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Biochemical characterisation of dairy yeasts and their application in cheese as anaerobic adjunct cultures

A thesis presented in partial fulfilment of the requirements of the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand

Shantanu Das

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Abstract

Yeasts are traditionally used as part of the surface microflora in surface-ripened cheeses, where they contribute positively to the flavour of the cheese. The primary objective of this study was to investigate the potential of three dairy yeasts to provide attributes as adjuncts in anaerobically ripened cheeses. *Geotrichum candidum* (B9001), *Yarrowia lipolytica* (B9014) and *Candida kefyr* (B9006), obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, were studied. They showed diverse metabolic activities in laboratory media, which were influenced by the growth conditions. The metabolic activities of special interest were the lipase and proteinase activities and the production of volatile compounds, as these are important for cheese ripening and flavour development.

Lipase activity (*p*-nitrophenyl butyrate assay) and proteinase activity (fluorescein isothiocyanate β-casein assay) were determined in three fractions prepared from yeast cultures and designated as extracellular fraction, washed-cell fraction and intracellular fraction. Lipase activity of *G. candidum* was detected only in the extracellular fraction and increased five fold when induced by safflower oil in a shake culture (0.16 μM/min/mL supernatant at 24 h). Lipase expression was delayed in static cultures. *Y. lipolytica* showed lipase activity in extracellular, washed-cell and intracellular fractions under all conditions. Static cultures in both glucose and safflower oil media showed higher lipase activity than shake cultures. The lipase activity of *Y. lipolytica* was higher in the late stationary phase than in the log phase under all conditions tested. The highest lipase activity was detected in a 192 h static culture grown in safflower oil medium (0.13 μM/min/mg dry cell weight, 0.3 μM/min/mg dry cell weight and 4.29 μM/min/mL supernatant in the intracellular, washed-cell and extracellular fractions respectively). *C. kefyr* did not show any lipase activity (< 0.03 μM/min/mL culture) under any of the growth conditions tested.

Proteinase activity was detected in the intracellular fraction of 72 h shake cultures of *G. candidum* grown in both glucose medium and safflower oil medium (154 and 122 RFU/min/mg dry cell weight respectively) but was not detected in static cultures. Proteinase activity was absent in the *Y. lipolytica* cultures under all conditions tested

(< 10 RFU/min/mL culture). *C. kefyr* showed low proteinase activity (12–74 RFU/min/mL supernatant) in the extracellular fraction only in shake cultures grown in glucose medium.

Volatile compounds of the headspace were sampled and analysed using solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS). The concentrations of volatile compounds were highest in shake cultures grown in glucose medium for all three yeasts. All yeasts produced several alcohols. Several esters were also detected in the *G. candidum* and *C. kefyr* cultures whereas aldehydes were detected only in the *G. candidum* cultures.

G. candidum and *Y. lipolytica* were selected for cheese production trials because of their active cheese ripening enzymes. These yeasts, grown under different conditions, were added to Cheddar cheese (10 L vat). The yeast adjuncts influenced the cheese ripening by lipolysis [in terms of the production of free fatty acids (FFAs) analysed by gas chromatography–flame ionisation detector (GC–FID)] and the production of volatile compounds (SPME–GC–MS), whereas proteolysis (analysed by size-exclusion high performance liquid chromatography) by yeast enzymes was not obvious.

The influence of *Y. lipolytica* as an anaerobic adjunct to cheese ripening was dependent on the growth conditions used during its propagation in laboratory media. The concentration of total FFAs was very high (37.1 mg/g cheese at 6 months) when a 192 h *Y. lipolytica* culture grown in safflower oil medium was added to a cheese make, whereas the cultures grown in glucose medium did not have any detectable effect. Addition of *G. candidum* culture to the cheese curd was more effective than its addition to the cheese milk.

Both *G. candidum* and *Y. lipolytica* lipase(s) selectively hydrolysed the long-chain unsaturated fatty acids from the milk triglyceride in the cheese environment. Also, *Y. lipolytica* lipase exhibited some selectivity towards hydrolysis of butyric acid from the milk fat in the cheese.

2-Heptanone, 3-methyl-2-butanone and 2-nonanone were detected (1–10 x 10^6 relative peak area) only in the cheeses with yeast adjuncts but not in the control cheese.

Enhancement of the production of both conjugated linoleic acid (CLA) and ethyl esters in a washed-curd, dry-salted cheese (375 L vat), made with *G. candidum*, *Y. lipolytica*, *Propionibacterium freudenreichii* ssp. *shermanii*, *Lactobacillus fermentum* and *Lb. rhamnosus*, was only partially successful. Higher concentrations of ethyl esters (> five fold; analysed by SPME–GC–MS) were produced in the cheeses made with yeast adjuncts. However, the concentration of total CLA (free plus esterified; analysed by GC–FID) did not increase although a higher concentration of free linoleic acid (> 10 fold), the substrate for CLA synthesis, was produced in the cheeses made with yeast adjuncts.

A study of the formation of aromatic volatile compounds by *C. kefyr* in a medium containing L-phenylalanine (L-phe) showed that the yeast's ability to produce phenyl ethanol, phenyl ethyl acetate and benzaldehyde (analysed by SPME–GC–MS) was enhanced with an increase in the initial L-phe concentration (in the experimental range; analysed by enzymatic assay using phenylalanine ammonia lyase), but the yield was very low (20–27%). The initial concentration of glucose (in the experimental range; analysed by enzymatic assay using Peridochrom glucose reagent) did not affect the production of these aromatic volatile compounds.

This study successfully showed that the yeasts *G. candidum* and *Y. lipolytica*, when used as anaerobic adjuncts, can influence the ripening and flavour development in Cheddar and washed-curd, dry-salted cheeses. The study also showed the capability of *C. kefyr* to produce aromatic volatile compounds from amino acid fermentation but the yields need to be increased by further manipulation of the medium components and the culture conditions before this capability can be used commercially.

To my wife Jinita and daughter Nilotri
For your love and support

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Chapter 1. Introduction

Yeasts are widely present in cheese, where they can be used to contribute positively to ripening and flavour development (Fleet, 1990). Yeasts are known to mediate the production of flavour compounds primarily by hydrolysis of the fats and proteins and also by subsequent metabolism of released amino acids and fatty acids. The metabolic potentials of yeasts, especially *Geotrichum candidum* and *Yarrowia lipolytica*, have been widely studied in laboratory media, milk, cream and cheese slurry (Baillargeon *et al.*, 1989; Jollivet *et al.*, 1994; Pereira-Meirelles *et al.*, 1997; Martin *et al.*, 2001).

A number of yeasts are active lipase producers (Pereira-Meirelles *et al.*, 1997). Some of the yeast lipases have special characteristics. For example, the lipases of some strains of *G. candidum* have specificity towards unsaturated fatty acids (Baillargeon *et al.*, 1989). Some *Y. lipolytica* strains are effective in converting unsaturated fatty acids to C10 and C12 γ lactones, which can contribute to cheese flavour (Ercoli *et al.*, 1992).

The proteolytic activities of different yeast strains are well known (Gueguen and Lenoir, 1976; Suzzi *et al.*, 2001). *Y. lipolytica* proteinase was found to hydrolyse β-casein (Guerzoni *et al.*, 1996), which was not hydrolysed by chymosin (Bogenrief and Olson, 1995). Aminopeptidase of *G. candidum* can reduce the bitterness in cheese (Molimard *et al.*, 1994), which is caused by the excess accumulation of bitter peptides (McSweeney, 2004).

The yeasts of dairy origin are known to produce a range of volatile compounds. These yeasts, especially *G. candidum*, produce a range of volatile sulphur compounds such as methanethiol, dimethyldisulphide, dimethyltrisulphide and S-methyl thioesters (Berger *et al.*, 1999; Demarigny *et al.*, 2000). Such sulphur compounds are very important constituents of the aroma of ripened cheese (Urbach, 1993). Fatty acid esters with fruity odours were identified when *G. candidum* was grown on breadcrumbs (Daigle *et al.*, 1999) and several free fatty acids and primary alcohols were produced when this yeast was grown in a milk and cheese slurry medium (Jollivet *et al.*, 1994). Fermenting cream using *Y. lipolytica* was shown to produce a cheesy flavour (Kalle *et al.*, 1976).

These metabolic activities of the yeasts make them potential candidates for producing novel flavours and accelerating cheese ripening.

The primary features of cheese ripening involve the transformation of the two principal constituents, proteins and lipids (Fox *et al.*, 2000). Lipolysis results in breakdown of milk fat triglycerides to release free fatty acids, which are key flavour compounds themselves and are also precursors of flavour compounds such as methyl ketones and lactones. Degradation of caseins by the combined action of rennet enzymes, plasmin and cell envelope proteinases and peptidases from lactic acid bacteria yields small peptides and amino acids. The amino acids are precursors of various alcohols, aldehydes, acids, esters and sulphur compounds (Smit *et al.*, 2002).

Microorganisms play important roles during both cheese manufacture and ripening. They can be divided into two main groups based on their functions in cheese: starters and secondary flora. The starter flora is responsible for acid development during cheese manufacture (Beresford *et al.*, 2001). Adjunct cultures are the secondary flora that is deliberately added to the cheese milk to contribute to the development of cheese flavour and functionality following curd formation. They are generally added at low levels (< 0.01%) and do not influence the acid development. The adjunct cultures can be grouped into aerobic and anaerobic microorganisms according to their use. Traditional aerobic adjunct cultures (*Penicillium*, *Geotrichum*, *Arthrobacter*, *Brevibacterium* and *Micrococcus*) are important to the flavour of Brie, Camembert, Blue-veined and smear-ripened cheeses. Traditional anaerobic adjunct cultures include *Leuconostoc* and *Propionibacterium*, which contribute to flavour, and body and texture developments in Gouda and Swiss-type cheeses respectively (Crow *et al.*, 2002).

Yeasts are used traditionally as a part of the surface microflora in surface-ripened cheese (Reps, 1993), where they contribute to cheese flavour development. Because the majority of the cheeses in New Zealand are ripened anaerobically, there is interest in exploiting the ripening potential of yeasts as anaerobic adjuncts. However, there is a scarcity of published information regarding the use of yeasts as anaerobic adjuncts in cheese. Guerzoni *et al.* (1996) reported significant flavour intensification and accelerated texture development in Cheddar-type cheese manufactured with a *Y. lipolytica* culture. Deiana *et al.* (1984) found faster proteolysis, higher concentrations of

free fatty acids and lower amounts of acetic acid in Pecarino Romano cheese when *Debaryomyces hansenii* was used as an adjunct culture. Ferreira and Viljoen (2003) produced a strongly flavoured Cheddar-type cheese using *Y. lipolytica* and *D. hansenii* as adjunct cultures. They found that the numbers of the yeasts decreased during the ripening. Crow *et al.* (2002) also reported similar results, *i.e.* a decrease in the yeast count in the cheese during ripening. This was not unexpected considering the requirement of oxygen for the growth of many yeasts.

Given the potential effects of yeast adjuncts on cheese ripening, and at the same time recognising the decreasing viability of yeasts inside the cheese, it has been hypothesised that, without significant growth in the cheese, the yeast adjuncts would influence the ripening mainly by the enzymes already produced in the cultures before their addition to the cheese. Therefore, the culture preparation for the yeast adjuncts, the addition quantity and the stage of addition could be important for the biochemical changes in the cheese.

The first part of the present study was designed to test this hypothesis (first objective). Three dairy yeasts from the collection of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, *Geotrichum candidum* (B9001), *Yarrowia lipolytica* (B9014) and *Candida kefyr* (B9006), were cultured under different conditions to identify the optimum conditions for the production of ripening enzymes and volatile compounds.

Based on the enzymatic activities shown by the yeasts in the laboratory media, *G. candidum* and *Y. lipolytica* were selected for use as cheese adjunct cultures. *G. candidum* and *Y. lipolytica* cultures, grown under different conditions, were used in Cheddar cheeses as anaerobic adjuncts and the biochemical changes in the cheeses over the ripening period were monitored.

The preferential release of long-chain unsaturated fatty acids including linoleic acid by *G. candidum* and *Y. lipolytica* in Cheddar cheese (present study) and the capacity of some strains of propionibacteria for converting free linoleic acid to conjugated linoleic acid (CLA) (Jiang *et al.*, 1998), a bioactive compound, led to the hypothesis that the concentration of CLA could be enhanced in a ripening cheese by using these yeasts and

bacterial adjuncts together. This hypothesis was the basis for producing a washed-curd, dry-salted cheese, with increased CLA content and elevated concentrations of ethyl esters to mask the undesirable soapy flavour effects of long-chain free fatty acids. The microbial production of ethyl esters was studied by combining yeast and bacterial adjuncts. The development of this cheese constituted the second objective of the study.

A study of the production of volatile flavour compounds by *C. kefyr* in laboratory media through L-phenylalanine metabolism was a further objective of the study.

Thus, the present work investigated the ripening potential of two dairy yeasts in Cheddar cheese, the possibility of combining yeast and bacterial adjuncts to produce targeted bioactive and flavour compounds in a washed-curd, dry-salted cheese, and the potential of a dairy yeast for producing flavour compounds through specific amino acid metabolism.

Chapter 2. Review of the literature

2.1 Introduction

Traditionally, and also from an economic point of view, yeasts are the most important microorganisms exploited by human beings. Yeasts are used in the production of different food products (bread, wine and beer), animal feeds, fuel alcohol and pharmaceutical grade biochemicals (Jakobsen and Narvhus, 1996). Yeasts are present in different dairy products and have two potential roles; they may act as spoilage organisms or they may contribute to the product positively, especially to the ripening and flavour development of cheese. The capacity of yeasts, compared with the majority of the bacteria, to grow at low temperatures and at low water activity enables them to grow in cheese. Capacity for lactose fermentation and assimilation, capacity for assimilation of organic acids, and lipolytic and proteolytic activity are some other characteristics of yeasts that help their growth in cheese (Fleet, 1990). The lipolytic and proteolytic capacities of yeasts along with the capacity for aroma production mainly through amino acid catabolism make them potential candidates as cheese ripening agents.

General characteristics of yeasts, including their classification based on fermentation and assimilation characteristics, are briefly discussed in Section 2.2 as background information.

In Section 2.3, yeasts associated with or used in cheese and the properties of yeasts that may contribute to cheese ripening and flavour development are discussed.

Yeasts have the potential to provide enzyme(s) that could work together with bacterial enzyme(s) to produce conjugated linoleic acid (CLA), a bioactive compound. Microbial production of CLA is reviewed in Section 2.4 to provide useful background information for the experimental work.

Amino acid degradation products are important for cheese flavour. Yeasts have been shown to produce flavour compounds from different amino acids including L-phenylalanine, L-methionine, leucine and isoleucine. The fermentation of L-

phenylalanine by yeasts to produce volatile aroma compounds is reviewed in Section 2.5, as it is also pertinent to one of the objectives (Chapter 3) of the present work.

2.2 Classifications and characteristics of yeasts

The classification of yeasts is very complex and can be based on their morphology, reproduction or fermentation and assimilation. For a study of the suitability of yeasts as cheese adjuncts, the most useful classification is on the basis of fermentation and assimilation. Glucose, fructose and mannose are utilised by the more than 400 species of yeast. In contrast, great differences between species in the utilisation of other carbon sources including other sugars are encountered. The common theme in the metabolism of carbohydrates is the conversion of glucose-6-phosphate into pyruvate by the Embden-Meyerhof-Parnas pathway. The subsequent fate of the sugar depends on both the sugar and the yeast. On the basis of the destiny of the pyruvate arising from glucose, yeasts may be divided into two groups, namely obligate aerobes and facultative anaerobes. Obligate aerobes are unable to utilise glucose in the absence of oxygen and their metabolism is exclusively respiratory, whereby the pyruvate is funnelled into the tricarboxylic acid (TCA) cycle to be completely oxidised. *Debaryomyces hansenii* belongs to this group. Facultative anaerobes are able to utilise glucose under aerobic and anaerobic conditions. In the latter case, they metabolise glucose to ethanol. During aerobic growth, both fermentation and respiration may contribute to the catabolism of glucose. On this basis, they are divided into two groups, *i.e.* fermentative yeasts and respiratory yeasts. During the aerobic growth of fermentative yeasts, only 10% of the available glucose is catabolised in respiration. *Saccaromyces* and *Brettanomyces* genera and some species of other genera also belong to this group. However, the great majority of yeasts belong to the respiratory yeast subgroup. During aerobic growth, less than 30% of the available glucose is fermented. *Candida* and *Kluyveromyces* belong to this category (Gancedo and Serrano, 1993).

In general, yeast cells are larger than most bacteria but they do vary considerably in size, ranging from 1 to 5 μm in width and from 5 to 30 μm or more in length. They are commonly ovoid, but some are elongated and some are spherical. Each species has a characteristic shape, but even in pure culture there is considerable variation in the size and shape of individual cells, depending on age and environment. Yeasts have no

flagella or other external organelles of locomotion (Pelczar *et al.*, 1986). Some fungi are dimorphic, *i.e.* they exist in two forms. Some fungi pathogenic to humans and animals have a unicellular and yeast-like form in their host, but have a filamentous mould form when growing saprophytically in soil or on a laboratory medium (Pelczar *et al.*, 1986). Two examples of dimorphic fungi are given below.

Geotrichum candidum, a yeast commonly found in soft cheeses, is dimorphic. In its teleomorphic state, this fungus has a yeast-like form and can produce ascospores. In the filamentous state, development occurs by vegetative multiplication involving large quantities of arthrospores produced by the disarticulation of the mycelium (Jollivet *et al.*, 1994). Gueguen and Jacquet (1982) studied the cultural characteristics of a number of *G. candidum* strains growing in different solid media and classified them according to morphological models depending upon physiological features and biochemical activities. Model 1: Cream-coloured colonies, yeast-like cells, an optimum growth temperature between 22 and 25°C, with lower growth at 30°C, copious production of arthrospores, giving few mycelia, acidifying, and low proteolytic activity. Model 2: Intermediary. Model 3: White-coloured colonies, more or less felting, a higher optimum temperature (25–30°C), with lower growth at 22°C, production of a few arthrospores, mycelial structures formed, alkalinising, and stronger proteolytic activity.

Debaryomyces hansenii exhibits yeast to mycelium dimorphism in the continuous fermentation of xylose-containing media (made from the acid hydrolysate of barley bran) depending upon the availability of oxygen. Low aeration caused the transition from oval cells to hyphae, and further increases in the dissolved oxygen concentration resulted in recuperation of the oval shape. Under the operational conditions used, xylitol was the major fermentation product in both morphological forms, whereas the production of ethanol increased when the yeast grew under hyphal morphology and oxygen limitation (Cruz *et al.*, 2000).

2.3 Yeasts in cheese and their potential for cheese ripening

Low water activity, low pH, low temperature and an anaerobic environment are some of the environmental attributes in cheese. The effects of these conditions on growth will determine whether a yeast strain is able to grow in cheese and contribute to maturation. The growth characteristics, the presence and importance of yeasts in different cheese varieties, their lipolytic and proteolytic activities, their capacities for sugar and organic acid assimilation, and their capacity for producing flavour compounds are discussed in this section.

2.3.1 Growth characteristics of yeasts

The effects of environmental factors, such as water activity, pH, temperature, oxygen and nutrients, on the growth of yeasts are discussed below.

Water activity

The majority of yeasts are less sensitive to the lowering of the water activity (a_w) than most bacteria. The yeasts from the genus *Zygosaccharomyces* are the most tolerant to low a_w. The most common is *Zygosaccaromyces rouxii*, strains that show growth at a_w as low as 0.76. The minimum a_w value for growth is influenced by the nature of the solute and by other ecological factors. Growth of *Z. rouxii* ceases at an a_w of 0.85 when salt is used to lower the a_w, whereas the minimum value for growth is less in the presence of sugars. Conversely, *D. hansenii* strains are noted for their high salt tolerance. The physiological basis of xerotolerance (resistance to low a_w) in yeasts is attributed to the synthesis and intracellular accumulation of polyols (glycerol, arabitol and erythritol) at low a_w values. Polyols are thought to be compatible solutes, both compensating for the concentration difference across the cell membrane and allowing the metabolic enzymes to function (Deak, 1991). At 10°C (pH 4.0–6.0), strains of *D. hansenii* were able to grow at a_w values of 0.92–0.99 whereas strains of *Yarrowia lipolytica* were highly affected by low a_w (a_w = 0.94) and no growth was observed at an a_w of 0.92 (van den Tempel and Jakobsen, 2000). *G. candidum* showed no growth in a medium containing 4% NaCl (van den Tempel and Nielsen, 2000).

pH

In general, yeasts prefer a slightly acidic medium, with an optimum pH between 4.5 and 6.5 (Pitt, 1974). van den Tempel and Nielsen (2000) also found that medium pHs of 4.5 and 6.5 were of minor importance for the growth of *G. candidum*, *D. hansenii*, *Penicillium roqueforti* and *Penicillium caseifulvum*. The pH of the environment affects appreciably other physiological behaviours of the yeast cell, such as heat tolerance and resistance to chemical compounds (Deak, 1991). However, most of the yeasts tolerate a wide range of pH and grow readily at pH values between 3 and 8.

Temperature

The vast majority of yeasts live in a range of diverse habitats with temperature domains falling between 0 and 45°C. Accordingly, most yeasts can be classified as mesophilic. A few yeasts are capable of growing at a temperature around 0°C as well as at 25–30°C. Some yeasts, *e.g. Candida* and *Cryptococcus* species, exhibit optimum growth at temperatures below 20°C. They are considered to be psychrophilic. A few yeasts, mainly associated with the digestive tract of animals, have a minimum temperature for growth above 20°C and a maximum temperature for growth of about 46–48°C, and are called thermophiles. The temperature for growth is influenced by other environmental factors. It is a general phenomenon that the optimum growth temperature is elevated in solutions of high sugar or salt concentration (Deak, 1991). Kassim and Khan (1996) reported 30°C to be the temperature optimum for growth of *G. candidum*. Gueguen *et al.* (1983) found that the temperature optima of yeast-like and matted cultures of *G. candidum* respectively were 22–25°C and 25–30°C.

Oxygen

Response to the gas composition of the environment could determine the survival of a yeast strain inside or on the surface of cheese. van den Tempel and Nielsen (2000) reported that the strains of *G. candidum* were the most resistant, among the microorganisms tested, towards 25% carbon dioxide. This effect was independent of the oxygen level. Strains of *D. hansenii* did not grow in 25% carbon dioxide in the presence of 4% NaCl, but grew well in the presence of 4% NaCl in a normal environment. They found that environmental conditions with 21% oxygen, 0% carbon dioxide and 0% salt were the most suitable for the growth of *G. candidum*, *D. hansenii*, *P. roqueforti* and *P.*

caseifulvum. Bretanomyces species have been found to be the yeasts most tolerant of carbon dioxide and are the main spoilage yeasts of carbonated beverages (Deak, 1991).

Nutrients

The most important nutrients for yeasts are carbohydrates, which serve as sources of energy. The aerobic utilisation of a substrate for growth is called assimilation, and the anaerobic metabolism of carbohydrate to ethanol and carbon dioxide is called fermentation. Yeasts can ferment only a few sugars, mostly hexoses and oligosaccharides. The range of carbon sources that are utilised aerobically is much wider, and includes hexoses, pentoses, alcohols, organic acids and some other carbon compounds. The ability to decompose complex carbohydrates is restricted to a few yeast species (Deak, 1991).

Amino acids, amines and urea are suitable nitrogen sources for practically all yeasts, as are inorganic ammonium salts.

In addition to basic carbon and nitrogen sources, inorganic microelements and small amounts of complex organic growth factors, mostly vitamins, may be required for the growth of yeasts. These requirements are normally fulfilled adequately in natural substances and foods. Yeasts vary widely in their requirements for minerals and growth factors. Many species synthesise all of the necessary vitamins for growth and propagate vigorously in vitamin-free media. Others require certain vitamins, and this can be used for identification purposes (Barnett *et al.*, 1990). Biotin appears to be the most commonly required vitamin; some species require niacin, thiamine, pantothenic acid, riboflavin or minocitol (Deak, 1991).

The cheese surface offers an amicable environment for the growth of yeasts and gives competitive advantages against bacteria, which need relatively high a_w and pH for growth. The a_w of cheeses is in the range 0.9–0.99. The common pH range of freshly made cheeses is 4.5–5.3. Cheeses are generally coagulated at 30–32°C and cooked at 37–38°C. The ripening temperatures for cheese vary from 6°C (Cheddar cheese) to 20°C (Emmental). Cheese is a good source of nutrients, containing residual lactose, lactic acid, protein and fat (Fox *et al.*, 2000A). The oxygen-reduction potential of cheese is

about –250 mV (a positive value indicates an oxidative state and a negative value indicates a reduced state). Because of the anaerobic environment inside cheese, the yeasts grow mostly on its surface (Fox *et al.*, 2000A). The yeast population of a Camembert cheese made from raw milk was 4-log cycles higher on the surface than in the inside (Devoyod, 1990).

2.3.2 Presence of yeasts in cheese

The occurrence of yeasts in cheese is not unexpected because of their tolerance towards low pH, low moisture content and elevated salt concentration (Fleet, 1990). Proteolytic and lipolytic capacities are the other attributes of yeasts that facilitate their growth in dairy products including cheese.

Yeasts contribute to spoilage in some cheeses, but make a positive contribution to flavour development during maturation in other cheeses. Assessment of the spoilage of cheese by yeasts is complicated by subjective judgements on whether yeast activity during maturation is detrimental or beneficial to product quality. Over-ripening during maturation could be interpreted as spoilage. Thus, continued lactose fermentation by yeasts at this stage could lead to increased acidity, gassiness and fruity flavours, and continued hydrolysis of protein and fat could contribute to bitter and rancid flavours as well as to softening of the product texture (Fleet, 1990). Yeasts that contribute to desirable qualities in dairy products, and cheeses in particular, have been dealt with in several investigations, but not in a systematic way, and have to only a very limited extent been brought to the point of active use, such as the application of yeasts as starter cultures (or adjunct cultures) in dairy products. Potential advantages resulting from the use of yeasts in the production and maturation of dairy products are evident. The advantages comprise beneficial microbial interactions between different desirable organisms including starter cultures, production of aroma components, acceleration of maturation processes by enzymatic activities, probiotic effects and inhibitory effects against spoilage organisms (Jakobsen and Narvhus, 1996).

Yeasts associated with particular groups of cheeses and their contributions towards maturation and flavour development are discussed under the following subheadings:

Blue and Camembert cheese, bacterial surface-ripened cheese, hard cheese and cheese flavour concentrate.

2.3.2.1 Yeasts in Blue and Camembert cheese

Yeasts are frequently found within the microflora of Camembert and Blue-veined cheeses. In a comprehensive study of 256 samples of Blue-veined cheeses, representing mostly Danablue, Roquefort and Gorgonzola varieties, de Boer and Kuik (1987) recorded a very high incidence of yeasts. The majority of the Gorgonzola and Roquefort samples (87% and 77% respectively) contained yeast populations exceeding 10^6 cfu/g, with some samples having 10^7–10^8 cfu/g. *D. hansenii* was the most frequent species, but isolates of *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Y. lipolytica* were also obtained.

The yeasts impact on cheese quality through their production of lipolytic and proteolytic enzymes, their fermentation of residual lactose, their utilisation of lactic acid and their autolysis (Fleet, 1990). High intracellular protease activities were found to occur in *D. hansenii* (Lenoir, 1984), which could be significant in ripening after autolysis (Roostita and Fleet, 1996). The frequent occurrence of *Candida lipolytica* (*Y. lipolytica*) and *Candida catenulata* in the cheese correlated with their very strong lipolytic and proteolytic properties (Roostita and Fleet, 1996A; Pereira-Meirelles *et al.*, 1997; Suzzi *et al.*, 2001), ability to utilise citric and lactic acids and growth at lower temperatures (Roostita and Fleet, 1996, 1996A). The key property of *K. marxianus* leading to its growth in cheese is its very strong ability to ferment lactose. However, the proteolytic and lipolytic activities of this yeast were found to be very weak (Roostita and Fleet, 1996, 1996A).

Mourgues *et al.* (1983) added *G. candidum* to Camembert cheese and found that it grew on the cheese surface; counts were 7.5 x 10^5 cfu/g after 24 h and 2.3 x 10^7 cfu/g after 15 days. Addition of *G. candidum* reduced bitterness and enhanced the flavour characteristics of traditional Camembert cheese. Although the pH of the cheeses during ripening was increased by the presence of *G. candidum*, there was no difference from the control after ripening. This suggests that *G. candidum* could be useful for improving the organoleptic characteristics of Camembert cheeses. Vassal and Gripon (1984)

reported that inoculation of the cheese milk with *G. candidum* in addition to *Penicillium caseicolum*, in the manufacture of Camembert cheese, resulted in a higher pH on the cheese surface, slower growth of *P. caseicolum* and less bitter peptide formation. *G. candidum* has a higher aminopeptidase activity than *Penicillium camemberti*; it may be the pathway by which *G. candidum* decreases bitterness (Molimard *et al.*, 1994). Kawai and Nakazawa (1997) produced Camembert cheese using a strain of *G. candidum*. This cheese produced more free amino acids, free fatty acids, volatile organic acids and aroma constituents than the control. *G. candidum* caused a significant inhibition of fungal contamination on Camembert cheese (Nielsen *et al.*, 1998).

2.3.2.2 Yeasts in bacterial surface-ripened cheese

The yeasts represent an important part of the microflora of the bacterial surface-ripened cheeses. Species from the genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Hansenuela*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Yarrowia*, *Zygosaccaromyces* and *Geotrichum* have been found among the yeast flora of surface-ripened cheeses (Corsetti *et al.*, 2001).

Assimilation of lactate, formation of alkaline metabolites, liberation of bacterial growth factors, fermentation of lactose, lipolysis, proteolysis and formation of aroma compounds are some of the activities of yeasts that are considered to be important for the typical characteristics of the smear surface-ripened cheeses. The increase in pH by yeasts helps the function of surface-growing bacteria (Fleet, 1990; Corsetti *et al.*, 2001). Proteinases from smear cheese microflora, such as *Coryneforms* and *Micrococci*, generally show alkaline pH optima (Corsetti *et al.*, 2001). Yeasts also synthesise substances that stimulate the growth of the bacteria (Fleet, 1990). It has been shown that *Brevibacterium linens* isolated from Limburger cheese requires pantothenic acid for growth and the yeasts from the same cheese synthesise considerable amounts of pantothenic acid and other vitamins useful for bacterial growth (Purko *et al.*, 1951; cited in Corsetti *et al.*, 2001). Specific types of peptides produced by certain types of yeasts may promote growth for certain types of bacteria (Corsetti *et al.*, 2001).

2.3.2.3 Yeasts in hard cheese

Fleet and Mian (1987) reported that 48% of 23 samples of Australian Cheddar cheese had yeast counts in the range 10^4–10^6 cfu/g. The most frequently isolated species from the cheeses were *Candida famanta*, *K. marxianus* and *Candida diffluens*. Viljoen (1995) examined sources of yeast contamination in Cheddar and Gouda cheesemaking and isolated 187 representative yeast isolates from the factory environment, working surface, brine and workers' hands and aprons. The most prevalent isolates belonged to the genera *Debaryomyces* and *Candida*. Other genera encountered were *Cryptococcus*, *Rhodotorula*, *Yarrowia*, *Pichia*, *Trichosporon*, *Torulaspora*, *Issatchenkia*, *Saccharomyces* and *Zygosaccharomyces*.

Only a limited amount of published information on yeasts used as adjunct cultures in the manufacture of hard-type cheese is available. About half of the studies were published after the present work was started in 2001. Deiana *et al.* (1984) added *D. hansenii* to ewe's milk at the same time as the cheese starter in the manufacture of Pecorino Romano cheese. The yeast developed well in the cheese and resulted in more rapid proteolysis and overall ripening; the organoleptic score was not affected by the presence of the added *D. hansenii*. Analysis of samples from different parts of the wheel showed that the L (+) lactic acid concentration decreased during ripening (240 days), particularly in cheeses containing higher contents of yeast cells, whereas the D (–) lactic acid concentration increased during the first 30–60 days of ripening and then decreased slowly. Production of free fatty acids in the cheeses was higher, and that of acetic acid was lower, in the presence of added *D. hansenii*. Guerzoni *et al.* (1996) added *Y. lipolytica* to the curd individually and in combination with lactic acid culture and compared this with the traditional cheese. Fourier transform infrared (FTIR) spectroscopy showed remarkable differences between 2-month-old cheeses with and without *Y. lipolytica* adjunct, which were attributed to higher lipolytic and proteolytic activities of the yeast adjunct. A preliminary panel test, conducted for 15-day-, 1-month- and 2-month-old cheeses, gave the highest score for flavour, body and texture to the cheeses obtained using *Y. lipolytica* as a starter adjunct without lactic acid culture, followed by the cheese inoculated with both *Y. lipolytica* and lactic acid culture. The samples were characterised by a significant intensification of flavour and an accelerated desired textural development. Ferreira and Viljoen (2003) added *Y. lipolytica* and *D.*

hansenii as starter adjuncts in Cheddar-type cheese. The yeast species when added individually contributed to the development of bitter flavours despite accelerated development of strong Cheddar flavours. However, when both species were incorporated as part of the starter culture, the cheese had a good strong flavour after 4 months of ripening. The cheese had a clean, slightly sweet, pleasant taste and still retained this good flavour 9 months after production. Delforge (1997) reported on the 'Swing' range of microbial starters launched by Chr. Hansen. The starters contain combinations of a range of microorganisms including a few species of yeasts such as *Candida valida*, *D. hansenii*, *K. marxianus* and *G. candidum* and were targeted at improving curd texture and cheese flavour during ripening.

Yeast cultures did not grow in hard cheeses during ripening. In Cheddar cheese, *D. hansenii* decreased from 10^5 cfu/g initially to 10^2 cfu/g after 6 months, whereas *Y. lipolytica* decreased from 10^4 cfu/g initially to beyond detection after 4 months (Ferreira and Viljoen, 2003). Crow *et al.* (2002) also reported die-off of yeast adjuncts from 10^7 cfu/g to < 10^3 cfu/g in 24 h and then beyond detection after 2–4 weeks in cheeses in which yeasts had been used as anaerobic adjuncts. However, Guerzoni *et al.* (1996) reported that *Y. lipolytica* was able to grow up to 10^8 cfu/g and 10^7 cfu/g in the cheese rind and in the cheese centre respectively.

The yeast inocula did not inhibit the growth of lactic acid bacteria but rather activated their growth. For example, *Y. lipolytica* did not show any inhibition of both inoculated and naturally occurring lactic acid bacteria in cheese; in contrast, one of the *Y. lipolytica* strains seemed to stimulate the proliferation of lactic acid bacteria compared with the control (no yeast) (Guerzoni *et al.*, 1996). The utilisation of lactose was faster in cheeses with yeast adjunct cultures than in the control (no yeast). It has been postulated that the reason for the increase in lactose utilisation could be the increased lactic acid bacterial activity in the presence of the growth factors that were produced by yeast metabolism (Ferreira and Viljoen, 2003). Yeast extract, as an additive to the Cheddar curd, has been reported to improve the growth of non-starter lactic acid bacteria (NSLAB), which eventually improved and accelerated cheese flavour development (Rehman *et al.*, 2003).

Not only the yeast adjunct cultures but also crude yeast enzyme preparations and yeast extracts have been used to improve and accelerate cheese flavour in different studies. Most of these studies were carried out with Cheddar cheese. Grieve (1982) reported the use of yeast protease to accelerate the ripening of Cheddar cheese. Crude, cell-free protease extracts of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* were prepared, and were found to be active towards casein at the cheese pH and did not produce bitter casein hydrolysis products. The extract contained acid endopeptidase, serine carboxypeptidase and serine endopeptidase activity. Small amounts of aminopeptidase activity were also present in the extracts. Initially, three experimental cheeses were treated with *S. cerevisiae* protease extracts (added to the cheese milk). Differences in maturity between the test and control cheeses were estimated. Both proteolysis in the cheese and acceleration of ripening varied when different preparations of similar total proteolytic activity were added. When a 2.2-fold increase in trichloroacetic-acid-soluble nitrogen and a 2.5-fold increase in phosphotungstic-acid-soluble nitrogen were obtained in an experimental cheese, the grading results indicated 6 weeks advancement in ripening after 3 months of storage at 8°C. In contrast, when only a 1.5-fold increase in trichloroacetic-acid-soluble nitrogen and no increase in phosphotungstic-acid-soluble nitrogen were obtained, no acceleration of ripening after 3 months of storage was observed. However, details of the different preparations mentioned were not available. Mucchetti *et al.* (1983) also reported enhanced ripening and flavour of cheese with crude enzyme preparations of *G. candidum*.

2.3.2.4 Yeasts in cheese flavour concentrate

Kalle and Deshpande (1977) developed a process for producing processed cheese with bland curd and flavoured cream fermented with *C. lipolytica* (*Y. lipolytica*). The unique advantage of the approach was that the process itself was suitable for the Indian (the place of their study) situation where production of natural cheese was not a major industry and unnecessary investment in long term ripening times could be avoided. Mazas *et al.* (1998) described the production of cheese flavour with *C. lipolytica* (*Y. lipolytica*). Cheese whey was homogenised with 15% (v/v) butter oil, adjusted to pH 6.5 and fermented in a shake culture at 30°C with *C. lipolytica* (*Y. lipolytica*). A strong well-defined cheese flavour was noted after 48 h, and a rancid flavour with proteolysis was detected after 72 h.

To summarise the 'presence of yeasts in cheese', it can be said that yeasts are present in most cheeses. Yeasts are deliberately added as surface microflora in surface-ripened cheeses for their positive contributions towards cheese ripening. A limited number of studies in which yeasts have been used as adjunct cultures in hard varieties of cheese have been reported.

2.3.3 The lipolytic activity of yeasts

Lipolysis is important for cheese flavour because different fatty acids have different flavours themselves, and they are transformed into esters, lactones or ketones, which are also constituents of cheese flavour (McSweeney, 2004). The specificity of yeast lipase might be used to develop a novel cheese. In this regard, a review of the literature on yeast lipase is relevant. This section of the literature review deals mostly with the lipolytic capacities of *G. candidum* and *Y. lipolytica*, the lipolytic yeasts studied in the present work. These two yeasts were selected for their high ripening potential in terms of enzyme activity and production of volatile compounds, studied by different groups in laboratory media or in cheese slurry. Another yeast, *Candida kefyr*, was also selected for the present study on the basis of its potential for lactose utilisation and flavour synthesis (personal communication with Vaughan Crow, Fonterra Co-operative Group Ltd, Palmerston North, New Zealand). However, published literature on the enzymatic system of this yeast was not available.

2.3.3.1 *Geotrichum candidum* lipase

The extracellular lipase secreted by *G. candidum* has been studied because of its ability to lipolyse the fat in dairy products and because of its specificity for the unsaturated fatty acids (Tahoun *et al.*, 1982).

Effects of media components

The constituents of the growth media have an effect on the enzyme activities of microorganisms. Most of the studies demonstrated induction of *G. candidum* lipase by fatty acids or triglycerides and inhibition by sugars. Baillargeon *et al.* (1989) examined three stains of *G. candidum* (ATCC 34614, NRRL Y-552 and NRRL Y-553) for lipase production and activity. For all three strains of *G. candidum* studied, lipase production

was stimulated by the presence of oil or glycerol. In contrast, glucose and sucrose inhibited lipase production. El-Shanawany (2000) reported different effects of different fats on the lipase activity of *G. candidum*. Lipase activity was stimulated by adding 0.1% butter or sunflower oil to the growth medium, whereas corn, olive and palm oils reduced the production of lipase. Shimada *et al.* (1992) demonstrated that long-chain fatty acids and their methyl esters induced lipase production, whereas middle and short-chain fatty acids and their esters did not. Lipase production increased with the chain length of the fatty acids, and long-chain unsaturated fatty acid esters were the most effective. They postulated that the fatty acids, as a metabolic product of the hydrolysis of glyceryl esters, stimulate the expression of the *G. candidum* lipase gene.

However, some of the relatively older studies showed contrasting effects (compared with the results in the previous paragraph) of oil and sugar on the *G. candidum* lipase. Chander and Klostermeyer (1983) studied various media constituents affecting lipase production by *G. candidum*. They found that *G. candidum* showed the best growth and maximum lipase production in the presence of sucrose, followed by maltose, galactose, xylose, glucose, fructose and mannitol in decreasing order in the medium. They noted maximum growth and lipase synthesis in the presence of peptone in the medium whereas minimum lipase yield was recorded in the presence of ammonium nitrate and ammonium sulphate. They reported that synthetic triglycerides, butter oil, corn oil, mustard oil, olive oil and sunflower oil caused a reduction in lipase synthesis. Tahoun *et al.* (1982) studied the effects of different carbon sources on the amount of mycelial lipase and extracellular lipase produced by *G. candidum*. They found that glucose or olive oil (1%) caused an increase in extracellular lipase activity. In contrast, a remarkable increase in mycelial lipase activity was observed when the fungus was grown in the presence of Tween 80 (0.2%) or oleic acid (0.1%).

Effects of culture conditions

Not only the constituents of the growth medium but also the culture conditions affect the lipase activity of *G. candidum*. Baillargeon *et al.* (1989) reported that lipase production for all three strains (ATCC 34614, NRRL Y-552 and NRRL Y-553) grown on oil was highest and most rapid at 30°C and a neutral pH with 300 rev/min. Chander and Klostermeyer (1983) also found that maximum lipase production by *G. candidum* occurred at 30°C and pH 7.0 after 3 days. They observed that the increased mycelial

growth and lipase production were obtained in shake cultures rather than static culture. In contrast, Tahoun *et al.* (1982) achieved maximum lipase production by *G. candidum* culture under static conditions at 30°C and pH 5.5 after 7 days.

Stability of lipase

The stability of microbial enzyme activity is important for cheese ripening, especially if the enzyme is produced before the culture is added to the cheese. The lipase activity in *G. candidum* culture filtrates decreased with time and the decrease has been attributed to the presence of proteases (Baillargeon *et al.*, 1989). Tahoun *et al.* (1982) also reported the instability of the *G. candidum* lipase.

Fatty acid specificity

Lipases of *G. candidum* are known to show specificity for the hydrolysis of unsaturated fatty acids with a *cis* double bond at the 9-position (oleic, linoleic and linolenic acids) over the corresponding saturated fatty acid (stearic acid) (Jensen, 1974). *G. candidum* contains two types of lipase. No officially recognised nomenclature exists for distinguishing lipases with or without specificity towards fatty acids. Different workers have described the fractions in different ways. Charton *et al.* (1992) introduced a nomenclature in the following way: lipase type A, lipase without high specificity for unsaturated substrates with *cis*-9 double bonds; lipase type B, lipase with high specificity for esters of fatty acids containing *cis*-9 double bonds. Cloning of the lipase genes from four different strains of *G. candidum* revealed that all strains contained two genes coding for two types of lipase (Bertolini *et al.*, 1994). Some differences in the specificity among different strains have been reported. Jacobsen and Poulsen (1995) compared the type A lipase and the type B lipase of different strains of *G. candidum* and indicated that the specific activity for *cis*-9 fatty acids was related to the peptide pattern of the type B lipase.

Baillargeon *et al.* (1989) evaluated different strains of *G. candidum* for their fatty acid specificity and found that the lipase from the strain NRRL Y-553 and lipase 26557 RP displayed preferential specificity for hydrolysing oleic acid (C18:1) esters, whereas the lipases from strains ATCC 34614 and NRRL Y-552 and lipase GC-4 failed to discriminate between palmitic acid (C16) and oleic acid. Lipases from *G. candidum* strain NRRL Y-553 are of interest because of their unique specificity for *cis*-9

unsaturated fatty acids relative to both stearic acid (C18) and palmitic acid. The specificity of the crude lipases for the hydrolysis of 4-methyl-umbelliferyl esters of oleic acid over palmitic acid was 20 to 1. The specificity of the purified, partially separated lipases was similar to that of the crude preparation (Baillargeon and McCarthy, 1991). Baillargeon (1990) purified a crude commercial *G. candidum* lipase preparation (Amano GC-20) by hydrophobic interaction chromatography on octyl sepharose and found that the purified enzyme was a micro-heterogeneous glycoprotein, containing isozymes varying in molecular weight, pI and specificity. It consisted of 64, 62 and 59 kDa species as determined by denaturing polyacrylamide gel electrophoresis (PAGE). Five isozymes (pI 4.4, 4.47, 4.58, 4.67 and 4.72) were detected by isoelectric focusing. All the isozymes were specific for oleate esters over stearate esters; one isozyme (pI 4.72) was also specific for oleate over palmitate.

In recent years, fats rich in unsaturated fatty acids are becoming popular for health reasons but many of the natural triglycerides contain significant amounts of palmitic acid (C16) and stearic acid (C18). Thus, to selectively produce unsaturated fatty acids from agricultural fats and oils, a lipase that selectively releases the *cis*-9 unsaturated fatty acids in preference to both C16 and C18 is required. The lipase of *G. candidum* NRRL Y-553 was used to selectively hydrolyse *cis*-9 unsaturated fatty acids from rapeseed oil, tallow and soy oil (Baillargeon and Sonnet, 1991).

Diks and Lee (1999) evaluated the selective hydrolysis of sunflower oil using *G. candidum* B (GCB) lipase from the strain CMICC 335426 as a means to produce a very low saturated fatty acid concentrate. They observed up to 60% hydrolysis within 6–8 h. Because of the high specificity of the GCB lipases, the level of unsaturated fatty acids in the free fatty acid (FFA) fractions was > 99% w/w. After separation of the oil phase, the unsaturated fatty acids were recovered from the mixture by evaporation and were reconverted to triglycerides by enzymatic (immobilised *Rhizomucor miehei* lipase) esterification with glycerol to produce an oil with less than 1% saturated fatty acids (SAFA), whereas the starting sunflower oil originally contained 12% SAFA.

The specificity of *G. candidum* lipase has been used for the production of different, physiologically active polyunsaturated fatty acids (PUFA), such as docosahexanoic acid

(22:6, DHA), eicosapentanoic acid (20:5, EPA) (Shimada *et al.*, 1994, 1995) and γ linoleic acid (Fogila and Sonnet, 1995).

Specificity towards CLA isomers

Conjugated linoleic acid (CLA) is the name given to a group of isomers of octadecadienoic fatty acids containing a pair of conjugated double bonds in either the *cis* or *trans* configuration, located at various positions on the carbon chain. The commercially available CLA is a mixture of different isomers. The CLA isomers are of interest because of their physiological effects; however, different CLA isomers have different physiological effects. The physiological effects of CLA and its natural sources are discussed in Section 2.4. There has been interest in separation of CLA isomers for clinical trials to understand the effect of individual isomers. A unique property of *G. candidam* lipase is its ability to preferentially attack the *cis*-9, *trans*-11 isomer over other isomers of CLA, which could be utilised to obtain pure CLA isomers.

Haas *et al.* (1999) examined the abilities of preparations of *G. candidum* lipase to distinguish between the various CLA isomers. Both the enzymatic esterification of mixed free CLA with alcohols to form simple esters and the hydrolysis of mixed CLA esters were studied. The commercial methyl ester preparation of CLA was resolved into 12 CLA peaks using three Ag^+-HPLC (high performance liquid chromatography) columns in series. On the basis of relative peak areas of the detector chromatogram, the *cis*-9, *trans*-11 CLA isomer constituted 34% of the total CLA content. Hydrolysis with *G. candidum* lipase B caused progressive reduction of the *cis*-9, *trans*-11 C18:2 isomer to about 20% after 30 min and to 8% after 4 h. The resultant FFA fractions contained 95% *cis*-9, *trans*-11 C18:2 FFA. Incubation of a commercial preparation of CLA containing at least 12 different isomers with methanol and *G. candidum* lipase B in 3-heptanone resulted in the selective esterification of the *cis*-9, *trans*-11 isomer. In the FFA fraction, the isomer was reduced from 34 to 25% during 22.5 h of incubation, with no apparent reductions in the amounts of other CLA isomers in the FFA fraction. Thus, they found high specificity of *G. candidum* lipase B in both hydrolysis and esterification. McNeill *et al.* (1999) demonstrated that a lipase from *G. candidum* esterified the *cis*-9, *trans*-11 isomer more rapidly than the *trans*-10, *cis*-12 isomer. The selectivity was exploited at a kilogram scale to prepare an ester fraction with a content

of 91% *cis*-9, *trans*-11 CLA and an unreacted FFA fraction consisting of 82% *trans*-10, *cis*-12 CLA, based on the total CLA content.

Positional specificity

G. candidum lipase has also been shown to have positional specificity. Sugihara *et al.* (1991) found that a *G. candidum* strain, isolated in their laboratory, produced four molecular forms of lipase (I, II, III, IV) and that one (IV) showed some preference for the 2-position ester bond. Hydrophobic interaction chromatography of *G. candidum* lipase on a Butyl Toyopearl 650S column resolved the enzyme into four components, one major component (I) and three minor components (II, III, IV). About 84% of the total activity was ascribed to lipase I, and only 1.4% was ascribed to lipase IV. Sugihara *et al.* (1994) reported that lipase III had positional specificity similar to that of lipase IV. They found that the ratio of the hydrolysis rate of the 2-position ester bond of triolein to that of the 1- and 3-position ester bonds was 2:1. Although amino acid analysis failed to discriminate lipase III from lipases I and II, Edman degradation demonstrated that lipase III had an N-terminal sequence that was different from those of lipases I and II. They concluded that lipase III was derived from a gene distinct from those of lipases I and II (Sugihara *et al.*, 1993).

To summarise *G. candidum* lipase, it can be said that the lipase activity is affected by medium composition as well as by growth parameters. Triglycerides were able to induce lipase activity of *G. candidum* in most of the studies. *G. candidum* lipase has been found to have fatty acid specificity towards unsaturated fatty acids, isomer specificity for *cis*-9, *trans*-11 CLA and positional specificity towards 2-position ester bonds.

2.3.3.2 *Yarrowia lipolytica* lipase

The overall lipolytic activity and fatty acid specificity of a *Y. lipolytica* strain are important for its potential use in cheese, as not only the total lipolytic activity but also the specific release of fatty acids will affect the development of cheese flavour.

Fatty acid specificity and effect of isolation source

Y. lipolytica strains are different in their total lipolytic activity as well as in their fatty acid specificity according to their isolation source. Suzzi *et al.* (2001) reported a diversity of lipolytic activity among *Y. lipolytica* strains isolated from different dairy products. In particular, the degree of specificity for saturated and unsaturated fatty acids as well as for fatty acids with an even or odd number of carbon atoms varied among the strains. They studied the FFA production by *Y. lipolytica* in butter agar media after 3 and 6 days of incubation at 25°C. In terms of total lipolytic activity, two different behaviours were observed. Some strains showed very high lipolytic activity over the first 3 days of growth, producing the highest amounts of total FFA. In samples inoculated with other strains, a lower lipolytic activity was observed after 3 days of growth and the total FFA content increased after 6 days of incubation.

However, the specificity of the individual fatty acid release was not associated with this preliminary grouping. They noted that the short-chain FFAs (C4–C10) were produced by all strains at low levels, corresponding to only about 1–2% of the total FFAs. Short-chain FFAs were generally released during the initial days of incubation and later, with one exception, they disappeared. A decrease in previously released long-chain FFAs was observed after 6 days of incubation for some strains. In all strains, the major FFA released was C18:1 followed by C16, with the exception of two strains in which C16 represented the most prominent fatty acid released, followed by C18:1. The other major FFAs were myristic acid (C14) and C18. All the strains hydrolysed the fats with the liberation of high concentrations of even-carbon-number FFAs, whereas odd-carbon-number FFAs represented only a limited proportion of the total FFAs. The fatty acid profile was found to be totally modified after 6 days of incubation, compared with the profile after 3 days, for one strain of *Y. lipolytica*. A relevant increase in lauric acid (C12), pentadecanoic acid (C15) and margaric acid (C17), presumably at the expense of C14, C16 and C18:1, was observed (Suzzi *et al.*, 2001). They suggested that transformation of fatty acids to other fatty acids catalysed by yeast enzymes could be a reason for the changed profile. Different researchers have also suggested some mechanisms for the transformation of one fatty acid to another by yeasts. Ercoli *et al.* (1992) suggested that the yeasts could oxidise fatty acids by a conventional process of β-oxidation to yield fatty acids having two atoms of carbon less than the parent.

Guerzoni *et al.* (2001) also reported variation in lipolytic activity among the *Y. lipolytica* strains isolated from different sources. They found that C18:1 fatty acid was the principal fatty acid released in butter inoculated with the majority of the strains. It accounted for as much as 76.7% of the FFAs for one strain but its presence did not exceed 15% for some other strains. In contrast, butter samples inoculated with the latter strains were characterised by a high proportion of free linoleic acid (C18:2) and C18 fatty acid. Moreover, a selective release of C17 and heptadecanoic acid (C17:1) was achieved by one of these strains. Roostita and Fleet (1996A) reported that the main FFAs produced by *C. lipolytica* (*Y. lipolytica*) and *C. catenulata* were C18:1, C8 (caprylic), C12, C6 (caproic), C14 and C16, with their concentrations increased by 5–10-fold after 12 days in milk.

Effect of the constituents of the media and growth conditions

The constituents of the media and growth parameters, such as lipid source, oxygen pressure, pH and water activity, as well as the cell morphology of *Y. lipolytica* are regarded as determinants of lipase biosynthesis (Guerzoni *et al.*, 2001).

The lipolytic activity of *Y. lipolytica* strains has been found to be influenced by the constituents of the growth medium. Pereira-Meirelles *et al.* (1997) demonstrated an increase in lipase activity of *Y. lipolytica* in the presence of long-chain fatty acids or triacylglycerides (TAGs) containing long-chain fatty acids. A TAG containing only short-chain fatty acids was not as efficient (as an inducer of lipase activity) as a TAG containing long-chain fatty acids. The lipase activity of *Y. lipolytica* was found to show glucose depression. The depression of the lipase activity by glucose occurred irrespective of the presence of an inducer. Peptone was found to be a more efficient nitrogen source than inorganic nitrogen sources for lipase production by *Y. lipolytica*. There was a correlation between the maximum lipolytic activity and the increase in biomass concentration during cultivation of *Y. lipolytica* at 29°C with 160 rev/min. However, no correlation between the specific growth rate and lipase activity was found (Pereira-Meirelles *et al.*, 1997).

Environmental conditions, such as the presence of NaCl and the pH of the medium, could affect the lipase production of *Y. lipolytica*. Guerzoni *et al.* (2001) studied the effect of NaCl and lactic acid on the lipase activity of *Y. lipolytica* and modelled the

effects. They showed that both NaCl concentration and lactic acid concentration had an individual stimulating effect on lipolytic activity, particularly on total FFA release. In contrast, synergistically both factors inhibited lipolytic activity. Growth temperature also has an effect on the lipase activity of *Y. lipolytica*. Carini *et al.* (1975) noted that hydrolysis of milk fat by *Candida* sp. at 15°C gave a greater release of short-chain fatty acids [C4 (butyric) and C6)] whereas at 35°C there was a greater production of long-chain fatty acids.

Lipase location

Enzyme location (inside or outside the cell) is an important characteristic, as it may determine the possibility of substrate--enzyme contact. Pereira-Meirelles *et al.* (2000) assayed different fractions of the *Y. lipolytica* enzyme and found that the location of the enzyme changed with different phases of growth. At the beginning of the cultivation, in olive oil medium, only a minimal level of lipase was observed. Lipid was present in large amounts in the culture medium and lipase activity was induced. In the first exponential phase, the enzyme molecules were mostly located at the cell surface, until about 50% of the substrate was consumed. As soon as the carbon source fell below this level, the lipase started to be released into the culture medium, *i.e.* in the second stationary phase. Maximum extracellular lipolytic activity was detected in the late stationary phase; when cell growth stopped and the substrate was exhausted, the enzyme molecules, still cell bound, were released, and only a basal level remained inside.

Thus, the main characteristics of *Y. lipolytica* lipase, noted from the above review, are: the lipase activity of *Y. lipolytica* is dependent on the isolation source and is induced by the presence of lipids in the medium. For most of the strains, the predominant FFA produced is C18:1. Lipase activity is at its highest in the late stationary phase and is mostly extracellular.

2.3.4 The proteolytic activity of yeasts

Breakdown of casein is an indicator of cheese ripening. Casein is broken down by proteolytic activities of the coagulating enzyme and milk's native enzymes as well as by the enzymes of the added microorganisms. Peptides, the initial breakdown products of proteolysis, are further broken down to smaller peptides and amino acids by peptidase

activity imparted mainly by the added microorganisms. The amino acids are further converted into a range of compounds, which are important for cheese flavour (McSweeney, 2004). For consideration of a yeast strain as an adjunct culture in cheese manufacture, knowledge of its proteolytic, peptidolytic and amino-acid-converting properties is important. This section reviews some published information on the proteolytic and peptidolytic properties of yeasts, concentrating on *G. candidum* and *Y. lipolytica*. However, the catabolism of the amino acid L-phenylalanine by yeasts is discussed in detail in Section 2.5.

2.3.4.1 The proteolytic system of *G. candidum*

Chen and Ledford (1972) found that *G. candidum* was proteolytic on the α_s and β fractions of casein. Gueguen and Lenoir (1975) demonstrated variable proteolytic activities in different strains of *G. candidum* depending upon their isolation source. A classification of the strains according to their origin showed that most of those isolated from soft cheeses had higher growth and proteolytic activity than those isolated from hard cheeses. In contrast, Baillargeon and McCarthy (1991) did not find any proteolytic activity in the crude broth of *G. candidum* NRRL Y-553 using an agar diffusion method with casein as a substrate.

Gueguen and Lenoir (1976) studied the characteristics of the extracellular and intracellular proteolytic enzymes of six strains of *G. candidum*. The maximum casein hydrolysis by the extracellular system of all strains occurred over the pH range 5.5–6.0 at an optimum temperature of 55°C. For the intracellular enzymes, the optimum conditions were also in the pH range 5.5–6.0 and a temperature range of 50–55°C. The stabilities of the two systems were different, the extracellular system being more thermo labile and more rapidly inactivated at pH 4.0.

The peptidolytic activity of yeasts might also play an important role in the breakdown of bitter peptides by releasing smaller peptides and amino acids (Lenoir *et al.*, 1985). In particular, *G. candidum* is known to show such activity (Devoyod, 1990). Hannan and Gueguen (1985) reported the endopeptidase activity to be mostly intracellular. They found that glucose might exert a depressive effect on the endopeptidase activity; activity increased sharply when glucose was almost fully consumed. Optimum endopeptidase

activity occurred in the stationary phase. The endopeptidase activity of *G. candidum* is thought to be effective for reducing the bitterness in cheese.

Greenberg and Ledford (1979) studied the metabolism of two major casein amino acids, glutamic acid and aspartic acid, by *G. candidum*. Growth of *G. candidum* in an HCl-acidified amino-acid-containing medium elevated the pH of the medium from 3.5 to above 7. The organism grew in a low protein medium; however, the pH of the medium was not shifted upwards significantly. The result was similar with the use of lactic acid as acidulant. They hypothesised that, in the ripening of cheeses of the Limburger type, *G. candidum* not only metabolises lactic acid, a generally accepted role, but also neutralises the acidity by protein-associated changes involving ammonia. To determine the substrates for the ammonia liberation, intracellular extracts were incubated with different amino acids and glutamic acid and aspartic acid were identified as the source. The deamination of glutamic acid was presumed to involve two reactions: the isomerisation of glutamic acid to β-methylaspartic acid, followed by deamination of this compound to ammonia and mesaconic acid. The greatest amount of activity, which required vitamin B_{12}, magnesium and potassium, occurred in phosphoric acid buffer in the vicinity of pH 7.6. The maximum activity for deamination of aspartic acid to fumaric acid and ammonia in phosphate buffer occurred at pH 7.0; magnesium was required for this aspartase activity. They found the temperature optima to be in the vicinity of 25°C.

Thus, strain dependence of proteinase activity, de-bittering activity by endopeptidase and production of ammonia through amino acid catabolism are the main features discussed in the review of the '*G. candidum* proteolytic system'.

2.3.4.2 The proteolytic system of *Y. lipolytica*

Suzzi *et al.* (2001) compared eight strains of *Y. lipolytica* from different isolation sources for their proteolytic activity. The yeasts were inoculated in skim milk and incubated at 10°C and 25°C. Proteolytic activity was very weak for all strains after 8 days, whereas a marked increase in proteolytic activity was observed between the 8th and 14th days. The incubation temperature markedly affected the proteolytic activity. The proteolytic activity of all strains was higher at 25°C than at 10°C in skim milk

medium. van den Tempel and Jakobsen (2000) found proteolytic activity in four out of six experimental strains at 10°C with shaking at 200 rev/min for 10 days in an agar diffusion method. High pressure capillary electrophoresis (HPCE) showed that these strains degraded all casein components. Kalle *et al.* (1976) reported proteinase activity in the C_1B strain of *Y. lipolytica* var. *planta*. Guerzoni *et al.* (1996) studied the action of *Y. lipolytica* extracellular proteinase activity *in vitro* on α_{s1}- and β-caseins. The breakdown of these proteins at pH 7.0 by the 72 h culture supernatants of 12 strains was analysed after 15 h of contact at 30°C with urea PAGE and breakdown of α_{s1}-casein was observed. They grouped the strains into four according to the pattern of hydrolysis. Reverse phase HPLC confirmed the differences already observed using PAGE.

Hydrolysis of β-casein was found to be another characteristic of *Y. lipolytica* proteinase. The PAGE of β-casein hydrolysis showed five different band patterns (Guerzoni *et al.*, 1996). The protein is, in general, not degraded by chymosin in cheeses. In fact, because of hydrophobic interactions, there is no access to specific hydrolysis sites for chymosin. Moreover, plasmin, which is able to hydrolyse β-casein, is very much at a variable level in milk. Thus, the hydrolysis of β-casein, which has an important role in the rheological properties of cheese, is much slower than that of α_{s1}-casein during the ripening of cheese. In Cheddar cheese, the half-life of α_{s1}-casein was 2 days whereas that of β-casein could be 37 weeks (Bogenrief and Olson, 1995). The predominant amino acids produced by *C. lipolytica* (*Y. lipolytica*; isolated from cheese) in milk were leucine, glutamic acid, valine, phenylalanine and arginine (Roostita and Fleet, 1996A).

While reviewing the proteolytic system of *Y. lipolytica* in the above paragraphs, the salient features noted were that the proteolytic capability of *Y. lipolytica* is strain dependent and the *Y. lipolytica* proteinase is able to break down β-casein, which is broken down at a slower rate than α-casein in cheese.

2.3.4.3 The proteolytic systems of other yeasts

Different researchers have studied the proteolytic systems of several yeasts other than *G. candidum* and *Y. lipolytica*. Only a few relating to dairy products are reviewed in this section. *K. lactis* was found to have proteolytic activity and the rate of hydrolysis of the

individual caseins by cell-free extracts of a *K. lactis* strain followed the order of κ- > α_s- > β-casein (Grieve *et al.*, 1983).

