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Cheese flavour development by enzymatic conversions of peptides and amino acids

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Abstract

During ripening of cheese, many biochemical processes take place, which are essential for flavour development. The breakdown of caseins is a prerequisite for flavour development. A good balance between proteolysis and peptidolysis prevents the formation of bitterness in the cheese. For this reason, it is necessary to focus on starter cultures with highly active peptidases, which should be active in the cheese matrix. Amino-acid-converting enzymes (AACEs) are involved in the degradation of amino acids, which are liberated during proteolysis. Their activity results in various volatile (flavour) components; most notably the degradation of methionine results in flavour-active sulphur compounds. AACEs involved in degradation of methionine and other amino acids were identified and their role in (cheese) flavour formation is described. At least two pathways leading to the formation of sulphur compounds were identified. Overproduction of one of the enzymes involved, results specifically in a higher formation of sulphur compounds. This result, together with the observation that flavour production is highly strain-specific amongst various lactococcal bacteria, offers a new potential for industrial applications.

Keywords: Flavour formation; Proteolysis; Bitter; Amino acid converting enzymes; Cheese

1. Introduction

Flavour of food products is one of the key attributes for the consumer. In fermented products, e.g. dairy products, flavours are derived from milk components by enzymatic activities of micro-organisms. In cheese for instance, various flavour compounds have been identified as being essential and many of them are derived from casein degradation. Other enzymatic processes, such as lipolysis, are also involved, most notably in cheeses where fungi are involved in the ripening process, e.g. Camembert and Roquefort cheese. In addition, lactose fermentation might lead to flavour compounds such as propionic acid (Adda, 1986; Fig. 1).

Proteases and peptidases play an important role in cheese ripening. Proteolysis results in an increase in peptides and free amino acids. The former are directly involved in bitterness, whereas the latter contribute to the basic cheese flavour (Engels & Visser, 1994). Following proteolysis, the free amino acids can be converted into volatile flavour components through the action of amino-acid-converting enzymes (AACEs). The latter pathways are particularly interesting since the enzymes involved seem to be rate-limiting in flavour production. Moreover, depending on the enzymes present in the cultures used, different flavours can be obtained. Therefore, knowledge of these pathways and their regulation might directly add to flavour diversification and reduction of ripening times.

In contrast to the role of proteases and peptidases, relatively little is known about amino-acid-converting enzymes involved and their regulation. The degradation of amino acids by non-starter organisms from surface-ripened cheeses, e.g. *Brevibacterium linens* and *Pseudomonas*, and from blue cheese, e.g. *Penicillium roquefortii*, has been reported (Hemme, Bouillanne, Metro & Desmazeaud, 1982; Law, 1984). Research at our laboratory has shown that during the ripening of hard-type cheeses, such as Gouda, enzymes of mesophilic starter lactococci are probably also involved in the conversion of amino acids to aroma components (Engels & Visser, 1996). Degradation products of both methionine and leucine were detected after incubation with cell-free extracts of *Lactococcus lactis* subsp. *cremoris* B78.

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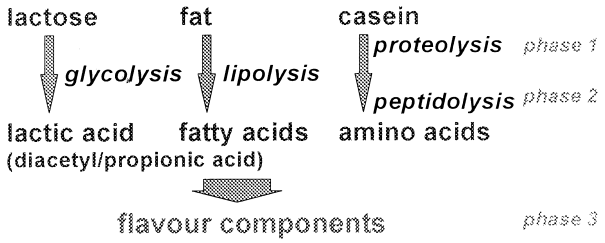

Fig. 1. Flavour formation in dairy products (cheese). In cheese ripening the proteolytic and peptidolytic pathways are called phase 1 and 2, respectively, and the conversions of amino acids, fatty acids and so on, leading to the actual cheese flavours, are known as the third phase of ripening.

Recently, Dias and Weimer (1998) reported the conversion of methionine to thiols by lactococcal enzymes.

