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Flavour formation in cheese

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22.1 Introduction

Micro-organisms play a number of major beneficial roles in the food industry. They are used to transform organic matter in foods, e.g. in fermented beverages, milk, meat and vegetables, and thereby they contribute to the preservation of food, but also to flavour and texture. Fermented milk products play an important if not dominant role in any discussion on food fermentations, which is no surprise in view of the size of the world's annual consumption, more than 15 billion kilograms (Kosikowski and Mistry, 1997). A variety of lactic acid bacteria and other micro-organisms are used in the production of a wide range of fermented dairy products. The main purpose of their use is to ensure a proper preservation of the fermented product. The rapid conversion of lactose, present in milk, into lactic acid is the most important feature of lactic acid bacteria in this respect. The resulting reduction of pH, a concomitant lowering of the redox potential and the absence of lactose inhibit growth of undesired bacteria in the dairy product, e.g. cheese.

In addition to the production of lactic acid, organisms used in fermented dairy products also determine flavour of the product. Milk is a rather bland product with regard to its flavour. This means that in fermented dairy products, the flavour compounds are nearly all generated during the fermentation and ripening of the product. Flavour development in fermented dairy products is a complex and, in the case of cheese ripening, slow process involving chemical and biochemical conversions of milk components (Engels and Visser, 1996; Engels *et al.*, 1997). Lactic acid bacteria (LAB), e.g. *Lactococcus lactis, Lactobacillus* species, *Streptococcus thermophilus* and *Leuconostoc mesenteroides*, form the main microflora in dairy products and are essential for the biochemical

conversions that determine the specific flavour. However, additional cultures are also used, such as *Propionibacterium* in the case of Swiss-type and Maasdammer-type cheeses, and various aerobic cultures, e.g. *Brevibacterium*, *Arthrobacter*, *Staphylococcus*, *Penicillium* and *Debaromyces*, for surface-ripened cheeses (Bockelmann, 2002; Molimard and Spinnler, 1996). As well as these starter organisms, endogenous milk enzymes, added enzymes (rennet) and mesophilic lactobacilli can also play a role. The latter, originating from the milk environment, might grow in the dairy products and thereby be a source of enzyme activities leading to the formation of flavours. Flavour compounds are formed by three processes: the conversions of lactose and citrate (glycolysis and/or pyruvate metabolism), fat (lipolysis), and caseins (proteolysis).

Although lactose is mainly converted to lactate by LAB, a fraction of the intermediate pyruvate can alternatively be converted to various flavour compounds such as diacetyl, acetoin, acetaldehyde or acetic acid, some of which contribute to typical yoghurt flavours. Lipolysis results in the formation of free fatty acids, which are precursors of flavour compounds such as methyl ketones, alcohols, lactones and esters (Molimard and Spinnler, 1996; Gallois and Langlois, 1990). Lipolysis is mainly due to mould activity, and much less to LAB activity (Molimard and Spinnler, 1996; Gripon, 1987). Fat hydrolysis is particularly important in soft cheeses like Camembert and blue cheeses.

The degradation of milk proteins is the major biochemical pathway for flavour formation in hard-type and semi-hard-type cheeses (van Kranenburg *et al.*, 2002; Engels, 1997). The activities of rennet enzymes, the cell-envelope proteinase and the peptidases from LAB yield small peptides and free amino acids from casein. These small peptides and amino acids are responsible for the important background flavour in a matured cheese, e.g. savoury, brothy and salty. However, the actual cheesy flavour is formed by amino-acid-converting enzymes and is superimposed on that basic flavour (Engels and Visser, 1994). This further conversion of amino acids yields various alcohols, aldehydes, acids, esters and sulphur compounds, facilitating specific flavour developments.

This chapter focuses on the main pathways involved in flavour formation from amino acids. The mechanisms of conversion of amino acids to volatile flavour compounds will be discussed in detail with examples of how fundamental knowledge of the flavour production pathways can be used to improve the flavour of a given cheese. The chapter summarises various aspects which have been reviewed by van Kranenburg, Smit, Yvon and their respective colleagues.

22.2 Amino acid conversion

22.2.1 Introduction

The volatile aroma components of various cheeses have received a great deal of attention and a large number of volatiles have been detected in individual types of cheese. Various methods have been applied for extraction of the volatiles

from WSF (water soluble fraction) and subsequent gas-chromatographic analysis of the compounds. Banks *et al.* (1992) used steam distillation and gas chromatography–mass spectrometry (GC–MS) for extraction and identification of volatile compounds from Cheddar cheese. The use of high-vacuum distillation, combined with gas-chromatographic analysis, has also been reported (Gallois and Langlois, 1990; Moio *et al.*, 1993). Headspace techniques (Bosset and Gauch, 1993; Wood *et al.*, 1994; Yang and Min, 1994) are used frequently nowadays. These methods measure all the volatile compounds while only a small fraction of volatiles are odour-active. In order to identify the odour-important compounds in cheese, analytical methods that combine gas chromatography and olfactometry have been developed (Acree *et al.*, 1984; Taylor *et al.*, 2000; Engels *et al.*, 2001). Some of the potent odorants result from lactose fermentation or citrate degradation and from lipolysis, while many other compounds result from amino acid conversion, mainly degradation of branched-chain and aromatic amino acids and methionine. In Table 22.1 typical examples of volatile compounds in various types of ripened cheese are shown. The volatiles can be divided in six major groups: fatty acids, esters, aldehydes, alcohols, ketones and sulphur compounds. Most of these compounds are present in all types of cheeses, though their concentrations show distinct differences.