D. hansenii was not able to hydrolyse casein at 10°C in an agar diffusion test (van den Tempel and Jakobsen, 2000). Kumura *et al.* (2002) isolated a strain of *D. hansenii* from Blue-veined cheese and found that the proteolytic activity was mostly in the intracellular fraction. They suggested that the contribution of yeast to cheese ripening was dependent on the susceptibility of the yeast cell to lysis, in addition to proteolytic activity.

Klein *et al.* (2002) studied the peptidases of four yeast species frequently encountered in dairy products (*K. lactis*, *S. cerevisiae*, *D. hansenii* and *Pichia anamola*) and compared them with six bacteria species (*Lactobacillus helveticus*, *Lactobacillus plantarum*, *Leuconostoc lactis*, *Pediococcus pentosaceus*, *Bifidobacterium bifidum* and *Brevebacterium linens*). All four yeast species were able to degrade β-casein peptides, and the bacteria species except *Lb. helveticus* showed weaker hydrolytic activities. They noted that the yeast species tested were less efficient than *Lb. helveticus* in proline release. In contrast, the yeasts were more efficient in releasing aspartic acid. They suggested inter- and intra-species differences in peptidase specificity and activity of the yeasts.

2.3.5 Assimilation of sugar and organic acids by yeasts

The capacity for assimilation of sugars and organic acids by yeasts is an important factor in their survival in a cheese environment, especially for the yeasts that are not either lipolytic or proteolytic. The change in pH by assimilation of organic acid may help the growth and metabolic activities of the bacterial strains present in cheese. Some studies related to sugar and organic acid assimilation by yeasts are reviewed in this section.

2.3.5.1 Assimilation of sugar (lactose) by yeasts

The lactose utilisation capacity of yeasts is dependent on the type of yeast. *K. marxianus* and *D. hansenii* are two examples of lactose-utilising yeasts. *K. marxianus*

was able to utilise lactose in milk at 25°C and produced glucose, galactose, ethanol and glycerol. Glucose and galactose produced in the early stages of growth were utilised in the later stages. The utilisation of lactose decreased at lower temperatures and in the presence of NaCl (Roostita and Fleet, 1996A). Fleet and Mian (1987) reported that *K. marxianus* could utilise approximately 60% of milk lactose and produced about 1.5% ethanol. Lactose and galactose were assimilated by all experimental strains of *D. hansenii* (van den Tempel and Jakobsen, 2000). The assimilation of lactose was strongly inhibited by salt contents higher than 4–6% (w/v), with the exception of one strain. The assimilation of galactose was less affected by NaCl and occurred for almost all strains at 12% NaCl (w/v) (van den Tempel and Jakobsen, 2000).

Y. lipolytica is one example of the non-lactose-utilising yeasts. The experimental strains of *Y. lipolytica* did not assimilate lactose or galactose (van den Tempel and Jakobsen, 2000). Sinigaglia *et al.* (1994) reported that the utilisation of D-galactose, D-ribose, cellobiose, salicin and arbutin was also negative in all experimental strains of *Y. lipolytica*. However, the assimilation of N-acetylglucosamine, sorbitol and gluconate was dependent on the isolation source of the *Y. lipolytica*.

2.3.5.2 Assimilation of organic acids by yeasts

The main organic acid present in milk is citric acid (3–4 mg/mL). This is followed by lesser amounts of lactic (0.6 mg/mL), formic (0.4 mg/mL) and succinic (0.15 mg/mL) acids (Roostita and Fleet, 1996A). Yeasts have varied capacity to assimilate these organic acids. All strains of *D. hansenii* and *Y. lipolytica* studied by van den Tempel and Jakobsen (2000) assimilated citric acid, except one strain of *D. hansenii*. *Y. lipolytica* was more sensitive to NaCl than *D. hansenii* in assimilating citric acid. Roostita and Fleet (1996A) reported *Candida catanulata* to be a better utiliser of citric acid than *C. lipolytica (Y. lipolytica)*. *C. catanulata* consumed 80% of the citric acid after 10 days of growth at 25°C in the absence of NaCl, whereas *C. lipolytica* (*Y. lipolytica*) utilised only 15–20% of the citric acid under the same conditions. They reported that the growth of *K. marxianus* in milk was characterised by partial utilisation of citric acid (approximately 30% utilised for growth at 25°C and without NaCl).

The capacity of lactic acid utilisation by *Y. lipolytica* (Sinigaglia *et al.*, 1994; Roostita and Fleet 1996A; van den Tempel and Jakobsen, 2000) and *D. hansenii* (van den Tempel and Jakobsen, 2000) has been reported. *C. lipolytica* (*Y. lipolytica*) and *K. marxianus* are also able to utilise formic and succinic acids (Roostita and Fleet, 1996A).

Thus, some dairy yeasts including *Y. lipolytica* are capable of utilising organic acids, which may lead to an increase in the pH of the medium.

2.3.6 Aroma compounds produced by yeasts

Yeasts can produce aroma compounds by various biochemical pathways. Fatty acids produced by lipolysis are themselves aroma compounds and can be transformed to various ester, lactone and ketone compounds, which could also contribute to cheese aroma. Different enzymes catalyse reactions leading to various flavour compounds. Also, different enzymes can produce the same compound. For example, esterase, acetyltransferase (Kallel-Mhiri and Milco, 1993) and lipase (Leblanc *et al.*, 1998) from yeast can produce esters. Proteolysis and peptidolysis lead to the production of peptides and amino acids. Yeast enzymes can further catabolise the amino acids. Amino acid catabolism could lead to the production of many compounds important for cheese flavour (Smit *et al.*, 2000, 2002). The capacity of producing aroma compounds is one of the most important considerations for selecting a yeast strain to be used as an adjunct culture.

Wyder and Puhan (1999) divided yeasts into three groups on the basis of flavour development in aseptic cheese slurries. One group consisting of *Clavispora lusitaniae*, *Pichia jadinii* and *Williopsis californica* was characterised by the ability to ferment glucose, to utilise lactate and to increase the pH significantly despite only weak proteolytic activity. They produced primarily an alcoholic, acidic, fermented and fruity aroma. The second group was composed of the non-fermenting species *Galactomyces geotrichum* (*G. candidum*), *Trichosporon ovoids* and *Y. lipolytica.* Even though these species are proteolytic and assimilate lactate, they did not affect the pH levels at all. Their contribution to aroma was characterised as cheesy. Only one species had combined properties of both groups: *D. hansenii*, which was fermentative, increased the

pH considerably and was proteolytic. It generated an alcoholic, acidic, fruity and also cheesy aroma.

The production of aroma compounds by yeasts is reviewed in this section, focusing on the yeasts of the second group mentioned above (*G. candidum* and *Y. lipolytica*) and co-cultures of yeasts and bacteria.

2.3.6.1 Aroma compounds produced by *G. candidum*

G. candidum produces aroma by its enzymatic activities. Lipase specificity and the catabolism of FFAs and amino acids are some of the features of *G. candidum* that can be important for the aromatic characterisation of cheese. The production of volatile flavour compounds by *G. candidum* strains in different media is reviewed in the following paragraphs. Then the production of sulphur compounds is reviewed in more detail as they are very important components of cheese flavour.

Aroma compounds produced in different media

Jollivet *et al.* (1994) studied the production of volatile compounds in model milk and cheese media by eight strains of *G. candidum*. In the model milk medium, all the strains produced four compounds: 2-methyl propanol, 3-methyl butanol, butanoic acid and 2-methyl propanoic acid. The two primary alcohols were quantitatively more important. Depending on the strain, they constituted from 50 to 85% of the total of each aromatic extract. Sources of these primary alcohols could be the branched chain amino acids (Yvon and Rijnen, 2001). In this model milk medium, *G. candidum* produced very low concentrations of methyl ketones and secondary alcohols. Only one strain produced phenyl ethyl alcohol, which has a floral aroma (Jollivet *et al.*, 1994).

In a model cheese medium, five compounds were produced by all the strains: propanol, 2-methyl propanol, 3-methyl butanol, 2-methyl pentanol and butanoic acid. Another five components were produced by seven out of eight strains: 2-pentanone, 3-pentanone, hexanoic acid, octanoic acid and phenyl ethyl alcohol. In this medium, compared with the milk medium, the yields of methyl ketones and secondary alcohols were increased, but the yields of fatty acids and primary alcohols were reduced. Analysis of the volatile compounds liberated by *G. candidum* strains in the two model

media revealed seven different fatty acids, *i.e.* butanoic acid, 2-methyl propanoic acid, 2-methyl butanoic acid, 3-methyl butanoic acid, pentanoic acid, hexanoic acid, and octanoic acid.

Kawai *et al.* (1998) identified different aroma compounds produced by nine strains of *G. candidum* in a laboratory medium containing glucose, yeast extract and peptone. Some strains had similar chromatographic profiles by gas chromatography–mass spectrometry (GC–MS) and these strains might share a similar metabolic pathway. Acetaldehyde, ethanol, ethyl acetate, 2-methylpropanol, ethyl propanoate, 3-methyl butanol, 2-methyl butanol, phenyl acetaldehyde and phenyl ethyl alcohol were identified from one strain cultured in a laboratory medium. Ethyl 2-methyl propanoate, ethyl butanoate, ethyl 2-methyl butanoate, ethyl 3-methyl butanoate, 3-methyl butyl acetate and ethyl 3-hydroxy-3-methylbutanoate were also identified from a different strain in the same medium. This strain produced an apple-like aroma.

Daigle *et al.* (1999) studied the production of aroma compounds by *G. candidum* on waste breadcrumbs. They compared *G. candidum* with a few other types of yeast and found that most of the cultures containing *G. candidum* produced a more pleasant aroma than the other yeasts. They identified different fatty acid esters, such as ethyl acetate, ethyl butanoate, ethyl butyrate, ethyl caproate, ethyl isobutyrate, ethyl isovalerate, ethyl propionate and ethyl valerate; all have some fruity notes. The production of aroma compounds was optimal after about 48 h, which corresponded to the stationary phase of growth. Among the esters monitored, ethyl propionate was produced in the highest concentrations, with about 150 mg/kg of medium, followed by ethyl acetate with about 50 mg/kg of medium. About 30–50 and 10–25 mg/kg respectively of ethyl isobutyrate and ethyl butyrate were produced after fermentation for 48–72 h. It was found that incubation at 30°C was more favourable for growth as well as for the production of volatile compounds than incubation at 20°C. Shaking was found to be a necessary factor for the production of the aroma compounds. *G. candidum* did not produce significant volatile compounds on meat extract (Jacobsen and Hinrichsen, 1997).

Production of sulphur flavour compounds

Sulphur compounds, which are constituents of many food flavours, are found commonly in a range of dairy products (Berger *et al.*, 1999). Sulphur compounds are

very important constituents of ripened cheese aroma (Urbach, 1993). Yeasts, especially *G. candidum*, are found to produce volatile sulphur compounds. Berger *et al.* (1999) studied the production of sulphur flavours by ten strains of *G. candidum* in a liquid cheese model medium. Dynamic headspace analysis of the samples showed that dimethyl disulphide (DMDS) was the major sulphur compound present in cultures of all strains tested, at concentrations ranging from 0.1 ppm to a maximum of 1.93 ppm. With one exception, it was shown that methanethiol (MTL) was also generated by all cultures of *G. candidum*, suggesting that it was the likely precursor of DMDS. The observation that the strain that did not generate significant levels of MTL also produced the smallest quantities of DMDS further strengthened the view. *G. candidum* also produced dimethyl trisulphide (DMTS), and the highest yields were obtained with those cultures that produced the greatest amounts of MTL and DMDS. The strains of *G. candidum* that proved to be the most useful for the production of sulphides were also capable of generating S-methyl thioacetate (MTA), S-methyl thiobutyrate (MTB) and other thioesters. However, with the exception of MTA, the thioesters were produced at 10–40-fold lower concentrations than the sulphides. It was also shown that the most suitable strains for the generation of thioesters were those that produced the highest concentrations of dimethyl sulphide (DMS), DMDS and DMTS. It was concluded from the findings that *G. candidum* might play an important role in the development of cheese flavour and that these sulphur compounds were correlated with specific flavour notes (*e.g.* cabbage), identified in their previous trial of Camembert cheese with *P. camemberti* and specific strains of *G. candidum*. Among the thioesters, MTA, which has the flavour of cooked cauliflower, is the most commonly found thioester in cheese (Yang and Min, 1994). Direct precursors for S-methyl thioesters are believed to be MTL and fatty acids, both of which are generated by microorganisms during cheese ripening (Weimer *et al.*, 1999). Acyl-CoA and MTL accumulate through the catabolism of fatty acids, sugars or amino acids. S-methyl thioesters are subsequently generated from acyl-CoA and MTL, either enzymatically giving essentially MTA, or spontaneously giving rise to a much wider variety of S-methyl thioesters (Helinck *et al.*, 2000).

Both MTL and DMS were produced from methionine, but two different pathways were used by *G. candidum* (Demarigny *et al.*, 2000). Spinnler *et al.* (2001) studied *G. candidum* as well as a few other yeasts for their capacity to produce sulphur

compounds. They found that, in the media inoculated with *G. candidum* and *Y. lipolytica*, the production of DMS (9898 and 6818 μg/kg respectively) was much higher than with the non-inoculated medium in the presence of S-methylmethionine. However, DMS did not increase in *K. lactis*, *D. hansenii* and *S. cerevisiae* cultures in comparison with the non-inoculated culture in the presence of S-methylmethionine. When L-methionine was added to the culture, the production patterns of the volatile sulphur compounds varied greatly. Although produced in different amounts, all strains produced MTL, DMDS, DMTS and MTA, whereas DMS was produced only by *G. candidum.* MTL was best produced by *G. candidum* and *Y. lipolytica*; the highest DMDS and DMTS yields were also achieved by these cultures. MTA production was, by far, highest with *G. candidum* (2900 μg/kg), whereas *S. cerevisiae* and *K. lactis* produced only 120 and 70 μg/kg respectively of this metabolite. The different enzymes responsible for the production of sulphur compounds were assayed. No deaminase activity was found in any of the yeasts. Demethiolase activity was detected in all strains. With the notable exception of *D. hansenii*, this activity was found to be enhanced in cultures supplemented with L-methionine, which indicates a possible inductive effect of the substrate on this activity. The highest demethiolase activities were obtained with *S. cerevisiae* (250 nM/g/s) and *G. candidum* (210 nM/g/s) in the PDB (potato dextrose broth) medium. No transaminase activity was detected in *K. lactis*. Apart from *D. hansenii*, for which transaminase activity was detected only when cultivated in PDB medium, transaminase activity was increased in cellular extracts from PDB + methionine medium. *G. candidum* demonstrated the highest transaminase activities both in PDB medium (583 nM/g/s) and in PDB + methionine medium (1738 nM/g/s).

Thus, the above paragraphs reviewing the production of aromatic compounds by *G. candidum* show that this yeast is capable of producing alcohols, aldehydes, FFAs, esters and a range of sulphur compounds, which are important for cheese flavour.

2.3.6.2 Aroma compounds produced by *Y. lipolytica*

The large variability observed among the *Y. lipolytica* strains regarding the release of fatty acids can have an indirect effect on the subsequent transformation of the fatty acids into aroma compounds. In particular, the release of PUFAs can have a relevant role in

the aroma of surface-ripened cheeses. Linoleic acid could act as a precursor of 10-carbon oxo and hydroxy acids (Jelen and Wasowicz, 1998). In contrast, the monounsaturated C16 and C18 carbon chain fatty acids (C16:1 and C18:1) are precursors of hydroxy acids which, in turn, are converted to γ and δ lactones (Guerzoni *et al.*, 2001). Kalle *et al.* (1976) fermented milk cream with *Y. lipolytica* to produce cheese-like flavour. Flavour development in cream closely followed the increase in FFAs and was maximum in about 50 h. They associated the flavour development with lipolytic activity. They compared the distribution of fatty acids between the aforesaid fermented cream and Cheddar cheese, and found that the lipolysed cream had all the major fatty acids reported in Cheddar cheese.

From the above review of 'aroma compounds produced by *Y. lipolytica*', it can be summarised that *Y. lipolytica* primarily affects cheese flavour by its lipolytic activity, although this yeast, like *G. candidum*, can also produce volatile sulphur compounds, as mentioned in the previous section.

2.3.6.3 Aroma compounds produced by co-cultures of yeasts and bacteria

As ripened cheeses are produced with mixed cultures, the co-cultures provide more relevant information than do pure cultures. Martin *et al.* (1999, 2001) studied aroma production by co-cultures of four yeast species — *Y. lipolytica*, *K. lactis*, *D. hansenii* (one strain each) and *G. candidum* (three strains) — and four species of bacteria (one strain of *Corynebacterium glutamicum*, *Staphylococcus lentus* and *Arthrobacter globiformis*, and two strains of *Brevibacterium lenens*). They found that *K. lactis*, associated with bacteria, yielded a curd with intense fruity (banana) and solvent flavour. The association of bacterial cultures with *Y. lipolytica* and *G. candidum* produced more intense savoury flavour (cabbage). *D. hansenii* associated with bacteria yielded an intermediate odour without dominant flavour notes. It was found that, associated with specific yeasts, five bacteria induced very similar odours.

K. lactis produced significantly larger concentrations of aroma compounds, such as acetaldehyde, ethyl acetate and 3-methyl butanol, than the other yeasts. These metabolites explain the perceived fruity flavour. *Y. lipolytica* was characterised by the production of high concentrations of 2-propanol, 2-butanone and 2-pentanone. Co-

culture of *Y. lipolytica* and bacteria produced 2-propanol and ketones (acetone, 2-butanone and 2-pentanone). *D. hansenii* produced the highest quantity of 2-methyl propanal. The curds ripened with *D. hansenii* and any of the bacteria were characterised by the strongest production of alcohols (2-methyl propanol and 3-methyl butanol) and aldehydes (2-methyl propanal and 3-methyl butanal). These branched chain aldehydes produced by *D. hansenii* might be responsible for the chocolate flavour notes in bacterial surface-ripened cheeses (Martin *et al.*, 2001). Strains of *G. candidum* produced the highest quantities of DMDS, butanol and DMTS.

Associations including *Y. lipolytica* and *G. candidum* were thought to be useful for cheese flavour improvement and intensification as a result of the production of sulphides (DMS, DMDS and DMTS), esters (ethyl acetate and ethyl butanoate) and other compounds (2-heptanone and pinene). As yeasts are capable of producing ethyl esters, production of ethyl alcohol (substrate) in high concentration would increase the production of ethyl esters. The production of high concentrations of ethyl alcohol in cheese by *Lactobacillus fermentum* has been demonstrated (Crow *et al.*, 2002). One of the problems with the assessment of the influences of the yeast adjunct cultures on cheese flavour is the variability in cheese flavour because of adventitious NSLAB. Control of the NSLAB population in cheese is possible by deliberate addition of a strain of NSLAB (*e.g. Lactobacillus rhamnosus*) in cheese (Crow *et al.*, 2001, 2002). This can make the determination of the influence of the yeast adjunct cultures on cheese flavour more accurate.

2.4 Natural conversion of free linoleic acid to CLA

The capability of the yeast lipase to selectively hydrolyse long-chain unsaturated fatty acids from lipid is discussed in Section 2.3.3. Free long-chain unsaturated fatty acids are not desirable for cheese flavour, but one free long-chain unsaturated fatty acid, linoleic acid (C18:2), can be converted to CLA, a bioactive compound. The capacity of some dairy cultures to convert free linoleic acid to CLA has been studied over the last few years (Jiang *et al.*, 1998; Lin *et al.*, 1999). The studies were mostly carried out in laboratory media or milk with added linoleic acid. The synthesis of CLA by the dairy cultures in a cheese environment with an internal source of free linoleic acid would be of interest. In this context, a short review of the sources and biological attributes of CLA and a more detailed review of the natural conversion of linoleic acid to CLA is appropriate.

2.4.1 CLA: isomers and structures

CLA refers to a group of positional and geometric isomers of octadecadienoic acids with conjugated double bonds. The predominant isomer in food is the *cis*-9, *trans*-11 isomer. In total, 24 isomers of CLA are possible. Among them, *cis*-9, *trans*-11 is the most abundant form. The CLA isomer *trans*-10, *cis*-12 occupies the next position in terms of existence in foods such as cheese (Ha *et al.*, 1989). CLA can be produced chemically by an alkaline-catalysed reaction using linoleate as a substrate. The product is a racemic mixture of various CLA isomers. Different isomers of CLA have different physiological functions. Among the CLA isomers, the *cis*-9, *trans*-11 isomer has been found to be the most important in terms of anti-carcinogenic activity. Only this isomer was incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al.*, 1990). The *trans*-10, *cis*-12 isomer of CLA was found to be more active in controlling body fat deposition (Park *et al.*, 1999). The chemical structures of linoleic acid and the major CLA isomer (*cis*-9, *trans*-11) are shown in Figure 2.1.

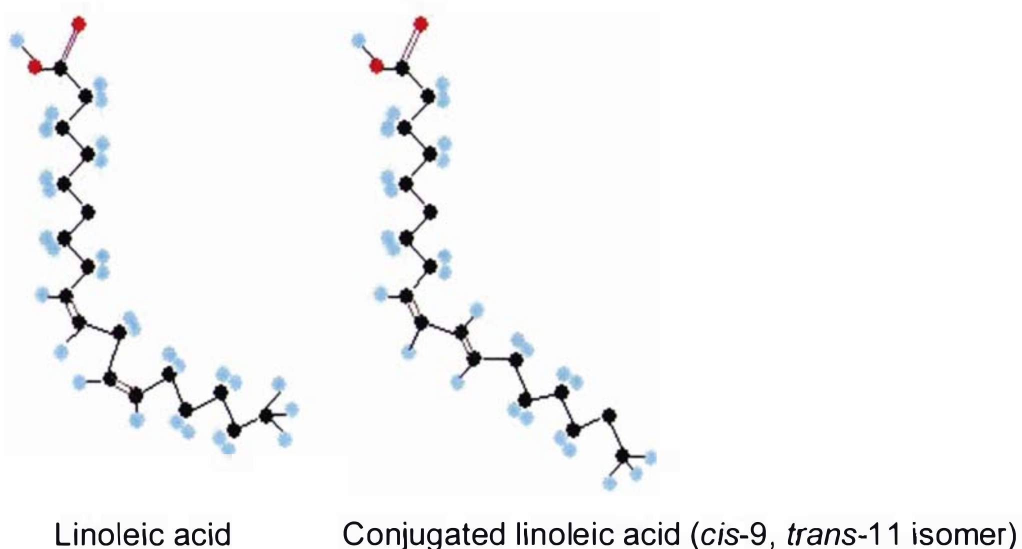


Figure 2.1 Chemical structures of linoleic acid and CLA (the *cis*-9, *trans*-11 isomer).

2.4.2 Biological activities of CLA

Many articles on the biological activities of CLA have been published during the last 25 years. A detailed review of those works is beyond the scope of this review. However, the main biological activities of CLA along with a few references are summarised in Table 2.1.

Table 2.1 Biological activities of CLA

Biological activity	Experimental subject	Reference
Anticarcinogenic activity	Rat	Ip *et al*., 1991
	Human cancer cell line	Choi *et al*., 2002
Hypocholesterolaemic activity	Rabbit	Lee *et al*., 1994
Antidiabetogenic activity	Rat	Houseknecht *et al*., 1998
Immunomodulation	Chicken, rat	Cook *et al*., 1993
Body composition	Rat	Park *et al*., 1999
	Human	Smedman and Vessby, 2001
Bone health	Chicken	Watkins and Seifert, 2000

2.4.3 Sources of CLA

The major CLA isomer in natural sources is the *cis*-9, *trans*-11 isomer. Ruminant fats are the most important natural sources of CLA, which is why the most abundant natural CLA isomer (*cis*-9, *trans*-11) is given the name 'rumenic acid'. Dietary sources of CLA include milk fat, dairy products and meat products (Ha *et al.*, 1989; Chin *et al.*, 1992; Shantha *et al.*, 1994; Jiang *et al.*, 1997). The fats of ruminant origin generally contain more CLA than those from non-ruminants. For instance, the fat of bovine milk, lamb or beef contains 0.5–1% CLA, whereas pork, salmon and egg yolk contain only about one-tenth of that amount of CLA (Fogerty *et al.*, 1988; Chin *et al.*, 1992). Plant oils, such as canola, corn and olive oils, contain trace levels of CLA (Chin *et al.*, 1992).

2.4.3.1 Biosynthesis of CLA in the rumen

CLA is synthesised in the cow's rumen during the biohydrogenation process. Initial forage lipids, which consist primarily of galactolipids and phospholipids, are hydrolysed to liberate FFAs. Next, a linoleic acid isomerase (EC 5.2.1.5) from the rumen bacteria *Butyrivibrio fibrisolvens* transposes the *cis* double bond at position 12 in forage-derived linoleic acid (*cis*-9, *cis*-12 C18:2) to position 11 and in doing so assumes the *trans* configuration. The transformation produces *cis*-9, *trans*-11 C18:2 (CLA) as a stable first intermediate in the biohydrogenation process. Further biohydrogenation reduces the CLA to *trans*-11 C18:1, a predominant fatty acid in milk fat. The *trans*-11 C18:1 is hydrogenated by other rumen bacteria to stearic acid (C18). Some of the CLA and *trans*-11 C18:1 evade further biohydrogenation. They are absorbed from the intestine and then transported to adipose tissue and the mammary gland (Parodi, 2003). The intestinal flora of rats is also found to be capable of converting free linoleic acid to the *cis*-9, *trans*-11 isomer of CLA (Chin *et al.*, 1992).

2.4.3.2 CLA in milk and dairy products

Table 2.2 shows the range of levels of the *cis*-9, *trans*-11 isomer of CLA in some dairy products, determined by various investigators.

Table 2.2 Comparison of the concentrations of *cis*-9, *trans*-11 C18:2 (CLA) in dairy products from different studies (table adapted from Jiang *et al.*, 1997)

Source	*cis*-9, *trans*-11 C18:2 concentration (mg/g fat)			
	Chin *et al.* (1992)	Lin *et al.* (1995)	Shantha *et al.* (1995)	Jiang *et al.* (1997)
Fresh milk	5.5	4.1–4.5	3.4–5.9	5.8–5.9
Fermented milk	1.77–5.4	3.8–4.7	3.7–7.3	4.5–6.2
Cream products	4.6–6.1	4.1–4.2	6.1–6.4	6.1–6.2
Cheese	2.9–7.1	3.6–9.0	3.9–5.2	5–7.1

The cow's diet appears to be the major determinant of milk CLA levels, whereas breed, age and stage of lactation exert only a minor influence (Parodi, 2003). Jiang *et al.* (1996) reported that the concentration of CLA could be increased in bovine milk by changing the dietary regime. They recorded a *cis*-9, *trans*-11 CLA range between 2.47 and 17.68 mg/g milk fat in cows fed with different ratios of forage and concentrate. The CLA concentration in milk varies with the season, with the highest values occurring during the summer period when the cow grazes pasture. The concentration of CLA is higher in the milk in countries like New Zealand and Australia, where cows graze on fresh pasture, than in Western Europe and North America, where cows are mainly grain fed (Parodi, 2003). The mean CLA concentration in New Zealand milk fat is about 11 mg/g (1.1% of the total fatty acids) with a range from 7 to 15 mg/g. The New Zealand CLA values are two to three times higher than the values from countries where pasture is not predominant (MacGibbon and Hill, 1998).

Several studies were carried out to determine the effect of processing on the CLA content in dairy products. Shantha *et al.* (1995) found that processing milk or cream into low fat and regular yoghurt, sour cream, cheese and ice cream did not result in substantial increases in the CLA concentration. They did not find any change in the CLA concentration in dairy products during storage. Werner *et al.* (1992) reported that different starter cultures, processing conditions and aging periods had a negligible influence on the total CLA concentration of Cougar Gold, Cheddar and Viking cheeses. However, aging may have affected the CLA isomer distribution, because the concentration of *cis*-9, *trans*-11 C18:2 was greater in the aged cheeses than in the fresh

cheeses. In contrast, Ha *et al.* (1989) suggested a positive relationship between the aging period and the CLA content of four cheeses aged between 3 and 10 months. Oxidative reactions have been postulated to promote CLA formation by causing the formation of linoleate radicals, resulting in a shift in the double bonds to form a conjugated system (Ha *et al.*, 1989). Zlatanos *et al.* (2002) determined the CLA concentrations and fatty acid compositions of Feta cheese and other Greek cheeses. Greek cheese contains up to 1.9% CLA, with an average of 0.8% of the fat. They found that, compared with cheeses that are produced from cow's milk, Greek cheeses, which are produced from sheep and goat milk, contain higher percentages of CLA in the fat. There was no apparent relationship between the CLA content and linoleic acid. This indicates that the CLA content is not influenced by the initial quantities of linoleic acid in milk. Also, no relationship between the CLA and other unsaturated fatty acids in cheese was found. Lavillonniere *et al.* (1998) reported 5.3–15.8 mg CLA/g cheese fat in French cheeses and suggested that the difference in CLA content depends primarily on the milk (influenced by season and geography) and somewhat on the production process.

From the different studies carried out to determine the CLA concentration in milk and dairy products, it is evident that the natural CLA concentration of milk is the main factor for the variation in the CLA concentration in dairy products, not the processing.

2.4.4 Conversion of linoleic acid to CLA by dairy cultures

The potential of dairy cultures to convert linoleic acid to CLA has been a subject of interest for the last 15 years. Several researchers have studied different dairy cultures. A few *Propionibacterium* and *Lactobacillus* species showed positive results. The capacity for the conversion of linoleic acid to CLA was strain specific. Table 2.3 summarises the available literature on the dairy bacteria that showed the capacity to convert linoleic acid to CLA, the medium used and the source of the linoleic acid.

Table 2.3 Dairy cultures capable of converting linoleic acid to CLA, the media used and the sources of the linoleic acid: studied by various researchers. * MRS = Man-Rogosa-Sharpe

Reference	Dairy culture	Medium	Linoleic acid source
Jiang *et al.*, 1998	*Propionibacterium freudenreichii* ssp. *freudenreichii* ATCC 6207 (PFF)	Laboratory media (MRS*)	External
	Propionibacterium freudenreichii ssp. *freudenreichii* Propioni-6 (PFF6)		
	Propionibacterium freudenreichii ssp. *shermanii* 9093 (PFS)		
Lin *et al.*, 1999, 2000	*Lactobacillus acidophilus* (CCRC14079)	Skim milk	External
	Lactobacillus delbrueckii ssp. *lactis* (CCRC14078)		
	Lactobacillus delbrueckii ssp. *bulgaricus* (CCRC 14009)		
	Lactococcus lactis ssp. *cremoris* (CCRC12586)		
	Lactococcus lactis ssp. *lactis* (CCRC10791)		
	Streptococcus salivarius ssp. *thermophilus* (CCRC12257)		
Ogawa *et al.*, 2001	*Lactobacillus acidophilus* AKU 1137	Laboratory media (MRS)	External
Rainio *et al.*, 2002	*Propionibacterium freudenreichii* ssp. *shermanii* strain JS (DSM 7067)	Laboratory media (whey permeate media)	External
Kishino *et al.*, 2002	*Lactobacillus plantarum* AKU 1009a	Laboratory media (MRS)	External
Kim and Liu, 2002	*Lactococcus lactis* I-01	Whole milk	Sunflower oil mixed with milk
Anon., 2002	*Bifidobacterium dentium*, *Bifidobacterium breve* (NCFB2257, NCFB2258, NCTC 11815, NCIMB 8815, NCIMB 8807)	Laboratory media (MRS)	External
Lin, 2003	Mixed culture of *Lactobacillus acidophilus* (CCRC 14079), yoghurt bacteria comprising *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*	Yoghurt	External

2.4.4.1 CLA conversion by *Propionibacterium* sp.

Propionibacteria are commonly characterised by their capacity to produce propionic acid and carbon dioxide and are used in Swiss cheese as an adjunct. Lactate is transformed into propionate and acetate along with carbon dioxide and water in the following proportion (Steffen *et al.*, 1993).

3 Lactate $\rightarrow$ 2 Propionate + Acetate + CO_2 + H_2O

Propionibacteria strains have another attribute of converting free linoleic acid to CLA. Jiang *et al.* (1998) screened 19 strains of bacteria, used as dairy cultures, and found that three strains of *Propionibacterium freudenreichii* (Table 2.3) were capable of producing CLA. CLA was present mainly in extracellular liquid and the *cis*-9, *trans*-11/*cis*-11, *trans*-9 isomer comprised 70–90% of the total CLA. Different strains of propionibacteria showed differences in CLA conversion as well as in tolerance of linoleic acid concentration. For strain PFF, the concentration of *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2 increased from 2.6 μg/mL in the control culture (no free linoleic acid) to 17.6 μg/mL culture in a medium containing free linoleic acid at 100 μg/mL but the CLA concentration decreased significantly when the free linoleic acid concentration reached 200 μg/mL in the medium. At 500 μg/mL in the medium, the growth was significantly inhibited and no CLA was formed. For strain PFS, the formation of *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2 increased with the amount of free linoleic acid in the medium up to 200 μg/mL. Production of *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2 was nearly constant between 200 and 600 μg/mL free linoleic acid in the medium. Strain PFF6 produced the highest level of *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2 (246.4 μg/mL of medium) at a free linoleic acid concentration of 750 μg/mL in the medium. Even at a concentration of free linoleic acid as high as 1500 μg/mL, *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2 was produced although at a decreased level. Rainio *et al.* (2002) studied a strain of *Propionibacterium freudenreichii* ssp. *shermanii* (Table 2.3) and found that the final amount of CLA increased as the initial concentration of the linoleic acid was increased although the specific growth rate and the final cell weight decreased slightly. Even with an initial linoleic acid concentration of 2000 μg/mL, the yield of CLA was 80%. Of the CLA formed, 85–95% was the *cis*-9, *trans*-11 isomer and 98% was extracellular.

2.4.4.2 CLA conversion by *Lactobacillus* sp.

Some *Lactobacillus* species were also found to carry out the conversion of free linoleic acid to CLA although the amount of CLA produced varied extensively in different studies. Lin *et al.* (1999) studied a range of *Lactobacillus* species (Table 2.3) and found that all were capable of producing CLA in sterilised skim milk. Jiang *et al.* (1998) could not find this capacity of *Lactobacillus* in MRS broth. The reasons for the positive results in the skim milk medium were probably the neutralisation of the inhibitory effects of fatty acids by milk protein and prevention of CLA oxidation by alkyl radicals formed from the milk proteins (Lin *et al.*, 1999). They noted a significant increase in CLA when 1000 μg/mL linoleic acid was added to the medium. The highest level was achieved for *Lb. acidophilus* (105.5 μg/mL) after 24 h in a medium with an initial linoleic acid concentration of 1000 μg/mL. Increasing the level of linoleic acid to 5000 μg/mL did not increase the level of CLA significantly. In another study, the CLA synthesis capacity of *Lb. acidophilus* was much higher. *Lb. acidophilus* converted 95% of the added linoleic acid in a medium containing 5000 μg/mL added linoleic acid (Ogawa *et al.*, 2001). CLA was accumulated as intracellular or cell-associated lipids in free form. The dominant CLA isomer was *cis*-9, *trans*-11/*trans*-9, *cis*-11 C18:2. Lin (2003) reported that inoculation of mixed cultures comprising *Lb. acidophilus* and yoghurt bacteria (Table 2.3) with 0.1% linoleic acid addition produced significant increases in the *cis*-9, *trans*-11 CLA content without decreasing the product acceptability. The highest *cis*-9, *trans*-11 CLA concentration recorded in the yoghurt was 2.95 μg/g yoghurt.

2.4.4.3 CLA conversion by *Bifidobacterium* sp.

The genus *Bifidobacterium* also showed the capacity to produce CLA. The capacity for CLA production exhibited considerable interspecies variation, with *B. breve* and *B. dentium* (Table 2.3) being the most efficient producers, converting up to 65% of linoleic acid to CLA at linoleic acid concentrations of 200–1000 μg/mL in MRS medium (Anon., 2002).

2.4.4.4 Effect of the phase of growth on CLA synthesis

P. freudenreichii ssp. *shermanii* showed the capacity of CLA synthesis in both growth and stationary phases, although the production rate was higher in growing cells (Rainio *et al.*, 2002). Lin *et al.* (1999) studied CLA conversion by some lactic acid bacteria (Table 2.3) and found that most of the conversion took place within the first 24 h. Between 24 and 48 h, the changes in CLA concentration were insignificant. Kishino *et al.* (2002) used washed cells of *Lb. plantarum* and found that the cells in the late log phase showed significant productivity, but further cultivation resulted in a decrease in productivity. Ogawa *et al.* (2001) successfully used washed cells of a 3-day-old *Lb. acidophilus* culture for CLA conversion.

2.4.4.5 Effect of additives on CLA synthesis

Different researchers have studied the effects of different additives on CLA conversion by different bacteria. The presence of certain surface-active substances, such as Tween-80 or proteins, plays an important role in countering the inhibitory effects of free linoleic acid on the growth of propionibacteria and, hence, on the production of CLA (Jiang *et al.*, 1998). The growth-preventing effect of linoleic acid was overcome by adding micellar linoleic acid stock solution into polyoxyethylene-sorbitan-monooleate-supplemented whey permeate medium (Rainio *et al.*, 2002).

Lin (2000) observed a significant decrease in CLA conversion in all lactic acid bacteria cultures (Table 2.3) in the presence of 10 g/L NaCl. The CLA conversion was decreased significantly in the presence of sucrose, lactose and fructose in all lactic acid bacteria except for *Lactococcus lactis* ssp. *cremoris* (Table 2.3). Lin (2003) reported that fructo-oligosaccaride addition had little or no effect on *cis*-9, *trans*-11 CLA enhancement of yoghurt.

2.4.4.6 Mechanism of CLA conversion

Different mechanisms for the conversion of linoleic acid to CLA have been suggested. The antimicrobial effect of linoleic acid has been known for many years. Jiang *et al.* (1998) suggested that the conversion of free linoleic acid to CLA might function as a

detoxification mechanism. They found that, among the free-CLA-producing strains of propionibacteria (Table 2.3), a positive correlation existed between CLA production and the ability to tolerate free linoleic acid. Rainio *et al.* (2002) supported the detoxification mechanism with their study and postulated that the detoxification of linoleic acid via conversion to CLA was a probable physiological role for the isomerisation in the *P. freudenreichii* ssp. *shermanii* strain (Table 2.3). Lin *et al.* (2002) suggested the potential for CLA production by *Lb. acidophilus* (CCRC 14079) and *P. freudenreichii* ssp. *shermanii* (CCRC 11076) through linoleic acid isomerase. Catalysis of the isomerisation reaction by an ultrafiltration retentate, using a membrane with a 100 kDa molecular weight cut-off, indicates that the molecular weight of the linoleic acid isomerase is higher than 100 kDa (Lin *et al.*, 2002). Ogawa *et al.* (2001), while studying CLA synthesis by *Lb. acidophilus*, indicated that the transformation of linoleic acid to CLA is not a one-step isomerisation of a non-conjugated diene to a conjugated diene. Rather, the transformation involves the production of hydroxy fatty acids, *i.e.* 10-hydroxy-*trans*-12-octadecaenoic acid and 1-hydroxy-*cis*-12-octadecaenoic acid.

Summarising the microbial conversion of linoleic acid to CLA, it can be said that several species of dairy bacteria of the genera *Propionibacterium* and *Lactobacillus* can convert free linoleic acid to CLA in laboratory media. The efficiency of the conversion is higher in the growth phase and in the presence of surface-active substances. The conversion process has been postulated to be a detoxification mechanism.

2.5 Production of phenyl ethyl alcohol and related aromatic compounds by yeasts

Phenyl ethyl alcohol (PE or 2-phenylethanol or phenylethanol) and its esters are very important flavour compounds in foods. With their pleasant rose-scented aroma, PE and its ester phenyl ethyl acetate (PEA) are known to be responsible for the aromatic character of soft cheeses (Adda *et al*., 1978). Lee and Richard (1984) observed that, among the different microorganisms developing in raw milk Camembert, only yeasts were able to produce PE from phenylalanine. Investigation of the capacity of the production of these aromatic compounds by the yeasts is in the scope of the present study. In that context, a literature review of the biosynthesis of PE is presented in this section.

PE is a higher aromatic alcohol with a rose-like odour. The fragrance of rose is very popular and widely used in soaps and perfumes. It is also used in foods such as soft drinks, candy and cookies. Its esters, especially PEA, are also valuable flavour and fragrance compounds. Natural sources of PE are different flowers and plants, such as rose, hyacinths, jasmine, narcissi and lilies. Fermented food products, such as tea leaves, cocoa, coffee and wine, also contain this aromatic alcohol (Clark, 1990; Etschman *et al.*, 2002). The majority of the commercial production of PE is carried out by chemical synthesis. Since the first synthesis of PE by the reduction of phenyl acetaldehyde with sodium amalgam in aqueous alcohol in 1876, numerous laboratory- and industrial-scale chemical synthesis processes have evolved (Bedoukian, 1967; Etschman *et al.*, 2002). The consumer preference for natural food additives led the way towards the biotechnological production of PE. The price of PE can vary widely according to the source. PE labelled as 'natural' could cost as much as US$1000/kg, whereas PE without any further description is available at around US$3.5/kg (Etschman *et al*., 2002).

2.5.1 Biological production of PE: pathways of phenylalanine degradation

PE can be produced in yeast by *de novo* synthesis or by metabolism of L-phenylalanine (L-phe). In *de novo* synthesis, the production of PE is very low and can be increased by the addition of L-phe in the medium (Fabre *et al*., 1998). Figure 2.2 shows different possible pathways of PE production and possible routes of L-phe metabolism in yeasts.

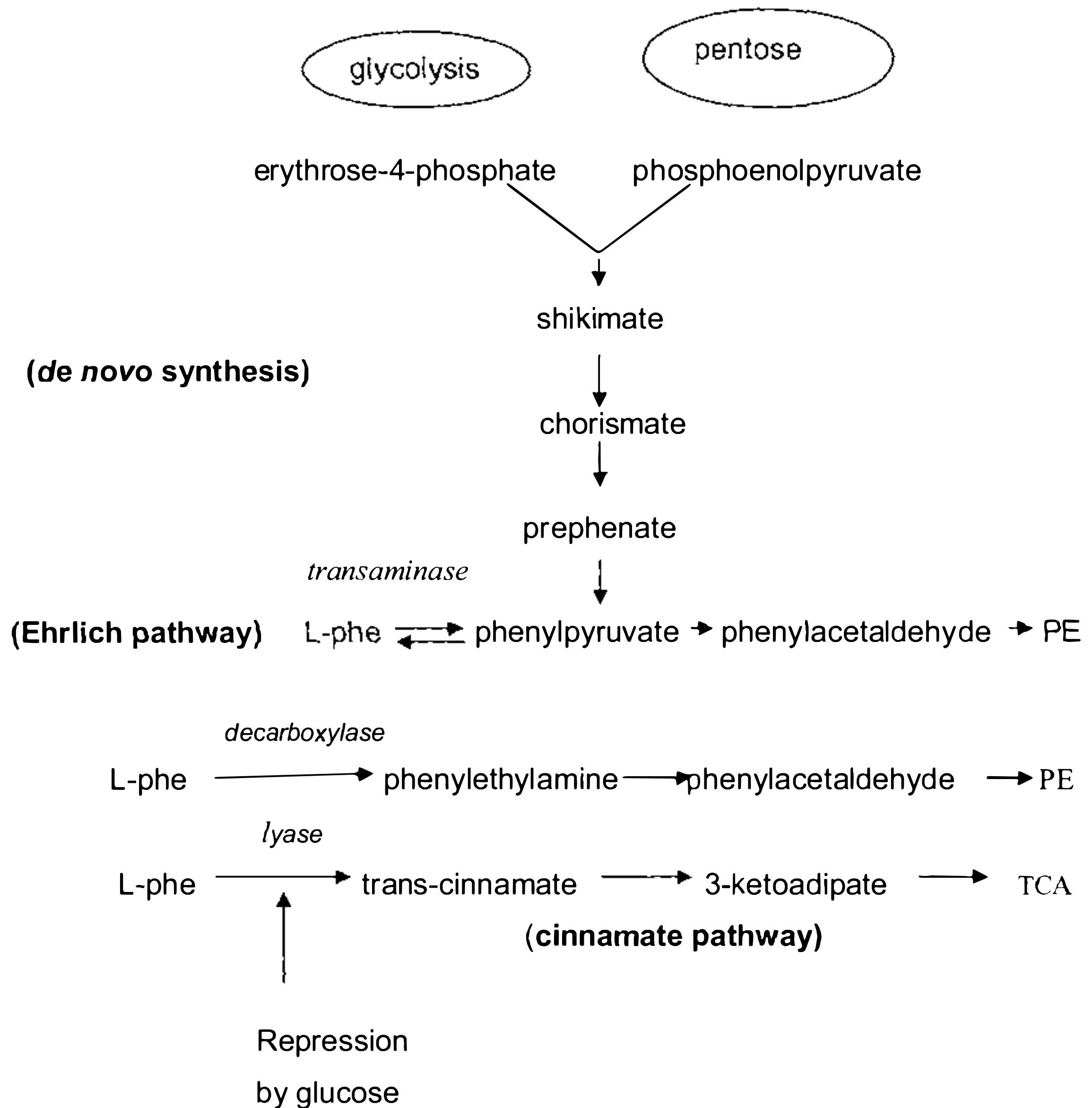


Figure 2.2 Catabolism of L-phe and biosynthesis of PE in yeast (Etschmann *et al.*, 2002)

The most likely route from L-phe to PE is by transamination of the amino acid to phenylpyruvate, decarboxylation to phenylacetaldehyde and reduction to PE, first described by Ehrlich and consequently named after him (Ehrlich, 1907; Etschmann *et al.*, 2002). The Ehrlich pathway for PE synthesis is shown in Figure 2.3 (a).

(a)

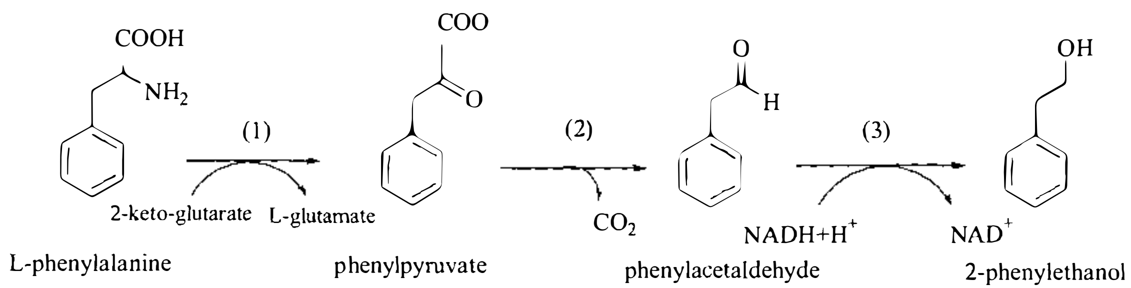


Figure 2.3 (a) Ehrlich pathway for PE synthesis. Enzymes: (1) transaminase, (2) decarboxylase and (3) dehydrogenase.

L-phe metabolism in yeast may take another pathway, called the cinnamate pathway. The first step in this pathway is the removal of an amino group by phenylalanine ammonia lyase (PAL) to produce trans-cinnamate. The formation of ammonia and trans-cinnamate was first described in the yeast *Sporobolomyces roseus* and later PAL was purified from *Rhodotorula glutinis*. The enzyme is induced in the presence of L-phe. For yeasts that use these amino acids as a nitrogen source, no further degradation is necessary. Those that can use the amino acid as the sole carbon source as well as the sole nitrogen source are further catabolised via benzaldehyde, benzoate, protocatechuate, maleylacetate and ketoadipate into the tricarboxylic acid (TCA) cycle (Large, 1986). The cinnamate pathway in yeasts is shown in Figure 2.3 (b). PE can be synthesised in yeasts by *de novo* synthesis via the shikimate pathway (Figure 2.2). However, there are no indications that this reaction is of any significance (Etschmann *et al.*, 2002).

(b)

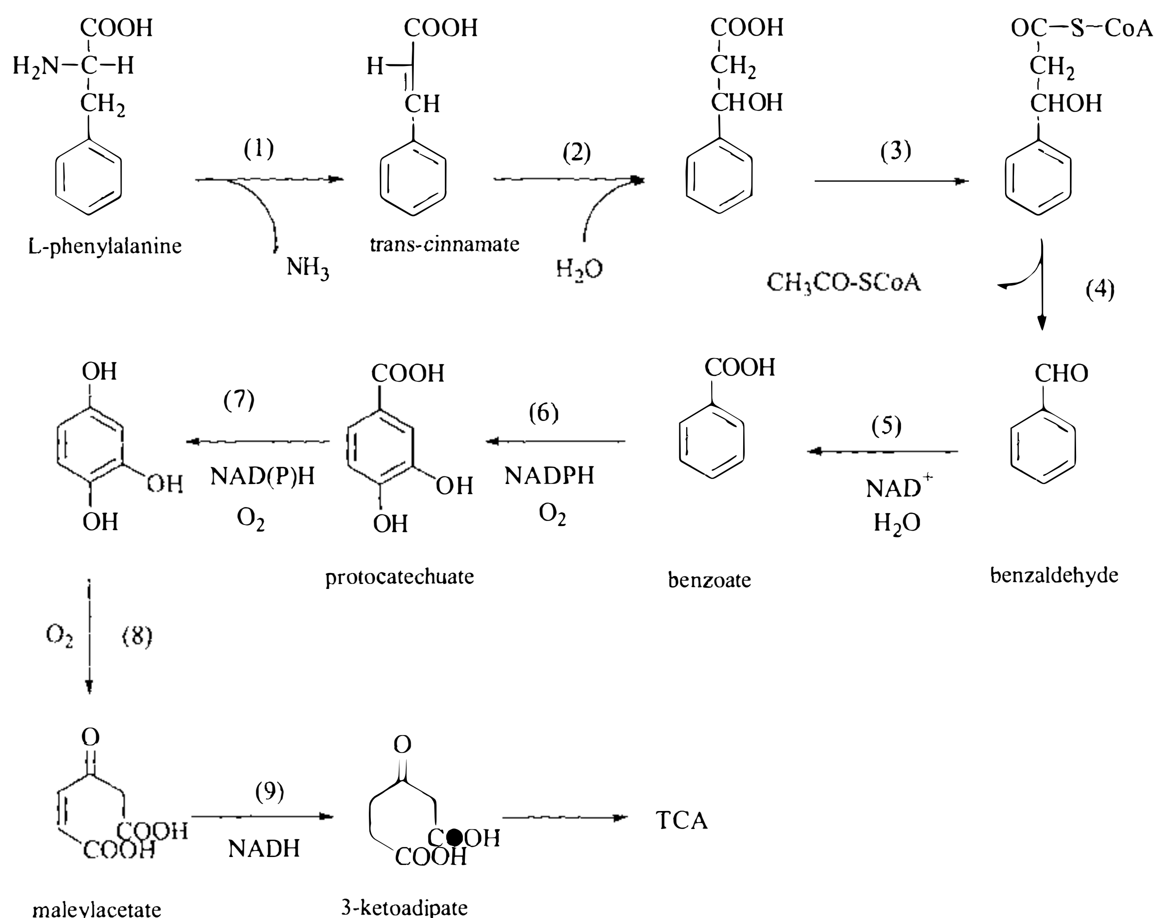


Figure 2.3 (b) L-phe degradation in yeasts via the cinnamate pathway. Enzymes (1) phenylalanine ammonia lyase, (2) trans-cinnamate hydratase, (3) β-(4-hydroxy-phenyl)-hydracryloyl-coenzyme A synthatase, (4) hydroxyphenylhydracryloyl-coenzyme A lyase, (5) 4-hydroxybenzaldehyde dehydrogenase, (6) 4-hydroxybenzoate hydroxylase, (7) protocatechuate hydroxylase, (8) hydroxyquinol dioxygenase, (9) maleylacetate reductase (Large, 1986; Etschmann *et al.*, 2002).

Some bacteria, such as *Microbacterium* sp. and *Brevibacterium* (Jollivet *et al.*, 1992), and some fungi, such as *Phellinus ignarious* and *Ischnoderma benzoinum* (Etschmann *et al.*, 2002) have been shown to produce PE. *Lactobacillus plantarum* has been shown to produce benzaldehyde from phenylalanine conversion (Groot and de Bont, 1998). However, the most prominent producers of PE are yeasts. Yeasts have been shown to produce other phenylalanine breakdown products, such as esters of PE and benzaldehyde. Table 2.4 summarises some published results regarding the production of PE and associated metabolites by yeasts.

Table 2.4 Production of aromatic volatile compounds from phenylalanine by different yeasts. All studies used the L-phe isomer except for [1] where the isomer was not specified. Phenylalanine was added externally except for [2] where it was sourced from glucose–yeast extract medium

Yeast	Products detected	Reference
Saccharomyces cerevisiae (isolated from Camembert cheese)	PE, phenyl pyruvate	Lee and Richard, 1984
Kluyveromyces lactis (isolated from Camembert cheese)	PE, phenyl pyruvate	Lee and Richard, 1984
Kluyveromyces fragilis (isolated from Camembert cheese)	PE, phenyl pyruvate	Lee and Richard, 1984
Torulopsis sphaerica (isolated from Camembert cheese)	PE, phenyl pyruvate	Lee and Richard, 1984
Debaryomyces hansenii (isolated from Camembert cheese)	PE, phenyl pyruvate	Lee and Richard, 1984
Candida pseudotropicalis (isolated from Camembert cheese)	PE	Lee and Richard, 1984
Hansenula anamola CBS110	PE, PEA	Albertazzi *et al*., 1994
Kloeckera saturnus CBS 5761	PE, PEA	Albertazzi *et al*., 1994
Pichia etchelsii CBS2011	PE	Albertazzi *et al*., 1994
Pichia pastoris CBS704	PE, PEA	Albertazzi *et al*., 1994
Saccharomyces cerevisiae NCYC 739	PE	Albertazzi *et al*., 1994
Saccharomyces delbrueckii CBS1146	PE, PEA	Albertazzi *et al*., 1994
Kluyveromyces lactis CBS 5670	PE, PEA, phenyl ethyl propionate, phenyl ethyl isobutyrate, phenyl ethyl butyrate	Jiang, 1995[2]
Kluyveromyces marxianus (laboratory collection)	PE	Fabre *et al*., 1998
Pichia fermentans L-5	PE, PEA	Huang *et al*., 2000[1] Huang *et al*., 2001[1]

2.5.1.1 The effect of the constituents of the medium on the production of PE and other aromatic volatile compounds

The effects of the concentration of the medium components, including phenylalanine and other components of the medium, on the production of PE and other aromatic volatile compounds are reviewed in the following paragraphs.

Effect of phenylalanine

Several researchers studied the production of PE and PEA by different yeasts in phenylalanine-supplemented laboratory media. Fabre *et al.* (1998) found that only the natural isomer L-phe is utilised by the yeast (*K. marxianus*). D-phe is not consumed. They found that, when an equal mixture of the L and D isomers of phenylalanine was used, *K. marxianus* could use only 50% of the mixture. That was explained by the yeast's capacity to use only one isomer.

Phenylalanine does not affect the growth of yeasts and is exclusively involved in the production of metabolites including PE (Fabre *et al.*, 1998; Huang *et al.*, 2000). The exogenous PE was toxic for yeast strains (*e.g. K. marxianus*). PE significantly inhibited growth at a concentration of 0.5 g/L and the viability decreased to 37% and 0% in the presence of 1 and 2 g/L of PE respectively (Fabre *et al.*, 1998).

Production of PE by *P. fermentans* L-5 increased significantly as the concentration of phenylalanine in the medium was increased to 0.1%. Further increase in the concentration in the medium did not significantly increase the production of PE by the yeast. In the medium containing 0.1% phenylalanine, the production of PE (453.1 mg/L) was about 22.5 times higher than that in the control medium (without added phenylalanine). The metabolism of phenylalanine by the yeast was proposed to be the reason for the increase in PE. The phenylalanine initially present in the medium (0–0.05%) was completely consumed by *P. fermentans* L-5 after 16 h of fermentation. The percentage consumption of phenylalanine decreased as the initial content of phenylalanine in the medium increased. The molar yield of PE decreased with an increase in the concentration of phenylalanine (Huang *et al.*, 2000). Albertazzi *et al.* (1994) reported the production of 1728 mg/L and 1573 mg/L PE by *H. anamola* and *S. cerevisiae* respectively in a medium containing 0.2% L-phe. *P. etchelsii*, *K. saturnus*

and *S. delbrueckii* produced lesser concentrations of PE (92, 190 and 725 mg/L respectively) in the same medium. In a medium with 0.2% added L-phe, *K. marxianus* produced 700 mg/L PE (Fabre *et al.*, 1998). Lee and Richard (1984) reported the production of 390.4 mg/L PE by *K. lactis* in a medium containing 0.08% L-phe. *K. fragilis*, *T. sphaerica*, *D. hansenii* and *S. cerevisiae* produced 292.8–366 mg/L PE in the same medium. Jiang (1995) reported the production of PE and its esters by *K. lactis* in glucose–yeast extract medium without any added phenylalanine. Yeast extract could act as a source of L-phe that may be metabolised to these compounds. Some of the PE could be a result of *de novo* synthesis.

Roger *et al.* (1988) studied the effect of spraying phenylalanine on the surface of Camembert cheese. Maximum production of PE was after 7 days for the cheeses both with and without phenylalanine spraying; the maximum for PEA was recorded after 7 days in a cheese sprayed with phenylalanine and after 14 days in the control. These maxima were twofold higher in cheese sprayed with phenylalanine. However, at the end of the ripening process, the values were almost the same in the control and test cheeses.

Effect of carbon and nitrogen sources

Although the precursor of PE and PEA is L-phe/phenylalanine (isomer not defined), other constituents of the medium, such as carbon and nitrogen sources, may have influences on the formation of these aromatic compounds. The production of PE and PEA by different yeasts in media containing different sources and concentrations of carbon and nitrogen has been studied. Fabre *et al.* (1998) studied the effect of different carbon sources, such as glucose, ethanol, fructose, galactose, glycerol, lactose, maltose, sucrose and xylose, on the production of PE by *K. marxianus* and found that glucose was the most efficient for the production of PE. Huang *et al.* (2000) also demonstrated that carbon sources in the media affected the molar yields of PE and the percentage of phenylalanine consumed by *P. fermentans* L-5. *P. fermentans* L-5 produced a significantly higher amount of PE in media containing glycerol or sucrose as the carbon source than in media containing other carbon sources (glucose, fructose, galactose, xylose, maltose, lactose, soluble starch, sorbitol and ethanol). The molar yields of PE ranged between 0.3 and 0.89 M/M in the media depending on the carbon source used. With glucose, fructose or glycerol as the carbon source, the amount of phenylalanine originally present in the medium was completely consumed after 16 h of fermentation

by the test organism. In media containing other carbon sources, the consumption of phenylalanine ranged from 44.5 to 72.9%.

The production of PE increased as the sucrose concentration in the medium increased up to 18%. In the medium containing 18% sucrose, the production of PE was found to be the highest among the various concentrations (0–30%) tested. Increasing osmotic pressure as a result of the increasing sucrose concentration in the medium may have been the cause of this phenomenon. Increasing osmotic pressure has been reported to stimulate the biosynthesis of intracellular metabolites participating in osmoregulation. Increasing the sucrose concentration above 18% in the medium resulted in a reduction in PE production. This may have been due to enzyme inhibition caused by too high an osmotic pressure. At 18% sucrose concentration, the consumption of phenylalanine was 100% and the molar yield was the highest (Huang *et al.*, 2000). However, Fabre *et al.* (1998) found that changing the glucose concentration (10–50 g/L) in the medium did not affect the production of PE.

A significantly higher amount of PEA was detected in media containing glucose and fructose compared with media containing other carbon sources. The PEA present in the medium was considered to be formed from acetyl-CoA (due to glucose or fructose metabolism) and PE by alcohol acetyltransferase (Yoshioka and Hashimoto, 1981; Akita *et al.*, 1987). Albertazzi *et al.* (1994) noted production of a high concentration of PEA by *K. saturnus* and *P. pastoris* in a medium containing 0.2% L-phe (1763 and 734 mg/L respectively). In an attempt to increase the level of acetate, they fed the growing culture of two yeast strains with increasing amounts of the alcohol, but in no case was the level of ester affected. They also postulated that the formation of PEA was due to an alcohol acetyltransferase activity rather than an esterase activity.

Lee (1998) (cited in Huang *et al.*, 2000) reported yeast extract to be the best nitrogen source for the production of PE and PEA by *P. fermentans* L-5. Huang *et al.* (2000) investigated the effect of the concentration of yeast extract on the production of PE by *P. fermentans* L-5. The highest production of PE was found in the medium containing 0.25% yeast extract. In this medium, the phenylalanine was completely utilised and the molar yield of PE was the highest. Further increasing the level of yeast extract in the

medium resulted in a decrease in the production of PE by the test organism. Yeast extract serves as a source of both nitrogen and vitamins.

2.5.1.2 The effect of the culture conditions on the production of PE and other aromatic volatile compounds

The production of PE and other aromatic compounds can be affected not only by the components of the medium but also by different growth conditions. PE and PEA are produced from phenylalanine through enzyme-mediated reaction; the activities of the enzymes may be influenced by the change in pH. Huang *et al.* (2001) studied phenylalanine metabolism by *P. fermentans* in media with an initial pH range from 4.0 to 11.0 and found that the production of PE as well as the consumption of phenylalanine were the highest in the medium with pH 8.5. The production of PE was drastically decreased above pH 10 whereas the concentration of PEA was the highest at pH 10.

The temperature of cultivation may also have an effect on the formation of PE and PEA. The production of PE by *P. fermentans* was the highest between 25 and 35°C. The production of PEA was higher at 20 and 40°C than at 25, 30 and 35°C (Huang *et al.*, 2001). Fabre *et al.* (1998) and Albertazzi *et al.* (1994) detected PE and PEA produced by yeasts at 30°C and 28–30°C respectively.

The production of PE as well as the consumption of phenylalanine by yeasts can be influenced by shaking. An increase in the shaking speed increased the production of PE by *P. fermentans* and was the highest at 250 rev/min (Huang *et al.*, 2001). In contrast, PEA was not detected in the culture with a shaking speed of 250 rev/min although it was present in cultures with a slower rate of shaking. It was postulated that, at higher availability of oxygen, the formation of unsaturated fatty acids is favoured, which might inhibit the activity of alcohol acetyltransferase (Huang *et al.*, 2001).

In most of the published studies, it was found that the aromatic compounds PE and PEA were produced in the growth phase of yeast cultures in a medium containing phenylalanine. Huang *et al.* (2001) reported a simultaneous increase in biomass and PE that reached a peak after 16 h of incubation. The concentration of PE was stable up to 40 h and then started to decrease. PEA was produced at the growth stage and reached its

maximum concentration at 10 h and then disappeared after 14 h. Albertazzi *et al.* (1994) and Fabre *et al.* (1998) cultured the yeasts (Table 2.4) for 24 h and reported the production of high concentrations of PE and PEA. However, they did not study other time periods for comparison. Jiang (1995) studied the pattern of production of PE and PEA by *K. lactis* in a medium without added phenylalanine. The concentration of PE increased gradually over 14 days of incubation, whereas the concentration of PEA reached its peak after 2–3 days and then started decreasing.

