Formation of Amino Acid Derived Cheese Flavour Compounds

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Contents

Chapter 1

General introduction

1.1. <u>Abstract</u>

Fermentation of food can contribute greatly to its nutritional value, perception and texture besides to longer and safer use. Especially nutritious foods like milk are often fermented to a wide variety of products by various micro-organisms. Lactic acid bacteria like *Lactococcus lactis* are often used for the fermentation of milk into various cheeses. Most lactic acid bacteria convert lactose into lactic acid, thereby lowering the pH. This decrease in pH, together with the addition of salt and the removal of water results in a stable product. Besides production of acid, micro-organisms like lactic acid bacteria often produce other metabolites from milk sugar, protein and fat. The reactions primary take place for own maintenance and growth, but the resulting low molecular compounds have sometimes strong flavour characteristics. The most dominant flavour compounds (key-flavours) can be isolated and identified. Their subsequent recombination usually results in a good impression of the original product-flavour. Many key flavours in semi-hard cheeses like Gouda, are derived from protein degradation. The biochemical conversion from protein to flavour compounds starts with proteolysis and peptidolysis, which results in free amino acids. These can be converted in several ways, leading to a large variety of (flavour) compounds. This thesis focuses on the production of one class of these compounds as model system: aldehydes, in particular the key-flavour 3-methylbutanal, which are derived from the amino acid leucine. Only few lactic acid bacteria are known to produce considerable amounts of this aldehyde. The aim of this research is to gain understanding on the formation pathway of this kind of flavour compounds, in order to improve the control of flavour development during fermentation. Targeted strain selection is one of these tools, which might open opportunities for application in flavour diversification and/or acceleration of the cheese ripening process.

<u>Key-words</u>

1.2. <u>**Fermented dairy products**</u>

Milk and dairy products are an important part of a well balanced diet all over the world. Milk contains many nutrients, for nursing and sustaining a newborn animal. The high nutritious value of milk makes it sensitive to spoilage by bacteria when keeping it for longer times. In some cases (controlled) spoilage, fermentation, leads to a more stable product, often due to the production of conserving acids by lactic acid bacteria. More generally fermentation can be defined as the change of the properties of a raw material by micro-organisms or enzymes to improve its safety, shelf life, nutritious value or perception (Steinkraus, '94). Major factors to achieve a well-controlled fermentation process are the use of only selected, well-characterised micro-organisms, the pre-treatment of raw material, and the control of process parameters such as temperature, pH, mixing intensity etc. Examples of the very diverse group of fermented dairy products are cheese, butter, buttermilk, kefir, yoghurt and quarg (labneh). Other products derived from dairy fermentations are lactic acid and whey, which are applied as food and non-food ingredients.

The long history of the first fermented dairy products with an improved shelf life has presumably started together with the domestification of animals. These fermented milks most probably resembled products like yoghurt. Although the appearance of these products did not change very much over time, the focus on yoghurt fermentations has changed from preservation to improvement of characteristics like texture, flavour and health promoting activity. The latter focus started around the 1900's, when Metschnikoff studied the health effects of fermented milks, in relation the longer life expectance of people on the Balkan (Metschnikoff, '08). Nowadays several types of yoghurts have been developed with an improved health imago, structure and flavour.

By removing a part of the water phase, the dry matter content and the appearance of the fermented milk changed largely to a more or less cheese-like product. The earliest records of cheese making are probably from the ancient Egypt and from around 300 BC also more textured cheese-like products, like Domiati had become established (Robinson, '95). Due to regional characteristics, like climate, distinctive varieties of cheese emerged. For example, it appears that there is a distinct relationship between climate and the bacteria associated with the fermented product of that region. In northern Europe, mainly mesophilic bacteria have been used, and in warmer climates the major part of the flora consisted of thermophiles. Later, in the Middle Ages, human activities played a dominant role in the development of cheese varieties. Transporting cheese from production area to large conurbations and trading it there demanded cheese with a longer shelf life. Therefore harder and drier cheeses became common, although soft cheeses retained their position in some areas. The advent of refrigeration and fast, reliable transport changed this pattern again, and especially in the USA soft cheeses claim a major part of the

market. Nowadays, a large range of cheese types is available and, from an economical point of view, cheese is the most important fermented milk product. In Table 1.1 some popular cheeses are categorised based on hardness and use of an additional culture (Ridgway, '99). The most common categorisation of cheese is based on their water content, which can be noticed by the hardness of the cheese. The main source of bacteria in these modern cheese varieties is the primary starter, which is a mixture of selected lactic acid bacteria (LAB). In addition to this starter, some other micro-organisms (secondary starter) might be present in the cheese, and these might add largely to the product characteristics of the cheese. The introduction of new microbial strains in cheese making is a powerful tool to change product characteristics. In Gouda cheeses produced with non-dairy LAB (wild strains) large differences in flavour formation were observed (Ayad *ea.*, '00). The background of the formation of these differences however is still unclear.

Table 1.1 Some major types of cheese, categorised on hardness and secondairy starter used. (Edited after Ridgway, '99)

Category	Secondary starter	Examples	Country
Extra Hard	-	Parmesan	Italy
		Grana Padano	Italy
Hard	-	Cheddar	England
		Manchego	Spain
		Edam	The Netherlands
Semi-Hard	-	Gouda	The Netherlands
		Port Salut	France
	Eye formers	Emmental	Switzerland
		Maasdam	The Netherlands
	Surface bacteria (smear)	Gruyère	Switzerland
		Limburger	Germany
		Tilsit	Germany
		Kernhemmer	The Netherlands
Soft		Cottage cheese	USA
		Fromage frais	France
		Mozerella	Italy
		Feta	Greece
	Mould-veined (blue)	Danish blue	Denmark
		Gorgonzola	Italy
		Roquefort	France
	Surface mould (white)	Brie	France
		Camembert	France

This thesis will focus on the formation of some of these flavour compounds present in Dutch-type cheeses like Gouda. Therefore, the production of Gouda cheese will be discussed next, and more detail on the micro-organisms will be discussed in section 1.2.

The Dutch dairy industry produced about 640.000 tons of cheese in 2002, of which about 77% was exported. The major types produced are Gouda, Edam, Maasdam (Walstra *ea.*, '93; Productschap Zuivel, '03). The production of Gouda cheese is schematically shown in Figure 1.1. The essence of this process, like for all other cheeses, is the separation of milk protein (casein) and fat from the rest (mainly water). This is achieved by coagulation of the caseins, which is in the case of Gouda cheese initiated by the specific action of proteolytic enzymes (in rennet) on κ-casein. The resulting para-casein coagulates into a casein gel, which entraps the fat and bacteria present in the milk, as well as part of the sugar, salts and rennet. The gel is cut or stirred to promote syneresis (extrusion of the whey from the gel (future curd)). The syneresis is further
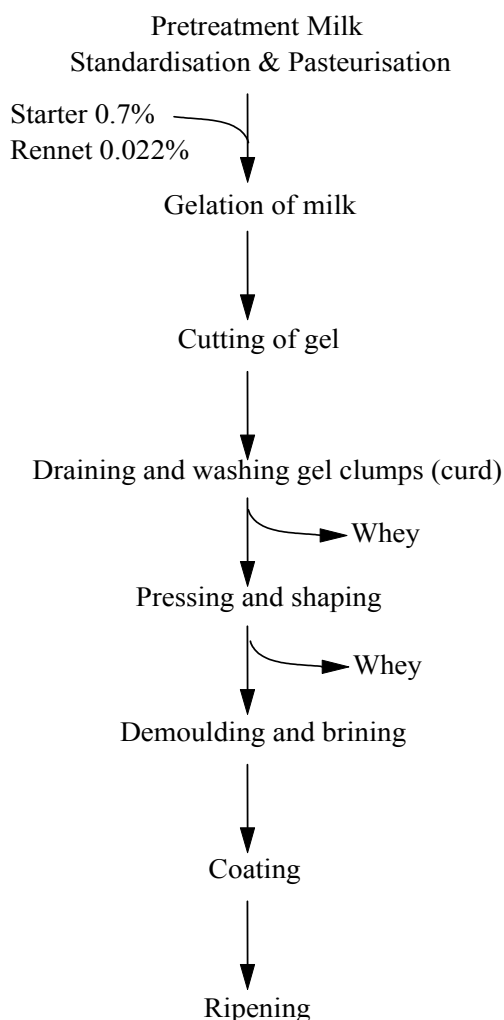

Figure 1.1 Simplified schematic overview of the production of Gouda cheese.

promoted by heating the curd/whey mixture (cooking). In the production process of Gouda cheese, syneresis is promoted by adding hot water. In the next steps of cheese production process,

the whey is removed by draining and pressing, resulting in fresh cheese with a closed rind. During the pressing phase, the subsequent holding and salting stage, the fermentation of the residual lactose by LAB results in a decrease of the pH to 5.2-5.4 within 24 hours for Gouda cheese. Dutch-type cheeses are salted by brining them for about 6 hours (Walstra *ea.*, '93; Kosikowski *ea.*, '97).

Besides the conversion of lactose during the fermentation stage, oxygen is consumed resulting in a low redox potential of the cheese (Galesloot, '60; Walstra *ea.*, '93). After production Gouda cheeses are ripened up to 2 years at 12-15°. In this time microbial, enzymatic and chemical activities change the cheese from soft and bendable (long) without much flavour, to a dryer, harder, breakable (short) product with strong flavour characteristics (Kanawjia *ea.*, '91). The age of the cheese is expressed as young (6 weeks), mature (3 months) or old (>6 months). Although the properties of the milk do influence the properties of the final product, the most important way to control the flavour formation is the selection of microbial strains with desired characteristics. The next paragraph focuses on these micro-organisms, and the current knowledge on cheese flavour compounds and their formation will be addressed in sections 1.4 and 1.5.

1.3. <u>Dairy related micro-organisms</u>

Many different organisms are used in milk fermentations. This large biodiversity results in large differences in the product characteristics, such as flavour. As mentioned in section 1.2, historically the fermentation occurred spontaneously, with the flora present in the milk, or obtained from the environment (Ridgway, '99). Nowadays, selected organisms form the major flora. The primary starter culture may be defined as a mixture of LAB, which is added to the milk in order to obtain fast acidification of the milk. By converting sugar (lactose) to lactic acid (lactate), these bacteria are able to lower the pH down to 4.2 in the case of some fermented milks. Secondary starters (adjunct cultures) are used to add extra fermentation characteristics to the final product. Besides these starters also the environment may still be a source for the micro-organisms present in the product. In some cases this is desired, but in other cases this might lead to spoilage of the product. This balance is more critical in cheeses made from raw milk. Apart from inoculation by adding a starter to the cheese milk, a starter can also be applied on the surface of the cheese. In that case the smear of ripened cheese is used as inoculum for the surface of new cheeses; the term old-new smearing is used (Bockelmann *ea.*, '97a). An overview of the main microbial genera associated with dairy fermentations, with their major characteristics is given in Table 1.2. All micro-organisms present in the fermented dairy product will convert milk ingredients like sugar, fat and protein into various metabolites, lactate only being one of them. Of these, the metabolites with strong flavour impact are discussed in the next sections. As a result of all the conversions the product (perception) changes with time. Since the product is an environment where various micro-organisms are present, the growth of certain micro-organisms

is influenced by the changes occurring, which results in differences in populations over time (population dynamics). This is an important aspect of mixed microbial fermentations, like most dairy fermentations. All the interactions between organisms and the product are important, and are possible control points for regulating the cheese ripening process. In Table 1.2 some important benefits of the micro-organisms mentioned are also briefly addressed. The micro-organisms used in dairy fermentations (Table 1.2) have very diverse characteristics, but almost all of them have a long history of safe usage in foods, the so called GRAS status (Salminen *ea.*, '98). Although the micro-aerophilic LAB are most important in the primary starter, a large range or micro-organisms, among them several prokaryotes and eukaryotes, are used as secondary starter. LAB obtained their name, because they are able to produce lactic acid very efficiently. This definition excludes the dairy associated strains of the genera *Propionibacterium* and *Bifidobacterium* from LAB. LAB are classified according to their ability to ferment glucose solely to lactate, or also to additional products, i.e. as homofermentative or hetrofermentative (Schlegel, '97). All LAB are strictly fermentative and are grown under anaerobic conditions, although they are mostly aero tolerant. LAB lack cytochromes and catalase, they are Gram-positive, non-sporulating, acid tolerant and nutritionally fastidious (Schlegel, '97; Axelsson, '98).

Also within the group of LAB large biodiversity exists and this offers many opportunities for exploitation, aiming at changes in product and process characteristics. Some important (metabolic) characteristics are bacteriocin production, phage resistance, nutritional requirements, and temperature sensitivity (Boutibonnes *ea.*, '95; Allison *ea.*, '98; Ayad *ea.*, '99; Hyronimus *ea.*, '00). In addition to the chromosomal DNA, most LAB carry plasmid DNA, which may also code for several for fermentation relevant characteristics, such as lactose and citrate metabolism, cell envelope proteinase and antibiotic resistances (Libudzisz *ea.*, '91; Perreten, '96). The characteristics coded for on these plasmids are less stable than chromosomal coded characteristics, and might be lost during cultivation or even transferred to other strains by conjugation (Guedon *ea.*, '00).

The diversity of LAB as a group can also be illustrated using modern taxonomical methods. Taxonomy of bacteria used to be based on their physiological and morphological characteristics, rather than on actual genetic relatedness of strains. With modern techniques like 16S or 23S rRNA sequencing, the use of fluorescent oligonucleotide probes, DNA restriction analysis and DNA-DNA hybridisation, phylogenetic trees based on evolutionary relatedness of LAB can be created (Collins *ea.*, '91; Klein *ea.*, '91; Salama *ea.*, '91; Schleifer *ea.*, '95; Axelsson, '98; Rademaker *ea.*, '04). As a result of this well-defined way of taxonomy, and large interest in exploration of the natural biodiversity, several new species of LAB are described each year and many lactobacilli have been reclassified.

Table 1.2   Micro-organisms used in dairy fermentations. (Examples taken from Oritz de Apodaca *ea.*, '93; Molimard *ea.*, '96; Bockelmann *ea.*, '97b; Kosikowski *ea.*, '97).

Organism		Type of product	Benefits
BACTERIA			
Lactococcus lactis	*lactis*	Most types of cheeses, sour cream, buttermilk	Acid, flavour
	cremoris	Most types of cheeses, sour cream, buttermilk	Acid, flavour
	lactis biovar. *diacetylactis*		Acid, flavour, eyes
Streptococcus	*thermophilus*	Yoghurts	Acid
		Cheese	Acid
Lactobacillus	*helveticus*	Semi hard cheeses	Acid, flavour
	acidophilus	Fermented and sweet acidophilus milk (semi) hard cheeses	Acid, flavour, probiotic
	thermophilus	Semi hard cheeses	Acid, flavour
	plantarum	Cheese adjunct culture	Structure, probiotic
	delbrueckii subsp.*bulgaricus*	Yoghurt, kefir, Koumiss, (semi) hard cheeses	Acid, flavour
	casei	Cheese adjunct culture	Flavour, structure
	rhamnosus	Cheese adjunct culture	
	buchneri	Found in ripened cheeses	
	fermentum	Found in ripened cheeses	
	kefir	Kefir	Acid
Leuconostoc	*mesenteroides*	Semi hard cheeses, buttermilk, sour cream	Flavour
Propionibacterium	*freudenreichii* subsp. *shermanii*	Maasdam and Swiss type cheeses	Favour, eye formation
	acidipropionici	Cheese	Flavour, eye formation
Arthrobacter	spp.	Smear cheeses	Flavour, colour
Bifidobacterium	spp.	Yoghurt, sweet-milk	Acid, flavour, health promoting
Brevibacterium	*linens*	Smear cheeses, soft cheeses	Flavour, colour
	casei	Smear cheeses	Flavour, colour
Corynebacterium	spp.	Smear cheeses	
Pediococcus	*pentosaceus*	Reduced fat cheese	Flavour
	acidilacti	Ripened cheese	
Micrococcus	spp.	(Smear) cheeses	
Enterococcus	*feacalis*	Raw milk cheeses	
	faecium	Raw milk cheeses	
Staphylococcus	spp.	Smear cheeses	
FUNGI and YEASTS			
Penicillium	*roqueforti*	Soft-cheeses (veined)	Flavour, colour
	camemberti	Soft-cheeses (surface)	Flavour, texture, colour
Geotrichum	*candidum*	Smear cheeses, Camembert	Flavour
Kluyveromyces	*lactis*	Smear cheeses	Enhancement of other micro-organisms
Saccharomyces	spp.	Smear cheeses	Enhancement of other micro-organisms
Debaryomyces	spp.	Smear cheeses	Enhancement of other micro-organisms

Lactotococcus is the main species of commercial interest, and is the most frequently used organism in the production of semi-hard type of cheeses, like Gouda, Edam and Cheddar, but is also present in many other cheese varieties. This genus is divided in the species *L. lactis, L. piscium, L. graviae* and *L. raffinolactis. L. lactis* is subdivided in *L. lactis* subsp. *lactis,* subsp. *cremoris* and subsp. *hordniae* (Schleifer *ea.*, '87; Tailliez, '01). *Lactotococcus* spp. are cocci that occur singly, in pairs or in chains, and grow at temperatures between 10°C and 45°C. The metabolism of lactococci is relative simple. They obtain their energy by a homo-fermentative route from sugars to lactic acid. The citric acid cycle is not functionally present, and no respiratory enzymes have been found (Schlegel, '97). Due to this relatively short and simple pathway only little energy is produced from one sugar molecule. To obtain enough energy, the flux through this pathway is high, which results in the fast acidification of the dairy products mentioned earlier. This is a competitive advantage over other micro-organisms. In the anabolism, abilities to produce metabolites like vitamins and amino acids are very limited, and, therefore, these substrates are preferentially taken up from the environment (Mitchell *ea.*, '41; Reiter *ea.*, '62; Burns *ea.*, '63; Otto, '81; Keefe *ea.*, '95). The inability to synthesise amino acids might be due to mutations in some synthetic enzymes (Godon *ea.*, '92; Godon *ea.*, '93). Typically, industrial *L. lactis* subsp. *cremoris* strains require more amino acids for growth than (dairy and non-dairy) wild strains (Ayad *ea.*, '99). The absence of some amino acid biosynthetic pathways in dairy lactococci might be a consequence of their adaptation to milk, 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 their growth and survival. 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 dairy strains (Engels *ea.*, '03).
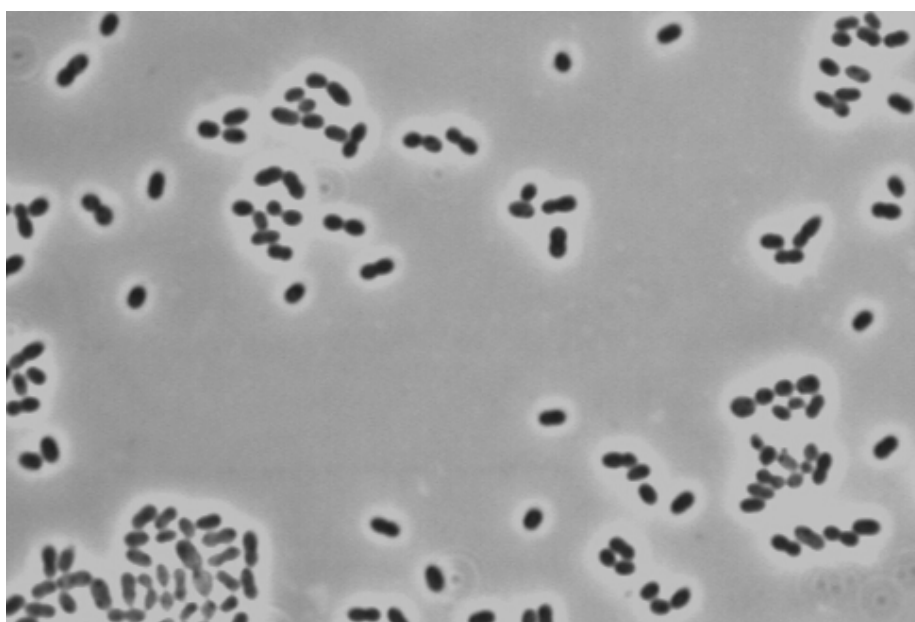

Figure 1.2 Microscopic photograph (100x) of cells of *L. lactis* B1157

The simple metabolic machinery is energetically relatively cheap to maintain, which also results in a competitive advantage for *Lactococcus* over other micro-organisms. On the other hand this limited metabolic machinery limits their occurrence mainly to substrate-rich niches like milk. Still large differences in the metabolism within the species *Lactococcus* exist. *L. lactis* spp. *cremoris* strains, for example, have generally more requirements for growth than strains belonging to *L. lactis* subsp. *lactis* and only strains belonging to *L. lactis* subsp. *lactis* biovar. *diacetylactis* are able to metabolise citrate, because they posses an additional plasmid (Reiter *ea.*, '62; Otto, '81). *L. lactis* is the main species of commercial interest, and is the most frequently used organism in the production of semi-hard type of cheeses, like Gouda, Edam and Cheddar, but is also present in many other cheese varieties. Selection and application of strains that do not have a history in cheese making (wild strains) is an important approach for changing product characteristics. The application of the wild strain *L. lactis* subsp. *cremoris* B1157 in cheese making revealed large differences in the flavour perception of these cheeses (Ayad *ea.*, '00). B1157 will therefore be the main model organism in this thesis, and a microscopic photograph is shown in Figure 1.2.

