10
The Influence of Biotechnological Developments on Cheese Manufacture

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Introduction

It has often been observed that biotechnology existed before biotechnologists; the long-term existence of fermentation-based industries illustrates the point very clearly. In particular, the use of biological phenomena in the production of cheese has always encapsulated many of the science disciplines making up biotechnology, and the modern industry is now encouraging the pursuit of every conceivable biotechnological approach, including molecular genetics and protein engineering.

Cheese production is a particularly good example of a biotechnology because it has always relied on a combination of microbial physiology and enzyme biochemistry to convert bland liquid milk into highly flavoured solid cheese, and on bioseparation science and biotransformations to deal with the major by-product, whey. With the advent of modern biotechnological sciences, the traditional biotechnology is able to cope with its transition from a cottage industry to a major commercial operation involving heavy investment by multinational companies in the engineering and food sectors. The contribution of biotechnological sciences in smoothing this transition has, until now, been in developing improved fermentation techniques for the bulk production of pure starter cultures, providing precise DNA-based methods of typing the cultures and their bacteriophages (which attack them in the cheese vat), identifying and producing new enzymes for coagulation of milk and controlling cheese maturation and, finally, fractionating those components of cheese whey which can be used in pharmaceutical and food ingredient applications. Future applications for biotechnology will come from monoclonal antibody and DNA probe-based diagnostic methods for the detection of pathogens and toxins, recombinant DNA technology for starter cultures to enhance flavour production and anti-pathogen activity and protein engineering to modify the range of activities of coagulating and ripening enzymes.

Abbreviations: diACA, L-alanyl-β-chloro-alanine; FDA, Food and Drug Administration; LPS, lactoperoxidase; pepXP, X-prolyl dipeptidyl aminopeptidase

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Monitoring and controlling milk quality

In any industrial process, the quality of the raw materials is critical in determining the quality of the product. The raw material of dairy fermentations is, of course, milk and, being a biological secretion of high nutritional potential, it is subject to spoilage, principally by contaminating bacteria from the cow's environment. The reduction of spoilage potential of milk at source has not been a target of biotechnological interest, but rather one of husbandry, antisepsis and engineering. However, there have been developments in enzyme technology and nucleic acid technology which are relevant to the monitoring and control of milk contamination by spoilage bacteria and pathogens.

ENZYME APPLICATIONS FOR MILK PRESERVATION

The overt spoilage of raw (unpasteurized) milk, in the form of souring by lactic acid bacteria or physical breakdown by sporeformers, is prevented by bulk storage at refrigeration temperatures (ideally *c.* 4°C). However, this does not prevent the growth of psychrotrophic bacteria which produce heat-resistant proteinases and lipases which can reduce the 'manufacturing quality' of the milk, causing flavour taints, yield losses and texture defect in cheese and whey-based products (Law, 1979). Milk contains a natural enzyme system that can kill psychrotrophs (and other Gram-negative bacteria) but it requires activation to be of practical use. This system is based on lactoperoxidase, which uses H_2O_2 to oxidize indigenous thiocyanate to a bactericidal intermediate capable of interfering with membrane energization (Law and John, 1981).

However, the availability of substrates in milk is often low. Thus the lactoperoxidase (LPS) in raw milk requires an exogenous source of hydrogen peroxide (H_2O_2) to kill bacteria. In many countries this cannot legally be added directly and effects are short-lived. Xanthine oxidase (Bjorck and Claesson, 1979) and glucose oxidase (Bjorck and Rosen, 1976) have been used to generate H_2O_2 *in situ* at such a rate that it does not accumulate, but is used by the LPS as it is produced. Although the glucose oxidase method has been successful in preserving milk for cheesemaking, the excess glucose resulted in a fast, over-acid cheese and the expensive glucose oxidase was irretrievable (Reiter and Marshall, 1979). Bjorck and Rosen (1976) avoided direct additions to the milk by immobilizing the glucose oxidase on glass beads and generating the glucose in small amounts from the milk lactose using immobilized β-galactosidase. When the mixed enzyme-associated glass beads are packed in a column, the system forms the basis of a continuous flow, cold sterilization unit.

EXPLOITING ENZYMES FOR DETERMINING MILK QUALITY

Assays of the activities of several enzymes as indicators of milk quality have been used extensively for many decades. Enzyme assays are simple, cheap

and rapid to perform and obtain results, and they usually require minimal equipment and interpretative skills. They can thus be ideal screening methods to indicate whether more detailed analyses are required. That useful information can be produced so readily, is due to the biochemical specificity of enzymes and this section will deal with the more commonly recognized, and possible new, practical methods; many more have been suggested than can be dealt with here.

Direct assays using enzymes

Two basic approaches have been developed. The first relies on assays of the activities of indigenous milk enzymes. The other measures the activities of microbial enzymes to indicate the presence of the micro-organisms, which are known to affect milk quality.

Indigenous enzymes. The phosphatase test is the most widely used of this type of assay. Raw milk contains many different enzymes, but by chance the thermal destruction characteristics of alkaline phosphatase allow it to be used to indicate whether the correct time/temperature treatment for achieving pasteurization has been applied (Lück 1972; Anon., 1983). The enzyme can be assayed quickly and cheaply, and, together with the sensitivity of the test, this has proved extremely useful over many decades. Problems can be encountered with apparent reactivation of enzyme activity, probably due to production of phosphatases by micro-organisms, but no other enzymes appear to be suitable—with the possible exception of lactoperoxidase (Griffiths, 1986).

Mastitis can lead to major alterations in the chemical composition of milk, which can then affect milk quality and derived dairy product quality. Assays for several milk enzymes have been suggested as possible screening tests for mastitis, the most notable being catalase and *N*-acetyl-β-D-glucuronidase (Kitchen, 1981).

Bacterial enzymes. Dye reduction tests have been used for many decades for assessing the bacteriological quality of raw and pasteurized milk. They rely on the reduction of redox dyes, usually resazurin or methylene blue (although tetrazolium salts can also be used), by bacterial dehydrogenases, which relates in some way to microbial activity and cell numbers. Although originally criticized, these tests were widely adopted and are still in use in the dairy industry. However, it is now generally accepted that they are of little value as they are not able to grade milk samples successfully. This has been mainly due to the widespread introduction of refrigeration during the transport and storage of raw milk. This not only reduced microbial activity, but also shifted the general composition of the microflora, so that Gram-negative psychrotrophic bacteria are now predominant, which are apparently intrinsically poor at dye reduction (Lück, 1982; Kroll, 1989). Some rapid, more sensitive, electrochemical assays measuring microbial reducing activity have recently been developed, but results to date suggest that these may be of

limited value in their present form (Swain, 1988; Patchett, Kelly and Kroll, 1989).

Other simple enzyme tests for detecting bacterial enzymes have been developed. Most of these methods have been suggested relatively recently and they are not widely used. Either the methods have not yet been fully developed, or the potential benefits to milk processors have not been sufficient, or sufficiently appreciated, to install the methods for routine use. The nitrate reduction test was used in New Zealand for some years and probably does have some value for screening raw milk samples for high levels of psychrotrophs or coliforms (Lück, 1982; Kroll, 1989). Assays of bacterial cytochrome *c* oxidase or catalase have been suggested as quick, simple methods for assessing psychrotrophs in pasteurized milk, but may also have value for raw milk (Kroll and Rodrigues, 1986; Phillips and Griffiths, 1987; Kroll, Frears and Bayliss, 1989). Similarly, microbial lipases and proteases are known to affect the quality of liquid milk and milk products (Fairbairn and Law, 1986; Stead, 1986). Some effective cheap enzyme tests have been developed but do not appear to be widely used, perhaps due to an under-appreciation of the role of these heat-stable enzymes in the quality of dairy products.

Indirect assays using enzymes

Because of their biological specificity and activity, enzymes are finding increasing uses in novel assay procedures which use enzymes as specific secondary reporter molecules or direct participants in a specific reaction to measure an analyte of interest.

Primary enzyme involvement for bacterial detection. An attractive rapid assay expected to give results equivalent to total bacterial counts is the assay of microbial ATP. This uses firefly luciferase and a product of the reaction—light—is detected by photometry. Good correlations of the light emitted with bacterial numbers in pure cultures have been demonstrated. However, the application to milk and dairy products has been problematic due to background interference by non-microbial ATP. This has been improved by sample pretreatment steps, including a filtration step and the application of an enzyme, apyrase, for degrading non-microbial ATP (Webster *et al.*, 1988). These modifications have increased sensitivity substantially and the method may soon be sensitive enough for practical applications.

An analogous and rather elegant approach exploits the detection of another light-emitting enzyme, this time a bacterial luciferase. The gene encoding the enzyme is cloned into a phage which will specifically infect an organism of interest, e.g. *Salmonella* or *Listeria*. When the foreign DNA is ingested into the target cell, the enzyme is expressed, and the energy stored (as reducing equivalents) in the cell is used to drive the reaction. The target organism lights up and can be detected (Stewart, 1990). Preliminary reports have shown that this system is very sensitive and detects the target organisms in a mixture of other organisms and other interfering materials. A primary

requirement for the success of this method is that phage specificity is total, which may be difficult to achieve in practice. Nevertheless commercial development of this system is under way.

Other bacterial products have been investigated as measures of milk quality, the most notable being enzymatic assays of pyruvate (Cousins, Rodrigues and Fulford, 1981). However, this has proved to be too insensitive to be of use.

For analyte detection. Enzyme-based specific assays for sugars, organic and amino acids have been in use for some time. However, a particularly interesting development has been the development of enzyme electrodes. These are a type of biosensor which transduces biological specificity into direct electrical signals, having many potential adventages in terms of ease of use, rapid or in-line monitoring and automation. Most interest has centred on the measurement of blood glucose, using glucose oxidase as the specific enzyme immobilized at an electrode surface. Both microelectronic and laboratory-scale systems have been produced, which have the required sensitivity, selectivity and stability (Scheller *et al.*, 1987). It is obvious that this approach could be adopted to develop electrodes specific for many analyses of interest to the dairy industry (e.g. lactose, lactate) and some prototypes have been produced.

The presence of antibiotics in milk, particularly penicillin, is of particular concern. Antibiotic therapy is widely used for the treatment of mastitis and trace amounts entering the milk supply can inhibit the growth of dairy starter organisms or may cause allergenic reactions in consumers. A simple rapid test used carboxypeptidases, D-amino acid oxidase and peroxidase to give a quantitative chromogenic reaction, which is inhibited in the presence of β-lactam antibiotics (Thorogood and Ray, 1984). Penicillin-specific enzyme electrodes have been suggested (Blackburn, 1987), but this method does not appear to be in widespread use.

Secondary enzyme involvement. The development of reliable, rapid, sensitive, and accurate methods for detecting pathogenic organisms in milk and dairy products is a priority for research. Traditional microbiological methods rely on largely cultural procedures involving pre-enrichment, selective enrichment, plating on diagnostic media and a battery of biochemical and serological tests. This is not only labour intensive and expensive, but the results may take many days or weeks to be obtained and the accuracy of the results is sometimes questionable.