The importance of PE and PEA in food flavours including cheese flavour and the increasing demand for natural food flavour makes microbial production of PE and PEA an interesting research topic. Genetic engineering could be an option for increasing the production of PE and related compounds by microorganisms, but consumers might not accept those products. So far, the most promising option for increasing the production of PE and related esters by yeasts is through the Ehrlich pathway, by feeding L-phe at the optimum concentration and optimising the other growth conditions. It was seen from the literature that glucose and yeast extract are the best carbon and nitrogen sources respectively, and that alkaline pH, 30°C and shaking are suitable growth conditions for the production of volatile aromatic compounds from phenylalanine by yeasts.

2.6 Conclusions

The literature review consolidates published information on the lipolytic, proteolytic and aroma-producing capacities of yeasts with special reference to *G. candidum* and *Y. lipolytica*. The review of the contributions of yeasts to cheese ripening as aerobic adjuncts and the information on their enzymatic potential provide a good background for the potential of yeasts as anaerobic adjuncts in cheese. A review of the microbial transformation of linoleic acid to CLA provides a background for designing a cheese with natural enrichment of CLA. The information relating to the production of PE and associated compounds by yeasts helps in setting up experiments for producing these aromatic flavour compounds through L-phe metabolism by yeasts.

Chapter 3. Objectives

Yeasts are traditionally used as aerobic adjunct cultures in surface-ripened cheeses, such as Camembert and Limburger (Reps, 1993). The yeasts influence cheese ripening by their flavour producing capabilities including the activities of their hydrolytic enzymes (Fleet, 1990). However, as the majority of cheeses in New Zealand are ripened anaerobically, there is interest in using yeasts as anaerobic adjuncts for their ripening potential. This study was carried out to investigate the possibility of using yeasts in this role. The influences of some technological aspects (the growth conditions of the adjunct culture, the point of adjunct addition during the cheesemaking process, the temperature of ripening and the level of added adjunct) on the ripening of cheeses made with yeast adjuncts, and the synthesis of conjugated linoleic acid (CLA) and selected flavour compounds in the cheese by combining yeasts and bacterial adjuncts were studied. Also, the capacity of yeasts for producing aromatic volatile compounds from the amino acid L-phenylalanine was studied in laboratory media.

It was hypothesised that, without significant growth in the cheese, the yeast adjuncts would influence the ripening mainly by the enzymes already produced in the cultures before their addition to the cheese.

The specific objectives of the study were as follows.

Objective 1. To study the effects of the growth conditions on the lipase and proteinase activities of three dairy yeasts (*Geotrichum candidum*, *Yarrowia lipolytica* and *Candida kefyr*) in laboratory media and their ability to produce volatile aroma compounds in the same media.

Objective 2. To show how yeasts, when used as anaerobic adjuncts, influence the ripening of cheese. This objective was divided into four parts.

- To study the effect of the growth conditions of the yeast cultures on the ripening of Cheddar cheese.
- To study the effect of the cheese manufacturing step at which the yeast culture is added on the ripening of Cheddar cheese.

- To study the effect of the ripening temperature on the ripening of a washed-curd, dry-salted cheese made with yeast and bacterial adjuncts.
- To study the effect of the level of the yeast culture added on the ripening of a washed-curd, dry-salted cheese made with yeast and bacterial adjuncts.

Objective 3. To produce a cheese with increased CLA concentration and targeted flavour compounds (ethyl esters) using yeast and bacterial adjuncts.

Objective 4. To study the production of volatile flavour compounds from L-phenylalanine by *C. kefyr* growing in laboratory media.

Chapter 4. Characterisation of three dairy yeasts for their potential use as flavour cultures in cheese

4.1 Introduction

Fresh cheese curd is a mixture of proteins (predominantly casein), lipids, residual lactose, lactic acid, citric acid, sodium chloride and water. The primary features of cheese ripening involve the transformation of the two principal organic constituents, proteins and lipids, although the metabolism of lactose and citrate is also important (Fox *et al.*, 2000). Degradation of caseins by the action of rennet enzymes and plasmin and the activity of the cell envelope proteinases and peptidases from lactic acid bacteria, incorporated in the curd, yields small peptides and amino acids. The amino acids are precursors of various alcohols, aldehydes, acids, esters and sulphur compounds (Smit *et al.*, 2002). Lipolysis results in the breakdown of milk fat triglycerides to release free fatty acids, which are precursors of flavour compounds such as methyl ketones and lactones (McSweeney, 2004).

Microorganisms are essential components of all natural cheese varieties and play an important role during both cheese manufacture and ripening. They can be divided into two main groups: starters and secondary flora. The starter flora is responsible for acid development during cheese manufacture (Beresford *et al.*, 2001). Adjuncts are the secondary flora that is deliberately added to the cheese milk to contribute to the development of cheese flavour and functionality following curd formation. As they are generally added at low levels (< 0.01% of cheese milk), adjunct cultures do not influence acid development. Adjuncts may be divided into two groups according to their tolerance of oxygen. Firstly, traditional aerobic adjuncts (*Penicillium*, *Geotrichum*, *Arthrobacter*, *Brevibacterium* and *Micrococcus*) grow on the surface of the cheeses and are important to the flavour of the soft surface-ripened cheeses, such as Brie, Camembert, Blue-veined and smear-ripened cheeses. Secondly, traditional anaerobic adjuncts are incorporated within the cheese and include *Leuconostoc* and *Propionibacterium* in Gouda and Swiss-type cheeses respectively (Crow *et al.*, 2002).

The metabolic potential of adjunct cultures, particularly their ability to metabolise the primary components of cheese (protein, lipids and carbohydrates), determines their ability to influence cheese flavour. Yeasts are known for their hydrolytic impact on lipids and proteins. A number of yeasts are active lipase producers (Pereira-Meirelles *et al.*, 1997). *Geotrichum candidum* lipase has specificity towards unsaturated fatty acids (Baillargeon *et al.*, 1989). Ercoli *et al.* (1992) showed that some *Yarrowia lipolytica* strains are effective in converting unsaturated fatty acids to C10 and C12 γ lactones, which can contribute to cheese flavour. The proteolytic activities of different yeast strains are well known (Gueguen and Lenoir, 1976; Suzzi *et al.*, 2001). *Y. lipolytica* proteinase was found to hydrolyse β-casein (Guerzoni *et al.*, 1996). During cheese manufacture, bitter peptides can be produced by coagulants, thus creating problems if these peptides accumulate in excess (McSweeney, 2004). *G. candidum* has aminopeptidase activity, which offers a mechanism by which this yeast could reduce bitterness (Molimard *et al.*, 1994). Sulphur compounds are very important constituents of ripened cheese aroma (Urbach, 1993). Yeasts, especially *G. candidum*, produce a range of sulphur compounds such as methanethiol, dimethyldisulphide, dimethyltrisulphide and S-methyl thioesters possibly through amino acid metabolism (Berger *et al.*, 1999; Demarigny *et al.*, 2000). Other volatile compounds are also known to be produced by yeasts of dairy origin. Fatty acid esters with fruity odours were identified when *G. candidum* was grown on breadcrumbs (Daigle *et al.*, 1999) and several free fatty acids and primary alcohols were produced when this yeast was grown in a milk and cheese slurry medium (Jollivet *et al.*, 1994). Fermenting cream with *Y. lipolytica* was shown to produce a cheesy flavour (Kalle *et al.*, 1976). Thus the lipolytic, proteolytic and peptidolytic properties of yeasts and their ability to produce volatile aroma compounds make them potential candidates for use in flavour development and accelerated ripening of cheese.

Yeasts are traditional aerobic adjuncts in surface-ripened cheeses (Reps, 1993). However, this study was initiated to investigate their potential to act as anaerobic adjuncts in hard cheese. The first step of the study was to characterise yeast cultures for their ability to produce ripening enzymes (lipases and proteinases) and to synthesise volatile flavour compounds. This chapter describes the characterisation of three dairy yeasts from the culture collection of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, for these biochemical properties.

4.2 Materials and methods

4.2.1 Growth experiments

Cultures used

Yeasts: *Geotrichum candidum* B9001 (CMICC 335426), *Yarrowia lipolytica* B9014 (SPUE 73) and *Candida kefyr* B9006 (NCYC 143) were obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand. The cultures were kept frozen in Malt–Yeast extract–Glucose–Peptone medium (described in the following paragraphs) at –70°C.

Materials

1. Components of medium: Malt extract (Merck KGaA, 64271, Darmstadt, Germany), yeast extract (DIFCO Laboratories, Detroit, MI 48232-7058, USA), D (+) glucose (BDH Laboratory Supplies, Poole, BH15 1TD, England), peptone from casein pancreatically digested (Merck, KGaA, 64271, Darmstadt, Germany), safflower oil containing 76.0–80.0% linoleic acid (Bronson and Jacobs Pty Ltd., New Zealand Division)
2. Phosphate buffer (0.05 M, pH 7.5) – prepared using K_2HPO_4 and KH_2PO_4 (BDH Laboratory Supplies, Poole, BH15 1TD, England)

Method

G. candidum, *Y. lipolytica* and *C. kefyr* cultures were grown in either glucose medium or safflower oil medium as shake (200 rev/min) or static cultures incubated at 30°C.

The glucose medium contained malt extract, 0.3% w/v; yeast extract, 0.3% w/v; D (+) glucose, 1.0% w/v; and peptone, 0.5% w/v. The components were dissolved in water, the pH was adjusted to pH 6.0 with 1 M HCl (BDH) and the mixture was autoclaved for 15 min at 121°C in 250 mL Erlenmeyer flasks. Each flask contained 100 mL of medium and was capped with a cotton plug, covered with aluminium foil. The safflower oil medium was prepared according to the procedure used for the glucose medium except that 1.0% (w/v) safflower oil replaced 1.0% (w/v) glucose.

Frozen stock culture (10^9 cfu/mL; personal communication with Shao Liu, Fonterra Co-operative Group, Palmerston North) was thawed (30 min) and inoculated into 10 mL of glucose medium [2.0% (v/v)] in a laminar flow chamber and incubated at 30°C with shaking at 200 rev/min. After 24 h, the culture was used to inoculate [2.0% (v/v)] 100 mL of the experimental medium in a 250 mL Erlenmeyer flask. The cultures were incubated in a 30°C room. A shaker supplied by New Brunswick Scientific Co., Edison, NJ, USA, was used for shake cultures. Static cultures were grown on a shelf at 30°C.

Growth was assessed by measuring the optical density (OD) of the cell suspensions. The culture broth was mixed thoroughly before sampling. The culture was diluted with blank medium so that the OD at 600 nm did not exceed 1.0. A Hitachi U-2000 spectrophotometer (supplied by Hitachi Ltd., Tokyo, Japan) was used to read the OD at 600 nm. The OD of the blank medium was subtracted from the OD reading of the culture broth. The adjusted readings were used to prepare growth curves.

The following procedure was followed to establish a correlation between OD_{600} and dry cell weight. Culture was grown in glucose medium at 30°C with shaking at 200 rev/min for 36 h. The cells were harvested by centrifuging for 15 min at 13 700 *g*. The pellet was washed with 0.05 M phosphate buffer, pH 7.5, and centrifuged for 15 min at 20 000 *g*. The washing was repeated twice. Then a thick cell suspension (100 mL/L culture) was made by re-suspending the washed pellet in 0.05 M phosphate buffer, pH 7.5. A part of the suspension was used for OD_{600} measurements and another part was used for dry cell weight determinations. The first part was diluted to different dilutions with phosphate buffer and OD values at 600 nm were read for all dilutions, as described earlier. A defined volume of the second part of the suspension was filtered through a pre-dried, pre-weighed 0.45 μm filter membrane (Millipore Corporation, Bedford, MA 01730, USA). The residue was dried at 105°C until the weight became constant. The weight of the membrane and cells minus the weight of the membrane gave the weight of the dry cells for the known volume. The calculated weight per unit volume for different dilutions and the corresponding OD were plotted. A correlation equation between dry cell weight and OD was calculated using Microsoft Excel (Appendices 4.1, 4.2 and 4.3 for *G. candidum*, *Y. lipolytica* and *C. kefyr* respectively).

4.2.2 Enzyme activities

Lipase and proteinase activities of the yeast cultures were determined at different stages of culture growth under various growth conditions.

4.2.2.1 Sample preparation

Materials

Phosphate buffer was prepared as described in Section 4.2.1.

Method

The culture broth was centrifuged at 20 000 *g* for 15 min at 4°C. The supernatant was designated the extracellular fraction. The pellet was washed with 0.05 M phosphate buffer (pH 7.5, 4°C). The quantity of buffer was equivalent to the initial culture volume. The suspension was centrifuged for 15 min at 20 000 *g* (4°C). Washing was repeated. The pellet obtained after the second washing was diluted with 0.05 M phosphate buffer (pH 7.5, 4°C), taking the final volume to 75% of the original broth volume. This fraction was designated the washed-cell fraction. Ballotini glass beads (0.45 mm; B. Braun Biotech International GmbH, Melsungen, Germany) were added to the suspension and the mixture was placed in a Ballotini glass bead shaker capsule (B. Braun). The capsule was shaken for 20 s at low speed and for 40 s at high speed. The shaker was cooled with liquid carbon dioxide. The disintegrated cell mass was centrifuged for 30 min at 27 000 *g* (4°C) and the supernatant was filtered through 0.45 μm filter paper (Millipore Corporation, Bedford, MA 01730, USA). This filtrate was designated the intracellular fraction. The preparation of extracellular, washed-cell and intracellular fractions is shown in Figure 4.1A. For convenience of the presentation, the enzymes present in these three fractions are termed extracellular, washed-cell and intracellular respectively (schematic diagram in Figure 4.1B). There was the possibility of cross contamination between the fractions during sample preparation; for example, some intracellular enzymes may have come out of the cells because of cell damage or a part of the substrate may have transported into the intracellular space. Definitive tests for the enzyme locations were not carried out in this study, as it focused on the possibility of using the enzymatic capabilities of the yeasts for cheese ripening.

However, the experiments provided a useful indication of the possible cellular location(s) of the enzyme(s) of interest.

Culture

Centrifugation

Supernatant (**Extracellular fraction**)

Pellet

Washing, centrifugation and re-suspension

Cell suspension (**Washed-cell fraction**)

Glass bead shaking and centrifugation

Supernatant (**Intracellular fraction**)

Pellet/cell debris

Figure 4.1A Preparation of fractions from yeast cultures, designated extracellular, washed-cell and intracellular fractions.

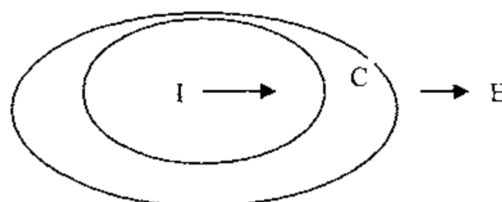


Figure 4.1B Schematic representation of enzyme location during cultivation of yeast cells: enzymes located in intracellular (I), washed-cell (C) and extracellular (E) fractions (diagram adapted from Pereira-Meirelles *et al.*, 2000).

4.2.2.2 Lipase assay

Materials

1. *p*-Nitrophenyl butyrate (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA)
2. Methanol (BDH Laboratory Supplies, Poole, BH15 1TD, England)
3. Phosphate buffer was prepared as described in Section 4.2.1.

Method

p-Nitrophenyl butyrate hydrolysis was measured as an indicator of intracellular, washed-cell and extracellular lipase activity (Lee and Lee, 1989) using a time scan program (wavelength used 410 nm) on a Hitachi U-2000 spectrophotometer (supplied by Hitachi Ltd., Tokyo, Japan), set at 30°C. These measured activities also included esterase activities. However, this enzyme is called lipase throughout this thesis because of the capability of the yeast cultures to hydrolyse milk fat (Chapters 5 and 6). The activity was expressed as μM/min/mg dry cell weight for intracellular and washed-cell lipases and as μM/min/mL supernatant for extracellular lipase. The following formula was used to calculate the lipase activity.

Activity = 1/z x 2/1 x 1000/y x Δabsorbance/min

z = *p*-Nitrophenyl millimolar extinction coefficient (12.2)

y = amount of sample added (μL)

4.2.2.3 Proteinase assay

Materials

FITC-labelled β-casein was made using the procedure described in Twining (1984), using fluorescein isothiocyanate (FITC) (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA) and β-casein (supplied by Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA).

Method

An FITC β-casein assay was carried out for intracellular, washed-cell and extracellular proteinase (Twining, 1984), using a Perkin Elmer (Model LS50B) fluorometer (Perkin-

Elmer Ltd., Beaconsfield, Buckinghamshire, England). The activity was expressed as relative fluorescence units RFU/min/mg dry cell weight for intracellular and washed-cell proteinase activity and as RFU/min/mL supernatant for extracellular proteinase activity.

4.2.3 Estimation of volatile compounds

Six grams of culture were weighed into a glass vial and sealed, and the volatile compounds in the headspace of the culture were analysed by solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) using the methodology described in Liu *et al.* (2003). The relative concentrations were expressed as relative peak area x 10^6.

4.2.4 Statistical analysis

Statistical analyses were carried out using SAS software version 8.2 (SAS Institute Inc., NC, USA).

4.3 Results

G. candidum, *Y. lipolytica* and *C. kefyr* cultures were grown in either glucose medium or safflower oil medium as shake (200 rev/min) or static cultures incubated at 30°C. Growth characteristics, lipase and proteinase activities as well as volatile compounds from the culture headspace were studied over the incubation period. Measurements of growth, lipase activity and proteinase activity were from two independent fermentations. However, only a single sample was studied for volatile compounds. This was considered to be acceptable as the intention was to monitor trends in the level of volatile compounds (in relative peak area units) rather than the absolute quantities. Also, analysis of a single sample showed a high level of repeatability (± 10% for three analyses of the single sample).

4.3.1 Characterisation of *G. candidum*

Growth characteristics, lipase and proteinase activities of *G. candidum* and volatile compounds produced by *G. candidum* are described in this section.

4.3.1.1 Growth characteristics of *G. candidum*

The *G. candidum* shake cultures in both glucose medium and safflower oil medium produced a homogeneous dispersion. However, a white layer was formed on the top of the cultures grown in both media under static conditions (Figure 4.2). The layer formed could not be mixed properly, and reading of the OD at various growth stages was not possible for these static cultures. Dry cell weights of *G. candidum* shake cultures grown in glucose medium and safflower oil medium over 168 h are shown in Figure 4.3.

The shake cultures grown in glucose medium and safflower oil medium attained the highest dry cell weight after 48 h and 96 h of incubation respectively. The highest dry cell weight of *G. candidum* grown in safflower oil medium (11 mg/mL) was 1.8 times higher than that in glucose medium (6.1 mg/mL). However, the maximum growth rates were similar for the cultures in both media (0.13 and 0.12 mg/mL/h respectively).

Figure 4.2 *G. candidum* grown in glucose medium as a shake or static culture over 72 h of incubation at 30°C.

The initial pH of all media was adjusted to pH 6.0 (Figure 4.4). The pH of the *G. candidum* cultures in glucose medium (both shake and static) showed a similar trend, dropping initially and then increasing. However, the changes occurred more rapidly in the shake cultures, with the pH reaching a minimum of pH 4.4 at 24 h, rising to a plateau of pH 7.6 at 72 h and then increasing slowly to pH 8.4 at 168 h. In contrast, the pH of the cultures in safflower oil medium did not vary as much, increasing slowly to reach pH 7.2 (shake cultures) and pH 7.0 (static cultures) by 168 h.

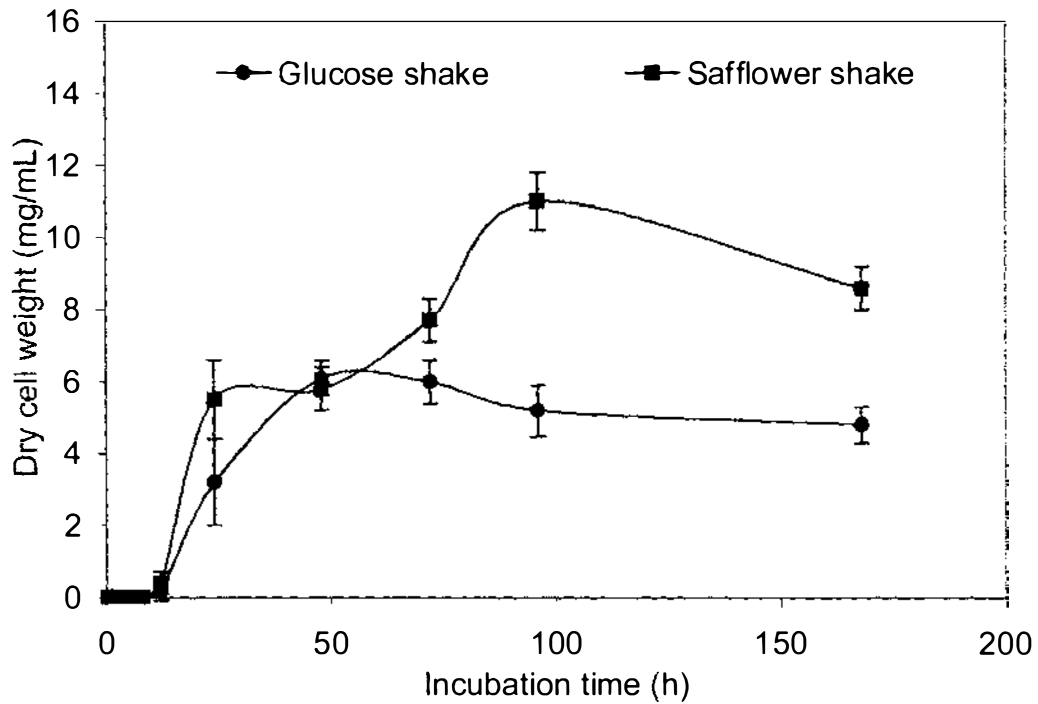


Figure 4.3 Dry cell weights of *G. candidum* shake cultures grown in glucose medium and safflower oil medium over 168 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

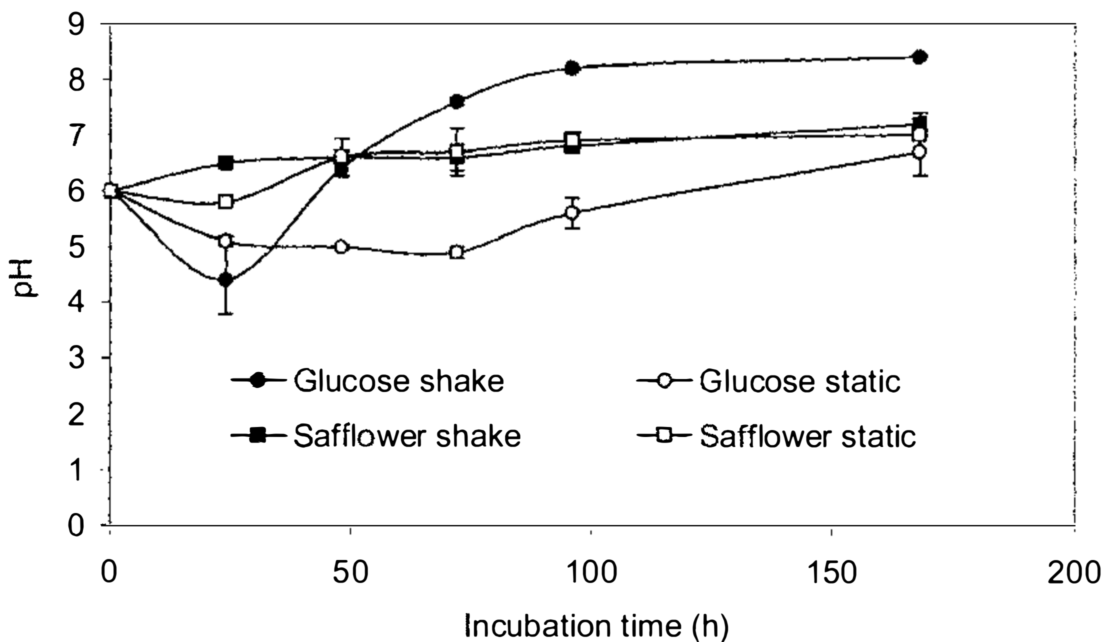


Figure 4.4 Changes in pH of *G. candidum* shake and static cultures grown in glucose medium and safflower oil medium over 168 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

4.3.1.2 Lipase activity of *G. candidum*

Intracellular, washed-cell and extracellular lipase activities of *G. candidum* under different treatments were determined. *G. candidum* cultures did not show any intracellular or washed-cell lipase activity under the experimental conditions. The extracellular lipase activities of *G. candidum* cultures are shown in Figure 4.5.

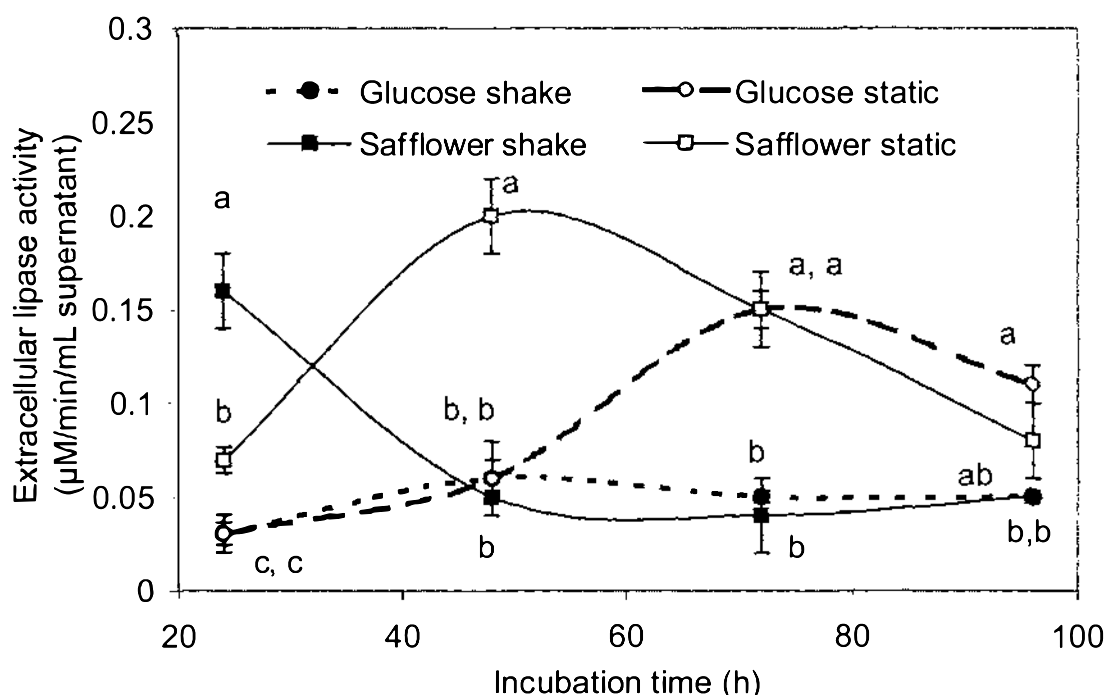


Figure 4.5 Extracellular lipase activities of *G. candidum* shake and static cultures grown in glucose medium and safflower oil medium at 30°C. The letters a, b, c at each time point indicate treatments that are significantly different ($p \leq 0.05$). The error bars represent standard deviations between two independent fermentations.

G. candidum shake cultures in glucose medium showed minimal extracellular lipase activity (≤ 0.06 µM/min/mL supernatant) over the 96 h period of study. When the glucose in the medium was replaced with safflower oil, the extracellular lipase activity was 5 times higher, at 24 h of incubation (0.16 µM/min/mL supernatant). The extracellular lipase activity of the shake cultures in safflower oil medium disappeared after 48 h of incubation (≤ 0.05 µM/min/mL supernatant). Unlike the shake cultures, the static cultures in glucose medium showed lipase activity. After 72 h of incubation, the extracellular lipase activity of the static cultures was 3 times higher than that of the shake cultures (0.15 µM/min/mL supernatant) in glucose medium. *G. candidum* static

cultures grown in safflower oil medium attained the highest lipase activity (0.2 μM/min/mL supernatant) at 48 h, whereas shake cultures grown in the same medium attained the highest lipase activity (0.16 μM/min/mL supernatant) at 24 h.

4.3.1.3 Proteinase activity of *G. candidum*

The intracellular, washed-cell and extracellular proteinase activities of *G. candidum* cultures under different conditions were determined. Intracellular proteinase activity was detected in *G. candidum* shake cultures both in glucose medium and in safflower oil medium and is presented in Figure 4.6.

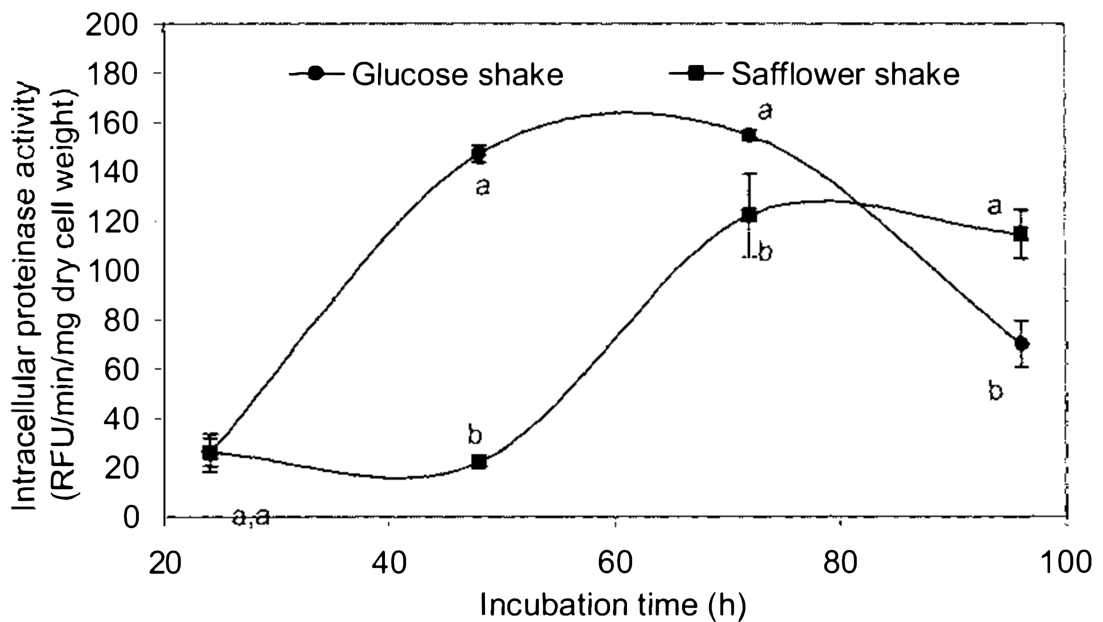


Figure 4.6 Intracellular proteinase activities of *G. candidum* shake cultures grown in glucose medium and safflower oil medium at 30°C. The letters a, b at each time point indicate treatments that are significantly different ($p \leq 0.05$). The error bars represent standard deviations between two independent fermentations.

The intracellular proteinase activities of *G. candidum* reached a peak after 72 h of incubation in shake cultures grown in both media (154.7 and 122.2 RFU/min/mg dry cell weight in glucose medium and safflower oil medium respectively). No intracellular proteinase activity was detected in the static cultures in either medium.

None of the cultures showed any proteinase activity in the extracellular or washed-cell fractions.

4.3.1.4 Production of volatile compounds by *G. candidum*

A range of alcohols, esters, ketones, aldehydes and acids were detected in headspace analysis of *G. candidum* shake cultures grown in glucose medium. Many of the compounds are important for cheese flavour and are discussed in detail in Section 4.4. The relative peak areas of most of the volatile compounds were at a maximum at 48 h and had disappeared ($< 1 \times 10^6$ relative peak area) at 72 h. A fruity/floral odour was perceived in the 48 h shake culture in glucose medium but this disappeared along with the volatile compounds after 72 h. The different volatile compounds detected in the headspace analysis of *G. candidam* shake cultures in glucose medium along with their relative peak areas are given in Table 4.1.

Many of these volatile compounds in the shake culture of *G. candidum* grown in glucose medium were detected in the static culture grown in glucose medium, but the relative peak areas were different (Appendix 4.4). In the cultures grown in safflower oil medium, most of the volatile compounds including ethanol and esters were absent, although some alcohols were present (Appendix 4.4).

Table 4.1 Volatile compounds detected in *G. candidum* shake culture grown in glucose medium at 30°C at different points of incubation

Volatile compounds	Relative peak area (x 10^6)		
	24 h	48 h	72 h
Alcohols			
Ethanol	50.4	37.8	nd*
2-Methyl propanol	19.5	20.0	nd
2-Methyl butanol and 3-Methyl butanol	131.4	171.7	nd
4-Butoxy butanol	nd	1.4	nd
Phenyl ethyl alcohol	7.4	30.6	15.6
Esters			
Ethyl acetate	0.7	2.0	nd
2-Ethyl, methyl propanoate	nd	10.4	nd
2-Methyl, propyl acetate	nd	1.7	nd
Ethyl butanoate	1.5	4.6	nd
2-Ethyl, methyl butanoate	nd	16.8	nd
3-Ethyl, methyl butanoate	nd	7.4	nd
3-Butyl, methyl butanoate	nd	1.6	nd
1-Hydroxy-3-methyl butylacetate	5.4	5.5	nd
Ketone			
2-Hydroxy-3-pentanone	nd	2.4	nd
Aldehydes			
2-Methyl propanal	nd	10.7	nd
2-Methyl butanal	nd	17.2	nd
3-Methyl butanal	nd	46.4	nd
Benzaldehyde	nd	1.3	nd
Acids			
Acetic acid	2.9	nd	1.0
2-Methyl propanoic acid	3.6	2.8	nd
3-Methyl butanoic acid	4.1	nd	nd

* = Not detectable.

4.3.2 Characterisation of *Y. lipolytica*

Growth characteristics, activities of lipase and proteinase of *Y. lipolytica* and production of volatile compounds by *Y. lipolytica* are described in this section.

4.3.2.1 Growth characteristics of *Y. lipolytica*

Y. lipolytica was grown in glucose medium or safflower oil medium under shake (200 rev/min) or static conditions at 30°C for 11 days (264 h). The changes in the dry cell weights under different treatments are shown in Figure 4.7. Unlike *G. candidum*, no surface layer was formed.

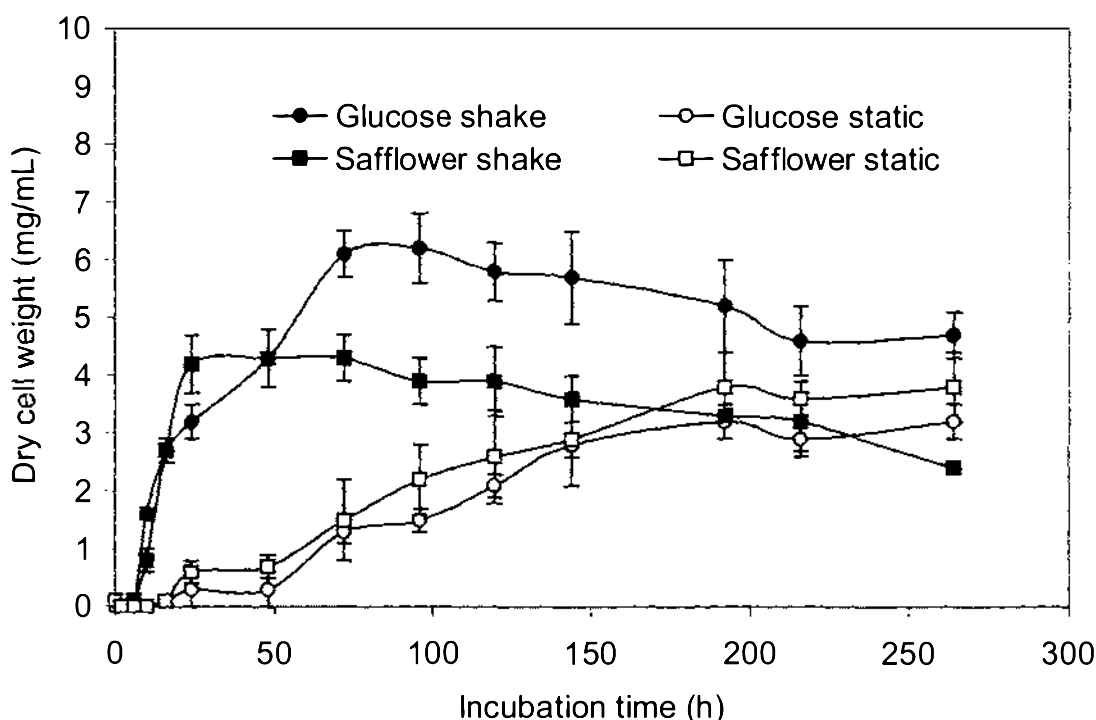


Figure 4.7 Dry cell weights of *Y. lipolytica* shake and static cultures grown in glucose medium and safflower oil medium over 264 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

The highest dry cell weight attained by *Y. lipolytica* was in the shake cultures in glucose medium (6.2 mg/mL). The highest dry cell weight attained by *Y. lipolytica* shake cultures was lower in safflower oil medium (4.3 mg/mL) than in glucose medium. However, the highest dry cell weights attained under static conditions were similar in

glucose medium (3.2 mg/mL) and in safflower oil medium (3.8 mg/mL). The maximum growth rates were higher in shake cultures (0.09 mg/mL/h) than in static cultures (0.02 mg/mL/h) in either medium. The results indicate a requirement for oxygen for faster growth.

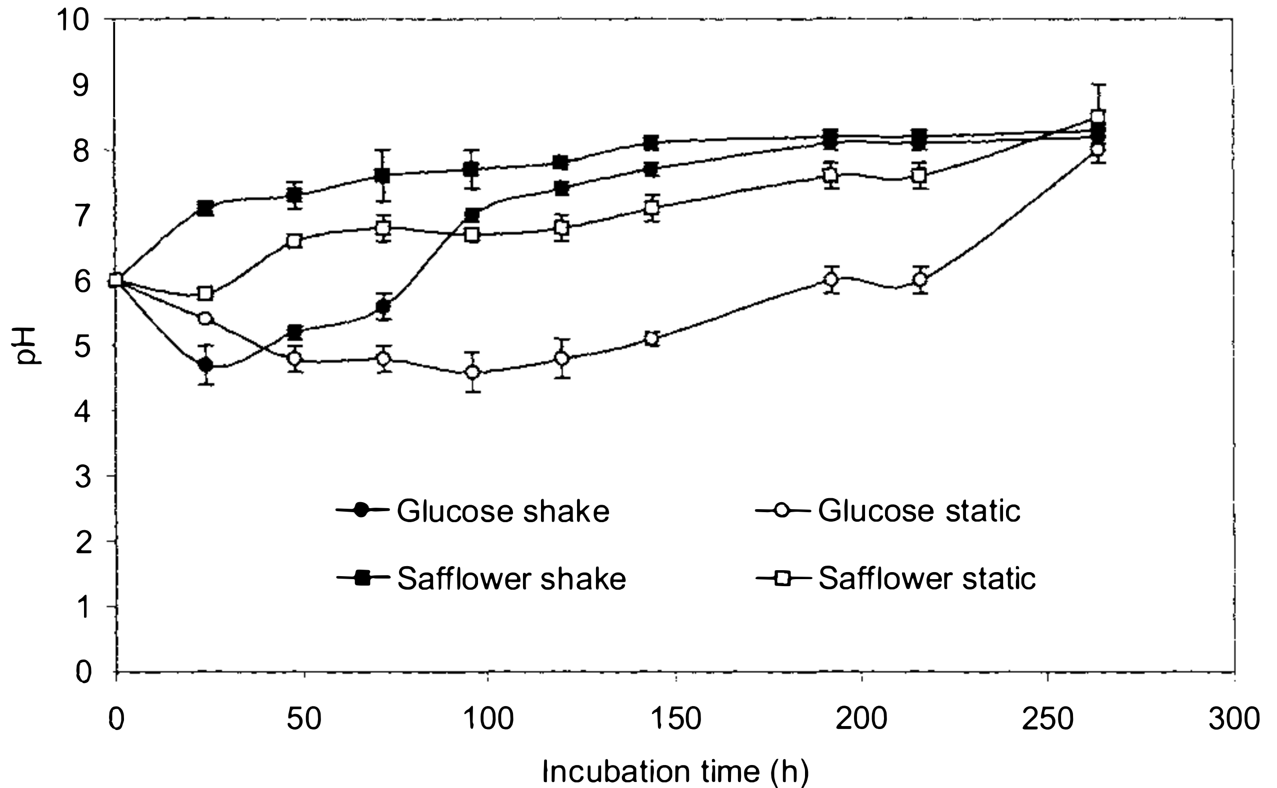


Figure 4.8 Changes in pH of *Y. lipolytica* shake and static cultures in glucose medium and safflower oil medium over 264 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

The changes in pH of *Y. lipolytica* cultures under different treatments are presented in Figure 4.8. The patterns of the changes in pH of *Y. lipolytica* cultures were similar to those of the *G. candidum* cultures. The initial pH of all media was adjusted to pH 6.0. The pH of *Y. lipolytica* cultures in glucose medium under either shake or static conditions decreased initially and then started increasing. However, the changes were faster in shake cultures than in static cultures. The pH dropped to 4.7 at 24 h in shake cultures and to 4.6 at 96 h in static cultures. In contrast, the pH of the cultures in safflower oil medium did not vary as greatly, but increased slowly over the period. However, the pH values were similar in all cultures after 11 days and ranged between 8.0 and 8.5.

4.3.2.2 Lipase activity of *Y. lipolytica*

Y. lipolytica showed lipase activities in the intracellular, washed-cell and extracellular fractions under all treatments. The data are presented in Figures 4.9A, 4.9B, 4.9C and 4.9D.

In glucose medium, *Y. lipolytica* showed higher levels of lipase activity in the static cultures than in the shake cultures. The maximum values of intracellular, washed-cell and extracellular lipase activities in the static cultures were 0.71 μM/min/mg dry cell weight, 0.49 μM/min/mg dry cell weight and 0.48 μM/min/mL supernatant respectively compared with 0.12 μM/min/mg dry cell weight, 0.08 μM/min/mg dry cell weight and 0.34 μM/min/mL supernatant in the shake cultures, both in glucose medium. In shake cultures, the intracellular and washed-cell lipase activities were the highest at 216 h and the extracellular lipase activity was the highest at 264 h. The lipase activities of the *Y. lipolytica* static cultures in glucose medium followed the same trend. Intracellular and washed-cell lipase activities increased between 192 and 216 h and then decreased between 216 and 264 h. An increase in extracellular lipase activity was observed between 216 and 264 h.

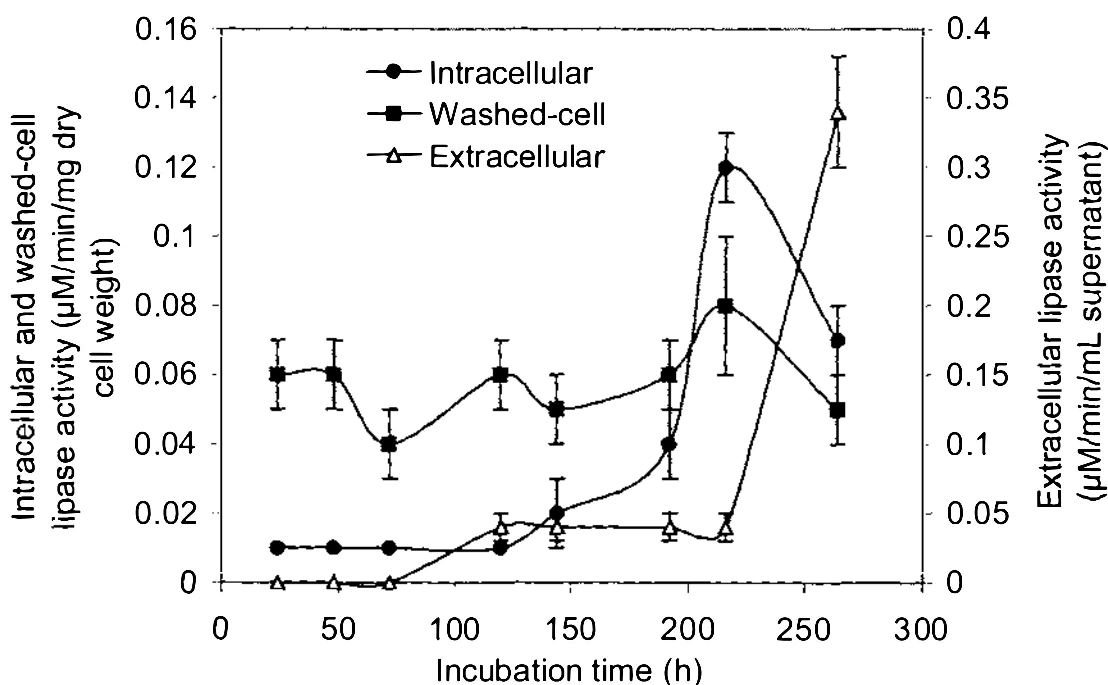


Figure 4.9A Intracellular, washed-cell and extracellular lipase activities in *Y. lipolytica* shake cultures in glucose medium incubated at 30°C. The error bars represent standard deviations between two independent fermentations.

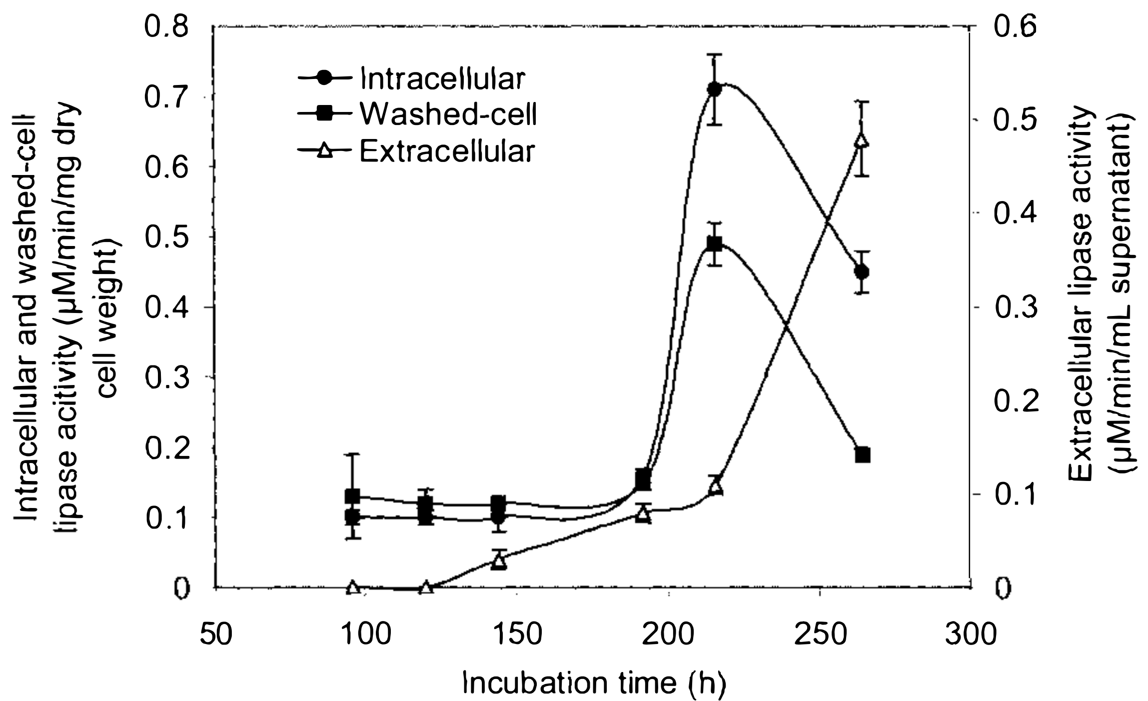


Figure 4.9B Intracellular, washed-cell and extracellular lipase activities of *Y. lipolytica* static cultures in glucose medium incubated at 30°C. The error bars represent standard deviations between two independent fermentations.

In the shake cultures of *Y. lipolytica* in safflower oil medium, the highest levels of intracellular, washed-cell and extracellular lipase activities were observed at 192, 120 and 192 h respectively (Figure 4.9C). The highest extracellular lipase activity recorded in these cultures was less than that of the shake cultures in glucose medium, although the levels of the intracellular and washed-cell lipase activities were higher in safflower oil medium than in glucose medium. The lipase activities were followed up to 192 h; at that point, the lipase activities in the intracellular and extracellular fractions were still increasing, indicating a possibility of further increase.

Y. lipolytica showed the highest lipase activity in the static cultures grown in safflower oil medium (Figure 4.9D). Extracellular lipase activity was minimal in the cultures before 120 h ($\leq$ 0.05 µM/min/mL supernatant), increased to 4.3 µM/min/mL supernatant at 192 h and then decreased slightly to reach 3.7 µM/min/mL supernatant at 264 h. Intracellular and washed-cell lipase activities were minor by comparison. The intracellular lipase activity decreased from 0.24 µM/min/mg dry cell weight to a minimal level ($\leq$ 0.05 µM/min/mg dry cell weight) between 120 and 264 h. An increase

in extracellular lipase activity and a decrease in intracellular lipase activity over the same period of incubation suggest leaching of the lipase from the yeast cells.

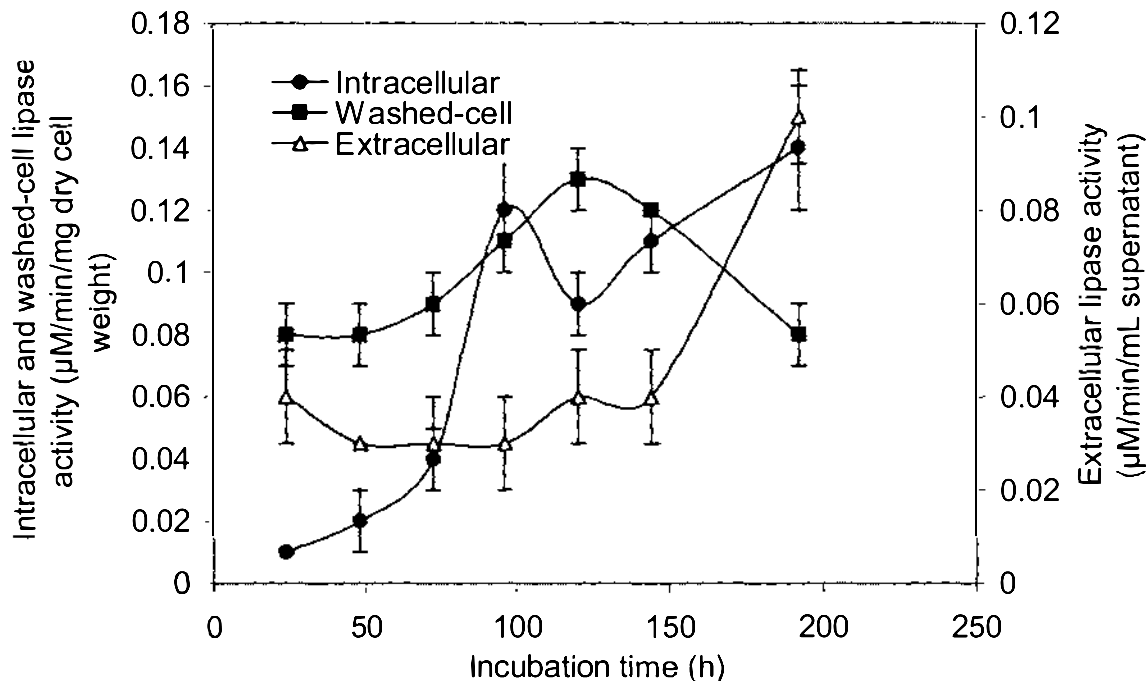


Figure 4.9C Intracellular, washed-cell and extracellular lipase activities of *Y. lipolytica* shake cultures in safflower oil medium incubated at 30°C. The error bars represent standard deviations between two independent fermentations.

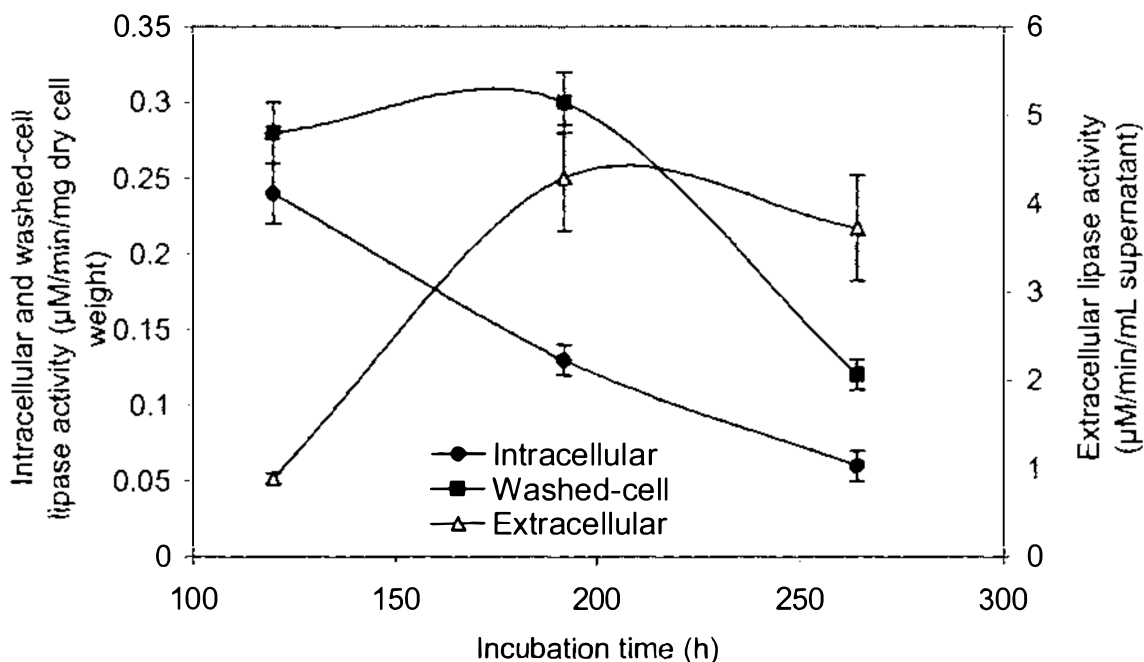


Figure 4.9D Intracellular, washed-cell and extracellular lipase activities of *Y. lipolytica* static cultures in safflower oil medium incubated at 30°C. The error bars represent standard deviations between two independent fermentations.

Y. lipolytica static cultures showed higher lipase activities in safflower oil medium than in glucose medium. Induction of lipase activity by oil could be an explanation for this. In either medium, lipase activity was higher in the static cultures than in the shake cultures. Dry cell weight and maximum cell growth were higher in the shake cultures than in the static cultures. Stress conditions possibly influenced the lipase activity of *Y. lipolytica* positively.

4.3.2.3 Proteinase activity of *Y. lipolytica*

Proteinase activity was absent in intracellular, washed-cell and extracellular fractions of *Y. lipolytica* under all treatments.

4.3.2.4 Production of volatile compounds by *Y. lipolytica*

A range of alcohols was detected in headspace analysis of *Y. lipolytica* cultures. Ethyl acetate was present only in the 24 h shake cultures in glucose medium (Table 4.2). Unlike *G. candidum* cultures, aldehydes and ketones were not detected. Ethanol was present only in the cultures in glucose medium, not in safflower oil medium. Concentrations of the volatile compounds were less in the static cultures than in the shake cultures (Appendix 4.5).

Table 4.2 Volatile compounds detected in *Y. lipolytica* shake culture grown in glucose medium at 30°C at different points of incubation

Volatile compounds	Relative peak area (x 10^6)		
	24 h	48 h	72 h
Alcohols			
Ethanol	87.9	115.3	3.2
2-Methyl propanol	11.7	39.3	5.5
2-Methyl butanol and 3-Methyl butanol	219.5	475.8	179.3
Phenyl ethyl alcohol	5.9	21.9	35.6
Ester			
Ethyl acetate	4.1	nd*	nd
Acids			
Acetic acid	5.0	nd	nd
3-Methyl butanoic acid	1.0	nd	nd

* = Not detectable.

4.3.3 Characterisation of *C. kefyr*

Growth characteristics, lipase and proteinase activities of *C. kefyr* and volatile compounds produced by *C. kefyr* are described in this section.

4.3.3.1 Growth characteristics of *C. kefyr*

The dry cell weights of *C. kefyr* cultures under different treatments (glucose medium or safflower oil medium under shake or static conditions) over an incubation of 148 h at 30°C are presented in Figure 4.10. Dry cell weight was higher under shake conditions than under static conditions in both media. The highest dry cell weight (3.9 mg/mL) was achieved in glucose medium under shake conditions at 72 h.

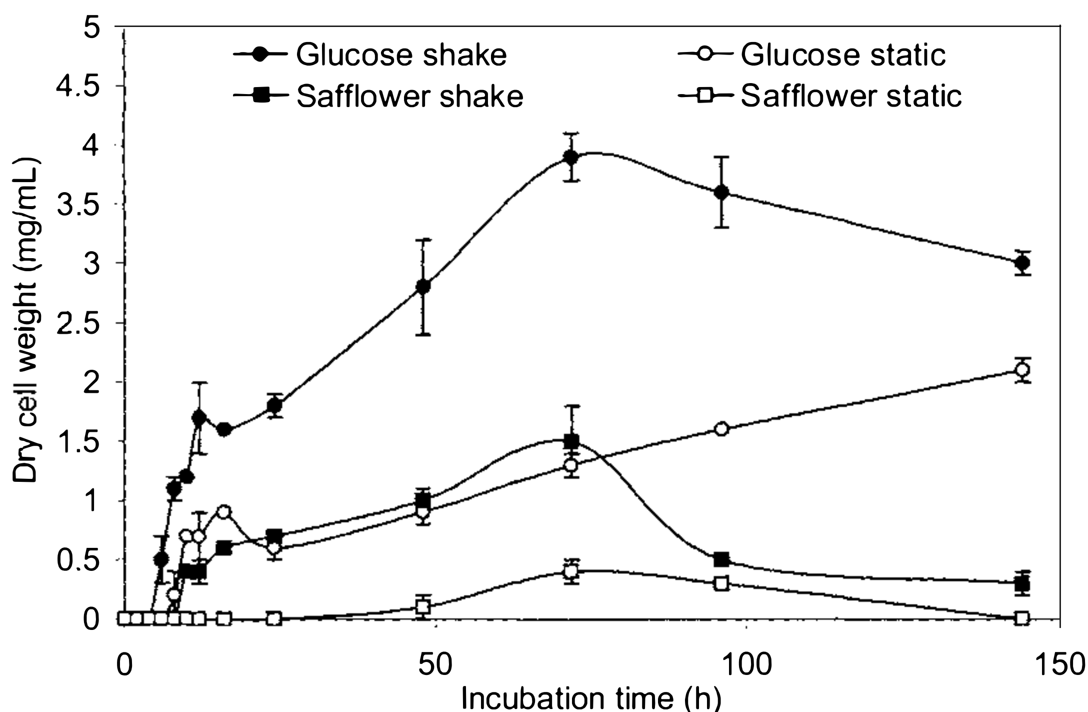


Figure 4.10 Dry cell weights of *C. kefyr* shake and static cultures grown in glucose medium and safflower oil medium over 148 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

The changes in the pH values of the *C. kefyr* cultures under different treatments over the incubation period are shown in Figure 4.11.

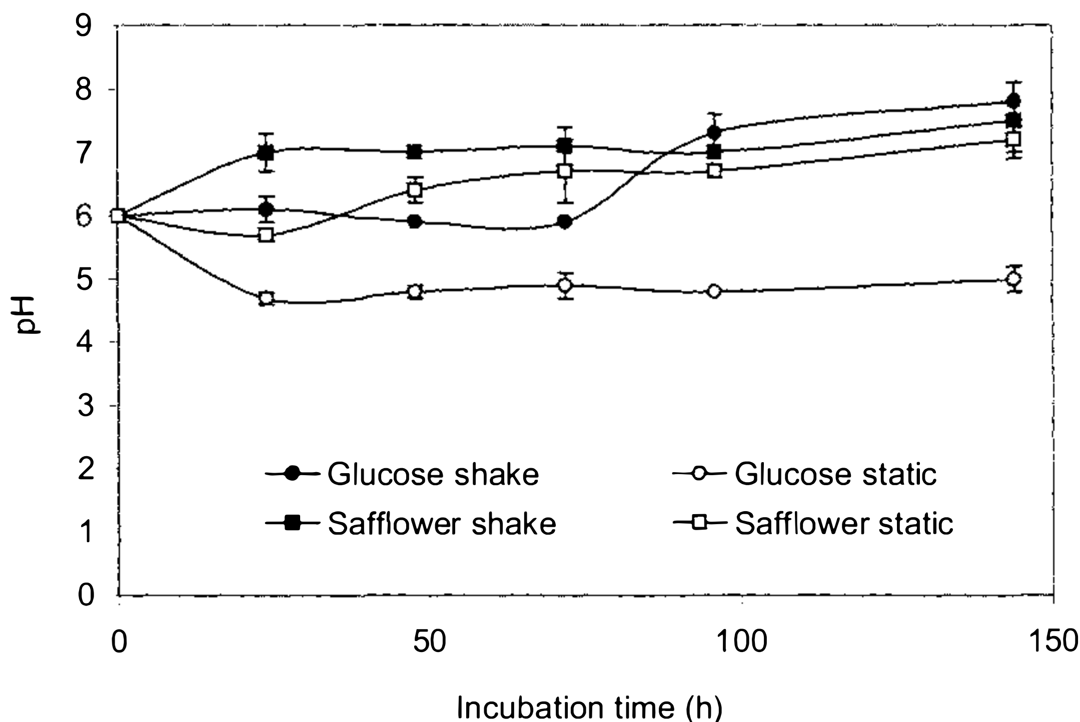


Figure 4.11 Changes in pH of *C. kefyr* shake and static cultures grown in glucose medium and safflower oil medium over 148 h of incubation at 30°C. The error bars represent standard deviations between two independent fermentations.

The initial pH of both the glucose medium and the safflower oil medium was adjusted to 6.0. The pH of *C. kefyr* shake cultures in glucose medium was unchanged in the growth phase (72 h) and then increased to 7.8 at 144 h, as the dry cell weights dropped. The pH of the static cultures in glucose medium was below pH 6.0 (pH 4.7–5) between 24 and 144 h. The pH of the *C. kefyr* cultures in safflower oil medium increased throughout, reaching 7.5 and 7.2 in the shake and static cultures respectively at 144 h.

4.3.3.2 Lipase activity of *C. kefyr*

Lipase activity was absent in the intracellular, washed-cell and extracellular fractions of *C. kefyr* cultures under all treatments tested (in glucose medium and in safflower oil medium under shake and static conditions).

4.3.3.3 Proteinase activity of *C. kefyr*

Proteinase activities detected in the different fractions of the *C. kefyr* shake cultures in glucose medium are given in Table 4.3. The highest activity was recorded at 96 h.