The protein catabolism in relation to flavour formation by LAB and in particular *Lactococcus* is reviewed in section 1.5; first the identification and appearance of flavour compounds in cheese will be discussed.

1.4. <u>Flavour compounds</u>

The sensory perception of a food is a very important product characteristic. Sensory perception is a complex process, which is influenced by many factors, such as content of flavour compounds, the texture, and appearance of the product, but also several characteristics of the individual consuming the product in a certain environment. Flavour (perception) is defined as the sensation arising from the integration or interplay of signals produced as a consequence of sensing chemical substances by smell, taste and irritation stimuli from food or beverage (Laing *ea.*, '96). Regarding flavour, in the mouth only basic differences like sweet, acid, bitter, salt and unami are sensed by taste-receptor cells, while in the nose many different neurones are able to respond to many different volatile compounds (Yamaguchi, '79; Chauf *ea.*, '90; Brand *ea.*, '94; Laing *ea.*, '96; Ninomiya, '02). Perceiving the flavour of a cheese as positive, or as off-flavour is a very delicate balance between a large number of compounds (Mulder, '52; Bosset *ea.*, '93). In the first instance, studying flavour focuses on the most dominant flavour compounds in a certain product. The dominance of a certain flavour compound depends on the concentration and characteristics of the flavour in regard to the product, but also the characteristics of the subjects sensing the flavour.

A commonly used step in the determination of the dominant compounds of a flavour can be done by GC-O, which is a combination of separation of the compounds by gas chromatography (GC) in combination with analytical identification and human detection of the individual compounds (olfactometry) (Curioni *ea.*, '02). Generally, various dilutions of a product or extract are prepared, analysed on the GC, and detected by the subject. The compounds which are still sensed in the highest dilution (= FD-factor) of the product are regarded key-flavour compounds (Acree *ea.*, '84; Grosch, '93; Mistry *ea.*, '97; Grosch, '01). In order to be able to apply a food product on the GC, the flavour compounds are often extracted or isolated from the product. Several methods have been developed for this purpose, such as direct extraction methods, supercritical fluid extraction, steam distillation (stripping), high vacuum distillation and static as well as dynamic headspace techniques (Curioni *ea.*, '02). The GC-O data generated can be evaluated, using several types of analysis (Curioni *ea.*, '02), which might lead to differences in the interpretation of the data. The method is based on detection of separated flavours in air, and therefore only results on single compounds are obtained. These results contain information about the nature of the flavour and the sensitivity of receptors, but do not include aspects like product characteristics and some other human characteristics influencing the flavour perception.

To meet some of these concerns, the term OAV (Odour Activity Value) was introduced by Grosch and co-workers ('01). The OAV is the ratio between the concentration of a compound in a product and the nasal or retro nasal threshold of the compound, as it is present in the matrix of the product, or a matrix that closely resembles this product (Grosch, '01). In this way, also the matrix interactions are included in determining key-flavour compounds. This approach does not yet take into account the possible interactions between volatiles, that might effect their perception also in concentrations below their threshold (Blank, '97). Although the determination of key-flavours compounds has some flaws, recombination of the key-flavour compounds usually gives a good imitation of the product studied, although the concentrations needed in this mix for similar perception might largely differ from the concentrations in the original product (Blank, '97; Mistry *ea.*, '97).

Several important flavour compounds of several cheeses are shown in Table 1.3. Since not all products were analysed by GC-O, not all flavour compounds may be called key-flavours. The flavour compounds are categorised by the metabolic pathway they are most likely derived from, as will be discussed in the next section. A whole range of other cheese varieties has also been analysed on their flavour compound composition. As an indication the following references are given: Limburger (Parliment *ea.*, '82; Lindsay *ea.*, '86; Urbach, '93), Gruyère (Rychlik *ea.*, '01a; b), Gorgonzola (Moio *ea.*, '00), Mozzarella (Moio *ea.*, '93), Parmigiano (Bosset *ea.*, '93; Qian *ea.*, '02), Grana Padano (Moio *ea.*, '98), Mahón, Fontina, Comté, Beaufort and Appenzeller (Bosset *ea.*, '93).

Table 1.3 Examples of key- and other important flavour components in four types of cheese

Metabolism	Gouda	Cheddar	Camembert	Swiss-type (and Maasdam)
Amino acid	3-Methylbutanal 3-Methylbutanol Methanethiol Dimethylsulphide (DMS) 2-methylpropanol Dimethyltrisulphide (DMTS)	3-Methylbutanal Isovaleric acid Methional Methanethiol DMS DMTS	3-Methylbutyrate 3-Methylbutanal Methional Methanethiol DMS Benzaldehyde	Methional 3-Methylbutanal Skatole
Sugar .	Diacetyl	Propionic acid Diacetyl	2,3-Butanedione	Propionic acid Diacetyl
Fat	Butyric acid Butanon Hexanal Pentanal	Butyric acid Acetic acid 1-Octen-3-one Butanon	1-Octen-3-ol Butyric acid 1-Octen-3-one 2-Undecalactone γ-Decalactone	
Rest & Combined pathways	Ethyl butyrate Limonene	Ethyl butyrate Ethyl hexanoate	Phenylethyl acetate	Ethyl butyrate Ethyl hexanoate Ethyl-3-methylbutanoate Phenylethyl acetate
Reference	(Engels, '97)	(Dacremont *ea.*, '94; Christensen *ea.*, '95; Milo *ea.*, '97; Curioni *ea.*, '02)	(Kubickova *ea.*, '97)	(Zourari *ea.*, '92; Preininger *ea.*, '94; Curioni *ea.*, '02)

As illustrated in Table 1.3 a very important group of flavour compounds in semi-hard cheeses like Gouda are derived from the amino acid metabolism. Several of these compounds such as 3-methylbutanal, 3-methylbutyric acid, methional, methanethiol, benzaldehyde, etcetera are also important flavour compounds of other fermented products like beer, sourdough-bread, dry sausages etc, (Schieberle *ea.*, '91; Perpete *ea.*, '00). Gas chromatographic analysis of the concentrations of key-flavours in cheeses during the ripening, showed that these compounds are formed during this period. For influencing formation, it is also important to realise which sensation arises from the compounds in the route, since several compounds can interact, in regard to the sensation. Knowing the odour threshold, can also lead to more targeted approach in changing the flavour formation. The sensations arising from some relevant flavour compounds and their odour thresholds are given in Table 1.4. Aldehydes like 3-methylbutanal generally have a much lower odour threshold than their corresponding organic acids (3-methylbutyric acid) or alcohol (3-methylbutanol).

Table 1.4 Description of some important key-flavous and their odour tresholds. (Badings, '84; Dunn *ea.*, '85; Griffith *ea.*, '89; Leffingwell *ea.*, '89; Dacremont *ea.*, '94)

Flavour compound	Description	Odour threshold in ppb (μM)	
		Water	Oil
2-Methylpropanal	Banana, malty, chocolate-like	0.1-2.3 (0.002-0.03)	-
3-Methylbutanal	Malty, powerful, cheese	1 (0.01)	13 (0.15)
3-Methylbutanol	Fresh cheese, breathtaking, alcoholic	250 (2.8)	-
3-Methylbutyric acid	Rancid, sweat, cheese, putrid	120-700 (1.2-6.9)	-
Butyric acid	Sweaty, butter, cheese, strong, acid	240 (2.7)	-
Propionic acid	Pungent, sour milk, cheese	$2 \cdot 10^4$ (270)	-
Ethylbutyrate	Fruity, buttery, ripe fruit	1 (0.008)	-
Diacetyl	Buttery, strong	2.3 (0.026)	50 (0.56)
Acetaldehyde	Yoghurt, green, nutty, pungent	15 (0.34)	-
Methional	Cooked potato, meat like, sulphur	0.05-10 ($1 \cdot 10^{-4}$-0.9)	0.2 (0.002)
Methanethiol	"Rotting" cabbage, chees, vegetative, sulphur	0.02-2 ($3 \cdot 10^{-4}$-0.3)	-
Benzaldehyde	Bitter almond oil, character. sweet cherry	350 (3.3)	-
Phenyl acetate	Rough, lily-jasmin with metallic note	-	-

Since flavour perception is very complex in which many flavour compounds play a role in the flavour of a product, and slight changes in the balance may already be noticed by the consumers, regulation of flavour formation is an important topic in food fermentation processes. The work on flavour formation in cheese has two sides: Increasing all, or at least all key-flavours will lead to acceleration of cheese ripening, while increasing one or a few flavour compounds can lead to flavour diversification. This diversification may lead to new varieties of cheese, but may also result in an unbalance in flavour perception, a so-called off-flavour. This dual effect has also been reported for 3-methylbutanal (Dunn *ea.*, '85; Ayad *ea.*, '00). To control flavour formation their formation pathways have to be known. In the next paragraph current knowledge on these (bio-) chemical pathways will be reviewed, and this thesis will deal with expanding the knowledge on some relevant reactions in these pathways.

1.5. <u>(Bio-)chemical routes leading from proteins to flavour</u>

In previous sections, flavour has been introduced as an important product characteristic of the fermented dairy product, cheese. Flavour compounds in cheese arise from the action of enzymes from the rennet(-substitute), the milk, the (secondary) starter and non-starter bacteria, together with non-enzymatic conversions (Walstra *ea.*, '93; Kosikowski *ea.*, '97; Skeie *ea.*, '00). In cheese making, selected starter cultures are of main importance for controlling flavour formation. Most

compounds with a strong flavour impact have a low molecular weight and are found in all parts of the metabolism (sugar, fat and protein) of the starter micro-organisms. In this section, some flavour forming pathways in fermented dairy products will be reviewed after a short introduction.

The main metabolic product of LAB, lactate, originates from sugar metabolism. Although the flavour impact of lactate is limited, the production of lactate leads to a low pH and thereby affects the growth of other (flavour-producing) micro-organisms. Well studied flavour compounds derived from sugar metabolism are diacetyl and propionic acid, which are of major importance for the characteristic flavour of respectively butter and Maasdam / Swiss type cheeses (Hugenholtz *ea.*, '93; Sarkar *ea.*, '95; Thierry *ea.*, '02a). Conversion of fat is particularly extensive in hard Italian varieties, smear-cheeses, and blue mould-veined cheeses and generally leads to characteristic flavour compounds, which can be described as sharp or rancid. Although fat degradation in semi-hard types of cheese is much less prevalent, the process is essential, and cheeses made with skimmed milk do not develop the correct flavour (Ohren *ea.*, '67). (See McSweeney *ea.*, '00; Collins *ea.*, '03 for reviews). The third group of flavour compounds originates from proteins and is important for almost all cheese-varieties.

Flavour forming pathways originating from proteins are combined in Figure 1.3. After a short description of this figure, each part of the route will be reviewed separately. Amino acids are primary needed for protein synthesis, but *Lactococcus lactis* is not able to produce all amino acids from the central metabolism (Fig.1.3 [12, 13]) (Andersen *ea.*, '53; Reiter *ea.*, '62). Certain genes coding for enzymes involved in the amino acid biosynthesis seem to be disrupted (Deguchi *ea.*, '92; Van Kranenburg *ea.*, '02). To obtain all essential amino acids, *L. lactis* is able to take up small peptides and to a lesser extend amino acids from the environment. This uptake can be preceded by extracellular degradation of proteins (proteolysis and peptidolysis, Fig. 1.3 [A – D]) (Konings *ea.*, '89; Smid, '91). Intracellularly most amino acids can be converted by transaminases / aminotransferases [1 in Fig. 1.3] to their corresponding α-keto acids. Other types of deaminating enzymes have not been found in LAB. α-Keto acids are central intermediates, and can be converted to hydroxy acids [2], aldehydes [3] and CoA-esters [8]. These reactions are mostly enzymatic, but some chemical conversion steps have also been described, like the formation of benzaldehyde from phenylpyruvic acid (Nierop Groot *ea.*, '98). The aldehydes formed can generally be dehydrogenated [4], or hydrogenated [5] to their corresponding alcohols or organic acids (Zourari *ea.*, '92; Libudzisz *ea.*, '93; Jensen *ea.*, '01), which are in their turn substrates for esterases and acyltransferases [7], leading to (thio)-esters (Christiani *ea.*, '01). One of the biological roles of these amino acid degrading pathways is the generation of precursors, which are needed for example in the sterol and branched-chain fatty acids synthesis (Oku *ea.*, '88). On the other hand the hydrogenation of the α-keto acids may act as sink for excessive redox potential (NADH). The conversion of amino acids to alcohols via α-keto acids as described

above was first identified for the formation of fusel alcohols (short-[branched-]chain alcohols) in yeast, where it is called the Ehrlich's pathway (Ehrlich, '07).

Another important conversion route of amino acids is initiated by lyases [10], like cystathionine ß-lyase, which is able to convert methionine to methanethiol (Alting *ea.*, '95; Dias *ea.*, '98a; McSweeney *ea.*, '00). Threonine aldolase (EC 4.1.2.5)[9] belongs to another class of lyases, and is able to convert threonine directly to acetaldehyde (Bell *ea.*, '73; Lees *ea.*, '76; Raya *ea.*, '86a; Hugenholtz *ea.*, '00; Ott *ea.*, '00).

A third conversion pathway for amino acids is the deimination/decarboxylation to amines. These reactions are studied intensively, in regard to the health risk of biogenic amines (Joosten, '87; Joosten *ea.*, '87; Leuschner *ea.*, '98). The direct decarboxylation of amino acids explains the presence of most of the amines found, but not the formation of secondary and tertiary amines (Adda *ea.*, '82).
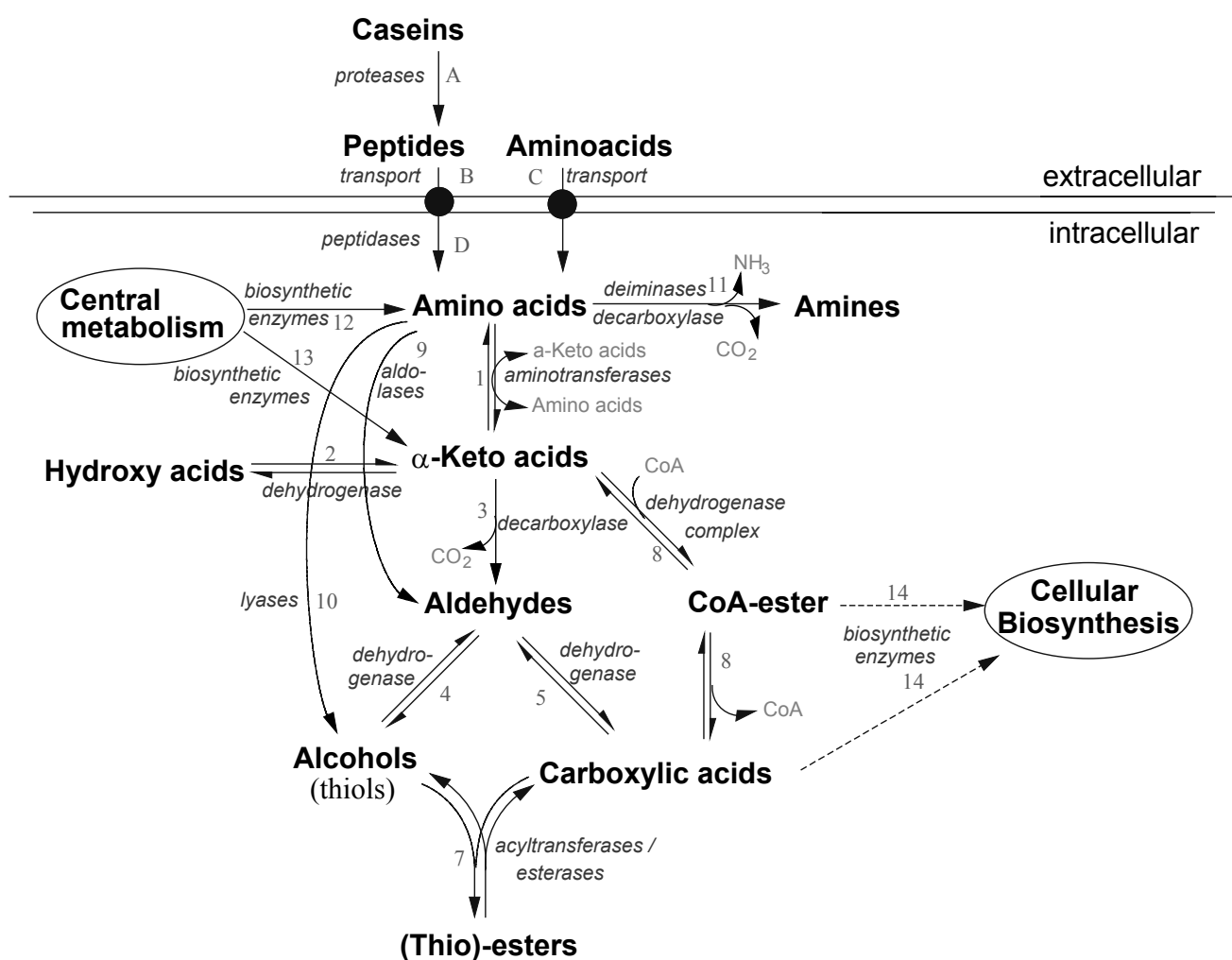

Figure 1.3 Summary of general protein conversion pathways relevant for flavour formation. Numbers and letters besides reactions are used for pointing out these reactions in the text.