The products of methionine breakdown are considered very relevant for cheese flavour. Volatile sulfur compounds have been detected in various cheese types e.g. Parmesan, Cheddar and Gouda (Engels & Visser 1996; Urbach, 1995). Their formation is usually attributed to the action of enzymes from non-starter organisms in cheese. Tanaka, Esaki and Soda (1977) purified and characterized methionine-γ-lyase from *Pseudomonas ovalis*, a pyridoxal 5′-phosphate (PLP) dependent enzyme, which is regarded as an essential enzyme in bacterial methionine metabolism. The enzyme catalyzes the simultaneous deamination and dethiomethylation of methionine. The possible role of aminotransferases in flavour formation in cheeses has received recent attention. These PLP-dependent enzymes catalyze the transfer of the aminogroup from an amino acid to an α-keto acid. The transamination of aromatic amino acids by*Brevibacterium linens* was investigated by Lee and Desmazeaud (1985). In the present paper, an overview is presented about the current knowledge concerning the conversion of casein to flavour compounds in cheeses.

2. Materials and methods

Strains of *Lactococcus lactis* collected from commercial starter cultures, artisanal origin and natural niches were obtained from the culture collection of NIZO food research, Ede, The Netherlands. Cell free extracts and enzyme preparations were obtained as described by Alting, Engels, Van Schalkwijk and Exterkate (1995). Debittering activity of starter cultures was measured according to Smit, Kruyswijk, Weerkamp, de Jong and Neeter (1996). Flavour analysis was performed according to Engels and Visser (1994). Protein purification was performed according to Alting et al. and Engels (1997). The food-grade expression system (NICE system) was used for the overexpression of the gene encoding cystathionine β-lyase (De Ruyter, Kuipers, & de Vos, 1996).

3. Results and discussion

3.1. Proteolysis

Proteolysis in cheese is caused by the action of the rennet retained in the curd, plasmin of the milk and proteases of the starter cultures used. Protease activity leads to a large amount of peptides, the so-called soluble-nitrogen fraction in a cheese. Subsequently, these peptides are further degraded to small peptides and free amino acids, the amino-nitrogen (AN) fraction in cheese. Protease and peptidase activities are dependent on the starter culture used. For this reason a lot of attention has been paid to the use of (adjunct) starter cultures, which would enhance ripening and flavour formation in cheese. Particularly the use of thermophilic lactic acid bacteria as an additional culture (adjunct starters) to the mesophilic starter cultures has become common practice in cheese making for accelerated ripening or flavour modification (Exterkate, 1987; Fox, Wallace, Morgan, Lynch, Niland & Tobin, 1996). The effect of such adjunct cultures on the formation of amino-nitrogen is shown in Fig. 2, with *L. acidophilus* T72 as adjunct culture. The casein degradation is increased by 100% in the presence of the adjunct culture. However, a relatively common problem with the use of this and other cultures is development of bitterness in the cheese. Bitterness is one of the most common off-flavours in cheese (Lemieux, Puchades & Simard, 1989; Visser, Slangen, Hup & Stadhouders, 1983). Therefore, we focussed on the possibilities of predicting and controlling the bitter-degrading abilities of cheese (adjunct) cultures. The bitter-tasting C-terminal part of β-casein, the so-called C-peptide (a.a. 193–209), formed by the action of rennet and starter organisms, is a major cause of bitterness in Gouda cheese (Stadhouders, Hup, Exterkate & Visser, 1983; Visser et al., 1983) as well as in Cheddar cheese (Lemieux et al., 1989). It has been found that the use of

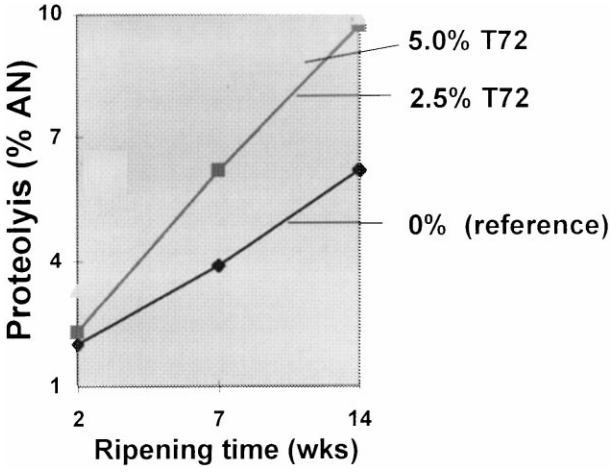

Fig. 2. Effect of adjunct culture *L. acidophilus* T72 on proteolysis during cheese ripening.

certain starter cultures and/or adjunct cultures especially give rise to the development of a bitter taste in cheese and they therefore are characterized as 'bitter' strains (Visser et al., 1983). A better understanding of bitter formation and degradation was needed to select cultures with a high debittering activity.