The development of immunological methods provided an alternative approach and offered the potential of sensitive assays, which could simultaneously give both identification and detection, using a microbial antigen as the target for a specific immunological reaction. Although originally developed as radioimmunoassays, fluorescent antibody methods were soon developed and pathogens could be detected directly and identified from enrichment broths, the use of radiolabels not being acceptable for routine food analyses. Despite many advantages, fluorescent antibody methods were

not widely adopted for routine analyses, primarily due to the incidence of false positive results and microscope fatigue in the operators (Thompson, 1981).

Two recent developments have transformed this situation and brought about the commercial availability of several excellent immunoassay kits for detecting different pathogens (e.g. salmonellae and *Listeria*), which are gaining use in practical application. First, the development of monoclonal antibodies has enabled the specificity of detection to be improved. Secondly, methods were developed where enzymes could be coupled to the secondary antibody to act as reporter molecules, so that colorimetric assays could be produced. With the primary antibody immobilized in the wells of microtitre plates, this made for relatively simple, sensitive and rapid assays (Beckers *et al.*, 1988; Mattingley *et al.*, 1988). As with enzyme assays for analytes, prototype immunologically specific assays for bacterial pathogens, which produce direct electrical signals, have been developed, some of which employ enzymes as reporter molecules (Mirhabibollahi, Brooks and Kroll, 1990; Prusak-Sochaczewski, Luong and Guilbault, 1990).

Another exciting area is the development of oligonucleotide probes specific to base sequences in bacterial RNA or DNA. A limitation with antibody based methods is that they rely on the phenotypic expression of cell-surface markers. These can be subject to natural variation and may also be entirely genus- or species-specific surface antigens, so that immunological assays cannot always be produced. Because the nucleic acids contain all the primary information that specifies the complete make-up and characteristics of an organism, probes should be able to be designed which are not only entirely specific, but related to the organism's genealogy and are specific at the different taxonomic levels of genus and species, or for a particular gene of interest, such as a pathogenic factor. Many nucleic acid probes have now been produced, mainly in research laboratories, although some have been commercialized (Klinger *et al.*, 1988; Barry, Powell and Gannon, 1990). However, probe hybridization is invariably measured using ^{32}P, and, as with immunoassays, radioactive detection is not acceptable in food analyses. Several different non-radioactive detection systems, using enzymes as reporter molecules, have been developed (Evans and Towner, 1990). Initial reports look most encouraging and rapid, sensitive and specific assays look to be a possibility. A further notable aspect is the development of the polymerase chain reaction. This involves the use of a thermostable polymerase enzyme, which can be used to multiply the target sequences of probes many thousandfold in a few hours (Chen *et al.*, 1989; Olive, 1989). Results with pure cultures have shown that this can substantially increase the sensitivity of detection, although whether this can be applied directly to milk and milk products remains to be established.

The enzymic coagulation of milk

This section concentrates on recombinant chymosin, rather than on the microbial coagulants, which are already established in a small sector of the market.

DEVELOPMENT OF RECOMBINANT CHYMOSIN

Chymosin (rennin) is an aspartyl protease found in the fourth stomach of the unweaned calf. This enzyme catalyses a single cleavage between phenylalanine and leucine in κ-casein, which releases a charged glycomacropeptide, destabilizing the colloidal suspension of casein micelles. The gross effect of these events is that the milk casein forms a gel, which is the structural basis of cheese curd. This process is essential for cheesemaking, but the limited availability of the enzyme from calf stomach made it a prime candidate for recombination DNA research. The native enzyme is produced as a zymogen precursor (prochymosin) and a 42 amino acid residue N-terminal propeptide is autocatalytically cleaved at low pH. There is also a further presequence of 16 amino acids which acts as a leader. The procedures followed to obtain the gene were the standard methods for isolation of messenger DNA. Then a reverse transcriptase was used to produce copy DNA. In the approach of Emtage *et al.* (1983) the gene was reconstructed *in vitro* from a series of restriction fragments of the cDNA clone. This was then inserted into a bacterial plasmid immediately downstream from the strong *Escherichia coli trp* promoter, a functional ribosome binding site and ATG by means of synthetic DNA fragments. The prochymosin gene was used for this procedure, as preprochymosin would require *E. coli* to recognize the eukaryotic signal peptide and process it accurately to prochymosin. Although *E. coli* processes the rat preproinsulin in this manner, apparently it does not process preinterferon-α or -β or pregrowth hormone.

The production of chymosin directly would lead to a molecule with a methionine as the first amino acid, whereas prochymosin could be cleaved by use of acid incubation. Ninety per cent of *E. coli* proteins are precipitated under acidic conditions, which helps in the purification of chymosin. In addition, 14 bases downstream of the ribosome-binding site is an initiator, ATG, followed immediately by restriction sites and the terminator *E. coli* RNA polymerase from bacteriophage T7. When the Shine–Dalgarno to ATG distances were optimized, authentic chymosin was expressed. As usual with heterologous expression in *E. coli*, 1–5% of the total protein was chymosin and it formed inclusion bodies. These were seen as advantageous by Emtage *et al.* (1983), as they assisted in purification of the enzyme. Final purification, after solubilization of the inclusion bodies with 9 M urea, refolding in neutral buffer and DEAE-cellulose chromatography, resulted in an authentic milk-clotting chymosin. The formation of inclusion bodies can be seen as both an advantage and a disadvantage. Taking the latter view, Goff *et al.* (1984) tried to transform *Saccharomyces cerevisiae*. Apart from the fact that yeasts are common food constituents and therefore should be ideal for producing

food-grade enzymes, the fermentation conditions for this organism are well developed on an industrial scale. The use of the *gal1* promoter results in good amounts of chymosin being produced in the cells. Although there was no obvious inclusion body formation, the extractable cellular contents did not account for all of the enzyme and there seemed to be absorption within the cells. The next advance was to obtain controlled expression and secretion of this enzyme using a host which is known to secrete large amounts of protein. Cullen *et al.* (1987) chose *Aspergillus niger* because this filamentous fungus normally secretes large amounts of protein into the extracellular medium. (It has been shown that >5 g l^{-1} of glucoamylase can be secreted.) Various improvements to the yeast system have brought the yield up to 20 mg l^{-1} of chymosin (Smith, Duncan and Moir, 1985) and it was hoped that the use of the glucoamylase promoter from *A. niger* would improve upon this. Stable transformants of *A. niger* are only obtained when vector sequences are integrated into the chromosome. Autonomously replicating vectors have not been described for *Aspergillus*. Integration of some constructs was demonstrated and the expression levels did not directly correlate with gene copy numbers. The signal sequence of chymosin was equally as good as the signal sequence of glucoamylase in promoting expression. More than 90% was secreted into the culture medium and the majority was enzymically active. The prochymosin was cleaved correctly and these experiments indicated that chymosin could be produced commercially using the *Aspergillus* glucoamylase promoter.

More recently, Berka *et al.* (1991) have reported that the expression of bovine chymosin and *Mucor miehei* proteinase (an approved fungal alternative to chymosin) can be improved to commercially viable levels in *A. niger* var. *awanori*. Although *Aspergillus nidulans* and *Aspergillus oryzae* can produce both enzymes, most of the investigative work has been done with the *A. niger* strain. These authors showed that the major obstacle to high-level production of heterologous proteins is the failure of *secretion* of the foreign gene products, despite the organism's known efficiency at secreting native proteins. Interestingly, their fungal strain secreted the *Mucor* enzyme much more efficiently than the bovine enzyme, and the phenomenon was associated with the fact that the *Mucor* enzyme, but not the bovine, was glycosylated. However, even the introduction of an *N*-linked glycosylation site into chymosin, though producing some improvement, did not result in anything approaching the secretion levels of the *Mucor* enzyme. Yields of bovine chymosin from *A. niger* have been improved by a different approach; that of expressing it as a fusion protein with the efficiently produced native glucoamylase. Chymosin can be released by autocatalytic cleavage of its propeptide.

As yet another option, a host/vector system has been developed to express chymosin in *Trichoderma reesei* (Harkki *et al.*, 1989). The organism grows by breaking down cellulosic substrates and the promoter and terminator for the major cellulase (*cbh1*) have been used to construct an expression vector for chymosin A which produces chymosin at 40 mg l^{-1}.

It is to be hoped that use of these techniques will overcome the world

shortage of chymosin for use in the cheese industry. Cheddar cheesemaking trials on recombinant calf chymosin expressed in *E. coli* (Green *et al.*, 1985), *Kluyveromyces* sp. (Bines, Young and Law, 1989) and *Aspergillus* sp. (Law, unpublished results) clearly show that such enzyme preparations can replace the traditional source in the successful manufacture of hard cheese, but no long-term commercial trial results are available yet. However, recombinant chymosins have been approved in the USA for use in commercial cheesemaking and their efficacy will no doubt be realized in due course.

CHYMOSIN PROTEIN ENGINEERING

The availability of expression systems capable of making correctly folded chymosin molecules has opened up the possibility of using site-directed mutagenesis to modify the activity of the enzyme. To date, the only commercially relevant example of protein engineering of coagulants has been provided by a chemically oxidized version of *M. miehei* coagulant. Its heat lability was increased in order to prevent its persistence in pasteurized whey, which is used in many food and feed formulations. However, there are opportunities for modified chymosin, especially with respect to its specificity. For example, the fungal enzymes are easier to produce than calf chymosin in high yield by recombinant DNA technology, but their general proteolytic activity is higher than their specific (Phe-105–Met-106 cleavage) milk-clotting activity. This can result in bitterness in long-hold cheese. Any protein engineering strategy based on enhancement of Phe–Met cleavage, and attenuation of other bond-cleavage activities would produce a technologically better enzyme. A modification which could give benefit in cheese maturation would involve mutations to give a pH/activity shift involving a change in specificity, such that at milk-clotting pH (6·5) the tight Phe–Met specificity was maintained, whereas at the lower pH values found in cheese (4·8–5·4) specificity was relaxed to allow for the release of small flavour-enhancing peptides and amino acids. Although this type of modification is some way away, Quinn *et al.* (1991) have reported chymosin mutants with pH shifts (Thr-218 → Ala and Asp-304 → Ala/Thr-218 → Ala) and a substrate-specificity pocket variant (Gu-288 → Lys). Similarly, Beppu (1990) examined chymosin mutants produced by linker DNA insertion into restriction sites of cDNA and demonstrated a role for Tyr-75 (located in the active-site flap structure) in both catalytic function *and* specificity determination. Other mutants at sites that interact with residues more distant from the scissile bond binding region showed marked changes in specificity of potential technological value. For example, Lys-220 → Leu showed a marked shift in pH optimum to the acidic side, and a Thr-218 → Ser variant had a higher ratio of milk-clotting to proteolytic activity (0·81) than the native enzyme (0·23). If similar changes could be made to fungal coagulants, their potential to produce bitter peptides in cheese would be reduced; of course, any increase in specific activity with respect to milk clotting (Phe–Met cleavage) would also allow a reduction in the amounts of coagulant used in cheesemaking.