The most potent flavour compounds in Figure 1.3 are the aldehydes, alcohols, carboxylic acids and esters (Table 1.3 and 1.4). Especially important are the aldehydes, alcohols, carboxylic acids and esters derived from the amino acids methionine, phenylalanine, threonine and the branched-chain amino acids. The importance of these amino acids for the cheese flavour is a combination of their abundance, their conversion rates, and the odour threshold of the compounds derived from them. Parts of Figure 1.3 will be discussed in detail in the next subsections.

1.5.1. <u>Proteolysis and Peptidolysis</u>

Proteolysis and peptidolysis (Fig. 1.3 [A-D]) constitute to the primary step in protein degradation, and these steps result in free amino acids. The proteolytic system has been studied intensively, and is initiated by a single cell envelope-bound serine proteinase (Prt). The lactococci that lack this protease (Prt $^-$), can only grow in a free amino acid-rich environments, or in protein containing media in co-operation with Prt$^+$-strains (Laan *ea.*, '89; Kunji *ea.*, '96). Two genetically related types of Prt, present in the cell envelope of *Lactococcus lactis* subsp *cremoris* strains are called the HP-type and AM$_I$-type (Exterkate *ea.*, '87; Kok *ea.*, '88; Exterkate *ea.*, '89). The HP-type preferably converts β-casein and appears in two catalytically active forms, the stable PI and relatively unstable PII type (Exterkate *ea.*, '89). The AM$_I$-type (=PIII) also cleaves α$_{S1}$-casein (Visser *ea.*, '86). Another protease, HtrA, is also identified in a *Lactococcus* strain, but this enzyme does most probably only play a role in maturation of secreted proteins (Poquet *ea.*, '00).

Two oligopeptide transport systems and two ABC transporters transport small peptides over the cell membrane (Konings *ea.*, '89; Tynkkynen *ea.*, '93; Detmers *ea.*, '98). Amino acid transporters usually transport structurally similar amino acids (Konings *ea.*, '89; Konings *ea.*, '91). Peptidases, converting the peptides intra- and extracellularly to amino acids (Exterkate, '84), were studied intensively (reviews by Kunji *ea.*, '96; Christensen *ea.*, '99). These peptidases can be divided in aminopeptidases, endopeptidases, di-/tri-peptidases, and proline peptidases. Carboxy peptidases have never been found in LAB. Gene inactivation and gene over-expression studies in cheese model systems have shown that peptidases like PepN, PepW, PepT, PepX, and PepQ play an important role in determining the overall level of amino acids (Guinec *ea.*, '00; Courtin *ea.*, '02). Although peptides and amino acids do have specific flavour characteristics, like sweet, bitter, or malty, (Mulder, '52; Haefeli *ea.*, '90; Lemieux *ea.*, '92; Engels *ea.*, '94), it is generally believed that they only add to the basic taste of cheese. Stimulation or over-expressing of several proteolytic enzymes and also the addition of amino acids to cheese did hardly influence the positive flavour perception of the product (Wallace *ea.*, '97). However, unbalanced proteolysis might lead to excess of bitter peptides, which can lead to decreased cheese flavour perception (Stadhouders *ea.*, '83; Smit *ea.*, '98). The use of selected strains can prevent excess bitterness by breaking down these peptides (Visser *ea.*, '83; Smit *ea.*, '96). The concentrations of amino acids in Gouda and Cheddar are given in Table 1.5. These concentrations are high enough to provide the substrates for successive reactions, and the pattern in concentrations is roughly the same as

the composition of casein (Creamer, '03). All together the conclusion might be drawn that although proteolysis and peptidolysis are important for flavour formation, they are generally not rate-controlling in flavour formation from proteins.

Table 1.5 Free amino acid concentrations (mmol/kg cheese) in Gouda and Cheddar (after 6 months ripening, n.d. = not determined)(Jarrett *ea.*, '82; Joosten, '87).

Amino acid	Gouda	Cheddar	Amino acid	Gouda	Cheddar
Alanine	5.3	5.3	Leucine	23.5	22.8
Arginine	4.1	8.2	Lysine	14.0	14.5
Asparagine	15.4	16.8	Methionine	3.6	n.d.
Aspartic acid	3.3	n.d	Phenyl alanine	9.4	10.5
Cysteine	n.d.	n.d	Proline	3.6	2.9
Glutamine	<0.5	n.d	Serine	3.7	6.6
Glutamic acid	25.1	30.9	Threonine	4.9	6.0
Glycine	4.3	2.85	Tryptophan	n.d.	n.d.
Histidine	2.4	n.d	Tyrosine	4.4	3.9
Isoleucine	5.8	7.6	Valine	11.4	9.3

1.5.2. <u>Transaminase-pathway</u>

Methional, 3-methylbutanal, isovaleric acid, and benzaldehyde are examples of key flavour compounds which are formed by a (initially) similar pathway, which is initiated by a transaminase (AT=aminotransferase). In Table 1.6 specific flavour compounds derived from some relevant amino acids are specified, corresponding to the class of chemical compounds, used in Figure 1.3. The AT-pathway is very important for the formation of many flavours, and not much is known on rate-controlling steps in this pathway in relation to the formation of several flavours by dairy related micro-organisms. In the next subsections individual reactions of this pathway are reviewed.

Table 1.6 (Flavour) compounds derived from amino acids via α-keto acids by the transaminase pathway. Compounds in italic are formed by non-enzymatic reactions.

Amino Acid	Keto acid	Aldehyde	Organic acid	Alcohol (thiol)	Esters (example)
Ile	α-keto-3-methyl-pentanoic acid	2-Methylbutanal	2-Methyl butyric acid	2-Methylbutanol	
Leu	α-ketoisocaproic acid	3-Methylbutanal	3-methylbutyric acid	3-Methylbutanol	Ethyl-3-methylbutanoate
		2-Methylpropanal	2-Methyl propanoic acid	2-Methylpropanol	
Val	α-ketoisovaleric acid	2-Methylpropanal	2-Methyl propanoic acid	2-Methylpropanol	Ethyl isobutanoate
Phe	Phenyl pyruvate	*Benzaldehyde* Phenylacetaldehyde	Benzoic acid Phenylaceticacid	Phenylmethanol Phenylethanol	Ethyl benzoate Phenylethyl acetate
Trp	Indole-3-pyruvate	Indole-3-acetaldehyde	Indole-3-acetic acid		
Met	α-keto methylthio butyrate	Methional	Methylthiobutyric acid	Methionol	Ethyl-3-methylthio propionate
		Methylthio-acetaldehyde		Methanethiol	Methylthioacetate

Transaminase

Transaminases [1 in Fig. 1.3] are widely distributed among micro-organisms, and use pyridoxal-5'-phosphate as cofactor for catalysis. Transaminases catalyse the conversion of an amino acid to its corresponding α-keto acid, and as such transamination is the first step of the amino acid catabolism. The enzymes are also able to catalyse the reversed reaction, and in that role they are the last enzyme in the amino acid anabolism. For the conversion of several types of amino acids, such as the branched-chain amino acids (BcAA), specific transaminases have been identified and characterised (Rollan *ea.*, '89; Gao *ea.*, '98b; Rijnen *ea.*, '99a; Engels *ea.*, '00; Yvon *ea.*, '00). The enzymes, however, have overlapping substrate specificities, which also leads to the conversion of for example methionine, an amino acid for which no specific transaminase has been identified (Jensen *ea.*, '81; Engels, '97; Yvon *ea.*, '01). The BcAA transaminase identified by Engels was stable and active under cheese ripening conditions (Engels, '97). Knocking out the aromatic amino acid transaminase (ArAA-TA) gene, resulted in a lack of Phe-derived flavour compounds in semi-hard cheese, which proved that transamination is essential for the formation of Phe-derived flavours, and also that the aromatic amino acid transaminase is the major Phe-transaminating enzyme (Rijnen *ea.*, '99b).

α-ketoglutarate is generally the preferred amino group acceptor (co-substrate) for transamination reactions. Improving the availability of this co-substrate leads to increased conversion of amino

acids. This was shown *in situ* by increased amino acid conversion in several types of cheese, when adding α-ketoglutarate to the curd (Yvon *ea.*, '98; Banks *ea.*, '01). This increased conversion led to increased concentrations of the α-keto acids, α-hydroxy acids, and carboxylic acids. Besides externally adding the α-ketoglutarate, the availability of α-ketoglutarate could also be increased by introduction of a glutamate dehydrogenase gene from *Peptostreptococcus* in *L. lactis* (Rijnen *ea.*, '00). The introduction of this gene not only resulted in increased transamination, but also flavour active acids, like isovaleric acid were produced in higher amounts (Rijnen *ea.*, '00).

The α-keto acids are central intermediates in the AT-catabolic pathway, and can be hydrogenated to the corresponding α-hydroxy acid, decarboxylated to the corresponding aldehyde, converted chemically, or dehydrogenated / oxidative decarboxylated by a dehydrogenase complex, resulting in the corresponding CoA-ester. The CoA ester can in many organisms be used as building block for several compounds, but can also be converted to the flavour compound isovaleric acid. These conversions are discussed next.

Hydroxy acid dehydrogenase

Hydroxy acids are not major flavour compounds, and are not known as precursors of flavour compounds (Yvon *ea.*, '01). The hydrogenation of keto acids to hydroxy acids [2 in Fig. 1.3], can lead to low α-keto acid concentrations, thereby negatively affecting the flux towards flavour compounds such as aldehydes. The hydroxy acids derived from BcAA's, ArAA's and methionine have been observed in many dairy fermentations (Hummel *ea.*, '85; Gao *ea.*, '98a; Gummalla *ea.*, '99). Several 2-hydroxy acid dehydrogenases, such as lactate dehydrogenase (LDH), hydroxy isocaproate dehydrogenase (HicDH), mandelate dehydrogenase, have been identified and characterised from several LAB (Schütte *ea.*, '84; Hummel *ea.*, '85; Yamazaki *ea.*, '86; Hummel *ea.*, '88; Bernard *ea.*, '94). The best known enzyme is LDH, which substrate specificity is mainly restricted to pyruvate. Several different enzymes are collectively called hydroxy isocaproate dehydrogenase (HicDH), since α-ketoisocaproate is often their preferred substrate. These enzymes have thus, in contrast to LDH, a broad substrate specificity (Yvon *ea.*, '01), and catalyse the stereo specific hydrogenation of α-keto acids, using NADH as hydrogen donor. HicDH enzymes appear in two forms, the D and L form, with the latter being rather unusual. In LAB, hydroxy acid dehydrogenases play an important role in maintaining the intracellular redox balance, by converting the excess NADH from the glycolysis.

Since hydroxy acids are the major amino acid degradation products found in semi hard cheeses made with lactococci (Yvon *ea.*, '98), inactivation of the genes encoding for some of these dehydrogenases could allow a better use of α-keto acids for producing aroma compounds.

Decarboxylase

The decarboxylation [3 in Fig. 1.3] of the branched-chain keto acids results in aldehydes with a malty/chocolate-like flavour and a very low odour threshold. The production of these characteristic malty flavour compounds has only been shown in few strains belonging to the following species: *Carnobacterium piscicola, Lactobacillus casei, L. lactis maltigenes* and "wild" *L. lactis* (Morgan *ea.*, '66; Tucker *ea.*, '67; Miller *ea.*, '74; Weerkamp *ea.*, '96; Ayad *ea.*, '99; Larrouture *ea.*, '99). Most lactococci produce only small amounts of these aldehydes, suggesting that these strains weakly express the decarboxylase gene, that in these strains a less efficient pathway may exist, or that the aldehyde is converted relatively fast to other compounds (Yvon *ea.*, '01).

Despite the interest of many research groups, the decarboxylating enzyme has only been partially characterised. Partial purification based on the methionine and leucine degradation pathway revealed that a thiamine diphosphate (TPP) dependent enzyme is present in *L. lactis* IFPL730. This enzyme has a broad substrate specificity, and has an optimal pH of about 6.3 (Amarita *ea.*, '01). Several α-keto acid decarboxylases with TPP as cofactor have been described in micro-organisms, e.g. pyruvate decarboxylase (PDC, EC 4.1.1.1) (Singer *ea.*, '52), phenylpyruvate decarboxylase (EC 4.1.1.43) (Asakawa *ea.*, '68; Vuralhan *ea.*, '03), branched-chain α-keto acid decarboxylase (BcKAD, EC 4.1.1.72) (Oku *ea.*, '88; Amarita *ea.*, '01), 2-oxoglutarate decarboxylase (EC 4.1.1.71) (Shigeoka *ea.*, '91; Palaniappan *ea.*, '92) and indole-3-pyruvate decarboxylase (IPD, EC 4.1.1.74) (Koga, '95). The most commonly known keto acid decarboxylase is PDC, which plays an important role in glycolysis in many organisms. In the genome of *Bacillus subtilis* ATCC 14579 (Kunst *ea.*, '97) genes for 3 iso-enzymes are found, but in the *L. lactis* SK11 (Weimer *ea.*, '03) and IL1403 (Bolotin *ea.*, '01) genomes no PDC genes are found. In addition to this, the substrate specificity of PDC is generally very narrow, although some papers also report the conversion of α-ketoisovaleric acid by yeast PDC (Yvon *ea.*, '01). The only α-keto acid decarboxylases, identified by homology in the genomes of *L. lactis* IL1403 and SK11 were IPD and 2-oxoglutarate decarboxylase. IPD was purified and characterised from *Enterobacter cloacae* (Koga *ea.*, '92). Like PDC, this enzyme consists of four monomers of approximately 60kDa, and the main difference with PDC is the substrate specificity (Koga *ea.*, '92). Since the major substrate of the partially purified enzyme from *L. lactis* is α-ketoisocaproic acid, the enzyme might be a BcKAD, but this enzyme has totally different structural characteristics than PDC and IPD (Oku *ea.*, '88; Koga, '95). To conclude, only one keto acid decarboxylase has partially been purified from a specific *Lactococcus* strain, and it still remains unclear whether this enzyme is responsible for the formation of 3-methylbutanal by a limited number of LAB.

Alcohol, aldehyde and keto acid dehydrogenases

Hydrogenation of a branched-chain aldehyde by aldehyde dehydrogenase [5 in Fig. 1.3] leads to the corresponding branched-chain organic acid. The reaction equilibrium is close to this organic

acid, and the enzyme uses NAD$^+$ as hydrogen acceptor (Nosova *ea.*, '00). Branched chain organic acids are generally believed to be the substrates for the formation of branched-chain fatty acids. In addition to the decarboxylase in combination with the aldehyde dehydrogenase, a dehydrogenase complex ([8] in Figure 1.3) is most probably present in LAB, which is able to convert the α-keto acid directly to the corresponding organic acid. Although both routes might be active under other conditions, with different rates, the existence of two routes probably indicate that one can be missed, without effect on the growth. This would explain the absence of decarboxylating activity in many LAB. The dehydrogenase enzyme complex performs the oxidative decarboxylation of α-keto acids resulting in the formation of organic acids, without transitory formation of aldehydes. Although this dehydrogenase complex has not been identified or characterised in LAB yet, the reaction proceeds in lactococci (Gao *ea.*, '97; Yvon *ea.*, '98), but also in propionibacteria and micrococci (Yvon *ea.*, '01). A similar dehydrogenase complex for branched-chain α-keto acids has been characterised in *Bacillus subtilis* (Namba *ea.*, '69). This enzyme complex clearly differs from pyruvate dehydrogenase (PDH). The dehydrogenase complex consists of 3 catalytic components, being a keto acid dehydrogenase, dihydrolipoyl transacylase and a lipamide dehydrogenase. The oxidative decarboxylation of α-keto acids by this complex in LAB is also relevant for flavour formation in cheese, since carboxylic acids like isovaleric acid are important flavour compounds (see section 1.4). Furthermore, these carboxylic acids are precursors for other aroma compounds, such as esters, thioesters, cresol and skatole. Cresol and skatole can chemically as well as enzymatically be formed from the amino acids, tyrosine and tryptophan (Yokoyama *ea.*, '81; Urbach, '95).

Alcohol dehydrogenase [4] is identified in most LAB (Morgan *ea.*, '66; Zourari *ea.*, '92; Libudzisz *ea.*, '93; Nosova *ea.*, '00; Jensen *ea.*, '01). Although the reaction equilibrium of this reaction is far to the side of the alcohol, in many fermented dairy products, aldehyde concentrations are stable at relatively high concentrations. This might be explained by the relatively low activity of this enzyme activity in LAB. The flavour intensity of aldehydes is higher than that of their corresponding alcohols (Table 1.4), and therefore this conversion to alcohols might not be favourable, when maximal flavour intensity is desired.

Esterases and acyltransferases

Esters, such as ethylbutyrate, contribute to Cheddar and Gouda flavour, although, an excess of esters in proportion to other flavour compounds could be responsible for the fruity defect of Cheddar (Bills *ea.*, '65). In Camembert, phenylacetaldehyde, 2-phenylethanol and the derived ester phenylethyl acetate, which all result from phenylalanine degradation, are identified in fractions with floral rose-like odour (Kubickova *ea.*, '97), and could cause the pleasant floral note of this cheese (Roger *ea.*, '88). Esters are formed in a reaction between an alcohol and an organic acid [7,8], which also might be activated by coupling to CoA. Besides amino acid metabolism, also sugar and fat metabolism provide substrates for ester formation (Molimard *ea.*, '96; Yvon

ea., '01). Although ester formation in generally considered to be an enzymatic catalysed reaction, the reaction between acetyl-CoA and methanethiol is spontaneous (Helinck *ea.*, '00). Esterases and lipases are serine hydrolases capable of synthesising or hydrolysing esters, depending on the environmental conditions, while alcohol acetyltransferases only catalyse ester synthesis. By knocking out the esterase gene (*est*A) in *L. lactis,* Fernandez *et al*. ('00a) showed that all ester hydrolysing activity in *L. lactis* was lost and that this organism most probably had only one enzyme with esterase activity. Later, this EstA-enzyme has been found to be responsible for the formation of short chain fatty acid esters *in vitro* (Nardi *ea.*, '02). The extrapolation of these data to cheese is not directly possible, since the reaction equilibrium for these kind of esterifications depends strongly on environmental parameters like water activity.

1.5.3. <u>Lyase pathway</u>

Conversion of methionine by LAB can occur via the aminotransferase-initiated pathway as described above, but also via an α,γ-elimination of methionine by the lyase activities of cystathionine β-lyase (CBL) or cystathionine γ-lyase (CGL) (Alting *ea.*, '95; Bruinenberg *ea.*, '97; Dias *ea.*, '98b; Smacchi *ea.*, '98; Fernandez *ea.*, '00b; Fernandez *ea.*, '02). In *Brevibacterium linens* methionine γ-lyase (MGL) plays a central role in the methionine and cystathione metabolism (Dias and Weimer, 1998b). In contrast to the transaminase pathway in which methanethiol is formed in several steps, lyase activity on methionine results directly in methanethiol.

Although cystathionine lyases are active under cheese-ripening conditions (Alting *ea.*, '95; Smacchi *ea.*, '98), their activity towards methionine could not be detected using ^{13}C nuclear magnetic resonance (Gao *ea.*, '98a). 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, where found to be able to degrade methionine, indicating the potential of this enzyme in the production of sulphury flavours. The specificity of CBL (Alting *ea.*, '95) is a particular advantage in this respect, since one might expect that only sulphury flavour compounds will increase in strains with high CBL activity.

The physiological role of CBL is the conversion of cystathionine to homocysteine, which is the penultimate step in the biosynthesis of methionine. This indicates that amino acid converting enzymes (AACEs) can in fact be involved in the biosynthesis of amino acids rather than catabolism only. 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*) (Fernandez *ea.*, '00b), thus genetically linking the methionine and cysteine biosynthesis pathways. The expression of the *metC-cysK* gene cluster is strongly influenced by the amounts of methionine and cysteine in the culture medium (Fernandez *ea.*, '02). 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 most likely very important in the control of flavour forming enzymes in starter cultures and adjunct cultures.