Table 22.1 Major groups of volatile compounds formed during cheese ripening (after Engels, 1997)

Compounds	Typical examples	Cheese type where especially found
Fatty acids	Acetic acid	Gruyère, Parmesan, Camembert
	Propionic acid	Emmental, Maasdam
	Butyric acid	Gruyère, Parmesan, Camembert
Esters	Ethyl butanoate	Gruyère, Parmesan
	Ethyl decanoate	Roquefort
Aldehydes	3-Methyl-butanal	Proosdij, Parmesan
	2-Methyl-butanal	Parmesan
	Benzaldehyde	Comté
Alcohols	1-Butanol	Gruyère, Parmesan, Maasdam
	3-Methyl-1-butanol	Edam, Maasdam, Parmesan
	Phenylethanol	Camembert
Ketones	2-Heptanone	Roquefort, Camembert
	2-Nonanone	Roquefort, Camembert
	2-Butanone	Edam
Sulphur compounds	Dimethyldisulphide	Limburger, Cheddar, Gouda
	Methional	Cheddar, Emmental
Various components	Phenol	Limburger
	Limonene	Fontina
	δ-Decalactone	Emmental
	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	Emmental

It is important to realise that there is not a single compound or class of compounds which is responsible for the full flavour of cheese. The flavour is indisputably due to the presence of numerous components as already mentioned. Most of these compounds originate from lactose and the casein and fat fractions of the milk and are formed by the enzymatic action of the starter cultures used. Furthermore, chemical conversions of intermediate compounds occur concomitantly.

22.2.2 Amino acid biosynthesis and requirements

Flavour formation from amino acids by LAB depends on a very complex network of reactions, and many factors may contribute to the balance of various flavour compounds. In general, the main processes are:

- Generation and uptake of amino acids, i.e. formation of the intracellular pool of amino acids
- Conversion of amino acids
- Regulation of these pathways.

Lactococci have a limited capacity for biosynthesis of amino acids, which explains their complex nutritional requirements. They require essential amino acids for growth, and the number of essential amino acids is strain-dependent (Andersen and Elliker, 1953; Reiter and Oram, 1962; Chopin, 1993; Jensen and Hammer, 1993; Ayad *et al*., 1999). Most dairy *Lactococcus* strains need glutamate, valine, methionine, histidine, serine, leucine and isoleucine. Typically, industrial *L. lactis* ssp. *cremoris* strains require more amino acids for growth than dairy and non-dairy wild strains (Ayad *et al*., 1999). Wild *L. lactis* ssp. *cremoris* and ssp. *lactis* strains generally require fewer than three amino acids. The absence of some amino acid biosynthethic pathways in dairy lactococci might be a consequence of their adaptation to dairy products, since amino acids are readily available by the proteolysis of caseins, and as a result amino acid auxotrophy will not have a negative influence on cell growth. Strains isolated from natural niches are usually not associated with a rich environment such as milk, which makes them more dependent on their own synthesis of amino acids compared to industrial strains.

Since the concentrations of free amino acids and peptides are very low in milk, the starter cultures depend heavily for growth in milk on their proteolytic systems. The degradation of milk proteins (caseins) yields peptides and free amino acids, which can subsequently be taken up by the cells (Kunji *et al.,* 1996; Christensen *et al*., 1999). Proteolysis is initiated by a single cell-wall-bound extracellular proteinase (Prt), which can be either chromosomally or plasmid-encoded (Exterkate *et al*., 1993). While most dairy LAB strains contain such an extracellular proteinase, several do not and these strains are mainly dependent on other strains in the starter culture for the production of peptides and amino acids. Such dependency of strains is rather common in starter cultures, and it is expected that, in order to be able to develop stable starter

cultures, knowledge of the population dynamics between strains is essential (Ayad *et al.*, 2001a).

Peptide and amino acid transport systems have been studied extensively in lactococci, but far less is known for other LAB such as lactobacilli (for a review, see Kunji *et al.*, 1996). Peptide uptake occurs via oligopeptide transport systems and di-/tri-peptide transporters. The oligopeptide transporter is an ABC transporter capable of transporting peptides ranging in size from four to 18 amino acids (Detmers *et al.*, 1998). Also various amino acid transport systems have been identified with a high specificity for structurally similar amino acids (Konings *et al.*, 1989).

Following uptake, the peptides are degraded intracellularly by a variety of peptidases, which have been extensively studied in both lactococci and lactobacilli (reviews by Kunji *et al.*, 1996; Christensen *et al.*, 1999). These peptidases can be divided into endopeptidases, aminopeptidases, di-/tri-peptidases, and proline-specific peptidases. The specialised peptidases in LAB for hydrolysis of Pro-containing peptides have been postulated to be important for the degradation of casein-derived peptides, since these are known to have a high proline content.

The balance between the formation of peptides and their subsequent degradation into free amino acids is of particular importance for the taste of cheeses, since accumulation of peptides might lead to a bitter off-flavour (Stadhouders *et al.*, 1983; Visser *et al.*, 1983; Smit *et al.*, 1996, 1998). Various bitter-tasting peptides have been identified and especially these peptides should be degraded rapidly in order to prevent bitterness (Stadhouders *et al.*, 1983; Visser *et al.*, 1983; Smit *et al.*, 1998). Specific cultures have been selected with high bitter-tasting-peptide degrading abilities (Smit *et al.*, 1998) and such cultures are nowadays frequently used in the preparation of various types of cheese.