Table 4.3 Proteinase activities in different cell fractions of *C. kefyr* shake cultures grown in glucose medium at 30°C. The values are mean ± SD from two independent fermentations

Cell fractions	24 h	48 h	96 h	144 h
Extracellular (extracellular proteinase) (RFU/min/mL supernatant)	nd*	12 ± 5	74 ± 11	63 ± 13
Washed-cell (washed-cell proteinase) (RFU/min/mg dry cell weight)	nd	nd	nd	nd
Intracellular (intracellular proteinase) (RFU/min/mg dry cell weight)	nd	nd	16 ± 4	nd

* = Not detectable.

C. kefyr static cultures in glucose medium did not show any proteinase activity. The assays of proteinase activity in the different fractions of the cultures grown in safflower oil medium showed very low or no activity. As there was a large variation in duplicates for these measurements, the data are not shown. The cause of the large assay error for these samples is not known.

4.3.3.4 Production of volatile compounds by *C. kefyr*

A range of alcohols and esters were detected in *C. kefyr* cultures in glucose medium under shake or static conditions. Table 4.4 tabulates the relative peak areas of volatile compounds detected in the shake culture in glucose medium. The amounts of the volatile compounds, as indicated by relative peak areas, were lower under static

conditions than under shake conditions in both media (Appendix 4.6). Ethanol, esters and acetic acid were absent in safflower oil medium (Appendix 4.6).

Table 4.4 Volatile compounds detected in *C. kefyr* shake culture grown in glucose medium at 30°C at different points of incubation

Volatile compounds	Relative peak area (x 10^6)		
	24 h	48 h	72 h
Alcohols			
Ethanol	37.3	16.7	nd*
2-Methyl propanol	3.3	4.6	2.0
2-Methyl butanol and 3-Methyl butanol	144.0	146.0	137.9
Phenyl ethyl alcohol	50.7	52.4	51.2
Esters			
Ethyl acetate	41.3	29.9	7.8
1-Hydroxy-3-methyl butylacetate	24.8	28.6	3.5
1-Hydroxy-3-methyl butylpropanoate	3.4	4.2	1.2
2-Phenyl ethyl acetate	25.8	21.8	12.3
2-Phenyl ethyl propionate	5.9	5.7	nd
Acid			
Acetic acid	1.1	1.0	nd

* = Not detectable.

4.4 Discussion

4.4.1 Growth

The two culture types observed for *G. candidum* – a homogeneous dispersion in shake cultures and a white layer of mycelium in static cultures – can be explained by dimorphism (Jollivet *et al.*, 1994). Yeast morphology is reversibly affected by dissolved oxygen: low aeration causes the transition from single cells to hyphae (Cruz *et al.*, 2000). Although this conclusion was reported for *Debaryomyces hansenii*, it can be

extrapolated to *G. candidum*. This kind of transition from single cell to hyphae was not observed for *Y. lipolytica* or *C. kefyr*. The biomass production by *G. candidum* in the present study was much greater than has been reported previously by Amrane and Prigent (1999): 6.1 mg dry cell weight/mL culture compared with 1.5 mg dry cell weight/mL culture. The reason could be vigorous aeration in the current study, with shaking at 200 rev/min, compared with a low aeration rate in the study of Amrane and Prigent (1999). The maximum growth rate of *Y. lipolytica* in shake cultures was about 5 times higher than in static cultures in either glucose medium or safflower oil medium, which suggests that *Y. lipolytica* needs oxygen for faster growth. The maximum dry cell weights of *G. candidum*, *Y. lipolytica* and *C. kefyr* cultures were 6.1, 6.2 and 3.9 mg/mL respectively in glucose medium under shake conditions. The change in biomass accumulation as a result of substitution of the glucose with safflower oil was different for these three yeasts. *G. candidum* attained 80% more dry cell weight in safflower oil medium than in glucose medium (shake cultures). In contrast, *Y. lipolytica* and *C. kefyr* attained 29% and 62% less dry cell weight respectively in safflower oil medium than in glucose medium (shake cultures). Thus, among the yeasts studied, the capacity to utilise oil for growth was highest for *G. candidum*.

The decrease in pH in the shake cultures of *G. candidum* and *Y. lipolytica* in glucose medium during the first 24 h may have been due to the formation of acetic acid, which was detected by GC–MS in the 24 h cultures (Tables 4.1 and 4.2) or due to non-volatile acids, not detected by GC–MS. The acetic acid disappeared by 48 h in these cultures. The drop in the pH values during the first 96 h of incubation in the static cultures of *G. candidum* and *Y. lipolytica* grown in glucose medium could be related to the presence of acetic acid in those cultures for a longer period (1×10^6 and 3.2×10^6 relative peak area for *G. candidum* and *Y. lipolytica* respectively at 72 h). Unlike the cultures in glucose medium, the cultures in safflower oil medium did not show a pH drop in the early stage of growth. The absence of acetic acid in the headspace analysis might explain this. Acetic acid is probably a fermentation product of glucose (Roostita and Fleet, 1996A). The pH of the *C. kefyr* static cultures in glucose medium was always lower than the pH of the shake cultures in the same medium. This is not explained by the volatile acids detected by GC–MS and is probably due to the production of non-volatile acids. Detection of the non volatile acids in the yeast cultures could be carried out in future studies. The increase in pH in later stages in all cultures could have been due to

liberation of ammonia from the deamination of amino acids. Although the headspace analysis used in this study did not detect ammonia, a range of amino acid breakdown products, including 3-methyl butanol, 2-methyl butanol, 2-methyl propanol, 3-methyl butanal, 2-methyl butanal and 2-methyl propanal, were detected (Tables 4.1, 4.2 and 4.4).

4.4.2 Lipase activity

Lipase activity was minimal in the *G. candidum* shake cultures in glucose medium. Replacing the glucose with safflower oil increased the lipase activity by 5 times, at 24 h. It is evident that safflower oil, which is a substrate of lipase, does have an induction effect on lipase activity. However, literature reports regarding the induction of lipase activity are contradictory. Baillargeon *et al.* (1989) found stimulation of lipase activity by oil in *G. candidum.* Shimada *et al.* (1992) demonstrated induction of lipase production by long-chain fatty acids. They postulated that the fatty acids, as a metabolic product of their glyceryl esters, stimulate the expression of the *G. candidum* lipase gene. Conversely, Chander and Klostermeyer (1983) reported a reduction in lipase activity by oils. The extracellular lipase activity in the *G. candidum* shake cultures in safflower oil medium decreased by 69% between 24 and 48 h. The reason for the reduction in the lipase activity could have been the impact of the proteinase activity of the microorganism (Pereira-Meirelles *et al.*, 1997). In the *G. candidum* shake culture in safflower oil medium, the proteinase activity started to increase after 48 h (Figure 4.6), and the existence of the lipase in extracellular liquid and of the proteinase inside the cell makes it difficult to explain how the proteinase would attack the lipase. However, the existence of considerable lipase activity even after 72 h in the static cultures, which had either a very low level or no detectable proteinase activity, supports the 'destruction of lipase enzyme protein by the proteinase' theory. *G. candidum* static cultures grown in glucose medium showed distinct lipase activity only after 72 h of production, which suggests that glucose may have a repressive effect on the lipase activity and that lipase activity is expressed only after the glucose has been exhausted. When both safflower oil and glucose were present in the medium, lipase activity was found only after 72 h, compared with 24 h for the oil-only medium (shake cultures; data not shown). This also supports the repressive activity of glucose. Baillargeon *et al.* (1989) also reported

inhibition of lipase production by glucose, whereas Tahoun *et al.* (1982) reported an increase in extracellular lipase activity by glucose, in *G. candidum.*

Y. lipolytica showed the highest lipase activity among the three yeasts studied. The lipase activity of *Y. lipolytica* was highest in the late stationary phase under all conditions studied. The delay in lipase expression cannot be explained by glucose repression (Pereira-Meirelles *et al*., 1997), as the time for the highest lipase activity was independent of the glucose in the medium.

4.4.3 Proteinase activity

Intracellular proteinase activity was found in *G. candidum.* Reports regarding the proteinase activity of *G. candidum* are contradictory. Baillargeon and McCarthy (1991) could not detect any proteinase activity, whereas Chen and Ledford (1972) found *G. candidum* to be proteolytic on the α_s and β fractions of casein. Gueguen and Lenoir (1976) mentioned intracellular as well as extracellular proteolytic systems of *G. candidum*. In the present study, only very weak proteinase activity was detected in the extracellular fraction after 96 h of culture growth and that was less than 5% of the total proteinase activity. The absence of any whole protein in the medium could have been a reason.

No proteinase activity was found in the *Y. lipolytica* culture used in the present study. This is contradictory to literature reports (Kalle *et al*., 1976; Guerzoni *et al*., 1996). Suzzi *et al.* (2001) reported a marked increase in proteolytic activity in *Y. lipolytica* after 8 days of incubation. In the present study, proteolytic activity was measured up to 9 days. Thus, the possibility of activation (or induction) of proteinase in the later stages of the culture is also very remote. The absence of proteinase activity in glucose medium and in safflower oil medium could have been due to the absence of whole protein. Another possibility is that the present strain could be non-proteolytic. Strain-dependent proteolytic activity was shown by van den Tempel and Jakobsen (2000).

4.4.4 Volatile flavour compounds

Ethanol was present in cultures of all three yeasts grown in glucose medium. Ethanol can be produced by glucose fermentation (Barnett *et al.*, 1990), which explains the absence of ethanol in the cultures grown in safflower oil medium (no glucose). Two primary alcohols, 3-methyl butanol and 2-methyl butanol, were identified by GC–MS in relatively large quantity and constituted a major part of the total volatile compounds in the cultures of all three yeasts under all conditions. Another alcohol, 2-methyl propanol, was present in all shake cultures grown in glucose medium. Jollivet *et al.* (1994) reported that 2-methyl propanol and 3-methyl butanol constituted 50–85% of the total volatile compounds of different strains of *G. candidum* in a model milk medium. They reported these alcohols to be products of amino acid catabolism. The alcohols 3-methyl butanol, 2-methyl butanol and 2-methyl propanol could be produced from leucine, isoleucine and valine respectively (Yvon and Rijnen, 2001). Phenyl ethyl alcohol (PE) was detected in the cultures of all three yeasts under all conditions tested. With its pleasant rose-scented aroma (Kubickova and Grosch, 1997), PE is known to contribute to the aromatic character of soft cheeses (Adda *et al.*, 1978). The possible precursor for this aromatic alcohol is L-phenylalanine (Lee and Richard, 1984). Kawai *et al.* (1998) identified PE in a culture of one strain of *G. candidum*, although Lee and Richard (1984) reported that *G. candidum* was not capable of producing PE. Kawai *et al.* (1998) reported an apple-like aroma in *G. candidum* culture that contained detectable PE. In the present study, floral/fruity aromas were perceived in the *G. candidam* cultures at 48 h and in the *C. kefyr* cultures throughout the 72 h of incubation, when these yeasts were grown in glucose medium with 200 rev/min. That could be associated with the detection of the higher relative peak areas of PE ($> 30 \times 10^6$).

Esters were present only in the yeast cultures grown in glucose medium. The presence of ethanol only in the cultures grown in glucose medium could be a possible reason for the absence of ethyl esters in the cultures grown in safflower oil medium.

Aldehydes, such as 2-methyl propanal, 2-methyl butanal and 3-methyl butanal, detected in the *G. candidum* shake culture (in glucose medium) could be the products of amino acid catabolism. Catabolism of leucine and isoleucine could result in the formation of 3-methyl butanal and 2-methyl butanal respectively (Smit *et al.*, 2000). In some cheese

types, 3-methyl butanal was found to be a key flavour component, imparting sweet and caramel flavour (Neeter *et al.*, 1996).

2-Methyl propanoic acid and 3-methyl butanoic acid, detected in *G. candidum* shake cultures grown in glucose medium, were also reported in *G. candidum* cultures in a model milk medium (Jollivet *et al.*, 1994).

4.5 Conclusions

The findings of the present study demonstrate the diverse metabolic capacity of yeasts, which may impact on their application as flavour adjuncts in cheese. *G. candidum* has both lipase and proteinase activity. *Y. lipolytica* has lipase activity but no detectable proteinase activity. *C. kefyr* has no detectable lipase activity and very weak proteinase activity. All the yeasts studied produced volatile flavour compounds, including alcohols, esters, aldehydes and acids. *C. kefyr* produced higher amounts of esters and an aromatic alcohol (PE) than the other two yeasts. The amounts of enzyme activity as well as the volatile compounds produced were influenced by the growth conditions. Thus, when using these yeast cultures as flavour adjuncts in cheese, the conditions of the culture preparation could be important. Also, the indications regarding the locations of the yeast enzymes could be useful in determining whether the yeast cultures will be added to milk or curd during cheese manufacture.

Two of the three yeasts studied, *G. candidum* and *Y. lipolytica*, were selected for cheese production trials (Chapters 5 and 6) because of their active hydrolytic enzyme(s). The third yeast, *C. kefyr*, was selected for further study of the production of aromatic volatile compounds in laboratory media (Chapter 7) because of its capacity to produce PE.

Chapter 5. Yeasts as adjunct cultures in Cheddar cheese

5.1 Introduction

The manufacture and the ripening of curd to produce consumer or ingredient cheese products involve two groups of microorganisms: starter cultures and secondary microbial flora (Crow *et al.*, 2002). The starter cultures are involved primarily in acid development during curd manufacture whereas the secondary flora is more important in curd ripening (Beresford *et al.*, 2001). The secondary flora can be either adventitious flora [mainly the non-starter lactic acid bacteria (NSLAB)] or deliberately added microbial cultures. The term adjunct refers to the secondary flora that is deliberately added to the cheese milk to contribute to the development of cheese flavour and functionality following curd formation. As adjuncts are generally added at low levels (< 0.01% of cheese milk), they do not influence acid development (Crow *et al.*, 2002). Adjunct cultures can be aerobic or anaerobic. Traditional aerobic adjuncts (*Penicillium*, *Geotrichum*, *Arthrobactor*, *Brevibacterium* and *Micrococcus*) are important to the flavour of Brie, Camembert, Blue-veined and smear-ripened cheeses, in which aerobic ripening conditions are present. Traditional anaerobic adjuncts include *Leuconostoc* and *Propionibacterium* in Gouda and Swiss-type cheeses respectively, which are ripened anaerobically (Crow *et al.*, 2002). Market demands for cheese with diversified flavour characteristics and functionality have prompted researchers to investigate options for new adjunct cultures.

Yeasts are known for their hydrolytic impact on fat and protein as well as for their capacity for producing flavour compounds. Several research groups, as discussed in Section 4.1, have studied the metabolic potential of yeasts, especially *Geotrichum candidum* and *Yarrowia lipolytica*, in laboratory media, milk, cream and cheese slurry. Yeasts were found to possess lipolytic, proteolytic, peptidolytic and amino acid degradation capabilities. Yeasts are frequently present as adventitious microorganisms in almost all types of cheese (Fleet, 1990). Yeasts are traditionally used as part of the surface microflora in surface-ripened cheeses. There is a scarcity of published information on the use of yeasts as anaerobic adjuncts in cheese such as Cheddar. Guerzoni *et al.* (1996) reported significant acceleration of flavour and texture

development in Cheddar cheese using a *Y. lipolytica* adjunct culture. Ferreira and Viljoen (2003) produced a good strong-flavoured Cheddar cheese using *Y. lipolytica* and *Debaryomyces hansenii* as adjunct cultures. Deiana *et al.* (1984) found faster proteolysis, higher levels of free fatty acids and a lower level of acetic acid in another anaerobically ripened cheese, Pecarino Romano, when *D. hansenii* was used as an adjunct culture.

Many of the dairy yeasts are not expected to grow inside an anaerobically ripened cheese, as oxygen is required for growth. This is supported by previous work (Crow *et al.*, 2002; Ferreira and Viljoen, 2003). Thus, it was hypothesised that, without growth inside the cheese, the ripening potential of a yeast adjunct will be solely determined by the inoculum culture; the conditions of the culture preparation will therefore be important. For the same reason, the quantity of yeast adjunct inoculum required in an anaerobically ripened cheese will be higher than the quantity of an anaerobic microbial adjunct that will grow in the ripening cheese.

The *G. candidum* strain used in the present study showed lipase and proteinase activity, whereas the *Y. lipolytica* strain showed only lipase activity. Both strains also had the capacity to produce aroma compounds in laboratory media (Chapter 4). The enzyme activities and the production of aroma compounds were influenced by the growth conditions (Chapter 4). The objective of the work described in this chapter was to study the effect of *G. candidum* and *Y. lipolytica* cultures as anaerobic adjuncts on Cheddar cheese, including whether the conditions of culture preparation for the yeast adjunct cultures and the stage of yeast adjunct culture addition had any effect on cheese ripening. The cheeses were manufactured once and all the analyses were performed in duplicate. The averages of the two analytical results are presented.

5.2 Materials and methods

5.2.1 Cheese manufacture

Materials

1. Milk (pH 6.61 and composition: fat 4.32%, protein 3.34%, lactose 5.52%) was obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand.
2. Starter cultures – mesophilic starters A (made up of four defined strains of *Lactococcus lactis* ssp. *cremoris*) and B (made up of three defined strains of *Lactococcus lactis* ssp. *cremoris*) were obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, and were grown for 16 h at 30°C in 10% w/v heat-treated (95°C for 90 min) reconstituted skim milk. The viable cell count in the starter culture is 10^8 – 10^9 cfu/mL (personal communication with Howard Heap, Fonterra Co-operative Group, Palmerston North).
3. Yeast adjuncts – the growth conditions of *G. candidum* (B9001) and *Y. lipolytica* (B9014) (obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand) for use in different vats of cheese are presented in Tables 5.1 and 5.2 respectively.
4. Rennet – Australian double strength rennet (Dairy Meats NZ Ltd, CPO Box 2240, Shortland Street, Auckland, New Zealand) was used.
5. Salt was obtained from Dominion Salt, Totara Road, Mt. Maunganui, New Zealand.

Table 5.1 Culture conditions of *G. candidum* adjunct cultures and the manufacturing steps in which the adjunct cultures were added

Vat	Code	Quantity of *G. candidum* culture added (mL)	Culture condition	*G. candidum* added to
1 (Control)	G1	0	NA	NA
2	G2	100	24 h shake culture grown in safflower oil medium	Cheese milk (10 L)
3	G3	100	24 h shake culture grown in safflower oil medium	Cheese curd (made from 10 L milk)
4	G4	100	72 h shake culture grown in safflower oil medium	Cheese milk (10 L)

Table 5.2 Culture conditions of *Y. lipolytica* adjunct cultures and the manufacturing steps in which the adjunct cultures were added

Vat	Code	Quantity of *Y. lipolytica* culture added (mL)	Culture condition	*Y. lipolytica* added to
1 (Control)	Y1	0	NA	NA
2	Y2	100	96 h static culture grown in glucose medium	Cheese milk (10 L)
3	Y3	100	192 h static culture grown in glucose medium	Cheese milk (10 L)
4	Y4	100	192 h static culture grown in safflower oil medium	Cheese milk (10 L)

Method

Cheese was manufactured in small vats containing 10 L of cheese milk at the pilot plant of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand. A standard protocol for Cheddar cheese was followed (Kosikowski, 1982). Pasteurised milk was used. Rennet at 1.14 mL per vat and mesophilic starter culture at 250 mL per vat were added to the cheese milk. Starter cultures A and B were used for *G. candidum* and *Y. lipolytica* cheeses respectively (different starter cultures were used for the two cheese trials depending on their availability). Yeast adjunct (100 mL per vat) was added to the cheese milk or to the cheese curd of the experimental cheeses (Tables 5.1 and 5.2). For the control cheese, 100 mL of sterilised glucose medium (recipe described in Section 4.2.1) was added to the cheese milk. The set (rennet coagulation) temperature was 32°C. The set time was 40 min. Cutting was carried out with two rotating knives per vat. The cooking temperature was 37°C. The whey was drained at pH 6.2–6.3 for the different vats. The curd was transferred into covered plastic buckets (separate buckets for different vats) and incubated at 37°C. Cheddaring, milling and salting were carried out in the plastic buckets. Salt was added at 22 g/kg curd after the curd pH had dropped to 5.2–5.3. After pressing (14 psi) overnight, each block was cut into five portions of approximately 200 g and vacuum packed separately in cheese bags (barrier bags, obtained from Sealed Air Corporation (NZ), Private Bag 50901, Porirua, New Zealand). To ensure that anaerobic conditions were maintained, each piece of cheese was vacuum

packed in three layers of cheese bag. The cheese blocks were ripened at 13°C for up to 6 months.

5.2.2 Estimation of free fatty acids (FFAs) in cheese

Materials

The reagents n-heptane, diethyl ether (DEE), H_2SO_4, Na_2SO_4, chloroform and isopropyl alcohol (IPA) were supplied by BDH Laboratory Supplies, Poole, BH15 1TD, England. Tridecanoic acid was supplied by Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA, and formic acid was purchased from RdH Laborchemikalien GmbH & Co., KG.D – 30926, Seelze, Germany. All chemicals used were of analytical grade.

Method

The estimation of FFAs involved three stages.

Stage 1. Cheese extraction: 1–2 g of grated cheese (the quantity of sample varied with the concentration of FFAs) was taken into a Nalgene® solvent-resistant SS34 centrifuge tube. Heptane/DEE, 1:1 (10 mL), 6 g of oven-dried Na_2SO_4, 0.3 mL of 5 M H_2SO_4 and 100–400 μL of tridecanoic acid (C13) internal standard solution (2.0 mg/mL) were added. The quantity of internal standard depended on the amounts of FFAs in the samples. The tube was securely capped, shaken well and centrifuged for 5 min at 2000 *g*. The supernatant was decanted into a Kimax® tube and the pellet was washed twice with 3.0 mL of heptane/DEE (1:1). All supernatants were combined.

Stage 2. Solid phase extraction: A 200 mg aminopropyl SPE cartridge (International Sorbent Technology Ltd, Dyffryn Business Park, Hengoed, Mid Glamorgan, UK) was conditioned with 9.0 mL of heptane. Then heptane/DEE cheese extract (2–13 mL) was loaded on to the cartridge, the volume depending on the expected concentrations of FFAs. Neutral lipids were eluted with 3.0 mL of chloroform/IPA (2:1) and the eluate was discarded. FFAs were eluted with a total of 4.0 mL of 6% formic acid in heptane/DEE (2:1). Approximately 1 mL of this eluate was transferred to a chromatography vial. The operations in this step were carried out sequentially by an

automated solid phase extraction system (Gilson, 3000 W. Beltline Hwy, P.O. Box 620027, Middleton, WI 562-0027, USA).

Stage 3. Gas liquid chromatography (GC) for FFAs: FFA analysis was performed on a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with a split/splitless injector, a flame ionisation detector (FID) and an AOC-20i auto-injector. The GC column was a 30 m x 0.53 mm internal diameter EC-1000 (1.2 μm film thickness) column (Alltech Associates Inc., 2051 Waukegan Road, Deerfield, IL 60015, USA). The carrier gas was helium at a flow rate of 6 mL/min. The initial oven temperature was held at 100°C for 1.5 min, followed by a thermal gradient to 245°C (10°C/min). The holding time at 245°C was 24 min. The FFA peaks were identified by retention time and quantified using data-handling software (Shimadzu Class-VP, Shimadzu Corporation) and tridecanoic acid (C13) as an internal standard.

5.2.3 Estimation of total fatty acids (free plus esterified) in cheese

Materials

The reagents sodium methoxide, methanol, boron trifluoride, hexane, di-potassium hydrogen phosphate and potassium hydroxide were obtained from BDH Laboratory Supplies, Poole, BH15 1TD, England. The internal standards tridecanoic acid (C13) and tridecanoin (triglyceride of C13) were obtained from Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA. All chemicals were of analytical grade.

Method

Sample preparation: Extraction of FFAs and lipids from cheese was carried out according to the method described in Section 5.2.2, omitting the solid phase extraction step. The total lipids (triglyceride/FFA mixture) were mixed with 1 mL of sodium methoxide reagent (0.5 M sodium methoxide in methanol as a 20% solution in ether/isooctane), held for approximately 5 min at 40°C and then cooled in a water bath at room temperature. Boron trifluoride (14% in methanol) reagent (1 mL) was added, mixed and held for 10 min at room temperature. Hexane (10 mL) was added and the neutralising solution [5% w/v di-potassium hydrogen phosphate (K_2HPO_4) and 1.5% w/v potassium hydroxide (KOH) in Milli-Q water] was added after 2 min, mixed

thoroughly and centrifuged at 175 *g* at room temperature for 5 min. The upper layer (1 mL) was transferred to a 2 mL autosampler vial and capped immediately.

Gas liquid chromatography: Fatty acid analyses were performed on a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with an on-column injector, an FID and an AOC-20i auto-injector. The GC column was a 50 m x 0.22 mm internal diameter BPX-70 (0.25 μm film thickness) column (SGE International Pty Ltd, www.sge.com) with a 1 m x 0.53 mm internal diameter deactivated pre-column. The carrier gas was hydrogen at a flow rate of 1.2 mL/min. The initial oven temperature was 50°C for 1.5 min, followed by a gradient to 220°C (3°C/min). The holding time at 220°C was 10 min. The injection volume was 0.2 μL. The on-column injector was held at 80°C for 0.1 min, and then programmed at the maximum rate to 220°C and held for 2 min. Quantification of fatty acid peaks was carried out using data-handling software (Shimadzu Class-VP, Shimadzu Corporation) and tridecanoic acid (C13) and tridecanoin (triglyceride of C13) as internal standards.

5.2.4 Estimation of peptides in cheese

Estimation of peptides by size-exclusion high performance liquid chromatography (SE-HPLC) was used to study the protein breakdown in cheese.

Materials

1. One litre of alkaline urea buffer (pH 8.4) was made by dissolving 10.8 g tris (hydroxymethyl) methylamine, 360 g urea, 0.92 g ethylene diamine-tetra-acetic acid (EDTA) (BDH Laboratory Supplies, Poole, BH15 1TD, England) and 5.5 g boric acid (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA) in milli-Q water. The pH was adjusted to 8.4 with 1 M HCl (BDH).
2. Acetonitrile (CH_3CN) (BDH Laboratory Supplies, Poole, BH15 1TD, England).
3. Trifluoroacetic acid (TFA) (BDH Laboratory Supplies, Poole, BH15 1TD, England).

All chemicals used were of analytical grade.

Method

Proteins and peptides were extracted from the cheese samples using an alkaline urea extraction buffer (pH 8.4). Grated cheese sample (0.03 g) was added to 15 mL of extraction buffer and incubated at 40°C for 1 h. This mixture was then homogenised with an Ultra-Turrax T25 homogeniser (Janke and Kunkel Ika-Labor-Technik, Staufen, Germany) for 20 s at high speed and centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was decanted and stored at −20°C for HPLC analysis.

The HPLC equipment was a Hewlett Packard 1050 module fitted with an auto-injector. The HPLC system was equipped with a diode array detector and was operated by HP Chemstation software (Hewlett-Packard Co., Palo Alto, CA, USA). SE-HPLC was carried out by injecting 8 μL of extracted sample on to a TSKgel G2000 SW_{xl} size-exclusion column (Tosoh Corporation, Tokyo, Japan). The column was held at a constant 25°C. The sample was fractioned from the column with 36% (v/v) CH_3CN/0.1% (v/v) TFA at a flow rate of 0.5 mL/min for 35 min (isocratic elution). The absorbance of the eluate was monitored continuously at 214 and 280 nm. The following series of standard proteins of known molecular mass was analysed using the SE-HPLC column: glyceraldehyde-3-phosphate dehydrogenase (36 000 Da), carbonic anhydrase (29 000 Da), trypsinogen (24 000 Da), egg white lysozyme (14 300 Da), aprotinin (6500 Da), insulin B-chain (3400 Da), bacitracin(1420 Da), glutathione oxidized (612 Da) and phenylalanine (165 Da). A calibration curve relating retention time and molecular mass was drawn (Appendix 5.1).

5.2.5 Estimation of volatile compounds

Six grams of grated cheese was used and headspace analysis was performed according to the method described in Section 4.2.3 using the same equipment.

5.2.6 Composition analysis

Compositional analyses were carried out using standard methods. The references of the methods are listed below.

Cheese pH: pH values of the cheese samples were determined using the method described in New Zealand Technical Manual 3, NZTM 3.2.8.

Cheese moisture: Moisture was determined by drying the grated cheese samples at 105°C for 16 h (NZTM 3.12.6).

Cheese non-protein-nitrogen (NPN): The method used for NPN was based on NZTM 3.15.3 (milk and milk powders). This method was appropriate for cheese as well.

Cheese salt: The salt contents of the cheese samples were determined by auto-titrator (NZTM 3.9.6).

Cheese fat: The fat content of the G1–G4 cheeses was measured using the Schmid-Bondzynski-Ratzlaff gravimetric method (International Dairy Federation Standard 5B: 1986). The fat content of the Y1–Y4 cheeses was measured using the Babcock method (*American Public Health Association Standard Methods for the Examination of Dairy Products*, 16th edn, Section 15.8A).

Cheese calcium: The calcium content of the G1–G4 cheeses was measured by inductively coupled plasma optical emission spectrometry [ICP–OES; NZTM 3.9.21 (draft)]. The calcium content of the Y1–Y4 cheeses was measured by a titration method (Pearce, 1977).

5.3 Results

The cheese composition and the results for lipolytic and proteolytic breakdown and production of volatile compounds in the Cheddar cheeses made with *G. candidum* and *Y. lipolytica* adjunct cultures during ripening are presented in Sections 5.3.1 and 5.3.2 respectively.

5.3.1 Cheddar cheese with *G. candidum* adjunct culture

5.3.1.1 Composition and pH

The pH and the composition of the 1-day-old Cheddar cheeses from the four vats are summarised in Table 5.3.

Table 5.3 pH and composition of 1-day-old Cheddar cheeses with (G2, G3 and G4) and without (G1) *G. candidum* adjunct culture

Cheese	pH	Moisture (% w/w)	NPN (% w/w)	Salt in moisture (% w/w)	Fat (% w/w)	Calcium (mg/kg)
G1	5.29	36.1	0.135	4.6	30.9	7240
G2	5.23	35.1	0.144	4.3	30.8	7670
G3	5.12	37.1	0.089	3.6	30.6	7280
G4	5.33	35.2	0.124	4.9	30.1	7340

The pH values as well as the compositions of the G1 (without yeast adjunct), G2 and G4 (with yeast adjunct) Cheddar cheeses were similar at 1 day after manufacture. Thus, there were no compositional differences between these three cheeses, and the only variable was the yeast adjunct. However, the G3 Cheddar cheese had lower salt in moisture (S/M) content than the other three cheeses. The lower S/M and NPN could have been due to the addition of yeast adjunct culture to the curd compared with its addition to the milk in the other vats. The lower S/M could be another parameter other than addition of yeast adjuncts that was likely to make a difference in the ripening of the

G3 cheese compared with the control cheese (G1). See the proteolysis results (Section 5.3.1.3) for a discussion on whether the low S/M of the G3 cheese had any effect on ripening.

5.3.1.2 Lipolysis

The changes in the total FFA concentrations measured by GC–FID in the G1, G2, G3 and G4 cheeses over the 6 months of ripening are shown in Figure 5.1. The total FFAs refers to those FFAs originating from milk fat and includes fatty acids with an even number of carbon atoms [the predominant fatty acids in milk have a straight carbon chain with an even number of carbon atoms and may be saturated or unsaturated (Fox *et al.*, 2000B)] between C4 and C18:2 (C4, C6, C8, C10, C12, C14, C16, C18, C18:1 and C18:2, as shown in Figure 5.2).

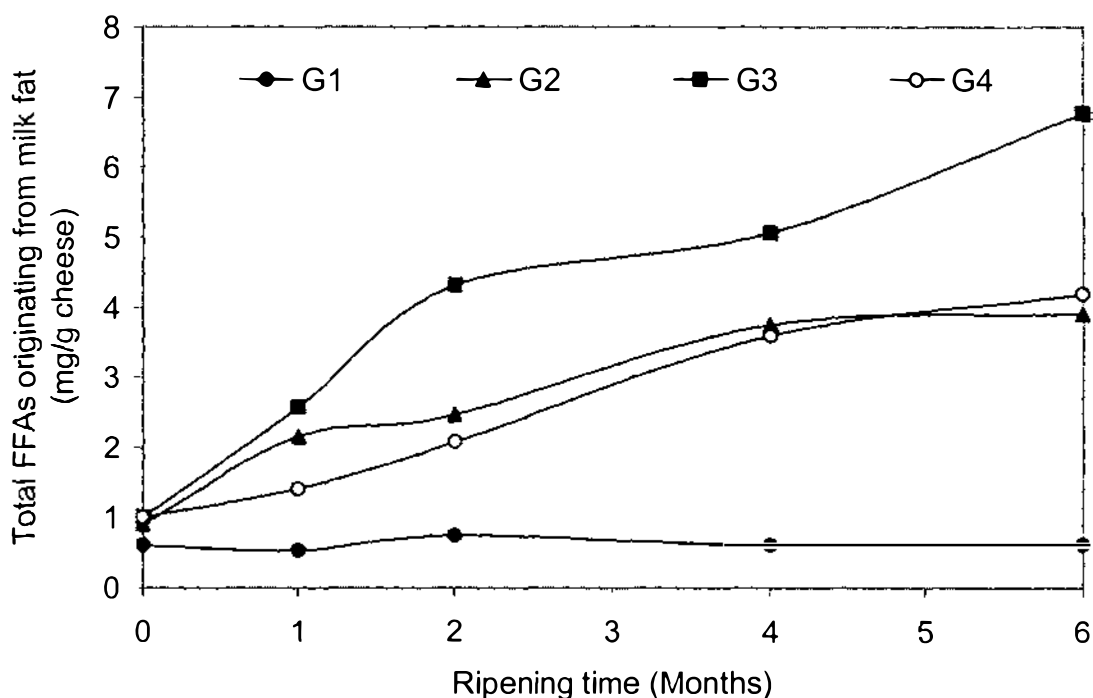


Figure 5.1 Production of total FFAs from four vats of cheeses over 6 months of ripening at 13°C. G1 is the control cheese without any yeast adjunct culture; G2, G3 and G4 are cheeses with *G. candidum* adjunct cultures, grown under different conditions and added at different steps as summarised in Table 5.1.

The total FFA concentrations in the control cheese (G1) were in the range 0.53–0.74 mg/g cheese without any significant change during ripening. The total FFA concentrations in the three cheeses made with *G. candidum* adjunct increased

considerably during ripening. The total FFA concentrations were 3.9, 6.8 and 4.2 mg/g cheese in 6-month-old samples of the G2, G3 and G4 cheeses respectively. The highest FFA concentration was detected in the G3 cheese, which contained a 24 h *G. candidum* shake culture grown in safflower oil medium and added to the cheese curd.

The concentrations of the individual FFAs for all four cheeses were quantified to determine if the *G. candidum* lipase had any selectivity. All the FFAs with an even number of carbon atoms between C4 and C18 were determined. Also, total fatty acids (free plus esterified) in the cheeses were determined. The cheeses made from milk of similar origin had similar total fatty acid profiles irrespective of the addition of yeast adjuncts and the fatty acid profiles were similar to the fatty acid profile of New Zealand bovine milk, reported by Creamer and MacGibbon (1996). Hence, the same values for the total fatty acid (free plus esterified) profile is used for all comparisons in this study (Chapters 5 and 6) and, for convenience of presentation, the profile is called the total fatty acid (free plus esterified) profile of the reference cheese. Total fatty acid (free plus esterified) profile of reference cheese does not imply any particular cheese but does mean the typical fatty acid (free plus esterified) profile of New Zealand bovine milk fat, which is the starting material of the cheese manufactured in the study. The ratio between the long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) total fatty acids (free plus esterified) in the reference cheese was 0.5.

The individual FFAs in the G1, G2, G3 and G4 cheeses after 4 months of ripening are compared with the total fatty acid (free plus esterified) profile of the reference cheese in Figure 5.2. The FFA results at 1, 2 and 6 months (data not shown) followed the same trend. However, the concentrations of all the individual FFAs for all four cheeses at different times (1, 2, 4 and 6 months) are provided in Appendix 5.2A (as mg/g cheese) and Appendix 5.2B (as a % of total FFAs). The FFAs in the cheeses, especially the free linoleic acid (C18:2) at 1 day, were influenced by the FFAs present in the added cultures and thus could be misleading, which is why the FFA results of the samples at 1 day are not included.

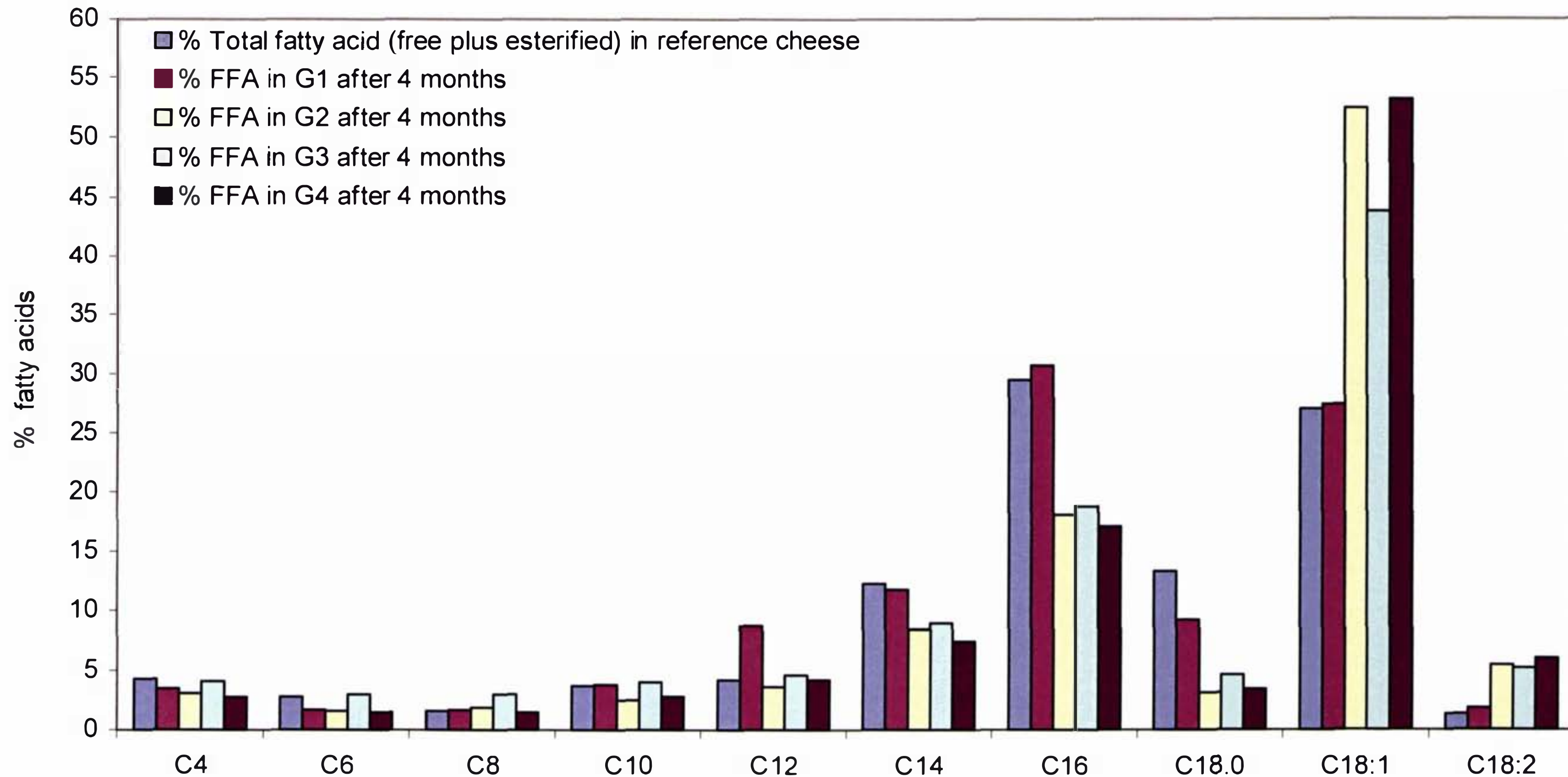


Figure 5.2 Comparison of the proportions of the individual FFAs expressed as a % of the total FFAs originating from milk fat in 4-month-old Cheddar cheeses containing *G. candidum* (G2, G3 and G4) with those of the 4-month-old control cheese (G1), as well as with the total fatty acid (free plus esterified) profile of the reference cheese.

The proportions of the FFAs originating from milk fat in the control cheese (G1) were similar to the proportions of the respective total fatty acids (free plus esterified) in the reference cheese, although there were some differences in C12 and C18. In the cheeses containing *G. candidum* (G2, G3 and G4), the proportions of the short- and medium-chain FFAs (C4 to C12) were similar to those of the respective total fatty acids (free plus esterified) in the reference cheese. This suggests that the *G. candidam* lipase is not selective for the C4 to C12 fatty acids of milk fat. However, the proportions of the long-chain FFAs (C14 to C18:2) in the G2, G3 and G4 cheeses were different from those of the respective total fatty acids (free plus esterified) in the reference cheese. The proportions of the free long-chain saturated fatty acids (C14, C16 and C18) in the G2, G3 and G4 cheeses were less than those of the respective total fatty acids (free plus esterified) in the reference cheese. The long-chain unsaturated FFAs (C18:1 and C18:2) in the G2, G3 and G4 cheeses showed the opposite trend, *i.e.* the proportions were greater than those of the respective total fatty acids (free plus esterified) in the reference cheese. These results indicate a selectivity of *G. candidum* lipase towards liberation of long-chain unsaturated fatty acids.

The G3 cheese had a lower S/M content than the other cheeses, but the FFA profiles were similar to those of the G2 and G4 cheeses, which indicates that the S/M content did not have any significant effect on the lipase specificity of *G. candidum*.

The ratio between the long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) total fatty acids (free plus esterified) in the reference cheese was 0.5. The ratios between the free long-chain unsaturated and saturated fatty acids in the control cheese (G1) were similar and ranged from 0.6 to 0.7. However, the ratios between the long-chain unsaturated and saturated FFAs were 2–5 times higher in the cheeses containing *G. candidum* compared with the ratio of the respective total fatty acids (free plus esterified) in the reference cheese. The ratios between long-chain unsaturated and saturated FFAs in the different cheeses between 1 and 6 months of ripening are presented in Table 5.4. It is notable that the ratios decreased in the later stages of ripening, especially at 6 months. Depletion of the substrate (esterified C18:1 or C18:2) in the later stages of ripening could be a possible explanation.

Table 5.4 Ratio between long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) FFAs in the different cheeses between 1 and 6 months of ripening at 13°C

Cheese	Month 1	Month 2	Month 4	Month 6
G1	0.6	0.7	0.6	0.7
G2	2.3	2.1	2.0	1.0
G3	1.9	1.8	1.5	1.5
G4	2.3	2.5	2.1	1.2

5.3.1.3 Proteolysis

Proteolysis leads to the breakdown of protein into lower molecular weight peptides and amino acids during the ripening of cheese. The molecular weight distribution patterns of proteins and peptides in the cheeses with and without *G. candidum* were determined by the SE-HPLC method to understand the effect of *G. candidum* on the proteolysis in the cheeses. Molecular weight distributions of the proteins and peptides in different cheese samples over the first 2 months of ripening are presented in Table 5.5. The large molecular weight fraction (> 16 000 Da, possibly protein) decreased at a similar rate during ripening for all four cheeses. In contrast, the six lower molecular weight fractions increased during ripening and the pattern was similar for the four cheeses. These data indicate that neither the *G. candidum* adjunct nor the lower S/M of the G3 cheese had an effect on the proteolysis of the cheese.

Table 5.5 Proteins and peptides as proportions of the total peak area in different cheeses (G1, G2, G3 and G4) at three sampling points (1 day, 30 days and 60 days) of ripening at 13°C. MW = molecular weight

MW range (Dalton)	G1			G2			G3			G4		
	1 day	30 days	60 days	1 day	30 days	60 days	1 day	30 days	60 days	1 day	30 days	60 days
> 16 100	79.4	64.1	61.1	80.8	65.0	63.1	79.1	65.0	60.0	81.6	64.3	60.0
16 100–3500	12.5	23.5	26.3	12.4	24.0	28.0	12.3	23.9	27.0	12.4	24.6	27.9
3500–2500	0.8	2.6	2.7	0.7	2.4	2.5	0.9	2.6	2.8	0.7	2.5	2.7
2500–1250	1.0	1.9	2.0	0.8	1.9	1.8	1.1	1.9	2.3	0.8	2.0	2.1
1250–900	0.4	0.8	0.8	0.0	0.8	0.7	0.5	0.7	0.9	0.0	0.8	0.9
900–400	1.3	2.0	2.0	1.0	2.0	1.5	1.4	1.8	2.2	1.0	2.1	2.2
< 400	4.5	5.2	5.1	4.1	4.1	2.5	4.8	4.1	4.6	3.6	3.7	4.3

5.3.1.4 Production of volatile compounds

The volatile compounds produced in the cheeses with (G2, G3 and G4) and without (G1) *G. candidum* adjunct were detected by headspace analysis using gas chromatography–mass spectrometry (GC–MS). The peaks with areas over a million units (10^6) were considered. Cheese samples after 1 day, 1 month, 2 months, 4 months and 6 months were analysed. The results for only the 6-month-old cheeses, in which four groups of volatile compounds were detected, are summarised in Table 5.6. In the 1-day-old cheeses, all the volatile compounds were absent except that acetic acid was present in similar concentration ($2–3 \times 10^6$) in all cheeses. After 2 and 4 months of ripening, all volatile compounds followed the same trend as shown for the 6-month-old cheeses but were present in lower concentrations.

Table 5.6 Volatile compounds produced in the cheeses without (G1) and with (G2, G3 and G4) *G. candidum* adjunct culture after 6 months of ripening at 13°C

Volatile compounds	Relative peak areas (x 10^6)			
	G1	G2	G3	G4
Alcohol				
Ethanol	5.1	4.8	3.7	5.3
Esters				
Ethyl butanoate	5.6	4.1	2.9	3.5
Ethyl hexanoate	1.3	1.2	2.2	1.5
Ketones				
2-Butanone	32.7	25.0	39.3	28.6
3-Methyl-2-butanone	nd*	1.3	1.2	1.3
2-Heptanone	nd	6.5	8.9	8.1
Acids				
Acetic acid	15.2	10.0	15.7	18.8
Butanoic acid	14.5	89.8	139.4	106.0
Hexanoic acid	4.2	44.0	108.6	60.0
Octanoic acid	nd	8.3	26.0	12.5
n-Decanoic acid	nd	nd	3.6	1.4

* = Not detectable.

The concentrations of the esters and ethanol were similar in all cheeses. Two of the three ketones, 3-methyl-2-butanone and 2-heptanone, were present in *G. candidum* cheeses but not in the control cheese, whereas 2-butanone was present in all cheeses. The concentration of acetic acid was similar in all cheeses, whereas the concentrations of fatty acids originating from milk fat were different in the cheeses with and without *G. candidum* adjunct. The pattern was similar to that of the FFAs detected by GC–FID (Figures 5.1 and 5.2). The concentrations of butanoic acid and hexanoic acid were higher in cheeses made with *G. candidum* adjunct than in the control cheese. Octanoic acid was present in the G2, G3 and G4 cheeses and n-decanoic acid was present only in the G3 and G4 cheeses, whereas both these acids were absent in the control cheese (G1). The concentrations of these acids were highest in the G3 cheese.

5.3.2 Cheddar cheese with *Y. lipolytica* adjunct culture

5.3.2.1 Composition and pH

The pH and the composition of cheeses made without (Y1) and with (Y2, Y3 and Y4) *Y. lipolytica* (added to the cheese milk) at 1 day after manufacture are summarised in Table 5.7.

Table 5.7 pH and composition of 1-day-old Cheddar cheeses without (Y1) and with (Y2, Y3 and Y4) *Y. lipolytica* adjunct culture

Cheese	pH	Moisture (% w/w)	NPN (% w/w)	S/M (% w/w)	Fat (% w/w)	Calcium (mM/kg)
Y1	5.26	36.0	0.102	4.7	35	158
Y2	5.19	35.8	0.088	4.1	35	168
Y3	5.27	35.4	0.088	4.7	33	161
Y4	5.27	35.9	0.087	4.5	36	135

All cheeses, with or without *Y. lipolytica* adjunct culture, had similar pH and composition at 1 day after manufacture. Thus, the *Y. lipolytica* adjunct culture was the main variable affecting the ripening of the cheeses.

The cheese blocks were ripened at 13°C for 6 months. Production of FFAs, peptides and volatile compounds was determined at different time points to monitor the ripening of the Cheddar cheeses.

5.3.2 2 Lipolysis

The changes in the total FFA concentrations measured by GC–FID in the Y1, Y2, Y3 and Y4 cheeses over the 6 months of ripening at 13°C are shown in Figure 5.3. The total FFAs refers to those FFAs originating from milk fat and includes fatty acids with an even number of carbon atoms between C4 and C18:2 (C4, C6, C8, C10, C12, C14, C16, C18, C18:1 and C18:2, as shown in Figure 5.4).

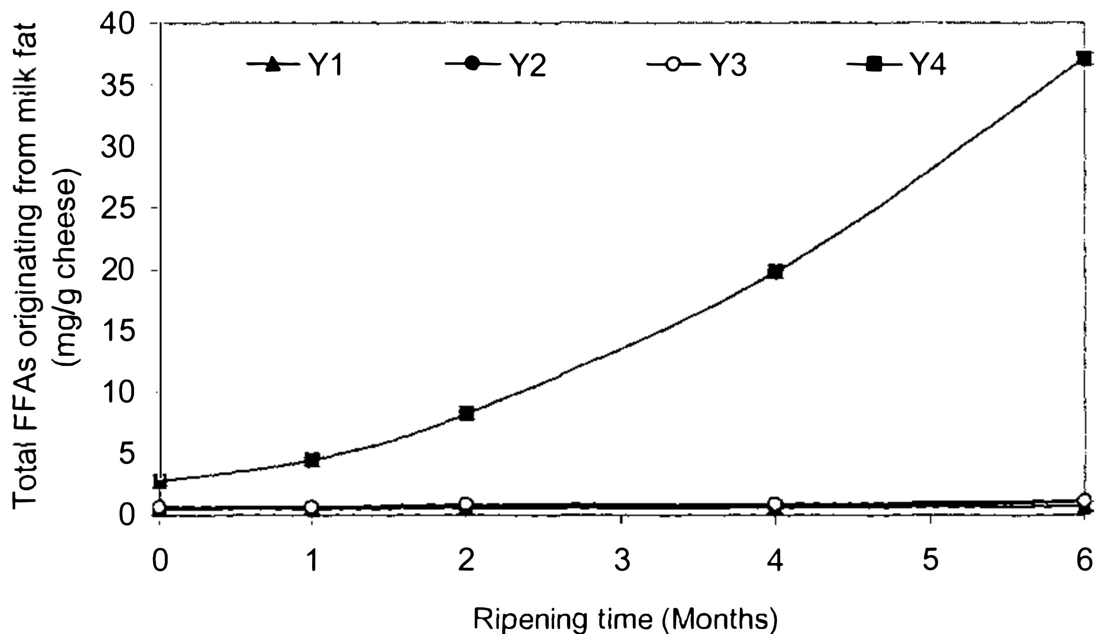


Figure 5.3 Production of total FFAs from four vats of cheeses over 6 months of ripening at 13°C. Y1 is the control cheese without any yeast adjunct culture; Y2, Y3 and Y4 are cheeses with *Y. lipolytica* adjunct cultures, grown under different conditions as summarised in Table 5.2.

The total FFA concentrations in the control cheese (Y1) and two of the three *Y. lipolytica* cheeses (Y2 and Y3) were similar – low and ranged between 0.51 and 1.2

mg/g cheese without any significant changes during ripening. The total FFA concentration in the Y4 cheese was higher than in the other three cheeses throughout the ripening period. In the 6-month-old samples, the total FFA concentration was 31–53-fold higher in the Y4 cheese than in the other three cheeses. After 6 months of ripening, the total FFA concentration in the Y4 cheese was 37.1 mg/g cheese, whereas the total FFA concentrations in the other three cheeses ranged between 0.7 and 1.2 mg/g cheese. Thus, it was found that not only the addition of *Y. lipolytica* culture but also the condition of the culture preparation had an impact on the production of FFAs in the cheese. The static culture of *Y. lipolytica* grown for 192 h in safflower oil medium at 30°C (the adjunct culture used in vat Y4) was capable of producing the highest concentration of FFAs from the milk fat in the cheese. *Y. lipolytica* grown under these conditions also showed the highest level of lipase activity among the conditions tested (Section 4.3.2.2).

The concentrations of the individual FFAs for all four cheeses were analysed throughout the ripening period to determine if the *Y. lipolytica* lipase had any selectivity. All the FFAs with an even number of carbon atoms between C4 and C18:2 were determined and compared with the total fatty acid (free plus esterified) profile of the reference cheese (Section 5.3.1.2). Figure 5.4 shows a comparison between the FFA proportions of the 4-month-old control cheese (Y1) and one of the cheeses containing *Y. lipolytica* (Y4) and the total fatty acid (free plus esterified) proportions in the reference cheese. The FFA results of the 1-, 2- and 6-month-old samples followed a similar pattern. Y4 was the only cheese in which *Y. lipolytica* influenced the lipolysis (Figure 5.3). The FFA concentrations of all four cheeses (Y1, Y2, Y3 and Y4) at different time points during ripening are tabulated in Appendix 5.3A (as mg/g cheese) and Appendix 5.3B (as a % of total FFAs). The FFA results of the 1-day samples are not included for the same reasons as described for the cheeses with *G. candidum* (Section 5.3.1.2). The FFA proportions of the control cheese were similar to those of the respective total fatty acids (free plus esterified) in the reference cheese, except for some differences in C12, C16 and C18 fatty acids. The FFA proportions of the Y4 cheese were distinctly different from those of the respective total fatty acids (free plus esterified) in the reference cheese. The short-chain fatty acids (C4, C6 and C8) were in higher proportions in the free form than in the total (free and esterified) fatty acids. The short-

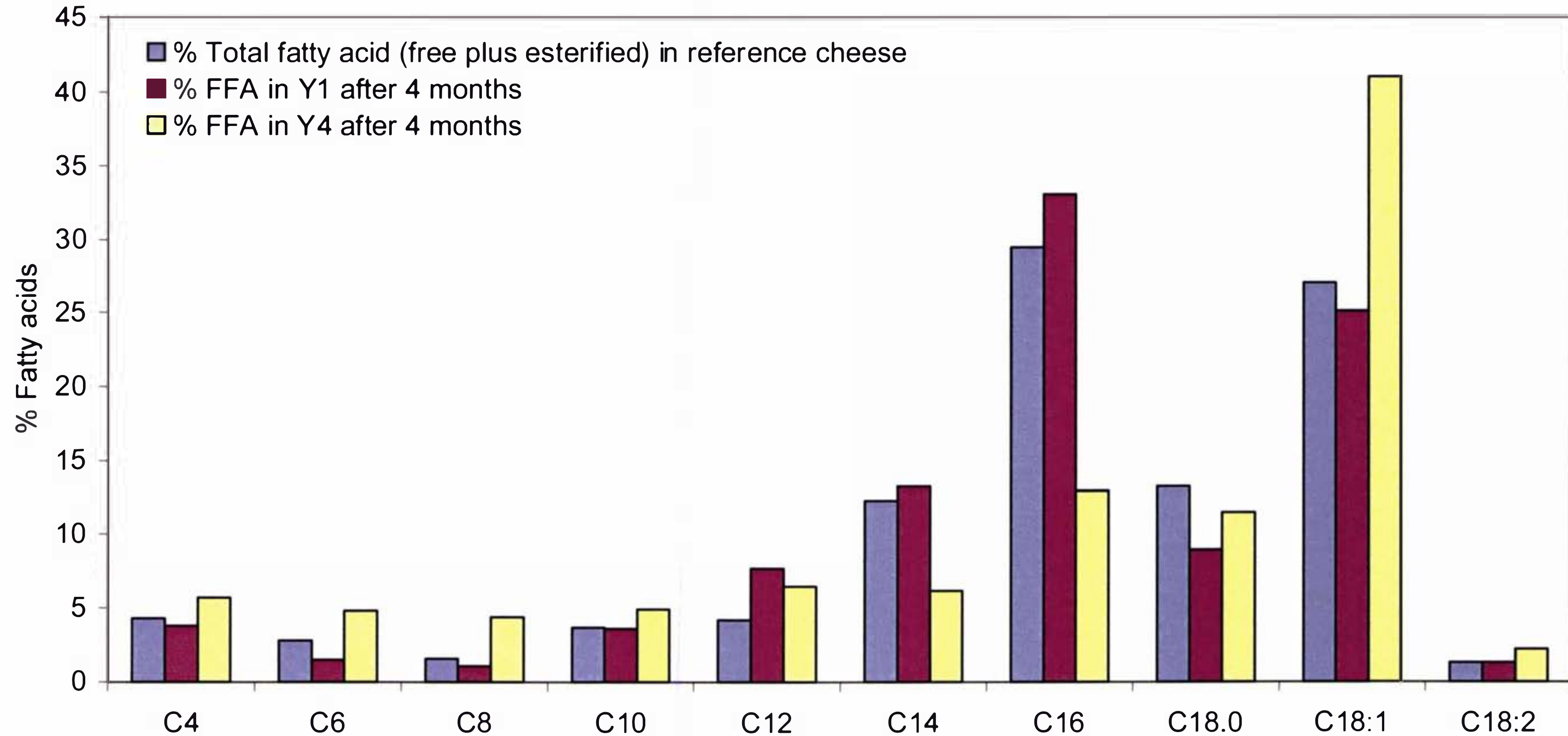


Figure 5.4 Comparison of the proportions of the individual FFAs expressed as a % of the total FFAs originating from milk fat in 4-month-old Cheddar cheese containing *Y. lipolytica* (Y4) with those of the 4-month-old control cheese (Y1), as well as with the total fatty acid (free plus esterified) profile of the reference cheese.

chain fatty acids (C4, C6 and C8) constituted 7.9% of the total fatty acids in the reference cheese, whereas these fatty acids constituted 14.2% of the FFAs in the Y4 cheese. These results indicate that *Y. lipolytica* may have one or more lipase(s) with some specificity towards liberation of the three short-chain fatty acids.

The proportions of two long-chain saturated fatty acids (C14 and C16) were notably less in the total FFAs of the Y4 cheese than those of the total fatty acids (free plus esterified) in the reference cheese. The percentage of the other long-chain saturated fatty acid C18 was only marginally less in the total FFAs of the Y4 cheese than in the total fatty acids (free plus esterified) in the reference cheese. In contrast, the proportion of the long-chain unsaturated fatty acid C18:1 was much higher in the total FFAs (41%) of the Y4 cheese than in the total fatty acids (free plus esterified; 27.1%) in the reference cheese. However, the proportion of C18:2 was only marginally higher in the total FFAs of the Y4 cheese. The ratios between the saturated long-chain (C14, C16 and C18) and unsaturated long-chain (C18:1 and C18:2) fatty acids were 0.5 in the total fatty acids (free and esterified) in the reference cheese as well as in the FFAs of the 4-month-old control cheese (Y1). However, this ratio was more than double (1.1–1.4) in the Y4 cheese over 6 months of ripening. The ratios in the Y2 and Y3 cheeses (0.6–0.7) were only marginally higher than in the control (0.5) as there was no contribution of *Y. lipolytica* to lipolysis in the Y2 and Y3 cheeses.

The results suggest the existence of lipase(s) in *Y. lipolytica* with selectivity towards short-chain (C4, C6 and C8) and long-chain unsaturated (C18:1 and C18:2) fatty acids from the milk fat in the cheese environment.

5.3.2.3 Proteolysis

Proteolysis during ripening of the cheeses with and without *Y. lipolytica* is shown as the molecular weight distribution patterns of proteins and peptides determined by SE-HPLC. The molecular weight distributions of the proteins and peptides in the different cheese samples over the first 2 months of ripening at 13°C are presented in Table 5.8. The large molecular weight fraction (>16 000 Da, possibly protein) decreased at a similar rate during ripening for all four cheeses. In contrast, the lower molecular weight fractions increased at similar rates for all four cheeses, although the < 400 Da fraction

did not show any particular pattern. These data indicate that the *Y. lipolytica* adjunct did not affect the proteolysis in the cheese.

5.3.2.4 Production of volatile compounds

The volatile compounds produced in the control cheese without *Y. lipolytica* adjunct (Y1) and in the cheeses with *Y. lipolytica* adjunct (Y2, Y3 and Y4) were detected by headspace analysis using GC–MS. Samples were analysed after 1 day, 1 month, 2 months, 4 months and 6 months. The volatile compounds detected in the 6-month-old samples are summarised in Table 5.9. The peaks with areas over a million units (10^6) were considered. Among these volatile compounds, only acetic acid ($1.1–1.3 \times 10^6$) was present at 1 day in the Y1, Y2 and Y3 cheeses. In the Y4 cheese, 2-heptanone and all the acids except n-decanoic acid were present but in lower concentrations. The concentrations of the volatile compounds increased with time.

The ethyl ester of butanoic acid was present in the Y2 and Y4 cheeses after 6 months of ripening. The other two esters, ethyl esters of hexanoic and octanoic acid, were present only in the Y4 cheese. The ketone pattern was similar for the first three cheeses (Y1, Y2 and Y3), being modest levels of 2-butanone and low levels of 2-heptanone. In contrast, the Y4 cheese had no 2-butanone, medium levels of 2-heptanone and low levels of two other ketones (2-nonanone and 3-methyl-2-butanone).

The acids were the most dominant among the volatile compounds. The concentration of acetic acid was lower in the Y4 cheese than in the other three cheeses, whereas the other acids showed the opposite trend. The concentrations of butanoic, hexanoic and octanoic acids were higher in the *Y. lipolytica* cheeses than in the control cheese. The concentrations were over 7 times higher in the Y4 cheese than in the other *Y. lipolytica* cheeses (Y2 and Y3). Heptanoic acid and n-decanoic acid were detected only in the Y4 cheese. GC–FID analysis did not detect any free C6 fatty acid in the Y2 and Y3 cheeses (Appendix 5.3A), but a relatively moderate concentration was detected by GC–MS, reflecting the different detection levels of the two methods.