Threonine aldolase (EC 4.1.2.5) belongs to the class of carbon-carbon lyases, and catalyses the conversion of threonine to glycine and acetaldehyde. The latter is an especially important flavour compound in yoghurt. Although several sources for acetaldehyde are known, this lyase pathway contributes largely to the acetaldehyde pool. This has intensively been studied and references suggested for reading are: (Raya *ea.*, '86b; Zourari *ea.*, '92; Beshkova *ea.*, '98; Ott *ea.*, '00).

1.5.4. <u>Non-enzymatic conversions</u>

Although most of the flavour forming reactions are enzymatic (proteolysis, transaminase- and lyase pathway), the α-keto acids of phenylalanine (phenylpyruvic acid) and methionine (KaMet) are (also) non-enzymatically converted to flavour compounds such as benzaldehyde and methylthioacetaldehyde. The existence of the chemical conversion of phenylpyruvic acid was demonstrated by Villablanca *et al*. ('87) and by Nierop Groot *et al*. ('98), the conversion of indole-3-pyruvate by Gao *et al*. ('97), and the conversion of KaMet to methylthioacetaldehyde was suggested by Yvon et. al. ('02). The spontaneous degradation of hydroxyphenylpyruvate to hydroxybenzaldehyde also occurs under simulated Cheddar cheese conditions (Gao *ea.*, '98a), and both benzaldehyde and hydroxybenzaldehyde were found in significant amounts in semi-hard cheeses (Yvon *ea.*, '98). The reaction is catalyses by several divalent metal ions (Nierop Groot *ea.*, '98). The conversion products of indole-3-pyruvate (indole acetic acid, indol-3-acetaldehyde and skatole) have been identified as off-flavours in Cheddar cheese (Gao *ea.*, '97). Besides these descriptions of the occurrence of chemical α-keto acid conversion, hardly any characteristics of this reaction have been published in relation to flavour formation in fermented food products.

Another important chemical reaction leading from amino acids to flavour compounds is the Strecker degradation. Generally the Strecker degradation is described as the reaction of the amino group of an amino acid with an α-dicarbonyl like a reducing sugar, and it is an important step in the Maillard reaction (Hofmann *ea.*, '00; Martins *ea.*, '00). However, at high temperatures also direct oxidative decarboxylation of amino acids can lead to the same aldehydes (Yaylayan *ea.*, '01; Yaylayan, '03). These reactions are especially intense at high temperatures, and therefore contribute largely to the flavour of baked products. Nevertheless, at lower temperatures the reactions have also been shown to proceed, e.g. in the case of cheese and beer production (Dunn *ea.*, '85; Perpete *ea.*, '00). Strecker degradation of leucine results in 3-methylbutanal, but it has

also been suggested that the conversion of valine results in this aldehyde as well (Perpete *ea.*, '00).

These non-enzymatic conversions result in flavour active compounds and therefore these reactions might also be relevant for the flavour formation in cheese. However, more research on the conversions under cheese ripening conditions is desired.

1.6. <u>Outline of this thesis</u>

This thesis focuses on the production of cheese flavour compounds. The main focus is on the production of the branched-chain aldehydes such as 3-methylbutanal and 2-methylpropanal, which can proceed both enzymatically as well as non-enzymatically. As discussed before, 3-methylbutanal and 2-methylpropanal are aldehydes with a low odour threshold (Table 1.4), and have been identified as key-flavour compounds in many types of cheese (Table 1.3). These compounds can be derived from the amino acid leucine, which is the most abundant branched-chain amino acid in cheese (Table 1.5). *Lactococcus lactis* B1157 was used as model organism, to investigate the enzymatic conversion. *Lactococcus* is the main genus in starter cultures for several types of cheeses, including Gouda and Cheddar. Previous work showed that this strain produces considerable amounts of 3-methylbutanal, while many industrial and laboratory strains like *L. lactis* SK110 and NZ9000 do not (Ayad *ea.*, '01). The enzymatic conversion of leucine is initiated by a transamination reaction to the corresponding keto acid (KaLeu), followed by a decarboxylation reaction to 3-methylbutanal. This pathway is similar for the conversion of several amino acids into important flavour compounds, and is therefore an obvious pathway for controlling flavour formation. Focussing on the formation of the aldehydes mentioned above, a more detailed analysis of this pathway was desired.

In Chapter 2 the identification of the rate-limiting step in the enzymatic pathway, a decarboxylating enzyme, is presented. Identification of this enzyme demanded faster methods for analysing 3-methylbutanal. Therefore, a high throughput screening (HTS) method for the detection of volatiles has been developed, which is presented and discussed in Chapter 3. An example shows that this HTS-method not only enables the analysis of more samples per time unit, but can also be used to measure several flavour compounds in the headspace above miniaturised fermentations. The latter application enables the screening of culture collections on the production of flavour compounds. This is very useful in exploiting the natural biodiversity, by selection and subsequent application of strains with specific metabolic properties with regard to flavour formation.

Being able to measure many samples in a short time also enables kinetic measurements on some reactions, like the non-enzymatic conversion of KaLeu. This reaction was identified while

studying the enzymatic decarboxylation of KaLeu. This non-enzymatic conversion resulted in the key-flavour compound 2-methylpropanal in stead of 3-methylbutanal. These results are presented in Chapter 4.

In Chapter 5 the identification of the gene coding for the keto acid decarboxylase is described. The identification was achieved by screening mutant libraries on the production of 3-methylbutanal. Cloning and characterisation of the identified gene and gene product proved that some lactococci have a specific branched-chain α-keto acid decarboxylase which is responsible for the conversion of several keto acids to aldehydes.

Studying the amino acid conversion pathways in more detail can lead to a more focussed and faster approach for controlling cheese flavour development. Since reactions described in this thesis are often not limited to one substrate or one strain, controlling these reactions might lead to a change of the formation of several flavour compounds. Therefore, in chapter 6 the conversions of the amino acids phenylalanine and methionine are discussed. These amino acids can be converted into several flavour compounds by similar pathways as described for the branched-chain amino acids. Furthermore, possible control points for flavour formation in the pathways studied in this thesis are discussed, aiming at possible applications and future research opportunities.

Chapter 2

Diversity of leucine catabolism in various micro-organisms involved in cheese ripening and identification of the rate controlling step in 3-methylbutanal formation

Abstract

Various micro-organisms, belonging to the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Bifidobacterium*, *Propionibacterium*, *Brevibacterium*, *Corynebacterium* and *Arthrobacter*, used in dairy fermentations such as cheese making, were analysed for their potential to convert leucine into flavour compounds, most notably 3-methylbutanal. A large variation between and within species was observed for various enzyme activities involved in the conversion pathway, e.g. transaminases, α-hydroxy acid dehydrogenase and α-keto acid decarboxylase. In particular, α-keto acid decarboxylase activity, leading to 3-methylbutanal, was found to be present in only two of the strains tested. It is proposed that this activity is rate-controlling in the conversion pathway leading to the flavour compound 3-methylbutanal.

Key-words

Lactococcus, Corynebacterium, Lactobacillus, Streptococcus, flavour, leucine, branched-chain aldehydes, decarboxylation, 3-methylbutanal, cheese, aroma.

Introduction

Flavour formation in cheese and other fermented dairy products results mainly from catabolism of milk proteins, sugar and lipids. Depending on the type of dairy product and the micro-organisms used for production, the importance of one or more of these pathways varies (Smit *ea.*, '02b). In semi-hard cheeses, like Gouda or Cheddar, degradation of protein into amino acids and subsequent conversion of amino acids by Lactococcus sp. and/or Lactobacillus sp. is crucial for flavour development (Visser, '93; Smit *ea.*, '00). Other examples of micro-organisms involved in the formation of very diverse flavours in dairy products are Propionibacterium sp. in Maasdammer and Swiss-type cheeses (Sarkar *ea.*, '95), *Streptococcus* sp., *Lb. bulgaricus* spp. and *Bifidobacterium sp.* in (health-promoting) yoghurts (Zourari *ea.*, '92; Nosova *ea.*, '00; Hugenholtz *ea.*, '02), and *Arthrobacter, Brevibacterium* and *Corynebacterium* sp. in surface-ripened cheeses (Bockelmann *ea.*, '01).

Protein degradation in semi-hard type cheeses starts with proteolysis by rennet enzymes and extracellular proteinases from starter lactic acid bacteria (LAB) (Visser, '93). This proteolysis results in peptides that can be taken up by LAB. Intracellularly, these peptides are further hydrolysed to free amino acids, which can subsequently be converted into various flavour compounds (Bockelmann *ea.*, '01; Yvon *ea.*, '01; Van Kranenburg *ea.*, '02). Some important amino-acid-derived cheese flavour compounds are methanethiol from methionine (Engels *ea.*, '94; Engels, '97; Weimer *ea.*, '99), acetaldehyde from threonine (Hugenholtz *ea.*, '00), benzaldehyde from phenylalanine (Nierop Groot *ea.*, '98), and branched-chain aldehydes from the amino acids valine, leucine and isoleucine (Engels, '97; Yvon *ea.*, '01).

The first step in the formation pathway of the flavour compounds mentioned above is the transamination of amino acids to the corresponding α-keto acids. A number of transaminases with different substrate specificities have been described (Yvon *ea.*, '97; Engels *ea.*, '00). These enzymes are reported for several *Lactococcus lactis, Lactobacillus, Streptococcus* and *Propionibacterium* species (Engels *ea.*, '00; Hansen *ea.*, '01; Thierry *ea.*, '02b). The specific activity of these enzymes under cheese-ripening conditions is relatively low, but significant, since cheese ripening is a long process. By adding α-ketoglutarate to the cheese curd, Yvon et al. (Yvon *ea.*, '98) showed that an increase in availability of this co-substrate for transamination led to an increase in the concentration a number of flavour compounds. However, this increase in amino acid degradation by transamination led mainly to an increase in the intermediary α-keto acid concentration.

α-Keto acids are central intermediates in the metabolism of most amino acids (Fig. 2.1) They can for example be (1) transaminated back to the corresponding amino acid, (2) hydrogenated to the corresponding hydroxy acid (Yvon *ea.*, '01), (3) coupled to CoA in the production of isovaleric acid (Thierry *ea.*, '02b) and for further use in cellular biosynthesis, or (4) decarboxylated directly

or indirectly to the corresponding aldehyde by a decarboxylase (Tucker *ea.*, '67; Oku *ea.*, '88; Ter Schure *ea.*, '98; Amarita *ea.*, '01). Upon formation, the aldehyde could be converted to the corresponding alcohol or organic acid. The alcohol is produced by alcohol dehydrogenase (ADH), which has been found to be active in most species used in the present study (Zourari *ea.*, '92; Libudzisz *ea.*, '93; Nosova *ea.*, '00; Jensen *ea.*, '01). Of these compounds, the aldehyde has the strongest flavour impact (Sable *ea.*, '97; Curioni *ea.*, '02).
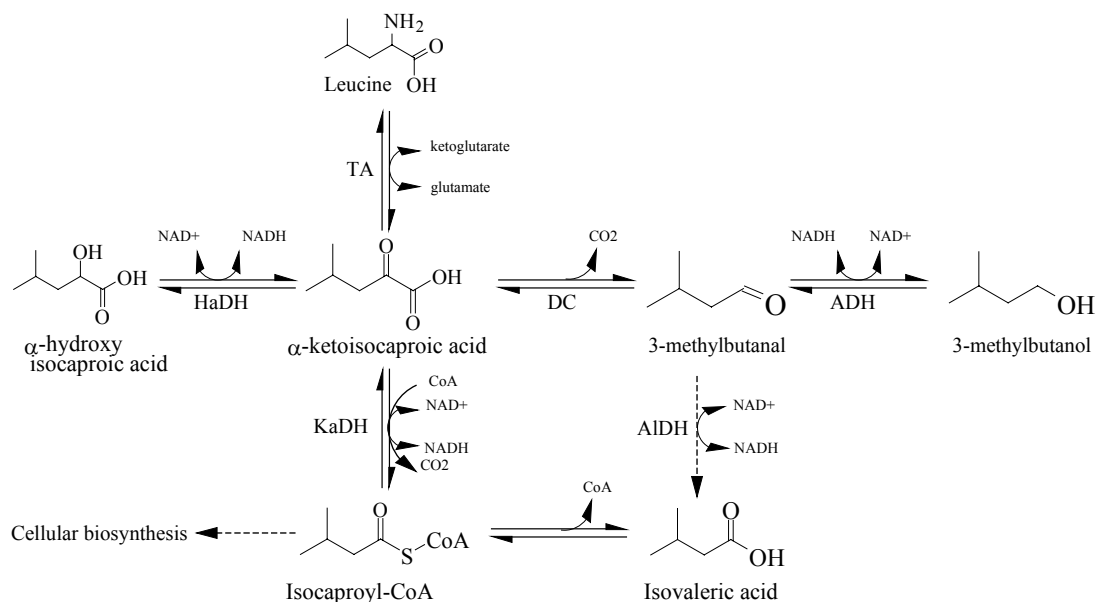
Figure 2.1 Reaction scheme of simplified leucine degradation pathway. TA=Transaminase, HaDH=hydroxy acid dehydrogenase, DC= α-keto acid decarboxylase, ADH=alcohol dehydrogenase, AlDH= aldehyde dehydrogenase, KaDH=keto acid dehydrogenase

In order to generate a stronger flavour, e.g. more aldehydes, it is essential to focus on the rate-controlling step in their formation. This paper focuses on identifying which step in the formation of 3-methylbutanal, the aldehyde derived from leucine degradation, is rate-controlling and how the various enzyme activities involved in its synthesis vary between different strains and species of starter cultures used in cheese making. The observed variation in enzyme activities, together with knowledge of the rate-controlling step in the process, will enhance further developments for flavour diversification and/or acceleration of cheese ripening.

Materials and Methods

Micro-organisms. All micro-organisms were obtained from the NIZO culture collection. The strains used and their growth conditions are summarised in Table 1. Strains were grown in 1 l M17-medium (Difco, Detroit, Mich.) with extra carbon source as indicated. Aerobic cultures were grown in 250 ml portions in 0.5 l Erlenmeyer flasks in a rotary shaker, shaking at 200 rpm. The other cultures were grown in closed flasks without headspace (HS; low oxygen).

Table 2.1 Strains and growth conditions

Strain	NIZO code	T (°C)	Time[a] (h)	O_2[b]	Carbon source
Lactococcus lactis subsp. *cremoris*	B1492	30	20	–	0.5% glucose
L. lactis subsp. *cremoris*	B697	30	20	–	0.5% lactose
L. lactis subsp. *cremoris*	B1157	30	20	–	0.5% lactose
Lactobacillus casei subsp. *casei*	B931	30	20	–	0.5% lactose
Lactobacillus helvetius	B217	37	20	–	0.5% lactose
Lactobacillus acidophilus	B232	37	20	–	0.5% lactose
L. acidophilus	B1221	37	20	–	0.5% lactose
L. acidophilus	B1222	37	20	–	0.5% lactose
Streptococcus thermophilus	B108	37	20	–	0.5% lactose
S. thermophilus	B883	37	20	–	0.5% lactose
Leuconostoc mesenteroides	B177	25	68	–	0.5% lactose
Leuconostoc mesenteroides	B1659	25	68	–	0.5% lactose
Bifidobacterium lacticum	B402	37	20	–	0.5% lactose
Bifidobacterium sp.	B1667	37	20	–	0.5% lactose
Propionibacterium freudenreichii subsp. *shermanii*	B369	30	68	–	1.5% lactate
P. freudenreichii subsp. *shermanii*	B368	30	68	–	1.5% lactate
P. freudenreichii subsp. *shermanii*	B1185	30	68	–	1.5% lactate
Brevibacterium linens	B875	25	44	+	0.5% lactose
B. linens	B1204	25	44	+	0.5% lactose
Corynebacterium ammoniagenes	B1506	25	44	+	0.5% lactose
Corynebacterium variabilis	B1380	25	30	+	0.5% lactose
Arthrobacter nicotianae	B1507	25	44	+	0.5% lactose
A. nicotianae	B1410	25	44	+	0.5% lactose

a) Approximate time for the culture to reach the stationary phase of growth

b) + Aerobic, – anaerobic (closed flask)

Flavour production by micro-organisms. Several samples were taken from cultures up to 72 h of incubation, and the OD_{600} was measured. A 3 ml aliquot of the sample was transferred to a 10 ml HS vial, incubated for 0.5 h at growth temperature, and subsequently stored at –25°C for HS analysis (see below).

Aliquots (50 ml) of cultures in stationary phase were harvested by centrifugation (15 min, 5,000 g, 15°C). Pelleted cells were washed in 25 mM sodium citrate buffer (pH 5.5) and centrifuged again. Finally, the cells were resuspended in this buffer to a final OD_{600} between 15 and 25. Aliquots (1 ml) of these cell suspensions were incubated with two different substrates for 8 h at 30°C in a closed 10 ml HS vial with a total volume of 3.0 ml. The final concentrations in the first set of incubations were 100 mM sodium citrate buffer (pH 5.5), 20 mM leucine, 10 mM α-ketoglutaric acid and 0.25% glucose. The final concentrations in the second set of incubations were 100 mM sodium citrate buffer (pH 5.5), 5 mM α-ketoisocaproic acid and 0.25% glucose. After these incubations, samples were stored at –25°C for HS analysis (see below).

Preparation of cell-free extract. All steps were performed between 0 and 4°C. Cells were grown as described above, and were washed three times in 25 mM sodium phosphate buffer (pH 7.0). Washed cells were resuspended to an OD600 of about 100 in the same buffer and disrupted in a French press twice at 20,000 psi cell pressure (SLM-instruments, Rochester, N.Y.). The resulting suspension was centrifuged (30 min, 31,000 g, 4°C) and the supernatants obtained were used for the enzyme assays described below.

Transaminase activity. Conversion of leucine to α-ketoisocaproic acid was assayed by incubation of different quantities of cell-free extract (CFE) with leucine for 2 h at 30°C. The reaction mixture (300 μl) contained 25 mM potassium phosphate (pH 7.5), 0.5 mM EDTA, 100 μM pyridoxal-5-phosphate, 20 mM leucine and 10 mM α-ketoglutaric acid. After incubation, the reaction was stopped by lowering the pH to between 2 and 3 with 40 μl 0.50 N HCl. The samples were then frozen at −20°C. Before analysis, the samples were thawed and centrifuged in an Eppendorf centrifuge at maximum speed for 15 min. Leucine and α-ketoisocaproic acid concentrations in 50 μl supernatant were determined by C_{18} reverse phase high performance liquid chromatography (RP-HPLC) as described below. Because of the non-linearity of the assay, each assay consisted of a series of diluted CFE for each strain. Only results with α-ketoisocaproic acid concentrations ranging from 100 to 500 μM were taken into account. The assay was performed three times on different days with different solutions and dilutions to obtain statistically justified results. The specific activity was expressed as micromoles of α-ketoisocaproic acid formed per milligram protein in 1 min.