A laboratory assay was developed to measure the ability of cultures to degrade bitter peptide(s) within hours (instead of months during cheese trials) (Smit et al., 1996). The assay was used to screen various (adjunct) starter cultures, as well as to test the effects of growth conditions on their debittering activity. For each strain tested, the debittering activity in the assay was expressed as the decrease in C-peptide per hour and per amount of cells. The strains were found to differ significantly in their ability to degrade the bitter-tasting C-peptide, ranging from no activity to very fast degradation. Cultures with strong activities obviously have the potential to be used as debittering starter/adjunct cultures. Thermophilic adjunct culture *L. acidophilus* I233 was selected for this purpose (Fig. 3).

The ability of several cultures to degrade the C-peptide was found to be dependent on their growth conditions. For example, it was found that pH-controlled growth conditions resulted in a higher debittering activity as compared to culturing under acidifying conditions. Therefore, cultures cannot simply be marked as 'bitter' or 'non-bitter'. This opens possibilities to use cultures which were previously disregarded. The results of current research reveal that sensitivity of the cells to lyse plays a major role in the differences observed and point to the underlying cellular mechanisms involved. This result corroborates the recent work of Meijer, van der Bunt, Twigt, De Jonge, Smit and Hugenholtz (1998), showing that a reduction in lysis sensitivity of a starter culture resulted in bitterness during cheese ripening.

In order to test whether results from the bitter assay can be used to predict bitterness, cheeses were made with adjunct cultures in conjunction with starter cultures which normally give rise to bitter cheeses (cultures A and B). As shown in Table 1, cheeses made with these cultures indeed resulted in bitter-tasting cheeses. However, bitterness was almost absent when culture I233 was added as adjunct culture. Moreover, in order to judge on overall flavour effects (apart from bitterness), cheeses made with I233 were also graded by consumer panels. The results revealed that consumers scored cheeses prepared with culture I233 significantly better than control cheeses, particularly in items like 'overall flavour', 'aftertaste' and 'full taste' (data not shown).

Taken together, the bitter assay is found to be a powerful tool for fast screening of cultures and growth conditions for prediction of bitter formation in cheeses. Bitterness in cheese can be controlled by adaptation of the growth condition and/or by the use of highly debittering cultures, such as culture I233.

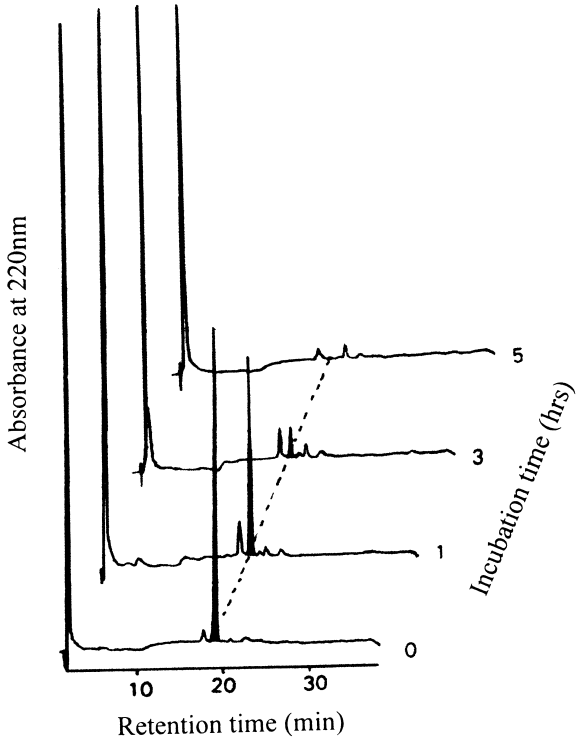

Fig. 3. Degradation of the bitter-tasting C-peptide during incubation with *L. acidophilus* I233 in the laboratory assay.

3.2. Flavour-forming enzymes

During the last decade, the focus of flavour formation has been primarily on the degradation of caseins via (bitter) peptides into free amino acids. Much knowledge has been obtained on proteases and peptidases involved (see for a review Visser, 1993). On the other hand, based on sensory evaluations and analytical chemical analysis of dairy products, various groups of volatiles were identified as being important for the final taste and aroma of cheese and other dairy products. Such compounds are: fatty acids, esters, aldehydes, alcohols, ketones and sulphur compounds (Badings, 1991; Bosset

Table 1
Debittering activity of debittering culture *L. acidophilus* I233 in cheese

Starter culture	Adjunct culture	Bitter score in cheese[a]
A	None	2.7
A	1233	0.2
B	None	1.5
B	1233	0.1

[a] Cheeses were graded after 3 months of ripening and bitter was scored on a scale from 0 (absent) to 4 (very strong).

