because the  $\beta$ -CN is hydrolyzed into peptides which in time are further hydrolyzed during the ripening process. The fractions  $\beta$ -CN (1-?)\* and  $\beta$ -CN (1-?) and fraction  $\beta$ -CN (45-?) are still more visible and clearly defined from approximately day 90 of ripening in the cheeses (T+Y) (fig. 4.16) and (T) (fig.4.17) than in the other cheeses.

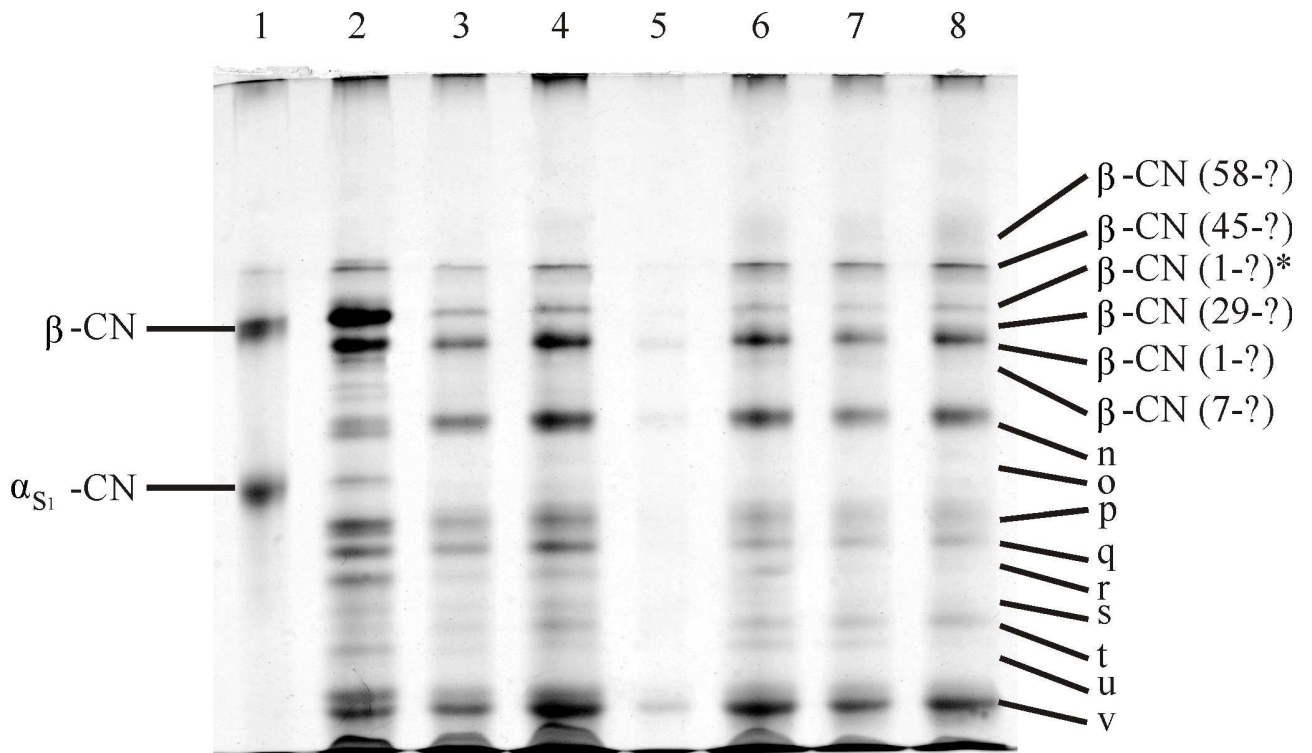
In the cheeses (D+Y), (T+Y), (Db+Y) and (Db) (fig. 4.13, 4.16, 4.18 and 4.19), an extra peptide band (L) develops during the first 60 days of ripening that is not visible in the other samples (fig. 4.12, 4.14, 4.15 and 4.17), but it disappears immediately after 60 days. It is possible that this peptide L is hydrolyzed at a faster rate in the latter cheeses so that it can no more be detected at 60 days ripening. The  $\beta$ -CN (29-?) fraction starts to develop approximately at day 150 as a very faint band above  $\beta$ -CN (1-?) in (S), (D+Y), (D), (Db+Y) and (Db) (fig. 4.12 – 4.14 and 4.18 – 4.19). But the  $\beta$ -CN (29-?) fractions develop at approximately day 60 for (Y), (T+Y), (T) (fig. 4.15 – 4.17).

In all the cheeses (fig. 4.12 – 4.19) the following peptides were affected as follows:

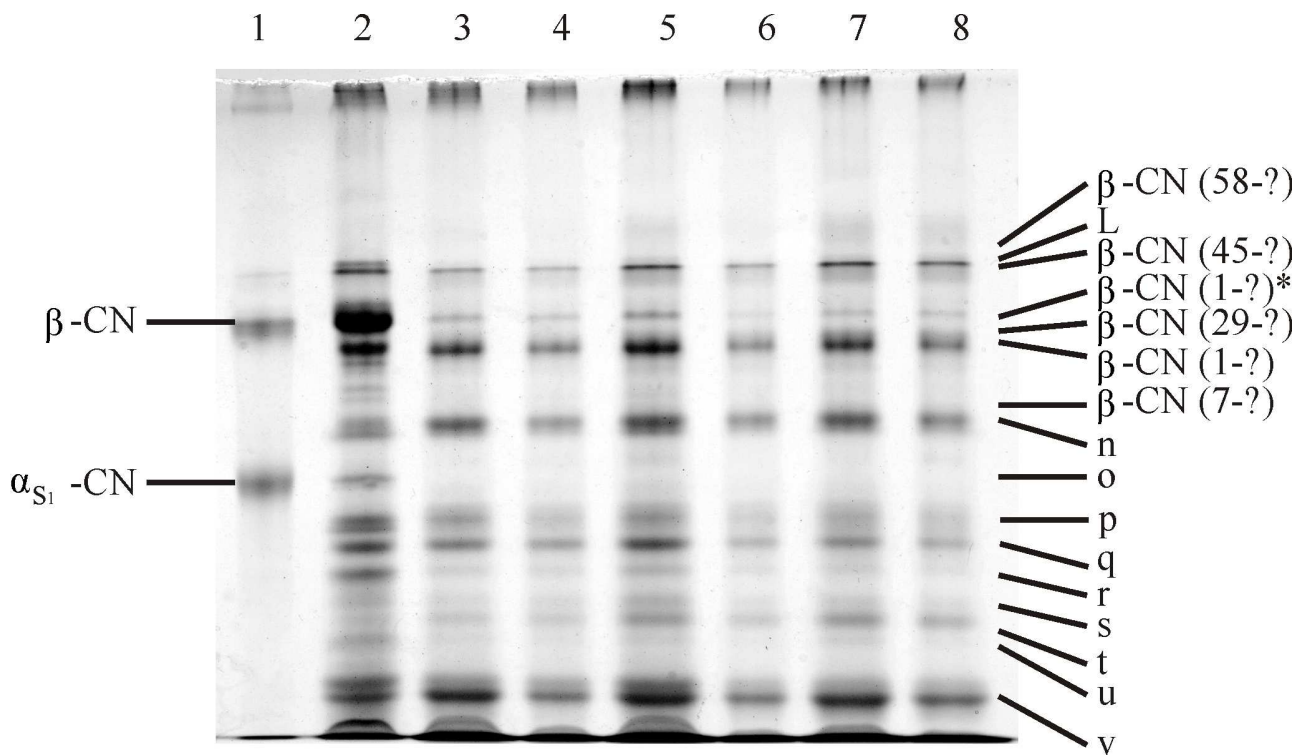
- o – is hydrolyzed immediately at day 0
- p – is hydrolyzed and the intensity decreases
- q – is not hydrozed
- r – is hydrolyzed and disappears



- s – is hydrolyzed and disappears
- t – is not hydrolyzed
- u – is hydrolyzed and fades away
- v – is not hydrolyzed