Table 5.8 Proteins and peptides as proportions of the total peak area in different cheeses (Y1, Y2, Y3 and Y4) at three sampling points (1 day, 30 days and 60 days) of ripening at 13°C. MW = molecular weight

MW range (Dalton)	Y1			Y2			Y3			Y4		
	1 day	30 days	60 days	1 day	30 days	60 days	1 day	30 days	60 days	1 day	30 days	60 days
> 16 100	78.7	67.9	45.7	79.1	68.6	60.1	82.5	63.4	59.0	76.0	68.4	56.0
16 100–3500	12.2	21.7	43.9	12.8	21.8	29.3	11.6	21.9	28.8	12.0	23.0	26.1
3500 –2500	0.9	2.3	2.9	1.0	2.3	2.9	0.0	2.4	2.9	1.1	2.3	2.6
2500–1250	1.3	1.5	2.1	1.1	1.7	2.0	2.8	2.0	2.4	1.3	1.8	2.9
1250–900	0.5	0.6	0.8	0.5	0.7	0.9	0.0	0.8	0.9	0.6	0.7	1.2
900–400	1.5	1.7	2.1	1.4	1.9	2.3	0.0	2.4	2.6	1.8	1.8	3.3
< 400	5.1	4.3	2.5	4.2	3.0	2.4	3.0	7.1	3.4	6.8	2.0	8.0

Table 5.9 Volatile compounds produced in the cheeses without (Y1) and with (Y2, Y3 and Y4) *Y. lipolytica* adjunct culture after 6 months ripening at 13°C

Volatile compounds	Relative peak areas (10^6)			
	Y1	Y2	Y3	Y4
Alcohol				
Ethanol	3.2	3.1	3.3	1.0
Esters				
Ethyl butanoate	nd*	1.3	nd	1.2
Ethyl hexanoate	nd	nd	nd	1.1
Ethyl octanoate	nd	nd	nd	2.2
Ketones				
2-Butanone	17.1	15.4	22.3	nd
2-Heptanone	1.4	1.7	1.8	10.1
3-Methyl-2-butanone	nd	nd	nd	1.0
2-Nonanone	nd	nd	nd	2.9
Acids				
Acetic acid	7.0	6.3	9.3	3.2
Butanoic acid	29.8	58.4	58.5	404.6
Hexanoic acid	nd	21.4	16.3	252.8
Heptanoic acid	nd	nd	nd	3.5
Octanoic acid	1.0	4.0	2.7	96.5
n-Decanoic acid	nd	nd	nd	20.8

* = Not detectable.

5.4 Discussion

Two groups of cheese were made – to study the effect of *G. candidum* and *Y. lipolytica* on ripening of Cheddar cheese. In each group, four cheeses were made; three of these contained yeast adjunct grown under different conditions or added at different stages of cheese making. The fourth cheese did not contain any yeast adjunct and was used as control. In composition (pH, fat, moisture, salt, calcium), the cheese were virtually identical (there was difference in salt content of G3 cheese, which did not effect the

cheese ripening and discussed in Section 5.4.1.), and in this respect, were therefore essentially replicates of each other, only difference being the yeast adjunct. Thus, although the study as a whole was not replicated, the design of the study provided sufficient internal controls to engender confidence in the data that were derived.

5.4.1 Lipolysis in cheese

The total FFA concentrations were higher in the cheeses with *G. candidum* adjunct than in the cheese without *G. candidum* adjunct. This suggests that the lipolytic system of *G. candidum* is able to hydrolyse milk fat in the cheese environment. The 24 and 72 h shake cultures of *G. candidum* grown in safflower oil medium were added to the cheese milk of the G2 and G4 cheeses respectively. The 24 h *G. candidum* culture showed higher lipase activity than the 72 h culture (0.16 and 0.04 μM/min/mL culture, Section 4.3.1.2) but the concentrations of FFAs in the G2 and G4 cheeses were similar. The possible reasons could be that the lower activity of *G. candidum* lipase was sufficient for a maximum catalytic effect on lipolysis or, more likely, the same amount of extracellular lipase was trapped by the curd. Partial loss of the extracellular lipase of *G. candidum* in the whey also explains the higher concentration of total FFAs in the G3 cheese than in the G2 cheese, both of which contained the same *G. candidum* culture added to the curd (in G3) and the milk (in G2) respectively. Another probable reason could be the modification/damage of the enzyme during cheese manufacture in the G2 cheese.

The S/M content of the G3 cheese (3.6%) was less than that of the G2 cheese (4.3%). The S/M values usually have more influence on proteolysis of the cheese than on lipolysis, as many of the lipases are independent of salt concentration (personal communication with Ross Holland, Fonterra Co-operative Group Ltd, Palmerston North, New Zealand). Similar patterns of proteolysis in these two cheeses (Section 5.3.1.3) suggest that the different S/M content was unlikely to have influenced the ripening of the cheeses. Thus the lipolysis was only influenced by yeast adjuncts.

The ratio between long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) fatty acids in the FFAs of the control cheese and in the total fatty acids (free plus esterified) in the reference cheese was similar. In contrast, this ratio was 2–5 times

higher in the FFAs of the cheeses made with *G. candidum* adjunct, which leads to the conclusion that the *G. candidum* lipase(s) preferentially hydrolyse(s) long-chain unsaturated fatty acids from the milk fat in the cheese environment. The lipase of *G. candidum* is known to show specificity for the hydrolysis of unsaturated fatty acids with a *cis* double bond at the 9-position (oleic, linoleic and linolenic acids) over the corresponding saturated fatty acid (stearic) (Jensen, 1974). Baillargeon *et al.* (1989) evaluated different strains of *G. candidum* for their fatty acid specificity in laboratory media and found that the lipase from NRRL Y-553 and lipase 26557RP displayed selectivity for hydrolysing oleic acid esters, whereas lipases from ATCC34614 and NRRL Y-552 and lipase GC-4 failed to discriminate between palmitic acid and oleic acid. The *G. candidum* lipase contains two types of lipase. No officially recognised nomenclature exists for distinguishing the fatty acid specificity of lipases. Charton *et al.* (1992) introduced a nomenclature by dividing *G. candidum* lipases into two groups: type A, lipase without high specificity for unsaturated substrates with *cis*-9 double bonds; and lipase B, lipases with high specificity for esters of FFAs containing *cis*-9 double bonds. Cloning of the lipase genes from four different strains of *G. candidum* revealed that all strains contained two genes coding for lipase A and lipase B (Bertolini *et al.*, 1994). The present study shows that the strain of *G. candidum* used possesses lipase B (defined above). It may also have some lipase A, but purification and further characterisation of the lipase would need to be carried out in future studies to confirm that.

Y. lipolytica cultures grown under different conditions were used as adjuncts in Cheddar cheese. *Y. lipolytica* static cultures grown in glucose medium for 96 and 192 h were added to the cheese milk of the Y2 and Y3 cheeses respectively. The total FFA concentrations in the Y2 and Y3 cheeses were similar to the total FFA concentration in the control cheese (Y1). This shows that *Y. lipolytica* grown under these conditions did not have any appreciable effect on the lipolysis of the cheese. In contrast, when a 192 h static culture of *Y. lipolytica* grown in safflower oil medium was added to the cheese milk of the Y4 cheese, the FFA concentration was over 10 times higher than in the other three cheeses over the ripening period. This is a reflection of the earlier results (Section 4.3.2.2), where different lipase activities were found for *Y. lipolytica* grown under different conditions. The *Y. lipolytica* cultures added to the Y2, Y3 and Y4 cheeses had lipase activities of 0.3, 1.1 and 7.9 μM/min/mL culture (Section 4.3.2.2). The higher

lipase activity in the 192 h static *Y. lipolytica* culture grown in safflower oil medium can explain the higher FFA concentration in the Y4 cheese although a portion of the extracellular lipase could have been lost in the whey. It is concluded that the effect of the lipase of the *Y. lipolytica* adjunct on cheese ripening was dependent on the culture preparation conditions. This result supports the hypothesis that yeasts influence cheese ripening through the enzymes produced in the culture before its addition to the cheese. Death of yeast cells in the cheese has been reported previously (Crow *et al.*, 2002; Ferreira and Viljoen, 2003) and was confirmed by enumerating the yeast counts (Chapter 6).

The FFA concentration was over 5 times higher in the Y4 cheese (highest FFA concentration among the *Y. lipolytica* cheeses) than in the G3 cheese (highest FFA concentration among the *G. candidum* cheeses) after 6 months of ripening. Higher lipase activity in *Y. lipolytica* culture than in *G. candidum* culture explains the difference in FFA concentrations. Another reason could be the lipase location. Lipase was located both in the cell and in the supernatant of the *Y. lipolytica* culture whereas the entire lipase was located in the supernatant of the *G. candidum* culture. Extracellular lipase is more likely to be lost in the whey (in the case of the G3 cheese, only white whey). A trial with the addition of *Y. lipolytica* culture to the cheese curd was not carried out as high FFA concentration was achieved by addition of the culture to the cheese milk and, unlike the *G. candidum* lipase, a part of the *Y. lipolytica* lipase was located inside the cell.

The results described in Section 5.3.2.2 indicate that the lipase of *Y. lipolytica* preferentially hydrolyses long-chain unsaturated fatty acids from milk fat in Cheddar cheese. Unlike *G. candidum* lipase, only limited published information on the selectivity of *Y. lipolytica* lipase is available. Suzzi *et al.* (2001) studied the lipolytic activity of several *Y. lipolytica* strains isolated from dairy products and found that in all strains the major FFA released was C18:1 followed by C16, with the exception of two strains in which C16 represented the most prominent fatty acid released followed by C18:1. Higher proportions of short-chain fatty acids (C4, C6 and C8) in the FFAs (14.2%) in the Y4 cheese than in the total fatty acids (free plus esterified; 7.9%) in the reference cheese indicate the existence of another lipase that can preferentially hydrolyse short-chain fatty acids from the milk fat in the cheese environment. The

source of the extra free butyric acid is unlikely to be a lactate fermentation product by *Clostridium tyrobutyricum* because no sign of blowing (Steffen *et al.*, 1993) was noticed in the cheeses and the extra free butyric acid was not detected in the other cheeses, which were not influenced by the *Y. lipolytica* lipase. Also, these cheeses were dry salted with an S/M content over 4% at 1 day, which is likely to protect the cheese from *Clostridium* growth. This enzyme of *Y. lipolytica* could be of interest as short-chain free fatty acids, especially butyric acid, are important for cheese flavour, especially in strong-flavoured ingredient cheeses. The capacity of preferential release of short-chain fatty acids by *Y. lipolytica* lipase is compared with that of a commercial lipase preparation containing a mammalian lipase in Chapter 6.

5.4.2 Proteolysis in cheese

No difference in the peptide profiles of the *G. candidum* and *Y. lipolytica* cheeses from the control cheeses indicates that these yeast adjuncts did not have any obvious proteolytic effect on the cheese. The 24 h shake culture of *G. candidum* grown in safflower oil medium and the *Y. lipolytica* cultures grown under any of the conditions did not show any proteinase activity (Sections 4.3.1.3 and 4.3.2.3). Absence of any proteinase activity in the yeast cultures and no effect of the yeast cultures on the proteolysis of the cheese correlate well in the light of the hypothesis that yeasts influence cheese ripening through the enzymes produced in the culture before its addition to the cheese. However, the 72 h shake culture of *G. candidum* grown in safflower oil medium showed proteinase activity (Section 4.3.1.3) but did not affect the proteolysis of the cheese at a detectable level. The quantity of proteinase may have been too little to affect the proteolysis of the cheese or the proteinase may not have worked because of the environmental conditions in the cheese (high S/M and low pH). Further work on other culture conditions in an attempt to induce higher proteolytic activities would be of value in future studies.

The published information on the effect of yeasts on biochemical changes in hard cheeses is limited. Guerzoni *et al.* (1996) reported higher lipolytic and proteolytic changes in Cheddar cheese with *Y. lipolytica* adjunct culture after 2 months of ripening. Deiana *et al.* (1984) reported rapid proteolysis in Pecarino Romano cheese when *D. hansenii* was used as an adjunct. Grieve (1982) reported accelerated ripening of

Cheddar cheese by crude cell-free protease extracts of *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

5.4.3 Production of volatile compounds in cheese

The detected levels of ethyl esters were not higher in the *G. candidum* cheeses than in the control cheese, which indicates that *G. candidum* enzyme was not contributing to ester formation in the cheese. *G. candidum* produced a range of ethyl esters in the glucose medium (Section 4.3.1.4) in which ethanol was detected in higher concentrations (37–50 x 10^6). Ethanol was detected in lower concentrations in these cheeses (3–5 x 10^6), which indicate that ethanol could be the limiting factor in the ethyl ester synthesis by *G. candidum* adjunct in the cheeses. The presence of ethyl esters only in the *Y. lipolytica* cheeses (highest in the Y4 cheese), but not in the control cheese, indicated that the *Y. lipolytica* enzymes, possibly the lipase, could contribute to the formation of ethyl esters in the cheese. Ethyl esters were present in the control cheese G1 but not in the control cheese Y1. The use of different combinations of starter cultures in the *G. candidum* and *Y. lipolytica* cheeses could be the reason.

Two ketone compounds, 3-methyl-2-butanone and 2-heptanone, were present in the *G. candidum* cheeses whereas these ketones were absent in the control cheese. In the cheese containing a 192 h static culture of *Y. lipolytica* grown in safflower oil medium (Y4), the concentrations of 2-heptanone and 2-nonanone were higher than in the control and the other *Y. lipolytica* cheeses. These ketone compounds are possibly the secondary products of lipolysis catalysed by yeast enzymes. Urbach (1993) reported that ketone compounds (2-butanone, 3-methyl-2-butanone, 2-heptanone, 2-nonanone and 2-tridecanone) in Cheddar cheese could influence the cheese flavour. An informal descriptive sensory analysis of the cheeses described one of the flavour aspects as blue cheese flavour only for the Y4 cheese (2 months onward). The presence of the ketone compounds could be responsible for this blue cheesy flavour (McSweeney, 2004).

The acids other than acetic acid were probably the products of lipolysis. Higher concentrations of these acids in the cheeses with yeast adjuncts were due to higher lipolytic activity in these cheeses. However, the concentrations of acetic acid in the different cheeses were similar.

5.5 Conclusions

The study showed that both *G. candidum* and *Y. lipolytica* could be used as adjuncts to provide obvious changes in the lipolysis of Cheddar cheese. However, growth conditions and the point of adjunct addition were important in determining their impact on cheese ripening.

Addition of the *G. candidum* adjunct to the cheese curd was more efficient than its addition to the cheese milk because all the lipase activity was extracellular. The *Y. lipolytica* culture, depending upon the conditions of culture preparation, had an effect on the lipolysis and the production of volatile compounds in the Cheddar cheese. A 192 h *Y. lipolytica* static culture grown in safflower oil medium was found to have the maximum effect on cheese ripening. The results support the hypothesis that the yeast culture influences the biochemical changes in the cheese by the enzyme(s) already produced in it before its addition to the cheese.

Both *G. candidum* and *Y. lipolytica* were shown to have a lipase that is capable of selectively hydrolysing unsaturated fatty acids with *cis*-9 double bonds from milk fat in the cheese environment. The results also indicated the selectivity of *Y. lipolytica* lipase towards short-chain fatty acids from milk fat in the cheese. Selectivity of yeast lipase and production of ketone and ester compounds from the fatty acids may have an influence on the cheese flavour. Although proteolysis of the cheeses was not affected by the yeast adjuncts, the lipolytic activity and the production of other related volatile compounds make these yeasts potential candidates for use in cheese with an aim to accelerate ripening and to develop new flavours.

G. candidum shake culture grown in safflower oil medium for 24 h and *Y. lipolytica* static culture grown in safflower oil medium for 192 h were selected for further cheese production trials (Chapter 6).

Chapter 6. Yeast adjuncts in a washed-curd, dry-salted cheese

6.1 Introduction

The lipase of *Geotrichum candidum*, a dairy yeast, is known for its selectivity towards unsaturated fatty acids with *cis*-9 double bonds (oleic, linoleic and linolenic acids) (Baillargeon, 1990). The major fatty acids released from milk fat by strains of another dairy yeast, *Yarrowia lipolytica*, were C18:1 followed by C16 (Suzzi *et al.*, 2001). Previous experiments with Cheddar cheese demonstrated that lipases of *G. candidum* and *Y. lipolytica* selectively released long-chain unsaturated fatty acids from milk fat in the cheese environment (Sections 5.3.1.2 and 5.3.2.2 respectively). Free long-chain saturated and unsaturated fatty acids are not desirable from a flavour point of view as they produce a soapy flavour defect, but the unsaturated fatty acid linoleic acid has the potential to be converted to conjugated linoleic acid (CLA), a bioactive compound. Certain strains of propionibacteria have been found to convert free linoleic acid to CLA (Jiang *et al.*, 1998). Free linoleic acid is toxic to many microorganisms and the conversion of free linoleic acid to CLA might function as a detoxification mechanism (Jiang *et al.*, 1998).

The term 'CLA' refers to a group of positional and geometric isomers of octadecadienoic acids with conjugated double bonds. The predominant isomer in food is the *cis*-9, *trans*-11 (*c*9,*t*11) isomer. CLA has attracted attention in recent years for its anti-carcinogenic properties (Ip *et al.*, 1991) and other physiological properties, such as improving the immune response (Cook *et al.*, 1993) and controlling the deposition of body fat (Park *et al.*, 1999). Linoleic acid can be converted to CLA chemically but natural production of CLA may be an attractive proposition. This is because, in the natural conversion, the major proportion of the CLA isomers is *c*9,*t*11 CLA (Jiang *et al.*, 1998), which is the most biologically active isomer (Ip *et al.*, 1991). In contrast, chemically synthesised CLA is an equal mixture of four isomers. Increasing the CLA content of milk by a controlled feeding regime of the cattle (Jiang *et al.*, 1996) and microbial conversion of free linoleic acid to CLA in the product are two possible approaches for enhancing the natural CLA concentration of dairy products.

In this chapter, a study that took a microbial conversion approach to enhancing the CLA content of cheese during cheese ripening is described. In a previous study at Fonterra, three out of five strains of propionibacteria screened were identified from the collection of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, as having high isomerisation activity (Das *et al*., 2005; Appendix 6.1). It was hypothesised that the use of yeast lipase and propionibacteria together might increase the concentration of CLA during the ripening of cheese.

One strain of *G. candidum* and one strain of *Y. lipolytica*, grown under optimum conditions for lipase production (Chapter 4), were used as yeast adjuncts in a washed-curd, dry-salted cheese. Two non-starter lactic acid bacteria (NSLAB) were also used as adjuncts. These were *Lactobacillus fermentum*, to produce ethanol and thus to promote ester formation (Crow *et al*., 2002) to mask the undesirable flavours of the long-chain free fatty acids (FFAs), and a strain of *Lb. rhamnosus* to control the growth of adventitious NSLAB flora (Crow *et al*., 2001, 2002). Three strains of *Propionibacterium freudenreichii* ssp. *shermanii* were added to three different 375 L cheese vats along with starter and the other adjuncts (the strains of propionibacteria had different capacities for CLA synthesis, as detailed in Appendix 6.1). The resulting blocks (10 kg) of cheese from the vats containing yeast adjuncts were matured at 20, 13 and 10°C for 4 months. The control cheese was ripened only at 20°C, a temperature commonly used for ripening Swiss-style cheeses containing cultures of propionibacteria (Steffen *et al*., 1993). Thus the specific objective of this chapter was to produce CLA and ester in the cheese using yeast and bacterial adjuncts together. Also, a commercial lipase preparation was added to another vat of cheese along with the starter cultures and bacterial adjuncts to compare the lipolytic effect of the exogenous lipase with that of the yeast cultures in the cheese.

It has been hypothesised that, without growth in the cheese, the yeast adjuncts will influence cheese ripening by the enzymes already produced in the cultures before their addition to the cheese (Chapter 5). Thus the level of yeast adjunct culture used could be important. Another objective of this study was to screen the influences of different levels of *G. candidam* and *Y. lipolytica* in the cheese by experimenting with 1 kg blocks from the control (C) 375 L vat (detail of the experimental design provided in Section 6.2.2).

6.2 Materials and methods

6.2.1 Culture preparation for cheese manufacture

G. candidum (B9001) cultures were grown in linoleic-acid-rich safflower oil medium (malt extract, 0.3% w/v; yeast extract, 0.3% w/v; linoleic-acid-rich safflower oil, 1.0% w/v; peptone, 0.5% w/v) at 30°C and 200 rev/min for 24 h. *Y. lipolytica* (B9014) cultures were grown in linoleic-acid-rich safflower oil medium at 30°C as static cultures for 192 h. *P. freudenreichii* ssp. *shermanii* strains B6022, B6027 and B6028 were grown separately in 10% w/v reconstituted skim milk (heat treated at 95°C for 90 min) containing 0.1% yeast extract for 24 h at 37°C. Mesophilic starter cultures (made up of three defined strains of *Lactococcus lactis* ssp. *cremoris*) and NSLAB adjunct cultures *Lb. fermentum* (FT2) and *Lb. rhamnosus* (DR20®) were grown in 10% w/v reconstituted skim milk (heat treated at 95°C for 90 min) for 16 h at 30°C. In the case of the NSLAB cultures, 0.1% w/v yeast extract was used in the skim milk medium. All strains were from the culture collection of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand.

6.2.2 Cheese manufacture

Washed-curd, dry-salted cheese was manufactured using 375 L of pasteurised milk. All five vats were inoculated with starter culture and adjunct cultures and were coagulated with rennet (Australian double strength, Dairy Meats NZ Ltd, Auckland, New Zealand) at 10 mL/100 L.

Lactococcal starter culture (6.75 L), *Lb. fermentum* (2 L), *Lb. rhamnosus* (10 mL) and propionibacteria (100 mL) were added to each of the five vats. In addition, vats E1, E2 and E3 were inoculated with 3.75 L of *G. candidum* and 3.75 L of *Y. lipolytica*, and vat M was mixed with a commercial preparation of lipase (3 g calf lipase and 0.02 g palatase; supplied by Novo Industries, Bagsvaerd, Denmark, and Danisco A/S, Copenhagen, Denmark, respectively (a mixture of palatase and calf lipase was used to get a balance in free fatty acid composition of cheese. Palatase does not have specificity for any fatty acid, whereas mammalian lipase has specificity for short chain fatty acids.). The propionibacteria strain used in vats C, E1 and M was B6022, and the strains

used in vats E2 and E3 were B6027 and B6028 respectively. The addition of adjunct cultures and enzymes to different cheese vats is summarised in Table 6.1A.

The set temperature was 32°C. Cutting was started at 40 min after the set time. A 12 mm knife size was used. After 15 min, 30% of the whey was drained and replaced by water (32°C). The curd–whey mix was cooked to 38°C for 30 min and held until draining. The whey was drained at pH 6.25. The curd was cheddared followed by milling (milling pH 5.46) and dry salting at 17 g/kg. After salting, the 40 kg of curd from each vat was treated in the following way. For the control vat (C), a 10 kg block was made, and rest of the curd was used for 1 kg blocks, which were mixed with yeast cultures according to Table 6.1B. Curd from vats E1, E2 and E3 were divided into four equal-sized blocks (approximately 10 kg). Curd for one block (10 kg) from each of these three vats was mixed with 250 g of linoleic-acid-rich safflower oil. The cheese blocks were pressed overnight (14 psi) and then vacuum packed in cheese bags (Amcor Flexibles, Auckland, New Zealand). Four 10 kg blocks were made from vat M, although only one was used for this experiment.

Table 6.1A Microbial adjuncts and extraneous enzymes added to different cheese vats

Adjuncts/enzymes	Vats				
	C	E1	E2	E3	M
G. candidum	No	Yes	Yes	Yes	No
Y. lipolytica	No	Yes	Yes	Yes	No
Propionibacteria	B6022	B6022	B6027	B6028	B6022
Lb. fermentum	Yes	Yes	Yes	Yes	Yes
Lb. rhamnosus	Yes	Yes	Yes	Yes	Yes
Commercial lipase	No	No	No	No	Yes

One block (10 kg) from each of vats E1, E2 and E3 was ripened at 20, 13 and 10°C for 4 months. The 10 kg block from vat C, the 10 kg blocks from vat M (only one used for

experimental purposes), the blocks with added safflower oil and all 1 kg blocks were ripened at 20°C for 4 months.

Table 6.1B Curd (1 kg) from vat C was mixed with different levels of yeast adjuncts

Cheese code	Type of yeast adjunct	Volume of yeast culture (mL)	Volume of blank medium (mL)
Control (C1)	NA	NA	100
GC20	*G. candidum*	20	80
GC100	*G. candidum*	100	0
GC500*	*G. candidum*	500	0
YL20	*Y. lipolytica*	20	80
YL100	*Y. lipolytica*	100	0
YL500*	*Y. lipolytica*	500	0

* 500 mL of culture was concentrated to 100 mL by ultrafiltration (molecular weight cut-off 10 000 Da) over approximately 16 h at 4°C.

6.2.3 Estimation of free CLA

Extraction of FFAs and neutral lipids (esterified fatty acids) from the cheese and isolation of FFAs by solid phase extraction were carried out according to the methods described in Section 5.2.2. The FFAs were methylated using boron trifluoride reagent (14% BF_3 in methanol). Boron trifluoride reagent (1 mL) was added to the sample, mixed and left for 5 min. Hexane (10 mL) was added, mixed and left for 2 min. Neutralising solution (5% w/v di-potassium hydrogen phosphate (K_2HPO_4) and 1.5% w/v potassium hydroxide (KOH) in Milli-Q water) was added (1 mL), mixed thoroughly by shaking and centrifuged at 175 *g* for 5 min. The upper layer (1 mL) was withdrawn and transferred to a 2 mL autosampler vial.

Gas liquid chromatography (GC): FFA analyses were performed on a Shimadzu GC-17A chromatograph (Shimadzu Corporation, Tokyo, Japan) equipped with an on-column injector, a flame ionisation detector (FID) and an AOC-20i auto-injector. The GC column was a 50 m x 0.22 mm internal diameter BPX-70 (0.25 µm film thickness) column (SGE International Pty Ltd, www.sge.com) with a 1 m x 0.53 mm internal diameter deactivated pre-column. The carrier gas was hydrogen at a flow rate of 1.2 mL/min. The initial oven temperature was 50°C for 1.5 min, followed by a gradient to 220°C (3°C/min). The holding time at 220°C was 10 min. The injection volume was 0.2 µL. The on-column injector was held at 80°C for 0.1 min, and then programmed at the maximum rate to 220°C and held for 2 min. Individual CLA isomers were identified and quantified with reference to injections of known amounts of pure standards of CLA isomers and using tridecanoic acid (C13) (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA) as an internal standard. Data analysis was carried out using Class-VP software (Shimadzu).

6.2.4 Microbial analysis

Microbial analyses of the cheeses were carried out using standard methods. The references for different methods are given below.

Yeast in cheese: Yeast in cheese was enumerated by the method described in New Zealand Technical Manual 2, NZTM 2.61.1.

NSLAB in cheese: NSLAB in cheese were enumerated by the method described in NZTM 2.54.1.

Propionibacteria: Propionibacteria numbers were enumerated on Trypticase-lactate agar (Crow & Turner, 1986).

Other methods of analysis used are described in Section 5.2 and include GC analysis for FFAs (Section 5.2.2), GC analysis for total (free plus esterified) fatty acids including total (free plus esterified) CLA (Section 5.2.3), estimation of volatile compounds (Section 5.2.5) and cheese composition analyses (Section 5.2.6).

6.3 Results

6.3.1 pH, composition and yeast count of cheeses (10 kg blocks)

All five vats of cheese had similar pH and composition values at 1 day after manufacture (Table 6.2). Thus, the main variable likely to affect the ripening of the experimental cheeses was the presence of the yeast adjuncts (E1, E2 and E3) or the exogenous enzyme (M), whereas the control cheese (C) did not contain any yeast adjunct or exogenous enzyme. The yeast count results (Table 6.3) show that at 1 day the cheeses from vats E1, E2 and E3 contained 10^5 cfu yeast/g cheese, whereas the yeast count in the cheeses from vats C and M were negligible. The other variable between the E1, E2 and E3 cheeses was the different strains of propionibacteria. The concentrations of propionic acid and acetic acid, FFAs originating from milk fat, free and total CLA, and volatile compounds were monitored over 4 months in all cheeses. Average values of results from the duplicate analyses are presented.

Table 6.2 pH and composition of the 1-day-old cheeses (average values from duplicate analyses)

Vat	pH	Moisture (% w/w)	Salt (% w/w)	Fat (% w/w)	Calcium (mM/kg)
C	5.13	38.8	1.39	32.5	177
E1	5.20	39.2	1.38	32.8	175
E2	5.24	39.3	1.22	32.3	178
E3	5.21	39.6	1.16	32.8	176
M	5.24	38.8	1.40	33.0	182

Table 6.3 Yeast counts (cfu/g cheese) in different cheeses at 1 day and 2 months (average values from duplicate analyses)

Time	Cheese				
	C	E1	E2	E3	M
1 day	12	2.3×10^5	6.3×10^5	5.7×10^5	64
2 months	nd*	3.3×10^3	1.2×10^2	4.3×10^2	nd

* = Not detectable.

6.3.2 Fermentation by propionibacteria in cheeses ripened at 20°C (10 kg blocks)

The concentrations of acetic acid and propionic acid, two fermentation products of propionibacteria produced during lactate fermentation (Steffen *et al.*, 1993), and measured by GC-FID are shown in Figures 6.1A and 6.1B.

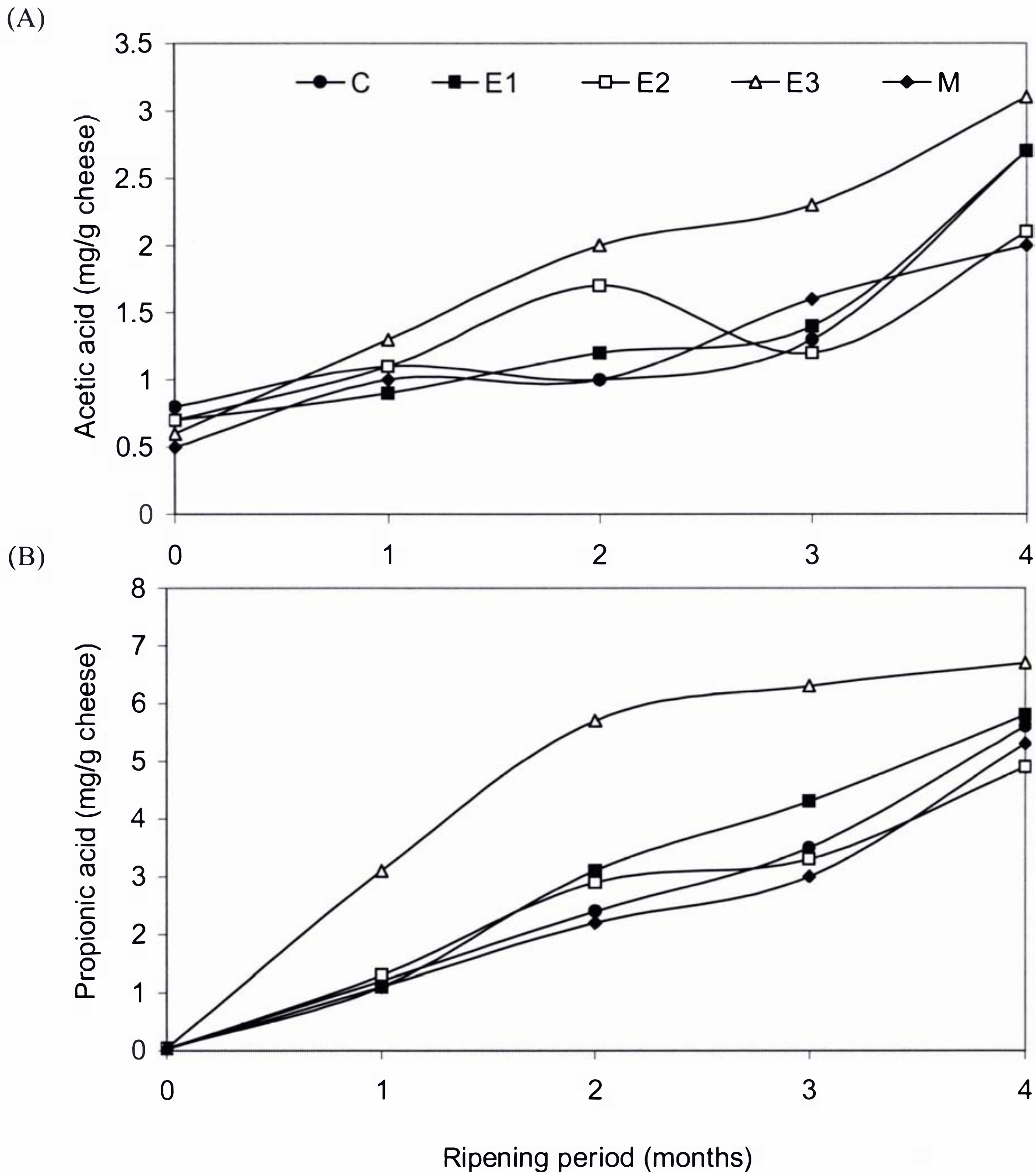


Figure 6.1 Concentration of acetic acid (A) and propionic acid (B) in the control cheese C and in the cheeses with yeast adjuncts (E1, E2 and E3) and a commercial enzyme preparation (M) over 4 months of ripening at 20°C.

These two fermentation products increased throughout ripening in all cheeses, indicating active lactate fermentation by propionibacteria throughout the 4-month period. The concentrations of propionic acid at 4 months were similar in the cheeses in which propionibacteria strain B6022 was used (5.6, 5.8 and 5.3 mg/g in the C, E1 and M cheeses respectively). This indicates that the high concentrations of FFAs in the E1 and M cheeses (Section 6.3.3) did not inhibit the lactate fermentation by propionibacteria. The concentration of propionic acid was slightly higher in the E1 cheese than in the C and M cheeses at 2 and 3 months (Figure 6.1), suggesting that the yeast adjunct cultures may have stimulated the activity of the propionibacteria. This is discussed later with more robust evidence (Sections 6.3.7.1.1 and 6.3.7.2.1). The propionibacteria population also increased in all cheeses during ripening (Table 6.4). The concentrations of both acetic acid and propionic acid were higher in the E3 cheese than in the other cheeses (Figure 6.1) and, as the densities of propionibacteria were similar in the E1, E2, E3 and M cheeses (Table 6.4), this suggests that strain B6028 fermented lactate more actively in the cheese than the other strains.

Table 6.4 Viable cell counts of propionibacteria (cfu/g cheese) in different cheeses at 1 day and 2 months (average values from duplicate analyses)

Time	Cheese				
	C	E1	E2	E3	M
1 day	7.7×10^6	1.5×10^7	5.6×10^6	2.0×10^7	6.1×10^6
2 months	5.6×10^7	1.7×10^8	2.2×10^8	2.0×10^8	1.3×10^8

6.3.3 Lipolysis in cheeses ripened at 20°C (10 kg blocks)

The changes in the total FFA concentrations in the control (C) and experimental cheeses (E1, E2, E3 and M) over the 4-month ripening period are shown in Figure 6.2. The total FFA concentrations in the control cheese (C) increased from 0.4 to 4.1 mg/g during ripening. The total FFA concentrations in the three cheeses made with yeast adjuncts

increased considerably more and were 27.7, 27.3 and 28.4 mg/g in the 4-month-old E1, E2 and E3 cheeses respectively. These obviously high total FFA concentrations in the cheeses made with yeast adjuncts compared with the control cheese demonstrate the lipolytic activity of the yeasts in the cheeses. The total FFA concentrations in the three cheeses containing yeast adjuncts with different propionibacteria strains were similar; this indicates that the lipolysis in the cheese was not influenced by the strains of *P. freudenreichii* ssp. *shermanii*.

The total FFA concentrations in the M cheese were higher throughout the ripening period than in the control cheese C, which was obviously due to the added lipase in the M cheese. The total FFA concentrations were higher in the cheeses made with yeast adjuncts (E1, E2 and E3) than in the cheeses made with added commercial lipase preparation (M) at 3 and 4 months. This shows that, at the later stage of ripening, the yeast lipase had more impact on the lipolysis of cheese than the commercial lipase preparation, which reached near saturation after 2 months.

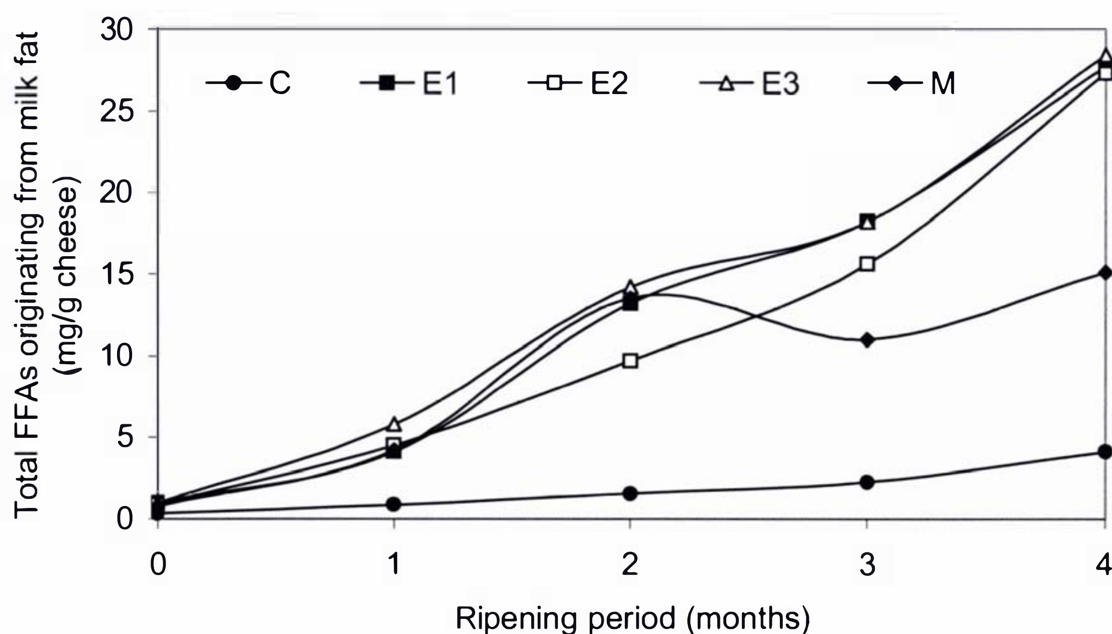


Figure 6.2 Concentrations of total FFAs in the control (C) and experimental (E1, E2, E3 and M) cheeses over 4 months of ripening at 20°C.

The concentrations of individual FFAs in these five cheeses were quantified to determine if the yeast lipase or the commercial lipase preparation had any selectivity for any particular fatty acid. All the FFAs with an even number of carbon atoms between C4:0 and C18:2 were quantified. The proportions of the individual FFAs in the control

(C) and experimental (E1, E2, E3 and M) cheeses at 4 months are compared with the total fatty acid (free plus esterified) profile in the reference cheese (Section 5.3.1.2) in Figure 6.3. The details of the FFA results for all cheeses over 4 months of ripening at 20°C are given in Appendix 6.2A (as mg/g cheese) and Appendix 6.2B (as a % of total FFAs).

The proportions of the individual FFAs in the control cheese were similar to those of the total fatty acids (free plus esterified) in the reference cheese. The proportion of the free C4 fatty acid in the cheeses with yeast adjuncts (6.1–6.3%) was higher than that of the total C4 fatty acid (free plus esterified) in the reference cheese (4.3%). This increase was not very high, but it may indicate that the yeast lipase has some selectivity for the butyric acid (C4) from milk fat in the cheese environment. The C8 fatty acid also showed the same pattern. Selectivity of *Y. lipolytica* lipase towards short-chain fatty acids (C4 and C8) was also shown in Cheddar cheese (Section 5.3.2.2). The proportions of C6, C10 and C12 fatty acids in the FFAs of the cheeses made with yeast adjuncts were similar to those of the total fatty acids (free plus esterified) in the reference cheese, suggesting no selectivity for these FFAs.

The long-chain saturated and unsaturated FFAs showed patterns opposite to each other. The proportions of the long-chain saturated fatty acids (C14, C16 and C18) were less in the FFAs of the cheeses made with yeast adjuncts than in the total fatty acids (free plus esterified) in the reference cheese. In contrast, the long-chain unsaturated fatty acids (C18:1 and C18:2) constituted higher proportions in the FFAs of the cheeses containing yeast adjuncts than in the total fatty acids (free and esterified) in the reference cheese. The ratio between the long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) fatty acids was 0.5 in the total fatty acids (free plus esterified) in the reference cheese, whereas the ratio was more than double (1.2–1.6) in the FFAs of the cheeses with yeast adjuncts. These individual FFA results indicate that the yeasts may have one or more lipase(s) that preferentially hydrolyse(s) long-chain unsaturated fatty acids from milk fat in the cheese environment. Selectivity of *G. candidum* and *Y. lipolytica* lipase(s) towards long-chain unsaturated fatty acids in Cheddar cheese was shown in Sections 5.3.1.2 and 5.3.2.2 respectively.

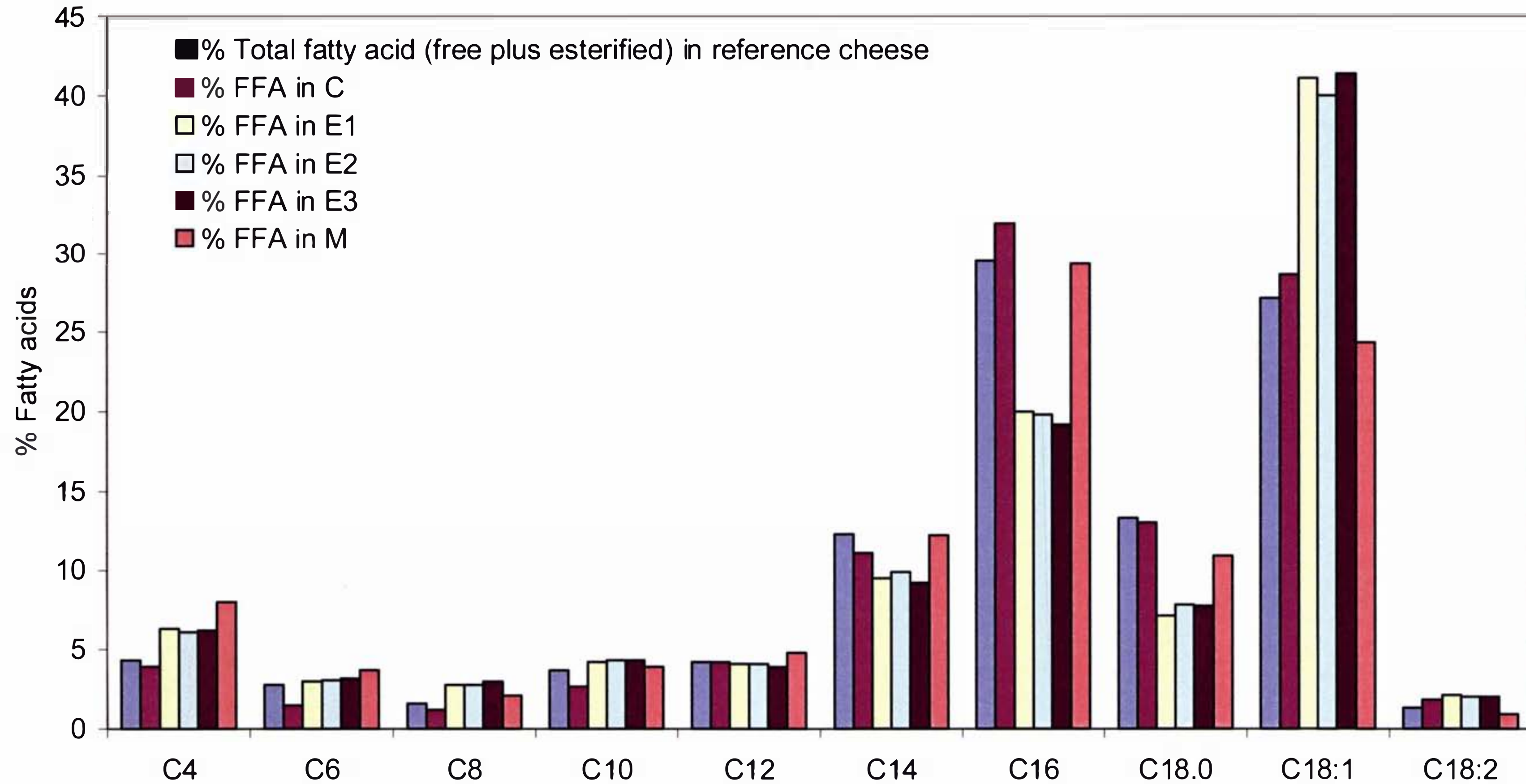


Figure 6.3 Comparison of the proportions of the individual FFAs expressed as % of the total FFAs originating from milk fat in 4-month-old cheeses (C, E1, E2, E3 and M) with the total fatty acid (free and esterified) profile of the reference cheese.

The proportion of C4 fatty acid in the total FFAs of the M cheese (8%) was higher than that of the cheeses containing yeast adjuncts (6.1–6.3%), whereas C4 fatty acid constituted 4.3% of the total fatty acids (free plus esterified) in the reference cheese. This suggests that the commercial lipase preparation used has selectivity for C4 fatty acid and that this selectivity may be higher than that of the yeast lipase. Unlike the yeast lipase, the commercial lipase preparation did not show any selectivity for long-chain unsaturated fatty acids. The proportions of the C18 and C18:1 fatty acids in the total FFAs of the M cheese were less than those of the C18 and C18:1 fatty acids in the total fatty acids (free plus esterified) in the reference cheese. The other fatty acids were more or less in the same proportions in the total FFAs of the M cheese as in the total fatty acids (free plus esterified) in the reference cheese.

The concentrations of linoleic acid (C18:2), the substrate for proposed CLA synthesis, were several times higher in the cheeses made with yeast adjuncts (E1, E2 and E3) than in the control cheese (C) and the cheese made with the commercial lipase preparation (M) throughout the ripening period (Figure 6.4). For example, after 2 months of ripening, the free linoleic acid concentrations were 0.28–0.47 mg/g in the cheeses containing yeast adjunct compared with 0.03 mg/g in the control cheese and 0.14 mg/g in the M cheese. The detailed data for free linoleic acid along with the other FFAs are provided in Appendix 6.2A.

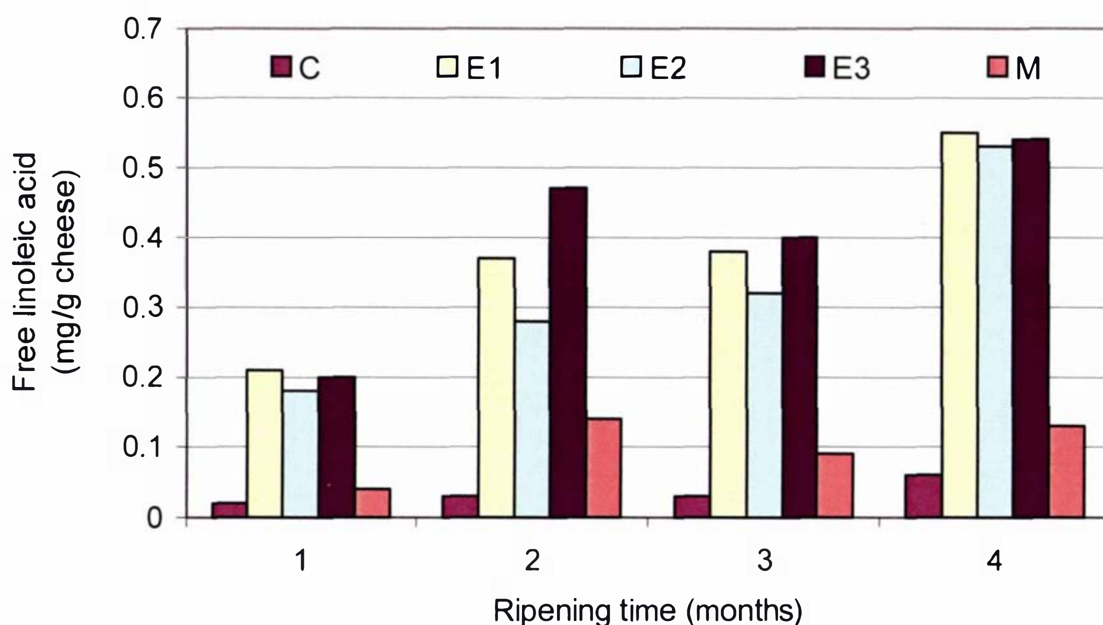


Figure 6.4 Concentrations of free linoleic acid in C, E1, E2, E3 and M cheeses.

6.3.4 CLA content in cheeses ripened at 20°C (10 kg blocks)

The concentrations of the free CLA isomers as well as their proportions of the total FFAs in the cheeses with and without yeast adjuncts are presented in Table 6.5.

Table 6.5 Concentrations of the free CLA isomers in the 2-month-old cheeses made with and without yeast adjuncts and ripened at 20°C

Free CLA	Cheese			
	C	E1	E2	E3
c-9,*t*-11 CLA (mg/g cheese)	0.01	0.34	0.25	0.4
cc-CLA (mg/g cheese)	0.00	0.01	0.00	0.0
tt-CLA (mg/g cheese)	0.00	0.07	0.06	0.1
Sum of free CLA isomers (mg/g cheese)	**0.01**	**0.42**	**0.31**	**0.5**
Free CLA as a % of total FFAs	**0.5**	**3.7**	**3.3**	**3.6**

The concentrations of free CLA were much higher in the cheeses with yeast adjuncts than in the cheese without yeast adjuncts and were similar in the three yeast-containing cheeses made with different strains of *P. freudenreichii* ssp. *shermanii.* The free CLA content was 0.01 mg/g in the cheese without yeast adjuncts (C) and ranged between 0.31 and 0.5 mg/g in the cheeses with yeast adjuncts (E1, E2 and E3). In terms of the CLA isomers as a percentage of the total FFAs, the percentage was 6.6–7.4 times higher in the cheeses with yeast adjuncts than in the cheese without yeast adjuncts. The possible source of the extra free CLA isomers could have been either the hydrolysis of CLA from milk triglycerides or from the conversion of free linoleic acid to CLA.

To establish the source of the extra free CLA in the cheeses with yeast adjuncts, the total CLA (free plus esterified) content of the cheeses was determined. The total CLA concentrations in the cheeses with and without yeast adjuncts are presented in Table 6.6. The CLA isomers as percentages of the total fatty acids (free plus esterified) were similar in all cheeses (1.6–1.8%). This result suggests that there was no conversion of free linoleic acid to CLA and that the reason for an increase in free CLA in the cheeses

with yeast adjuncts was the preferential release of unsaturated fatty acids by the yeast lipase.

Table 6.6 Concentrations of the total CLA isomers (free plus esterified) in the 2-month-old cheeses with and without yeast adjuncts and ripened at 20°C

Total CLA	Vat			
	C	E1	E2	E3
c-9,*t*-11 CLA (mg/g cheese)	4.0	3.8	5.2	3.4
cc-CLA (mg/g cheese)	0.3	0.2	0.3	0.2
tt-CLA (mg/g cheese)	0.8	0.8	1.0	0.7
Sum of total CLA isomers (mg/g cheese)	**5.1**	**4.8**	**6.5**	**4.3**
CLA as a % of total fatty acids	**1.7**	**1.6**	**1.8**	**1.6**

In an attempt to increase the potential for CLA synthesis in the cheeses, the linoleic acid (the substrate for CLA synthesis) concentrations in the cheeses were increased by adding linoleic-acid-rich (esterified) safflower oil to the cheese curd before pressing (Section 6.2.2). The concentrations of free linoleic acid and CLA in the cheeses containing yeast adjuncts with and without added safflower oil are compared in Figure 6.5.

There were considerable increases in the free linoleic acid concentration in the cheeses when safflower oil was added. The concentration of free linoleic acid ranged between 0.7 and 0.9 mg/g in the cheeses with added safflower oil compared with 0.28–0.47 mg/g in the cheeses without safflower oil. In spite of the increases in the concentration of free linoleic acid in the cheeses with added safflower oil, the concentrations of free CLA remained unchanged compared with those in the cheeses without added safflower oil. This result confirms that the free linoleic acid in the cheese was not converted to CLA. However, the acetic acid and propionic acid data (Appendix 6.3) show that the propionibacteria were active in the cheeses with added oil, although there were some decreases in the propionic acid concentrations (7–23%) in the presence of oil, which could have been due to the higher concentration of free linoleic acid.

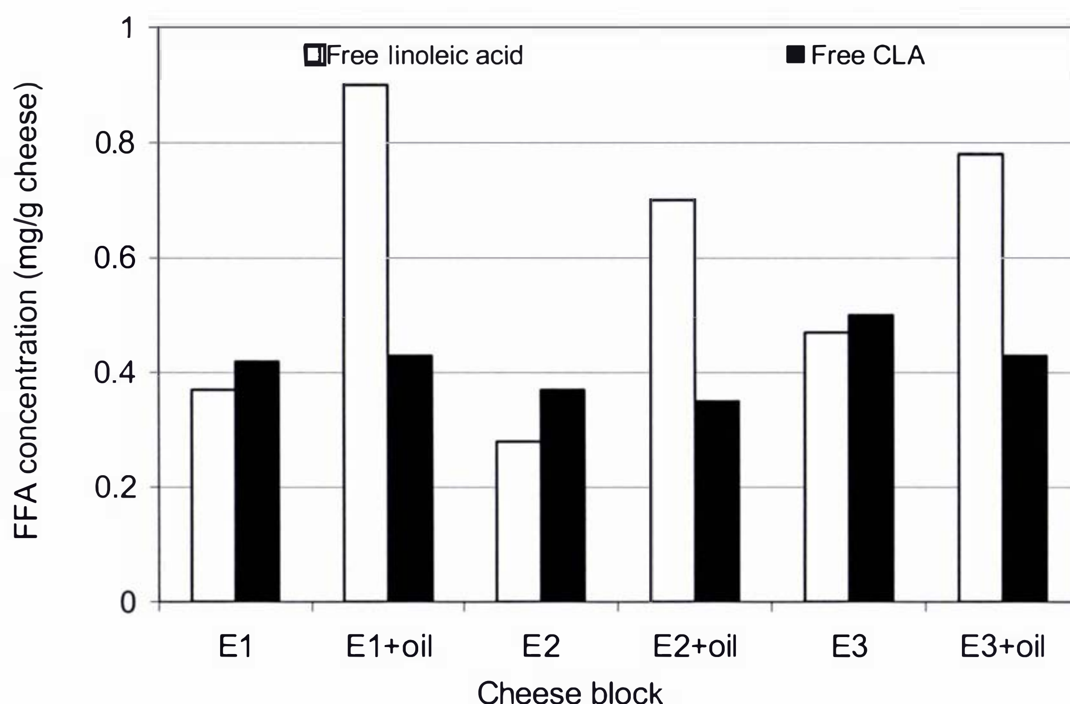


Figure 6.5 Concentrations of free linoleic acid and CLA in cheeses containing yeast adjuncts with and without added linoleic-acid-rich safflower oil after 2 months of ripening at 20°C.

As the ripening period might have an effect on CLA conversion, free and total CLA (free plus esterified) concentrations in the control cheese (C) and a cheese with yeast adjuncts (E1) were determined at different time points of ripening. The data are shown in Table 6.7. Although the concentration of free CLA increased with time in the presence of yeast adjuncts, the concentration of total CLA (free plus esterified) remained unchanged. The results show that no conversion of free linoleic acid to CLA occurred at any point during ripening.

Table 6.7 Free and total CLA content in the C and E1 cheeses over the ripening period

Cheese	Free CLA (mg/g cheese)			Total CLA (free plus esterified) (mg/g cheese)		
	1 Day	2 Months	4 Months	1 Day	2 Months	4 Months
C	0.01	0.01	0.01	5.2	5.1	5.1
E1	0.02	0.42	0.63	5.1	4.8	5.1

6.3.5 Volatile compounds in cheeses ripened at 20°C (10 kg blocks)

Headspace analyses of the C, E1, E2 and E3 cheeses were carried out at different points during ripening. At 1 day, only low concentrations (< 4 x 10^6 relative peak area) of ethanol and acetic acid were present in the cheeses. The concentrations of ethanol and acetic acid increased, and other volatile compounds appeared with ripening time. All volatile compounds detected in these four cheeses after 4 months of ripening at 20°C are listed in Appendix 6.4 A. The fatty acids of milk fat origin were in much higher concentrations in the cheeses with yeast adjuncts, following the same trend as seen in Sections 5.3.1.4 and 5.3.2.4. The concentrations of acetic acid and propionic acid were higher in the E3 cheese than in the other cheeses, which supports the GC–FID results (Figure 6.1) and suggests higher lactate fermentation ability of propionibacteria strain B6028.

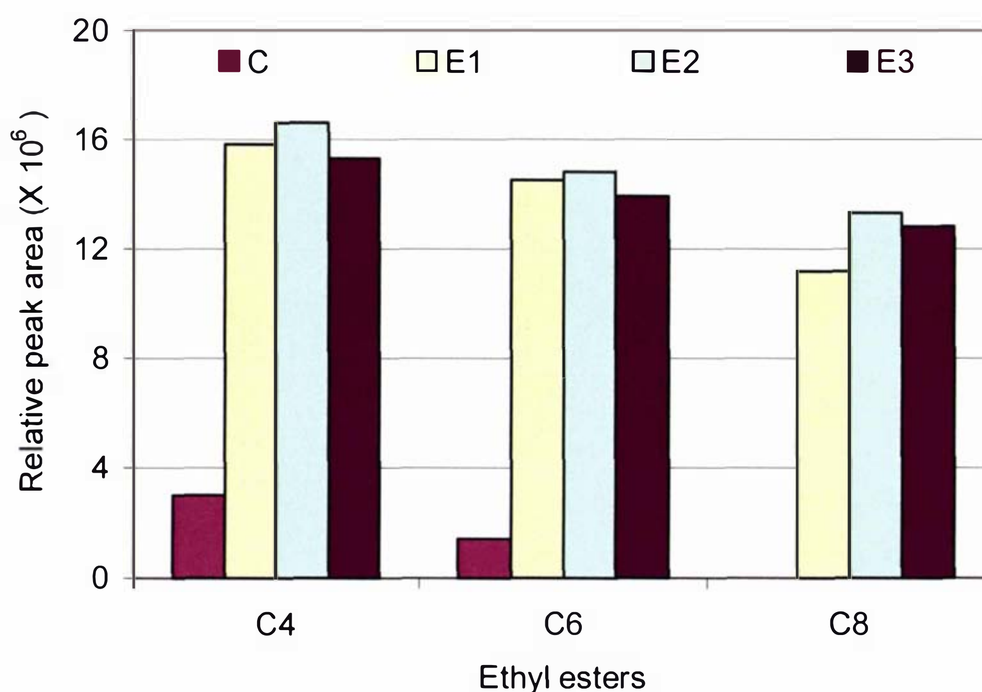


Figure 6.6 Relative concentrations of ethyl esters of butanoic acid (C4), hexanoic acid (C6) and octanoic acid (C8) in the control cheese C without yeast adjuncts and in the cheeses with yeast adjuncts, E1, E2 and E3, after 4 months of ripening at 20°C.

The relative concentrations of ethyl esters of butanoic (C4), hexanoic (C6) and octanoic (C8) acids were much higher in the cheeses made with yeast adjuncts than in the control cheese (Figure 6.6). However, the concentrations were similar in the E1, E2 and E3

cheeses containing different strains of propionibacteria. The control cheese as well as the cheeses made with yeast adjuncts had relatively high concentrations of ethanol (80–100 x 10^6 relative peak area after 1 month of ripening), suggesting that the *Lb. fermentum* strain was effective in producing ethanol in all vats. However, the higher concentrations of ethyl esters only in the cheeses containing yeast adjuncts suggest that yeast enzymes play an important role in producing esters.

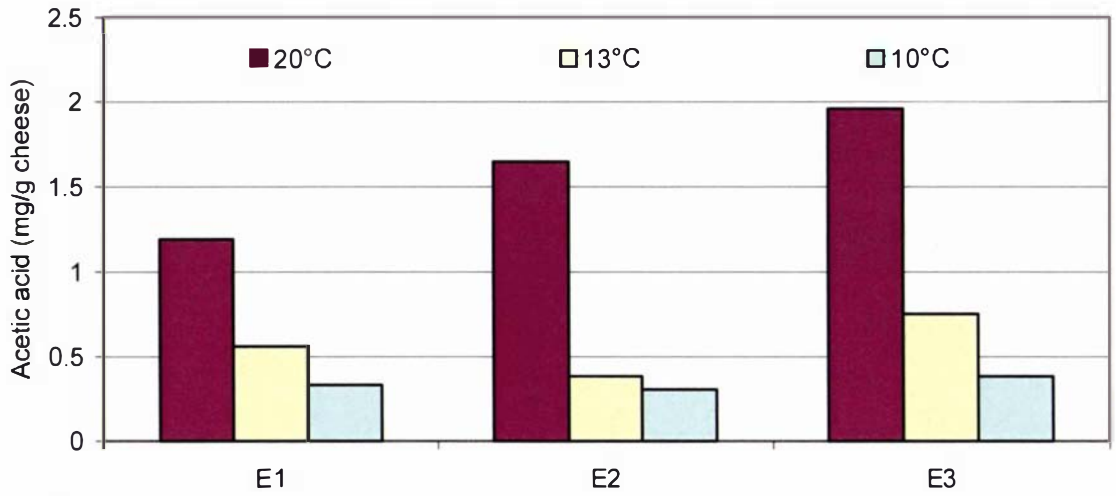
An expert panel performed sensory tastings of the cheeses at monthly intervals during the first 3 months of the ripening period. The sensory reports (see Appendix 6.4 B for detail) described the cheeses with yeast adjuncts as fruity. The fruity flavour perceived in the yeast cheeses can be correlated with the presence of ester compounds at high concentrations.

6.3.6 Effect of ripening temperatures on the cheeses with yeast adjuncts (10 kg blocks)

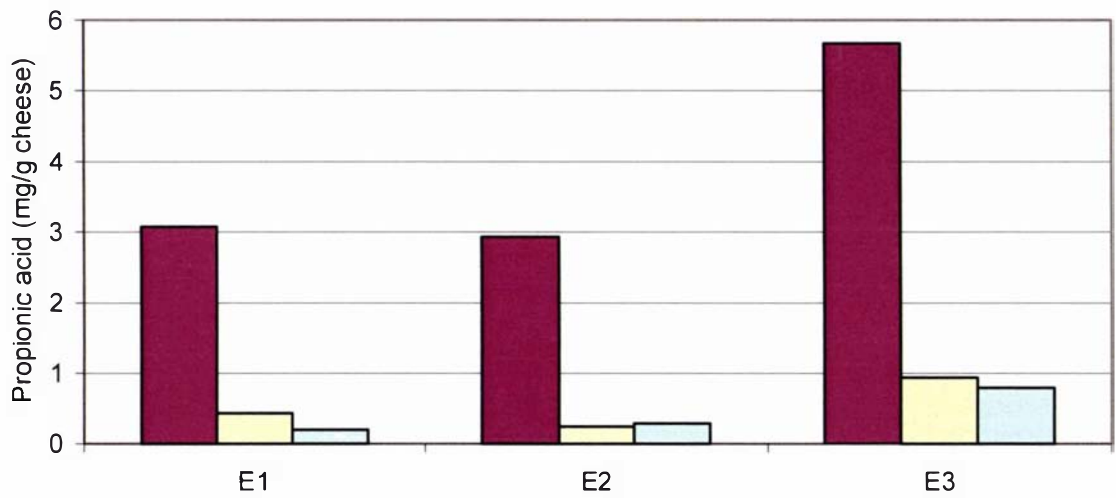
The cheeses made with combined yeast adjuncts *G. candidum* and *Y. lipolytica* were kept at different temperatures (20, 13 and 10°C) for ripening. The lactate fermentation products acetic acid and propionic acid and the FFAs of milk fat origin were determined after 2 and 4 months. The results for the 2-month-old cheeses are shown in the Figures 6.7A, 6.7B and 6.7C. The patterns were similar in the 4-month-old cheeses and are given in Appendix 6.5.