& Gauch 1993; Engels, Dekker, de Jong, Neeter & Visser, 1997; Urbach, 1995). Most of the volatile flavour components observed in cheese are formed during ripening of the cheeses (Fig. 4). Based on chemical identification of these compounds by gas-chromatography coupled to mass spectrometry, these compounds most likely result from conversion of lactose, caseins and fats. In cheesetypes such as Gouda and Cheddar, proteolysis of caseins is essential for sufficient formation of flavour. This implies that amino acids liberated during the proteolysis and peptidolysis (phases 1 and 2 of cheese ripening) are converted into volatile flavour compounds during the so-called third phase of cheese ripening.

Until recently, hardly anything was known about the enzymes involved in the formation of volatile flavour compounds from amino acids. In fact, it was rather unclear whether such conversions were caused by enzymatic or chemical reaction, and in the case of enzymatic conversions, what the origin of the enzymes was. It was often suggested that the natural -contaminating- microflora had the largest impact on the actual flavour formation. In order to establish the role of starter cultures in flavour formation, Engels and Visser (1996) developed a model system in which they were able to measure cheese flavour formation both chemically as well as organoleptically. For this purpose, water-soluble fractions (WSFs) of various cheeses were investigated and components that might contribute to the flavour were identified. From the organoleptic evaluations, it became clear that the WSFs contained the most essential components characteristic for the taste of each type of cheese. Based on this, Engels and Visser were able to mimic the flavour of cheese by incubations of cell-free extracts from a *Lactococcus lactis* starter culture and a mix of amino acids. Furthermore, it could be established that a number of amino acids were particularly important for the formation of cheesy flavours in the system; most notably methionine. These results showed that starter cultures have the ability to generate cheese flavours from amino acids and that these processes are, at least to a large extent, driven by the action of specific enzymes.

The next question was: which enzymes and substrates are the most important ones and how can their activities be regulated? Many enzymatic conversions are possible starting from amino acids. By omitting amino acids in the above mentioned mix, it could be established which amino acids are essential for flavour development. From this work it became clear that methionine plays a crucial role. Degradation of methionine results in the formation of several sulphur components and these conversions result, at least in part, from the action of enzymes from the starter bacteria. Two pathways were found to be present in starter bacteria resulting in the degradation of methionine (Fig. 5).

The first pathway is a direct demethiolation of methionine by the enzyme cystathionine β-lyase, this enzyme was found te be active under cheese conditions in a cheese model system (Alting et al., 1995; Smit, Braber, van Spronsen, van den Berg & Exterkate, 1995). In this first route methionine is simultaneously deaminated and demethiolated. As a result, methanethiol is produced, a very potent flavour compound and precursor for subsequent conversion into other sulphur