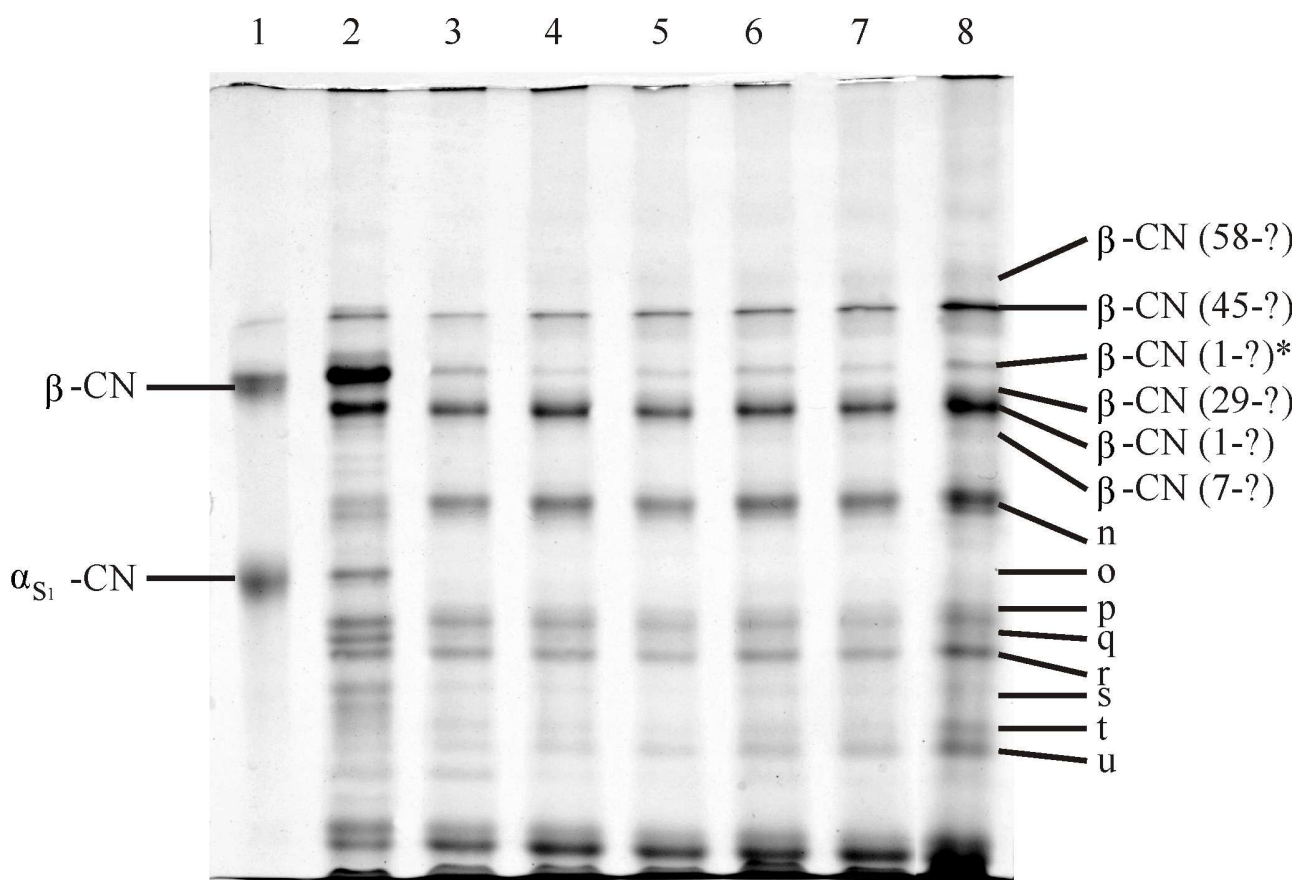


**Fig. 4.12** Urea-PAGE of the WSN fraction of standard Cheddar cheese. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

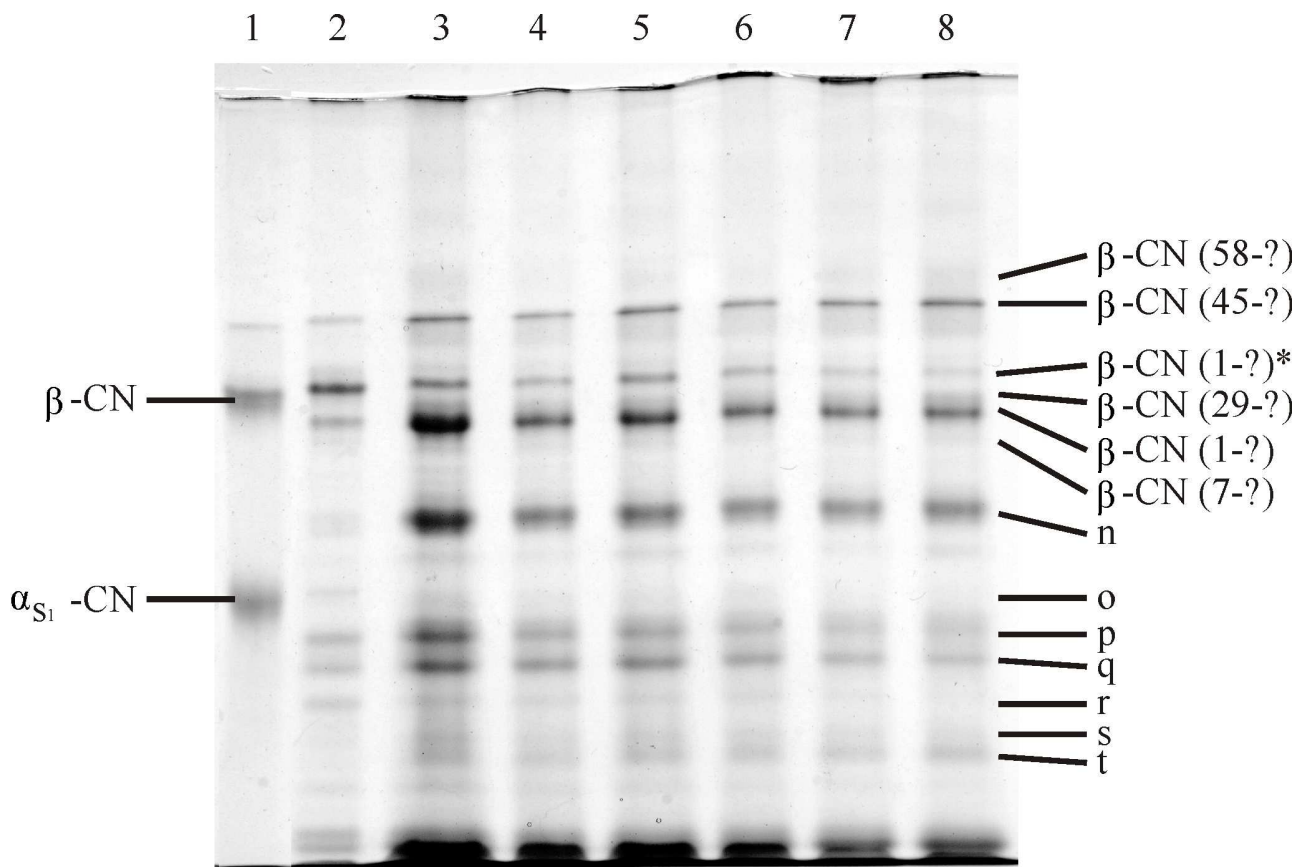


**Fig. 4.13** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Debaryomyces hansenii* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