22.3 Amino acid catabolism

Amino acids are the precursors of various volatile cheese flavour compounds that have been identified in cheese (Engels *et al.*, 1996, 1997, Yvon *et al.*, 1997; Yvon and Rijnen, 2001). They can be converted in many different ways by enzymes such as transaminases (aminotransferases), decarboxylases, deaminases and lyases (Fig. 22.1). Transamination of amino acids results in the formation of α-keto acids that can be converted into aldehydes by decarboxylation and, subsequently, into alcohols by reduction or into carboxylic acids by oxidation (Yvon and Rijnen, 2001; Engels *et al.*, 2000; Christensen *et al.*, 1999). Many of these components are odour-active and contribute to the overall flavour of the dairy product. Moreover, other reactions may occur, e.g. by hydrogenase activity towards α-keto acids resulting in the formation of hydroxy-acids, which hardly contribute to the flavour. Aromatic amino acids, branched-chain amino acids and methionine are the most relevant substrates for cheese flavour development.

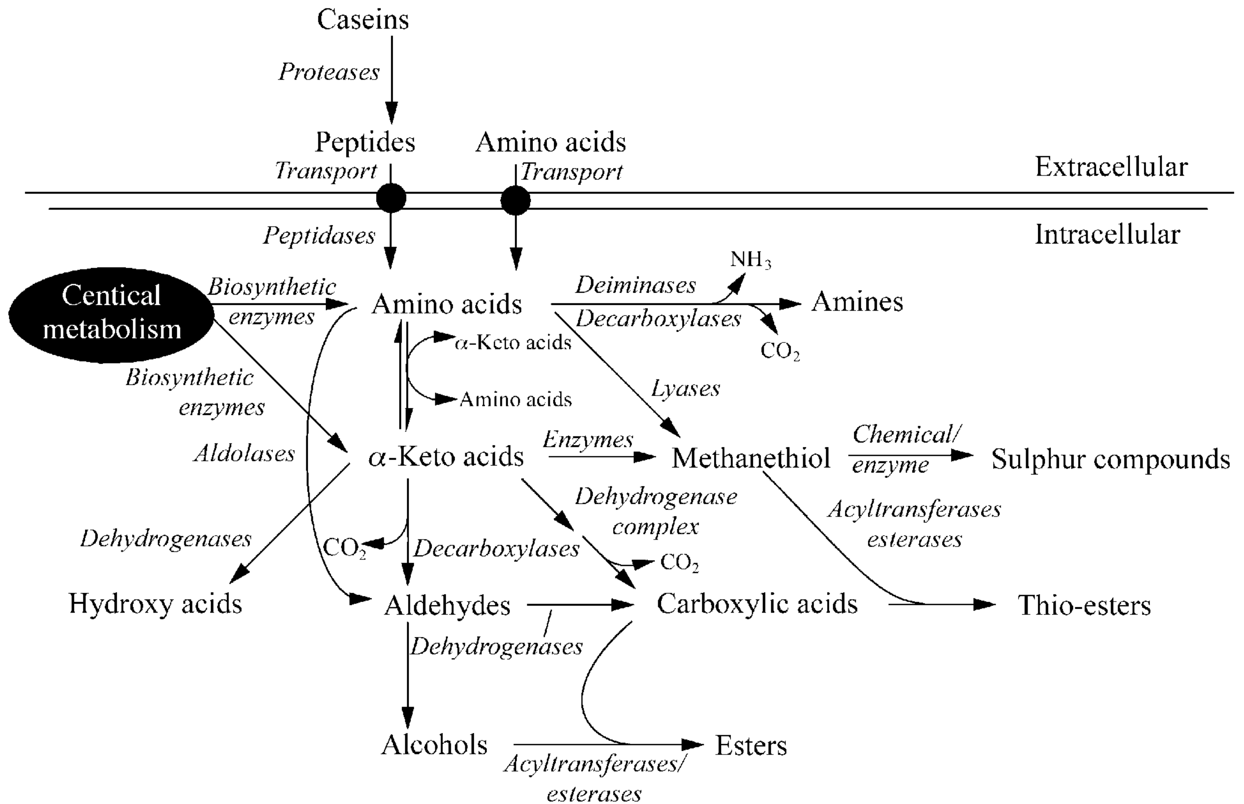

Fig. 22.1 Pathways of amino acid conversion leading to flavour compounds.

Table 22.2 Examples of products formed by breakdown of amino acids during cheese ripening

Amino acid	Volatile product	Flavour
Leu	3-Methyl-1-butanol	Fresh cheese, fruity
Ile	2-Methylbutanal	Malty, chocolate
Met	Methanethiol	Onion, cheese
Phe	Phenylacetaldehyde	Rose
Tyr	Phenol	Phenol, medicinal
Thr	Acetaldehyde	'Green', yoghurt
Val	2-Methylpropanal	Malty, chocolate

Compounds from catabolism of amino acids also include ammonia, amines, phenols and sulphur compounds. In Table 22.2, examples of amino acid-derived cheese volatiles are given. Methanethiol but also DMDS and DMTS contribute to the desirable garlic/sulphury note of the cheeses (Manning, 1974; Yvon and Rijnen, 2001; Weimer *et al.*, 1999). These sulphur compounds are highly odour-active and very volatile, and consequently they are mainly found in the headspace of cheese (Kubickova and Grosch, 1997; Milo and Reineccius, 1997). 2-Methylbutanal, but also 3-methylbutanal, have a green malty/chocolate-like odour which, when present in higher amounts caused unclean-harsh and dulling flavour sensations in Cheddar cheese (Dunn and Lindsay, 1985). However, in low concentrations the odour becomes fruity and rather pleasant. Isovaleric acid, derived from leucine, is more prevalent in Camembert than in Cheddar but it was also found in other cheeses such as Swiss cheese (Bosset *et al.*, 1993). It has a rancid, cheesy, sweaty and putrid odour that probably contributes highly to the very ripened-cheese aroma.