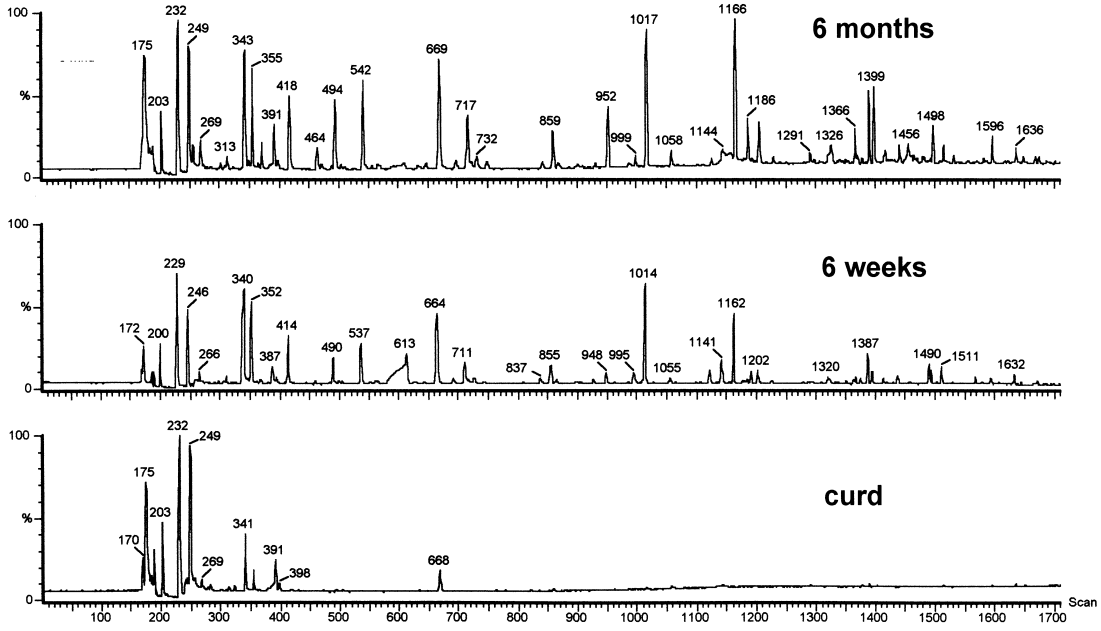

Fig. 4. Bioformation of volatile (flavour) components during Gouda cheese ripening.

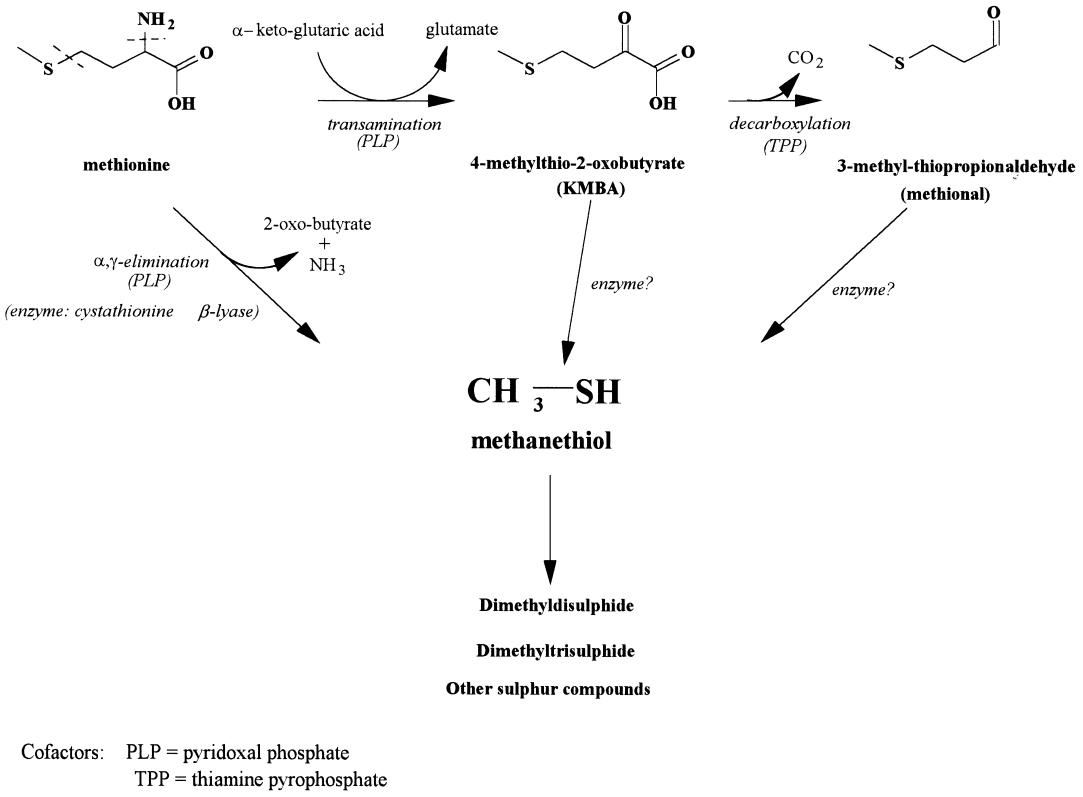

Fig. 5. Methionine degradation pathways by starter cultures.

components, e.g. dimethylsulphide, dimethyldisulphide (Lindsay & Rippe, 1986). A similar enzyme, cystathionine-γ-lyase, was purified from *Lactococcus lactis* subsp.*cremoris* SK11 by Bruinenberg, de Roo and Limsowtin (1997).