Decarboxylase activity. Conversion of α-ketoisocaproic acid to 3-methylbutanal was assayed by incubation of CFE in a reaction mixture with a total volume of 3.0 ml in a closed 10 ml HS vial at 30°C for 3 h. The reaction mixture contained 50 mM sodium citrate (pH 6.3), 100 μM thiamin pyrophosphate, 500 μM $MgCl_2$ and 20 mM α-ketoisocaproic acid. After incubation, the reaction was stopped by lowering the pH to between 2 and 3 with 70 μl 6.0 N HCl. The amount of 3-methylbutanal was determined by static HS gas chromatography (GC) (see description below). The specific activity was expressed as micromoles of 3-methylbutanal formed per milligram protein in one min. As with the transaminase assay, several independent experiments were performed, with a range of diluted CFE samples.

Hydroxyacid dehydrogenase activity. Conversion of α-ketoisocaproic acid to α-hydroxyisocaproic acid was assayed by incubation of different quantities of CFE in a reaction mixture with a total volume of 300 μl in a 300 μl 96-well plate at 30°C for 30 min, measuring the OD340 every 30 s. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1.1 mM NADH and 10 mM α-ketoisocaproic acid. The specific activity was calculated from the change in OD with time, corrected for the change in the blank without substrate. The specific activity was expressed as micromoles of NADH consumed per milligram protein in one min. Again, a minimum of three independent experiments was performed, with a range of diluted CFE samples.

Incubation of Lactococcus lactis subsp. cremoris B1157 CFE. Two series of incubation mixtures were prepared in 10 ml HS vials. The 5 ml reaction mixture in each vial contained 50 mM sodium citrate (pH 6.0), 100 µM thiamin pyrophosphate, 500 µM $MgCl_2$, 2 mM α-ketoisocaproic acid and 25 or 500 µl CFE of B1157. Series 2 also contained 2.0 mM NADH. The reaction was started by adding the substrate to the vial followed by tightly closing it with a septum and cap. At several time points the incubations were stopped by lowering the pH to between 2 and 3 with 100 µl 6.0 N HCl. After cooling the vial from 30°C to 4°C, 2 ml reaction mixture was withdrawn from the vial and frozen at –20°C for C_{18} RP-HPLC analysis as described below. The HS vials were also frozen at –20°C until static HS GC (see below) was performed. Production of compounds is expressed per milligram of protein.

Protein determination. Protein content of CFE, needed for calculation of the specific activities, was determined by the BCA protein assay reagent kit from Pierce (Rockford, Ill.). A 30 µl sample was mixed with 240 µl work reagent from the kit. After incubation at 37°C for 20 min the OD_{562} was measured. Triplicate measurements were performed independently.

Reversed phase high performance liquid chromatography. Determination of leucine, α-ketoisocaproic acid, α-hydroxyisocaproic acid and isovaleric acid was performed on an HPLC system consisting of two M6000A peristaltic pumps (Waters, Milford, Mass.), a type 680 gradient controller (Waters), a Spectro flow 783 UV detector at 220 nm (Kratos, Ramsey, N.J.) and a wide pore C_{18} 4.6×250 mm RP column preceded by a C_{18} guard column (Bio-Rad, Veenendaal, The Netherlands.). The column was equilibrated with 2% acetonitrile, 0.1% trifluor acetic acid (TFA) in water before applying 50 µl sample. Compounds were eluted at 30°C at a flow rate of 0.8 ml/min using a 20 min gradient from 2% to 12% acetonitrile +0.1% TFA. The column was washed after each run by a 5 min gradient from 12 to 60% acetonitrile +0.1% TFA. Concentrations were calculated by using calibration samples containing α-ketoisocaproic acid and leucine in water ranging from 100 to 500 µM.

Headspace gas chromatography. The equilibrium HS concentration of 3-methylbutanal and 3-methylbutanol was analysed by GC. A 1.0 ml HS sample was injected splitless on the column after 20 min of incubation at 60°C. The sample was concentrated on a Fisons MFA815 cold trap (CE Instruments, Milan, Italy) with the following settings: 300 s splitless time; 91 s waiting before heating the cold trap; –150°C initial temperature and final temperature at injection 200°C. The separation was performed on a GC-8000 top gas chromatograph (CE Instruments), equipped with a CP-SIL 5 CB low-bleed column (30 m × 0.25 mm, film thickness 1.0 µm, Chrompack, Middelburg, The Netherlands) and a flame ionisation detector. The oven temperature was initially 40°C for 5 min, then it was increased by 15°C/min to 150°C and kept at 150°C for 5 min. Inlet and detector temperatures were 250°C and 270°C, respectively. Carrier flow was 1.8 ml/min helium. Calibration was performed by measuring several dilutions of a 3-methylbutanal and 3-methylbutanol mix in water at concentrations ranging from 5 to 500 µM.

Results

3-Methylbutanal is a key flavour compound in (semi-)hard cheeses like Proosdij and Parmesan (Curioni *ea.*, '02). The formation of this flavour compound was studied to examine the enzymes involved in its formation/degradation and to study the diversity of various dairy-related bacteria with respect to their ability to produce it by transamination followed by decarboxylation. The actual conversion by some of the strains tested during growth on M17 broth is shown in Figure 2.2 by the sum of the concentrations of 3-methylbutanal and 3-methylbutanol, from which the latter is formed by a subsequent hydrogenation of 3-methylbutanal.
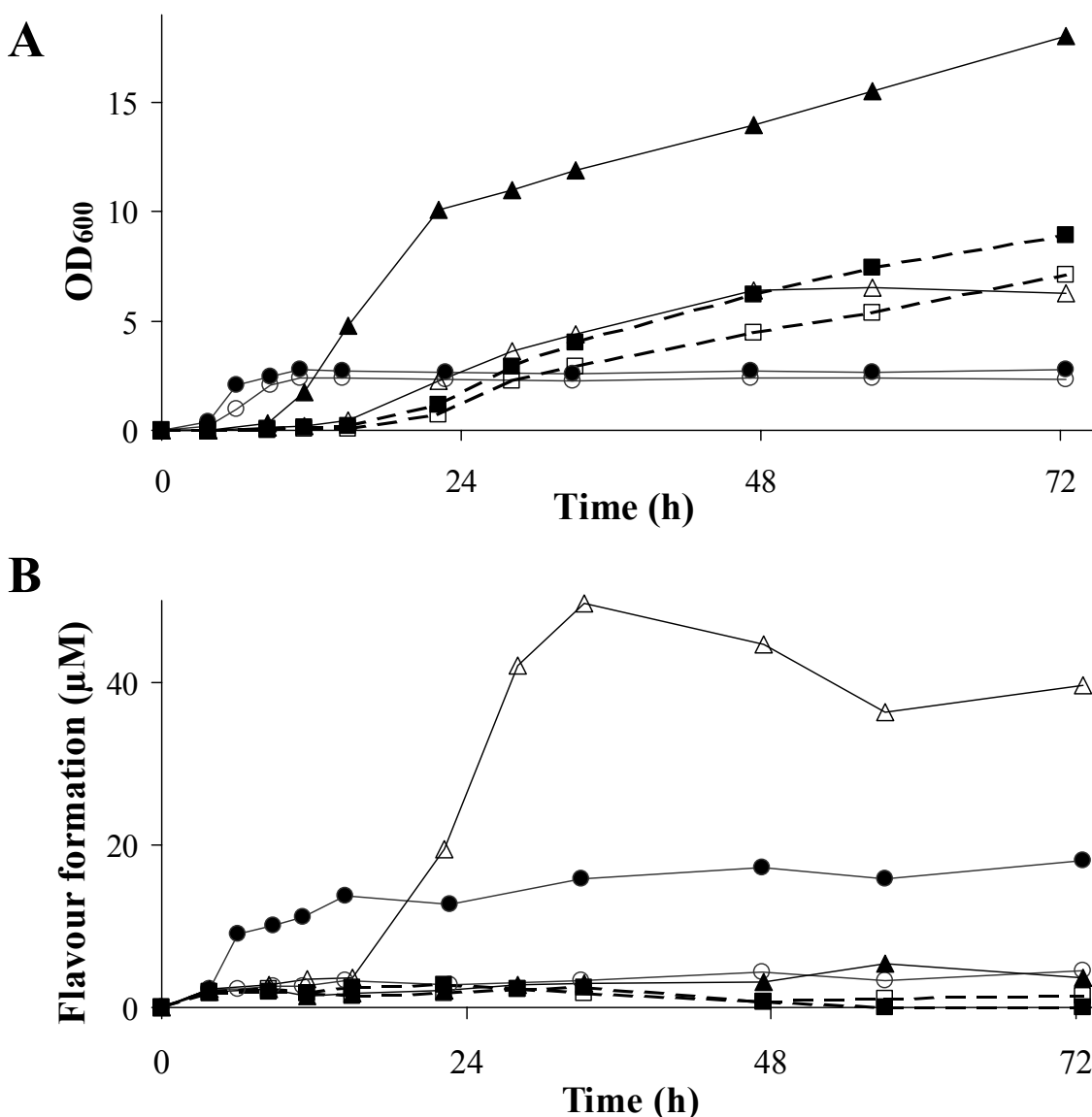
Figure 2.2 Growth (A) and flavour production (B) of six strains with distinct flavour (3-methylbutanal + 3-methylbutanol) production levels. Lactococcus lactis subsp. cremoris strains B697 (○) and B1157 (●), Corynebacterium ammoniagenes strains B1380 (▲) and B1506 (◊), and Arthrobacter nicotianae strains B1410 (□) and B1507 (■)

The production of isovaleric acid from 3-methylbutanal was not taken into account because this compound can also be produced directly from isocaproyl-CoA (Fig. 2.1)(Thierry *ea.*, '02b). *Corynebacterium ammoniagenes* B1506 and *Lactococcus lactis* B1157 were able to produce considerable amounts of 3-methylbutanal and 3-methylbutanol, while all other strains tested, including those closely related to the two above, lacked this ability.

To mimic the situation in cheese more closely, flavour formation by stationary-phase cells at pH 5.5 was studied. Washed cells from a stationary phase culture were incubated in two ways: with (1) leucine + α-ketoglutarate, and (2) α-ketoisocaproic acid. In both cases the pH was set at 5.5 and 0.25% glucose was added to energise the cells and to create reduction potential (NADH production). Also under these test conditions, only B1157 and B1506 were able to produce 3-methylbutanal and 3-methylbutanol. Addition of leucine and α-ketoglutarate resulted in about 200 and 80 μM of the flavour compounds, respectively, while 5-times higher flavour concentrations were found when α-ketoisocaproic acid was added. Isovaleric acid levels from incubations with leucine were similar for B1157, B697, B1506 and B1380, indicating that the majority of this compound is probably formed by the keto acid dehydrogenase pathway (data not shown). Apparently, the ability to convert leucine effectively into 3-methylbutanal and 3-methylbutanol is not a general property among cultures present in various dairy products.

Potential rate-controlling steps in 3-methylbutanal production were identified by measuring the individual enzyme activities involved in 3-methylbutanal formation. The proposed biochemical reactions are presented schematically in Figure 2.1.
First, the activity of branched-chain transaminase was measured by incubating CFE with leucine and an excess of the co-substrate, α-ketoglutarate. The transaminase activity was found to be present in all micro-organisms tested (Fig. 2.3A). The highest variation was observed in the Lactobacillus genus. *L. lactis* spp. and Streptococcus thermophilus showed the highest specific transaminase activity towards leucine and therefore potentially produce the highest concentrations of α-ketoisocaproic acid, which is the substrate for the formation of 3-methylbutanal.
Next, the hydrogenation of α-ketoisocaproic acid to the corresponding hydroxy acid (α-hydroxyisocaproic acid) was tested. This reaction is catalysed by an NADH-dependent enzyme, and it converts the substrate for the production of 3-methylbutanal into α-hydroxyisocaproic acid, a compound with hardly any flavour (Weimer *ea.*, '99; Helinck *ea.*, '00; Yvon *ea.*, '01). The hydroxy acid dehydrogenase varied strongly between species and also within species (Fig. 2.3B). In most anaerobic strains belonging to the genera *Lactococcus, Lactobacillus, Streptococcus* and *Leuconostoc*, specific hydroxy acid dehydrogenase activity was found to be 5- to 10-fold higher than the transaminase activity.
The decarboxylation of α-ketoisocaproic acid leads to the formation of 3-methylbutanal (Fig. 2.1). This decarboxylase activity was found in only two of the stains tested (B1157 and B1506) (Fig. 2.3C).
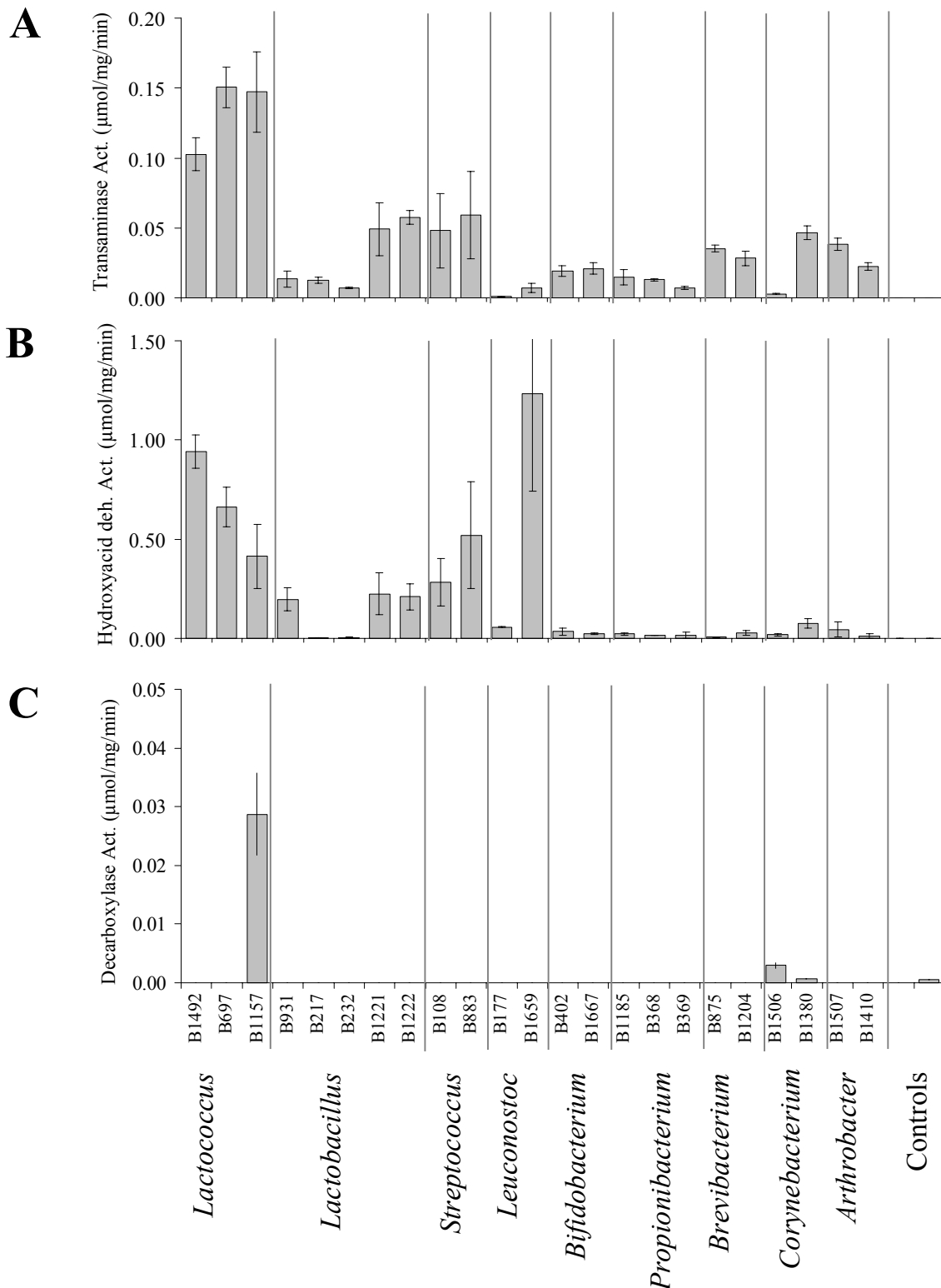
A

B

C

Figure 2.3A–C Specific enzyme activities of tested strains grouped by (sub)species. A) Transaminase activity, B) hydroxyacid dehydrogenase activity, C) keto acid decarboxylase activity. Error bars indicate the standard deviation of three independent measurements.

To study competition between hydroxy acid dehydrogenase and keto acid decarboxylase in more detail, CFE of *L. lactis* subsp. *cremoris* B1157 was incubated with α-ketoisocaproic acid in the presence and absence of NADH. These two cases mimic situations of relatively high and low

reduction potential in the micro-organism. LAB have excess reduction potential during growth. In neither case was 3-methylbutanol and 3-methylbutyric acid formation detected, and the sum of all compounds was about 1.7 mM during the whole experiment, indicating that no major leaks, e.g. as a result of the dehydrogenase complex, were present in the system. In the presence of NADH, a rapid decrease in α-ketoisocaproic acid and NADH concentration was accompanied by a rapid increase in α-hydroxyisocaproic acid concentration until most of the α-ketoisocaproic acid was converted to α-hydroxyisocaproic acid (Fig. 2.4A-B).
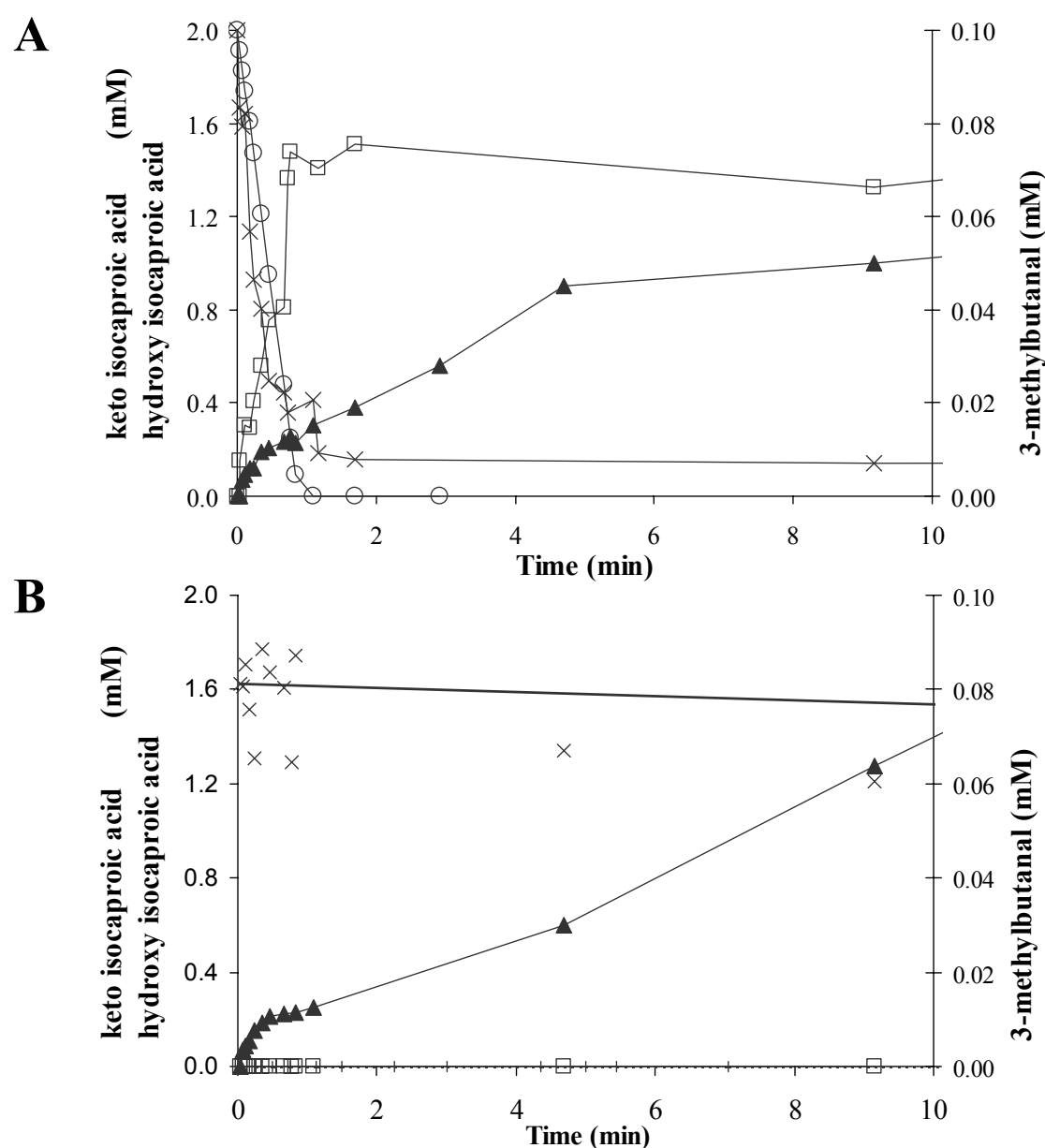
Figure. 2.4 Conversion of α-ketoisocaproic acid (×) to the corresponding hydroxy acid (□) and to the flavour component 3-methylbutanal (▲) in the presence (A) and absence (B) of NADH (○) by cell-free extracts (CFE) of *L. lactis* subsp. *cremoris* strain B1157.