Esters, such as ethylbutyrate, also contribute to Cheddar flavour, although an excess of esters in proportion to other flavour components could be responsible for the fruity defect of Cheddar (Bills *et al.*, 1965). In Camembert, phenyl acetaldehyde, 2-phenylethanol and the derived ester phenylethyl acetate, which all result from phenylalanine degradation, are identified in fractions with floral rose-like odour (Kubickova and Grosch, 1997) and could cause the pleasant floral note of this cheese. These compounds have been previously assumed to cause the pleasant floral note of Camembert (Dumont *et al.*, 1974; Roger *et al.*, 1988). However, the same degradation products of phenylalanine and metabolites of other aromatic amino acids such as p-cresol, indole and skatole were identified as responsible for unclean-utensil, rose-like off-flavours in Cheddar (Dunn and Lindsay, 1985, Christensen, 1999). Conversion of tryptophan or phenylalanine can also lead to benzaldehyde formation. This compound is found in various hard-type and soft-type cheeses and contributes positively to the overall flavour (Engels *et al.*, 1997; Molimard and Spinnler, 1996). Many of the reactions can also occur under cheese conditions and are highly dependent on the strain used (Gao *et al.*, 1997). LAB strains may vary greatly in their capacity to metabolise amino acids, depending on the enzymes

they possess and express. In the following sections, degradation of individual amino acids and the enzymes involved is described.

22.4 Methionine catabolism

The decomposition products of methionine are of crucial importance for cheese flavour development. The catabolism of methionine by starter organisms has been reported, and especially methanethiol, dimethyldisulphide and dimethyltrisulphide contribute significantly to cheese flavour (Dias and Weimer, 1998a; Urbach, 1995; Engels, 1997). In fact, a Gouda cheese-like flavour can be generated by incubation of methionine with cell extracts of *L. lactis* (Engels and Visser, 1996). Smear-ripened cheeses, such as Tilsit, Danbo, Limburger and Appenzeller, host a rather wide range of micro-organisms on their surface. These micro-organisms, primarily bacteria (such as *Brevibacterium linens* and staphylococci) and yeasts, are responsible for a relatively high production of volatile sulphur-containing flavour compounds in these cheese types. Conversion of methionine by LAB can occur via an aminotransferase-initiated pathway by branched-chain or aromatic aminotransferases, or via an α,γ-elimination of methionine by the lyase activities of cystathionine β-lyase (CBL) and cystathionine γ-lyase (CGL) (Fig. 22.2) (Alting *et al.*, 1995; Bruinenberg *et al.*, 1997; Dias and Weimer, 1998a, 1998b; Engels *et al.*, 2000; Gao *et al.*, 1998; Gao and Steele, 1998; Rijnen *et al.*, 1999; Yvon *et al.*, 1997, Fernández *et al.,* 2000, 2002). In *B. linens* methionine γ-lyase (MGL) plays a central role (Dias and Weimer, 1998b). Aminotransferase activity results in the formation of 4-methylthio-2-

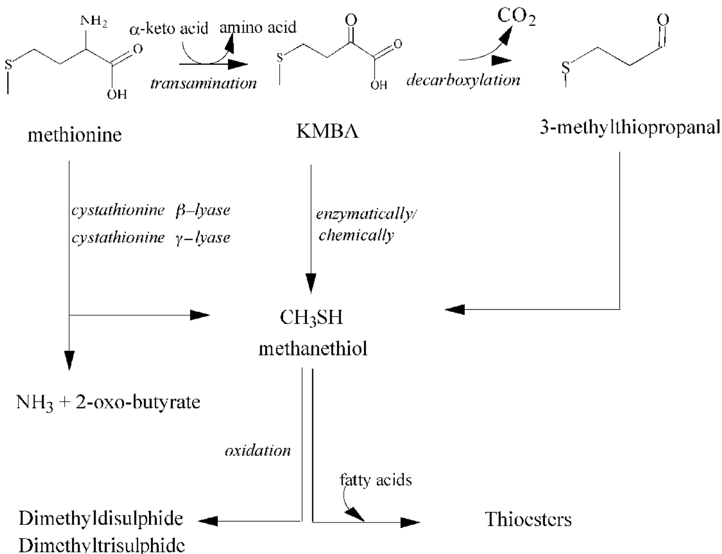

Fig. 22.2 Pathways of conversion of methionine to volatile sulphur compounds.