The lactic fermentation

THE PRODUCTION AND USE OF LACTIC STARTER CULTURES

The key stage in any milk fermentation is the conversion of lactose in milk to lactic acid by the 'starter' culture (*Figure 1*). These cultures are supplied to the industry either as undefined mixtures of many strains of the appropriate species or, increasingly, as pure defined strain cultures in the form of predetermined mixtures of a small number (2–4) of the correct organism. Cheeses are made with *Lactococcus lactis* (subsp. *lactis, cremoris* or *diacetylactis*) where curd cooking temperatures are below 40°C (e.g. Cheddar, Edam, soft cheeses) or a mixture of thermophilic lactobacilli (e.g. *Lactobacillus casei, Lb. helveticus*) and streptococci (*Streptococcus thermophilus*) where the curd temperature is raised >50°C (e.g. Emmental, Gruyère). Yoghurt is also made with thermophilic cultures. Since much of the biotechnology-driven advance in culture technology has been applied to cheesemaking, the following discussion concentrates on this, rather than the cultured milk sector.

The technology of producing consistent, pure cultures of starter lactic acid bacteria has advanced rapidly over the past 20 years with the introduction of frozen or freeze-dried, high biomass (10^{10} cfu ml^{-1} or g^{-1}) cultures which can be added directly to the cheese vat. This technique is gradually superseding the more traditional approach involving a sequence of mother culture, inoculation culture, bulk starter culture and, finally, vat inoculation

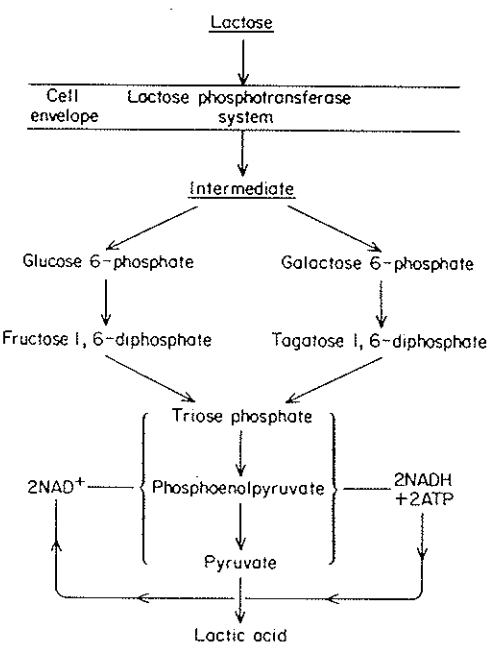

Figure 1. Pathway of lactose uptake and fermentation in lactococci used as cheese starter cultures.

(reviewed by Law, 1982). This development represents a success story for the traditional skills of biotechnologists, in that the production of strains that grow to high cell numbers, survive centrifugal harvesting, washing, freezing and drying has been achieved by using selection techniques, and their mass production was made possible by new bioreactor designs which used non-milk media (often produced using enzymic digestion) and highly automated fermentation control, particularly with respect to pH. The prevention of over-acidification of starter cultures, using pumped alkalis, solid neutralizers or even gaseous exchange, has developed into a technology in its own right.

Despite these successes, there are still problems associated with the use of lactic starter cultures which can only be solved in the medium- to long-term by the application of the 'new' biotechnologies, involving a detailed knowledge of cell biology, metabolic pathways, enzymology and genetics. The need for such basic understanding of starter bacteria arises from the extraordinary demands now being put on them in cheese factories. Capital investment in automated equipment for cheesemaking is now high, as are raw material costs, and profit margins are low (typically 5% for the manufacturer). To cope with this, the larger factories require starter cultures to produce lactic acid more quickly than traditional cultures in less automated units. For example, the time taken to fully coagulate and acidify milk (the 'rennet to mill' time) has been reduced from >5 h to about 3·5 h in many factories. This allows the cheese vats to be used several times a day (often round the clock) but brings its own problems. The most serious of these is caused by the existence of lytic bacteriophages (phages) which attack the lactic culture and slow down acid production. At best this interferes with the factory schedule, reducing output and profitability, and at worst, leads to under-acidified cheese of low quality (and value) which is also susceptible to invasion by pathogens. Most cheese in this latter category has to be discarded or used as a low-value ingredient in processed cheese.

DEVELOPMENT OF BACTERIOPHAGE-RESISTANT LACTIC CULTURES

Starter strains which produce acid quickly and are resistant to phages can be obtained by selection, and the problem is generally under control in the industry, but keeping it under control requires heavy technical back up, either within the industry itself, from Government Institutes, or from the culture supply companies. This back up takes the form of phage-unrelated strains for use in rotation, phage monitoring to ensure continuity of resistant strains, and the supply of starter culture media in which phage replication is inhibited. The prospects for a permanent solution to the phage problem arising from molecular and cell biology are increasing as knowledge of the phenotypic and genetic determinants of natural phage resistance increases.

Naturally ocurring phage-insensitive strains of lactic acid bacteria have been characterized and found to possess multiple defence systems which can operate at different stages of the lytic cycle to prevent adsorption, infection or replication of virulent phages (Klaenhammer, 1989). These defence systems are usually plasmid-encoded and numerous phage-resistance plasmids have

now been detected and characterized (Klaenhammer, 1991). Detailed information on the genotypes and phenotypes of phage-resistance plasmids has been derived from phage-'insensitive' strains which have been naturally selected to operate in culture systems within the industry. One of these strains, *Lac. lactis* ME2 (Klaenhammer and Sanozky, 1985) has a self-transmissible, phage-resistance plasmid, pTR2030, which has been extensively studied, and it provides a good example of how the determinants and mechanisms of phage resistance can be pin-pointed and exploited in transconjugants designed for use in the cheese industry. pTR2030 contains two regions which encode defence mechanisms that co-operate to prevent phage infection and proliferation. Their phenotypes are Hsp^+, manifested as an abortive infection mechanism, and R^+/M^+ restriction and modification activities. Although the overall phage-resistance phenotype exhibited by pTR2030 is highly dependent on the host strain of *Lactococcus* sp., the majority become completely resistant to the most common species of disruptive phage found in the factory environment.

The gene product of the *hsp* region of pTR2030 is a protein which appears to interfere with phage DNA replication, although its specific mechanism is not known (Hill, Miller and Klaenhammer, 1991). The R^+/M^+ phenotype is derived from a gene on pTR2030 encoding a type 11-A-methylase (designated LlaI; Klaenhammer, 1991). Combinations of the two pTR2030 defences are often found on a single plasmid or within one host strain as combinations of different plasmid replicons. Klaenhammer (1991) considers that the complementary action of the systems has the overall effect of minimizing the multiplication of infective agents at the same time as ensuring the survival of the maximum number of cells affected by the primary infection. The combination of defences also acts to minimize the potential for phage adaptation through mutation, recombination or host-dependent modification.

Although there are other examples of phage-resistance plasmids in the literature, pTR2030 is not only a useful example of how molecular biology is used in an investigative mode to find and define mechanisms, but it is also a prime example of how such genetic material can be exploited to construct commercial phage-resistant cultures for cheesemaking. In particular, Sanders *et al.* (1986) have transferred pTR2030 to such commercial strains, using phage resistance to select transconjugants, and plasmid analysis with DNA–DNA hybridization to confirm the acquisition of pTR2030. This strategy avoids the use of antibiotic-resistance markers, which are not favoured by food legislators. Indeed, Froseth and McKay (1991) have also addressed the problem of providing food-grade cloning vectors for use with lactic streptococci. They have developed a circularized *Eco*RI fragment, containing an origin of replication and a nisin-resistance determinant, which is capable of independent replication. It has been used to clone homologous DNA in *Lac. lactis* and transformants are selected by nisin resistance using a specially developed medium, designated M17-GTN.

The intraspecies nature of the DNA transfer by transconjugation is not classified as genetic engineering by regulating bodies. It is anticipated that

these defined genetic changes (unlike mutagenesis and selection) will produce phage-resistant variants which maintain other desirable phenotypic characteristics, such as good acid and flavour production, without interference from uncontrolled genetic events.

Klaenhammer (1991) points out that phage-resistant transconjugants having pTR2030 have been used successfully by the dairy industry since 1985 and that the small numbers of phages isolated from factories using the transconjugants are providing valuable insights into the development of phage counter-defences against host-cell resistance. Many of these are already known, but TR2030 avoidance has revealed the existence of phage acquisition of a functional region of a bacterial methylase gene which it uses as a counter-defence. The acquisition of a phage counter-mechanism to the pTR2030 R/M system is associated with a shift in the phage DNA origin of replication to avoid the abortive mechanism. Knowledge of such molecular events is essential for the molecular biologists to maintain starter biotechnology one step ahead of the phages.

MANIPULATION OF CARBOHYDRATE METABOLISM IN LACTIC CULTURES

The uptake mechanisms and metabolism of lactose and citrate in milk by lactic acid bacteria are well characterized (e.g. Law, 1982) and the DNA coding the enzymes involved has been localized with some precision. However, the scope for using this information is rather limited due to the complex interdependence of the gene products. It has been suggested that the loss of lactose metabolism in starter cultures, due sometimes to plasmid loss, could be reduced by incorporating key genes (such as those encoding the phosphoenolpyruvate-dependent phosphotransferase system for lactose transport) into the bacterial chromosome. However, *lac*$^-$ variants do not dominate lactose-grown cultures in any case, so there is little industrial interest in such a mission. Perhaps more relevant would be the prevention of revertants after selection of *lac*$^-$ variants; such variants are useful sources of additional biomass to hasten the maturation of cheese without interfering with acid production (Law, 1987). They are simple to construct and isolate, but do not always remain *lac*$^-$. Constructs which were inherently incapable of expressing acquired *lac*$^+$ plasmid DNA would be commercially useful.

Although this review is concerned chiefly with cheese, there are some useful examples of manipulations involving yoghurt starters (lactobacilli) which can ferment sucrose, and which do not produce acid in cold-stored yoghurt, thus avoiding the 'wheying-off' defect. For example, Mainzer *et al.* (1989) have introduced cold-sensitive mutations into the *Lactobacillus bulgaricus* β-galactosidase gene cloned in *E. coli*. Replacement of wild-type β-galactosidase genes in the lactobacillus by these mutated genes would produce a useful addition to 'live' yoghurt technology. Unfortunately, lactobacilli are not as receptive to DNA transfer techniques as lactococci, but this problem is being overcome.

CHARACTERIZATION AND MANIPULATION OF PROTEOLYTIC SYSTEMS IN LACTIC CULTURES

The lactococci used as cheese starter cultures are nutritionally fastidious, requiring exogenous sources of nucleotides, vitamins and amino acids. In order to achieve the rate of growth in milk that is necessary for the acidification of cheese, they depend on their proteolytic systems to break down the milk protein, casein, into peptides and essential amino acids. The proteolytic system of lactococci is made up of the cell-wall-associated proteinases and the peptidases (both intracellular and extracellular). Complementary transport systems are also present for the enzyme products (the amino acid transporters and the peptide transporters). As they all play an essential role in the nutritional capacity of lactococci, understanding the mechanisms involved is important for future biotechnological development of starter cultures. For example, there are wide phenotypic variations between starter strains in their rate of growth in milk, which may be related to their 'complement' and intracellular localization of peptidases and carriers, and the ability of the enzyme and carrier proteins to withstand the mechanical and physicochemical stresses of starter culture manufacture and packaging. Current research emphasis is on both the biochemical and genetic characterization of these systems, with a view to producing more efficient acid producers, more robust strains and strains that give enhanced development of savoury flavour in ripening cheese.