3-Methylbutanal was produced slowly under these conditions, and the production rate decreased after most of the α-ketoisocaproic acid was converted to α-hydroxyisocaproic acid, resulting in a maximum of approximately 50 µM 3-methylbutanal after 25 min (data not shown). In the absence of NADH, the α-ketoisocaproic acid concentration was almost constant, and 3-methylbutanal was produced to a final concentration of approximately 100 µM. These results indicate that competition for the availability of α-ketoisocaproic acid occurs between the hydroxy acid dehydrogenase and the keto acid decarboxylase, with the former activity being dominant.

Discussion

Among the dairy-related bacteria tested, a large biodiversity exists in the activity of enzymes involved in the pathway leading to the flavour compound 3-methylbutanal. For this specific route, both the difference within and between species is large. In fact, intra-species variation is in many cases larger than that between species. Due to this large biodiversity, selection of specific strains from a culture collection will be a useful approach for the development of new starter cultures. To be able to target this selection, more data about the rate-controlling step in this route is desired.

Protein degradation by proteolysis and peptidolysis leads to small peptides and free amino acids. The effect of this primary protein catabolism on flavour formation has been well studied (Igoshi, '86; Exterkate *ea.*, '90). The absence of proteolytic bacteria decreases the flavour perception of the product, while enhancing proteolysis may lead to the accumulation of bitter peptides and amino acids (Smit *ea.*, '98). Addition of selected amino acids to the cheese also did not result in enhanced flavour development, indicating that these conversions are not rate-limiting (Wallace *ea.*, '97).

The amino acid leucine is subsequently converted by a transamination reaction to α-ketoisocaproic acid, which is a central intermediate in amino acid catabolism. All the strains tested possessed this activity, although activities varied between strains, and was found to be very low in strain B177. Transamination can be stimulated in situ by adding α-ketoglutarate to the cheese curd, resulting mainly in increased accumulation of α-ketoisocaproic acid and α-hydroxyisocaproic acid (Yvon *ea.*, '98). The latter is formed by the direct hydrogenation of the α-keto acid. The accumulation of these compounds indicates that the main conversion under these conditions, as in standard cheeses, is conversion towards the hydroxy acid. Apparently, this conversion is slower than transamination since keto acids can still be measured in fair quantities (Yvon *ea.*, '98). However, in most of the strains tested, hydroxy acid dehydrogenase activity was higher than transaminase activity, indicating that large differences exist between conversion potential (specific activities of the strains) and actual conversion. In this case, this difference is most probably caused by a lack of reducing potential (NADH), leading to a decrease in dehydrogenase activity because, under cheese ripening conditions, the cells are no longer

growing. Because α-ketoisocaproic acid is converted mainly to α-hydroxyisocaproic acid in standard cheeses, this might limit other reactions that use α-keto acids as substrate, such as decarboxylation to 3-methylbutanal. A high hydroxy acid dehydrogenase activity is therefore probably not favourable for 3-methylbutanal production. Selection of strains without this activity could therefore be an interesting approach for enhancing flavour formation during fermentation of dairy products (Yvon *ea.*, '01). In line with this, *L. lactis* subsp. *cremoris* B697, a strain with a high hydroxy acid dehydrogenase activity compared to the transaminase activity (Fig. 2.3A, B), did not produce detectable amounts of 3-methylbutanal. Interestingly, *L. lactis* subsp. *cremoris* strain B1157, a strain reported to produce high levels of 3-methylbutanal (Ayad *ea.*, '99; Ayad *ea.*, '00), has transaminase and hydroxy acid dehydrogenase activities approximately similar to those of strain B697. The latter result indicates that the hydroxy acid dehydrogenase activity does not completely out-compete the decarboxylation reaction leading to 3-methylbutanal, even though specific decarboxylase activities were rather low compared to hydroxy acid dehydrogenase activities (Fig. 2.3).

In conclusion, the accumulation of α-ketoisocaproic acid should allow the decarboxylation to 3-methylbutanal (despite its low reaction rate) to proceed. Nevertheless, only two strains were found to be able to produce 3-methylbutanal or the reduced aldehyde, 3-methylbutanol. This 3-methylbutanal production clearly correlated with the presence or absence of decarboxylase activity. Again the difference between *L. lactis* B1157 and B697 is illustrative (Fig. 2.3), with B1157 being decarboxylase-positive and B697 negative. We therefore conclude that this step is critical in controlling 3-methylbutanal production by the strains tested. Testing for decarboxylase activity will therefore be the most obvious approach to increase the rate of aldehyde production.

The sum of 3-methylbutanal and 3-methylbutanol was taken as a measure of decarboxylation activity in this article, because the sum of these compounds gives the clearest presentation of the enzyme activity. For most of the organisms tested, it is known that ADH is present and active at low activities (Libudzisz *ea.*, '93; Nosova *ea.*, '00; Jensen *ea.*, '01). This could also be seen from the data: in *L. lactis* subsp. *cremoris* B1157 about 80% of the flavour formed in the incubation experiment was 3-methylbutanal, while for *C. ammoniagenes* B1506 this percentage was about 20% (data not shown).

Taken together, our results indicate that various strains and species used in the dairy industry vary significantly in the enzyme activities involved in the conversion of the amino acid leucine into flavour compounds like 3-methylbutanal. A rate-controlling step in the formation of 3-methylbutanal appears to be the decarboxylase activity, which was found to be present in only a few of the strains tested. Leucine degradation was taken as an example, but the specificity of the various enzymes involved in flavour formation varies. Control of cheese flavour formation by influencing enzymes with narrow substrate specificity might lead to a change in only one or a few flavour compounds, while influencing enzymes with a broad substrate specificity might lead to differences in several flavour compounds, or a whole flavour block. A better understanding of the essential enzyme activities needed for flavour formation and the potential of various strains in this respect is very important for selection of new starter cultures. Such new cultures can be used

for flavour diversification as well as the acceleration of the ripening process. Because decarboxylase is found to be of critical importance, future work will focus on the characterisation of the decarboxylase enzyme(s). The gene(s) for the decarboxylase(s) has not yet been identified but based on homology; several decarboxylase genes have been identified in *L. lactis* genomes. The knowledge obtained will be used in studying conversion pathways leading to other important flavour compounds and in developing high-throughput screening methods for fast and accurate selection of cultures with specific biochemical steps in the routes leading to flavour compounds. This is essential in order to apply these results in practice.

Chapter 3

Development of a high throughput screening method to test flavour-forming capabilities of anaerobic micro-organisms

Abstract

A commonly used method to control flavour formation in fermented food products is the selection of bacterial strains, which are able to produce the desired flavour compounds. Since large collections of strains are available for such screenings, studying biodiversity of micro-organisms on the level of metabolic routes is strongly facilitated by highly automated high throughput screening methods for measuring enzyme activities or production of metabolites. In this article, we present a HTS method based on measuring volatile metabolites by direct-inlet mass spectroscopy (DI-MS).

The method is based on the injection of a small headspace sample (100μl) from culture vials in a newly developed 96-well sample tray directly into the MS. In one day over 1500 samples could be analysed. The coefficient of variation for the response was less than 5%.

The effectiveness of the method is illustrated by screening of 72 strains belonging to the genus *Lactococcus* in quadruple on the production of the key-flavour compound 3-methylbutanal. Furthermore, knowledge of the biochemistry and physiology of 3-methylbutanal formation was used to optimise the composition of the growth medium to enhance 3-methylbutanal production, and thereby improve the screening. This method will be a useful tool for selecting flavour-producing strains and enhance starter culture development.

Key-words

High Throughput Screening (HTS), Direct-Inlet Mass Spectrometry, volatile compounds, flavour, 3-methylbutanal, *Lactococcus*.

Introduction

A well balanced flavour contributes highly to the perception of a food product. In fermented foods metabolic activity of the micro-organisms results in the formation of various flavour compounds. As the flavour formation capacity is strain dependent, the choice of starter cultures determines the flavour of the product. Careful screening of various culture collections is an important way for the selection of strains that control and direct flavour formation in fermented foods (Van den Berg *ea.*, '93; Smit *ea.*, '02b). This selection is usually based on the overall organoleptic properties of food produced with the organisms tested in pilot experiments (Ayad *ea.*, '00; '01), but as more knowledge on metabolic pathways becomes available, more specific selection methods are developed. These methods may rely on a specific property, such as specific enzyme activities or production of a specific volatile flavour compound. In order to screen large numbers of strains for these properties there is an increasing need for high-throughput screening (HTS) methods.

Many (HTS) enzyme assays are based on (indirect) colorimetric or fluorimetric measurements of product formation or substrate consumption during an *in vitro* reaction (Lavery *ea.*, '01; Cooper *ea.*, '02). Using *in vitro* systems often yields conversion potentials of an enzyme of choice at optimal pH, substrate concentration and cofactor availability, rather than actual *in vivo* conversion capacities. In a number of cases one would be interested in measuring volatile (flavour) metabolites directly. This is especially true for flavour formation routes, which are rather complex pathways comprising many enzymes. These pathways are often regulated and are desired to be active at unfavourable fermentation conditions such as low pH, high salt concentrations and low water activity. Therefore, the conversion in (miniaturised) fermentations is often more important than the conversion potentials of flavour forming micro-organisms under optimal conditions.

Most of the techniques for measuring volatile compounds presented in literature do not combine all characteristics needed for this kind of screenings, e.g., fast, small sample volumes and automation. For instance, fast-GC combines the specificity of chromatography and mass spectrometry in typical analysis times of 5 minutes (Klee *ea.*, '97; Lehotay *ea.*, '02). Mass spectrometry itself is fast, and is for example already in use for the on-line monitoring of flavour release, analysing air quality and for analysis of flavour quality or origin of production (Wong *ea.*, '95; Taylor *ea.*, '96; Pérès *ea.*, '02; Pillonel *ea.*, '03). However, most fast analysis methods are either not intended for headspace analysis or not for very small sample volumes. In this article we describe the development of an automated direct inlet mass spectrometric method for high throughput screening. The method is based on static headspace measurements in the standardised 96-well format, leading to simple and robust analysis of large amounts of microbial cultures on release of volatile compounds.

The measurement of the flavour compound 3-methylbutanal was chosen as an illustrative example for the HTS method. Branched-chain aldehydes like 3-methylbutanal are important flavour compounds in several fermented foods, e.g., cheese, bread and beer (Schieberle *ea.*, '91; Engels *ea.*, '97; Perpete *ea.*, '00; Curioni *ea.*, '02). Their formation pathway in dairy products has been well established. (See for reviews McSweeney *ea.*, '00; Yvon *ea.*, '01; Smit *ea.*, '02b; Van Kranenburg *ea.*, '02). Initially branched-chain amino acids are released from milk proteins by concerted action of proteases and peptidases (Kunji *ea.*, '96; Smit *ea.*, '00). Subsequently, the branched-chain amino acids are converted to the corresponding α-keto acids by amino transferases (transaminases). The α-keto acids can be decarboxylated in odour active aldehydes, dehydrogenated to the corresponding organic acids or hydrogenated to the corresponding hydroxy acids which have a minor flavour impact (Klein *ea.*, '01; Yvon *ea.*, '01; Smit *ea.*, '03). In this paper we focus on the production of 3-methylbutanal by lactococci, in order to identify strains with high production levels.

Materials and Methods

Detection of volatiles with direct-inlet MS (DI-MS). A 100-300 μl headspace sample of 30-50° was sampled by a Combi-PAL auto sampler (Interscience, Breda, The Netherlands) from stainless steel 96-well blocks (see below) or 10 ml headspace-vials (HS-vials). The sample was injected with 10-30 μl/s into a Trace GC (Interscience) equipped with 9 m x 0.1 mm deactivated silica column (Interscience). The column characteristics don't lead to separation of the sample into separate peaks, but to flow restriction in order to maintain the high vacuum of the MS. Helium was used as carrier with a constant column flow of 1-5 ml/min. The split ratio was 1:10 to 1:2 and temperature of the oven was set to 150°C constantly. Detection of the compounds was done with a Quadrupole Trace MS (Interscience) in single ion recording (SIR) mode sampling at a rate of 3/s. A calibration curve was prepared by analysing 10 ml HS-vials containing 3 ml calibration mixtures. These mixtures were prepared by adding 3-methylbutanal to M17 broth (Difco, Detroit, USA) with concentrations ranging from 5 to 500μM.

Organisms and Media. All organisms used belonged to the species *Lactococcus lactis*, and were obtained from the NIZO culture collection. To prepare the stocks, the strains were grown for 16 hours in a 96-well micro plate, containing 100 μl GM17 broth. GM17 was prepared by adding 20% glucose stock to M17 broth (Difco, Detroit, USA) to a final concentration of 0.5%. After growth 100 μl 60% glycerol was added and the micro plates were frozen at −80°C. The other media used were: GM17 + Leu, which was GM17 with addition of 5 mM filter-sterilised α-ketoglutarate and 5 mM L-leucine, and GM17 + KaLeu, which was GM17 with addition of 2 mM filter-sterilised α-ketoisocaproic acid (KaLeu).

Growth and screening of strains in a 96-well format. Strains were grown on the different media for 40 h at 30°C in a 96-well format stainless steel block. The 96-well block contained wells of 2 ml filled with 1 ml medium. The wells were closed airtight with a Teflon sheet (0.13 mm PTFE; Eriks, Alkmaar, The Netherlands), a 4 mm thick silicon sheet (60 g Shore A; Van de Berg, Barendrecht, The Netherlands) and a stainless steel cover (Fig. 3.2). 3-Methylbutanal production was determined by Direct Inlet MS (DI-MS) as described above and the optimal parameters are described in the results. Growth was measured after the screening by measuring optical density at 600 nm (OD_{600}).
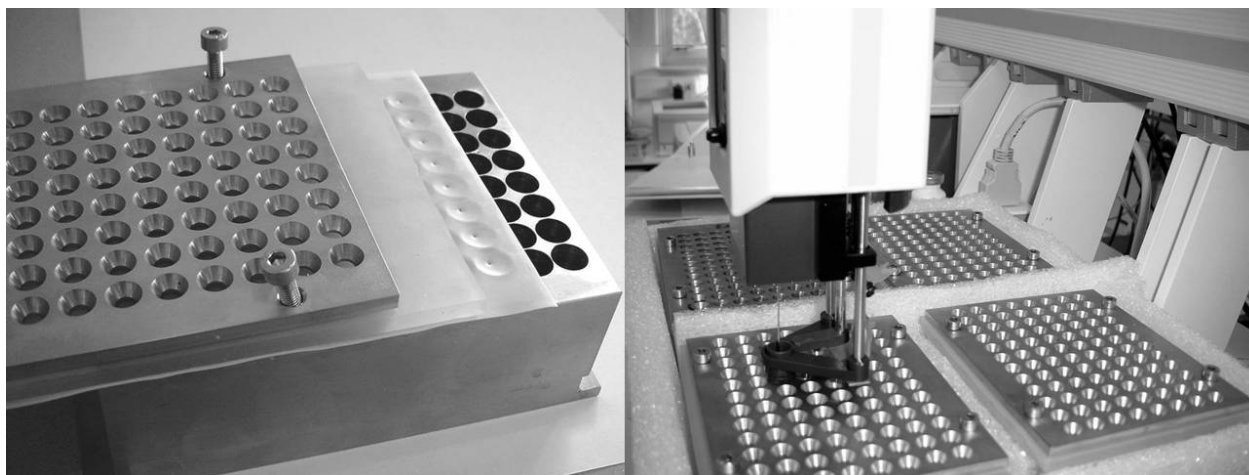
Figure 3.1. Photographs of the newly developed sample tray. Left the stainless steel block with Teflon and silicon sheets, covered with a stainless steel lid. Right sampling of the stainless steel blocks by a Combi Pal autosampler.

Control of the suitability of *m/z*=58 for the screening on 3-methylbutanal in microbial cultures with headspace GC (HS-GC-MS). *Lactococcus lactis* B1157 was grown in 3 ml GM17 and GM17 + KaLeu for 40 h at 30°C in 10 ml HS-vials. Analysis of the headspace was performed by using an GC-MS (Interscience). After heating the HS-vials at 60°C for 20 min, a 1.0 ml headspace sample was taken, and injected in the GC. The split ratio was 1:8. The separation was obtained with a CP-SIL 5 CB Low-bleed column (60 m x 0.32 mm, film thickness: 1.0 µm, Chrompack, Middelburg, The Netherlands). The oven temperature was initially 40 °C for 5 min, and then it was increased by 25 °C/min to 250 °C and kept at 250 °C for 5 min. Carrier flow was 1.5 ml helium per min. The total ion count (TIC, *m/z*=25-300) was acquired at a rate of 2 data points per second.

Control of the screening by headspace gas chromatography (HS-GC-FID). Four 3-methylbutanal-producing strains (B1157, B1231, B1236, B1152) and 4 non-producing strains (B40, B698, B78, B234) were grown in triplicate in 3 ml GM17 + KaLeu for 40 h at 30°C in 10 ml HS-vials. After growth, the vials were heated for 20 min at 60°C. A 1.0 ml headspace sample was injected splitless. The sample was concentrated on a Fisons MFA815 cold trap (CE

Instruments, Milan, Italy) with the following settings: 300 s splitless time; 91 s waiting before heating the cold trap; -150°C initial temperature and final temperature at injection 200°C. The separation was performed on a GC-8000top GC (CE Instruments), equipped with a CP-SIL 5 CB Low-bleed column (30 m x 0.25 mm, film thickness: 1.0 µm, Chrompack) and a flame ionisation detector (FID). The oven temperature was initially 40 °C for 5 min, then it was increased by 15 °C/min to 150 °C and kept at 150 °C for 5 min. Carrier flow was 1.8 ml helium per min. Calibration was performed by measuring several dilutions of a 3-methylbutanal in M17 broth with concentrations ranging from 5 to 500µM.