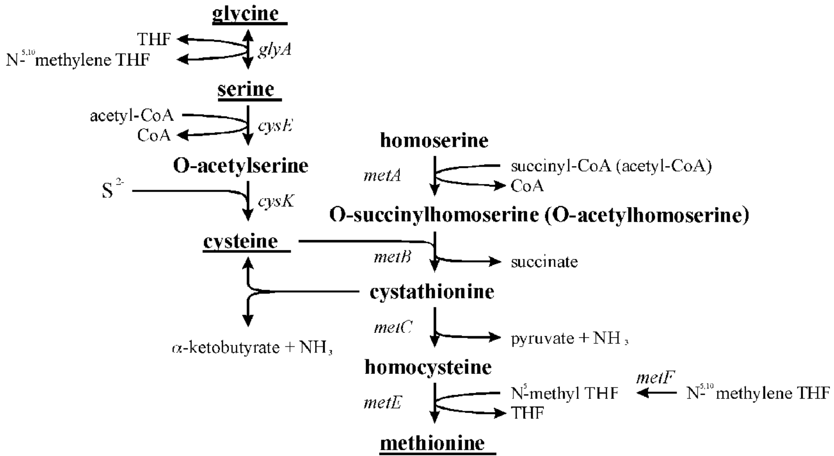

Fig. 22.3 Cysteine and methionine biosynsthesis pathways (van Kranenburg *et al.*, 2002).

ketobutyric acid (KMBA) which can be converted to methanethiol, probably via a thiamine pyrophosphate (TTP)-dependent decarboxylase that produces 3-methylthiopropanal (methional), and subsequent breakdown (Engels *et al.*, 2000).

Although cystathionine lyases are active under cheese-ripening conditions (Alting *et al.*, 1995; Ayad *et al.*, 1999; Smacchi and Gobbetti, 1998), their activity towards methionine could not be detected using ^{13}C nuclear magnetic resonance (Gao *et al.*, 1998). With this technique, only the aminotransferase-initiated pathway was observed, suggesting that this pathway is most prominent in methionine catabolism to produce methanethiol. On the other hand, strains that overproduce cystathionine β-lyase were found to be able to degrade methionine, indicating the potential of this enzyme in the production of sulphury flavours (Fig. 22.3). The specificity of CBL (Alting *et al.*, 1995) is a particular advantage in this respect, since one might expect that only sulphury flavour components will increase in strains with high activity. In the case of overproduction of other less specific enzymes such as transaminases more pathways will be influenced at the same time.

Biosynthesis and degradation of some amino acids are strongly linked pathways as illustrated in Fig. 22.3 for some amino acids. During cheese ripening, cystathionine β-lyase can convert methionine to various volatile flavour compounds, but in bacteria its physiological function is the conversion of cystathionine to homocysteine, which is the penultimate step in the biosynthesis of methionine (Fig. 22.3). This indicates that AACEs are in fact involved in the biosynthesis of amino acids. It is well known that biosynthesis of amino acids is highly regulated, and therefore the growth conditions of the starter cultures may affect their flavour-forming capacities.

For instance, in *L. lactis* the gene coding for cystathionine β-lyase (*metC*) is clustered together with a gene coding for cysteine synthase (*cysK*) (Fernández *et*

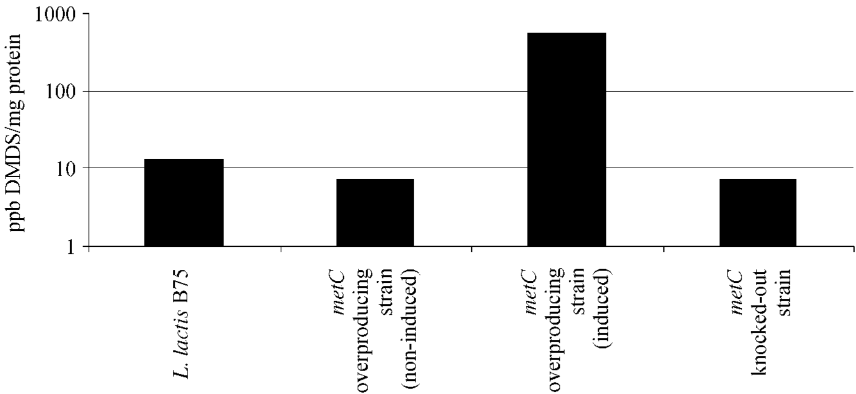

Fig. 22.4 Role of *metC* in production of sulphur flavours from methionine (DMDS = dimethyldisulphide).

al., 2000), thus genetically linking the methionine and cysteine biosynthesis pathways (Fig. 22.4). The expression of the *metC–cysK* gene cluster is strongly influenced by the amounts of methionine and cysteine in the culture medium (Fernández *et al.*, 2002). High concentrations of these amino acids completely abolish transcription and result in *L. lactis* cells almost deficient of cystathionine β-lyase activity. These regulatory aspects are probably very important in the control of flavour-forming enzymes in starter cultures and adjunct cultures.

22.5 Branched-chain and aromatic amino acid conversion

Amino acid transamination is a key step in the amino acid conversion to aroma compounds by cheese micro-organisms. In LAB, catabolism of aromatic amino acids (ArAAs), of branched-chain amino acids (BrAAs) and, at least in part, of methionine is initiated by a transamination reaction. It was demonstrated in lactococci (Gao *et al.*, 1997, 1998; Yvon *et al.*, 1997), in mesophilic lactobacilli such as *Lb. paracasei, Lb. casei, Lb. plantarum* and *Lb. rhamnosus* (Gummalla and Broadbent, 1996; Tammam *et al.*, 2000) and also in thermophilic lactobacilli such as *Lb. helveticus, Lb. delbrueckii lactis, Lb. delbrueckii bulgaricus, Streptococcus thermophilus* or *Propionibacterium freudenreicheii* (Gummalla and Broadbent, 1999; Thierry and Maillard, 2002) that an α-keto acid is used as amino group acceptor in transamination.