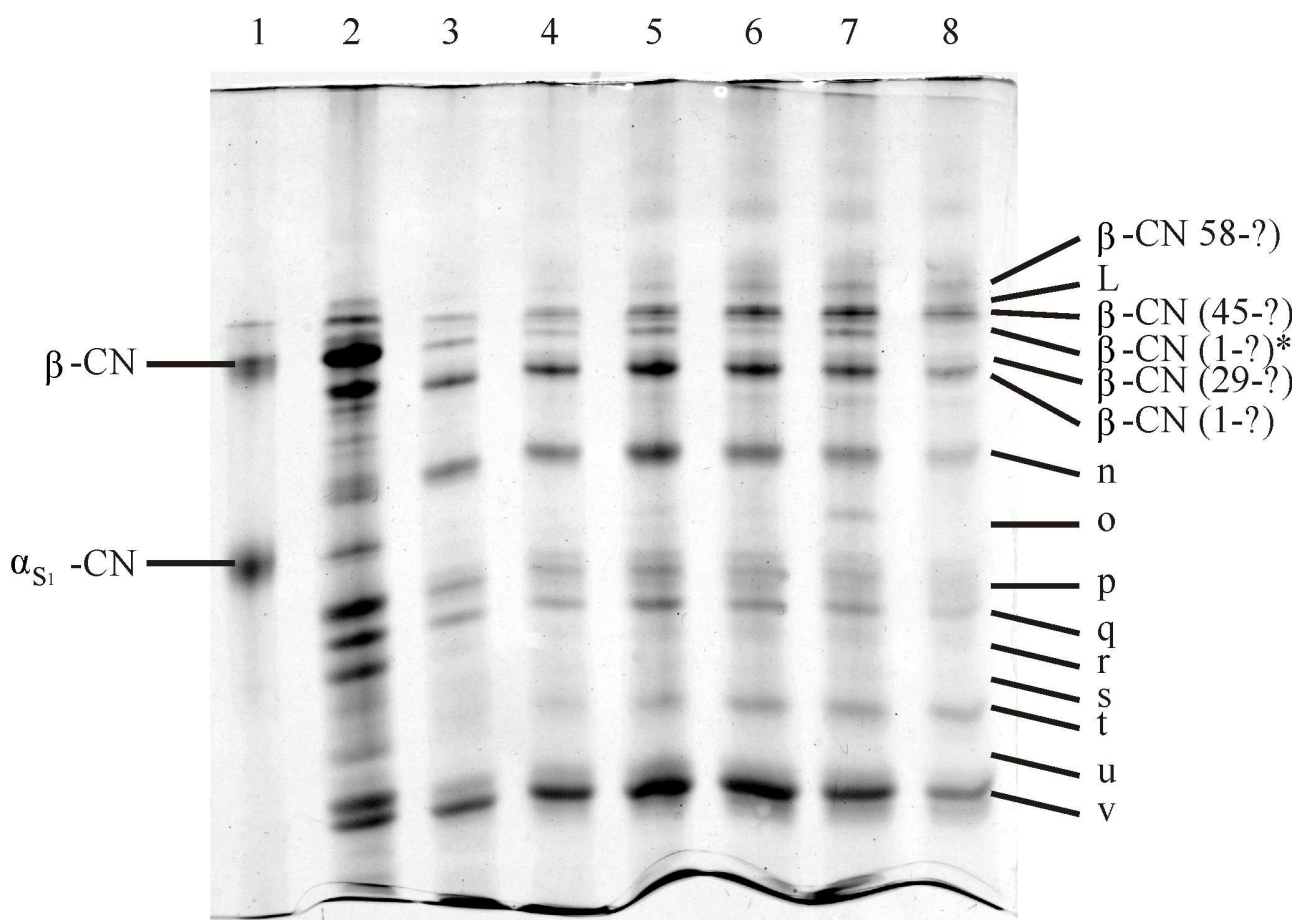




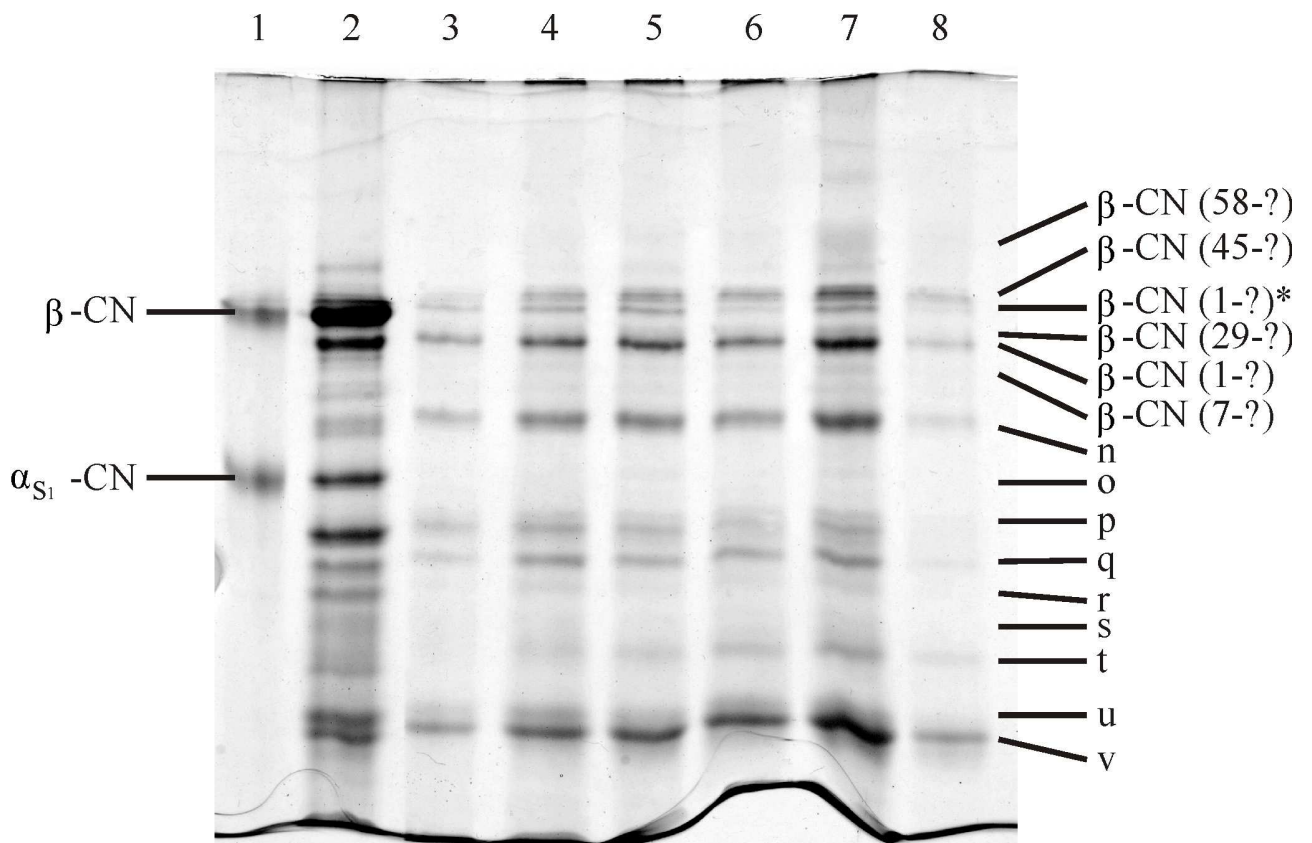
**Fig. 4.14** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Debaryomyces hansenii*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



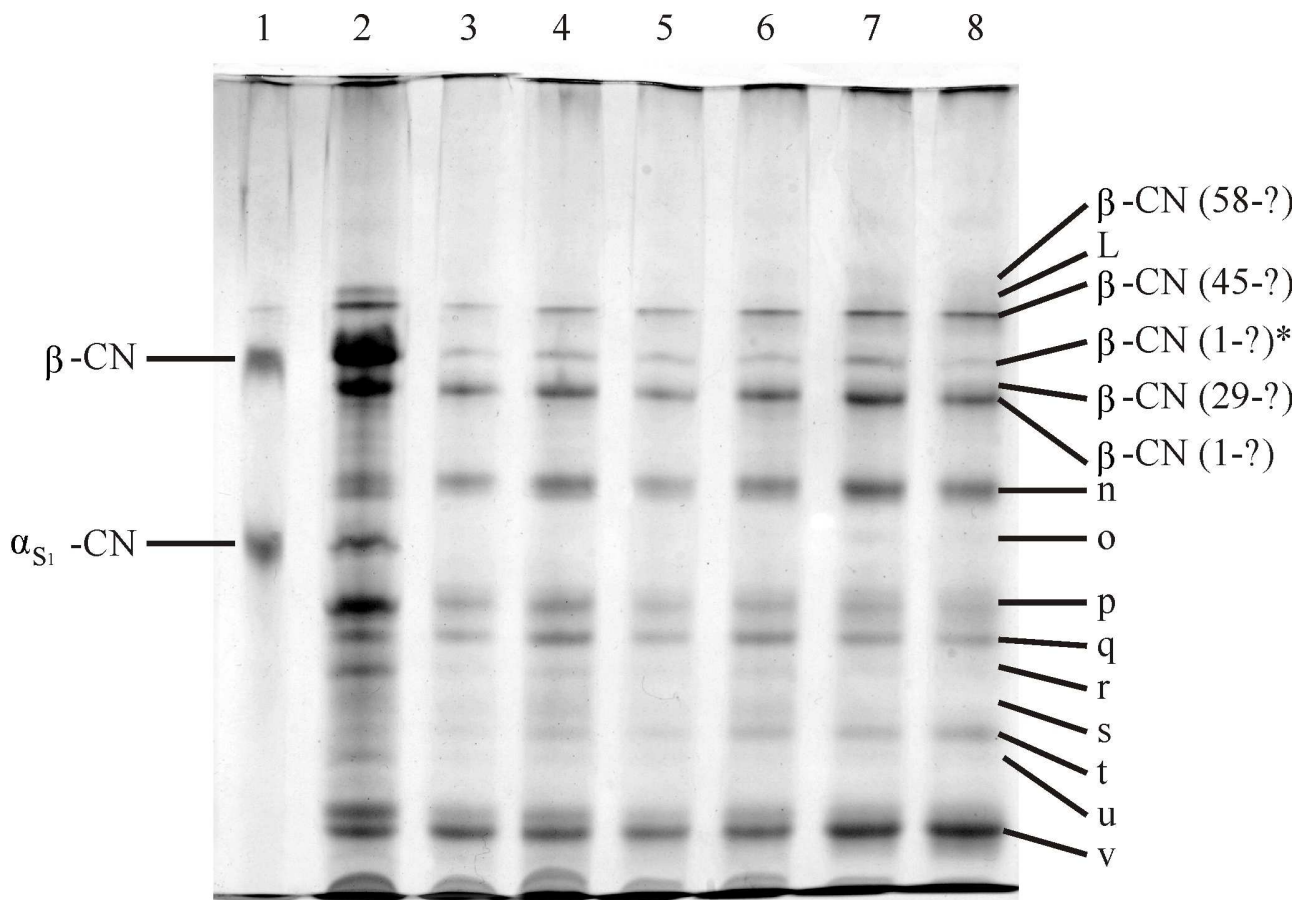
**Fig. 4.15** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