The concentrations of acetic acid and propionic acid were more than double in the cheeses ripened at 20°C than in the cheeses ripened at lower temperatures (13 and 10°C), whereas the concentrations were not appreciably different between the cheeses ripened at 13 and 10°C. These results indicate that a higher temperature (20°C) is required for the higher metabolic activities of propionibacteria. The possible reason for the high metabolic activity is the higher growth of propionibacteria at 20°C. The propionibacteria counts of the cheeses ripened for 2 months showed that the counts of viable cells were 1 log higher in the cheeses ripened at 20°C (1.7–2.2 x 10^8 cfu/g cheese; shown in Table 6.4) than in the cheeses ripened at 10°C (1.9–3.5 x 10^7 cfu/g cheese; not shown as a table) starting with the same count at 1 day.

(A)



(B)



(C)

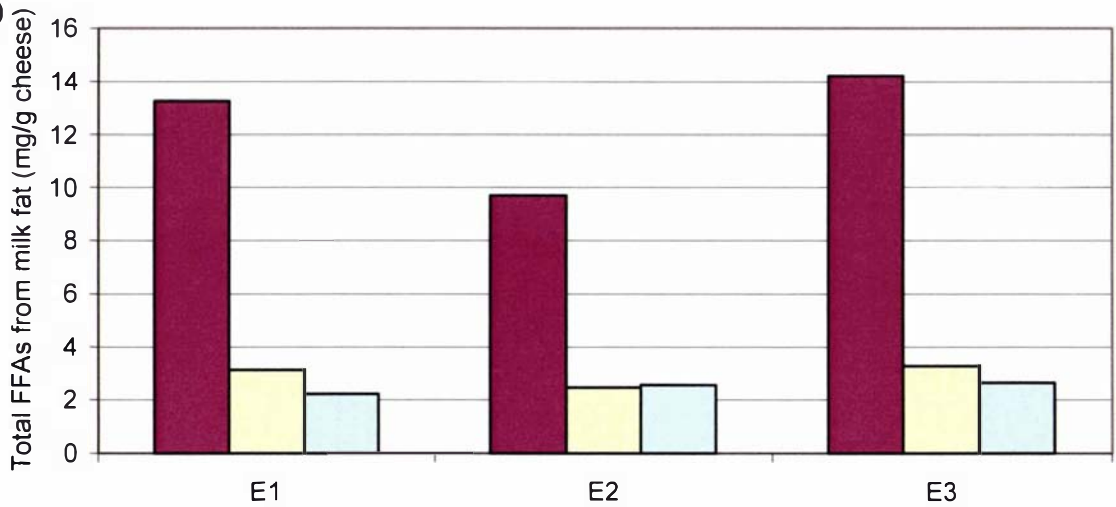


Figure 6.7 Concentrations of acetic acid (A), propionic acid (B) and total FFAs originating from milk fat (C) in the 2-month-old cheeses containing yeast adjuncts ripened at different temperatures.

The total FFA concentration of milk fat origin was more than 3 times higher in the cheeses ripened at 20°C than at the lower temperatures (13 and 10°C). However, the total FFA concentrations were similar in the cheeses ripened at 13 and 10°C. The results indicate higher activity of the yeast lipase at 20°C. The total FFA concentration was considerably less in the E2 cheese than in the E1 and E3 cheeses after 2 months of ripening at 20°C. However, the concentrations of total FFAs became similar in the E1, E2 and E3 cheeses at 4 months (Figure 6.2).

The individual fatty acids originating from milk fat (all fatty acids with an even number of carbon atoms between C4 to C18:2) in the cheeses at 20, 13 and 10°C were quantified after 2 and 4 months of ripening to determine the effect of ripening temperature on the specificity of the yeast lipase. The proportions of individual FFAs of the E1 cheese ripened at 20, 13 and 10°C for 4 months are compared with the total fatty acid (free plus esterified) profile in the reference cheese in Figure 6.8. The FFA data of the E1, E2 and E3 cheeses at 20, 13 and 10°C after 2 and 4 months of ripening are provided in Appendix 6.6A (as mg/g cheese) and Appendix 6.6B (as a % of total FFAs).

Higher proportions of C4 FFA in the E1 cheese ripened at 20°C, compared with the proportion of total C4 fatty acid (free plus esterified) in the reference cheese, indicated the selectivity of yeast lipase towards butyric acid (C4), which has been described earlier in Section 6.3.3. The proportions of C4 FFA in the E1 cheese at 13 and 10°C were similar to the proportion of total C4 fatty acid (free plus esterified) in the reference cheese, which did not indicate any selectivity towards C4 fatty acid. The possible explanation could be that the lipase(s) selective for C4 fatty acid is (are) more active at higher temperature (20°C). However, the *Y. lipolytica* lipase showed some selectivity towards C4 fatty acid at 13°C in Cheddar cheese (Section 5.3.2.2).

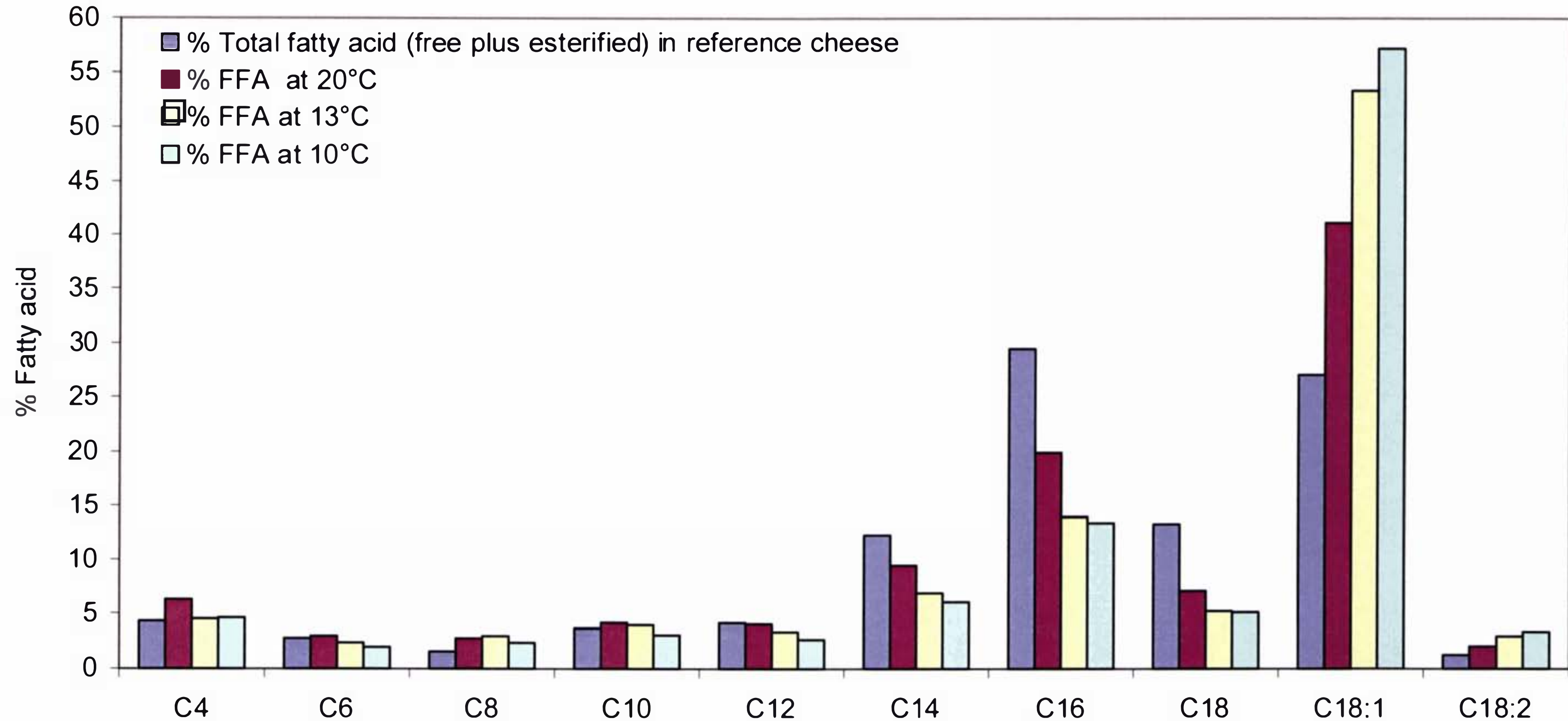


Figure 6.8 Comparison of the proportions of the individual FFAs expressed as a % of the total FFAs originating from milk fat in 4-month-old cheese containing yeast adjuncts (E1) ripened at 20, 13 and 10°C with the total fatty acid (free plus esterified) profile of the reference cheese.

The proportions of the long-chain unsaturated FFAs (C18:1 and C18:2) in the E1 cheese ripened at all temperatures were higher than those of the total (free plus esterified) long-chain unsaturated fatty acids in the reference cheese. In contrast, the proportions of the long-chain saturated FFAs (C14:0, C16:0 and C18:0) in the E1 cheese ripened at all temperatures were lower than those of the total (free plus esterified) long-chain saturated fatty acids in the reference cheese. The results show selectivity of yeast lipase towards long-chain unsaturated fatty acids at all temperatures. This selectivity is also described in Sections 5.3.1.2, 5.3.2.2 and 6.3.3.

The ratios between the long-chain unsaturated (C18:1 and C18:2) and saturated (C14:0, C16:0 and C18:0) FFAs in the yeast-containing cheeses ripened at different temperatures were more than twice the ratio (0.5) between the long-chain unsaturated and saturated total fatty acids (free plus esterified) in the reference cheese. The ratio was lower in the cheeses ripened at 20°C than in the cheeses ripened at 13 and 10°C (Table 6.8).

Table 6.8 Ratio between long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) FFAs in different cheeses after 2 and 4 months of ripening at 20, 13 and 10°C

Cheese	2 Months			4 Months		
	20°C	13°C	10°C	20°C	13°C	10°C
E1	1.5	2.3	2.1	1.2	2.2	2.2
E2	1.4	2.5	2.0	1.2	2.2	1.8
E3	1.6	2.1	2.0	1.2	1.9	1.8

The results suggest that the yeast lipase with selectivity for long-chain unsaturated fatty acids may be more active than the other lipases at lower temperatures (10 and 13°C) than at higher temperatures (20°C). Another notable point is the decrease in the ratios between 2 and 4 months for all three cheeses at all temperatures (exception the E1 cheese at 10°C). The same pattern was observed for Cheddar cheese (Section 5.3.1.2). The reason could be the diminishing concentrations of the substrates (glycerides with C18:1 and C18:2) following selective hydrolysis.

The concentration of free and total CLA was determined in the E1, E2 and E3 cheeses ripened at different temperatures to determine whether temperature had any effect on CLA production in the cheeses. The concentrations of free and total CLA in 2-month-old samples of the E1, E2 and E3 cheeses ripened at 20, 13 and 10°C are shown in Table 6.9. The concentrations of free CLA were higher in the cheeses ripened at 20°C than in the cheeses ripened at lower temperatures, whereas the concentrations of total CLA were similar in the cheeses ripened at different temperatures. The patterns were the same for the E1, E2 and E3 cheeses. These results suggest that the release of CLA from the milk fat by the yeast lipase was increased at higher temperature but that no CLA conversion (from free linoleic acid) occurred at any of the temperatures. The lower concentration of free CLA in the E3 cheese at 10°C compared with the other two cheeses at the same temperature was unusual and may have been an experimental error.

Table 6.9 Free and total (free plus esterified) CLA content in the C and E1, E2 and E3 cheeses after 2 months of ripening at different temperatures

Cheese	Free CLA (mg/g cheese)			Total (free plus esterified) CLA (mg/g cheese)		
	20°C	13°C	10°C	20°C	13°C	10°C
E1	0.42	0.17	0.12	4.8	5.0	5.1
E2	0.31	0.20	0.17	6.5	6.2	6.3
E3	0.50	0.17	0.03	4.3	4.2	4.2

6.3.7 Effect of different levels of yeast adjuncts on the ripening of cheeses at 20°C (1 kg blocks)

According to the initial hypothesis (Section 5.1), yeast adjuncts influence the ripening of cheese by the enzymes already produced before their addition to the cheese. Thus, the levels of the enzyme added to the cheese could be important. This section describes a screening study on the effects of different levels of *G. candidum* and *Y. lipolytica* cultures on the ripening of washed-curd, dry-salted cheese. Curd from the control vat C, which contained lactococcal starter culture, *Lb. fermentum*, *Lb. rhamnosus* and propionibacteria strain B6022, was used for this experiment. One kilogram of curd was

mixed with 20, 100 or 500 mL of yeast culture (experimental plan detailed in Table 6.1B) followed by pressing.

6.3.7.1 Cheeses with different levels of *G. candidum* adjunct culture

6.3.7.1.1 Fermentation by propionibacteria

Two of the fermentation products produced by propionibacteria from lactate (acetic acid and propionic acid) over 4 months of ripening at 20°C are shown in Figure 6.9A and 6.9B.

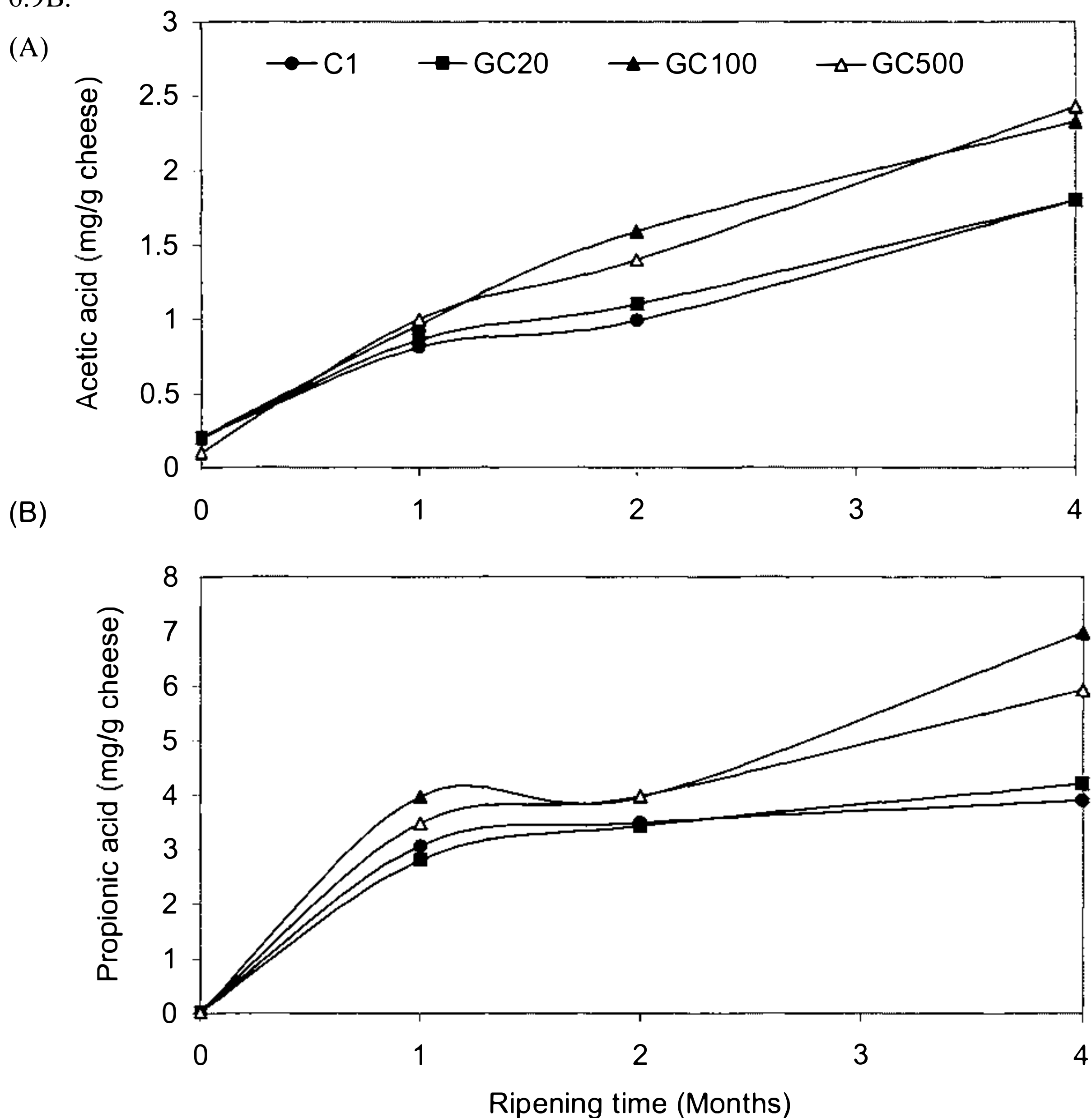


Figure 6.9 Production of acetic acid (A) and propionic acid (B) by propionibacteria in the presence of different concentrations of *G. candidum* adjunct culture over 4 months of ripening at 20°C.

The concentrations of both metabolites were higher in the cheeses containing 100 and 500 mL of *G. candidum* culture (GC100 and GC500) than in the control cheese (C1, no yeast adjunct), whereas the concentrations of acetic acid and propionic acid were similar in the C1 and GC20 cheeses. The results showed that higher concentrations of *G. candidum* adjunct culture (the yeast or the culture fluid) had some stimulating effect on the metabolism of propionibacteria and confirmed the observation made earlier for the C and E1 cheeses (Figure 6.1).

6.3.7.1.2 Lipolysis

The total FFA concentrations originating from milk fat detected by GC–FID in cheeses with different levels of *G. candidum* adjunct culture over 4 months of ripening at 20°C are presented in Figure 6.10.

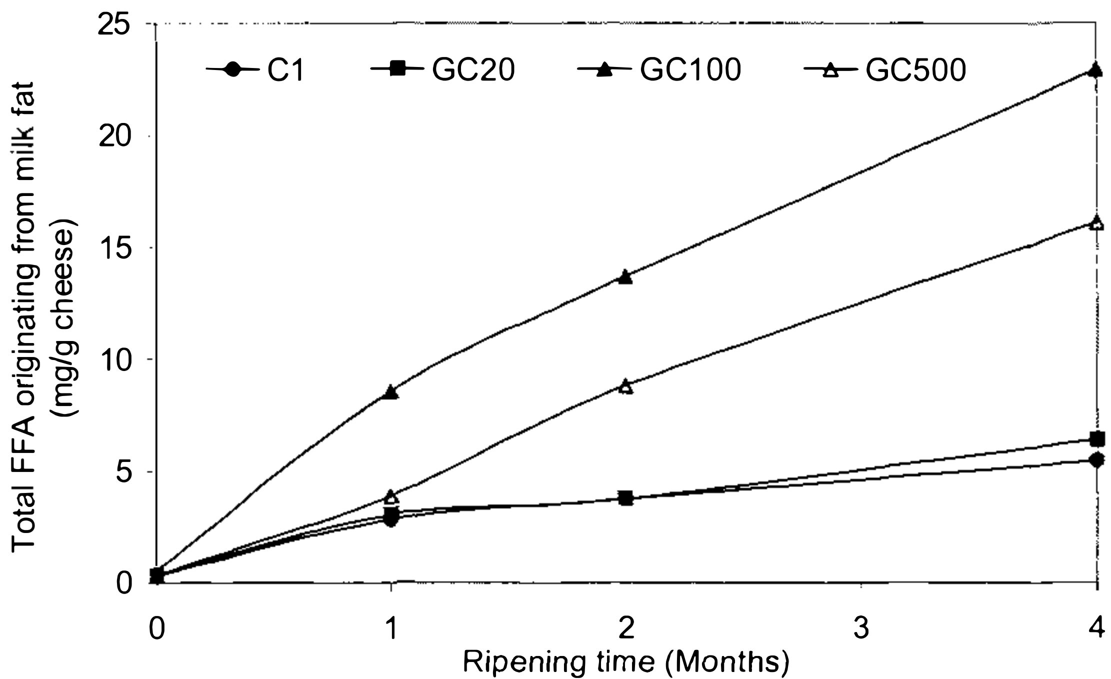


Figure 6.10 Production of total FFAs in cheeses with different levels of *G. candidum* adjunct culture over 4 months of ripening at 20°C.

The total FFA concentrations in the C1 and GC20 cheeses were similar throughout the ripening period. The total FFA concentrations after 4 months of ripening were 5.44 and 6.4 mg/g cheese respectively. The total FFA concentrations were higher in the GC100 and GC500 cheeses. After 4 months of ripening, the total FFA concentrations were 22.9 and 16.09 mg/g cheese in the GC100 and GC500 cheeses respectively. The total FFA

concentration was higher in the GC100 cheese than in the GC500 cheese, which was not expected and is discussed later (Section 6.4).

The concentrations of individual FFAs in the cheeses with different levels of adjuncts were studied to confirm the selectivity results obtained for Cheddar cheese (Section 5.3.1.2) and in the larger blocks of washed-curd, dry-salted cheese (Section 6.3.3). The FFAs with an even number of carbon atoms (C4 to C18:2) were determined after 1, 2 and 4 months of ripening. The long-chain unsaturated FFAs (C18:1 and C18:2) were selectively released from milk fat in the GC100 and GC500 cheeses, in which the lipolyses were influenced by *G. candidum* lipase. The data are not presented graphically as they follow the same pattern as shown in Figure 5.2. However, all the data for FFAs in the cheeses with different levels of *G. candidum* adjunct after 1, 2 and 4 months of ripening are provided in Appendix 6.7A (as mg/g cheese) and Appendix 6.7B (as a % of total FFAs).

The ratios between long-chain unsaturated and saturated FFAs in the C1 and GC20 cheeses were similar to the ratio between the respective total fatty acids (free plus esterified) in the reference cheese (0.5). This was expected because the *G. candidum* lipase did not affect the lipolysis in the GC20 cheese (Figure 6.10). The ratios in the GC100 and GC500 cheeses were around double the ratio between respective total (free plus esterified) unsaturated and saturated fatty acids in the reference cheese. There was no appreciable difference between the ratios in the GC100 and GC500 cheeses. The results confirm the selectivity of *G. candidum* lipase towards the long-chain unsaturated fatty acids, which was described in Section 5.3.1.2.

CLA was not determined in these cheeses because it was already concluded from Section 6.3.4 that propionibacteria did not produce CLA even though free linoleic acid was present.

6.3.7.1.3 Production of volatile compounds

Volatile compounds were detected by headspace analysis using GC–MS in all cheeses after 1 day, 1 month, 2 months and 4 months. The volatile compounds detected in 4-

month-old cheeses with different levels of *G. candidum* are shown in Table 6.10. Only the compounds with relative peak areas over a million (10^6) units were considered.

Table 6.10 Volatile compounds produced in cheeses with different levels of *G. candidum* after 4 months of ripening at 20°C

Volatile compounds	Relative peak area (x 10^6)			
	C1	GC20	GC100	GC500
Alcohol				
Ethanol	3.1	5.7	3.8	6.8
Esters				
Ethyl propanoate	1.5	1.3	1.4	1.6
Ethyl butanoate	nd*	1.3	2.4	2.6
Ethyl hexanoate	nd	1.1	2.4	2.8
Ethyl octanoate	nd	nd	nd	1.1
Ketones				
2-Methyl-2-butanone	nd	nd	2.1	1.6
5-Methyl-2-hexanone	4.3	4.1	9.1	7.6
Acids				
Acetic acid	79.6	151.0	136.0	155.0
Propanoic acid	232.0	327.7	346.0	350.0
3-Methyl propanoic acid, 2-Methyl propionic acid	7.2	8.1	2.2	2.9
Butanoic acid	48.4	86.6	243.0	164.0
2-Methyl butanoic acid	34.8	37.9	29.1	39.7
Hexanoic acid	26.1	58.6	174.0	135.0
Octanoic acid	4.2	9.3	46.3	31.7
n-Decanoic acid	nd	1.3	5.1	4.0

* = Not detectable.

The concentrations of the ethyl esters of propanoic acid were similar in all cheese samples, reflecting their likely origin to be from propionibacteria or starter activity. Ethyl esters of butanoic acid and hexanoic acid were not detected in the control cheese but were detected in the cheeses containing *G. candidum*. The concentrations were higher in the GC100 and GC500 cheeses than in the GC20 cheese. The ethyl ester of octanoic acid was present only in the GC500 cheese. The concentrations of 2-methyl-2-butanone and 5-methyl-2-hexanone were higher in the GC100 and GC500 cheeses than in the C1 and GC20 cheeses. The concentrations of acetic acid and propanoic acid were higher in the cheeses made with *G. candidum* adjunct. The results confirmed the patterns of acetic acid and propionic acid concentrations detected by GC–FID (Section 6.3.7.1.1). Propionic acid is definitely a product of lactate fermentation by propionibacteria, whereas acetic acid could originate from lactate fermentation by propionibacteria, amino acid fermentation, citric acid fermentation or metabolism of NSLAB. The concentrations of 3-methyl propionic acid and 2-methyl propionic acid were higher in the C1 and GC20 cheeses than in the GC100 and GC500 cheeses. The concentrations of butanoic, hexanoic, octanoic and n-decanoic acids followed the order C1 < GC20 < GC500 < GC100. These volatile compounds followed the same order as the total FFA concentrations (Figure 6.10). These acids were probably produced from the milk fat by hydrolysis.

6.3.7.2 Cheeses with different levels of *Y. lipolytica* adjunct culture

6.3.7.2.1 Fermentation by propionibacteria

Production of acetic acid and propionic acid from lactate fermentation by propionibacteria (for the data, see Appendix 6.8) followed the same trend as in the cheeses with *G. candidum* (Figure 6.9). The concentrations of these metabolites were higher in the cheeses manufactured with *Y. lipolytica* adjuncts than in the control cheese.

6.3.7.2.2 Lipolysis

The total FFA concentrations detected by GC–FID in the cheeses with different levels of *Y. lipolytica* adjunct over 4 months of ripening at 20°C are presented in Figure 6.11.

The total FFA concentration increased throughout the ripening period and the rate increased with an increase in the level of *Y. lipolytica* adjunct added. After 4 months of ripening at 20°C, the total FFA concentrations were 5.44, 15.52, 21.71 and 37.13 mg/g cheese in the C1, YL20, YL100 and YL500 cheeses respectively. This shows that the level of total FFAs had not reached its limit even with the addition of *Y. lipolytica* culture at 500 mL/kg curd.

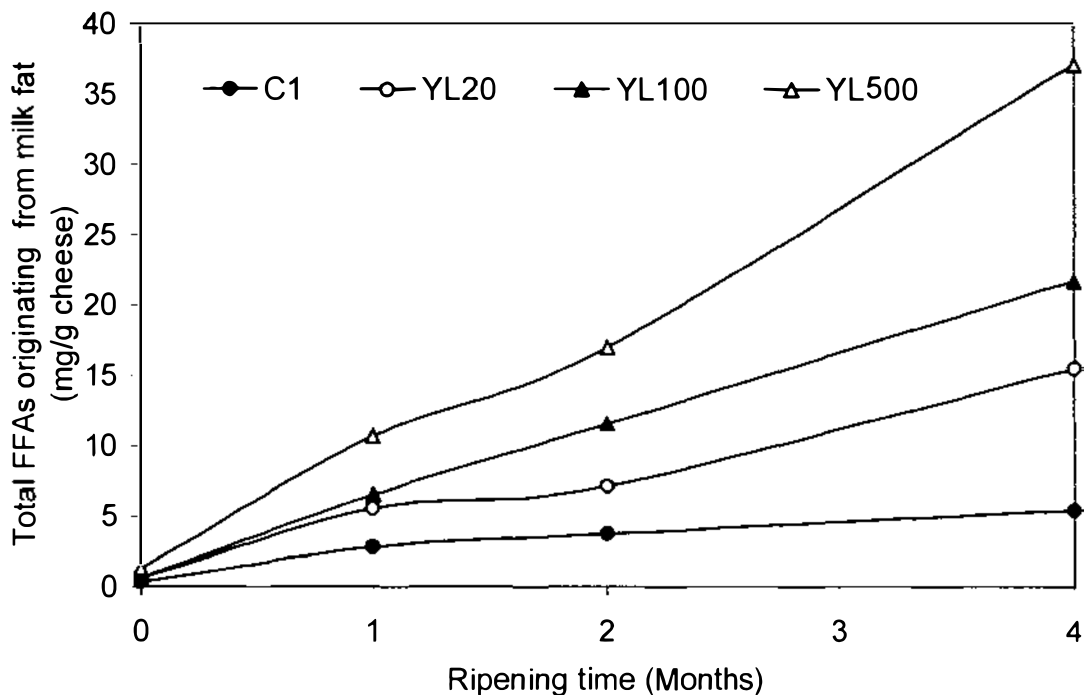


Figure 6.11 Production of total FFAs in cheeses with different levels of *Y. lipolytica* adjunct culture over 4 months of ripening at 20°C.

The concentrations of individual FFAs in the cheeses with different levels of *Y. lipolytica* were studied to confirm the selectivity results of the yeast lipase achieved in Cheddar cheeses (Section 5.3.2.2) and the large blocks of washed-curd, dry-salted cheese (Section 6.3.3). The FFAs with an even number of carbon atoms (C4 to C18:2) were determined after 1 month, 2 months and 4 months of ripening. A comparison of the proportions of the individual FFAs in the 4-month-old cheeses with the total fatty acid (free plus esterified) profile in the reference cheese is shown in Figure 6.12. The FFAs of the cheeses at 1 and 2 months followed the same pattern. The detailed FFA data for the cheeses at 1, 2 and 4 months of ripening are given in Appendix 6.9A (as mg/g cheese) and Appendix 6.9B (as a % of total FFAs).

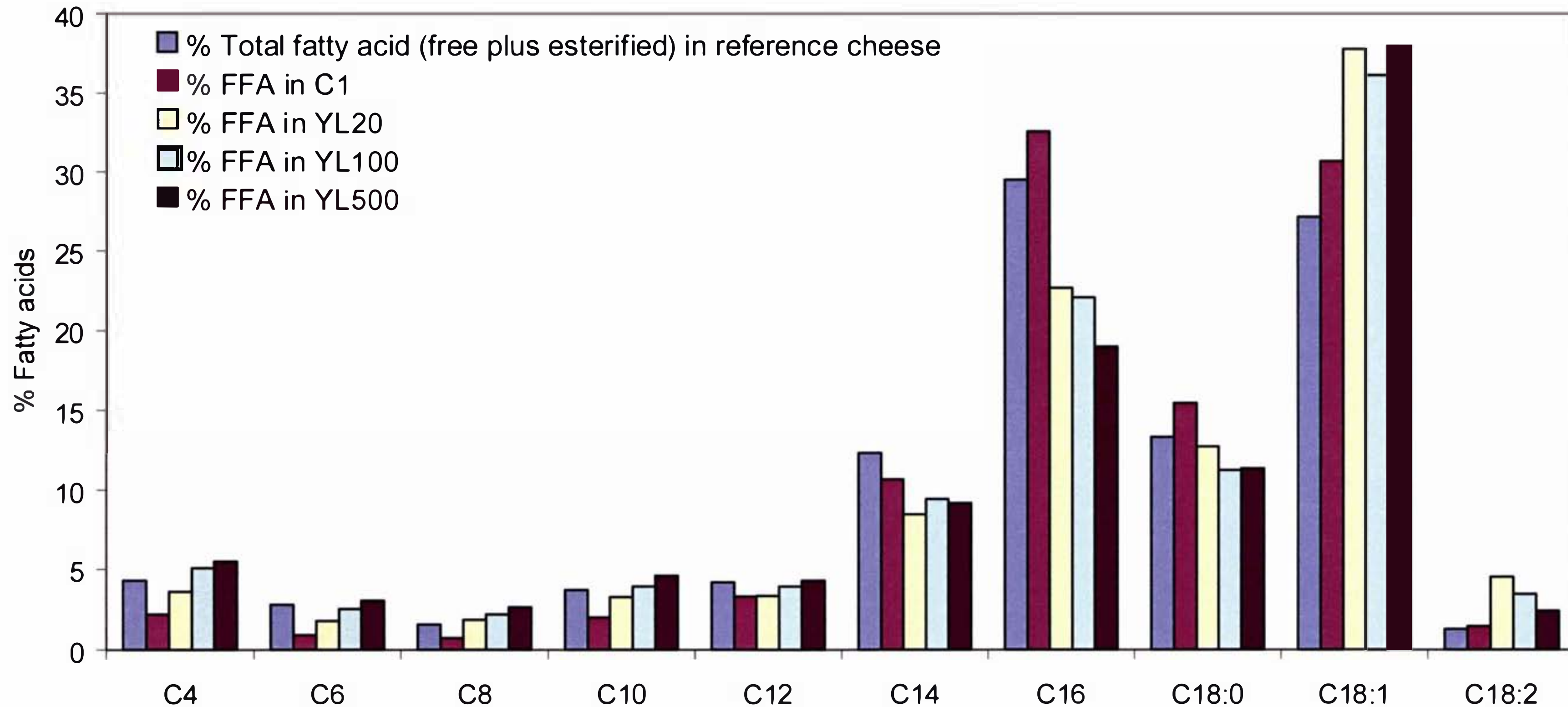


Figure 6.12 Comparison of the proportions of the individual FFAs expressed as a % of the total FFAs from milk fat in 4-month-old (ripened at 20°C) cheeses made without (C1) and with different levels of *Y. lipolytica* (YL20, YL100 and YL500) with the total fatty acid (free plus esterified) profile of the reference cheese.

The comparisons of the proportions of FFAs in the cheeses with *Y. lipolytica* adjunct with the proportions of the total fatty acids (free plus esterified) in the reference cheese followed the same pattern as shown for the Cheddar cheeses made with *Y. lipolytica* adjunct (Y4, Section 5.3.2.2). Selectivity of *Y. lipolytica* lipase towards the long-chain unsaturated fatty acids was noted in the cheeses with all levels of *Y. lipolytica* adjunct. The ratios between the long-chain unsaturated (C18:1 and C18:2) and saturated (C14, C16 and C18) FFAs ranged between 0.9 and 1.3 in the cheeses containing *Y. lipolytica*, whereas the ratio for the control cheese and the ratio between the respective total fatty acids (free plus esterified) in the reference cheese were 0.5. The proportion of C4 FFA was slightly higher in the YL100 and YL500 cheeses than the proportion of total C4 fatty acid (free plus esterified) in the reference cheese, as seen in the Y4 cheese (Section 5.3.2.2). However, there was no increase in the proportion of C4 FFA in the YL20 cheese. The results show that the selectivity of *Y. lipolytica* lipase was obvious at all concentrations and that the selectivity was retained after ultrafiltration.

6.3.7.2.3 Production of volatile compounds

Volatile compounds were detected by headspace analysis using GC–MS in all cheeses at 1 day, 1 month, 2 months and 4 months. The volatile compounds detected in 4-month-old cheeses with different levels of *Y. lipolytica* are presented in Table 6.11, showing compounds with peak areas of over a million (10^6) units.

Ethyl esters of butanoic acid and hexanoic acid were present only in the cheeses with *Y. lipolytica* adjunct. Ethyl octanoate and 2-methyl pentanal were present in the YL100 and YL500 cheeses but were absent in the C1 and YL20 cheeses. The concentration of 5-methyl-2-hexanone increased with an increase in the level of *Y. lipolytica* adjunct culture. The concentrations of acetic acid and propanoic acid were higher in the cheeses with *Y. lipolytica* adjunct culture than in the control cheese, which confirms the results of the GC–FID analyses (Section 6.3.7.2.1). The concentrations of the mixture of 3-methyl propanoic acid and 2-methyl propanoic acid and of 2-methyl butanoic acid were higher in the C1 and YL20 cheeses than in the cheeses with higher levels of yeast adjunct (YL100 and YL500). The concentrations of butanoic, hexanoic, octanoic and n-decanoic acids increased with an increase in the level of yeast adjunct.

Table 6.11 Volatile compounds produced in cheeses with different levels of *Y. lipolytica* after 4 months of ripening at 20°C

Volatile compounds	Relative peak area (x10^6)			
	Control	YL20	YL100	YL500
Alcohol				
Ethanol	3.2	4.1	4.5	2.9
Esters				
Ethyl propanoate	1.5	1.2	1.4	1.5
Ethyl butanoate	nd*	1.6	1.6	1.9
Ethyl hexanoate	nd	1.6	2.6	2.6
Ethyl octanoate	nd	nd	1.8	1.8
Ketones				
3- Methyl-2-butanone	1.9	1.7	1.2	2.1
5-Methyl-2 hexanone	4.3	6.6	6.9	12.0
Aldehyde				
2-Methyl pentanal	nd	nd	1.2	1.7
Acids				
Acetic acid	79.6	116.0	174.0	114.0
Propanoic acid	232.0	297.0	402.0	308.0
3-Methyl propanoic acid, 2-Methyl propanoic acid	7.2	6.1	3.0	1.0
Butanoic acid	48.4	149.0	251.0	317.0
2-Methyl butanoic acid	45.8	52.6	31.0	11.8
Hexanoic acid	26.1	122.0	191.0	327.0
Octanoic acid	4.2	38.5	77.0	93.5
n-Decanoic acid	nd	4.4	12.0	17.8

* = Not detectable.

6.4 Discussion

The results in Sections 6.3.3, 6.3.7.1.2 and 6.3.7.2.2 indicate the presence of one or more lipase(s) in *G. candidum* and *Y. lipolytica* that preferentially hydrolyse(s) long-chain unsaturated fatty acids from milk fat in the cheese environment. The lipase of *G. candidum* is known to show specificity for the hydrolysis of unsaturated fatty acids with a *cis* double bond at the 9-position (oleic, linoleic and linolenic acids) over the corresponding saturated fatty acid (stearic acid) (Jensen, 1974). Baillargeon *et al.* (1989) evaluated the fatty acid specificity of different strains of *G. candidum* and found that the lipases from strains NRRL Y-553 and 26557RP displayed preferential specificity for hydrolysing oleic acid esters, whereas lipases from strains ATCC34614 and NRRL Y-552 and lipase GC-4 failed to discriminate between palmitic acid and oleic acid. Thus, strains of *G. candidum* have two types of lipase. No officially recognised nomenclature exists to distinguish the fatty acid specificity of lipases. However, Charton *et al.* (1992) introduced a nomenclature for *G. candidum* lipases as types A and B. Lipase type A does not have specificity for unsaturated fatty acids with *cis*-9 double bonds, and lipase type B has high specificity for fatty acids containing a *cis*-9 double bond. Bertolini *et al.* (1994) found two different genes in *G. candidum* coding for lipases A and B. The specificity of *G. candidum* lipase towards long-chain unsaturated fatty acids was also noted in the Cheddar cheese study (Section 5.3.1.2).

Unlike *G. candidum* lipase, only limited published information on the selectivity of *Y. lipolytica* lipase is available. Suzzi *et al.* (2001) studied the lipolytic activity of several *Y. lipolytica* strains isolated from dairy products and found that in all strains the major FFA released was C18:1 followed by C16, with the exception of two strains in which C16 represented the most prominent fatty acid released, followed by C18:1. The specificity of *Y. lipolytica* lipase towards long-chain unsaturated fatty acids was also noted in the Cheddar cheese study (Section 5.3.2.2). The specificity of yeast lipase was shown earlier in synthetic media and vegetable oil; the present study establishes that these lipases with specificity towards particular fatty acid(s) can hydrolyse milk fat in the cheese environment.

The total FFA concentration was higher at a ripening temperature of 20°C than at 13 or 10°C. Temperature certainly has an effect on lipase (enzyme) activity. Baillargeon *et al.*

(1989) reported that the lipase of *G. candidum* was more active at 30°C than at 20 or 40°C. Sinigaglia *et al.* (1994) reported that interaction between the temperature and the enzymatic activities of *Y. lipolytica* depended on the source of isolation. They showed that the lipase activity of *Y. lipolytica* isolated from irradiated poultry meat decreased when the temperature was increased from 3 to 25°C but that the lipase activity behaved in the opposite way for a *Y. lipolytica* strain isolated from chilled foods. The higher proportions of unsaturated fatty acids in the total FFAs at lower temperatures in the present study (Table 6.8) suggest that the lipase(s), specific to the unsaturated fatty acids, is (are) more active at lower temperatures.

The results for the FFAs in 1 kg blocks of cheeses with different levels of yeast adjunct showed that there was no effect of *G. candidum* at 20 mL/kg cheese curd. The FFA concentration was higher in the cheese with 100 mL of *G. candidum* than in the cheese with 500 mL of *G. candidum*. This result was not expected. It has been hypothesised that the lipase, which is produced in the yeast cultures before their addition to the cheese, catalyses the measured lipolysis associated with the yeast adjuncts. A higher level of adjunct culture means a higher concentration of lipase, which is expected to produce higher concentrations of FFAs. However, a significant part of the lipase appeared to have been lost while concentrating 500 mL of culture to 100 mL by ultrafiltration (molecular weight cut-off 10 000 Da; at 4°C for approximately 16 h) before its addition to the cheese curd. This would need to be checked in further developments by assaying the lipase activity in the ultrafiltration concentrate and permeate. Baillargeon *et al.* (1989) found that lipase activity in the culture filtrate of *G. candidum* decreased with time and the decrease has been attributed to the presence of proteinase. Tahoun *et al.* (1982) also noted the instability of *G. candidum* lipase.

Y. lipolytica adjunct at all levels increased the concentration of total FFAs in the cheese. The total FFA concentrations increased with an increase in the level of *Y. lipolytica* adjunct. This was expected and agrees with the hypothesis that the enzymes, which are produced in the yeast cultures before their addition to the cheese, catalyse the biochemical changes during cheese ripening. It seems that, unlike *G. candidum* lipase, *Y. lipolytica* lipase is stable throughout the long ultrafiltration process. Pereira-Meirelles *et al.* (1997) demonstrated the stability of *Y. lipolytica* lipase, which remained 100% stable for at least 370 days at 5°C without any additives.

The concentration of free CLA increased in the cheeses when the yeast adjuncts were added but the total CLA concentration remained the same (Section 6.3.4). Likewise, whereas the free linoleic acid concentration in the cheeses containing yeast adjuncts showed a further increase with the addition of safflower oil, the free CLA concentration was similar to that in the cheese without added oil. This indicates that the free linoleic acid released from the milk fat and the higher concentration released from the addition of oil were not converted to CLA by propionibacteria. Jiang *et al.* (1998) proposed that the conversion of free linoleic acid to CLA could be a detoxification mechanism of the *P. freudenerichii* ssp. *shermanii* strains, as free linoleic acid is toxic to some strains of propionibacteria. The propionibacteria strains used in the present study were capable of converting free linoleic acid to CLA in laboratory media (Appendix 6.1). The densities of the propionibacteria strains were 10^8 cfu/g in the cheese after 2 months of ripening (Table 6.4) and these strains actively produced acetate and propionate throughout the ripening period (Figure 6.1), but there was no CLA conversion. There could be several reasons for the failure of the propionibacteria strains to convert free linoleic acid to CLA in the cheese environment. The most likely general reason is that the cheese environment, as opposed to the laboratory media, may not have been conducive to such conversion. The lower pH (Rainio *et al.*, 2002) or the lower water activity of the cheese may have adversely affected the activity of the isomerase. In the complex cheese matrix, free linoleic acid may not have come into contact with propionibacteria cells. The presence of the other FFAs may have affected the activity of the isomerase enzyme.

The yeast cultures may have had a stimulating effect on the metabolism of the propionibacteria, which was indicated in the larger cheese blocks (Section 6.3.2) and confirmed in the smaller blocks (Sections 6.3.7.1 and 6.3.7.2). The concentrations of acetic acid and propionic acid were higher in the cheeses containing yeast adjuncts (except GC20) than in the control (no yeast adjunct). The higher stimulating effect of the yeasts in the small blocks than in the large blocks can be explained by the ways in which the yeast adjuncts were added. The yeast adjuncts were added in the cheese milk for the large blocks and the putative stimulating factor(s) contained in the culture supernatant may have been drained out with the whey. In contrast, the yeast adjuncts were added to the cheese curd for the small blocks and more of the culture supernatant containing putative stimulating factors would have been retained. The enhanced survival of dairy bacterial cultures in the presence of yeast cultures in the cheese

environment has been reported previously (Ferreira and Viljoen, 2003). Yeast extract, as an additive to the Cheddar curd, has been reported to improve the growth of NSLAB, which eventually improved as well as accelerated the cheese flavour (Rehman *et al.*, 2003).

The relative concentrations of ethyl esters (C4–C8) were higher in the cheeses made with yeast adjuncts than in the control cheese (Section 6.3.5). One or more of the yeast enzymes – esterase, acetyltransferase (Kallel-Mhiri and Milco, 1993) or lipase (Leblanc *et al.*, 1998) – could have been responsible for producing the esters. The concentrations of the esters were higher (about 10 times) in the large blocks of the cheeses containing yeast adjuncts than in the small blocks of the cheeses containing yeast adjuncts. The limiting factor was probably the production of ethanol. Although both large and small blocks contained the same *Lb. fermentum* adjunct culture, the concentration of ethanol was approximately 10 times higher in the large blocks than in the small blocks. The sizes of the cheese blocks may have influenced the metabolism of the *Lb. fermentum* (the large blocks may have been more anaerobic than the small blocks).

Lb. rhamnosus culture was used to keep the NSLAB population consistent, so that the ripening of the cheese was not influenced by the different adventitious NSLAB population. At 1 day after manufacture, the number of NSLAB was high (10^6 cfu/g cheese; not shown) and the source of the NSLAB population was likely to have been the added NSLAB cultures (both *Lb. rhamnosus* and *Lb. fermentum*) considering the good manufacturing practices followed and the results of earlier cheese trials carried out in the same plant (Crow *et al.*, 2001, 2002). The similarity between the profiles of the volatile compounds (other than lactate fermentation products; Appendix 6.4) and the total FFA concentrations (Figure 6.2) in the three cheeses made with the same yeast adjuncts and different strains of propionibacteria indicate that the cheese ripening was not differentiated by the adventitious NSLAB flora.

6.5 Conclusions

The objective of producing high concentrations of esters to mask undesirable effects of free long-chain fatty acids was achieved. This gives a useful tool for producing targeted ester compounds in the cheese by the combined use of yeast cultures and bacterial

adjuncts. The use of yeast lipase and propionibacteria together to elevate the concentrations of CLA during the ripening of cheese was not successful. The first step of the conversion process, selective release of unsaturated fatty acids from milk triglycerides, was achieved but the second step of the conversion process, *i.e.* conversion of free linoleic acid to CLA, did not occur in the cheese environment (low pH and a_w). A pH-controlled cheese slurry medium could be tried in future to provide the propionibacteria with a near ideal environment (higher pH and a_w). Further study could be carried out using other dairy bacteria, such as *Lactobacillus*, which also showed capacity for CLA synthesis in laboratory media (Section 2.4.4.2).

The level of yeast adjunct added influenced the ripening of cheese, which is also proof of the hypothesis that the yeasts influence cheese ripening by the enzymes already produced before their addition to the cheese. The total FFA concentration was still increasing with the addition of 500 mL *Y. lipolytica*/kg curd, suggesting that the hydrolytic capacity had not reached a saturation level. Concentration of yeast culture beyond fivefold could be difficult to achieve by ultrafiltration; a freeze-dried form of the yeast cultures could be tried in future to determine the effect of higher levels of yeast culture (> 500 mL culture/kg curd) on cheese ripening.

Chapter 7. Production of volatile aroma compounds by *C. kefyr* through L-phenylalanine metabolism

7.1 Introduction

In the previous experiments on the characterisation of three dairy yeasts (Chapter 4), phenyl ethyl alcohol (PE) was detected by gas chromatography–mass spectrometry (GC–MS) (Section 4.2.3) in the cultures of all three yeasts studied — *Geotrichum candidum*, *Yarrowia lipolytica* and *Candida kefyr*. *C. kefyr* produced the highest relative peak area for PE and was the only yeast studied that produced phenyl ethyl acetate (PEA).

PE and PEA have a rose-like odour that is popular and desired, making PE the most-used fragrance chemical in perfume and cosmetics (Clark, 1990). It is also used in flavour compositions for foods such as candy, confectionery and beverages. PE can be synthesised chemically but naturally produced PE is preferred for food applications (Etschmann *et al*., 2002).

PE and PEA play very important roles in imparting the characteristic aroma of soft cheeses (Adda *et al*., 1978). Yeasts were found to be capable of producing these aromatic compounds in raw milk Camembert cheese (Lee and Richard, 1984).

Production of these aromatic compounds by yeasts is an obvious option for a natural source. Production of these aromatic compounds in cheese is not an option, because the yeasts in the cheese died out during ripening (Section 6.3.1), whereas these compounds are produced by metabolically active yeasts. This explains the absence of PE and PEA in the cheeses made with *G. candidum* and *Y. lipolytica* adjuncts (Chapters 5 and 6) even though these compounds were produced by both yeasts in laboratory media (Sections 4.3.1.4 and 4.3.2.4). There is some published information on the production of PE using different yeast types but no information on the use of *C. kefyr* was available. Thus a short investigation of PE and PEA production by *C. kefyr* in laboratory media was in the scope of the present study.

The most likely source of PE is the metabolism of the amino acid L-phenylalanine (L-phe) by the Ehrlich pathway, where L-phe is transformed to PE via phenylpyruvate and phenylacetaldehyde (Ehrlich, 1907; cited in Etschmann *et al.*, 2002). L-phe metabolism in yeast may be carried out by the cinnamate pathway. In this pathway, trans-cinnamate is either produced as an end product or catabolised further to 3-ketoadipate, which goes into the tricarboxylic acid (TCA) cycle (Large, 1986). PE can be synthesised in yeast by *de novo* synthesis via the shikimate pathway, although this pathway is not a significant route of PE synthesis (Etschmann *et al.*, 2002). These pathways are discussed in more detail in Section 2.5.1.

The production of PE and other aromatic compounds by yeasts is influenced by the components of the medium and the growth conditions, reviewed in Sections 2.5.1.1 and 2.5.1.2 respectively. The concentration of L-phe in the medium is the most important parameter influencing the production of PE and associated compounds by yeasts (Fabre *et al.*, 1998). Fabre *et al.* (1998) showed that yeasts can metabolise only the natural isomer L-phe but not the D-phe isomer. The source and the concentration of the carbon and nitrogen also have influences on the formation of these aromatic compounds. Glucose (Fabre *et al.*, 1998) and sucrose (Huang *et al.*, 2000) were found to be efficient for the production of PE by yeast strains. Yeast extract was reported to be the best nitrogen source for the production of PE and PEA by *Pichia fermentans* L-5 (Lee, 1998; cited in Huang *et al.*, 2000). Yeast extract serves as a source of both nitrogen and vitamins.

An experiment to study the effects of different initial concentrations of L-phe on the production of PE and PEA by *C. kefyr* in laboratory media was designed. The production of benzaldehyde, which is an intermediate compound produced in the cinnamate pathway, was also followed. Benzaldehyde is also an important aroma compound and imparts an almond flavour (Groot and de Bont, 1998). Major volatile compounds, not originating from L-phe, were also followed to delineate the effects of PE and associated compounds on the other reactions in the yeast cells. The catabolism of L-phe by *C. kefyr* in the presence of different concentrations of glucose was also studied. The growth parameters were kept the same as used in the earlier study (Chapter 4).

7.2 Materials and methods

7.2.1 Growing the cultures

Culture

C. kefyr (B9006) culture was obtained from the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, and was the same as described in Chapter 4.

Materials

Medium components: D-glucose, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, KH_2PO_4 and KH_2PO_4 were supplied by BDH Laboratory Supplies, Poole, BH15 1TD, England. Yeast extract and L-phe were supplied by Becton, Dickinson and Company (Sparks, MD 21152, USA) and Sigma Chemical Co. (P.O. Box 14508, St Louis, MO 63178, USA) respectively.

Method

x% (w/v) of glucose, y% (w/v) of L-phe (the percentage varied according to the experiment, detailed in Section 7.3), 0.25% (w/v) of yeast extract and 0.05 % of each salt were dissolved in milli-Q water. The pH of the solution was adjusted to 6.0 with 1 M NaOH or 1 M HCl. The solution was filter sterilised through a 0.22 μm sterile membrane (Millipore Corporation, Bedford, MA 01730, USA). The filtrate (100 mL) was aseptically transferred to a pre-sterilised 250 mL Erlenmeyer flask, capped with pre-sterilised cotton and covered by aluminium foil.

The growth studies and the measurement of dry cell weight were carried out according to the method described in Section 4.2.1. The correlation curve of OD_{600} values against dry cell weight is provided in Appendix 7.1.

7.2.2 Estimation of volatile compounds

Volatile compounds in the cultures were detected by GC–MS by the method described in Section 4.2.3 using the same instrument. The cultures were diluted with 0.05 M phosphate buffer (pH 6.0) to keep the peak areas of PE, PEA and benzaldehyde in the linear range of the correlation curve (Section 7.2.3).

7.2.3 Correlating the concentrations of metabolites and GC–MS peak areas

Correlation curves were derived for converting the GC–MS peak areas to the concentrations (mM) of PE, PEA and benzaldehyde.

Materials

1. PE (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA)
2. PEA (Fluka Chemie, Ag CH-9471, Buchs SG, Switzerland)
3. Benzaldehyde (Aldrich Chemical Co., Inc., Milwaukee, WI 53233, USA)
4. Phosphate buffer (0.05 M, pH 6.0) — prepared using K_2HPO_4 and KH_2PO_4 (BDH Laboratory Supplies, Poole, BH15 1TD, England)

Method

PE, PEA and benzaldehyde solutions of known concentration (mM) were prepared by diluting these chemicals in phosphate buffer (0.05 M, pH 6.0). The compounds in the solutions were detected by GC–MS (Section 4.2.3) and expressed as peak areas. Correlations between the peak areas and the concentrations were derived using Microsoft Excel. The standard curves along with the correlation equations are provided in Appendix 7.2A (PE), Appendix 7.2B (PEA) and Appendix 7.2C (benzaldehyde). The correlations were linear ($r^2 > 0.99$).

7.2.4 Estimation of L-phe

Estimation of L-phe was performed by enzymatic assay according to the procedure described in Shen and Abell (1985) with some modification.

Materials

1. Tris buffer (0.1 M, pH 8.75) and L-phe were supplied by Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA, and the reagents were made according to Shen and Abell (1985).
2. Phenylalanine ammonia lyase (PAL) from *Rhodotorula glutinis*, buffered aqueous glacial solution, grade I, 0.8–2 U/mg protein (one unit will deaminate 1.0 μM of L-phe to trans-cinnamate and NH_3 per minute at pH 8.5 at 30°C), was supplied by Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA. The enzyme was

diluted 20 times with 0.1 M tris buffer for use in the reaction mixture. The diluted enzyme solution was freshly prepared for each day's assay.

Method

Standard curve preparation: The reaction mixture contained 780 µL milli-Q water, 100 µL tris buffer, 100 µL L-phe solution and 20 µL PAL (total 1000 µL). The reaction mixture was incubated for 1 h at 30°C and then the absorbance was read at 290 nm. The absorbance readings were adjusted with the blank readings and were plotted against the concentrations of L-phe solution. The correlation equation was derived using Microsoft Excel. The correlation between the adjusted OD_{290} values and the concentrations of L-phe was linear ($r^2 > 0.97$) (Appendix 7.3).

Measurement of L-phe in the medium samples: The samples were diluted sufficiently and the same protocol, used for the standard curve preparation, was followed to obtain the adjusted readings. The concentrations of L-phe were calculated using the correlation equation.

7.2.5 Estimation of glucose

Estimation of glucose was performed by enzymatic assay, using Peridochrom glucose reagent.

Materials

1. D-glucose (BDH Laboratory Supplies, Poole, BH15 1TD, England)
2. Peridochrom glucose reagent (Boehringer Mannheim GmbH, Mannheim, Germany)

Method

Standard curve preparation: 100 µL of D-glucose solution was mixed with 1 mL of Peridochrom glucose reagent, vortexed and incubated at 25°C for 30 min, and the absorbance was read at 510 nm. The readings of the absorbances were adjusted with the reading of water and plotted against the concentrations of glucose solution (mM). A correlation equation was derived using Microsoft Excel. The correlation between the

adjusted OD_{510} values and the concentrations of glucose was linear ($r^2 > 0.99$) (Appendix 7.4).

Measurement of glucose in the medium samples: The samples were diluted sufficiently and the same protocol, used for the standard curve preparation, was followed to obtain the adjusted readings. The concentrations of glucose were calculated using the correlation equation.

7.2.6 Statistical analysis

Statistical analyses were carried out using SAS software version 8.2 (SAS Institute Inc., NC, USA).

7.3 Results

The dynamics of L-phe metabolism by *C. kefyr* and the effects of the initial concentrations of L-phe and glucose on the production of volatile compounds (PE, PEA, benzaldehyde, ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol) are described in this section. The fermentations were carried out at 30°C with 200 rev/min, and the initial pH values of the media were adjusted to 6.0.

The results for dry cell weight, pH, glucose and volatile compounds are the averages from two independent fermentations. However, the concentration of L-phe was determined in one of the two fermentations only.

7.3.1 Dynamics of L-phe metabolism by *C. kefyr*

C. kefyr was grown in a medium with an initial concentration of 13.9 mM L-phe and 61.2 mM D-glucose. Changes in the dry cell weight and the concentrations of glucose, L-phe, PE and PEA over 72 h are shown in Figure 7.1.

After a 12 h lag phase, the growth phase continued until 48 h, when the dry cell weight of *C. kefyr* reached its highest value (3.3 mg/mL), and then the dry cell weight started to decrease. The initial pH value of the medium was 6.0, dropped to 5.4 after 12 h and then increased to reach 7.7 at 72 h (pH values not shown in the figure). The initial drop in pH could have been due to the formation of organic acids in the fermentation of part of the glucose (Fabre *et al*., 1998). Acetic acid was detected in small concentrations (2–5 x 10^6 relative peak areas between 0 and 24 h) during the early stages and disappeared after 24 h. The increase in pH at the later stages could have been due to the production of NH_3 during catabolism of L-phe (Section 2.5.1). The yeast consumed 80.6% of the available L-phe (11.2 mM) and this occurred in the first 48 h. Most of the glucose was consumed between 12 and 24 h. The yeast consumed 6.4 mM of the glucose in the first 12 h (lag phase) and 54 mM between 12 and 24 h (growth phase). Continuing growth after the exhaustion of glucose suggests that the yeast might have used some of the metabolites produced from the glucose. Detection of ethanol (24 x 10^6 relative peak area) and

acetate (4 x 10^6 relative peak area) at 24 h and their disappearance at the later stages support this.

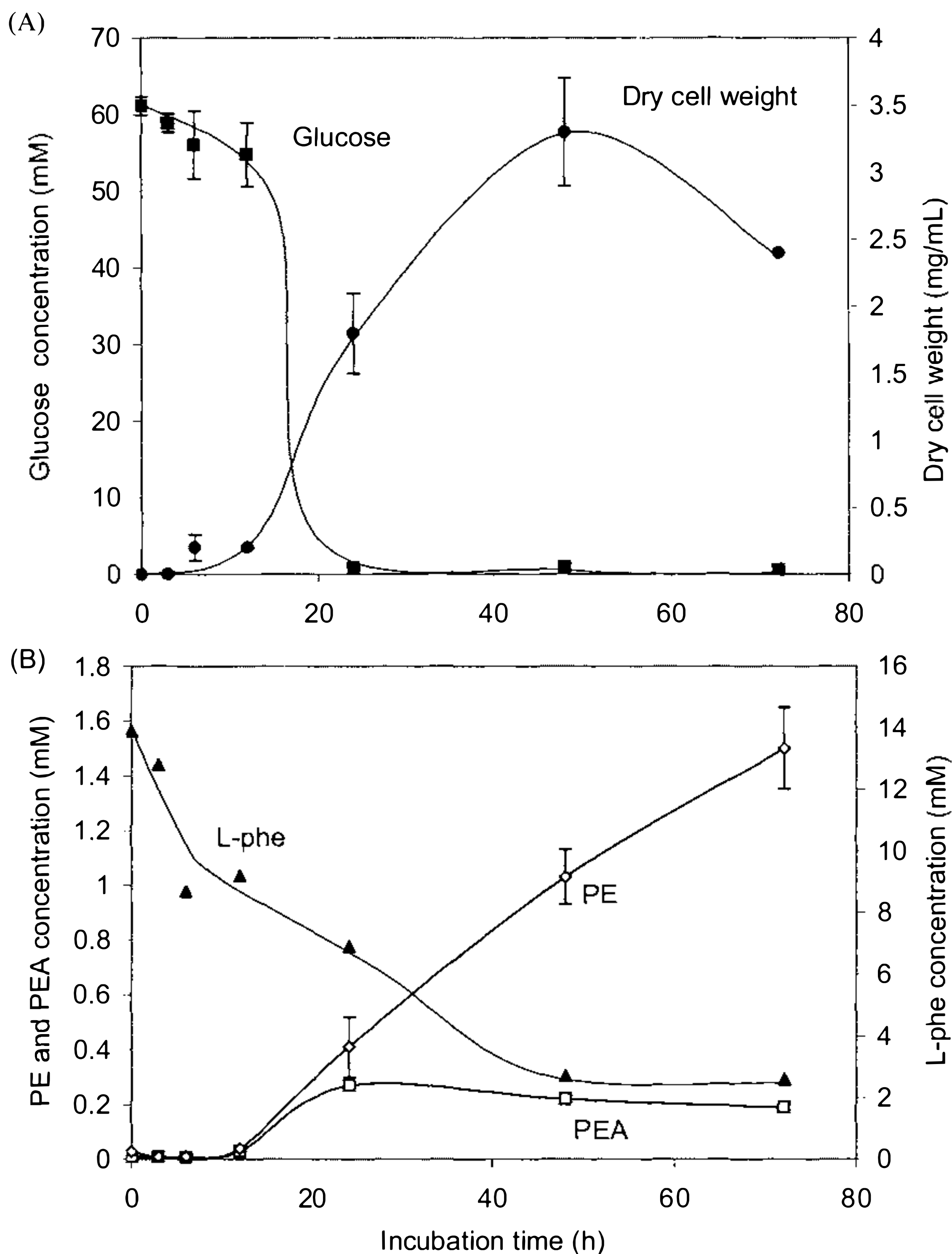


Figure 7.1 Dry cell weight and glucose concentration (A) and concentrations of L-phe, PE and PEA (B) in *C. kefyr* cultures over 72 h of incubation. The error bars represent standard deviations between two independent fermentations.

The concentration of PE was minimal (≤ 0.04 mM) until 12 h and then increased linearly (R^2 > 0.99) to reach 1.5 mM at 72 h. PEA was also first detected at 12 h, reached 0.27 mM at 24 h and then dropped to 0.19 mM at 72 h.

Only 15% of the L-phe consumed was transformed into PE and PEA (the conversion was calculated by dividing the number of moles of the metabolites produced by the number of moles of the substrate consumed). This suggests that the major portion of the L-phe consumed was converted to other products, presumably non-volatile compounds, which were not detected by GC–MS. L-phe was unlikely to have been used for energy as the growth data suggest that the yeast grew at a similar rate with or without L-phe in the medium (Appendix 7.5).