Cell-wall-associated proteinase

Proteinase activity is essential in lactococci required to show rapid growth in milk. The proteinase activity of lactococci has been known for many years to be unstable, with proteinase-positive strains (Prt^+) spontaneously converting to proteinase-negative strains (Prt^-). The Prt^- variants are able to reach only 10–25% of the maximum cell density attained by the Prt^+ organisms in milk culture (Lawrence, Thomas and Terzaghi, 1976). The production of the cell wall proteinase of lactococci is also subject to regulation by the growth media (Exterkate, 1979, 1985). Proteinase production in *Lac. lactis* subsp. *cremoris* AM_1 was effectively repressed by addition of peptides to milk media. The need to understand the mechanisms involved in this commercially important trait, has led to the proteinase activity being the most intensively studied system of lactic acid bacteria, the cell-wall-associated proteinase gene of *Lac. lactis* subsp. *cremoris* Wg2 being the first gene to be cloned from these organisms (Kok *et al.*, 1985).

The isolation and selection of plasmid-free strains of lactococci has played an important role in genetic studies on the lactococcal proteinase. Curing experiments had already indicated that in some strains of lactococci the proteinase activity was plasmid encoded, with the loss of plasmids associated with the loss of proteolytic activity (Gasson, 1983; McKay and Baldwin, 1974). This loss of activity could then be reversed by introducing into a plasmid-free Prt^-, strain, *Lac. lactis* subsp. *lactis* MG1363, the proteinase-

encoding recombinant plasmid pGVK500 (Kok *et al.*, 1985). The resulting organism was then able to grow normally in milk, producing a version of the proteinase capable of interacting with antibodies against the enzyme.

Most of the proteinase activity of lactococci is associated with the cell wall fraction (Exterkate, 1975; Thomas *et al.*, 1975). This activity was inhibited by phenylmethylsulphonyl fluoride, indicating that it was due to a serine proteinase, which by immunogold labelling, was found to be located at the outside of the cell wall (Hugenholtz *et al.*, 1987). Despite publications claiming the presence of several distinct proteinases in lactococci, based on biochemical characterization differences, it now appears that there is only one main external proteinase in lactococci. The reason for the apparent proliferation of proteinases is that the degradation products of the cell wall proteinase retain significant activity. These extracellular enzymes and other proteinase-associated fragments have now been shown to have the same amino acid sequence as the cell wall enzyme (Nissen-Meyer and Sletten, 1991).

In *Lac. lactis* subsp. *cremoris*, there are two genetic variants of the cell wall proteinase, PI and PIII, which differ phenotypically in their caseinolytic action. PIII is able to degrade both α- and β-casein, whereas PI cleaves primarily β-casein, with a specificity different from PIII (Visser *et al.*, 1986). The complete nucleotide sequences of the gene for both genetic variants have been determined; PI from *Lac. lactis* subsp. *cremoris* Wg2 (Kok *et al.*, 1988a) and PIII *Lac. lactis* subsp. *cremoris* SK11 (Vos *et al.*, 1989a). These show a 98% homology between the two sequences, differing in only 44 out of 1902 amino acids, with the SK11 enzyme containing an additional 60 amino acid duplication near the C-terminal end. The nucleotide sequence for the cell wall proteinase of *Lac. lactis* subsp. *lactis* NCDO 763 has also been determined and was found to differ in only 18 amino acids from the Wg2 strain enzyme (Kiwaki *et al.*, 1989). These nucleotide sequences encode proteins of ~ 200 kDa, and N- and C-terminal processing is believed to account for the discrepancy with the purified enzyme molecular size of ~ 135 kDa (Kok *et al.*, 1988a; Vos *et al.*, 1989a).

Analysis of the nucleotide sequences shows the proteinases to be synthesized as the preproenzyme, with, at the extreme N-terminal end, a 33 amino acid region with signal peptide characteristics. N-terminal sequence analysis of the purified enzyme showed the mature proteinase sequence to start as an aspartic acid at position 188 of the predicted amino acid sequence, with amino acids 34–187 the pro-sequence. In certain regions, the lactococcal cell wall proteinase is similar to the serine proteinase of the subtilisin family. This similarity applies especially in regions containing the amino acids of the subtilisin catalytic centre, Asp-32, His-64 and Ser-221. In the lactococcal proteinase, Asp-30, His-94 and Ser-443 form the catalytic triad. A major difference, however, between these enzymes is that the lactococcal proteinase is substantially larger than the subtilisins; the preprosubtilisin containing only 381 or 382 amino acids. This size difference is thought to be responsible for the different substrate specificity—subtilisin having a general proteolytic action, whereas the lactococcal proteinase action is highly bond-specific on caseins only.

Another major difference is that, unlike the subtilisins, which are secreted into the extracellular media, the lactococcal enzyme is located at the outer side of the cell membrane. Analysis of the extreme C-terminal region has shown a region, not found in subtilisins, that is similar to the membrane anchors of surface proteins of other Gram-positive bacteria. Deletion of this region prevents attachment of the proteinase to the cell, resulting in its extracellular release (de Vos *et al.*, 1989; Haandrikman *et al.*, 1989; Kiwaki *et al.*, 1989). This does not affect the activity of the enzyme. In both *Lac. lactis* subsp. *cremoris* Wg2 and SK11, deletion of ~300 amino acids from the C-terminal end can be made without affecting the caseinolytic specificity (Kok *et al.*, 1988b; Vos *et al.*, 1989a).

Immediately upstream of the gene encoding the proteinase in both Wg2 and SK11, is a highly conserved region that encodes a *trans*-acting protein involved in the processing of the proenzyme into active enzyme. This gene, *prtM*, transcribes from the same promoter region as the proteinase gene but in the opposite direction. The prtM protein is a lipoprotein, which deletion analysis showed is involved in the activation of the proenzyme. *Lac. lactis* subsp. *lactis* MG1363 transformants carrying the proteinase gene, but lacking the *prtM* gene were phenotypically proteinase deficient, but still expressed immunoreactive proteinase-antigen of higher molecular weight (Haandrikman *et al.*, 1989, 1991; Vos *et al.*, 1989b). Site-directed mutagenesis of the catalytically important Asp-30 → Asn-30 resulted in the formation of a similar high molecular weight, inactive proteinase, even if the *prtM* gene was present (Haandrikman *et al.*, 1991). *Figure 2* gives a schematic representation of these deletions and site-directed mutagenesis in the proteinase gene region of *Lac. lactis* subsp. *cremoris* Wg2 and their effects on the proteolytic activity and the cell attachment abilities of the resulting gene products.

Processing of the mature proteinase. Biochemical and genetic studies suggest that the lactococcal cell wall proteinase is processed in the following way. At the extreme N-terminal end of the preproenzyme there is a signal sequence and the pro-sequence which, by an autocatalytic mechanism involving a prtM maturation lipoprotein, is removed to form the active proteinase, starting at Asp-188 from the deduced nucleotide sequence. At the extreme C-terminal end a membrane anchor region holds the proteinase to the cell wall. Deletion of this membrane anchor region causes the proteinase to be secreted directly into the extracellular media. The purification of the proteinase involves the destabilization of the enzyme by removal of calcium associated with the enzyme. It is thought that this causes a conformational change exposing a cleavage point near the C-terminal membrane anchor, which, by intramolecular autoproteolysis, results in the release of the ~135 kDa mature proteinase into the extracellular media (Laan and Konings, 1989; Haandrikman *et al.*, 1991).

Protein engineering of the PI and PIII enzymes. The most recent work on the proteinase involves the construction of plasmids containing the proteinase gene from DNA fragments of the *prtP* genes encoding for PI and PIII (Vos *et*

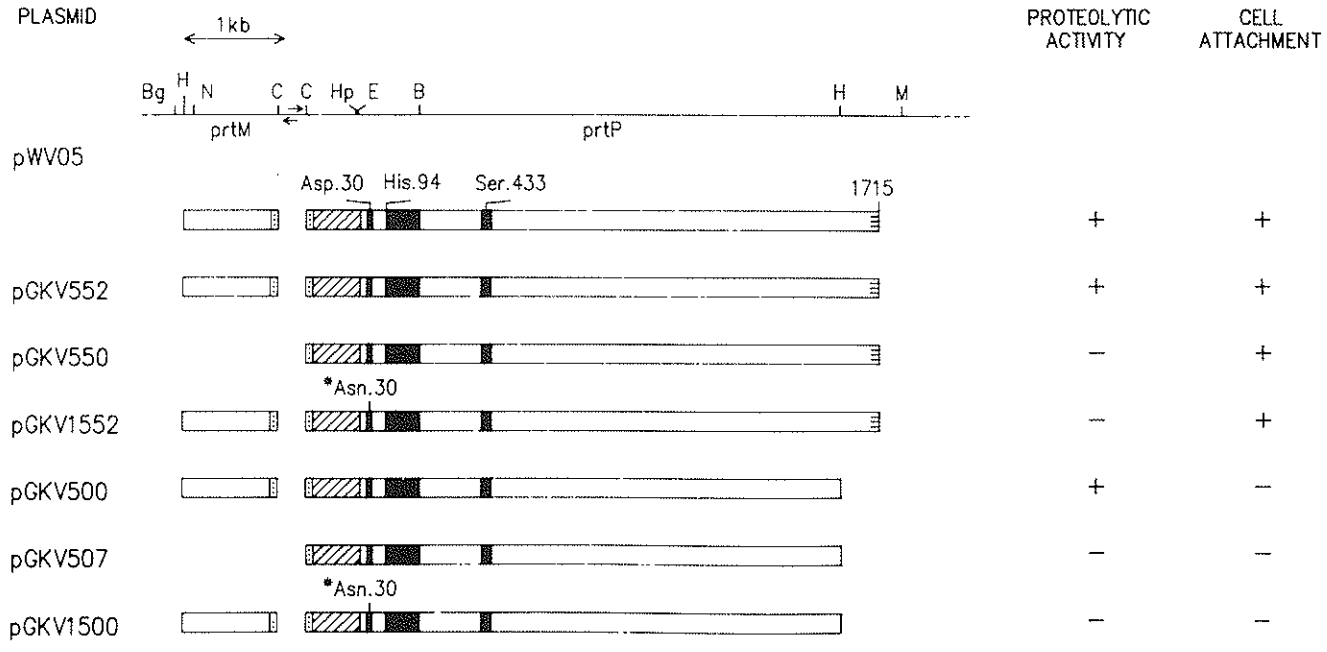

Figure 2. Schematic representation of the proteinase gene region of *Lactococcus lactis* subsp. *cremoris* Wg2 plasmid pWV05. The gene products of the proteinase gene *prtP* and maturation gene *prtM* and the plasmids used are indicated as bars. The three regions of homology of the proteinase with subtilisins are indicated by solid boxes. Signal sequences are indicated by stippled boxes. The proteinase membrane anchor is indicated by small, horizontally striped regions. The pro region is indicated by cross-hatched boxes. The locations of Asp-30, His-94 and Ser-344 of the active site are shown, as is the location of the Asp-30 → Asn-30 mutation. The presence of proteinase activity and the presence of proteinase attachment to lactococcal cells are indicated on the right. The promoter regions of *prtP* and *prtM* are indicated as small arrows. Abbreviations for restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RV; H, *Hin*dIII; Hp, *Hpa*I (not all sites shown); M, *Mlu*I; N, *Nru*I. (From Haandrikman *et al.*, 1991.)

al., 1991). By reciprocal exchange of specific regions identified as important in casein specificity, PIII-type activity was converted to PI-type activity, and vice versa. Two regions in the proteinase sequences were identified as especially important, and selective exchange of these regions resulted in the formation of hybrid enzymes with novel cleavage specificity towards α_{s1}-casein and β-casein.