Results

Objectives of the method

High throughput screening of culture collections on their capability to produce specific volatile compounds can be a valuable tool for selecting bacterial strains with specific improved flavour forming characteristics in fermented food products. To be able to perform such screenings efficiently, the following objectives were set: 1) it should be possible to use standard formats, like the 96- or 384-well format; 2) the applied headspace sample volume should be below 0.5 ml (to prevent vacuum in the sample); 3) The total time of analysis should be below 1 minute; 4) the detection limit should be below 5 µM; 5) the method should be robust for high numbers of samples and 6) the method should meet all requirements for standard headspace sampling, like the possibility of heating the samples and prevention of adhesion of flavour compounds to the equipment used.

Direct Inlet MS for detection of volatiles in headspace samples

Mass spectrometry was chosen, because it is a sensitive and fast method for detection of selected masses of fragmented molecules. Direct inlet refers to the direct injection of a sample in the MS, without separation of the sample by GC first. To maintain a stable vacuum in the mass spectrometer the inlet flow had to be restricted. Connecting a GC equipped with 9 meters of a deactivated GC column with a diameter of 0.1 mm was found to be a convenient way to obtain a pre-column pressure of about 75 kPa in combination with a column flow of 2 ml min^{-1}. A 10 ml HS-vial, filled with 3 ml distilled water containing 100 µM 3-methylbutanal was used for optimisation of the method. Since injection of a sample directly into the MS does not result in separation, or retention of the compounds present in the sample, the response of the MS almost instantaneously increases to a constant level proportional to the concentration of the selected compound in the carrier flow (Fig. 3.2). A small split flow was used to rinse the system after injection, leading to sharp and reproducible responses. The effect of variation of a number of relevant parameters is shown in Figure 3.2. An increase in the speed of injection (sample flow) of 50%, results in an increase in the response with 50%, however it was not possible to maintain a constant column flow in the system at high injection speeds. Decreasing the carrier flow (by

decreasing the pre-column pressure by 32%) results in an increase of the response of 35%. The system however is less stable if small carrier flows are used, and small carrier flows are unfavourable with regard to the split ratio. Increasing split ratios results in lower responses due to dilution of the sample. Decreasing the injection volume only leads to a shorter time the response can be measured. The combination of a split flow of 12 ml min^{-1}, injection with 30µl sec^{-1} and a column flow of 2.5 ml min^{-1} appeared to be optimal for the system used. With these settings, injection of 100µl headspace samples and an acquisition time of 30 s were sufficient for reproducible results. All together, the time for a complete analysis was as short as 55 s.

The optimal incubation temperature was found to be 45°C. Higher temperatures are favourable for the amount of compound present in the headspace, but at temperatures higher than 45°C, the water vapour concentration in the headspace started to disturb the measurements.
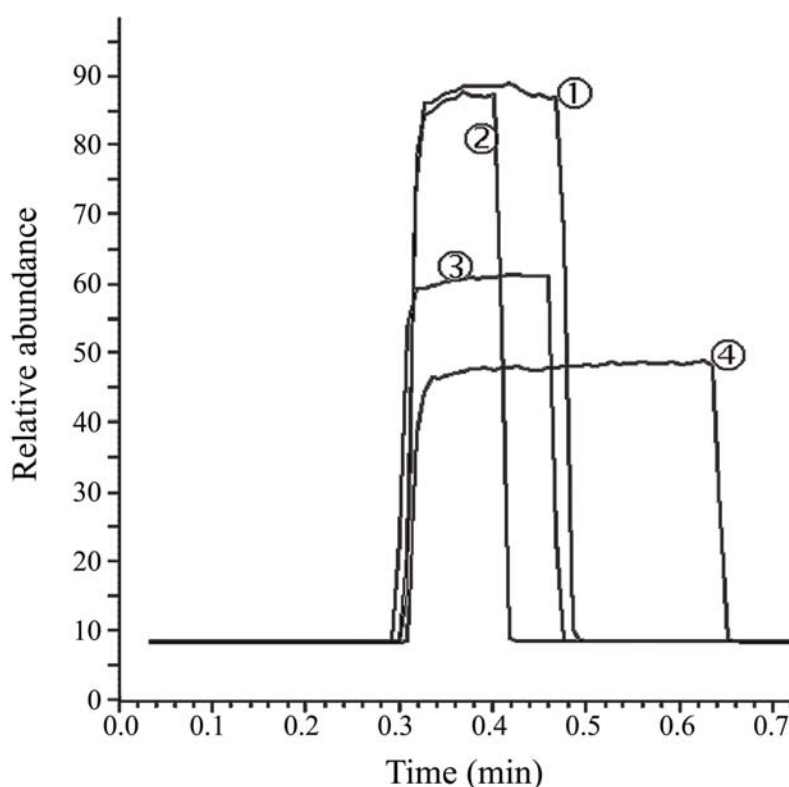
Figure 3.2. Effect of sample size, carrier flow and injection speed on response of MS. (1) 500µl sample containing 100µM 3-methylbutanal was injected with 50µl/sec into the system, which was running with a pre-column pressure of 15kPa and a split flow of 15 ml/min. (2) Injection volume changed to 300µl. (3) Pre-column pressure increased to 22kPa. (4) Injection speed decreased to 25µl/sec.

Development of sample tray and handling procedure

After optimising the conditions of analysis a sample tray with standard dimensions compatible with existing tools and equipment was developed to minimise the total handling time. A stainless steel block with cylindrical holes (2 ml) was constructed with the standard dimensions of a deep

well micro plate (96 wells) (see Fig. 3.1). Stainless steel was chosen because it is relatively inert for the compounds used. Moreover it can be used for heating of the samples before the analysis, and can be sterilised by autoclavation. In case studied chemicals do interact with the steel, standard shell vials of 40x8mm can be used as inserts. In our case the aldehyde was stable under the conditions used. Airtight closure of the vials was accomplished with a (sterile) Teflon sheet, a 4 mm thick sheet made of silicon and stainless steel cover. Using this tray over 1500 samples could be handled and analysed per day.

Data processing and reproducibility

The maximum response at a retention time of 0.25 minutes of the single-mass data was identified from the response data of the MS (see also Fig. 3.2). Smoothing was applied on the raw data to average small disturbances in response, resulting in unequivocal recognition and determination of the average maximum peak heights. Data were further processed in a spreadsheet program. The volatile compounds, 3-methylbutanal, 2-methylpropanal and benzaldehyde could already be distinguished from the background response down to the lowest concentration measured (0.5 µM $p < 0.05$, double sided student T-test) although the use of M17 broth gave especially in the case of 3-methylbutanal a high background response. These results are shown in Figure 3.3.

In case of a screening, the spreadsheet sorted the response data on strain ID, because the miniaturised fermentations were performed in quadruple and randomised over the blocks. Subsequently, average responses, standard deviations and concentrations of the samples were calculated based on these four independent fermentations. The coefficient of variation for the responses within one screening was found to be 4.5%.
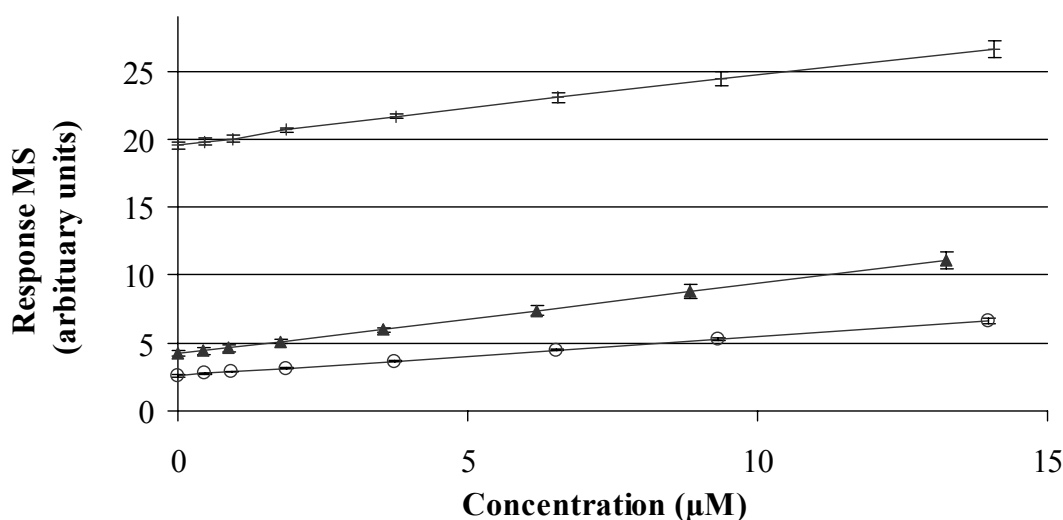

Figure 3.3. Detection of low concentrations of the pure flavour compounds 3-methylbutanal (*m/z*=86, +), 2-methylpropanal (*m/z*=72, ▲) and benzaldehyde (*m/z*=105+106, ○) dissolved in M17 broth. Error bars indicate standard deviations of five (*m/z*=86) or three (*m/z*=72 and *m/z*=105+106) measurements.

Screening lactococci for 3-methylbutanal production

To evaluate the newly developed screening method, 72 strains belonging to the species *Lactococcus lactis* were taken from the NIZO culture collection and screened on their ability to produce the key flavour 3-methylbutanal. Before the actual screening, a control experiment was performed to investigate whether 3-methylbutanal is the only compound present in the headspace of fermentation samples yielding an ion with *m/z*=58. Comparison of the total ion count (TIC) and the count of ions with *m/z*=58 in a headspace GC-MS chromatogram clearly shows that 3-methylbutanal is the major source of the latter ion (Fig. 3.4). However two other compounds, acetone and 2-methylbutanal, were also detected at *m/z*=58. Acetone is already present in small amounts in the medium and its production levels are only slightly influenced by fermentation, and therefore its presence will not influence the outcome of the experiment (data not shown). The flavour compound, 2-methylbutanal, is produced via the same metabolic pathway as 3-methylbutanal, has a similar flavour impact, and thus is also a marker for the same enzymatic activity that is screened for. Therefore it was concluded that *m/z*=58 was suitable for identifying branched-chain aldehyde producing strains.
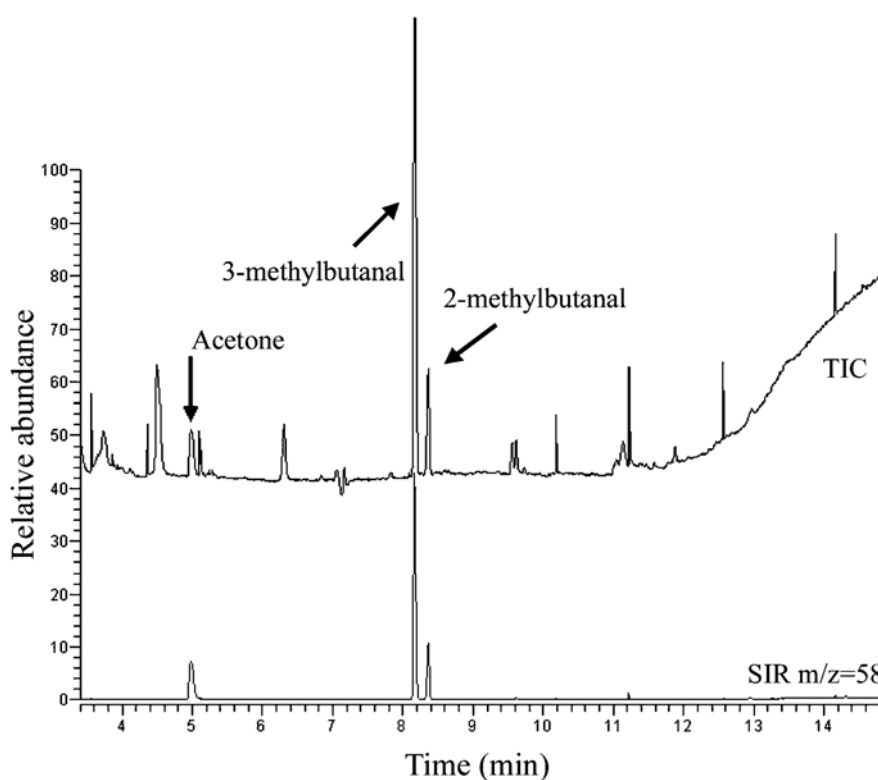
Figure 3.4.   Chromatograms (total ion count (TIC) and single ion recording (SIR) of *m/z*=58) of HS GC-MS analysis of the 3-methylbutanal producing strain, B1157, grown on GM17.

The screening was performed as described in the Materials and Methods section with the optimal parameters described above. Although the differences between strains grown on M17 are rather small, a limited number of strains were identified to produce relatively large amounts of 3-methylbutanal (Fig. 3.5, Panel A).
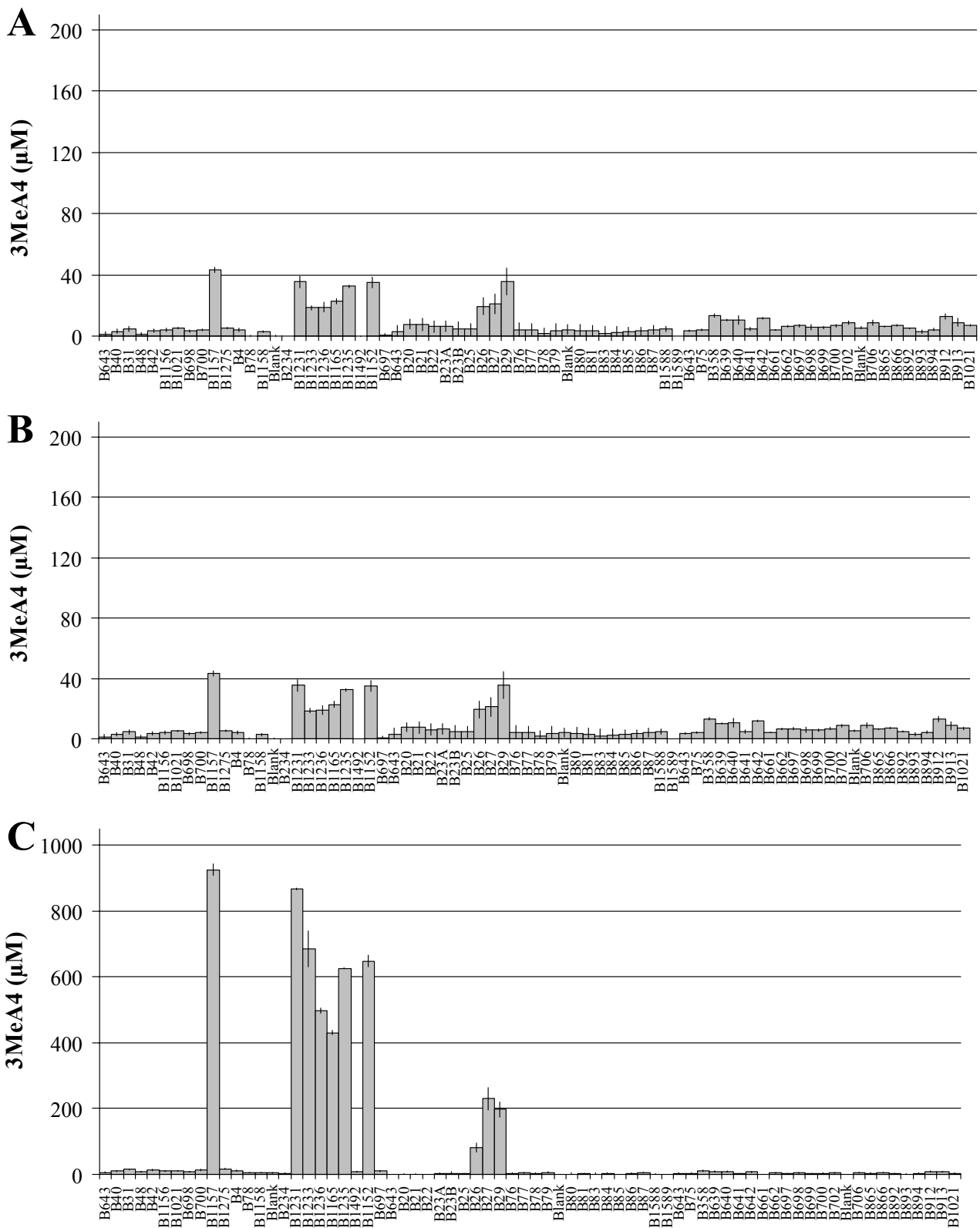
Figure 3.5. Screening of 72 strains belonging to the genus *Lactococcus lactis* on GM17-broth (A), GM17-broth with 5 mM leucine and 5 mM α-ketoglutarate (B) and GM17-broth with 2 mM α-ketoisocaproic acid (KaLeu)(C). The error bars indicate standard deviations of the quadruple measurements.

The aroma concentrations of these strains (B1152, B1157, B1165, B1231, B1233, B1235, B1236, B26, B27 and B29) differed significantly from all other strains tested without increased flavour production (p<0.05, Mann-Whitney U test). The aroma concentrations of these strains (B1152, B1157, B1165, B1231, B1233, B1235, B1236, B26, B27 and B29) differed significantly from all other strains tested without increased flavour production (p<0.05, Mann-Whitney U test). As described in the introduction, 3-methylbutanal is derived from leucine, which is liberated from proteins by proteolytic enzymes. In GM17 the main source for protein catabolism are peptides, therefore the production of 3-methylbutanal depends on the activity of several enzymes, and the presence of several cofactors. The rate-controlling step in this route under cheese conditions is probably not the availability of free amino acids (Wallace *ea.*, '97; Smit *ea.*, '03). Yvon *et. al.* showed that in cheese the transamination reaction from L-leucine could be increased by addition of α-ketoglutarate. This led to higher concentrations of KaLeu and flavour (Yvon *ea.*, '98). Therefore in a second experiment, we also added α-ketoglutarate (AKG) and leucine to GM17.

The results shown in Figure 3.5 panel B, indicate that the production levels of 3-methylbutanal by the same 3-methylbutanal producing strains indeed increased (maximal 165 µM), and thus improves the discrimination between producing and non-producing strains.

In the third medium, α-ketoisocaproic acid (KaLeu) was added. KaLeu is the substrate for the last conversion step to 3-methylbutanal, a keto acid decarboxylase. The results in Figure 3.5, Panel C show a further increase in 3-methylbutanal production up to 950 µM by the same strains that showed a high production levels in the previous experiments. Also, addition of the substrate KaLeu improved discrimination between producing and non-producing strains.