7.3.2 Effect of different initial concentrations of L-phe

C. kefyr was grown in media with different initial concentrations of L-phe. The medium for the control culture (A) did not have any added L-phe but had a background L-phe concentration of 0.1 mM, contributed by the components of the medium. The media for the experimental cultures had added L-phe; the initial concentrations of L-phe were 7.8 mM (B), 12.2 mM (C), 26.5 mM (D) and 50 mM (E). All media had the same initial concentration of glucose (61.2 mM) and pH (6.0).

The control culture (A) did not have any added L-phe and the background L-phe concentration was not detected after 24 h (Figure 7.2). The other cultures had different initial concentrations of added L-phe, which was consumed during the first 48 h of the incubation period; the consumption of L-phe did not continue after 48 h of incubation. At this incubation time, 7.2, 10.8, 16.3 and 22.5 mM L-phe had been consumed in cultures B, C, D and E respectively.

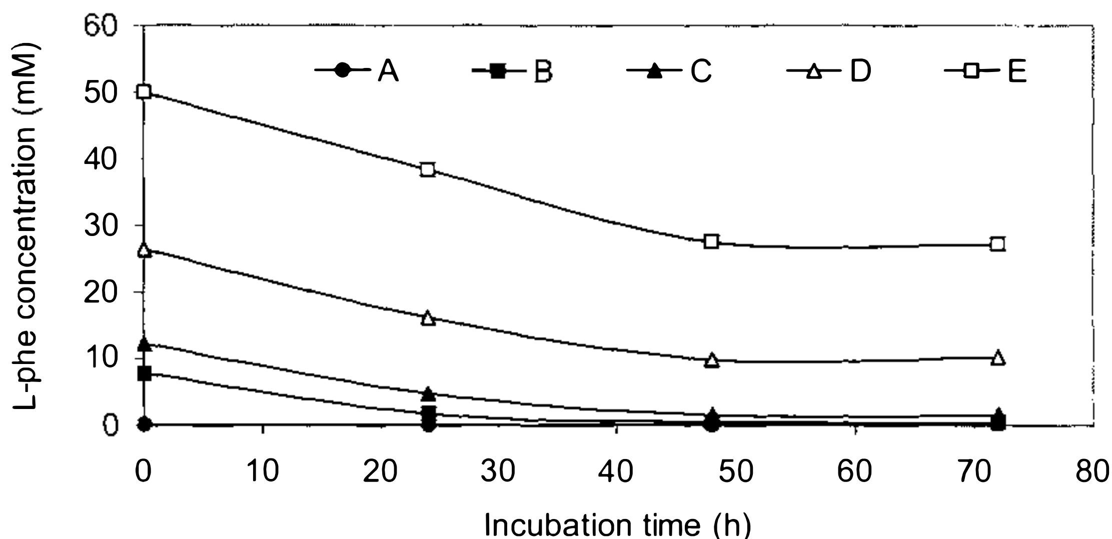


Figure 7.2 Concentrations of L-phe in *C. kefyr* cultures grown in media containing different initial concentrations of L-phe over 72 h of incubation. The initial concentrations of L-phe in the media for cultures A, B, C, D and E were 0.1, 7.8, 12.2, 26.5 and 50 mM respectively.

The concentrations of PE, PEA and benzaldehyde, the products of L-phe metabolism, were minimal ($\leq$ 0.1 mM) throughout the incubation period in the control culture A (Figure 7.3).

The concentration of PE increased until 48 h in all cultures with added L-phe. In cultures B and C, the PE concentration increased further until 72 h. The concentration was relatively unchanged between 48 and 72 h in culture D, and the concentration dropped during the same period in culture E.

The PE concentrations were significantly higher in the cultures with added L-phe (B, C, D and E) than in the control (A; $p \leq 0.01$) throughout the incubation period. The PE concentrations increased with an increase in the initial concentration of L-phe in the cultures. For example, at 72 h, the PE concentrations were 1.46 and 5.32 mM in the *C. kefyr* cultures containing 7.8 and 50 mM L-phe respectively. The exceptions were cultures B and C, which had similar PE concentrations (at 48 and 72 h) although they initially had different concentrations of L-phe (7.8 and 12.2 mM in cultures B and C respectively).

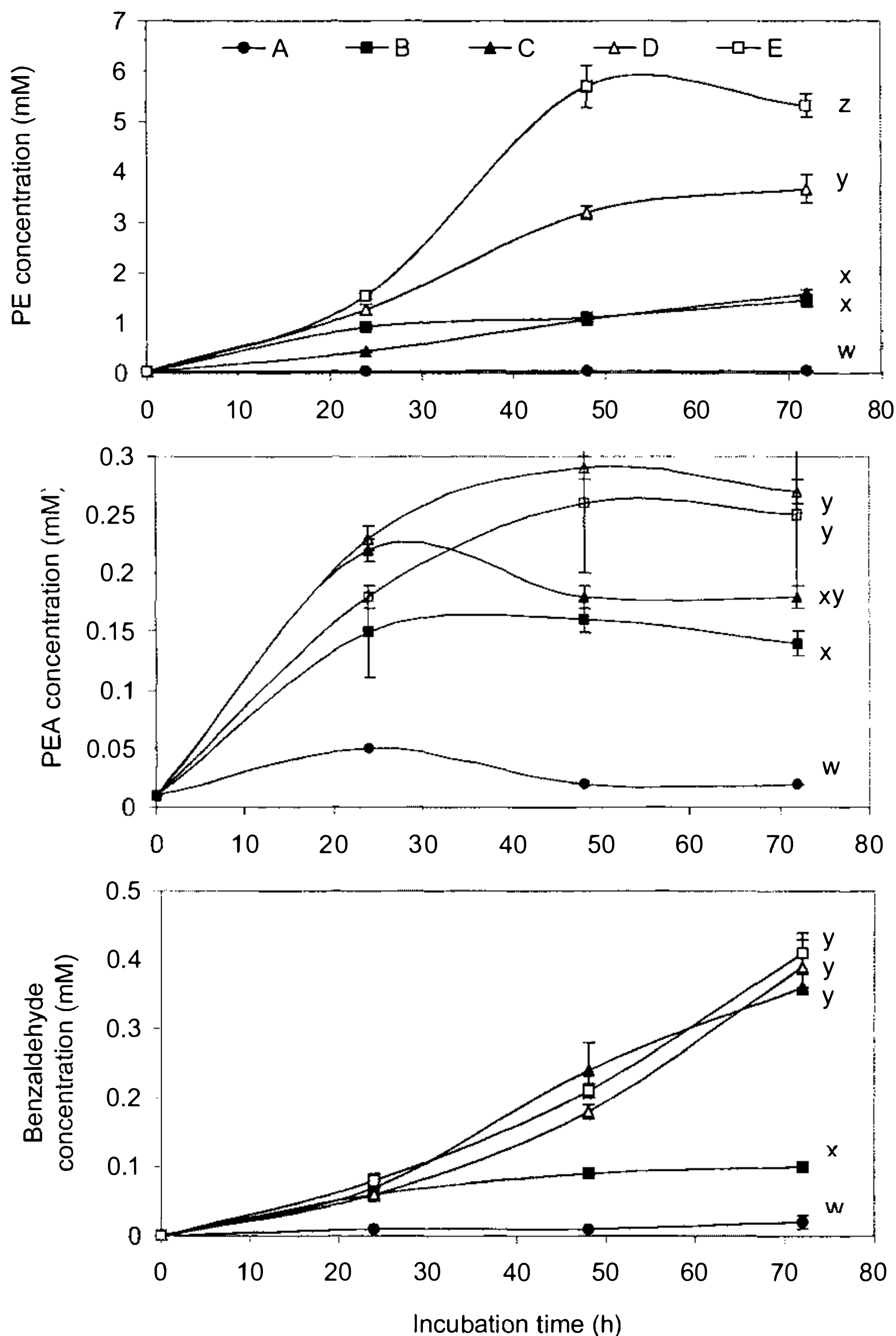


Figure 7.3 Production of PE, PEA and benzaldehyde in *C. kefyr* cultures with different initial concentrations of L-phe over 72 h of incubation. The letters w, x, y, and z indicate treatments that are significantly different at 72 h ($p \leq 0.01$). The error bars represent standard deviations between two independent fermentations.

In cultures B, D and E, the PEA concentrations increased until 48 h of incubation (0.16–0.29 mM at 48 h) and then reduced marginally between 48 and 72 h (0.14–0.27 at 72 h). The PEA concentration in culture C reached a maximum at 24 h (0.22 mM) followed by a drop between 24 and 48 h (0.18 mM at 48 h) and then remained unchanged. The PEA concentrations increased in the cultures with an increase in the initial concentration of L-phe, although there was no significant difference between cultures B and C and between cultures D and E at 72 h.

The benzaldehyde concentration in culture B increased until 48 h to reach 0.1 mM and then became relatively constant. However, in cultures C, D and E, the benzaldehyde concentrations increased throughout the incubation period to reach 0.36–0.41 mM at 72 h. Approximately fourfold concentrations of benzaldehyde in cultures C, D and E compared with culture B and similarity between the benzaldehyde concentrations in cultures C, D and E suggest that the initial concentration of L-phe is a limiting factor for benzaldehyde formation until a certain concentration of benzaldehyde (approximately 0.4 mM) is formed, after which a further increase in the initial concentration of L-phe does not result in an increase in benzaldehyde production.

After 72 h of incubation, 7.36, 10.69, 16.28 and 22.86 mM L-phe were consumed in cultures B, C, D and E respectively but only 23.1, 19.8, 26.6 and 26.2% of the L-phe consumed were used in the production of PE, PEA and benzaldehyde.

Ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol (Figure 7.4) were the three major volatile compounds detected in the cultures other than the volatile metabolites originating from L-phe (PE, PEA and benzaldehyde).

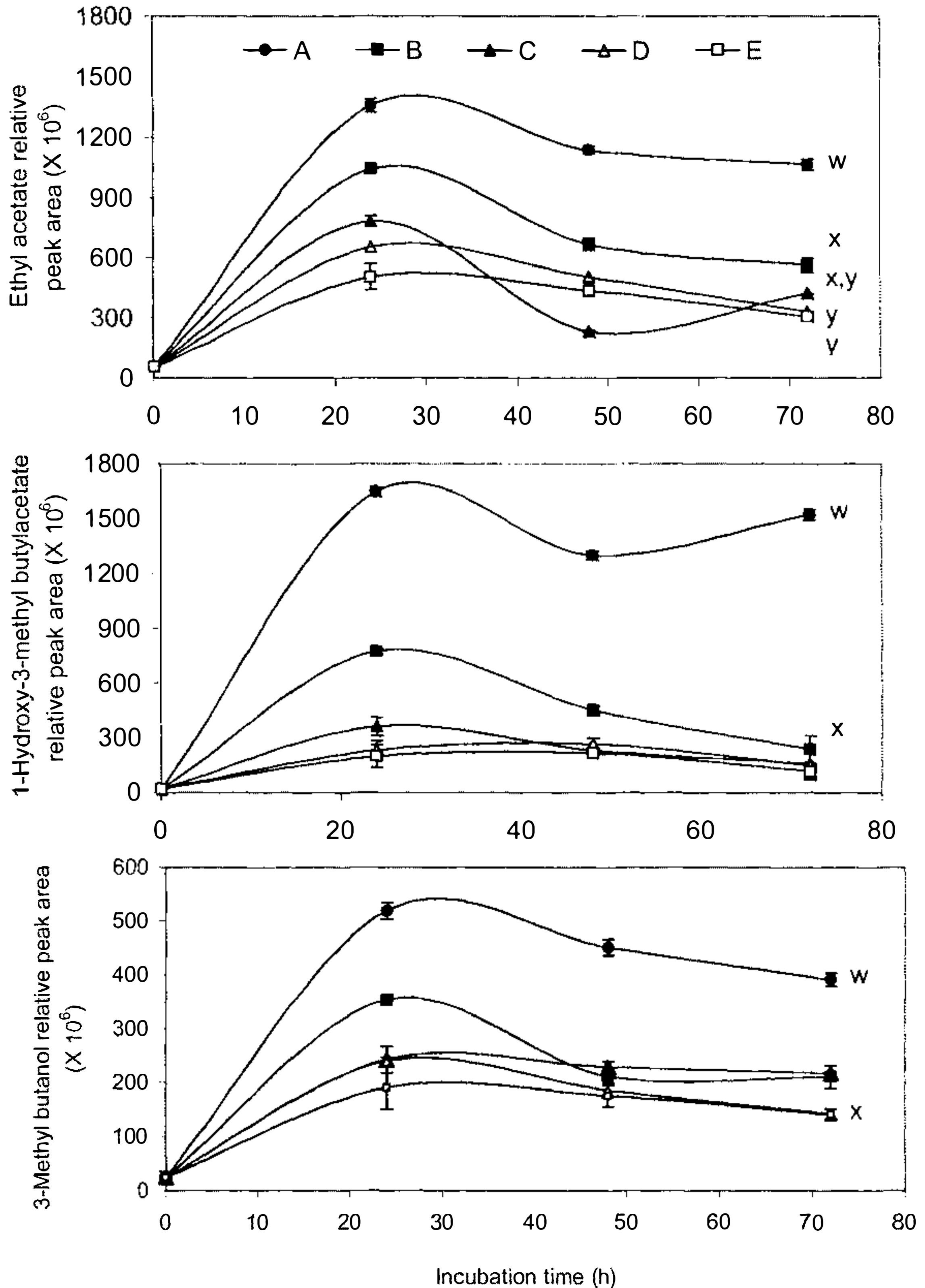


Figure 7.4 Concentrations of ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol in *C. kefyr* cultures over 72 h of incubation. The letters w, x, y indicate treatments that are significantly different at 72 h ($p \leq 0.05$). The error bars represent standard deviations between two independent fermentations.

Ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol followed the same pattern. The relative peak areas increased to reach maxima at 24 h, followed by decreases. The relative peak areas of all three compounds were highest in the control culture (A; no added L-phe). The relative peak area of ethyl acetate decreased with an increase in the initial concentration of L-phe. The relative peak areas of 1-hydroxy-3-methyl butylacetate and 3-methyl butanol in the cultures containing different concentrations of added L-phe were not significantly different from each other but were significantly less than in the control. This suggests that L-phe has a negative effect on the production of these volatile compounds. The extents of the inhibiting effects of L-phe were different for the three compounds. The relative peak areas of ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol decreased by 47–71.3%, 84.4–94.4% and 44.2–63.8% respectively in the presence of added L-phe at 72 h. Determination of the volatile compounds other than PE, PEA and benzaldehyde (three have been discussed above) and the non-volatile compounds would help to elucidate the metabolism of L-phe by *C. kefyr*. This may allow the development of a better strategy to improve the yields of PE and associated compounds.

7.3.3 Effect of different initial concentrations of glucose

C. kefyr was cultured in media with initial glucose concentrations of 0.9, 61.3, 125.1, 232.3 and 487.1 mM. The cultures were coded as F, G, H, I and J respectively. Culture F was the control without any added glucose. The glucose detected was endogenous in the medium, probably imparted by media components, and was exhausted in 24 h. All the media contained an initial L-phe concentration of 12.2 mM. The concentrations of glucose (Figure 7.5), PE and PEA (Figure 7.6) in all cultures over the 72 h of incubation were determined. The data for culture F are not shown because yeast did not grow in the culture and PE and PEA were at minimal levels only (≤ 0.03 mM).

The consumptions of glucose were 99 and 97% in cultures G and H respectively, and occurred during the first 24 h of incubation. In cultures I and J, 94.9 and 49% of the glucose were utilised respectively over the 72 h of incubation. The growth phase was continued until 48 h in the culture containing 61.2 mM glucose (Figure 7.1) but the growth phase of *C. kefyr* may have been extended beyond 48 h in the presence of excess glucose (cultures I and J).

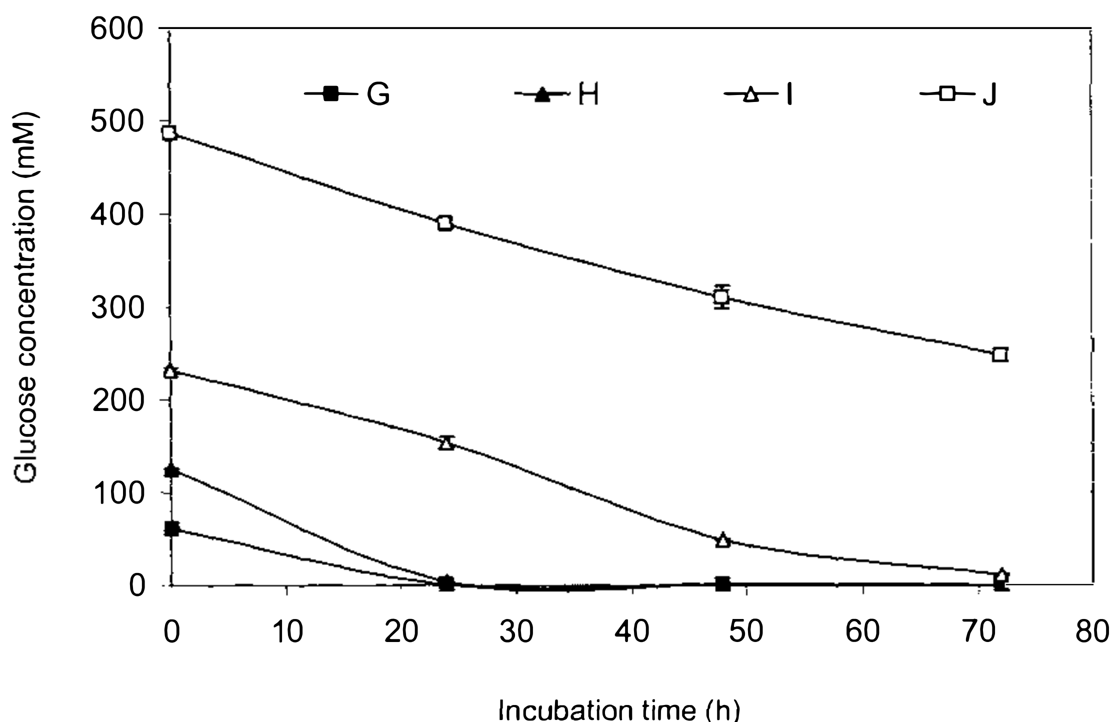


Figure 7.5 Changes in glucose concentrations in *C. kefyr* cultures in media with different initial glucose concentrations over 72 h of incubation. The initial concentrations of glucose in the media of cultures G, H, I and J were 61.3, 125.1, 232.3 and 487.1 mM respectively. The error bars represent standard deviations between two independent fermentations.

The PE concentration increased in cultures G, H, I and J during the incubation period. There was no significant difference between the PE concentrations in the cultures with different levels of added glucose, suggesting that concentrations of glucose between 61 and 487 mM do not have any influence on the production of PE.

The PEA concentrations reached maxima at 24 h in cultures G, H, I and J. The PEA concentrations dropped thereafter in cultures G and H, and remained relatively unchanged in cultures I and J. The increase in the PEA concentration in culture H between 48 and 72 h seemed to be unusual and was probably due to experimental error. Glucose was exhausted in cultures G and H after 24 h, but was present in cultures I and J. This may suggest that, in the absence of glucose, the yeast cells may use the PEA. At the maximum point (24 h), the PEA concentrations were not significantly different between the cultures containing different initial concentrations of glucose, which suggests that the concentrations of glucose in the experimental range (61–487 mM) do

not have an influence on the production of PEA although they may influence its stability.

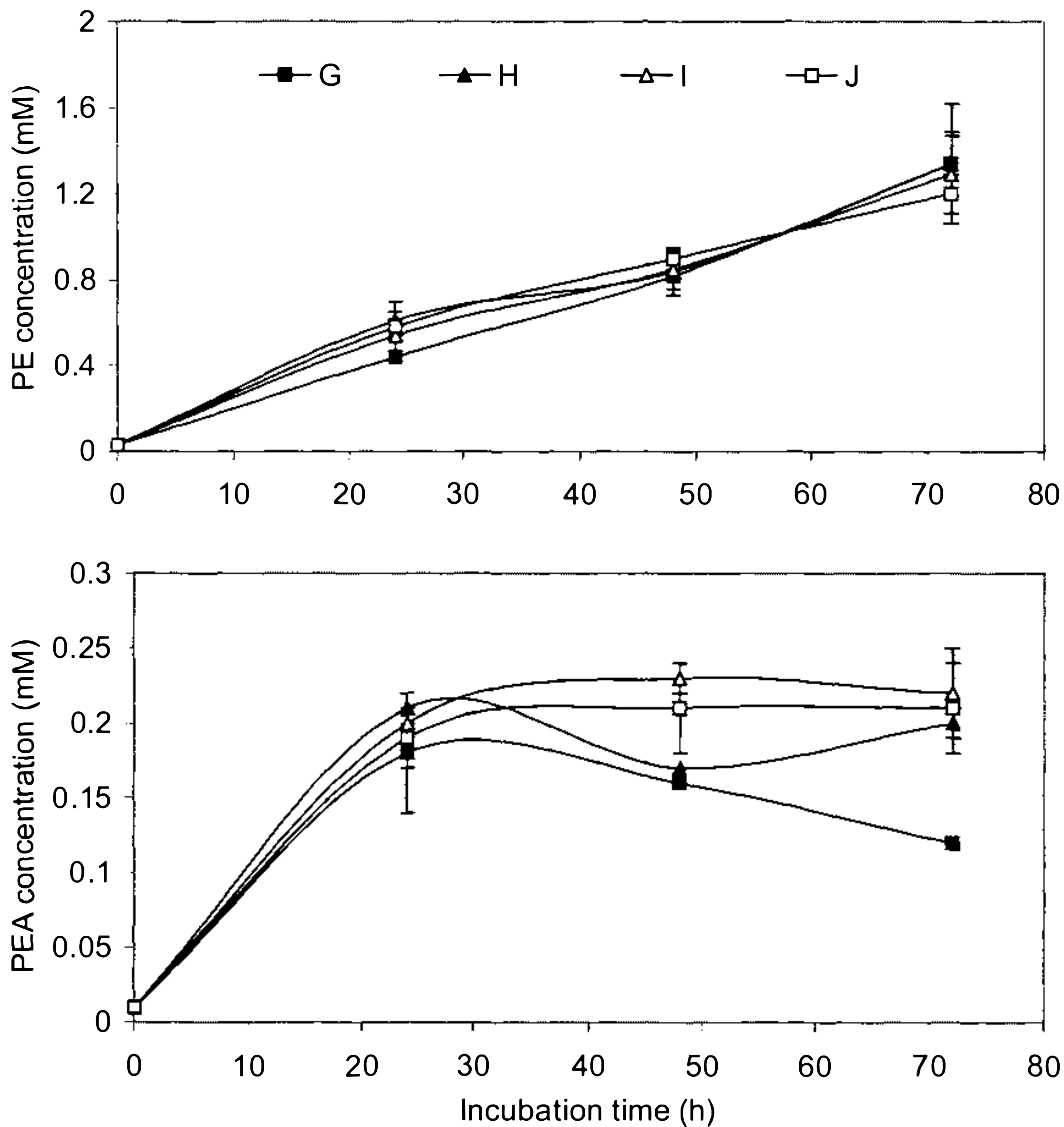


Figure 7.6 Production of PE and PEA in *C. kefyr* cultures with different initial concentrations of glucose over 72 h of incubation. The error bars represent standard deviations between two independent fermentations.

7.4 Discussion

The pattern of the production of PE by *C. kefyr* was different from those of some other yeasts. An increase in the PE concentrations during the death phase (48–72 h) in the *C. kefyr* culture (Figure 7.1) was unlike the situation in *Kluyveromyces marxianus* (Fabre *et al.*, 1998) and *Pichia fermentans* L-5 (Huang *et al.* 2001) cultures, where growth and biosynthesis of PE occurred simultaneously. The increase in the PE concentration during the death phase of the *C. kefyr* culture (between 48 and 72 h) could have been due to the release of the intracellular metabolites upon cell lysis. Jiang (1995) also observed a gradual increase in PE throughout the fermentation process (14 days) by *Kluyveromyces lactis.*

Table 7.1 Comparison of the production of PE by some yeasts in media supplemented with L-phe

Yeast	PE produced (mM)	Concentration of L-phe in the medium	Reference
K. lactis	3.2	0.08% (≈ 6.2 mM*)	Lee and Richard, 1984
S. cerevisiae	12.8	0.2% (≈ 12.2 mM)	Albertazzi *et al.*, 1994
P. etchelsii,	0.7	0.2% (≈ 12.2 mM)	
K. saturnus	1.6	0.2% (≈ 12.2 mM)	
S. delbrueckii	5.9	0.2% (≈ 12.2 mM)	
K. marxianus	5.7	0.2% (≈ 12.2 mM)	Fabre *et al.*, 1998
P. fermentans L-5	3.7	0.1%** (≈ 7.8 mM)	Huang *et al.*, 2000
C. kefyr	1.5	0.2% (12.2 mM)	Present study
	5.3	0.8% (50 mM)	

* = The mM values from other studies are based on the L-phe concentrations determined in the present study and thus are only approximate.
** = Phenylalanine isomer not stated.

C. kefyr produced a relatively lower amount of PE in media supplemented with L-phe than some other yeasts studied previously (Table 7.1). The conversion of the L-phe consumed to PE was 14.8–23.6% and to other metabolites (PEA and benzaldehyde) was 2.9–5.0% in the *C. kefyr* cultures containing 12.2–50 mM L-phe. In contrast, *P. fermentans* L-5 converted 47.4% of the available phenylalanine (isomer not stated) to

PE in a medium containing approximately 7.8 mM L-phe (Huang *et al.*, 2000). *Saccharomyces cerevisiae* NCYC 739 converted almost all of the initial L-phe to PE in a medium containing approximately 12.2 mM L-phe and no nitrogen source (Albertazzi *et al.*, 1994).

Some possible reasons for the low yield of PE from L-phe are discussed here. The most likely route of the production of PE from L-phe is by transamination of the amino acid to phenylpyruvate, decarboxylation to phenylacetaldehyde and reduction to PE (Ehrlich, 1907; cited in Etschmann *et al.*, 2002). The absence of adequate acceptor molecules may have retarded the transamination step and thus the production of PE. This suggests that an appropriate acceptor molecule in the medium (*e.g.* α-keto-glutarate; the chemical reaction is shown in Section 2.5.1) may improve the transamination and subsequently the production of PE. Supplementation of cheese curd with α-keto-glutarate has been reported to improve the flavour of cheese (McSweeney, 2004). Another possible reason for the low yield of PE and the other volatile metabolites (PEA and benzaldehyde) from L-phe could have been the formation of non-volatile compounds, which were not detected by GC–MS. Detection of non-volatile compounds in further studies will give a more complete picture of L-phe metabolism. Utilisation of L-phe by *C. kefyr* for energy was not obvious, because the experimental data showed that the growth of *C. kefyr* was not influenced by the absence or presence of L-phe in the medium (Appendix 7.5). L-phe also did not affect the growth of *P. fermentans* L-5 (Huang *et al.*, 2000) and *K. marxianus* (Fabre *et al.*, 1998). One of the limiting factors in the bioconversion of L-phe to PE is the cytotoxicity of PE (Fabre *et al.*, 1998; Etschman *et al.*, 2002). The accumulation of a higher concentration of PE is prevented by the toxic effect that PE has on microorganisms. The growth data of *C. kefyr* in the media with different concentrations of PE (cultures A, B, C, D and E; shown in Appendix 7.5) show that PE did not affect the growth at concentrations up to 5.5 mM. Another reason for the low conversion of L-phe to PE could be that part of the L-phe may have been transformed through the cinnamate pathway (the chemical reaction is shown in Section 2.5.1), which is supported by the detection of benzaldehyde (an intermediate product). Dependence of the metabolism of L-phe on the nitrogen source of the medium has been proposed (Akita *et al.*, 1990; Stark, 2001; cited in Etschman *et al.*, 2002). Only if amino acids represent the sole nitrogen source does the Ehrlich pathway predominate. If more readily digestible nitrogen sources are available, the

amino acid will be metabolised via the cinnamate pathway (Akita *et al.*, 1990; Stark, 2001; cited in Etschman *et al.*, 2002). Yeast extract in the present media could have provided an alternative source of nitrogen that may have diverted part of the L-phe from the Ehrlich pathway. A medium without any source of nitrogen other than amino acid could be used in the future to determine whether it improves the production of PE.

The PEA in the *C. kefyr* cultures may have been formed from acetyl-CoA (as a result of glucose metabolism) and PE by alcohol acetyltransferase (Huang *et al.*, 2000). *P. fermentans* L-5 (1.7 mM; Huang *et al.*, 2000) and *Kloeckera saturnus* (10.7 mM; Albertazzi *et al.*, 1994) produced more PEA than *C. kefyr* (maximum 0.3 mM) in media containing L-phe (the concentration of L-phe in the media is shown in Table 7.1). *S. cerevisiae* did not produce any PEA (Albertazzi *et al.*, 1994). Production of PEA by *K. marxianus* was not mentioned (Fabre *et al.*, 1998).

Benzaldehyde was produced by *C. kefyr* (Section 7.3.2) whereas this compound was not mentioned for *P. fermentans* L-5 (Huang *et al.*, 2000) or *K. marxianus* (Fabre *et al.*, 1998). Groot and de Bont (1998) reported the production of benzaldehyde in the fermentation of *Lactobacillus plantarum.* They recorded a benzaldehyde concentration up to 0.36 mM in *Lb. plantarum* culture supplemented with L-phe. *C. kefyr* also produced benzaldehyde in similar concentrations (0.36–0.41 mM) in media containing added L-phe. Various pathways of benzaldehyde production have been postulated. Benzaldehyde could be an intermediate product of the cinnamate pathway (Section 2.5.1). Groot and de Bont (1998) suggested that L-phe is initially converted to phenyl pyruvic acid by an aminotransferase and that the keto acid is further transformed to benzaldehyde. Formation of benzaldehyde through PE was suggested by Fabre *et al.* (1996).

The relative peak areas of ethyl acetate, 1-hydroxy-3-methyl butylacetate and 3-methyl butanol were much higher in the medium without added L-phe than in the media with added L-phe. Competition between ethanol and PE for the substrate could have been one reason for the reduced production of ethyl acetate and 1-hydroxy-3-methyl butylacetate. 3-Methyl butanol is possibly a product from branched chain amino acids. Higher concentrations of PE (in media with added L-phe) could have inhibited the production of some metabolites by the yeast. If more than one amino acid is present in

the medium, they compete with each other for entering the yeast cells. Competition for transportation with L-phe could inhibit the metabolism of branched chain amino acids.

There were no significant differences between the PE concentrations in the *C. kefyr* cultures with different initial concentrations of glucose (61–487 mM). Fabre *et al.* (1998) also reported that the production of PE by *K. marxianus* was independent of glucose concentration. In contrast, Huang *et al.* (2000) reported an increase in PE production by *P. fermentans* L-5 with an increase in the concentration of the primary carbon source (sucrose) up to 18%. They postulated that increasing osmotic pressure might have stimulated the biosynthesis of intracellular metabolites participating in osmo-regulation. But this stimulation did not occur in the *C. kefyr* cultures. An increase in the glucose consumption in some cultures for an extended period of time (72 h in cultures I and J against 24 h in cultures G and H; Figure 7.5) did not increase the production of PE in the medium containing 12.2 mM L-phe. A medium with glucose concentration similar to those in cultures I and J (232–487 mM) and an L-phe concentration higher than 12.2 mM could be studied in the future to determine whether the production of PE is increased.

7.5 Conclusions

This study showed that *C. kefyr* is capable of producing the flavour compounds PE, PEA and benzaldehyde using L-phe as a precursor. The concentrations of these compounds in the culture were increased by the addition of L-phe but the molar conversion of L-phe into these aroma compounds (20–27%) was very low. Further study is warranted to increase the yield by manipulating the media components and the growth conditions.

Chapter 8. Final conclusions

This study investigated the ripening potential of dairy yeasts as anaerobic adjuncts in cheese. It was hypothesised that, without growth of the yeasts in the cheese, the yeasts would influence the ripening of cheese by the enzymes produced in the cultures before their addition to the cheese. Thus, the growth conditions used to prepare the yeast adjuncts, the point of adjunct addition during the cheesemaking process and the amount of added culture would influence the ripening of cheese.

Objective 1 of the study was to investigate the effects of the growth conditions on the lipase and proteinase activities of three dairy yeasts (strains of *Geotrichum candidum*, *Yarrowia lipolytica* and *Candida kefyr*) in laboratory media and their capability to produce volatile compounds in the same media. This objective was achieved; the dairy yeasts studied demonstrated diverse metabolic capacities in the laboratory media under different conditions. *G. candidum* had lipase (extracellular) activity that was induced by safflower oil and its proteinase activity (intracellular) was present only in shake cultures (Section 4.3.1). *Y. lipolytica* had lipase activity (extracellular, washed-cell and intracellular), which was influenced by the growth conditions, but no detectable proteinase activity. The highest lipase activity of *Y. lipolytica* was detected in a 192 h static culture grown in safflower oil medium (Section 4.3.2). *C. kefyr* had no detectable lipase activity and only very weak proteinase activity but produced aromatic volatile compounds including the aromatic alcohol phenyl ethyl alcohol (PE), and its acetate and propionate esters, and the concentrations were highest in a glucose medium under shake conditions (Section 4.3.3). Two of the three yeasts studied, *G. candidum* and *Y. lipolytica*, were selected for cheese production trials (Chapters 5 and 6) because of their active hydrolytic enzyme(s). The third yeast, *C. kefyr*, was selected for further study of the production of aromatic volatile compounds in laboratory media (Chapter 7) because of its capacity to produce PE.

Objective 2 of the study was to investigate the influences of the yeasts on cheese ripening, when used as anaerobic adjuncts. This objective was achieved. *G. candidum* and *Y. lipolytica* were used in the cheese trials. These yeasts, when used as anaerobic adjunct cultures, had a distinct impact on the concentrations of the free fatty acids

(FFAs; Sections 5.3.1.2, 5.3.2.2, 6.3.3, 6.3.7.1.2 and 6.3.7.2.2) and the profiles of volatile compounds (Sections 5.3.1.4, 5.3.2.4, 6.3.7.1.3 and 6.3.7.2.3) although the effect on the peptide profile was not obvious (Sections 5.3.1.3 and 5.3.2.3). Further details of the lipolysis are discussed in later paragraphs. The number of living yeast cells decreased during ripening (Section 6.3.1), which showed that the yeasts did not grow in the cheese. Thus, it was concluded that the yeast adjuncts influenced cheese ripening by virtue of the enzymes already produced in the cultures before their addition to the cheese. This proves the hypothesis that yeasts can influence cheese ripening without active growth in the cheese. Use of the cell-free extracts of the yeasts in cheese ripening could be studied in future, which will allow utilisation of the ripening attributes of the yeast enzymes while keeping the yeast count within specification.

Studies of the effects of some technological parameters (growth conditions of the yeast adjuncts, point of adjunct addition, ripening temperature and level of yeast adjunct) on the influences of the yeast adjuncts were part of Objective 2. These studies successfully showed the importance of these technological parameters in cheese ripening.

The effects of the yeast adjunct cultures on Cheddar cheese ripening were influenced by the growth conditions of the cultures. A 192 h *Y. lipolytica* static culture grown in safflower oil medium was found to have the maximum effect on cheese ripening, whereas *Y. lipolytica* cultures grown under other conditions did not have any obvious effect (Section 5.3.2). *Y. lipolytica* also showed higher (>10 fold) lipase activity under this condition than under other conditions in laboratory media (Section 4.3.2.2). This result also supports the hypothesis.

The point at which the yeast adjunct was added influenced the ripening of Cheddar cheese, which is also proof of the hypothesis. The addition of the *G. candidum* adjunct to the cheese curd produced higher concentrations of FFAs in the cheese than its addition to the cheese milk (Section 5.3.1.2).

A comparison of ripening temperatures, which was part of the second objective, showed that ripening of washed-curd, dry-salted cheese at 20°C was more useful than ripening at 13 and 10°C in terms of the rate of lactate fermentation by propionibacteria and lipolysis in the cheese (Section 6.3.7).

The level of yeast culture influenced the total FFA concentration in the washed-curd, dry-salted cheeses. The FFA concentration increased with an increase in the inoculum of the *Y. lipolytica* adjunct used (Section 6.3.7.2.2). *G. candidum* adjunct also showed a similar pattern with one exception (Section 6.3.7.1.2; addition of 100 mL of culture produced higher FFA concentrations than addition of 500 mL of culture; possible reasons are discussed in Section 6.4). The results also support the hypothesis that the yeasts influence cheese ripening by the enzymes produced in the culture before their addition to the cheese. Concentrating the yeast cultures over fivefold (used in the present study) by ultrafiltration is difficult. Addition of freeze-dried cultures to the cheese could be tried to study the effects of higher levels of yeast adjunct cultures.

Comparison of the proportions of individual FFAs with the fatty acid profile of the cheese showed that *G. candidum* and *Y. lipolytica* have one or more lipases that preferentially hydrolyse(s) long-chain unsaturated fatty acids from milk fat (Sections 5.3.1.2, 5.3.2.2, 6.3.3, 6.3.7.1.2 and 6.3.7.2.2). The specificity of *G. candidum* lipase in laboratory media and vegetable oils has been reported by different researchers; the present study established that the lipase(s) with specificity towards the unsaturated fatty acids with double bonds at *cis*-9 positions is (are) capable of hydrolysing milk fat in the cheese environment. The specificity of *Y. lipolytica* lipase towards unsaturated fatty acids has been mentioned in only one or two published studies; the present study clearly showed this specificity of *Y. lipolytica* lipase. The *Y. lipolytica* lipase also showed some specificity towards butyric acid (Sections 5.3.2.2, 6.3.3 and 6.3.7.2.2); the *Y. lipolytica* lipase could be used to replace the mammalian lipase (used at present) in the production of ingredient cheeses, in which a high concentration of free butyric acid is desired. That may open new markets for these ingredient cheeses in countries where the use of mammalian lipase is prohibited. A further study of the characterisation and utilisation of this enzyme is warranted.

Producing a cheese with increased concentrations of conjugated linoleic acid (CLA) and ethyl esters was Objective 3 of this study. This objective was only partially achieved. The concentrations of the ethyl esters of butanoic, hexanoic and octanoic acids were higher in the cheeses made with yeast adjuncts than in the control (Section 6.3.5). However, the total CLA (free plus esterfied) content of the cheese did not increase. An approximately 10-fold increase in free linoleic acid occurred in the cheese but

conversion of the free linoleic acid to CLA did not occur, possibly because of an unfavourable *in-situ* cheese environment. Further study of CLA enrichment using other dairy bacteria and other dairy systems such as yoghurt and cheese slurry could be undertaken.

Objective 4 of the study was to produce aromatic volatile compounds from L-phenylalanine (L-phe) fermentation by *C. kefyr* in laboratory media. The objective was achieved by showing that *C. kefyr* is capable of producing PE, phenyl ethyl acetate (PEA) and benzaldehyde using L-phe as a precursor. The production was increased by the addition of increasing levels of L-phe (within the experimental range) in the media (Section 7.3.2). However, the yield of these aromatic volatile compounds was very low (20–27%). Developing a strategy to improve the production of these volatile compounds by manipulating the media components and the growth conditions (Section 7.4) will be important to optimise this fermentation.

Chapter 9. Future directions

The present study showed that yeasts have potential to be used as anaerobic adjuncts in cheese (Cheddar and washed-curd, dry-salted cheeses) for accelerating and diversifying flavour development. The present study suggested that further research work in different directions could be undertaken to explore the potentials clearly shown by these dairy yeasts to produce novel dairy products or ingredients. The important aspects are summarised below.

1. Cell-free extracts of the yeast cultures could be used in cheese to obtain the ripening effect of the yeast enzymes while keeping the yeast counts within specification.

2. Cheese experiments with *Yarrowia lipolytica* indicated a selective hydrolysis of butyric acid from milk fat. Free butyric acid is important for cheese flavour, especially for ingredient cheeses. Mammalian lipase has selectivity towards butyric acid and has been used in ingredient cheeses to produce sharp flavours. Because of religious restrictions in some countries, a microbial alternative would be of interest. A study of the characterisation and utilisation of this enzyme could be undertaken.

3. The freeze-dried form of the yeast cultures could be used to study the effect of higher concentrations of yeast adjuncts (beyond 500 mL/kg curd) on cheese ripening.

4. Attempts to increase the conjugated linoleic acid (CLA) content in cheese were unsuccessful. The yeast enzymes produced high concentrations of unsaturated fatty acids including linoleic acid, but the strains of propionibacteria used could not convert the free linoleic acid to CLA in the cheese, although they were able to do so in laboratory media. Further studies using other dairy bacteria (such as *Lactobacilli*) and other dairy systems, *e.g.* yoghurt and cheese slurry (higher water activity and more controlled pH), could produce interesting results.

5. The molar conversions of L-phenylalanine (L-phe) to aromatic volatile compounds by *Candida kefyr* cultures in L-phe-enriched growth media were low. Improvement in the yields of the aromatic compounds by manipulating the media components

(addition of α-keto-glutarate, replacing yeast extract with vitamins and addition of higher amounts of L-phe with higher amounts of glucose) or the growth conditions (temperature, pH and shaking) could be studied.

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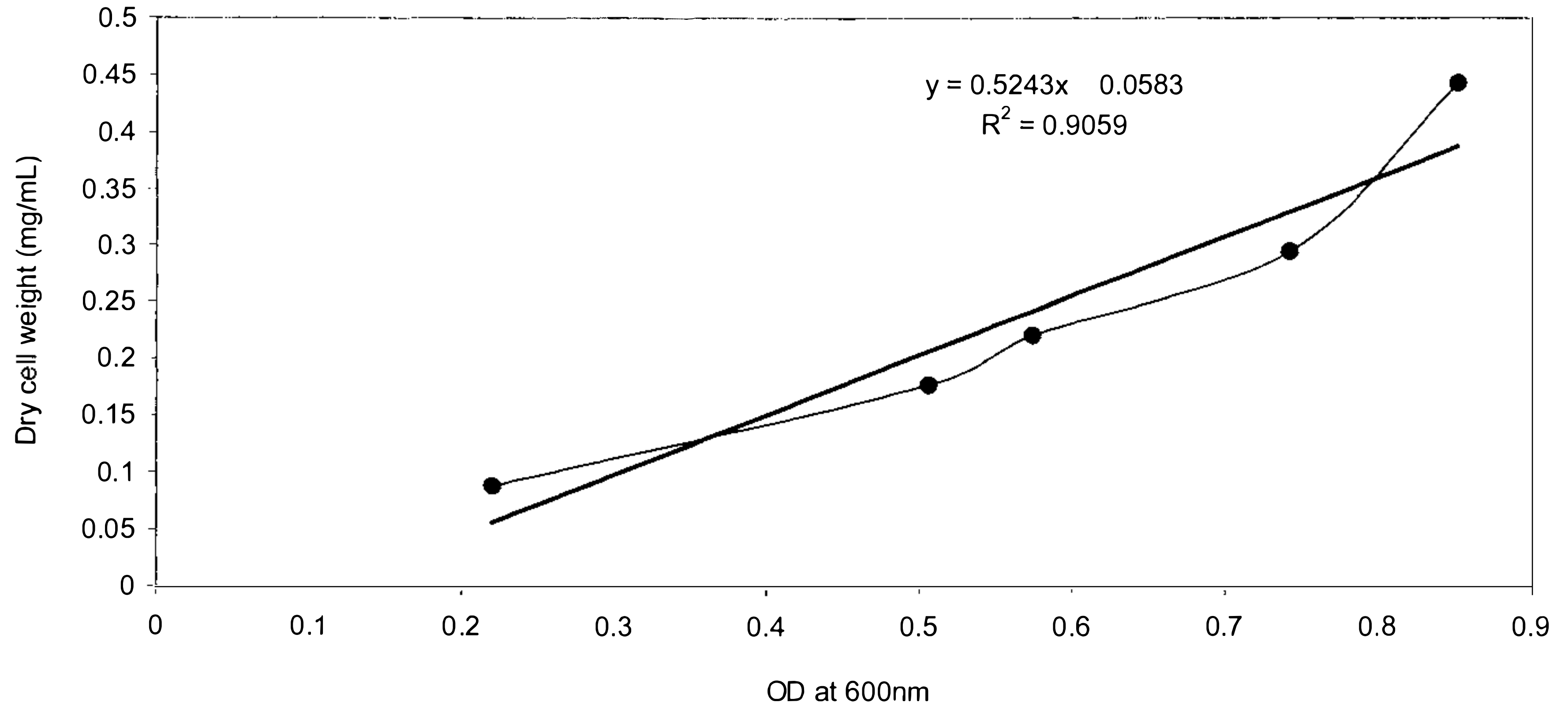
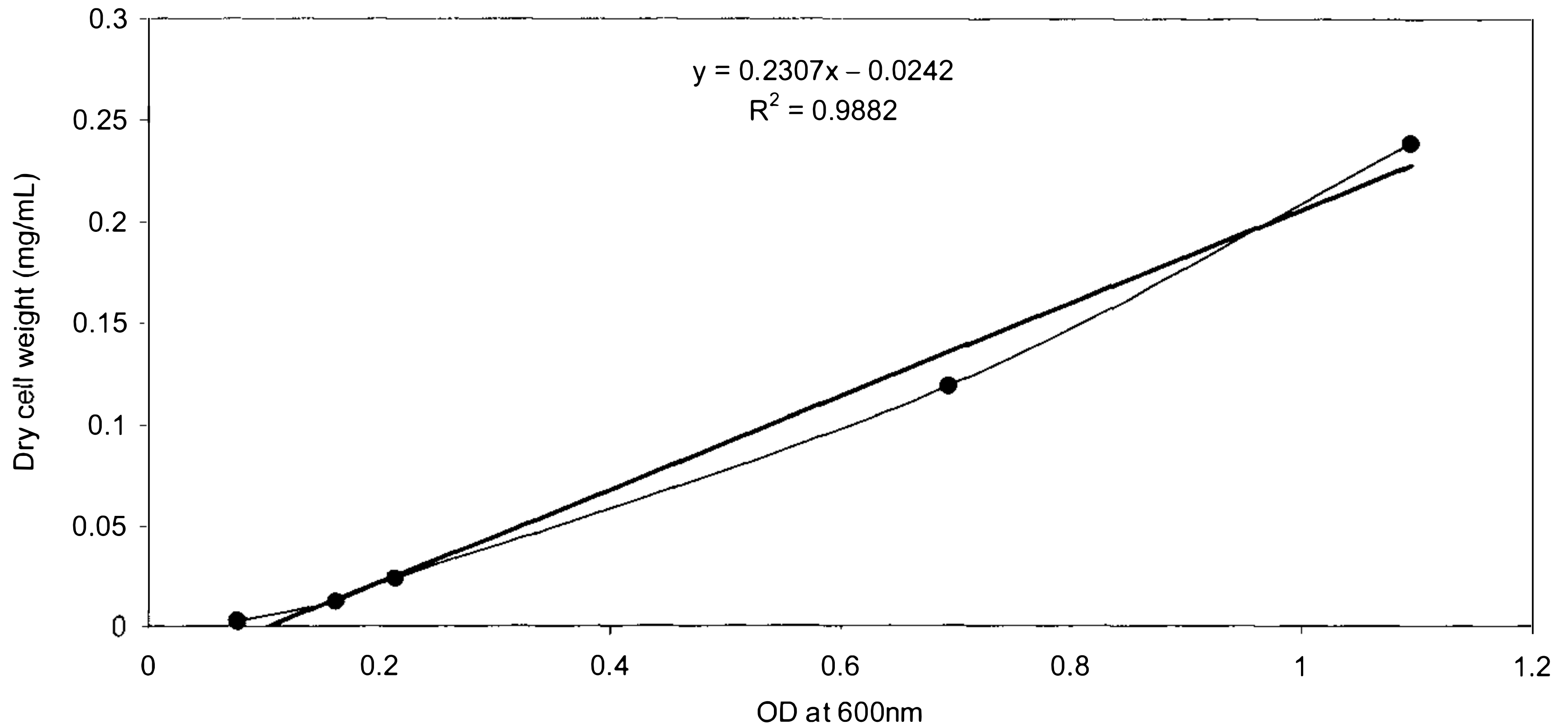
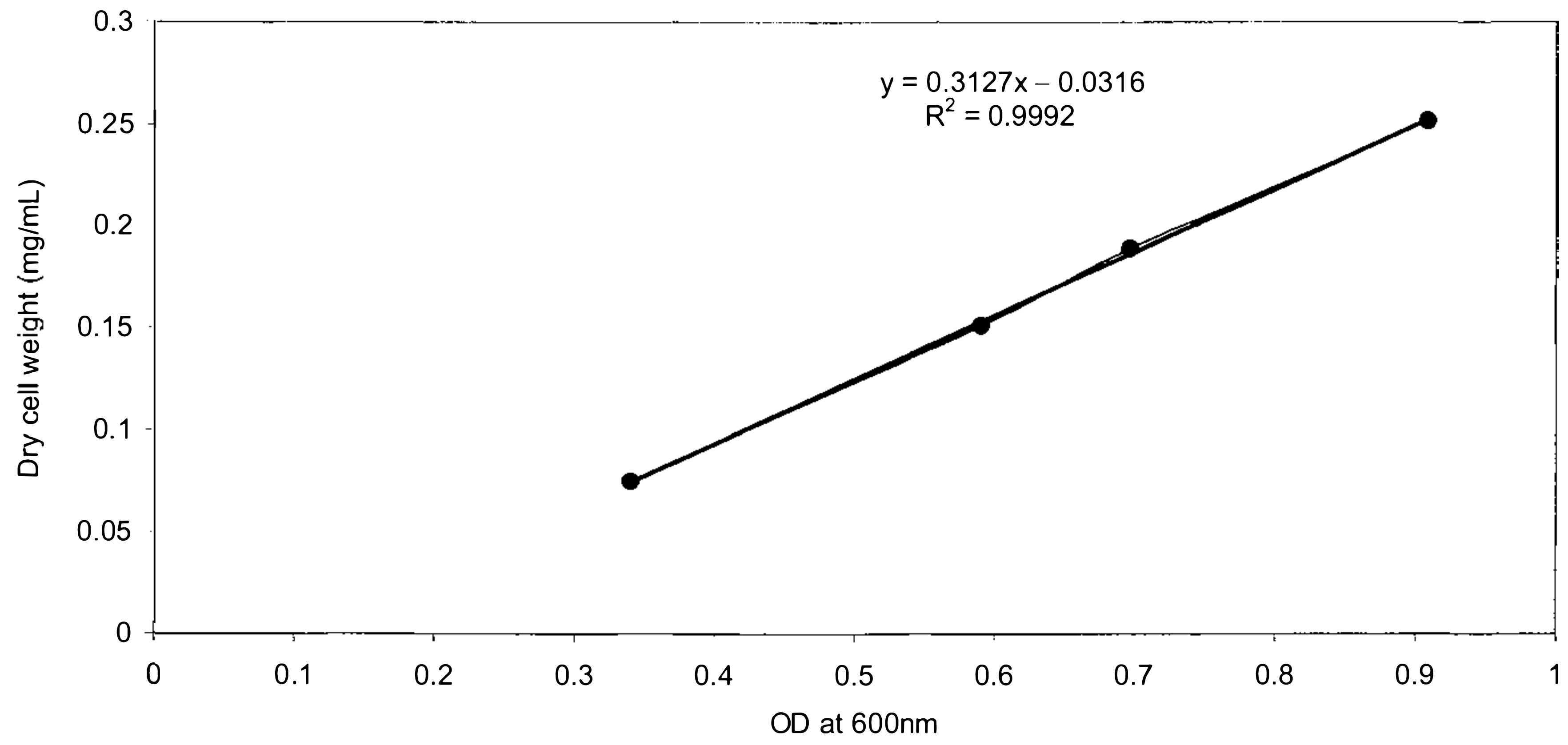
Appendices



Appendix 4.1 Correlation between OD_{600} and dry cell weight (mg/mL) of *G. candidum* culture.



Appendix 4.2 Correlation between OD_{600} and dry cell weight (mg/mL) of *Y. lipolytica* culture.



Appendix 4.3 Correlation between OD_{600} and dry cell weight (mg/mL) of *C. kefyr* culture.

Appendix 4.4 Volatile compounds detected in 24 h *G. candidum* cultures grown under different conditions

Volatile compounds	Relative peak area (x 10^6)			
	A[1]	B[2]	C[3]	D[4]
Alcohols				
Ethanol	50.4	22.1	nd*	nd
2-Methyl propanol	19.5	nd	nd	nd
2-Methyl butanol and 3-Methyl butanol	131.4	62.1	96.7	46.9
2-Ethyl butanol	nd	nd	3.2	1.1
Phenyl ethyl alcohol	7.4	1.9	4.3	1.2
Esters				
Ethyl acetate	0.7	nd	nd	nd
2-Ethyl, methyl propanoate	nd	23.0	nd	nd
Ethyl butanoate	1.5	3.4	nd	nd
2-Ethyl, methyl butanoate	nd	23.3	nd	nd
3-Ethyl, methyl butanoate	nd	2.3	nd	nd
1-Hydroxy-3-Methyl butylacetate	5.4	nd	nd	nd
Ethyl pentanoate	nd	1.2	nd	nd
Ethyl hexanoate	nd	2.3	nd	nd
Acids				
Acetic acid	2.9	1.2	nd	nd
2-Methyl propanoic acid	3.6	nd	nd	nd
3-Methyl butanoic acid	4.1	nd	nd	nd

[1]A = *G. candidum* shake culture grown in glucose medium.
[2]B = *G. candidum* static culture grown in glucose medium.
[3]C = *G. candidum* shake culture grown in safflower oil medium.
[4]D = *G. candidum* static culture grown in safflower oil medium.
* = Not detectable.

Appendix 4.5 Volatile compounds detected in 24 h *Y. lipolytica* cultures grown under different conditions

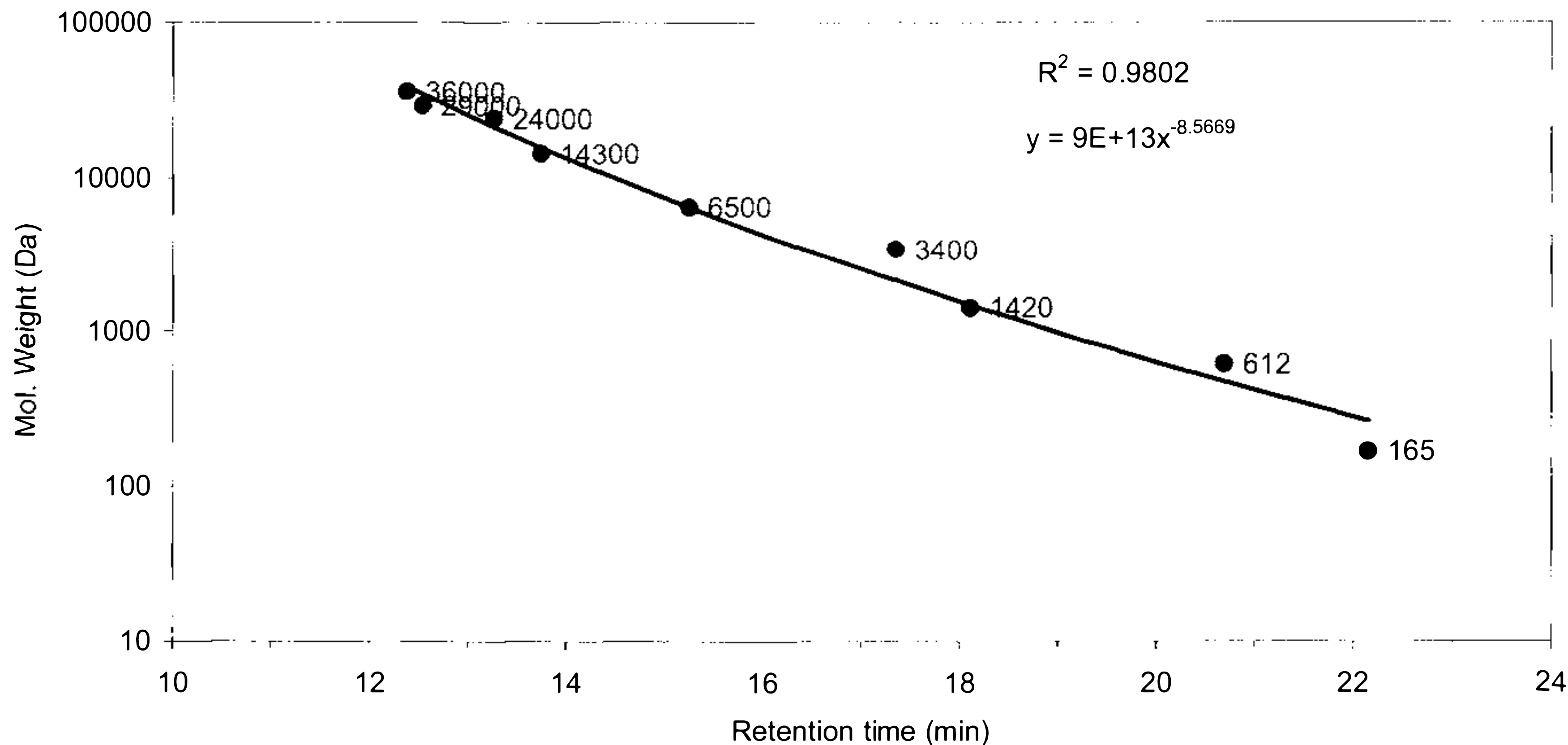
Volatile compounds	Relative peak area (x 10^6)			
	A[1]	B[2]	C[3]	D[4]
Alcohols				
Ethanol	87.9	23.6	nd*	nd
2-Methyl propanol	11.7	5.1	nd	nd
2-Methyl butanol and 3-Methyl butanol	219.5	66.6	131.6	85.2
Phenyl ethyl alcohol	5.9	1.2	15.7	8.1
Ester				
Ethyl acetate	4.1	nd	nd	nd
Acids				
Acetic acid	5.0	4.4	nd	nd
3-Methyl butanoic acid	1.0	nd	nd	nd

[1]A = *Y. lipolytica* shake culture grown in glucose medium.
[2]B = *Y. lipolytica* static culture grown in glucose medium.
[3]C = *Y. lipolytica* shake culture grown in safflower oil medium.
[4]D = *Y. lipolytica* static culture grown in safflower oil medium.
* = Not detectable.

Appendix 4.6 Volatile compounds detected in 24 h *C. kefyr* cultures grown under different conditions

Volatile compounds	Relative peak area (x 10^6)			
	A[1]	B[2]	C[3]	D[4]
Alcohols				
Ethanol	37.3	29.8	nd	nd
2-Methyl propanol	3.3	nd	nd	nd
2-Methyl butanol and 3-Methyl butanol	144.0	81.9	122.5	71.6
Phenyl ethyl alcohol	50.7	9.2	34.1	12.3
Esters				
Ethyl acetate	41.3	15.9	nd	nd
1-Hydroxy-3-Methyl butylacetate	24.8	21.1	nd	nd
1-Hydroxy-3-Methyl butylpropanoate	3.4	1.6	nd	nd
2-Phenyl ethyl acetate	25.8	14.7	nd	nd
2-Phenyl ethyl propionate	5.9	1.4	nd	nd
Acid				
Acetic acid	1.1	nd	nd	nd

[1]A = *C. kefyr* shake culture grown in glucose medium.
[2]B = *C. kefyr* static culture grown in glucose medium.
[3]C = *C. kefyr* shake culture grown in safflower oil medium.
[4]D = *C. kefyr* static culture grown in safflower oil medium.
* = Not detectable.



Appendix 5.1 Correlation between retention time and molecular weight in SE-HPLC. The standards used (molecular weight) were: glyceraldehyde-3-phosphate dehydrogenase (36 000 Da), carbonic anhydrase (29 000 Da), trypsinogen (24 000 Da), egg white lysozyme (14 300 Da), aprotinin (6500 Da), insulin-B-chain (3400 Da), bacitracin (1420 Da), glutathione oxidised (612 Da) and phenylalanine (165 Da).