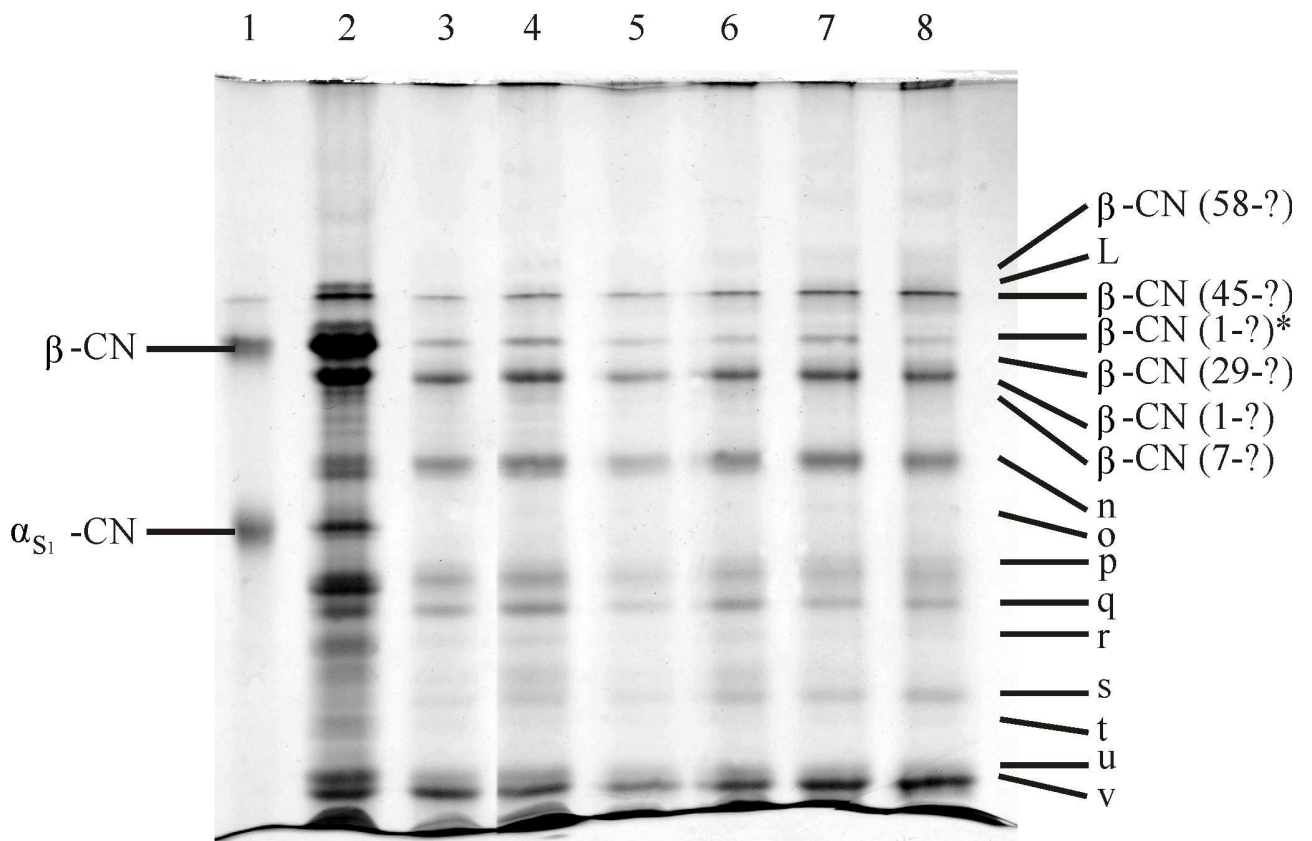
**Fig. 4.16** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Torulaspora delbrueckii* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.17** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Torulaspora delbrueckii*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.18** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Dekkera bruxellensis* + *Yarrowia lipolytica*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.



**Fig. 4.19** Urea-PAGE of the WSN fraction of Cheddar cheese inoculated with *Dekkera bruxellensis*. Lane 1: marker, Na-caseinate. Lane 2: Day 0. Lane 3: Day 60. Lane 4: Day 90. Lane 5: Day 120. Lane 6: Day 150. Lane 7: Day 180. Lane 8: Day 240.

#### 4.3.4 RP-HPLC analysis

Some differences in terms of peak development and degradation were observed among the eight cheeses during ripening (fig. 4.20). Chromatograms of cheese duplicates were obtained but only one chromatogram is shown for each cheese.

The chromatograms will be named as follows: (S), (D+Y), (D), (Y), (T+Y), (T), (Db+Y) and (Db). See section 4.2.1.

Few peptides are detected at day 0 and the elution profile of peptides did not differ amongst cheeses. For further discussion, the focus is mainly on the peptides that elute in the region of 10 – 20 min following ripening of the cheeses up to 240 days of ripening.

Specific peptide peaks visible at day 0 are those that elute at 10, 13, 17 and 19 min, the amounts of which are increasing during ripening. The peptide that elutes at 17 min reaches its peak amount at 60 days ripening, after which it disappears.

Two different peptides appear in its place, which elute at 17 and 18 min respectively. The development of these two peaks differ amongst the cheeses. In cheese (S) they are only visible at 120 – 180 days of ripening; for cheeses (D+Y) and (Db) they are only visible at 120 days; for cheese (D) at 90 – 120 days; for cheeses (Y), (T) and (Db+Y) at days 90 – 150; and for cheese (T+Y) only at 90 days. A single peak at 18 min elution time is detected for cheese (T+Y) at 120 – 150 days of ripening and 150 - 180 days for cheese (D).