The gene coding for cystathionine β-lyase from *L. lactis* B78 was isolated and sequenced. Using the NICE system (De Ruyter et al., 1996) the gene was overexpressed in *L. lactis* in a food grade manner (Fernandez et al., 2000). Induction of the gene resulted in a strongly increased formation of methanethiol and DMDS (Fig. 6). The construction of amino-acid-converting enzymes by genetically modified strains will facilitate further studies to elucidate the importance and regulation for flavour development in cheese and other fermented products. This result also showed that the absence of the right enzymes might be a rate-limiting step in the formation of these important flavour components. Whether this is the case in real cheese remains to be elucidated, since methionine itself might also be limiting under those conditions.

The second pathway is at least a two-step conversion with a transamination as the first step, leading to the formation of 4-methylthio-2-oxobutyric acid (KMBA), followed by a decarboxylation leading to methional. Methional is then converted (enzymatically?) to methanethiol. A number of transaminases involved have been purified (Engels, 1997; Gao & Steele, 1998; Roudot-Algaron & Yvon, 1998; Yvon, Thirouin, Rijnen, Fromentier & Gripon, 1997). Both cystathionine β-lyase and the transaminases are active under cheese-ripening conditions [pH 5.2–5.4 and 4% NaCl (w/v)] and can therefore play an essential role in the formation of cheese flavours.

Characterization of the transaminases clearly showed that the substrate specificity is rather low. In fact, the activity on methionine is much lower as compared to a number of other amino acids. Table 2 shows the relative

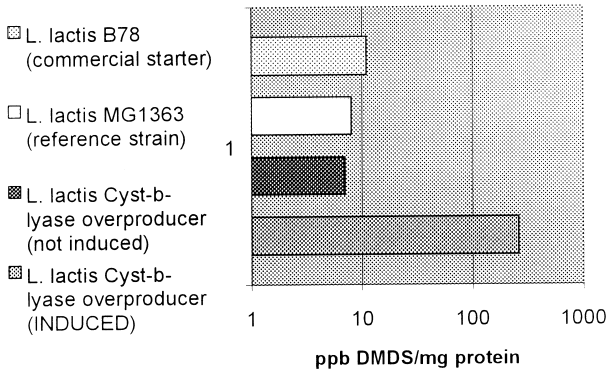

Fig. 6. Overexpression of cystathionine β-lyase in *L. lactis* results in higher production of volatile sulphur compounds.

Table 2
Substrate specificity of amino transferases from *L. lactis* strains

Substrate	Relative activity (%)[a]		
	B78-AtA	B78-AtB	NCDO763At (ref. 14)
L-Valine	100	98	0
L-Isoleucine	95	100	0
L-Leucine	87	85	100
L-Methionine	31	26	12
L-Phenylalanine	4	10	72
L-Tyrosine	0	6	73
L-Tryptophan	0	6	37
L-Aspartic acid	0	<1	0
L-Histidine	0	<1	0

[a] Relative activity as compared to the most preferred substrate.

activity of purified amino transferases towards a number of amino acids. The conversion of leucine, isoleucine and valine is also very important for flavour formation. For instance, conversion of leucine and isoleucine results in the formation of 3- and 2-methyl butanal, respectively. These aldehydes were found to be key-flavour components in some cheese types, such as Proosdij cheese (Neeter, De Jong, Teisman & Ellen, 1996).

From recent results, it becomes clear that flavour-forming abilities vary considerably within the species of*Lactococcus lactis*. Various strains, especially those isolated from artisanal and non-dairy environments, have the ability to produce flavour components distinct from industrial cultures (Ayad, Verheul, De Jong, Wouters & Smit, in press; Weerkamp, Klijn, Neeter & Smit, 1996). This might be explained by the presence of specific amino-acid-converting enzymes present in these strains and/or differences in their regulation. This hypothesis is supported by the observation that several wild lactococci strains required only a few amino acids for growth; indicating that these strains harbour more AACEs, since these enzymes are primarily involved in synthesis of amino acids, rather than degradation. These findings offer the possibility to develop tailor-made starter cultures for flavour diversification. One example of such a culture, isolated from raw milk, is given in Fig. 7. In contrast to commercial starter cultures, the activity of this strain to produce certain branched-chain aldehydes and the corresponding alcohols is very high.