Appendix 5.2A Concentrations of FFAs originating from milk fat (mg/g cheese) in Cheddar cheeses made without (G1) and with (G2, G3 and G4) *G. candidum* over 6 months of ripening at 13°C

Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18	C18.1	C18:2	Total
G1	1	0.01	0.01	0.01	0.02	0.05	0.06	0.16	0.05	0.14	0.01	0.53
	2	0.02	0.01	0.02	0.03	0.06	0.08	0.21	0.07	0.23	0.01	0.74
	4	0.02	0.01	0.01	0.02	0.05	0.07	0.18	0.06	0.17	0.011	0.60
	6	0.02	0.01	0.01	0.03	0.05	0.06	0.17	0.05	0.19	0.01	0.61
G2	1	0.03	0.02	0.03	0.04	0.07	0.13	0.39	0.07	1.24	0.14	2.16
	2	0.04	0.02	0.03	0.05	0.08	0.17	0.48	0.07	1.37	0.15	2.47
	4	0.12	0.06	0.07	0.09	0.13	0.32	0.68	0.12	1.96	0.20	3.75
	6	0.2622	0.13	0.14	0.17	0.31	0.43	0.88	0.16	1.18	0.25	3.91
G3	1	0.04	0.03	0.05	0.07	0.10	0.17	0.53	0.08	1.29	0.22	2.57
	2	0.10	0.08	0.09	0.13	0.17	0.33	0.88	0.15	2.11	0.30	4.32
	4	0.21	0.15	0.15	0.20	0.23	0.45	0.95	0.23	2.22	0.27	5.06
	6	0.40	0.29	0.26	0.28	0.34	0.59	1.22	0.30	2.81	0.28	6.77
G4	1	0.02	0.01	0.01	0.03	0.05	0.09	0.25	0.06	0.76	0.14	1.41
	2	0.03	0.02	0.02	0.04	0.07	0.13	0.34	0.07	1.20	0.16	2.08
	4	0.10	0.05	0.06	0.10	0.15	0.27	0.61	0.12	1.91	0.22	3.59
	6	0.26	0.15	0.14	0.18	0.26	0.43	0.87	0.17	1.46	0.27	4.19

Appendix 5.2B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in Cheddar cheeses made without (G1) and with (G2, G3 and G4) *G. candidum* over 6 months of ripening at 13°C

Cheese	Month	FFAs originating from milk fat (% of total FFAs)									
		C4	C6	C8	C10	C12	C14	C16	C18	C18:1	C18:2
G1	1	2.5	1.1	1.7	4.5	8.9	11.3	30.4	10.2	27.2	1.9
	2	2.8	1.8	2.2	4.6	8.1	11.1	28.4	8.9	30.5	1.8
	4	3.5	1.7	1.7	3.8	8.7	11.8	30.7	9.2	27.5	1.8
	6	3.8	1.8	1.3	5.1	8.2	10.2	28.2	7.7	31.6	2.3
G2	1	1.3	0.8	1.2	1.8	3.4	6.2	18.1	3.1	57.5	6.6
	2	1.6	1.0	1.3	1.9	3.3	6.8	19.3	3.0	55.3	6.2
	4	3.1	1.6	1.9	2.5	3.6	8.4	18.1	3.1	52.4	5.4
	6	6.7	3.4	3.5	4.3	8.0	11.0	22.5	4.0	30.3	6.3
G3	1	1.6	1.3	1.8	2.7	3.3	6.7	20.6	3.1	50.4	8.4
	2	2.2	1.7	2.0	3.0	3.9	7.6	20.3	3.4	48.9	6.9
	4	4.1	3.0	3.0	4.0	4.6	9.0	18.8	4.6	43.8	5.2
	6	5.9	4.3	3.9	4.2	5.0	8.7	18.1	4.4	41.6	4.1
G4	1	1.1	0.7	1.0	1.8	3.8	6.5	17.6	4.0	53.8	10.0
	2	1.5	0.9	1.1	1.8	3.3	6.3	16.2	3.3	57.6	7.9
	4	2.8	1.5	1.5	2.8	4.2	7.4	17.1	3.4	53.2	6.0
	6	6.1	3.5	3.2	4.2	6.3	10.3	20.8	4.1	34.9	6.5

Appendix 5.3A Concentrations of FFAs originating from milk fat (mg/g cheese) in Cheddar cheeses made without (Y1) and with (Y2, Y3 and Y4) *Y. lipolytica* over 6 months of ripening at 13°C

Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18	C18:1	C18:2	Total
Y1	1	0.01	0	0.01	0.02	0	0.07	0.2	0.05	0.15	0.01	0.51
	2	0.01	0	0.01	0.02	0	0.07	0.2	0.05	0.15	0.01	0.55
	4	0.02	0	0.01	0.02	0	0.08	0.2	0.06	0.15	0.01	0.61
	6	0.03	0	0.01	0.03	0.10	0.09	0.2	0.07	0.19	0.01	0.70
Y2	1	0.01	0	0.01	0.02	0	0.06	0.2	0.05	0.16	0.01	0.53
	2	0.03	0	0.01	0.03	0	0.08	0.2	0.05	0.21	0.02	0.66
	4	0.04	0	0.01	0.03	0	0.08	0.2	0.06	0.21	0.02	0.70
	6	0.06	0	0.03	0.08	0.10	0.13	0.3	0.09	0.34	0.02	1.12
Y3	1	0.02	0	0.01	0.02	0	0.07	0.2	0.06	0.22	0.02	0.66
	2	0.04	0	0.02	0.04	0.10	0.10	0.2	0.06	0.29	0.02	0.84
	4	0.05	0	0.02	0.04	0.10	0.10	0.2	0.07	0.29	0.02	0.89
	6	0.07	0	0.02	0.06	0.10	0.13	0.3	0.09	0.38	0.03	1.17
Y4	1	0.30	0.1	0.22	0.24	0.30	0.40	0.7	0.28	1.67	0.20	4.51
	2	0.74	0.4	0.46	0.48	0.70	0.76	1.3	0.48	2.75	0.18	8.26
	4	1.12	0.9	0.86	0.97	1.30	1.22	2.6	2.27	8.12	0.43	19.80
	6	2.24	1.5	1.50	1.67	1.80	2.97	6.0	4.49	14.20	0.70	37.10

Appendix 5.3B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in Cheddar cheeses made without (Y1) and with (Y2, Y3 and Y4) *Y. lipolytica* over 6 months of ripening at 13°C

Cheese	Month	FFAs originating from milk fat (% of total FFAs)									
		C4	C6	C8	C10	C12	C14	C16	C18	C18.1	C18:2
Y1	1	1.4	0.6	1.2	4.1	8.0	12.9	32.4	9.4	29.2	1.2
	2	2.4	0.9	1.3	3.6	8.2	13.5	32.4	9.6	27.6	1.3
	4	3.8	1.5	1.2	3.6	7.1	13.3	33.1	9.0	25.1	1.3
	6	4.6	1.9	1.5	3.9	7.7	13.0	30.6	9.3	26.7	1.6
Y2	1	2.6	0.8	1.3	3.6	7.6	11.9	30.2	8.9	30.2	2.3
	2	3.8	1.2	1.5	3.9	6.7	12.0	28.5	8.2	32.4	2.3
	4	5.1	1.6	1.4	3.6	6.4	12.0	28.7	8.0	30.3	2.3
	6	5.7	2.5	2.3	7.6	5.8	11.5	25.8	8.1	30.5	2.1
Y3	1	2.9	0.9	1.5	3.5	7.1	11.1	28.3	8.8	33.2	2.7
	2	4.3	1.5	2.1	4.3	6.8	11.4	25.7	7.5	33.9	2.5
	4	5.6	1.9	1.9	3.9	6.4	11.6	26.2	7.3	32.5	2.5
	6	5.8	2.1	2.1	5.1	5.8	11.2	25.5	7.8	32.7	2.3
Y4	1	6.7	3.2	4.8	5.4	7.4	8.9	16.0	6.2	37.0	4.4
	2	9.0	5.2	5.5	5.9	8.1	9.2	15.9	5.9	33.3	2.2
	4	5.7	4.7	4.4	4.9	6.5	6.2	13.0	11.5	41.0	2.2
	6	6.0	4.1	4.0	4.5	4.9	8.1	16.2	12.1	38.3	1.9

Appendix 6.1 Screening propionibacteria for CLA synthesis (results from work carried out by Ross Holland and Sharon Gibson, Fonterra Co-operative Group, Palmerston North, New Zealand, in 2000)

Materials and methods

Five strains of propionibacteria were from the collection of the Fonterra Co-operative Group Ltd, Palmerston North, New Zealand: *P. throenii* (B6011) and *P. freudenreichii* ssp. *shermanii* strains B6022, B6026, B6027 and B6028. Cultures were inoculated into Man-Rogosa-Sharpe (MRS) broth (pH 7.0; de Man *et al.*, 1960), containing D (+) glucose (0.5% w/v; BDH Laboratory Supplies, Poole, England), 1% v/v Tween-80, 5% v/v polyoxyethylene sorbitan monooleate (Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA) and 9, 12 linoleic acid (99% pure; Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178, USA) at different concentrations. Linoleic acid was added as a 5 mg/mL aqueous emulsion in 1% v/v Tween-80 (Jiang *et al.*, 1998). The cultures were incubated for 48 h at 30°C under anaerobic conditions. Samples were extracted at 48 h using methodology of Jiang *et al.* (1998) and were analysed by the method described in Section 6.2.3.

Results

The conversion of free linoleic acid to CLA by different strains of propionibacteria during 48 h of incubation in laboratory media containing different concentrations of free linoleic acid is shown in the following table. Increasing the level of free linoleic acid in the medium from 0.1 to 1.0 mg/mL drastically reduced (> 90%) the growth of strains B6022 and B6026, whereas strains B6011, B6027 and B6028 were not affected. *P. freudenreichii* ssp. *shermanii* strains B6022, B6027 and B6028 were able to synthesise CLA (> 10% conversion), whereas *P. freudenreichii* ssp. *shermanii* strain B6026 synthesised lower amounts (3–6% conversion) and the synthesis by *P. throenii* (B6011) was very low (< 1% conversion). The amount of CLA synthesised increased in cultures of *P. freudenreichii* ssp. *shermanii* strains B6027 and B6028 with increasing initial concentration of linoleic acid in the medium. For *P. freudenreichii* ssp. *shermanii* strain B6022, the amount of CLA synthesised decreased with increasing initial concentration of linoleic acid, reflecting growth inhibition of this strain by linoleic acid. However, the rate per unit cell mass increased for this strain (B6022) over the linoleic acid

concentration range tested (0.1–1.0 mg/mL). On the basis of these results, *P. freudenreichii* ssp. *shermanii* strains B6022, B6027 and B6028 were selected for use in cheese trials (Chapter 6).

Conversion of free linoleic acid (C18:2) by different strains of propionibacteria in MRS broth after 48 h of incubation at 30°C under anaerobic conditions

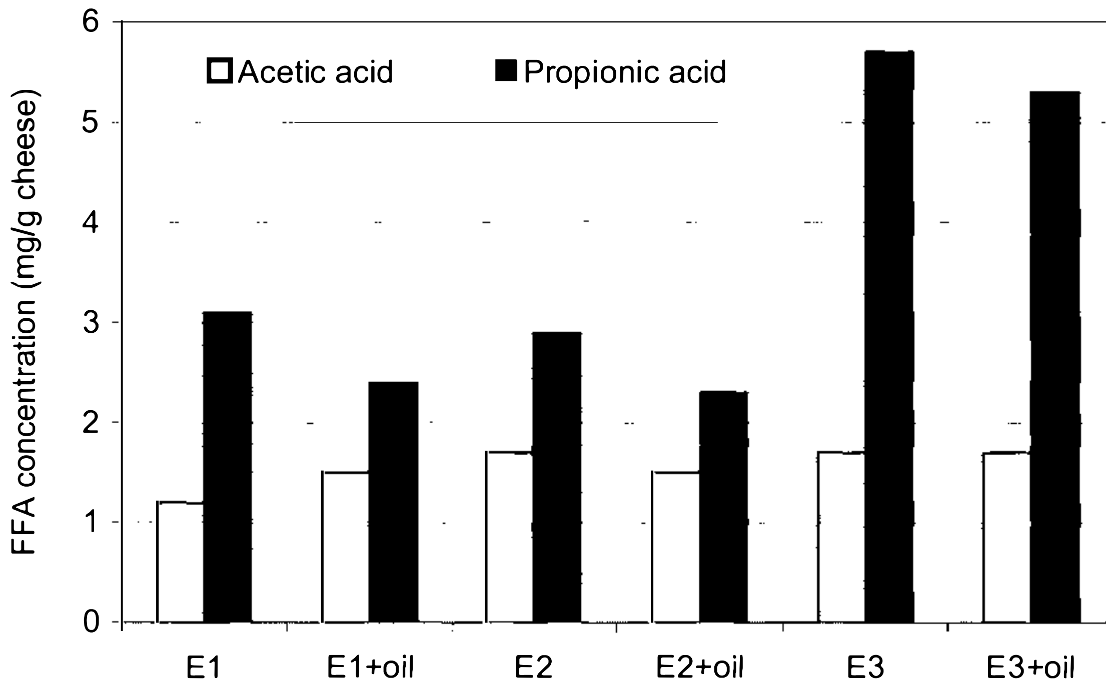
Propionibacteria strain	Initial concentration of free linoleic acid (mg/mL)	CLA recovered at 48 h (mg/mL)	Cell growth at 48 h (A_{600} nm)	CLA synthesis (mg/mL/unit OD)
B6011	0.1	0.001	2.99	0.0003
	0.5	0.001	3.10	0.0003
	1.0	0.001	3.05	0.0003
B6022	0.1	0.043	5.75	0.0075
	0.5	0.241	0.838	0.2876
	1.0	0.163	0.338	0.4822
B6026	0.1	0.004	6.48	0.0006
	0.5	0.030	1.42	0.0211
	1.0	0.033	0.55	0.0600
B6027	0.1	0.030	5.54	0.0054
	0.5	0.084	5.53	0.0152
	1.0	0.190	5.49	0.0346
B6028	0.1	0.020	3.71	0.0054
	0.5	0.065	3.80	0.0171
	1.0	0.132	3.69	0.0358

Appendix 6.2A Concentrations of FFAs originating from milk fat (mg/g cheese) in washed-curd, dry-salted cheeses made without yeast adjuncts or extraneous enzyme (C), with yeast adjuncts (E1, E2 and E3) and with commercial lipase (M) over 4 months of ripening at 20°C

Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18	C18.1	C18.2	Total
C	1	0.05	0.01	0.01	0.03	0.05	0.09	0.25	0.11	0.29	0.02	0.91
	2	0.06	0.02	0.02	0.04	0.06	0.16	0.48	0.23	0.50	0.03	1.60
	3	0.13	0.03	0.03	0.06	0.10	0.24	0.71	0.30	0.67	0.03	2.30
	4	0.13	0.05	0.04	0.09	0.14	0.37	1.06	0.43	0.95	0.06	3.32
E1	1	0.18	0.08	0.10	0.14	0.13	0.28	0.69	0.36	1.76	0.21	3.93
	2	0.66	0.27	0.27	0.39	0.38	0.89	2.36	1.20	6.45	0.37	13.2
	3	1.22	0.56	0.52	0.76	0.71	1.62	3.32	1.42	7.72	0.38	18.2
	4	1.69	0.80	0.74	1.13	1.09	2.54	5.38	1.90	11.04	0.55	26.9
E2	1	0.19	0.10	0.11	0.14	0.15	0.31	0.79	0.33	2.04	0.18	4.34
	2	0.48	0.23	0.21	0.30	0.30	0.74	1.92	0.81	4.43	0.28	9.70
	3	0.79	0.44	0.39	0.58	0.57	1.35	3.10	1.28	6.81	0.32	15.6
	4	1.61	0.83	0.75	1.15	1.09	2.62	5.24	2.07	10.58	0.53	26.5
E3	1	0.27	0.12	0.14	0.18	0.18	0.37	0.97	0.37	2.94	0.20	5.74
	2	0.74	0.31	0.31	0.43	0.41	0.93	2.42	1.26	6.94	0.47	14.2
	3	1.07	0.56	0.54	0.76	0.70	1.60	3.36	1.53	7.67	0.40	18.2
	4	1.70	0.88	0.82	1.18	1.08	2.55	5.32	2.13	11.45	0.54	27.7
M	1	0.35	0.15	0.09	0.18	0.20	0.43	0.89	0.81	1.01	0.04	4.15
	2	1.38	0.38	0.22	0.43	0.53	1.38	3.54	1.83	3.71	0.14	13.5
	3	1.01	0.46	0.25	0.45	0.55	1.36	3.34	1.42	2.90	0.09	11.8
	4	1.20	0.56	0.32	0.59	0.72	1.84	4.42	1.65	3.66	0.13	15.1

Appendix 6.2B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in washed-curd, dry-salted cheeses made without yeast adjuncts or extraneous enzyme (C), with yeast adjuncts (E1, E2 and E3) and with commercial lipase (M) over 4 months of ripening at 20°C

Cheese	Month	FFAs originating from milk fat (% of total FFAs)									
		C4	C6	C8	C10	C12	C14	C16	C18	C18.1	C18.2
C	1	4.5	1.1	1.1	3.3	5.5	9.9	27.5	12.1	31.9	2.20
	2	3.8	1.3	1.3	2.5	3.8	10.0	30.0	14.4	31.3	1.88
	3	5.7	1.3	1.3	2.6	4.4	10.4	30.9	13.0	29.1	1.30
	4	3.9	1.5	1.2	2.7	4.2	11.1	31.9	13.0	28.6	1.81
E1	1	4.6	2.0	2.5	3.6	3.3	7.1	17.6	9.2	44.8	5.34
	2	5.0	2.0	2.0	3.0	2.9	6.7	17.8	9.1	48.7	2.79
	3	6.7	3.1	2.9	4.2	3.9	8.9	18.2	7.8	42.4	2.08
	4	6.3	3.0	2.7	4.2	4.1	9.5	20.0	7.1	41.1	2.05
E2	1	4.4	2.3	2.5	3.2	3.5	7.1	18.2	7.6	47.0	4.15
	2	5.0	2.4	2.2	3.1	3.1	7.6	19.8	8.4	45.7	2.89
	3	5.1	2.8	2.5	3.7	3.7	8.6	19.8	8.2	43.6	2.05
	4	6.1	3.1	2.8	4.3	4.1	9.9	19.8	7.8	40.0	2.00
E3	1	4.7	2.1	2.4	3.1	3.1	6.5	16.9	6.5	51.2	3.48
	2	5.2	2.2	2.2	3.0	2.9	6.5	17.0	8.9	48.8	3.31
	3	5.9	3.1	3.0	4.2	3.9	8.8	18.5	8.4	42.2	2.20
	4	6.2	3.2	3.0	4.3	3.9	9.2	19.2	7.7	41.4	1.95
M	1	8.3	3.6	2.2	4.3	4.8	10.4	21.5	19.5	24.3	0.96
	2	10.2	2.8	1.6	3.2	3.9	10.2	26.1	13.5	27.4	1.03
	3	8.5	3.9	2.1	3.8	4.7	11.5	28.2	12.0	24.5	0.76
	4	8.0	3.7	2.1	3.9	4.8	12.2	29.3	10.9	24.3	0.86



Appendix 6.3 Concentrations of acetic acid and propionic acid in cheeses containing yeast adjuncts with and without added linoleic-acid-rich safflower oil after 2 months of ripening at 20°C.

Appendix 6.4A Volatile compounds produced in washed-curd, dry-salted cheeses made without (C) and with (E1, E2 and E3) yeast adjuncts after 4 months of ripening at 20°C

Volatile compounds	Relative peak area (x 10^6)			
	C	E1	E2	E3
Alcohol				
Ethanol	52.9	63.2	100.1	79.9
2-Methyl butanol	4.2	nd*	2.0	nd
Hexanol	1.5	1.6	1.7	1.9
Esters				
Ethyl acetate	1.0	1.0	1.2	0.6
Ethyl butanoate	3.0	15.8	16.6	15.3
Ethyl propanoate	3.5	1.5	1.3	1.30
Ethyl hexanoate	1.4	14.5	14.8	13.9
Ethyl octanoate	nd	11.2	13.3	12.8
Ketones				
1-Methoxy-2-propanone	9.7	nd	nd	nd
3-Methyl-2-butanone	1.1	2.3	1.8	1.8
2-Hexanone	2.8	2.1	3.0	1.7
5-Methyl-2-hexanone	nd	12.7	8.4	9.9
2-Heptanone	nd	11.2	10.9	9.1
Aldehyde				
2-Methyl pentanal	nd	2.0	2.1	2.1
Acids				
Acetic acid	88.9	58.2	100.2	192.6
Propanoic acid	288.4	223.0	254.5	388.7
2-Methyl propanoic acid	1.9	nd	1.56	1.03
Butanoic acid	48.3	283.4	284.8	316.0
3-Methyl butanoic acid	14.7	10.0	11.2	7.0
Hexanoic acid	22.9	204.1	212.9	231.9
Octanoic acid	3.4	85.1	93.5	98.5
n-Decanoic acid	nd	11.1	13.8	12.4

* = Not detectable.

Appendix 6.4B Sensory analysis of washed-curd, dry-salted cheeses ripened at 20°C

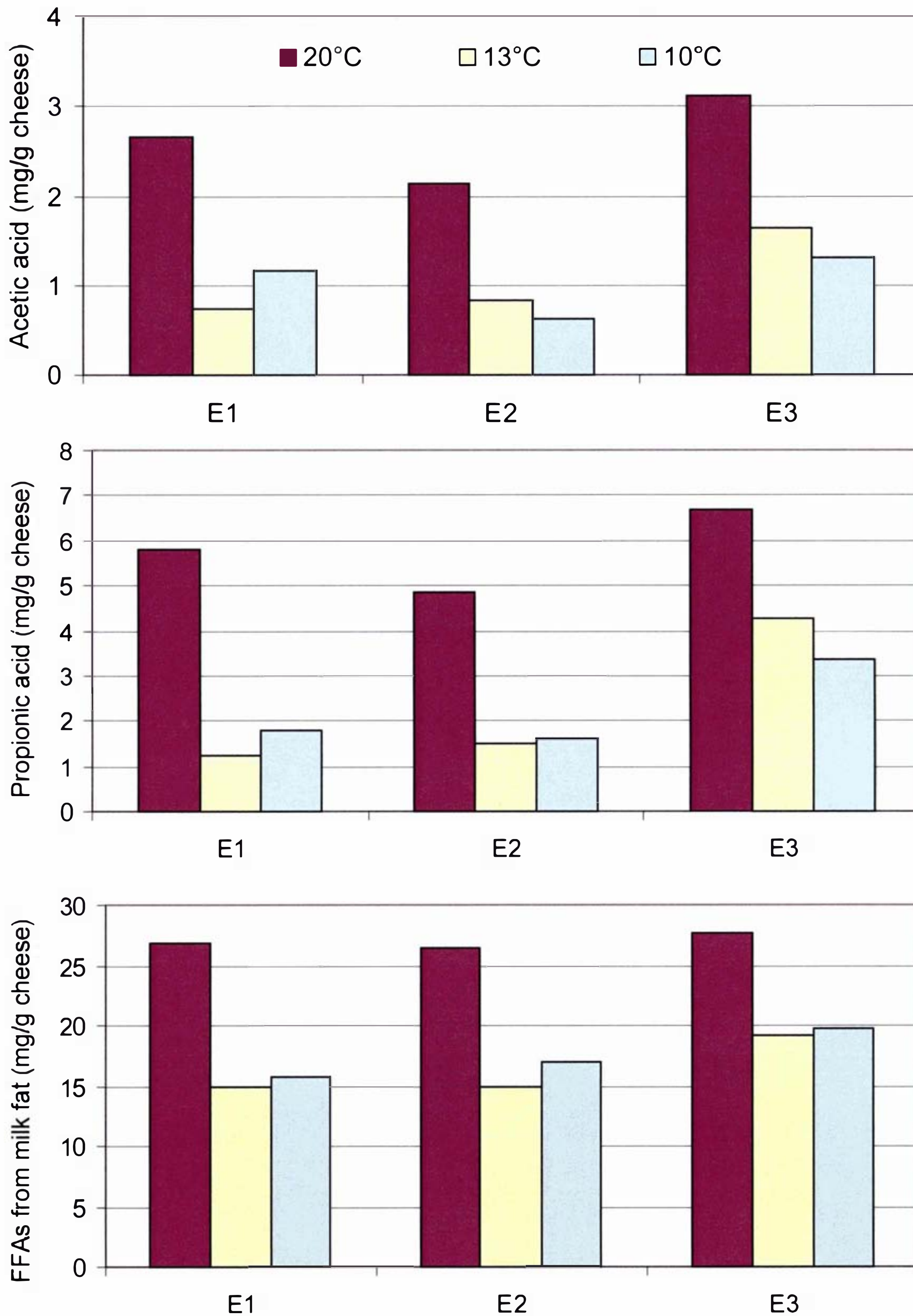
Sensory analysis of the washed-curd, dry-salted cheeses were carried out monthly by an expert panel at Fonterra, Palmerston North. There were 4 panelists. They were not trained for this particular cheese but they are active in cheese research and carry out the informal taste testing of cheese regularly. The sensory analysis was carried out in the office adjacent to the cheese ripening room. As this was not a formal sensory analysis, sensory booths were not used. Carbonated water was used to clean the palate between samples. Cheese samples were presented as plugs tempered to room temperature.

A quantitative descriptive sensory analysis was carried out using a 15 cm linear scale (the intensity increase from left to right). Seven flavour attributes: acid, bitter, fruity, savoury, nutty, butyric and soapy were assessed. The following table summarizes the sensory scores for the above mentioned flavour attributes of washed-curd, dry-salted cheeses over 3 months ripening at 20°C. The scores presented in this table are average values from the four panelists.

The score for the fruity flavour, which was a target, was higher for the cheeses made with yeast adjuncts at 2 and 3 months. The fruity flavour was most pronounced at 2 months. At that point the cheeses were most acceptable. The fruity note was described as desirable. At 3 months the flavour became more butyric and soapy and was not acceptable. Cheese ripening was continued for 4 months, in spite of the deteriorating flavour after 2 months, to see whether there was any change in CLA concentration at the later stage of ripening.

Sensory scores of some flavour attributes of washed-curd, dry-salted cheeses made without yeast adjuncts or exogenous enzyme (C), with yeast adjuncts (E1, E2 and E3) and with commercial lipase (M) over 3 months ripening at 20°C

Flavour attributes	1 Month					2 Months					3 Months				
	C	E1	E2	E3	M	C	E1	E2	E3	M	C	E1	E2	E3	M
Acid	3.3	4.5	4.7	4.5	2.9	4.2	3.7	4.7	3.9	4.5	4.3	5.5	5.1	6.5	4.2
Bitter	4.5	5.1	3.5	4.2	2.6	3.0	3.2	4.4	3.6	4.7	3.1	3.2	5.0	5.1	3.7
Fruity	4.8	4.1	4.4	5.0	5.0	6.2	8.1	8.1	9.7	5.5	6.6	7.5	8.2	7.1	6.5
Savoury	2.4	2.8	5.0	5.3	2.6	4.7	6.1	5.7	5.9	4.9	3.4	3.8	4.0	3.2	4.3
Nutty	2.7	1.9	3.6	5.6	2.0	5.8	5.5	7.4	7.5	4.6	4.8	5.7	6.5	6.2	4.7
Butyric	1.9	4.2	3.9	3.3	2.8	3.2	5.5	6.8	5.8	7.7	5.0	7.7	7.4	9.8	6.9
Soapy	0.3	3.1	2.6	2.9	4.1	2.2	3.8	3.6	4.7	5.6	2.9	5.0	3.8	6.7	5.3



Appendix 6.5 Concentrations of acetic acid (A), propionic acid (B) and FFAs originating from milk fat (C) in 4-month-old washed-curd, dry-salted cheeses containing yeast adjuncts at different ripening temperatures.

Appendix 6.6A Concentrations of FFAs originating from milk fat (mg/g cheese) in 2- and 4-month-old washed-curd, dry-salted cheeses containing yeast adjuncts and ripened at different temperatures

Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2	Total
20°C												
E1	2	0.66	0.27	0.27	0.39	0.38	0.89	2.36	1.20	6.45	0.37	13.24
	4	1.69	0.80	0.74	1.13	1.09	2.54	5.38	1.90	11.00	0.55	26.86
E2	2	0.48	0.23	0.21	0.30	0.30	0.74	1.92	0.81	4.43	0.28	9.70
	4	1.61	0.83	0.75	1.15	1.09	2.62	5.24	2.07	10.60	0.53	26.47
E3	2	0.74	0.31	0.31	0.43	0.41	0.93	2.42	1.26	6.94	0.47	14.22
	4	1.70	0.88	0.82	1.18	1.08	2.55	5.32	2.13	11.50	0.54	27.65
13°C												
E1	2	0.11	0.05	0.07	0.10	0.09	0.18	0.44	0.21	1.63	0.25	3.13
	4	0.68	0.36	0.45	0.60	0.51	1.03	2.10	0.80	8.00	0.45	14.98
E2	2	0.05	0.03	0.04	0.07	0.07	0.14	0.34	0.15	1.39	0.18	2.46
	4	0.69	0.37	0.43	0.56	0.49	0.99	2.10	0.81	8.05	0.43	14.92
E3	2	0.11	0.05	0.07	0.10	0.09	0.18	0.49	0.26	1.68	0.26	3.29
	4	1.03	0.53	0.65	0.86	0.68	1.37	2.85	1.09	9.53	0.58	19.17
10°C												
E1	2	0.05	0.03	0.04	0.06	0.07	0.15	0.36	0.12	1.21	0.14	2.23
	4	0.72	0.31	0.38	0.49	0.43	0.96	2.10	0.81	8.98	0.53	15.71
E2	2	0.09	0.04	0.06	0.09	0.08	0.18	0.42	0.14	1.31	0.15	2.56
	4	1.36	0.45	0.49	0.66	0.63	1.34	2.64	0.87	8.02	0.48	16.94
E3	2	0.10	0.05	0.07	0.10	0.09	0.19	0.43	0.14	1.35	0.15	2.67
	4	0.78	0.43	0.53	0.69	0.58	1.27	2.86	1.89	10.10	0.64	19.77

Appendix 6.6B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in 2- and 4-month-old washed-curd, dry-salted cheeses containing yeast adjuncts and ripened at different temperatures

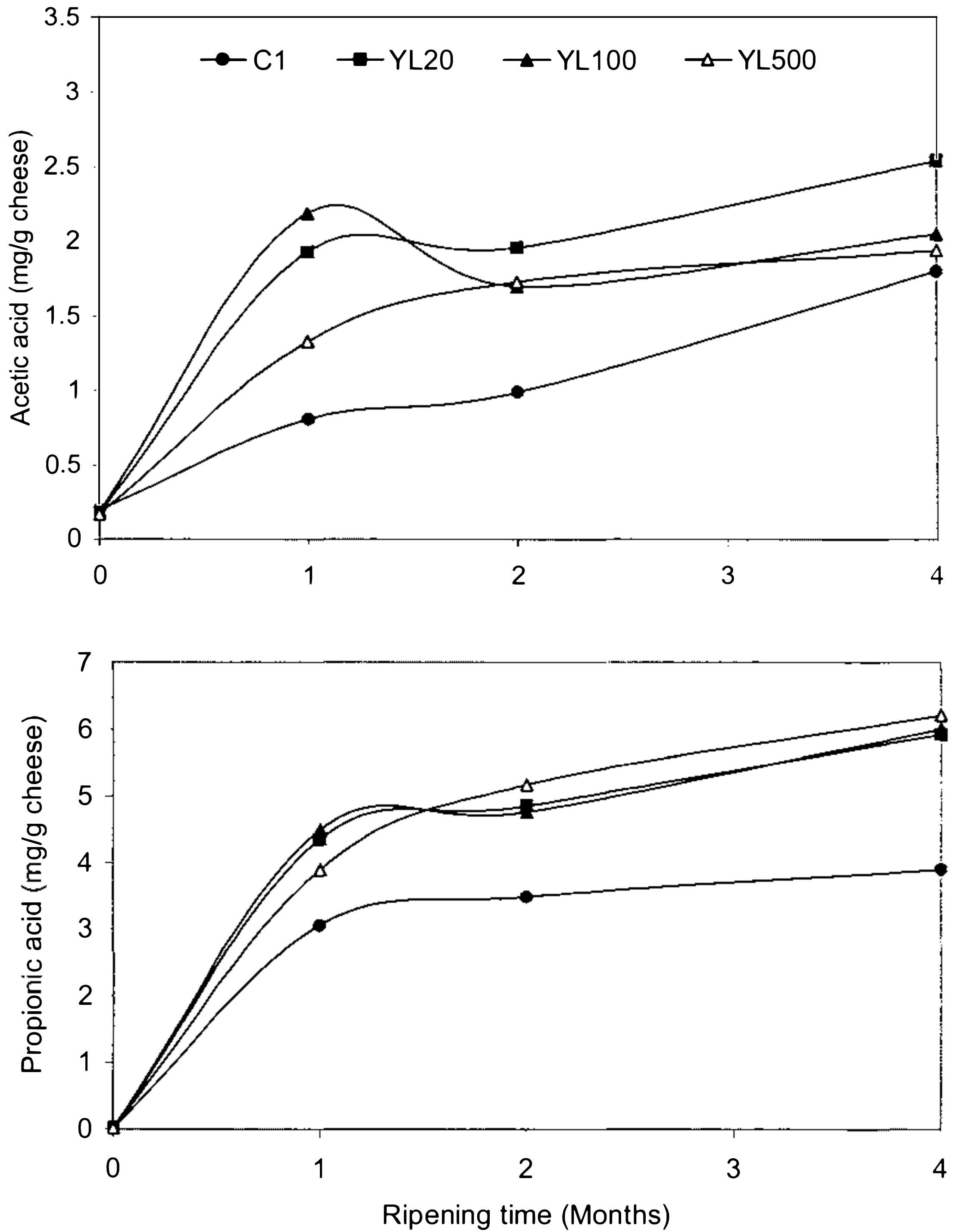
Cheese	Month	C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2
		FFAs originating from milk fat (% of total FFAs)									
20°C											
E1	2	5.0	2.0	2.0	3.0	2.9	6.7	17.8	9.1	48.7	2.8
	4	6.3	3.0	2.8	4.2	4.1	9.5	20.0	7.1	41.1	2.1
E2	2	5.0	2.4	2.2	3.1	3.1	7.6	19.8	8.4	45.7	2.9
	4	6.1	3.1	2.8	4.3	4.1	9.9	19.8	7.8	40.0	2.0
E3	2	5.2	2.2	2.2	3.0	2.9	6.5	17.0	8.9	48.8	3.3
	4	6.2	3.2	3.0	4.3	3.9	9.2	19.2	7.7	41.4	2.0
13°C											
E1	2	3.5	1.6	2.2	3.2	2.9	5.8	14.1	6.7	52.1	8.0
	4	4.5	2.4	3.0	4.0	3.4	6.9	14.0	5.3	53.4	3.0
E2	2	2.0	1.2	1.6	2.9	2.9	5.7	13.8	6.1	56.5	7.3
	4	4.6	2.5	2.9	3.8	3.3	6.6	14.1	5.4	53.9	2.9
E3	2	3.3	1.5	2.1	3.0	2.7	5.5	14.9	7.9	51.1	7.9
	4	5.4	2.8	3.4	4.5	3.6	7.2	14.9	5.7	49.7	3.0
10°C											
E1	2	2.2	1.4	1.8	2.7	3.1	6.7	16.1	5.4	54.3	6.3
	4	4.6	2.0	2.4	3.1	2.7	6.1	13.4	5.2	57.2	3.4
E2	2	3.5	1.6	2.3	3.5	3.1	7.0	16.4	5.5	51.2	5.9
	4	8.0	2.7	2.9	3.9	3.7	7.9	15.6	5.1	47.3	2.8
E3	2	3.8	1.9	2.6	3.8	3.4	7.1	16.1	5.2	50.6	5.6
	4	4.0	2.2	2.7	3.5	2.9	6.4	14.5	9.6	51.1	3.2

Appendix 6.7A Concentrations of FFAs originating from milk fat (mg/g cheese) in 1-, 2- and 4-month-old washed-curd, dry-salted cheeses containing *G. candidum* adjuncts at different levels and ripened at 20°C

Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2	Total
C1	1	0.08	0.03	0.03	0.07	0.12	0.32	0.92	0.38	0.83	0.04	2.82
	2	0.09	0.04	0.03	0.09	0.15	0.44	1.25	0.53	1.08	0.05	3.75
	4	0.12	0.05	0.04	0.11	0.18	0.58	1.77	0.84	1.67	0.08	5.44
GC20	1	0.10	0.03	0.03	0.08	0.12	0.33	0.95	0.39	0.90	0.10	3.03
	2	0.12	0.05	0.04	0.09	0.14	0.41	1.17	0.50	1.12	0.11	3.75
	4	0.19	0.07	0.06	0.13	0.20	0.62	1.86	0.87	2.13	0.27	6.40
GC100	1	0.40	0.20	0.15	0.24	0.28	0.71	1.85	0.71	3.59	0.41	8.54
	2	0.51	0.25	0.19	0.33	0.39	1.09	2.95	1.18	6.19	0.61	13.70
	4	1.28	0.61	0.40	0.69	0.75	2.03	4.73	1.67	10.12	0.62	22.90
GC500	1	0.13	0.08	0.06	0.11	0.14	0.37	1.01	0.40	1.50	0.07	3.87
	2	0.26	0.14	0.10	0.20	0.25	0.76	2.13	0.95	3.84	0.17	8.80
	4	0.54	0.27	0.19	0.35	0.41	1.19	3.25	1.64	7.93	0.32	16.10

Appendix 6.7B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in 1-, 2- and 4-month-old washed-curd, dry-salted cheeses containing *G. candidum* adjuncts at different levels and ripened at 20°C

Cheese	Month	FFAs originating from milk fat (% of total FFAs)									
		C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2
C1	1	2.8	1.1	1.1	2.4	4.3	11.3	32.6	13.5	29.4	1.4
	2	2.4	1.1	0.8	2.4	4.0	11.7	33.3	14.1	28.8	1.3
	4	2.2	0.9	0.7	2.0	3.3	10.7	32.5	15.4	30.7	1.5
GC20	1	3.3	1.0	1.0	2.6	4.0	10.9	31.4	12.9	29.7	3.3
	2	3.2	1.3	1.1	2.4	3.7	10.9	31.2	13.3	29.9	2.9
	4	3.0	1.1	0.9	2.0	3.1	9.7	29.1	13.6	33.3	4.2
GC100	1	4.7	2.3	1.8	2.8	3.3	8.3	21.7	8.3	42.4	4.8
	2	3.7	1.8	1.4	2.4	2.8	7.9	21.6	8.6	45.2	4.5
	4	5.6	2.7	1.7	3.0	3.3	8.9	20.7	7.3	44.2	2.7
GC500	1	3.4	2.1	1.6	2.8	3.6	10.0	26.1	10.3	38.8	1.8
	2	3.0	1.6	1.1	2.3	2.8	8.6	24.2	11.0	43.6	1.9
	4	3.4	1.7	1.2	2.2	2.5	7.4	20.2	10.2	49.3	2.0



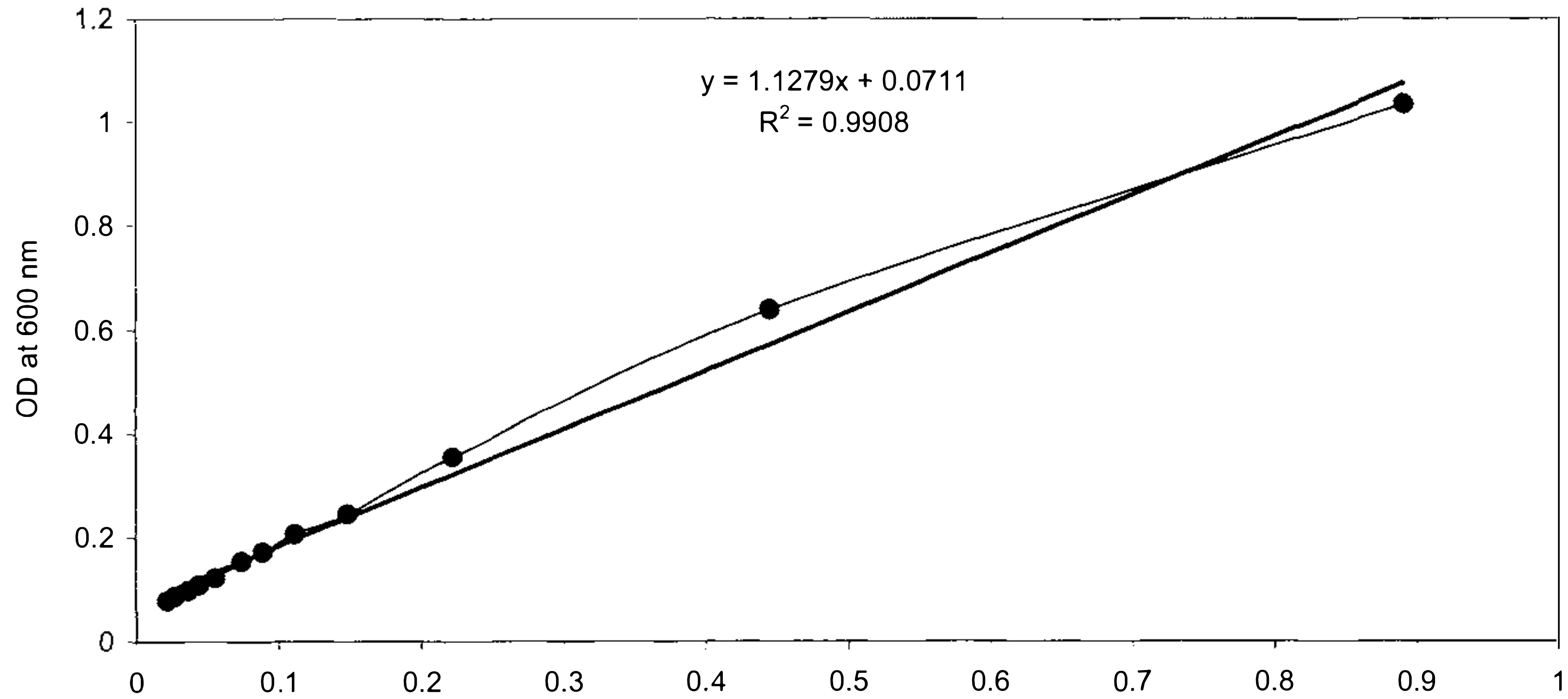
Appendix 6.8 Production of acetic acid and propionic acid by propionibacteria in washed-curd, dry-salted cheeses in the presence of different concentrations of *Y. lipolytica* adjunct over 4 months of ripening at 20°C.

Appendix 6.9A Concentrations of FFAs originating from milk fat (mg/g cheese) in 1-, 2- and 4-month-old washed-curd, dry-salted cheeses containing *Y. lipolytica* adjuncts at different levels and ripened at 20°C

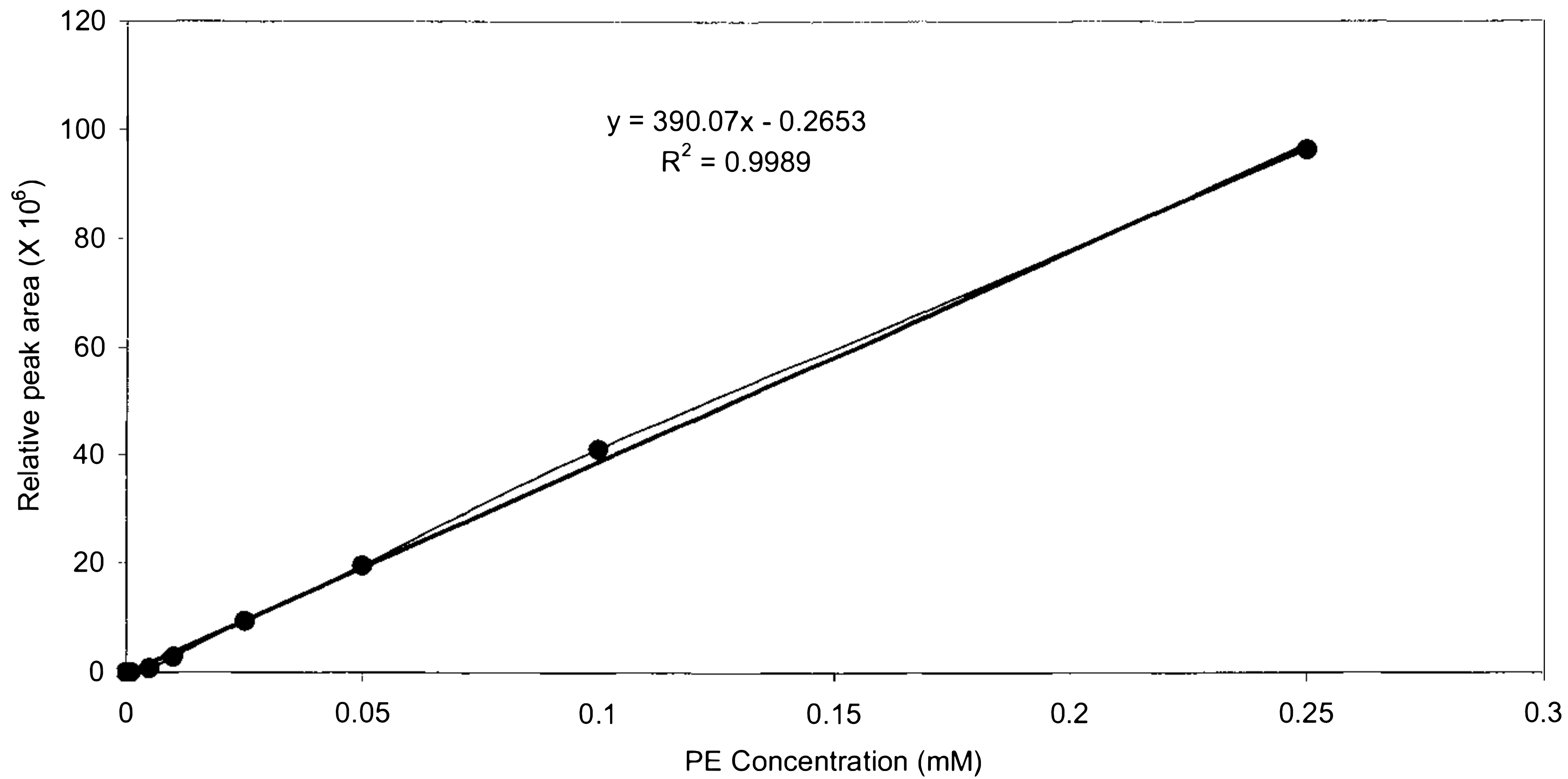
Cheese	Month	FFAs originating from milk fat (mg/g cheese)										
		C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2	Total
C1	1	0.08	0.03	0.03	0.07	0.12	0.32	0.92	0.38	0.83	0.04	2.82
	2	0.09	0.04	0.03	0.09	0.15	0.44	1.25	0.53	1.08	0.05	3.75
	4	0.12	0.05	0.04	0.11	0.18	0.58	1.77	0.84	1.67	0.08	5.44
YL20	1	0.17	0.08	0.08	0.15	0.19	0.51	1.42	0.62	1.94	0.37	5.53
	2	0.23	0.10	0.10	0.19	0.24	0.66	1.84	0.86	2.56	0.34	7.12
	4	0.56	0.28	0.29	0.51	0.52	1.31	3.52	1.97	5.86	0.70	15.52
YL100	1	0.32	0.14	0.14	0.25	0.27	0.69	0.83	0.83	2.64	0.39	6.50
	2	0.56	0.29	0.27	0.46	0.47	1.14	2.61	1.15	4.01	0.59	11.55
	4	1.10	0.55	0.48	0.85	0.85	2.05	4.80	2.44	7.84	0.75	21.71
YL500	1	0.58	0.30	0.30	0.50	0.48	1.08	2.33	0.93	3.83	0.32	10.65
	2	1.04	0.54	0.48	0.78	0.73	1.71	3.62	1.57	6.05	0.47	16.99
	4	2.04	1.14	0.99	1.71	1.60	3.41	7.04	4.22	14.08	0.90	37.13

Appendix 6.9B Concentrations of FFAs originating from milk fat (as a % of total FFAs) in 1-, 2- and 4-month-old washed-curd, dry-salted cheeses containing *Y. lipolytica* adjuncts at different levels and ripened at 20°C

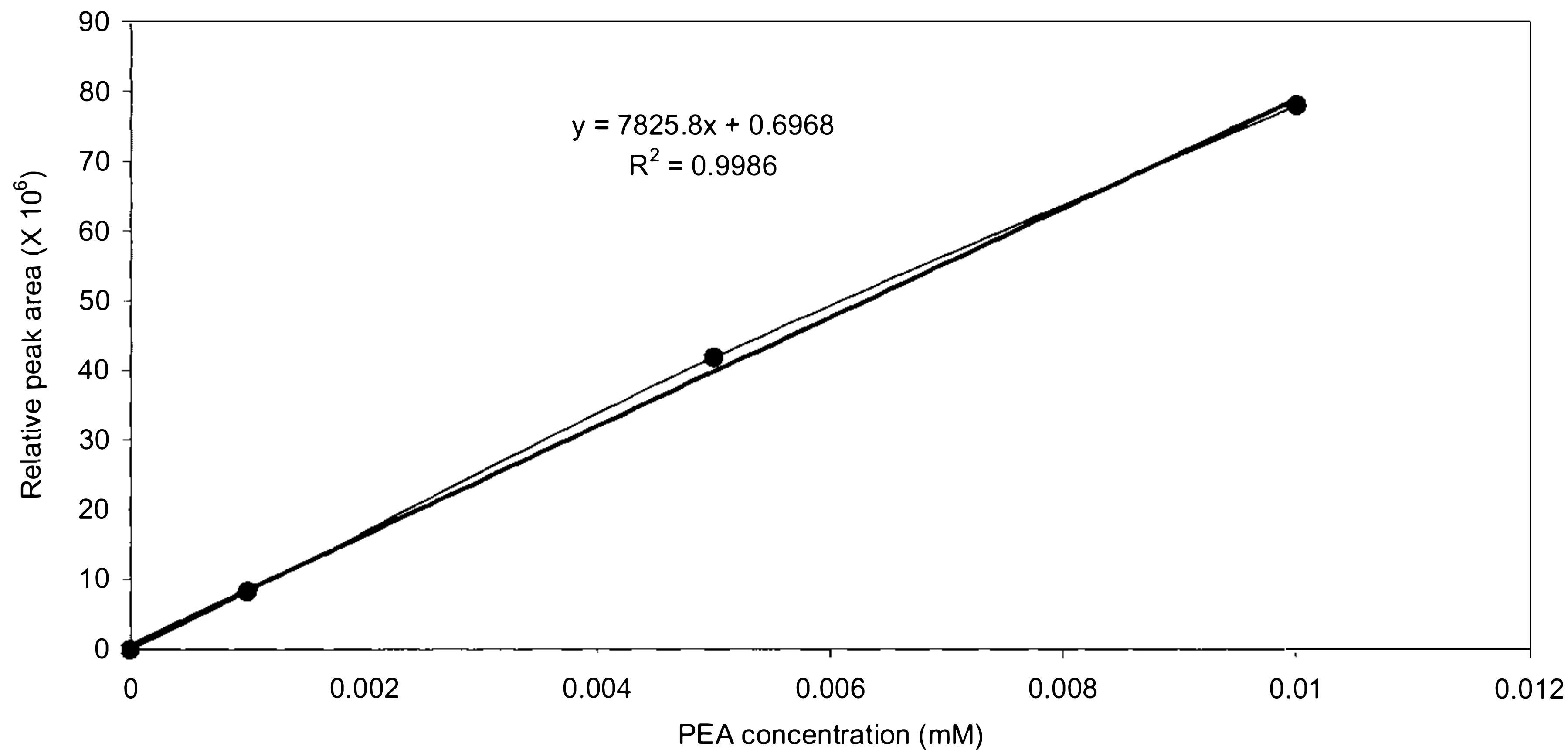
Cheese	Month	FFAs originating from milk fat (% of total FFAs)									
		C4	C6	C8	C10	C12	C14	C16	C18:0	C18:1	C18:2
C1	1	2.8	1.1	1.1	2.5	4.3	11.4	32.6	13.5	29.4	1.4
	2	2.4	1.1	0.8	2.4	4.0	11.7	33.3	14.1	28.8	1.3
	4	2.2	0.9	0.7	2.0	3.3	10.7	32.5	15.4	30.7	1.5
YL20	1	3.1	1.4	1.4	2.7	3.4	9.2	25.7	11.2	35.1	6.7
	2	3.2	1.4	1.4	2.7	3.4	9.3	25.5	12.1	36.0	4.8
	4	3.6	1.8	1.9	3.3	3.4	8.4	22.7	12.7	37.8	4.5
YL100	1	4.9	2.2	2.2	3.8	4.2	10.6	12.8	12.8	40.6	6.0
	2	4.8	2.5	2.3	4.0	4.1	9.9	22.6	10.0	34.7	5.1
	4	5.1	2.5	2.2	3.9	3.9	9.4	22.1	11.2	36.1	3.5
YL500	1	5.4	2.8	2.8	4.7	4.5	10.1	21.9	8.7	36.0	3.0
	2	6.1	3.2	2.8	4.6	4.3	10.1	21.3	9.2	35.6	2.8
	4	5.5	3.1	2.7	4.6	4.3	9.2	19.0	11.4	37.9	2.4



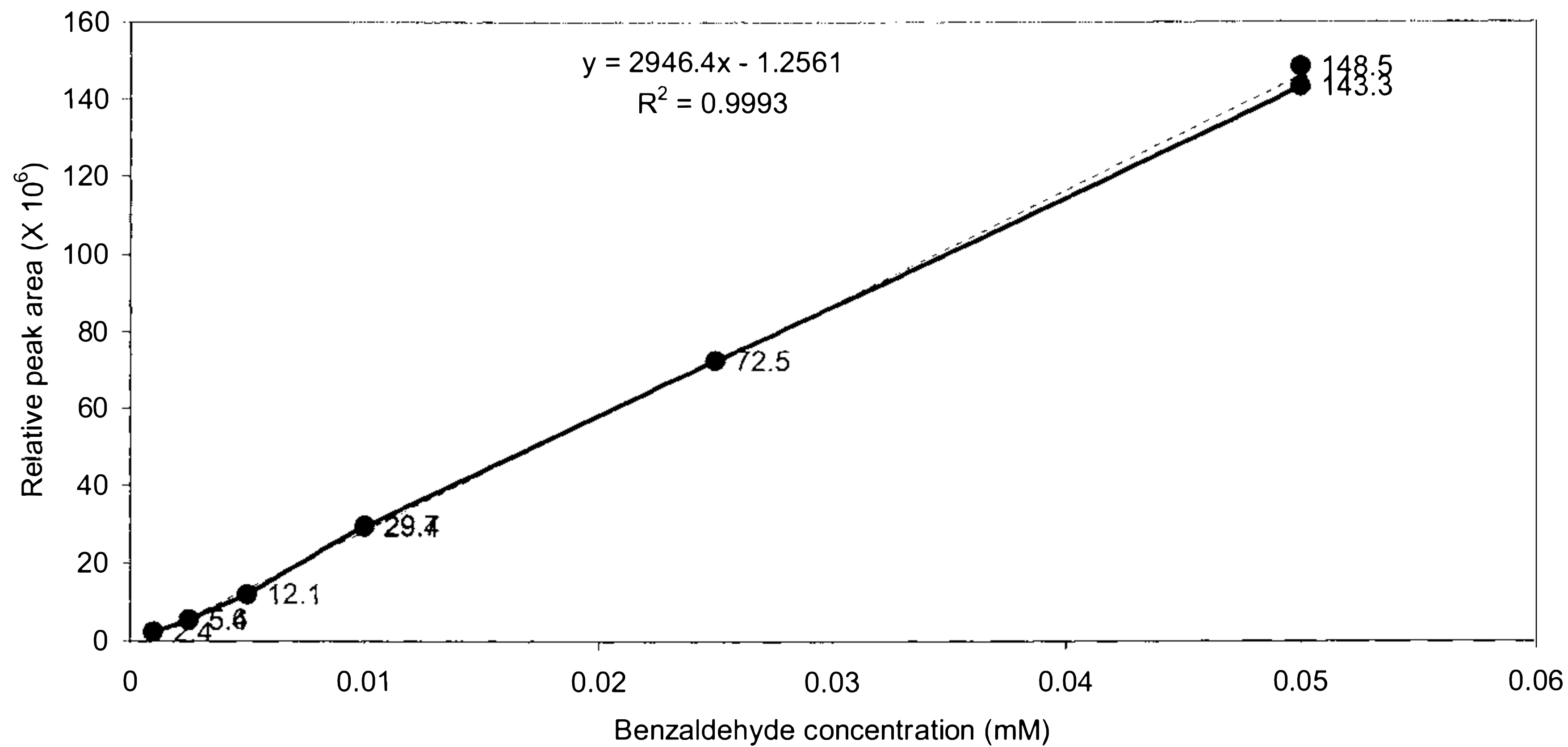
Appendix 7.1 Correlation between OD_{600} and dry cell weight (mg/mL) of *C. kefyr* in a medium containing 13.9 mM L-phe and 61.2 mM glucose (30°C and 200 rev/min).



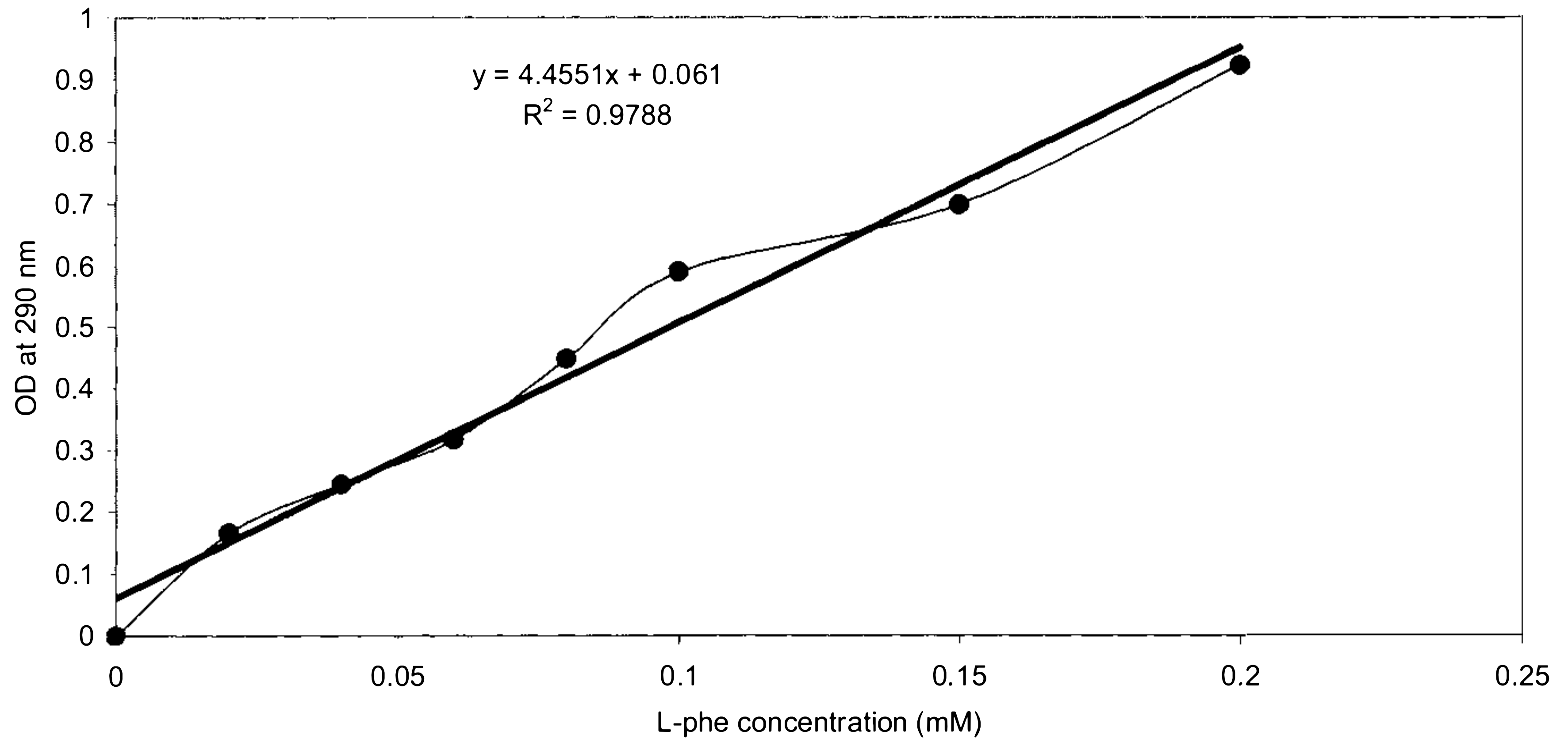
Appendix 7.2A Correlation between relative peak areas detected by GC–MS (10^6) and PE concentrations (mM).



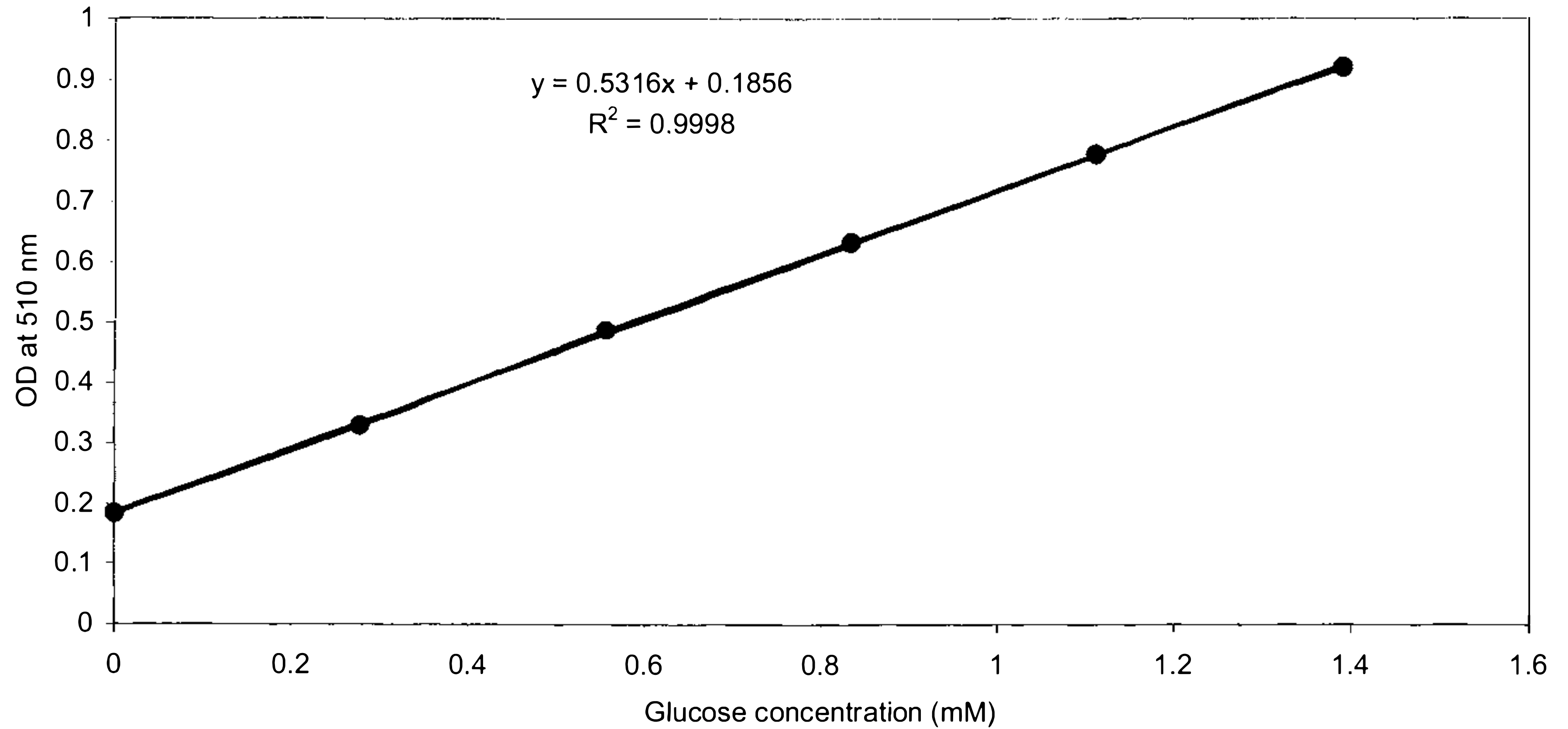
Appendix 7.2B Correlation between relative peak areas detected by GC–MS (10^6) and PEA concentrations (mM).



Appendix 7.2C Correlation between relative peak areas detected by GC–MS (10^6) and benzaldehyde concentrations (mM).



Appendix 7.3 Correlation between OD_{290} values and L-phe concentrations (mM).



Appendix 7.4 Correlation between OD_{510} values and glucose concentrations (mM).

Appendix 7.5 Dry cell weight and pH data for *C. kefyr* cultures grown in media containing different initial concentrations of L-phe at 30°C with 200 rev/min for 72 h (average results from two independent fermentations)

Incubation time (h)	Dry cell weight (mg/mL)					pH				
	A	B	C	D	E	A	B	C	D	E
0	0.0	0.0	0.0	0.0	0.3	6.00	6.00	6.00	6.00	6.00
24	1.6	1.6	1.8	1.6	1.7	5.46	5.97	5.95	5.85	5.76
48	2.9	2.8	3.1	2.5	2.8	5.63	5.89	5.93	5.88	6.00
72	2.6	2.9	2.9	2.6	3.0	6.37	5.88	7.28	7.22	7.20

Note: The control medium (A) did not have any added L-phe but had a background L-phe concentration of 0.1 mM contributed by components of the medium. The experimental media had added L-phe; the initial concentrations of L-phe were 7.8 mM (B), 12.2 mM (C), 26.5 mM (D) and 50 mM (E). All the media had the same initial concentration of glucose (61.2 mM) and pH (6.0).