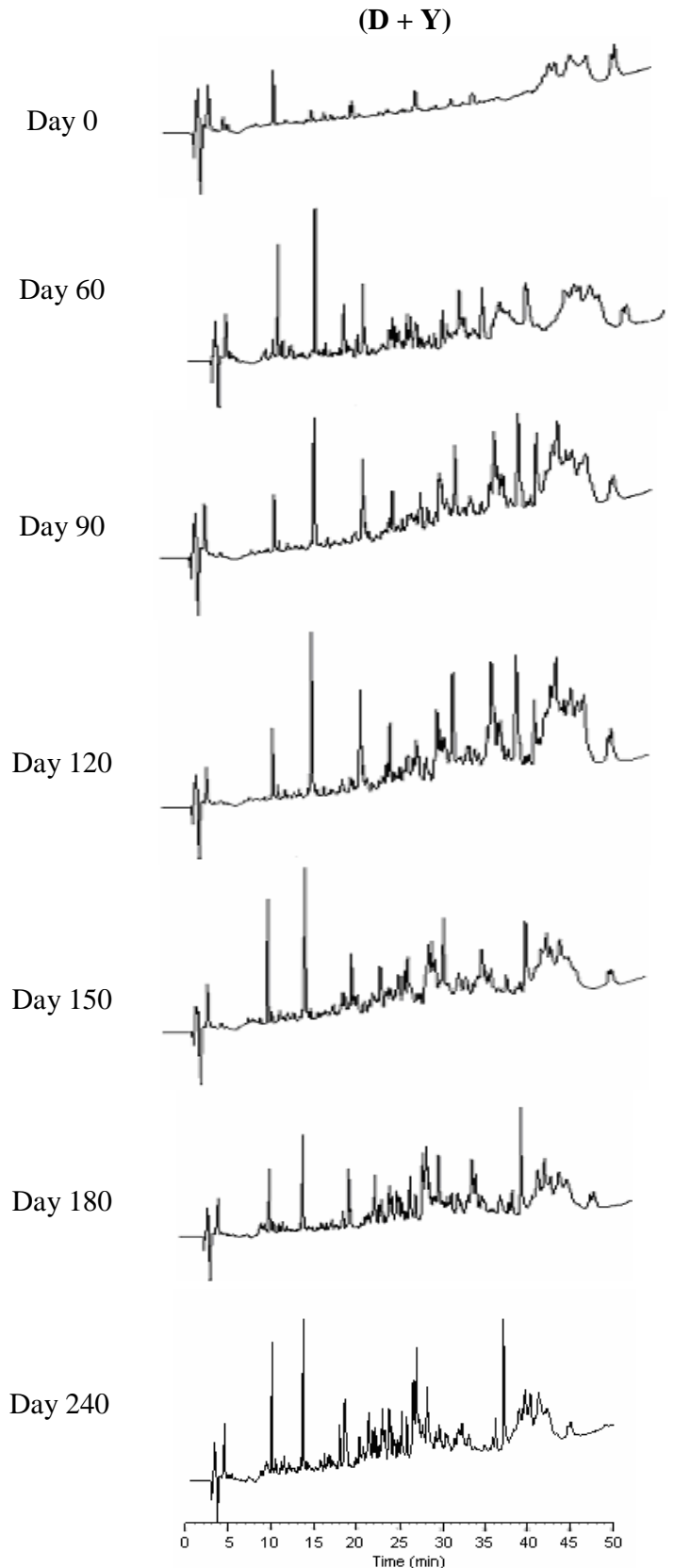
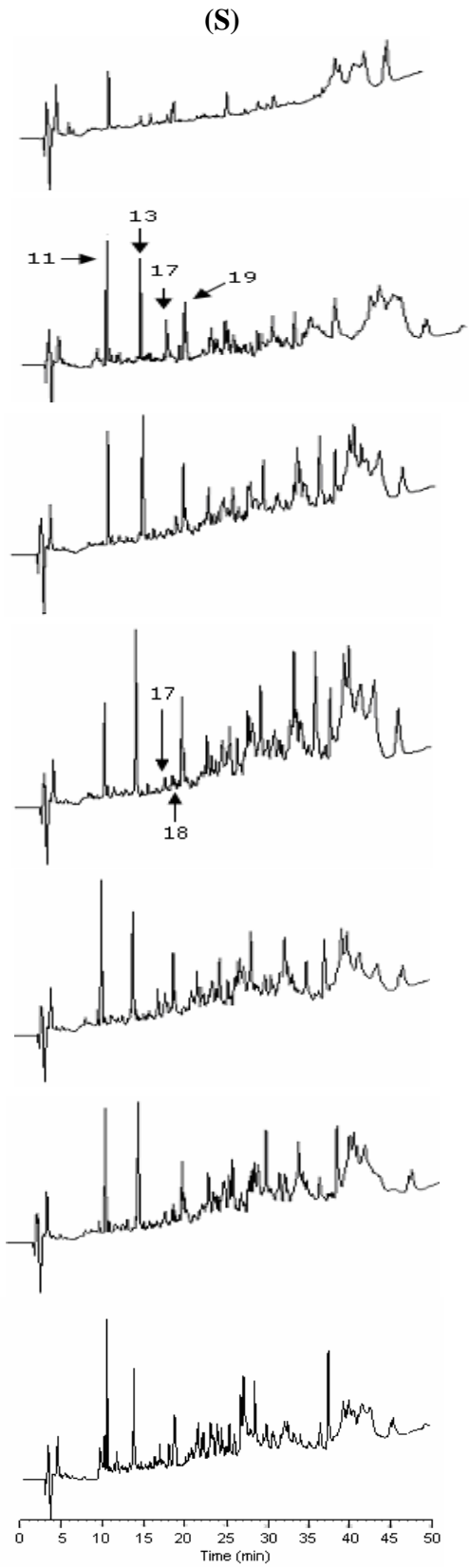
Looking at the relative peak heights of the peptides that elute at 10, 13, and 19 min, some differences are visible amongst the cheeses during ripening. In cheese (S), the peak at 10 min is always the highest and the peak at 19 min the lowest, except at day 90 when 10 and 13 min are almost equal and at 120 days of ripening when the peak at 13 min is the highest. In cheese (D+Y), the peptide eluting at 13 min is the highest throughout ripening. Cheeses which show a similar peptide peak development during early ripening

are cheeses (Db) (days 60), (Db+Y) (days 60 – 90) and (T+Y) (days 60 – 120). However, ripening beyond those stipulated times, the peptides eluting at 10 min became the highest and at 19 min the lowest. Cheeses (Y) and (T) also display very similar developments: at day 60, peptides eluting at 10 and 14 min are of equal height, and higher than the one at 19 min. At 90 days ripening, the relative heights are 13>10>19 and after 120 days the same order is observed as for cheese (Db), (Db+Y) and (D+Y).

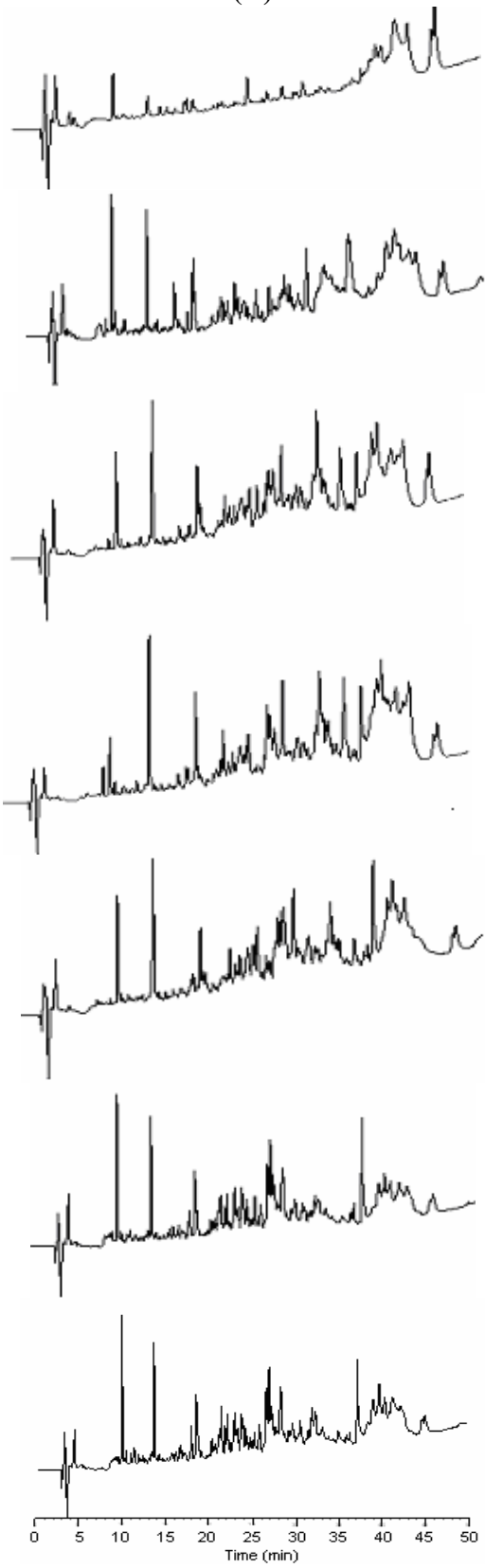
Based on these results, cheeses can be grouped as (S); (D+Y); (Y) and (T) which are very similar; (Db), (Db+Y) and (T+Y) being very similar.

The chromatograms that follow are named: Fig. 4.20 RP-HPLC profiles of the WSN fraction of cheeses during ripening.

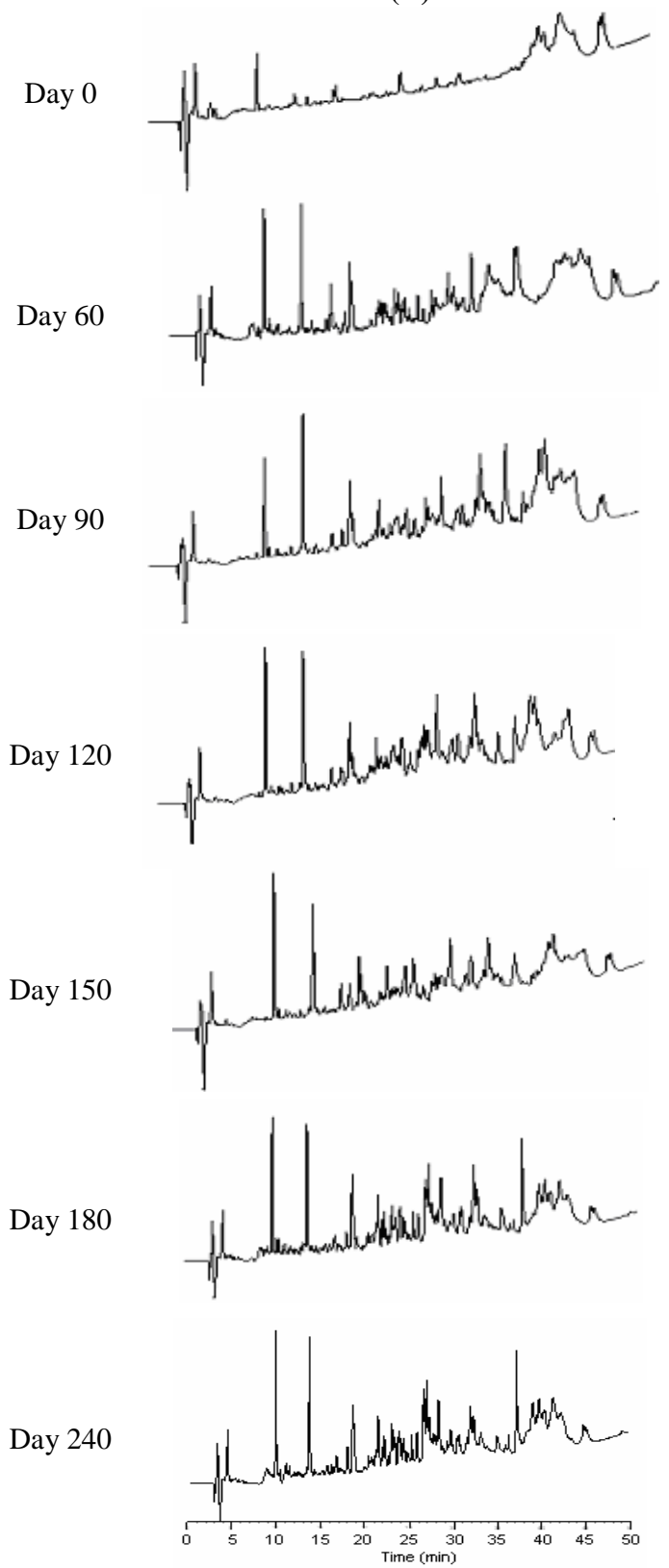




(D)