Cell wall proteinase genetic variants: their relevance in cheese ripening. The amino acid differences between proteinases PI and PIII are responsible for the different caseinolytic specificities of the two genetic variants. Six per cent more basic amino acids are present in PIII, and it has been suggested that the different binding and catalytic properties may be attributed to the different charge distribution between the genetic variants (Exterkate and de Veer, 1989). This may be of significance to the dairy industry as strains, later found to be exhibiting PIII activity, do not produce bitter off-flavoured flavours from caseins, whereas strains with PI activity produce bitter peptides from casein (Visser *et al.*, 1983). This is suggested as an explanation for the lower occurrence of bitter cheeses made with PIII-containing strains (Exterkate and de Veer, 1989).

In cheese, the action of the starter-culture cell wall proteinase on primary proteolysis of the caseins is not significant in comparison to the contribution of chymosin (Exterkate, 1987). The proteinase, however, is essential for the further hydrolysis of the products of chymosin action to amino nitrogen, which relates to flavour development. Although PI does not cleave whole α_{s1}-casein, it is active on fragments of α_{s1}-casein which are the product of chymosin action, namely the salt-soluble α_{s1}-casein (1–23)(Exterkate, Alting and Slanger, 1991). The specificity of PI to this substrate was very different from the action of the PIII proteinase, with three cleavage sites towards the N-terminal, positively charged section of the peptide, while three PIII cleavage sites were located towards the C-terminal end. Although the rules controlling the site of bond cleavage in casein are unclear, this result demonstrates that the PI proteinase is likely to play some role in proteolysis of α_{s1}-casein during cheese ripening.

The recent construction of hybrid enzymes, using DNA fragments from the proteinase genes of *Lac. lactis* subsp. *cremoris* Wg2 (PI) and SK11 (PIII) (Vos *et al.*, 1991), which have new casein-bond-cleavage specificity, may also be of potential use in the dairy industry.

Chromosomal stabilization of the proteinase gene. As noted earlier, proteinase activity is unstable in lactococci, due to being plasmid encoded. Examples of chromosomal integration of DNA into lactococci have been demonstrated (Chopin *et al.*, 1989; Leenhouts, Kok and Venema, 1989) and this has recently been achieved for the *L. lactis* proteinase genes, *prtP* and *prtM*, by a Campbell-like mechanism (Leenhouts *et al.*, 1991). Two chromosomally integrated, multiple copy transformants were obtained, both expressing active proteinase which, over 100 generations, remained stable compared

to a Prt^+ plasmid-encoded strain which lost over 50% of its Prt^+ phenotype after 20 generations.

The lactococcal plasmid, pWV01, has also recently been described as an integration vector for lactococci which can possibly be used for the development of a Campbell-like integration system derived exclusively of lactococcal DNA to generate stable multiple copies of any gene of interest in the lactococcal chromosome (Leenhouts, Kok and Venema, 1991). Although this vector still uses erthyromycin resistance as the selective marker, it is suggested that lactococcal genes, such as the nisin-resistance genes, could be used for this purpose, making the entire system composed of lactococcal DNA.

Peptidases and peptide uptake systems

In comparison to the lactococcal cell-wall-associated proteinase, less is known about the peptidases present in starter cultures. Their importance in processes such as cheese ripening, however, has been demonstrated clearly in accelerated ripening studies, both in cheese and cheese curd slurries, where a lactococcal peptidase-containing extract has been successfully combined with exogenous commercial proteinase to produce a normal-flavoured product more quickly (Law and Wigmore, 1983; Cliffe and Law, 1990; *see also* pp. 393–395).

Genetic studies on the lactococcal peptidases are less advanced than on the cell wall proteinase. Because of the proline-rich nature of β-casein, comprising 17% of the amino acids, the X-prolyl dipeptidyl aminopeptidase (pepXP) was considered to play an important role in lactococcal nutrition, and has been purified and characterized by several laboratories (Kiefer-Partsch *et al.*, 1989; Booth *et al.*, 1990; Zevaco, Monnet and Gripon, 1990; Lloyd and Pritchard, 1991). The pepXP gene (*pepXP*) from *Lac. lactis* subsp. *cremoris* P8-2-47 (Mayo *et al.*, 1991) and *Lac. lactis* subsp. *lactis* NCDO 763 (Nardi *et al.*, 1991) was the first lactococcal peptidase gene to be cloned and the DNA sequences determined, showing greater than 99% homology between the sequences obtained. No significant homology was found between the *pepXP* gene and other proteins, and no regions in the sequences were identified as a potential signal peptide, transmembrane α-helical domain or a membrane anchor. The amino acid N-terminal sequence of the purified enzyme matches the nucleotide sequence at the start of the *pepXP* gene, indicating that no post-translational modification of the N-terminus occurs.

The data suggest that pepXP is an intracellular enzyme (Nardi *et al.*, 1991), as indicated by most of the purification protocols, although one group postulated the enzyme to be, at least in part, extracellular (Kiefer-Partsch *et al.*, 1989). No conclusive immunohistochemical studies have been reported. The intracellular location, however, conflicts with the proposed scheme for utilization of the casein and prolyl-containing peptides by lactococci, which has the pepXP enzyme located extracelluarly (Smid and Konings, 1990; *see Figure 3*). Upstream of the *pepXP* gene in both sequences is a second gene that encodes a very hydrophobic protein with homology similar to the *E. coli*

glycerol facilitator protein, and which is thought to be a transmembrane protein. Although this plays no part in the activity of the protein, as active pepXP can be expressed in *E. coli* with this second gene deleted, it is tempting to speculate that it is involved in the extracellular translocation of pepXP.

Knowledge of the other peptidases is not as well advanced. The intracellular aminopeptidases purified from *Lac. lactis* subsp. *cremoris* strains AM2 (Neviani *et al.*, 1989) and Wg2 (Tan and Konings, 1990) have completely different physicochemical and immunological properties. They have, however, similar activities towards some synthetic substrates, although the former has a wider range of activity. Whether or not both enzymes are present in the same organism has yet to be determined. Recently the gene for the 95 kDa aminopeptidase from *Lac. lactis* subsp. *cremoris* Wg2 has been characterized and its intracellular location confirmed by nucleotide sequence analysis, showing no signal sequences, and by immunohistochemistry (van Alen-Boerrigter, Baankreis and de Vos, 1991). Cloning of the aminopeptidase gene on a multicopy plasmid in *Lac. lactis* resulted in a greatly increased expression of the aminopeptidase. This is the first example of significant overproduction of a homologous protein in lactococci and may be of importance in food biotechnology as a source of this potentially food-grade enzyme.

Aminopeptidase A, purified from *Lac. lactis* subsp. *cremoris* HP (Exterkate and de Veer, 1987) and *Lac. lactis* subsp. *lactis* NCDO 712 (Niven, 1991), has a specificity for N-terminal acidic amino acids. This activity is also found in the general aminopeptidase of *Lac. lactis* AM2 (Neviani *et al.*, 1989), but it is suggested that the aminopeptidase A is associated with the outside of the cell membrane (Exterkate, 1984). The presence of the glutamate–glutamine transporter would appear to support this, although no conclusive evidence of the cellular location of the aminopeptidase has yet to be demonstrated. The presence of an enzyme involved in the processing of peptides containing glutamate, a recognized flavour-enhancing agent, may be of considerable importance in both cheese ripening and flavour peptide studies (*see* pp. 389–390).

An intracellular dipeptidase from *Lac. lactis* subsp. *cremoris* H61 (Hwang, Kaminogawa and Yamauchi, 1981) and intracellular dipeptidase and tripeptidase from *Lac. lactis* subsp. *cremoris* Wg2 (van Boven, Tan and Konings, 1988; Bosman *et al.*, 1990) have been purified and characterized. Their nutritional role is probably the breakdown of peptides transported into lactococci by the di–tripeptide transporter, which has been shown to be the rate-limiting step regarding utilization of these peptides. Their role in cheese ripening has yet to be established, but it is suggested that if the rate of cell lysis can be increased the intracellular enzymes may be more effective in the ripening process.

Peptide and amino acid transport. Lactococci have at least three types of amino acid transport systems; proton motive force-driven transport for leucine, isoleucine, valine, alanine, glycine, serine, threonine and lysine, an

antiport system for arginine and ornithine and a phosphate-bond-linked transport system for glutamate, glutamine and asparagine (reviewed by Konings *et al.*, 1989). Separate transport systems exist for peptides, and a di–tripeptide transport system and an oligopeptide transport system have been identified.

Earlier studies demonstrated the role that peptide utilization can play in the growth of lactococci (Law, 1978). The role of the amino acid and peptide transporters in this process has now also been established. The glutamate–glutamine amino acid transport system is one reason why lactococci cannot grow in alkaline media (Poolman, Smid and Konings, 1987). Glutamate, as an essential amino acid, has to be acquired by the organism. The apparent affinity constant, K_T, for glutamate uptake is pH dependent, with values of 3·5, 11·2, 77 and 1200 μM at pH 4·0, 5·1, 6·0 and 7·0, respectively. The glutamate–glutamine transport system will only translocate glutamate with the γ-carboxyl as the free acid, not the anion salt. Therefore, at alkaline pH, the medium will have very little free acid present and transport is greatly reduced, as observed by the high K_T at pH 7·0. Poolman and Konings (1988) confirmed this, demonstrating the inability of lactococci to grow on chemically defined media at pHs above 7 in the presence of glutamate. The enzyme most likely to be responsible for the cleavage of glutamic acid from casein-derived oligopeptides is likely to be the externally located, membrane-associated aminopeptidase A, which releases N-terminal glutamate from peptides (Exterkate, 1984).

The lactococcal di–tripeptide transporter has also been shown to be of great significance to dairy fermentation biotechnology. Smid, Plapp and Konings (1989), using the toxic dipeptide analogue, L-alanyl-β-chloro-alanine (diACA), isolated lactococcal mutants that were resistant to this dipeptide. On investigation of the known proteolytic activities, the only one found to be significantly different was the greatly reduced rate of di- and tripeptide uptake by the mutant, compared to the wild type. The mutants were then found to be unable to grow on chemically defined media where the only source of nitrogen was casein. Growth could be restored by addition of amino acids, suggesting that the transportation of di- and tripeptides is essential for the growth of these organisms in milk.

A suggested explanation of this essential transporter activity is the utilization of proline by the lactococci (Smid and Konings, 1990). Proline is the most prevalent residue in β-casein but no transport system for the free imino acid exists in lactococci. In chemically defined media, millimolar concentrations of proline are required to sustain growth; significantly higher than the concentrations required of other amino acids. Transportation of proline is thought to be by simple passive diffusion across the cytoplasmic membrane using a concentration gradient to drive translocation, the gradient being maintained by utilization of the imino acid by the lactococci once inside the organism. By using di- and tripeptides as the sole proline source in a chemically defined medium, maximal growth could be achieved at 50 μM concentrations of the peptide, suggesting the significant effect of a transport system for proline-containing di- and tripeptides.