Amino acid transamination is catalysed by aminotransferases which are pyridoxal-S$'$-phosphate-dependent enzymes and are widely distributed in micro-organisms. Transamination results in the formation of α-keto acids while the α-keto acid acceptor is transformed to the corresponding amino acid, e.g. glutamate in the case of α-ketoglutarate. Aminotransferases are more or less specific for one group of amino acids (e.g. ArAAs, BcAAs) but often have

broad, overlapping substrate specificities (Yvon and Rijnen, 2001). Transaminations are physiologically very important because they play a crucial role in the biosynthesis as well as the catabolism of amino acids (Engels *et al.*, 2000; Yvon and Rijnen, 2001).

Despite their importance in amino acid conversion to aroma compounds, aminotransferases of cheese micro-organisms have been poorly studied. ArAA aminotransferases of *B. linens* were partially purified several years ago (Lee and Desmazeaud, 1985). More recently the aminotransferases active on ArAAs, BcAAs and Met were purified and characterised from *L. lactis* (Yvon *et al.*, 1997; Gao and Steele 1998; Engels *et al.*, 2000). The aromatic aminotransferases can convert aromatic amino acids, but also leucine and methionine, while the branched-chain aminotransferases can convert the branched-chain amino acids leucine, isoleucine and valine, but also methionine, cysteine and phenylalanine (Yvon *et al.*, 1997; Engels *et al.*, 2000). The physiological role of these aminotransferases in bacterial metabolism is to catalyse the last step in the biosynthesis of branched-chain or aromatic amino acids. Optimal pH for activity is around pH 7.5–8 and optimal temperature 35–50ºC (Yvon *et al.*, 1997; Engels *et al.*, 2000). The enzymes are still active under cheese ripening conditions (low pH and temperature, high salt). Expression of the *bcaT* gene is repressed by high concentrations of branched-chain amino acids or methionine (Yvon *et al.*, 2000). This illustrates that the selection of culture conditions can strongly influence the flavour-forming capacities of *L. lactis*.

Yvon and co-workers (1998) demonstrated that the amount of α-keto acids, functioning as co-substrate, is very important for high amino acid conversion rates. Overproduction of the transaminases alone did not lead to a strong increase in amino acid conversion without a simultaneous addition of keto acids as co-substrate (Yvon *et al.*, 1998). The introduction of a glutamate dehydrogenase gene, the dehydrogenase catalysing glutamate conversion to α-keto glutamate, from *Peptostreptococcus* in *L. lactis* resulted in a similar effect (Rijnen *et al.*, 2000). However, whether this activity also results in a strong increase in the desired flavour components remains to be determined. Recently, experiments were performed during which α-ketoglutarate was added to cheeses. The results suggested a positive effect on formation of cheese flavour compounds (Rijnen *et al.*, 1999; Banks *et al.*, 2001). Ayad *et al.* (2001b) found that also the presence of enzymes required for subsequent conversions of α-keto acids might be of crucial importance (see below). α-Keto acids resulting from amino acid transamination are further degraded to various compounds either by enzymatic reactions or by chemical reactions. Hydroxy acids, carbolic acids and aldehydes are resulting products. Moreover, KMBA, the methionine α-keto acid, can be degraded to methanethiol. Phenylpyruvic acid resulting from phenylalanine transamination is chemically converted to benzaldehyde in the presence of oxygen and manganese (Nierop Groot and De Bont, 1999). All these compounds, except hydroxy acids, are major aroma compounds.

The reactions and the enzymes involved in further degradation of α-keto acids have been only partially elucidated. Both conversion to aldehydes or

alcohols and conversion to carboxylic acids seem of importance. Formation of 3-methylbutanal and 3-methylbutanol by *L. lactis* can take place by enzymatic decarboxylation (followed by a reduction step in the case of the alcohol) of the α-keto acid from leucine, α-keto isocaproic acid (Smit, B.A. *et al.*, 2002, submitted). Production of carboxylic acids probably proceeds via a process of oxidative decarboxylation (Yvon and Rijnen, 2001). Conversion of KMBA by decarboxylation was already mentioned in the previous section. Several enzymes can thus be considered as being involved in degradation of amino acids, but also in biosynthesis, and α-keto acids are key intermediates.

22.6 Conversion of other amino acids

Threonine catabolism by LAB is initiated by threonine aldolase (serine hydroxymethyltransferase), an enzyme widely distributed in LAB. Threonine aldolase converts threonine to aceetaldehyde and glycine (Lees and Jago, 1976; Marshall and Cole, 1983). Aceetaldehyde is an important flavour compound of yoghurt, but also of cheese, butter and buttermilk (Ott *et al.*, 2000).

Two pathways for the catabolism of arginine have been described. The most common pathway for the catabolism of arginine in LAB is via the arginine deiminase (ADI) pathway (Cunin *et al.*, 1986; Konings *et al.*, 1995). This pathway results in the conversion of arginine into ammonia and ornithine and CO_2. Glutamic acid catabolism in LAB proceeds via transamination (to α-ketoglutarate) followed by decarboxylation or takes place directly by decarboxylation. In the latter case, γ-aminobutyrate (GABA) is the resulting product. Certain *Streptococcus thermophilus* strains present in starter cultures for, e.g., Parmesan-type cheese show high glutamate decarboxylase activity, resulting in excessive CO_2 production (Zoon and Allersma, 1996). Histamine production from histidine in fermented foods is of importance for health and safety concerns. In *Lactobacillus* histamine production could occur by histidine decarboxylases (Voight and Eitenmiller, 1978).