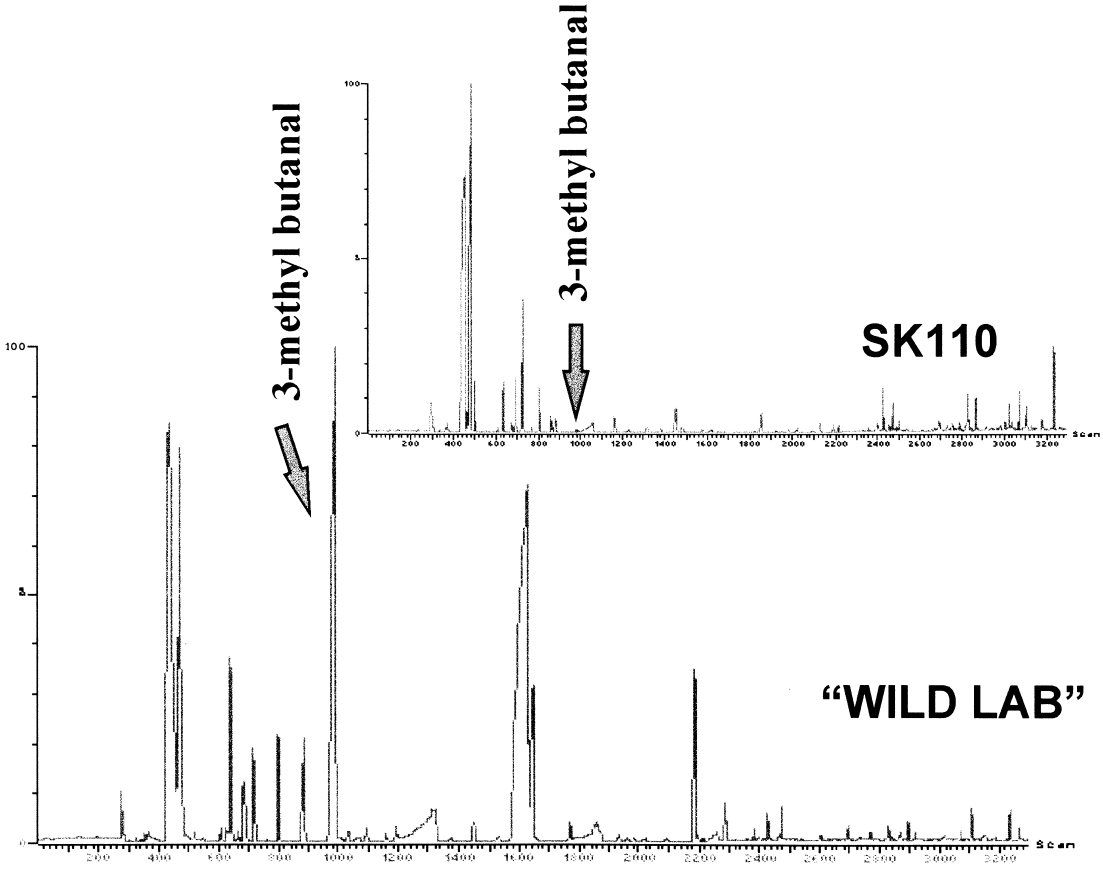

Fig. 7. Variation in aldehyde formation within the species *Lactococcus lactis*.

4. Conclusions

Taken together, significant progress has been made in the field of cheese ripening during the last years. The role of proteases and peptidases is evident, and a proper expression of such enzymes in cheese matrix is required for ripening. Subsequently, activity of AACEs results in the formation of the volatile flavour components, which are responsible for the real cheese flavour. A number of enzymes have already been purified and characterised from *Lactococcus lactis* strains. Construction of enzyme-overproducing strains as well as strains with a deletion of the genes encoding flavour-forming enzymes is ongoing and will reveal the importance of each enzyme in the pathways to flavour formation. Based on those results, bottlenecks in the pathways to key-flavour components will become clear. Genetically modified strains will become available and might possibly be used as starter cultures in the future. On the other hand, the biochemical and genetical knowledge of these enzymes will also permit faster selection of natural strains with specific desired flavour characteristics.

Based on the results obtained so far, it becomes evident that the current approach is promising, and that the results can be applied in various ways, ranging from product development to quality control. For instance, new cultures with specific/improved flavour forming abilities become available or can be selected in a directive manner. Moreover, diversification of flavour development and/or accelerated cheese ripening becomes possible as well as applications in other (fermented) dairy and other food products.

Acknowledgements

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