A balance of the correct nutrients is required by the lactococci to sustain maximal growth (Smid and Konings, 1990). Using chemically defined media with casein as the only amino nitrogen source, the further addition of dipeptides, including proline-containing peptides, to the media caused inhibition of maximal growth. The explanation offered for this result is that the caseins are releasing, by proteolysis, other essential or growth-stimulating peptides, which then have to compete with the added dipeptides for uptake via the di–tripeptide transport system.

With substantial work now proceeding on the developing genetically modified starter cultures, with the aim of elevating the levels of expression of the proteolytic enzymes, the results given here indicate that care must be taken to retain the balance of essential nutrients required by the organisms. Elevating the expression level of a peptidase may have the effect of releasing peptides, at concentrations above the nutritional requirements of the organism, that may compete with other essential or growth-stimulating peptides for the same transport system. This may be particularly relevant for peptidases located externally, whose products need to be transported into the cell.

Smid and Konings (1990) suggest that full hydrolysis and utilization of caseins by lactococci can be explained by our current knowledge of the proteolytic system. Based on this knowledge, *Figure 3* gives a scheme showing the likely localization of the proteolytic system of lactococci and its general action on casein breakdown. However, work still needs to be completed on the localization of the different peptidases to complete the picture and confirm that the overall plan of hydrolysis and uptake is valid.

Cheese maturation

There are so many different cheese varieties in the world that it would be impossible to catalogue all of the possibilities for biotechnology to influence their maturation. This discussion will therefore be confined largely to the major cheese types in terms of volume of production, i.e. the hard and semi-hard varieties typified by Cheddar and Gouda. There is another good reason for this emphasis: cheeses outside these categories often rely on complex secondary microfloras of yeasts, moulds and slime-forming bacteria to give them their characteristic flavour and appearance, and it is generally held that insufficient is known about 'cheese ecology' and interactive metabolism to warrant technological manipulation. Examples of biotechnological interventions into the making of these complex varieties will probably be confined to the application of immunologically based detection methods for mycotoxins in mould-ripened cheeses. Commercial strains of *Penicillium* moulds are screened for mycotoxin production but some concerns remain for cheeses on or in which adventitious moulds may grow as contaminants.

Economic motives for controlling the ripening of cheese range from the desire to shorten the maturation period to reduce costs, to the need to control defects that reduce the value of the cheese or make it unfit for consumption.

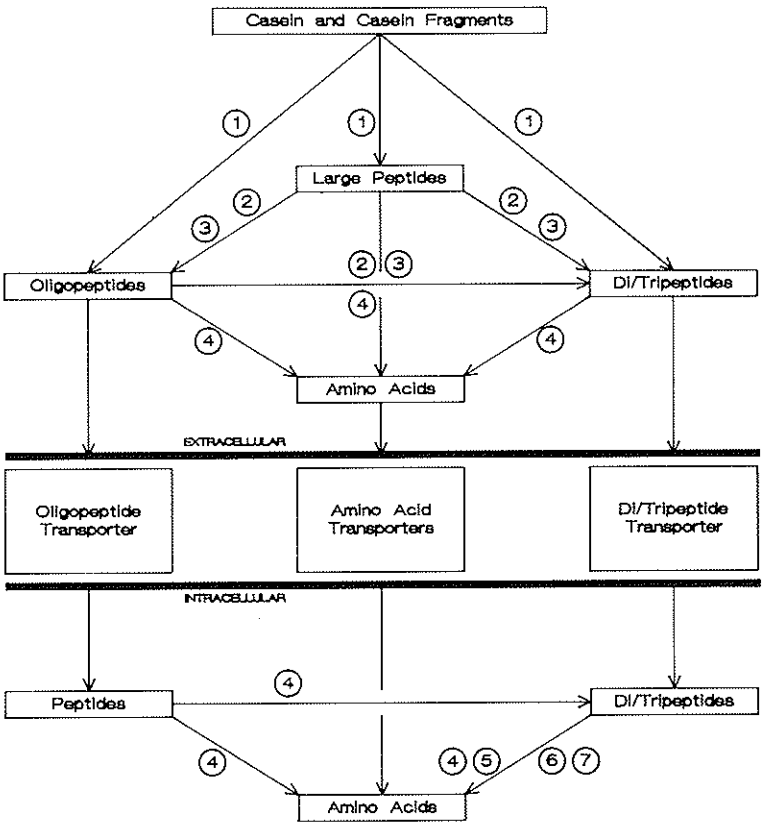

Figure 3. Possible location and action of the proteolytic system of lactococci on caseins. Key to enzymes: 1, cell-envelope proteinase; 2, endopeptidase; 3, X-Pro dipeptidyl peptidase; 4, aminopeptidase(s); 5, dipeptidase; 6, tripeptidase; 7, prolidase.

CONTROL OF PATHOGENS AND SPOILAGE ORGANISMS DURING MATURATION

The contamination of milk and cheese with pathogenic organisms has recently been highlighted by surveys of cheeses that have been found to contain *Listeria monocytogenes*. There is also a characteristic condition of cheese known as 'late blowing'. This is due to butyric acid fermentation brought about by growth of clostridial spores, especially *Clostridium tyrobutyricum*. Contamination of raw milk may be the source of these organisms, or in the case of *L. monocytogenes*, there seems to be an exceptional ability to colonize pasteurized or processed products. This latter pathogen can grow at 1–45°C and refrigeration alone is not sufficient to prevent growth in foods. Thus its presence may be currently unavoidable. Adding antimicrobial protection may be a way of preventing these sorts of contaminants.

Lysozyme (EC 3.2.1.17) is an enzyme widely distributed in animals and plants which was first described by Fleming (1922). The structure and catalytic mechanism of the enzyme are well known and its action on the cell walls of (particularly) Gram-positive bacteria confers antimicrobial properties

on it. Lysozyme is available in commercial quantities and this has led to its investigation as a possible antimicrobial enzyme to control cheese maturation. It was found that lysozyme is stable in cheese, 80–90% of the enzyme binding to the cheese curd. Five hundred units of lysozyme per ml of cheesemilk are sufficient to inhibit clostridia without inhibition of starter lactic acid bacteria. The use of lysozyme in cheese is legal in Germany, Italy and France (Anon., 1987). It has also been found that cheeses with lysozyme present initially had a fall in *Listeria* numbers, but although this was maintained for 31 days there was then an increase in growth (Hughey, Wilger and Johnson, 1989). The enzyme obviously has some antimicrobial activity in dairy products and protein engineering may increase its usefulness further. The possibility of targeting lysozyme more efficiently to bacteria within the water spaces of the cheese matrix, using liposome technology, is under investigation (Kirby, 1991).

Some biotechnology on natural antimicrobial activity in dairy products has already been described with the milk lactoperoxidase system. The antimicrobial activity of the lactic acid bacteria is also recognized as important in food preservation, with metabolic end-products, such as lactic acid, playing an essential role. The starter cultures also produce bactericidal proteins called bacteriocins. Recently, great attention has been given to these and a wide range have now been identified with a relatively broad spectrum of activity.

The best studied of these is the peptide antibiotic, nisin, produced by some strains of *Lac. lactis* subsp. *lactis*, which is bactericidal to Gram-positive bacteria in general and prevents outgrowth of *Clostridium* and *Bacillus* spores (Klaenhammer, 1988). It is now used frequently in Europe as an additive to prevent clostridial blowing in processed cheese (Lipinska, 1977) and has been accepted in the United States by the Food and Drug Administration (FDA) for use in certain pasteurized cheese spreads to prevent *Clostridium botulinum* contamination (FDA, 1988).

Nisin is produced by industrial-scale fermentation and downstream recovery, as a white powder to be added to food. There is also a wish to use nisin-producing starter cultures in combination with the usual industrial strains (mainly *Lac. lactis* subsp. *cremoris* strains) to control clostridial spoilage *in situ* in some cheeses. This mixture controlled clostrial growth but adversely affected the quality of the cheese, as the nisin also strongly inhibits the industrially important strains used (Lipinska, 1977; Hurst, 1981). The use of nisin-resistant mutants of these industrial strains, mixed with the nisin-producing strains also proved ineffective as the mutants suffered from a longer lag phase, increased phage sensitivity, lower heat resistance and lower acid production in milk; thus they lacked all these essential dairy traits. It is hoped that biotechnology will be able to develop nisin-resistant industrial strains that overcome these difficulties. Commercial exploitation of nisin as a general food preservative is also impeded by its low content in commercial preparation and its slow production by *Lac. lactis* subsp. *lactis* (Kaletta and Entian, 1989). Again, biotechnology is hoping to address these issues.

The nisin precursor gene, *nisA*, has been cloned, and the DNA sequence determined, although no production by the clones has been reported (Buch-

man, Banerjee and Hansen, 1988; Kaletta and Entian, 1989; Dodd, Horn and Gasson, 1990). This is due to the fact that nisin is synthesized as a prepropeptide which then undergoes post-translational modification, involving lanthionine-ring formation, to generate the mature antibiotic. Recently, however, three nisin-producing, nisin-immune (Nip^+) *Lac. lactis* subsp. *cremoris* strains have been obtained. This was achieved by direct plate conjugation of *Lac. lactis* subsp. *cremoris* strains, transformed by electroporation with plasmids pGK13 or pGB301 which contain resistant determinants to erythromycin and chloramphenicol, with nisin-producing *Lac. lactis* subsp. *lactis* strains (Broadbent and Kondo, 1991). Significantly these *Lac. lactis* subsp. *cremoris* transconjugants have retained their resistance to phage and are still strong acid producers in milk. It was also suggested that the undesirable antibiotic-resistance plasmids pGK13 and pGB301, initially used as genetic markers, can be removed by withdrawing the antibiotic pressure required for plasmid maintenance and selecting drug-sensitive isolates. This should result in *Lac. lactis* subsp. *cremoris* strains with the Nip^+ trait, suitable for use by the dairy industry, while retaining other desirable dairy fermentation characteristics.

CONTROLLING THE RATE OF CHEESE MATURATION

The redevelopment of biotechnological approaches to shortening maturation times and enhancing the typical flavour profile of Cheddar-type cheeses has received considerable attention over the past two decades and is worth considering here as an example of a range of approaches brought to bear on a single issue. The economic advantages associated with the development of new technologies that can enhance and speed up the natural maturation processes in cheeses are now well documented in reviews (e.g. Law, 1984; *Table 1*).

However, the balance between these advantages can change over the years and we would continue to stress the importance of knowledge-based control of cheese ripening in the face of rapid technological advances in other aspects of the industry, particularly in the fermentation stage. For example, the bulk producer of cheese (and therefore the biggest customer for cultures) will, quite correctly, put most emphasis on consistency and rate of acid production in the vat, rather than on the subsequent development of flavour during storage. This tendency has led some observers to the view that, although modern defined-strain starter systems are excellent in the factory, and the cheese they produce has a consistent, clean flavour, there remains a significant sector of the market which requires a more complex flavour profile which these highly efficient cultures cannot provide.