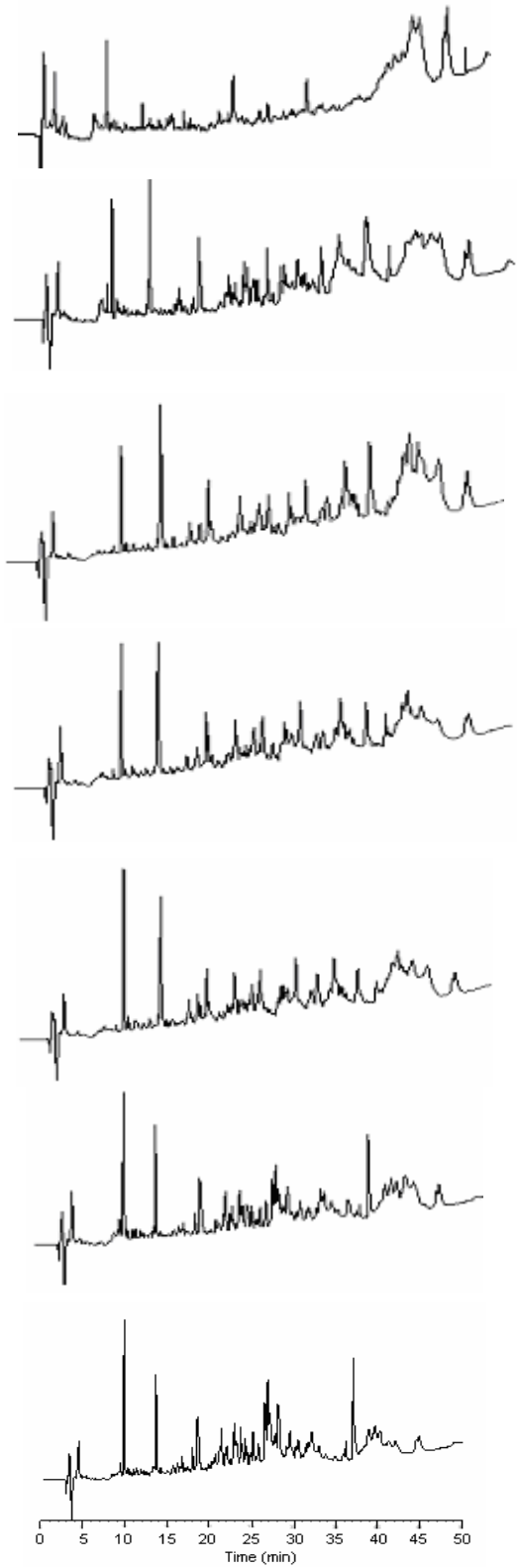


(Y)

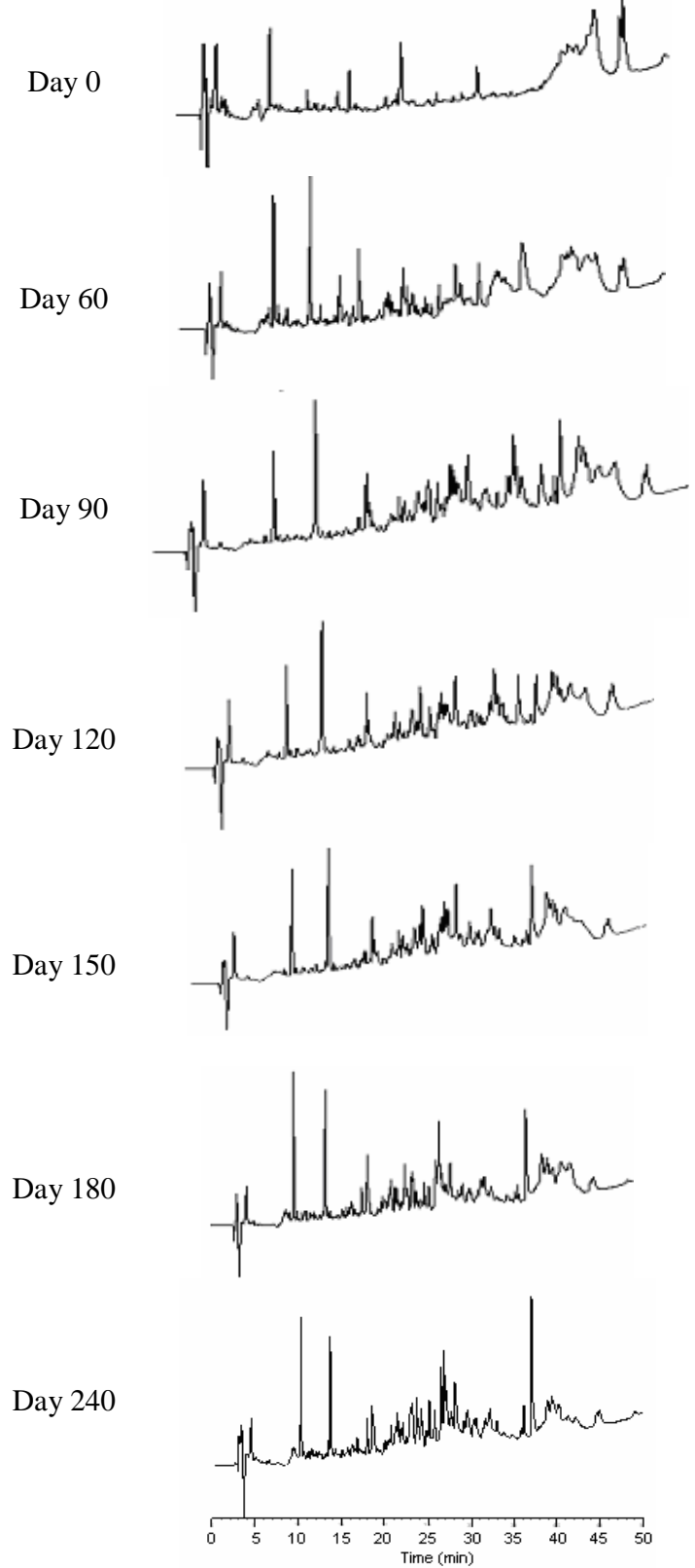




**(Db+Y)**



**(Db)**



#### 4.4 Discussion

Differences have been observed with UREA-PAGE gels as well as with the RP- HPLC chromatograms that might help to explain why some cheeses developed bitter compounds during ripening while other cheeses developed desirable flavours. PAGE of the WSN fraction of standard cheese (S) (fig.4.4) showed that it differed from the yeast inoculated cheeses with the development of peptide  $\beta$ -CN (1-?)\* after 150 days of ripening. The WSN fraction of (Y) (fig.4.15) and especially (T) (fig.4.17) and (T+Y) (fig. 4.16) developed differently regarding fraction  $\beta$ -CN (29-?). Peptide L developed in cheeses (D+Y), (T+Y), (Db+Y) and (Db) at day 0 (fig. 4.13, 4.16, 4.18 and 4.19). However, peptide L disappears during ripening and is no longer visible at 60 days.

No major differences in peptide development as analysed by UREA-PAGE and HPLC were observed. This is to be expected, because all cheeses were Cheddar type cheeses. For the HPLC chromatograms the focus was mainly on the peptide peaks that elute around 10 - 20 min. The relative sizes of the peptide peaks eluting at 10, 14, 17, 18 and 19 min is important in trying to explain sensory aspects. Cheeses (S) and (D+Y) seem to be unique, (Y) and (T) similar, while (Db), (Db+Y) and (T+Y) are similar at most times during ripening.

Since FAA and WSN as %TN was not found to be different in the cheeses during ripening, one will have to rely on the results of PAGE and HPLC. Considering this, at least cheeses (Y), (T) and (T+Y) showed similar results, which drastically differed from cheese (D+Y), (S), (Db) and (Db+Y). How these differences may be related to specific enzyme activity and sensory aspects will be discussed in chapter 5.