For the above-mentioned processes to take place, availability of free amino acids is of course essential. This requires a balanced proteolysis in cheese as well as (probably) lysis of starter cells to some extent (Visser, 1993). In contrast to the activity of peptidases (see above), where lysis generally enhances the activity, it probably depends on the type of enzyme (system) whether lysis will improve the activity or not. For instance, enzymes which require co-factors or co-substrates (e.g., PLP, NAD, NADP) might be negatively affected by lysis of the cells. The delicate balance, which is needed for the whole set of enzymes involved in flavour formation, will probably be affected by lysis (Smit, G. *et al.*, 2002).

The diversity observed in amino-acid-converting capacities of various strains, but also in lysis behaviour, implies that by targeted selection of starter bacteria the formation of desirable flavours can be influenced. A similar strain dependency is also found for enzyme activities which result in the formation

of undesired flavour compounds. This indicates that a strong potential for starter improvement exists. In the next section examples will be given of optimisation of flavour of dairy products by rational selection of strains.

22.7 Natural biodiversity and tailor-made starter cultures

Amino-acid-converting abilities of LAB are linked to the ability to synthesise amino acids. Lactococci used in dairy fermentations are known for their limited capacity for biosynthesis of amino acids, which explains their complex nutritional requirements. As described above, they require several amino acids for growth, and the number of essential amino acids is strain-dependent (Andersen and Elliker, 1953; Reiter and Oram, 1962; Chopin, 1993; Jensen and Hammer, 1993; Ayad *et al.*, 1999). Most dairy *Lactococcus* strains need glutamate, valine, methionine, histidine, serine, leucine and isoleucine. Industrial *L. lactis* ssp. *cremoris* strains require more different amino acids for growth than so-called wild lactococcal strains (Ayad *et al.*, 1999). The latter are more dependent on their own biosynthesis of amino acids.

The growth requirement for specific amino acids can result either from the absence of functional specific biosynthetic genes or from specific regulatory mechanisms (Chopin, 1993). For example, the existence of defects in biosynthesis of histidine and branched-chain amino acids has been established in *L. lactis* strains resulting from accumulated mutations and deletions within the genes coding for the biosynthetic enzymes (Delorme *et al.*, 1993; Godon *et al.*, 1993; Bolotin *et al.*, 2001). The involvement of regulatory mechanisms in amino acid requirements has also been demonstrated in *L. lactis*. For instance, the biosynthesis of the amino acids of the glutamate family (Glu, Gln, Arg and Pro) is dependent on the synthesis of glutamate itself which, in turn, can be affected by the ammonium ion concentration in the medium.

Interestingly, lactococci isolated from natural niches (wild strains) were found not only to have a larger potential in amino acid production, but concomitantly also to be able to produce rather unusual flavour components. This natural biodiversity could offer new possibilities when explored and applied in practice. Recently, it was demonstrated that the absence of parts of the flavour-forming pathways in individual strains can be complemented by using defined strain combinations (Ayad *et al.*, 2001b). For instance, the combination of artisanal lactococcal strain *L. lactis* B1157 and industrial lactococcal strain SK110 in milk resulted in a very strong chocolate-like flavour, due to high levels of 3-methyl butanal. In SK110, a highly proteolytic strain, the complete pathway from casein via leucine to 3-methyl butanal cannot proceed due to the lack of a decarboxylating enzyme in this strain (Fig. 22.5a). B1157 is a non-proteolytic strain and therefore unable to produce enough free amino acids that can serve as substrate for the subsequent transamination and decarboxylation steps (Fig. 22.5b). However, when B1157 and SK110 are incubated together, the strains complement each other with regard to their

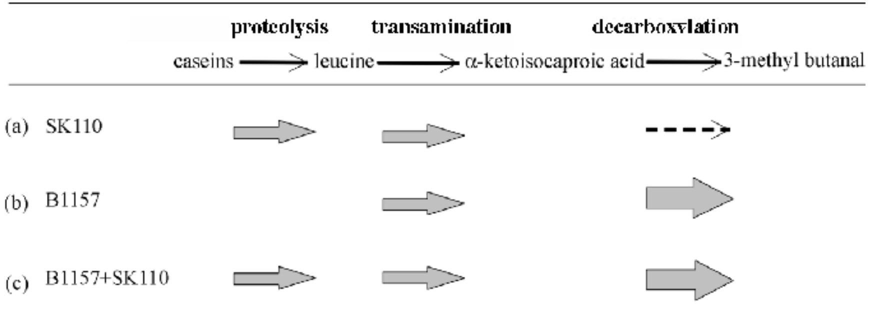

Fig. 22.5 Pathway of leucine comversion by individual and combined lactococcal strains (after Ayad *et al.*, 2001b).

enzyme activities, resulting in a high production of the chocolate flavour component 3-methyl butanal (Fig. 22.5c).