The overall effect of this trend on the emphasis of accelerated ripening

Table 1. Objectives of research in the accelerated ripening of cheese

Increase the profit margin for Cheddar manufacturers
Improve the reliability of mature flavour development
Compensate for flavour changes brought about by introduction of new technology

research has been to turn attention away from enzyme addition methods and towards starter-culture technology. This shift is enhanced in some countries by complications arising from legislation governing the use of enzymes for ripening (rather than manufacturing) cheese. Nevertheless, enzyme technology has made progress in the past few years, and the study of potential ripening enzymes, both in cheese micro-organisms and from other sources, cannot be divorced from starter-culture technology in the light of rapid advances in strain selection and genetic engineering techniques.

Enzyme technology for cheese ripening

Enzyme availability. The basic range of enzymes available to speed up cheese ripening has changed little over the past 10 years. Those innovations which have arisen have all been applicable to hard and semi-hard cheeses whose ripening does not involve complex secondary microfloras. Flavour profiles of the surface-ripened varieties appear to include oxidation products of milk fat (aldehydes and ketones) as well as derivatives of fatty acids and sulphur volatiles (thiol esters) for which there are no simple, well-characterized enzymic routes to exploit in technology. Even the recent partial purification of a methane thiol-producing enzyme from smear bacteria (*Brevibacterium linens*) by Collin and Law (1989), interesting though it is, does not advance our technology because the isolated enzyme is very unstable.

On the positive side, economically viable methods have been found to produce crude cell-free peptidase preparations from lactic cultures which, when added to cheese together with a food-grade proteinase, accelerate the formation of savoury flavour notes which have the effect of intensifying the overall flavour in the cheese. This technology originated from the authors' laboratory (Law and Wigmore, 1983) and was later developed to the product 'Accelase' by Imperial Biotechnology Ltd. It depends on the balanced breakdown of casein by a combination of endo- and exopeptidases so that bitter peptides do not accumulate. This technology could be further refined if the endopeptidase component could be selected or engineered to be more precise in peptide-bond cleavage, so that the protein-derived textural properties of the cheese (due to the α-casein network and water binding by β-casein; Creamer and Olson, 1982) were changed more slowly than the flavour properties. Protein engineering of proteinases is certainly likely to advance rapidly over the next decade, partly because more three-dimensional structures are being solved by crystallography and modelling and partly because more protein engineering groups are focusing their attention on the food sector.

There have been advances in the provision and use of lipases recently, which will influence the ripening of cheeses that have a recognizable fatty acid flavour note. Animal enzymes are already widely used to enhance the flavour of some Italian cheese varieties and to produce flavour-enhancing products for processed cheese. However, their specificity tends to accentuate 'sweaty'

and 'soapy' flavours due to short- and long-chain fatty acids, respectively. This effect is detrimental to most hard cheeses (e.g. Cheddar) so a new fungal lipase has been introduced which produces a greater proportion of medium-chain fatty acids, closer to a hard cheese flavour profile. The enzyme can be produced in a form that preferentially partitions into the cheese curd when the whey is separated (Arbidge and Silver, 1986).

Enzyme addition to cheese. The use of a particle-bound lipase to 'hold' enzymes in cheese curd is a relatively new concept (Arbidge and Silver, 1986) but it would not solve all the problems associated with the incorporation of proteolytic systems into cheese. The use of these systems poses certain problems concerning the way in which they interact with cheese proteins. The formation of cheese curd from milk involves complex physico-chemical changes which are not completely understood, but it is reasonable to suggest that exogenous enzymes would be incorporated homogeneously into cheese only if they were mixed with the cheesemilk and therefore present as the milk gel was formed. On the other hand, although enzymes added to the milk are likely to be better distributed, only a proportion is retained in the curd, and a significant amount is always lost into the whey at the stage of separation. For example, only 5–10% of Neutrase remains in Cheddar curd after pitching. This represents an economic loss of the enzyme itself, and it may also render the whey unsuitable for other uses without further processing, dependent on the temperature stability of the enzymes in use. For example, whey contaminated with lipases cannot be considered for ice-cream manufacture because the product contains fats that would be susceptible to degradation. Similar problems could arise in the case of proteolytic contamination where the whey was being used as a source of functional proteins in baked confectionary products. Proteinases added to milk also present a more direct threat to the economics of cheesemaking in that they can attack the milk proteins in the vat. As a result, some of the protein is lost as low molecular weight breakdown products into the whey. This obviously leads to reduced yields of cheese from a relatively expensive raw material. Methods developed to retain lipases in curd as particles may not be applicable to proteinases for this reason.

With careful mixing procedures, dry-salted cheese can be made by adding the enzyme to the curd before it is pressed and packed, after 'diluting' the enzyme powder in the salt and sprinkling onto the milled curd (Law and Wigmore, 1982). This step is necessary because amounts of enzyme are usually very small in relation to the curds to be treated. In granular form, the enzyme can also be handled safely and distributed reasonably evenly. However, the use of this addition technique is confined to cheese whose curds are milled and dry-salted before pressing. Many varieties of interest to European manufacturers have a curd-washing stage or partial drainage stage and are then surface salted or brine salted. In such cases, the enzymes would need to be added to the milk, or injected into the cheese.

Enzyme encapsulation offers the possibility of overcoming all of the problems outlined above. For example, proteinases can be kept separate

from milk proteins by the capsule material during the vat stage of cheesemaking. Also, suitably sized and charged capsules could be designed for maximum retention within the curd matrix as it formed during milk coagulation. Once entrapped, the encapsulated enzyme would be as well distributed as any other milk constituents and, when released from the capsules, in intimate contact with its substrates. A particularly successful approach to the need for an encapsulation system for proteolytic enzymes is based on the developing technology of liposomes. These are microscopic phospholipid vesicles first discovered in the sixties (Bangham, Standish and Watkins, 1965). Since the early work on these particles, they have been developed for use as drug delivery systems (Gregoriadis, 1977) and their composition offers a number of advantages over protein or fat capsules. For example, they can be made from lecithin, a common ingredient of foods, and their permeability/stability properties can be adjusted by small changes to this lipid composition. Since earlier work from the authors' laboratory had revealed the presence of phospholipase activity in cheese (Law *et al.*, 1973), a potential built-in release mechanism for liposome-entrapped enzymes was available. The general scheme for using the technique in cheesemaking is shown in *Figure 4*.

The first experiments were done with relatively crude liposomes, but they proved the principle to be correct even though enzyme entrapment was poor. Neutrase could be added to cheesemilk without attack on the milk proteins, then deposited and released into the curds of Cheddar cheese. In order to better exploit this idea, Kirby, Brooker and Law (1987) have adopted a new method for preparing liposomes with very high entrapment efficiency, using conditions that do not destroy the enzymes to be encapsulated. These liposomes are known as dehydration–rehydration vesicles. Using these particles, enzyme losses into whey are reduced from 95% to less than 10%. The liposomes are delivered evenly throughout the cheese and are trapped in water spaces within the casein matrix. This ensures that when the enzymes are released within the first few days of ripening (after rupture of the liposomes) they are in close proximity to their substrate.

Since the technical feasibility of this approach was demonstrated, the system has been progressively improved and a large number of Cheddar cheeses have been prepared and ripened using microencapsulated enzyme. Objective monitoring of protein degradation during the maturation process and subjective evaluation of flavour development by trained taste panels have shown the procedure to be as efficient as when enzyme is added in the 'free' form to the separated curd. Also, textural problems associated with the latter route, caused by high localized concentrations of enzyme around the curd boundaries, are completely avoided when the encapsulated enzyme is used. Efficacy trials have extended our technique to the more complex procedures involved in the manufacture of washed curd, brine-salted cheeses. Enzyme doses require adjustment to compensate for varying salt in moisture levels, but such cheeses can be made successfully and ripened with this technology.

Advances in understanding enzymic mechanisms. One of the major constraints on the further exploitation of enzymes in cheese ripening technology

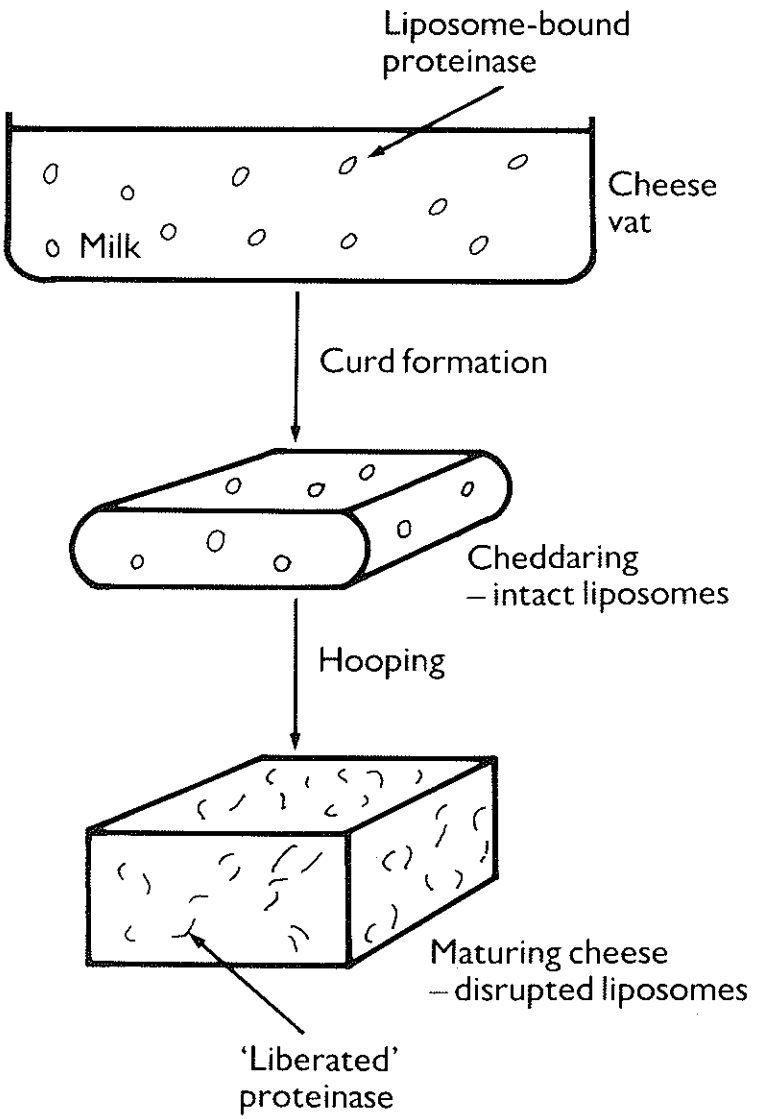

Figure 4. Schematic representation of enzyme addition to cheese for accelerated ripening, using liposomes as carriers.

is our lack of understanding of the underlying changes that take place in the chemical composition of cheese during maturation. Indeed, our knowledge of the origin of typical aroma, even in a major variety such as Cheddar, is so poor that we cannot hope to control any of the reactions involved. However, a general understanding of proteolytic events leading to the development of savoury flavour (reviewed by Law, 1987) has allowed the development of efficacious enzyme technology from a rather crude approach (p. 394) and this, in turn, has led to a more definitive study of the relationship between the

range of peptidases in lactic streptococci and the formation of 'flavour peptides' in cheese.