## Chapter 5

### CONCLUDING DISCUSSION

The rate and extend of proteolysis during Cheddar cheese ripening will have an effect on the quality as well as the flavour formation in cheese (Lane and Fox, 1997; Sousa *et al.*, 2001). A lot of research has been done towards the addition of adjunct yeast species to help improve ripening of surface ripened cheeses (Wallace and Fox, 1997; Kailasapathy and Lam, 2005). De Wit *et al.*, (2005) found that yeasts, when used as adjuncts in Cheddar cheese processing, may accelerate flavour development. Some yeast species are known for their proteolytic and lipolytic activities and are beneficial for the growth of the starter lactic acid bacteria (Viljoen, 2001). Inoculated yeasts were expected to grow and compete with other naturally occurring yeasts in the cheese and have a possible stimulating action when co-inoculated with starter lactic acid bacteria (Guerzoni *et al.*, 1998). The aim of this project was to screen yeasts associated with Cheddar cheese, like *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Dekkera bruxellensis*, for protease-, plasminogen activation-, plasmin- and glutamate dehydrogenase activity and to select certain yeasts to be inoculated with SLAB into model cheeses.

Intracellular protease (subtilisin-like) activity ( $0.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ) was detected in *Candida intermedia*, *Torulaspora delbrueckii*, *Candida zeylanoides* as well as trypsin like activity (tables 2.4 and 2.6). *Candida rugosa* displayed subtilisin-like (extracellular activity) for substrate N-Cbz-Gly-Gly-Leu-p-nitroanilide (table 2.4). *Candida intermedia* and *Torulaspora delbrueckii* displayed high chymotrypsin-like activity, *Debaryomyces hansenii* and *Candida rugosa* displayed lower chymotrypsin-like activity (table 2.5). Analysis of intracellular enzyme activity ( $>0.1 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ) of the yeasts displayed that *Yarrowia lipolytica*, *Candida rugosa*, *Candida zeylanoides* and *Torulaspora delbrueckii* possess the ability to activate plasminogen (PG) to plasmin (PL) (table 2.7). Yeasts such as *Yarrowia lipolytica*, *Candida intermedia*, *Rhodotorula mucilaginosa* and *Torulaspora delbrueckii* also displayed low PL activity ( $>0.2$

nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>) (table 2.8). The PG activation- and PL activities of *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Torulaspota delbrueckii* and *Dekkera bruxellensis* were confirmed by hydrolysis of  $\beta$ -casein in milk resulting in the production of peptide  $\beta$ -CN 29-?, of which the presence of trypsin-, chymotrypsin-, subtilisin-like activity in yeasts is not new (Kato *et al.*, 1995; Lesk and Fordham, 1996; Narinx *et al.*, 2003). However, this screening exercise added a few yeasts species to the list. *Yarrowia lipolytica* and *Torulaspota delbrueckii* both displayed higher PG activation- and PL activity than the other yeasts.

Intracellular GDH activity (>9 nmol.min<sup>-1</sup>.mg protein<sup>-1</sup>) was displayed in *Debaryomyces hansenii*, *Candida intermedia*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspota delbrueckii*, *Candida zeylanoides*, *Candida sake* and *Dekkera bruxellensis* (table 2.9).

A combination of *Yarrowia lipolytica* and *Debaryomyces hansenii* as adjuncts by de Wit *et al.* (2005) resulted in accelerated ripening, and it was speculated that the enzymes that contributed to PL activity and amino acid catabolism could be responsible. It therefore followed that yeast with PG activation-, PL- and GDH activity should be employed in further investigations.

Four yeast species were chosen according to their enzymatic activities: *Debaryomyces hansenii* and *Dekkera bruxellensis* for GDH activity, *Yarrowia lipolytica* for PG activation- and PL activity, *Torulaspota delbrueckii* for GDH- as well as PG activation- and PL activity. None of the *Candida* species were considered for the cheese making process because of their pathogenic tendencies. Model cheeses were manufactured and inoculated with these yeast species as follows: (S) (without yeasts and only LAB), (D+Y), (D), (Y), (T+Y), (T), (Db+Y) and (Db) (all with yeast and LAB). Sensory and chemical studies were carried out.

Sensory evaluation indicated that cheeses (D+Y), (D), (T), (Db+Y) and (Db) displayed acceptable sensory development after three months of ripening. Cheeses (D+Y) and (Db+Y) displayed acceptable sensory qualities at six months of ripening and at nine

months (S), (D+Y), (Db+Y) and (Db) (tables 3.3, 3.4 and 3.5). Cheeses inoculated with (Y), (T) (as separate inoculants) and (S) did not develop favourable flavours during ripening. Only cheeses (D+Y) and (Db+Y) developed Cheddar cheese flavour during nine months ripening. The moisture content turned out to be a problem which should be corrected in the future. Nevertheless this seems not to have had a major impact on the aims of this study. One could immediately derive that the high protease-, PG activation-, PL- and GDH activities of *Torulaspora delbrueckii* contributed to the negative sensory qualities. However, detailed chemical analysis was pursued.

Proteolysis during cheese ripening was monitored by determining WSN as %TN, FAA, electrophoresis of the WISN and WSN fractions and HPLC of the WSN fraction. Primary proteolysis as determined by analysis of the WSN as %TN did not show major statistical differences ( $p < 0.001$ ). A general increase of WSN as %TN from approximately 0.0003 to 0.0023 % (fig. 4.1) was observed and is in agreement with the results of Lane and Fox (1996) and de Wit *et al* (2005). FAA increases over ripening time (fig. 4.3) from an approximate value of 0.1 mg leucine g cheese<sup>-1</sup> at day 0 to an approximate value of 1.5 mg leucine g cheese<sup>-1</sup> which agrees with the results of Lane and Fox, (1996) and de Wit *et al*. (2005). No statistically significant differences ( $p < 0.001$ ) were observed in all the cheeses for FAA.

UREA-PAGE analysis of the WISN fraction (fig. 4.4 – 4.11) showed no major differences between the cheeses, except for cheese (S). The main difference is with the fractions  $\beta$ -CN ? 1,  $\beta$ -CN (f29-209),  $\beta$ -CN (f29-•), and  $\beta$ -CN (f30-•) that developed later during ripening in the standard cheese compared to the yeast inoculated cheeses. This would indicate that yeast inoculated cheeses develop differently compared to the standard cheese.

In a comparison of the WSN fraction, the main differences were seen in fractions  $\beta$ -CN (58-?), L,  $\beta$ -CN (1-?),  $\beta$ -CN (1-?)\* and  $\beta$ -CN (45-?) in the cheeses inoculated with (T+Y), (T), (D+Y), (Db+Y) and (Db) (fig. 4.13 – 4.19). The  $\beta$ -CN (58-?) fraction in all the cheese samples (fig. 4.12 – 4.15, 4.17 – 4.19) is barely visible, but for cheese (T+Y)



the fraction can be clearly seen (fig. 4.16). Cheeses (D+Y), (T+Y), (Db+Y) and (Db), develop an extra peptide band (L) during the first 60 days of ripening, which immediately disappears after 60 days. It is possible that this peptide L is hydrolyzed at a faster rate in these cheeses so that it can not be detected in the other cheeses at 60 days ripening. In all the cheeses, the intensity of fractions  $\beta$ -CN (1-?)\* and  $\beta$ -CN (1-?) decreased during ripening which might be because further hydrolysis of the  $\beta$ -CN fraction takes place over time. The  $\beta$ -CN (29-?) fraction, which is the result of PL activity, starts to develop approximately at day 150 as a very faint band above  $\beta$ -CN (1-?) in cheeses (S), (D+Y), (D), (Db+Y) and (Db), but developed earlier at approximately day 60, for cheeses (Y), (T+Y) and (T).

When WSN fractions were analysed by RP-HPLC, the focus was mainly on the peptide peaks that elute around 10 - 20 min. The relative sizes of the peptide peaks eluting at 10, 14, 17, 18 and 19 min were deemed important in explaining differences. Each cheese (S) and (D+Y), showed a unique pattern of peptide peak development. Furthermore were cheeses (Y) and (T) similar, while (Db), (Db+Y) and (T+Y) differed from the former during ripening.

Taking the overall chemical similarities and differences into account, it can be concluded that cheese (S) developed in a different manner than (D+Y) and (Db+Y), while these differ drastically from cheese (Y), (T) and (T+Y). When these results are compared with the sensory properties, it makes sense that the cheeses with the worst sensory quality after nine months of ripening, are cheeses (T) and (T+Y), which may be ascribed to a very high proteolytic activity, not only by PG activation- or PL activity, but also by trypsin-, chymotrypsin- and subtilisin-like activity, together with a high level of amino acid catabolism by GDH. Cheeses (D+Y) and (Db+Y) displayed the best sensory qualities, which could be attributed to a balanced contribution of PG activation- and PL activity by *Yarrowia lipolytica* and GDH by *Debaryomyces hansenii* and *Dekkera bruxellensis* and absence of other proteolytic enzymes.