The use of a selected mesophilic *L. lactis* strain with high activity to form 3-methyl butanal to improve the taste of Proosdij cheese is another example of the application of fundamental knowledge on flavour-forming abilities for the optimisation of flavour. Proosdij cheese is a Gouda-type cheese, prepared with a mesophilic starter culture in combination with a thermophilic adjunct culture. This cheese has a flavour profile with characteristics between Gouda and Parmesan cheese. One of the key flavours in the cheese is 3-methyl butanal (Neeter *et al.*, 1996; Engels *et al.*, 1997). The selected *L. lactis* strain (B851) was used in combination with the regular cultures (mesophilic starter Bos and thermophilic culture S1138) used for this type of cheese. The cheeses made with and without the selected strain were analysed for the production of 3-methyl butanal by headspace gas chromatography (Ayad *et al.*, 2001a) and graded by an expert panel (Ayad *et al.*, 2001b). It was found that the use of the selected adjunct strain in cheese resulted in an increase in both the key-flavour production (Fig. 22.6(b)) and the intensity of the Proosdij cheese flavour (Fig. 22.6(a)).

22.8 Future trends

Knowledge of the amino-acid-converting enzymes is currently expanding rapidly. Whole genome sequences of various lactic acid bacteria are becoming available, e.g. *L. lactis* (Bolotin *et al.*, 2001) and *L. plantarum* (Kleerebezem *et al.*, 2003) genomes, which will expand our knowledge of flavour-forming pathways and mechanisms in different bacteria even faster. Combined with the understanding of the microbial physiology of flavour formation and the use of genomics tools, it will soon allow prediction of the flavour-forming capacity of various lactic acid bacteria. This will make the rational design of improved tailor-made industrial cultures with attractive flavour-forming properties possible and lead to the design of probes for high-throughput screening and strain selection in the future.

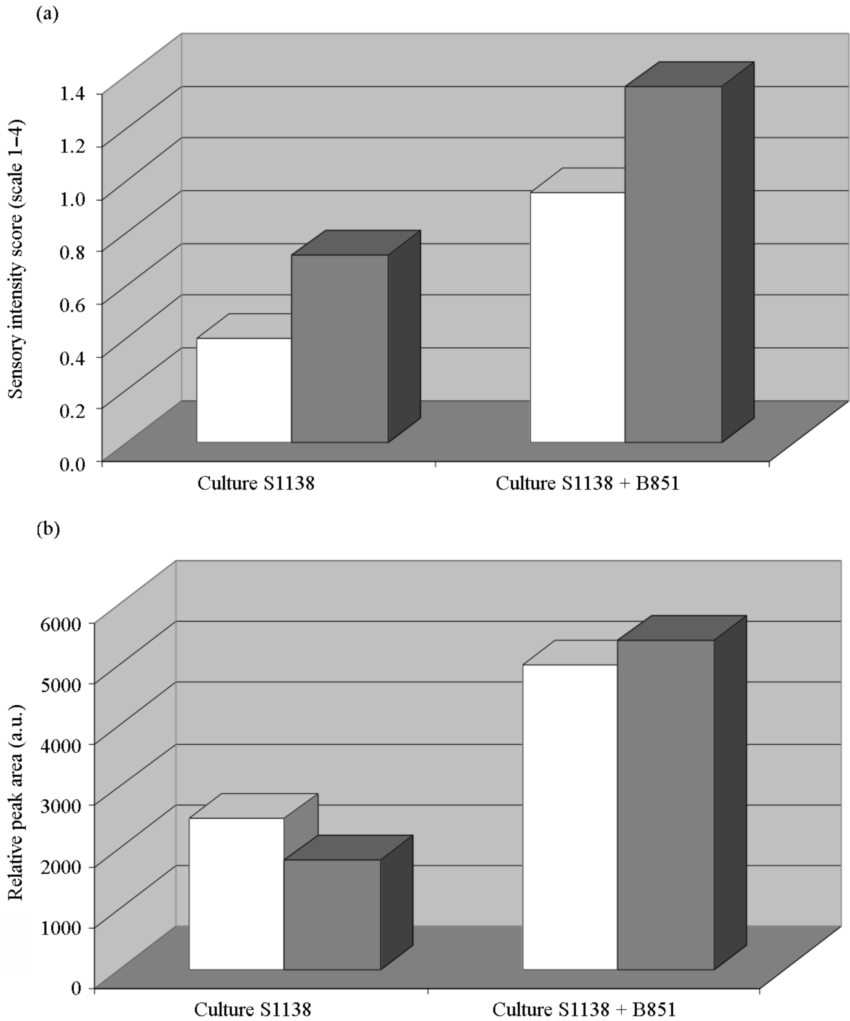

Fig. 22.6 Intensity of Proosdij cheese flavour (A) and relative amount of 3-methyl butanal (B) after 6 weeks' (open bars) and 3 months' (filled bars) ripening. Culture S1138: cheese made with starter Bos and adjunct culture S1138. Culture S1138: cheese made with starter Bos and adjunct culture S1138 and selected *L. lactis* strain B851.

New developments in the analysis of formation of flavours in fermented food products can be of great help for product development. By knowing the key flavours and their release patterns we can define more precisely the criteria for a successful product. An exciting perspective is the development of multifunctional starter cultures that combine excellent flavour-forming characteristics with a health-promoting effect such as the production of vitamins (Smid *et al.*, 2001). A multidisciplinary approach including key flavour component analysis, directed screening for flavour-producing starter organisms,

and tailored-flavour release, will be essential to accelerate development of these types of enhanced products.

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