Thus, Cliffe, Revell and Law (1989) developed a high-resolution liquid chromatographic method to resolve low molecular weight peptides in ripening cheese and showed that accelerated ripening by peptidase mixtures was characterized by the sequential production and breakdown of hydrophobic (bitter) peptides, to leave, in the best, most intensely flavoured cheese, a preponderance of hydrophilic peptides whose molecular weight profiles suggested that they were made up of 2–3 amino acid residues (Cliffe and Law, 1990, 1991). One peptide family in particular was present in concentrations that correlated well with enzyme-induced (*not time-induced*) flavour intensity, and this is under investigation at present.

Starter-culture technology

The study of enzymes involved naturally in cheese flavour development is likely to drive new developments in culture technology for improved and accelerated flavour. This can operate both at the strain selection level, and at the level of genetic engineering.

For example, researchers at Chr. Hansen's Laboratory (Anne-Maria Bech, personal communication) have used a cheese slurry method to screen commercial cultures from their vast collection on the basis of their ability to produce low molecular weight N (i.e. nitrogenous materials) in cheese curd and to generate savoury flavour notes (Law, 1990). By then selecting *lac*-variants of these cultures, it was possible to show that they could enhance the basic, clean, acid flavour produced by the most efficient, phage-resistant homofermentative cheese cultures.

As our understanding increases of the enzymes and enzyme products that are behind such phenomena, we have the opportunity to isolate, characterize biochemically and clone the enzymes, such that they are expressed more favourably in the most efficient, phage-resistant starters. The most promising enzymes for such cloning are already beginning to emerge (*see also* pp. 386–388). For example, several such enzymes have been purified from strains of *Lac. lactis* subsp. *cremoris*, including an aminopeptidase A (Exterkate and de Veer, 1987), a dipeptidase (Van Boven, Tan and Konings, 1988), a tripeptidase (Bosman *et al.*, 1990), an X-prolyl dipeptidyl aminopeptidase (Kiefer-Partsch *et al.*, 1989; Booth *et al.*, 1990) and aminopeptidases with activity against a wide range of dipeptides and tripeptides (Neviani *et al.*, 1989; Tan and Konings, 1990). Fewer studies have been carried out on the enzymes of *Lac. lactis* subsp. *lactis*, though Kaminogawa, Ninomiya and Yamauch (1983) used cluster analysis to demonstrate that *cremoris* and *lactis* subspecies of *Lac. lactis* formed distinct groups on the basis of peptidase profiles. It is therefore possible that there are significant differences in the characters and types of these enzymes between subspecies which contribute to their different cheesemaking properties. Further studies of the enzymes of *Lac. lactis* subsp. *lactis* are required if an adequate comparison is to be made.

It is clear that the subsp. *lactis* has been somewhat neglected in peptidase

research because its strains are not renowned for their flavour-producing abilities. However, this shortcoming appears to be due to their vigorous growth, and slow release of intracellular enzymes, rather than to any inherent lack of peptidases. Only two peptidases to date have been characterized from this subspecies, an X-Pro dipeptidyl peptidase (Zevaco, Monnet and Gripon, 1990; Lloyd and Pritchard, 1991) and an aminopeptidase which shows specific activity against acidic N-terminal residues (Niven, 1991).

The substrate specificity of the latter enzyme was tested against a substantial range of aminoacyl-alanine dipeptides and it was shown to be active against N-terminal aspartyl and glutamyl residues. It was also active against tripeptide substrates but did not cleave acidic C-terminal residues. It was therefore designated as an aminopeptidase A (EC 3.4.11.7). In addition, this enzyme also hydrolysed seryl alanine. An aminopeptidase A has previously been purified from *Lac. lactis* subsp. *cremoris* (Exterkate and de Veer, 1987) but activity against seryl residues was not reported, nor has this activity been observed in aminopeptidase A purified from mammalian sources (Benajiba and Maroux, 1980; Danielsen *et al.*, 1980; Tobe *et al.*, 1980). Many of the serine residues in caseins are phosphorylated (Eigel *et al.*, 1984) but it is possible that this enzyme may have activity against phosphorylated serine residues if the acidic side-chain causes them to act as aspartate analogues. This activity may therefore be of particular importance in the breakdown of casein-derived peptides and also in releasing glutamyl residues, which have known savoury properties.

Lactic cultures also produce dipeptidyl peptidases which release dipeptides from the N-terminal end of oligopeptide substrates. Such enzymes could be involved in the release of short, flavour-enhancing sequences, and attempts are already under way in a number of European laboratories to clone them for overproduction in commercial starters. Since some of the peptidase activity of starter cultures is intracellular, attempts to accelerate protein breakdown in cheese have included methods to hasten cell lysis in the early stages of maturation. Techniques range from the use of lysozyme (Law, Castanon and Sharpe, 1976) to the exploitation of phage lysin. The release of phages from the bacterial host cell can involve cell wall degradation by a lysozyme-like enzyme known as phage lysin. ØML3 is a prolate-headed phage that attacks *Lac. lactis* strains. The lysin gene from this phage has been cloned in *E. coli* and its DNA sequence determined (Shearman *et al.*, 1989). *Figure 5* shows the expression of the cloned lysin gene by λgt10 plaques overlaid with *Lac. lactis* subsp. *cremoris* cells. Phage lysin has now been expressed in *Lac. lactis* strains, which were found to be unaffected during exponential growth but lysed after reaching the stationary phase in GM17 media. In milk, the *Lac. lactis* subsp. *cremoris* strains that expressed lysin gene were found to be less viable than controls (Gasson, personal communication). This system may be of potential in accelerating cheese ripening, by causing early and increased cell lysis, releasing the intracellular peptidases to act on the milk protein peptides. It is suggested that this autolytic phenotype could also be exploited as a method of containing genetically manipulated

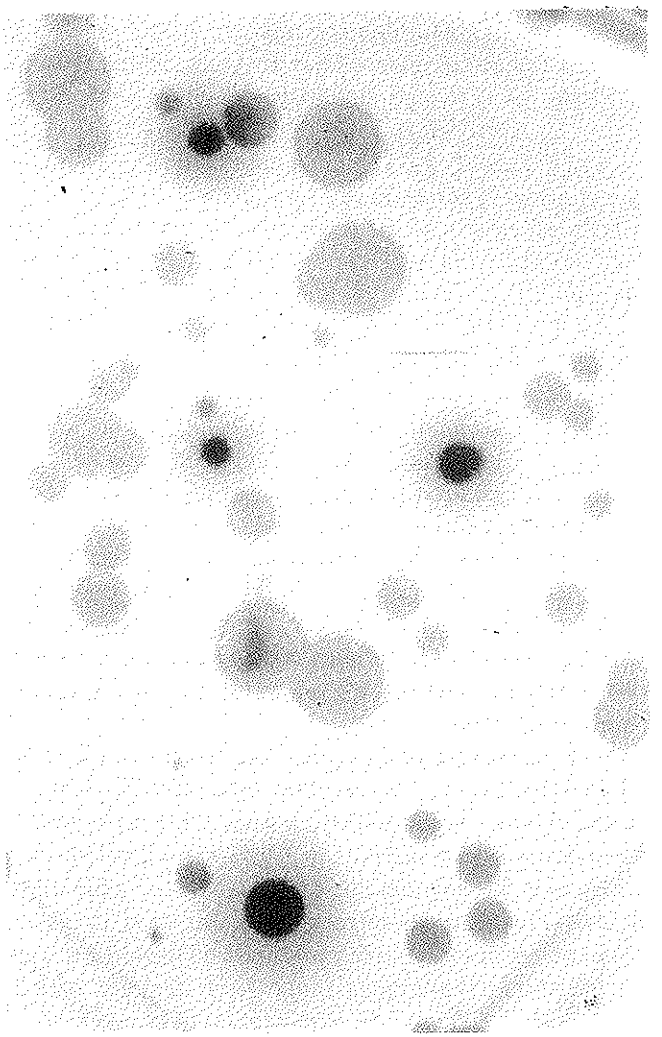

Figure 5. Expression of cloned lysin gene by λgt10 plaques. Plaques of the λgt10 with random cloned fragments of bacteriophage ØvML3 are overlaid with *Lactococcus lactis* subsp. *cremoris* cells. Lambda clones that express a cloned lysin gene produce a clear window and appear dark, whereas other lambda plaques retain a cloudy appearance. (From Shearman *et al.*, 1989.)

Lac. lactis strains by routinely inserting the lysin gene in a suicide vector (Shearman *et al.*, 1989).

Whey utilization

When cheesemaking was a cottage industry, the whey (liquid drained from cheese curd) was easily disposed of as a pig-feed base, or as a fertilizer by simply dumping it on the fields. However, the modern cheesemaking industry produces millions of litres of whey every year and, although some of it is dried

and used in food manufacture as a functional ingredient, there remains an excess production that cannot be dumped indiscriminately into the environment; whey has a particularly high BOD and quickly pollutes water courses if released. World whey solids production is about 7m tonnes, of which 5·5m tonnes is lactose. Although market outlets for whey solids have improved via advances in food processing technology, market forces and price fluctuations ensure that whey solids disposal remains an issue for the cheese industry.

Bioseparations science has provided advances in membrane filtration techniques (ultrafiltration, reverse osmosis) that ease the bulk transport problems inherent in whey utilization, and allow for the concentration and fractionation of emulsifying (β-lactoglobulin), nutritional (α-lactalbumin) and antimicrobial (lactoperoxidase, lactoferrin, immunoglobulin) proteins from whey.

Fermentation science has provided some methods for converting lactose in whey to higher-value compounds, and this is probably the major area in which biotechnology will help the cheese industry. For example, there are already a number of alcohol-producing plants operating on whey ultrafiltrate as a feedstock (Donnelly, 1990), and other biotransformations at various stages of development include the production of lactic acid, propionic acid (food preservatives), acetone/butanol, ascorbic acid, methane and single-cell protein (Sanderson and Reed, 1985). Donnelly (1990) discusses the possibilities for producing feed-grade amino acids from whey fermentation. However, although recombinant DNA technology exists and is being developed to improve pathway fluxes in amino acid synthesis by fermentative microorganisms, the emphasis has not been on lactose utilizers. When whey has to compete with alternative cheap feedstocks, there is little incentive to invest in basic research on its fermentative possibilities. However, new avenues may emerge from unexpected quarters, and the advent of low-water enzymology may yield new ways of derivatizing lactose to produce functional molecules, such as biosurfactants. This area of biotransformations is advancing rapidly in the field of monosaccharide esters (e.g. Vulfson, Fregapane and Sarney, 1991) and could be extended towards lactose utilization.

Concluding remarks

Compared with the influences of traditional biotechnology on cheese manufacture, modern biotechnological sciences have had little impact yet. However, the building of biochemical, biophysical and molecular biology knowledge and expertise in the areas of quality control, coagulants and by-product disposal will, without doubt, influence the industry in the future.

In particular, substantial resources are being directed at starter-culture technology and, as shown in the review, great advances have been made in our biochemical and genetic understanding of the lactococcal proteinase system. As our knowledge increases, similar advances are expected in the near future in other areas of proteolysis, the peptidases, and the amino acid and peptide transport systems. Other advances that can be expected are understanding of the mechanisms involved in resistance of starter cultures to

important antimicrobials, such as nisin, and to bacteriophages. Finally, over the next decade we can also expect to see the development of food-grade vectors that will allow the use of genetically manipulated starter cultures in cheese, which will have improved ripening properties.

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