It might be speculated whether lower amounts of *Torulaspota delbrueckii* should be inoculated to add to the desired enzymes, however, it has been shown that the presence of other proteolytic resulted in undesired flavours (Law, 2001). Similarly, it might be speculated that the introduction of higher amounts of *Yarrowia lipolytica* as adjunct should be advantageous, as it does not display high levels of other proteases. However, it may not be forgotten that flavour development is not only ascribed to the catabolism of protein, but also lactose and lipids, and *Yarrowia lipolytica* is known for lipase activity (Roostita and Fleet, 1996).

In the current study a biochemical explanation was reached for the accelerated ripening by (D+Y) as adjuncts. The explanation mainly focused on proteolysis. In future research, the catabolism of amino acids, not only by GDH but also by aminotransferase and  $\alpha$  keto-decarboxylase, should be attempted.

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## SUMMARY

Research in cheese technology is devoted towards the manipulation of glycolysis, lipolysis and proteolysis in order to accelerate ripening times or to improve flavour. Evidence suggests that certain yeast species, such as *Yarrowia lipolytica* and *Debaryomyces hansenii*, contribute to flavour and texture development during ripening of Cheddar cheese. The addition of yeast cultures as adjunct cultures may accelerate ripening, or lead to improved development of flavour and taste. This is brought about by an increase in primary proteolysis of the caseins as well as the break down of amino acids into flavour compounds. This research reports on the inclusion of yeasts as adjuncts in the processing of Cheddar cheese and a biochemical explanation of their utilisation of caseins.

Twelve yeast species associated with Cheddar cheese were screened for plasminogen activation-, plasmin- and glutamate dehydrogenase activity. They are *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Candida intermedia*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspora delbrueckii*, *Candida zeylanoides*, *Candida sake* and *Dekkera bruxellensis*. Four yeasts were chosen according to their enzyme activity to be inoculated into model Cheddar cheese together with the starter bacteria. *Yarrowia lipolytica* (for PG activation-/PL activity), *Debaryomyces hansenii* (for GDH activity), *Torulaspora delbrueckii* (for PG activation-/PL activity and GDH activity) and *Dekkera bruxellensis* (for GDH activity).

Yeasts that expressed both enzyme activities were added as single adjuncts, while combinations of yeasts with singular enzyme activity were employed. Changes during ripening were monitored by sensory and biochemical analyses. The latter includes proteolysis and enzyme activity.

Sensory tests showed that some of the cheeses developed bitter flavours (*Torulaspora delbrueckii* + *Yarrowia lipolytica*), others (*Debaryomyces hansenii* + *Yarrowia lipolytica*, *Dekkera bruxellensis* + *Yarrowia lipolytica*) developed desired flavours during nine months ripening. Analysis of primary proteolysis, by electrophoresis of water-soluble and insoluble peptides, and HPLC of water-soluble peptides, suggest that cheeses inoculated with *Yarrowia lipolytica* and *Torulaspora delbrueckii* alone or *Torulaspora delbrueckii* in combination with other yeasts differed from cheeses inoculated with *Debaryomyces hansenii* and *Dekkera bruxellensis*. Negative flavour development could be ascribed to high activity of general proteases, PG activation, PL and GDH, while positive flavour development to balanced activity of plasminogen activation, plasmin and glutamate dehydrogenase.

## OPSOMMING

Die rypwording van kaas bestaan uit `n reeks biochemiese prosesse wat, as hulle gebalanseerd en gesinchroniseerd is, lei tot die vorming van goeie smake en teksture in die kaas. As dit egter ongebalanseerd is, kan dit veroorsaak dat afsmake ontwikkel. Drie biochemiese prosesse (glikolise, lipolise en proteolise) is verantwoordelik vir die smaak vorming van kase.

Navorsing het bewys dat sekere gis spesies soos *Yarrowia lipolytica* en *Debaryomyces hansenii* kan bydrae tot die geur-, smaak- en tekstuurontwikkeling in kase, insluitend Cheddar. Die giste is bekend vir hulle proteolitiese en lipolitiese aktiwiteit en het ook `n stimulerende uitwerking op die groei van melksuurbakterieë.

Twaalf gis spesies, wat geassosieer word met Cheddar kaas, is getoets vir plasminogeen aktiverings -, plasmien- en glutamate dehidrogenase aktiwiteit. Hulle is *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Candida intermedia*, *Rhodotorula mucilaginosa*, *Rhodotorula minuta*, *Candida rugosa*, *Torulaspora delbrueckii*, *Candida zeylanoides*, *Candida sake* en *Dekkera bruxellensis*. Volgens die resultate is vier gis spesies gekies om saam met die melksuurbakterieë ge-inokuleer te word in model Cheddar kase. *Yarrowia lipolytica* (vir PG aktivering- /PL aktiwiteit), *Debaryomyces hansenii* (vir GDH aktiwiteit), *Torulasprora delbrueckii* (vir PG aktivering - /PL aktiwiteit en GDH aktiwiteit) en *Dekkera bruxellensis* (vir GDH aktiwiteit).

Giste wat twee of meer ensiem aktiwiteite getoon het, is alleen bygevoeg, terwyl giste met `n enkel ensiem aktiwiteit in kombinasies gebruik is. Veranderings gedurende rypwording is gemonitor deur sensoriese en biochemiese analises, wat proteolitiese- en ensiem aktiwiteite insluit.

Sensoriese analise het getoon dat sekere gis ge-inokuleerde kase (*Torulaspora delbrueckii* + *Yarrowia lipolytica*) bitter smake ontwikkel het, terwyl ander (*Debaryomyces hansenii* + *Yarrowia lipolytica*, *Dekkera bruxellensis* + *Yarrowia lipolytica*) goeie smake gedurende die nege maande rypwordings tyd gevorm het. Analise van primêre proteolise, deur elektroforese van water oplosbare en onoplosbare peptiede en HPLC van water oplosbare peptiede, het voorgestel dat kase geïnokuleer met *Yarrowia lipolytica* en *Torulaspora delbrueckii* alleen of *Torulaspora delbrueckii* in kombinasie met ander giste verskil het van kase wat met *Debaryomyces hansenii* en *Dekkera bruxellensis* geïnokuleer is. Negatiewe geur ontwikkeling kan wees weens hoë aktiwiteit van proteases, PG aktivering, PL en GDH, terwyl positiewe geur ontwikkeling kan wees weens gebalanseerde aktiwiteit van PG aktivering, PL en GDH.

## **ABSTRACT**

A great deal of research in cheese technology is devoted towards the manipulation of glycolysis, lipolysis and proteolysis in order to accelerate ripening times or to improve flavour. There is increasing evidence that certain yeast species contribute to flavour and texture development during ripening of certain cheeses, including Cheddar cheese. The addition of yeast cultures as adjunct cultures may accelerate ripening, or lead to a faster development of flavour and taste. This is brought about by an increase in primary proteolysis of the caseins as well as the break down of amino acids into flavour compounds. The respective enzymes involved seem to be proteases with plasmin or plasmin activator activity, and glutamate dehydrogenase.

This research reports on the inclusion of yeasts as adjuncts in the processing of Cheddar cheese. Yeasts that expressed both enzyme activities were added as single adjuncts, while combinations of yeasts with singular enzyme activity were employed. Changes during ripening were monitored by sensory and biochemical analyses. The latter includes proteolysis, lipolysis and enzyme activity.

**Addendum 1**

**DAIRY PRODUCT EVALUATION FORM**

**Subdivision.....**

**Class number.....**

INSCRIBE	POINTS ALLOCATION		FRAGRANCE OUT OF 11	JUDGE'S REMARK	TOTAL OUT OF 20
	APPEARANCE AND COLOUR	BUILD AND TEXTURE			

**JUDGE SIGNATURE .....**

**DATE.....**

	APPEARANCE	BUILD AND TEXTURE	SMELL
<b>VERY GOOD</b>	3	6; 5	11; 10; 9
<b>GOOD</b>	2	4; 3	8; 7; 9
<b>WEAK</b>	1	2; 1	5; 4; 3; 2; 1

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A great deal of research in cheese technology is devoted towards the manipulation of glycolysis, lipolysis and proteolysis in order to accelerate ripening times or to improve flavour. There is increasing evidence that certain yeast species contribute to flavour and texture development during ripening of certain cheeses, including Cheddar cheese. The addition of yeast cultures as adjunct cultures may accelerate ripening, or lead to a faster development of flavour and taste. This is brought about by an increase in primary proteolysis of the caseins as well as the break down of amino acids into flavour compounds. The respective enzymes involved seem to be proteases with plasmin or plasmin activator activity, and glutamate dehydrogenase.

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**KEYWORDS:** Yeast, Plasmin, Glutamate dehydrogenase, Cheddar cheese.