Validation

The results of the HTS screening were confirmed by growing four of the 3-methylbutanal producing strains and four non-producing strains in GM17 + KaLeu medium, in 10 ml headspace vials and analysing the headspace by standard HS-GC-FID analysis. In Figure 3.6 these results are summarised, together with the results for these strains from two independent screenings, performed in a different week. The results were in agreement, and acetone was only produced marginally, indicating that *m/z*=58 indeed is a discriminative mass for screening cultures on 3-methylbutanal production by using Direct Inlet MS. However, the results of the HTS seemed to be systematically lower than the values of traditional GC-FID. This difference might very well be caused by a small discrepancy in sample types between the actual screening and the calibration. The calibration samples are made in 10 ml vials, containing 3 ml liquid, and are heated in a closed incubator, while the screening takes place in the stainless the steel blocks. A degree difference in headspace temperature at 45°C for example already leads to an error over 4% (data not shown). However these small differences will not affect the aim of this kind of screenings; the selection of highly productive strains.
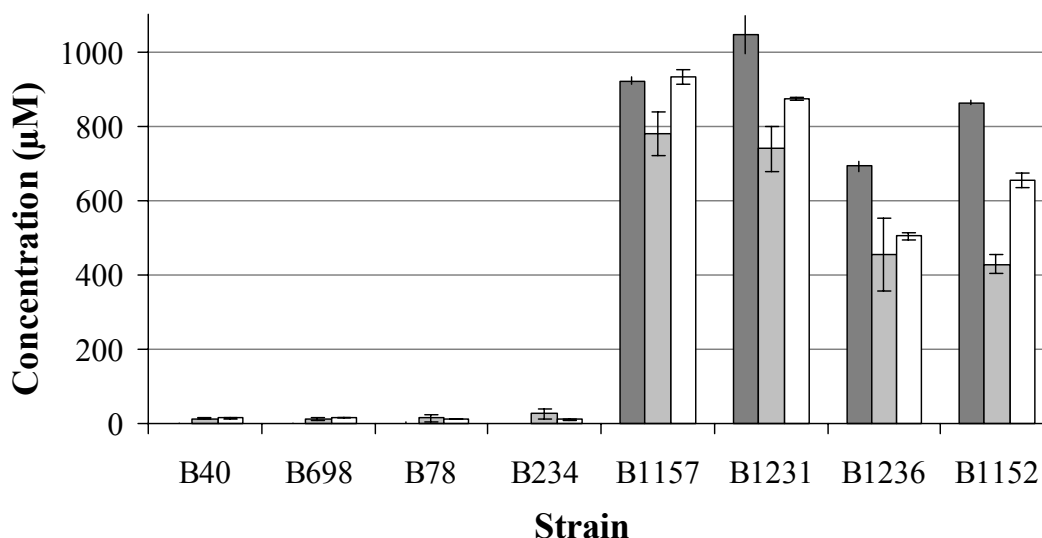
Figure 3.6. **Comparison of 3-methylbutanal production of eight (producing and non-producing) strains on GM17-broth with 2 mM α-ketoisocaproic acid by standard HS-GC-FID analysis (Black) and the newly developed Direct Inlet MS method, which was run twice totally independently (Grey and White). Error bars indicate standard deviations.**

Discussion

A fast screenings method and sample tray were developed for screening of volatile compounds by Direct Inlet MS. The method is based on the fast screening of specific compounds only using MS for detection. With the described method as much as 1500 measurements/day could be performed with a coefficient of variation in response of 4.5%. Several important flavour compounds could be measured with reasonably low detection thresholds in liquid media as M17 and milk. The detection threshold might be further improved by increasing the detector voltage. If desired, the throughput might be increased by small changes in the method and/or equipment (for example, no flushing of the syringe between measurements), since our system was limited by the handling time of the auto-sampler, rather than the analysis time of the MS.

The screening of lactococcal strains has shown that the newly developed HTS method for screening volatiles gives rapid and reliable results on the production of specific metabolic compounds like 3-methylbutanal in this test case. The model system with small, miniaturised fermentations was optimised by varying the medium composition. The addition of the substrates for transamination to the medium, resulted in higher 3-methylbutanal concentrations than in the case of standard medium (Fig. 3.5 Panel B). This could even be improved by the addition of KaLeu (Fig. 3.5 Panel C). Apparently, the concentration of KaLeu available for decarboxylation was lower than in the situation in which KaLeu was added directly to the medium. This might be caused by several factors. Firstly, it is known that free amino acids are not transported efficiently

into the cell by *Lactococcus lactis* (Kunji *ea.*, '96). Secondly, KaLeu might efficiently be converted to other metabolites e.g. 3-hydroxyisocaproic acid and isovaleric acid (Yvon *ea.*, '98; Smit *ea.*, '03). Thirdly, the capacity for transamination might be insufficient. These findings indicate that the choice of the correct model system can greatly enhance the discrimination between samples within the screening.

Remarkably, all 3-methylbutanal producing strains did not have a history in the dairy industry, but were so called "wild" strains, while most other strains tested were isolated from an industrial dairy setting. The absence of 3-methylbutanal production in the other strains confirms our previous finding that the presence or absence of α-keto acid decarboxylase, is a primary rate-controlling step in the conversion route to 3-methylbutanal (Smit *ea.*, '03).

Despite the fact that the current method was based on a flavour compound of which the conversion pathway from protein substrate to final compound is known, the developed method can also be applied for flavour compounds with unknown generation pathways, or for screening of other species, in which a certain pathway has not been shown to exist. This way, strain selection can greatly be enhanced. Moreover, selected strains can subsequently be used to study enzymes involved in their synthesis. Currently we are working on expanding the range of applications. For instance, the screening of mutant libraries, and thus for identification of genes involved in the production of volatile compounds. Moreover screening on cooperative effects when strains, cultured together (Ayad *ea.*, '01), can quickly be analysed. Such developments will eventually lead to an increased knowledge of flavour forming enzymes and improved starter cultures for the preparation of fermented food products.

Chapter 4

Non-enzymatic conversion of α-keto acids in relation to flavour formation in fermented foods

Abstract

Formation of flavour compounds from branched-chain α-keto acids in fermented foods like cheese is believed to be mainly an enzymatic process, while the conversion of phenyl pyruvic acid, which is derived from phenylalanine, also proceeds non-enzymatically. In this research the non-enzymatic conversion of α-keto acids to aldehydes with strong flavour characteristics was studied, with the main focus on the conversion of α-ketoisocaproic acid to the aldehyde 2-methylpropanal and a manganese catalysed reaction mechanism is proposed for this conversion. The mechanism involves keto-enol tautomerism enabling molecular oxygen to react with the ß-carbon atom of the α-keto acid, resulting in a peroxide. This peroxide can react in several ways leading to unstable dioxylactone or non-cyclic intermediates. These intermediates will break down into an aldehyde and oxalate or carbon oxides (CO and CO_2). All the α-keto acids tested, were converted at pH 5.5 and in the presence of manganese, although their conversion rates were rather diverse. This non-enzymatic reaction might provide new ways for controlling cheese flavour formation with the aim of acceleration of the ripening process, or diversification of the flavour characteristics.

Key-words

Introduction

Many flavours in fermented products are derived from amino acids. Examples of important amino acid derived flavour compounds in cheese are aldehydes like benzaldehyde, 2-methylpropanal, 3- and 2-methylbutanal, but also methionine-derived sulphur compounds, such as thioesters (Banks *ea.*, '92; Preininger *ea.*, '94; Engels *ea.*, '97; Rychlik *ea.*, '01a). Most flavour forming reactions in fermented products like cheese, are enzymatic (See Weimer *ea.*, '99; McSweeney *ea.*, '00; Smit *ea.*, '00; Yvon *ea.*, '01; Williams *ea.*, '02 for reviews). Protein degradation is initiated by proteolysis and peptidolysis, leading to free amino acids (Visser, '77). The amino acids are mostly enzymatically transaminated in the bacterial cell to the corresponding α-keto acid (Engels, '97; Gao *ea.*, '97). These α-keto acids can be converted to various metabolites, such as flavour compounds like the aldehydes, alcohols and thioesters mentioned above. The α-keto acid of leucine (KaLeu) for example, can enzymatically be decarboxylated by a number of *Lactococcus lactis* strains to 3-methylbutanal (Smit *ea.*, '03; '04b). Although most of these reactions are enzymatic, the α-keto acids of phenylalanine (phenylpyruvic acid = KaPhe) and methionine (KaMet) are (also) non-enzymatically converted to flavour compounds like benzaldehyde and methylthioacetaldehyde. The occurrence of the non-enzymatic conversion of KaPhe was demonstrated by Villablanca and by Nierop Groot, and the conversion of KaMet to methylthioacetaldehyde was suggested by Yvon *et. al.* (Yvon *ea.*, '02). The conversion of phenylpyruvic acid is an oxidation by molecular oxygen, and is catalysed by divalent kations like manganese (Villablanca *ea.*, '87; Nierop Groot *ea.*, '99).

The reactivity of ketones like α-keto acids is mainly due to the existence of their enol tautomers (Doy, '60; Pitt, '62; Carey *ea.*, '90b). Molecular oxygen is able to react with the enol, leading to the formation of a peroxide on the ß-carbon atom (Carey *ea.*, '90a). The nature of this peroxidation is not totally clear and might as well proceed directly as via a radical mechanism (Gersmann *ea.*, '71; Carey *ea.*, '90a). The very reactive peroxide intermediary of p-methoxyphenylpyruvate can react with the acid or keto carbon atom of the molecule resulting in a dioxylactone or dioxyethanol, although the latter reaction only occurs under basic, non nucleophilic conditions (Jefford *ea.*, '78). The instable dioxylactone formed from p-methoxyphenylpyruvate will decompose into an aldehyde and the carbon oxides (CO_2 and CO) (Jefford *ea.*, '78). A hydrophilic solvent, like water, is also able to react with the keto function of the peroxide intermediary resulting in a non cyclic intermediary (Jefford *ea.*, '78). Oxalic acid and water can be split off, leaving an aldehyde.

To summarize, the conversion of several keto acids depends strongly on the circumstances, e.g. solvent and pH, but the products of these reactions are an aldehyde and oxalate or carbon oxides (See also Fig. 4.4).

This paper focuses on revealing the characteristics and reaction mechanism of the non-enzymatic conversion of α-keto acids, leading to compounds relevant for fermented foods. It is important to note that the conversion of substrates present in the food takes place under mild conditions. The branched chain α-keto acid of leucine, α-ketoisocaproic acid, is chosen for this study, because this substrate is present in relatively high concentrations in fermented products like cheese, and its reaction product, 2-methylpropanal, has an important flavour impact in these products.

Materials and Methods

The non-enzymatic conversion was assayed in a 20 ml headspace vial with a reaction volume of 5 ml, or in 1 ml HPLC vials, with a reaction volume of 0.5 ml. Final concentrations of reactants in the reaction mixture were: 50 mM buffer, 10 mM metal chloride salt, 10 mM substrate. Standard conditions were: succinate buffer (pH=5.5), manganese chloride and α-ketoisocaproic acid as substrate. Used buffers were succinate (pH=3.5-6.5), glycerolphosphate (pH=4.5-7.2), bis-TRIS-propane (pH=6.4-7.4), HEPES (pH=6.4-7.8) and TRIS-HCl (pH 7-8). Metal salts: $MnCl_2$, $MnSO_4$, $MgCl_2$, $CaCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $CoCl_2$, $ZnCl_2$, NaCl. Substrates used in this study were: α-ketoisocaproic acid (KaLeu), 2-oxo-3-methylpentanoic acid (KaIle), α-ketoisovaleric acid (KaVal), α-ketohexanoic acid, α-ketopentanoic acid, α-ketobutanoic acid, pyruvic acid, 4-methylthio-2-oxobutanoic acid (KaMet), phenylpyruvic acid (KaPhe), 3-(4-hydroxyphenyl)-2-oxopropanoic acid (KaTyr), 3-indol-3-yl-2-oxopropanoic acid (KaTrp), 2-oxopentanedioic acid (KaGlu), 4-methylpentanoic acid, 3-methylbutanoic acid and 2-methylpropanoic acid. All substrates were analytical grade and obtained from Sigma (Zwijndrecht, The Netherlands) or Fisher (Landsmeer, The Netherlands). At t=0 substrate was added to the rest of the reaction mixture, the vial was closed, and placed in an incubator at 40°C.

Direct Inlet Mass Spectrometry (DI-MS) was used for the on-line monitoring of product formation. The method used was a slightly adapted version of the method published previously (Smit *ea.*, '04b). At regular intervals a 150µl headspace (HS) sample was taken from the incubator (Combi Pal, CTC analytics, Zwingen, Switserland) which was shaking at 500 rpm for 10s on / 5s off intervals at 40°C. The sample was injected with a 1.0 ml syringe of 60°C and 20 µl/s by the Combi Pal auto sampler (CTC analytics, Zwingen, Switserland) into the gas chromatograph (GC) (CE-instruments, Milan, Italy). The GC was equipped with an 8 m x 0.1 mm deactivated silica column (Interscience, Breda, The Netherlands). Helium was used as carrier gas, with a column flow of 2.5 ml/min and split flow of 10 ml/min. The oven temperature was 150°C constantly. Single ions at M/z = 58, 72 and 105 were recorded by a quadrupole mass spectrometer (Trace MS, CE-instruments, Milan, Italy). Acquisition time of the MS was 30 seconds. The height of the response at a certain m/z is directly correlated to the concentration of the compound. Quantification was done by using a calibration curve ranging from 10 to 1000 µM in the same buffer as used in the experiments, which was analysed before and after each

experiment in triplicate. The conversion rate is defined as the change of concentration in time, expressed in µM/h.

HPLC was used for the determination of α-keto acids and organic acids. The reaction mixture was prepared as described above and incubated in the auto sampler (model 717, Waters, Milford, MA) at 40°C. At regular intervals samples (25µl) were taken and injected to the system. For the analysis of polar molecules (pyruvic acid, 2-oxobutyric acid, KaGlu and oxalate) the system consisted of a LC-10AT pump (Shimadzu, Tokyo, Japan), pumping 0.01M methane sulfonic acid with an isocratic flow of 0.6 ml/min over a Rezex Fast Fruit pre-column (100 × 7.8 mm) and a double Rezex Organic Acid column (300 × 7.8 mm, Phenomenex, Torrance, Ca) at 30 °C. The other acids were analysed using the same system, but equipped with a wide pore C_{18}-RP column (250 × 4.6 mm, Bio-Rad, Veenendaal, The Netherlands), and compounds were eluted at 40 °C with a gradient from 5% to 30% acetonitrile in demineralised water containing 0.1% trifluoracetic acid (TFA). A Lamda-max detector (model481, Waters, Milford, MA) was used at 220 nm to detect the compounds in both system configurations. Concentrations were calculated by using separate calibration samples containing 4, 6, 8 and 10 mM of the acids in 50 mM succinate buffer (pH=5.5).

GC was used to determine the concentrations of O_2 and CO_2 in the headspace. The reaction mixtures were prepared as described above and incubated at 40°C. Before each measurement, samples were inverted 4-times and cooled to 25°C. During 5 seconds, a headspace sample was taken and analysed on a CP2001 gas chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a HayeSep A column (25 cm) and a MS 5A column (4 m). Because the sampled volumes were about 0.5 ml and samples were analysed in quadruplicate, a separate reaction vial was used each time interval. Concentrations were calculated by using air (21% O_2, 0.033% CO_2) and a calibration gas (1.00% CO_2, 1.00 % O_2) for calibration.

Results

The production of branched chain aldehydes from amino acids in cheese is believed to be a two-step process consisting of a transaminase and decarboxylase step. Leucine, the most abundant branched-chain amino acid in cheese, is transaminated to α-ketoisocaproic acid (KaLeu) which can subsequently be decarboxylated to 3-methylbutanal (Smit *ea.*, '03). Under certain conditions, fermented dairy products had unexpected high 2-methylpropanal concentrations (unpublished data). This flavour compound is believed to be produced from valine via a pathway similar to the one elucidated for leucine, with α-ketoisovaleric acid (KaVal) as the intermediate (McSweeney *ea.*, '00; Smit *ea.*, '00; Yvon *ea.*, '01). To control cheese flavour formation, it is important to understand the characteristics of these enzymatic conversions. Studying the enzymatic decarboxylation of KaLeu by static headspace measurements of incubations of cell free extract (CFE) with KaLeu revealed that also the concentration of 2-methylpropanal increased (data not shown), while no KaVal was added to these incubations. Repeating the experiment with cooked

CFE, hereby inactivating the decarboxylating enzyme, still led to the same increase of 2-methylpropanal concentration. This suggested that KaLeu could also be converted non-enzymatically with 2-methylpropanal as one of the products. This reaction was studied in more detail using Direct Inlet Mass Spectrometry, as described in the materials and methods section.

None of the tested metal-salts, except the mangenese-salts, were able to enhance the conversion at a concentration of 10 mM either at pH 5.5 or pH 7.5. Differences were observed in the enhancing effect of manganese chloride compared to manganese sulphate (Table 1). The smaller conversion rates of the chloride salt might be caused by partial manganese oxidation, which was noticed by slight browning of the manganese chloride stock solution. This might lead to a loss of catalytic capacity. Addition of 10 and 20 mM EDTA to reaction mixtures containing 10 mM $MnCl_2$ resulted in a decrease in the conversion rate with 88% and 96% respectively, indicating the need for free manganese ions. The correlation between the manganese concentration and the conversion rate is shown in Figure 4.1. The optimal manganese concentration (>10 mM) was much higher than expected for a catalyst. This might suggest that Mn^{2+} acts as reactant instead of catalyst, or only a fraction of the manganese dissolved is present in the catalytic active form.

Table 1. Catalytic effect of several metal-salts.

Metal (10 mM)	pH=5.5	pH=7.5
$MnCl_2$	100	100
$MnSO_4$	105	130
$MgCl_2$,	0	0
$CaCl_2$	0	0
$CuCl_2$	0	0
$FeCl_2$	0	0
$FeCl_3$	0	0
$CoCl_2$	0	n.d.
$ZnCl_2$	0	0
NaCl	0	0
$MnCl_2$ + 10 mM EDTA	12	n.d.
$MnCl_2$ + 20 mM EDTA	3	4

n.d.= not determined
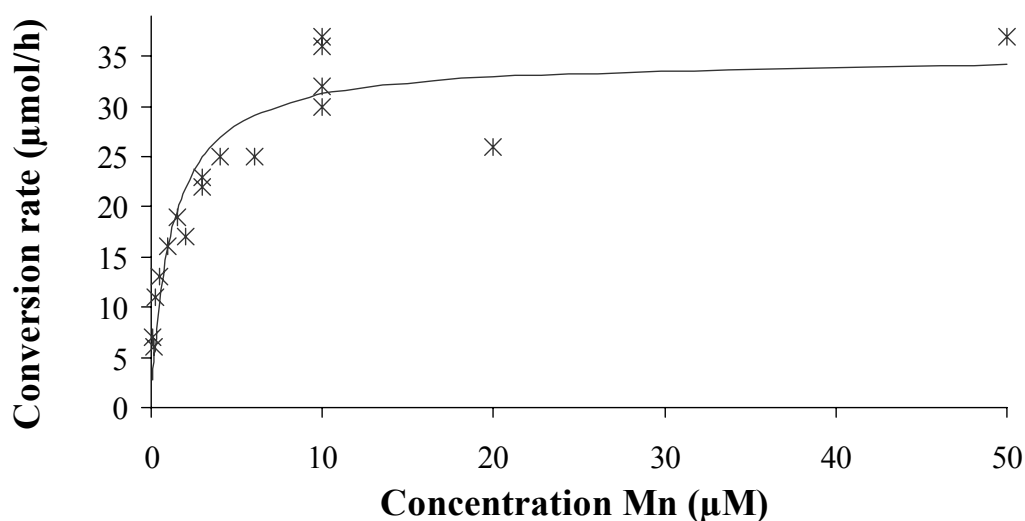Values are relative to $MnCl_2$.

Figure 4.1. Effect of manganese concentrations on the conversion rate of KaLeu to 2-methylpropanal (Succinate buffer, pH=6.0).

The correlation between pH and conversion rate was determined using several buffers for pH values, which are relevant for fermented dairy products (pH 4-8)(Fig.4.2). The pH of Gouda cheese is about 5.5 (Walstra *ea.*, '93), the intracellular pH of the micro-organisms in cheese is close to neutral (Molenaar *ea.*, '91; Hugenholtz *ea.*, '97) and the pH of mould and smear ripened cheeses can get close to pH=8 (Bockelmann, '03). A local maximum conversion was found at pH 5.5, but at pH 8 the conversion rate was even higher. At pH>8 even higher conversion rates were expected, but the formation of a brown precipitate (MnO_2) at this pH resulted in non-interpretable results.
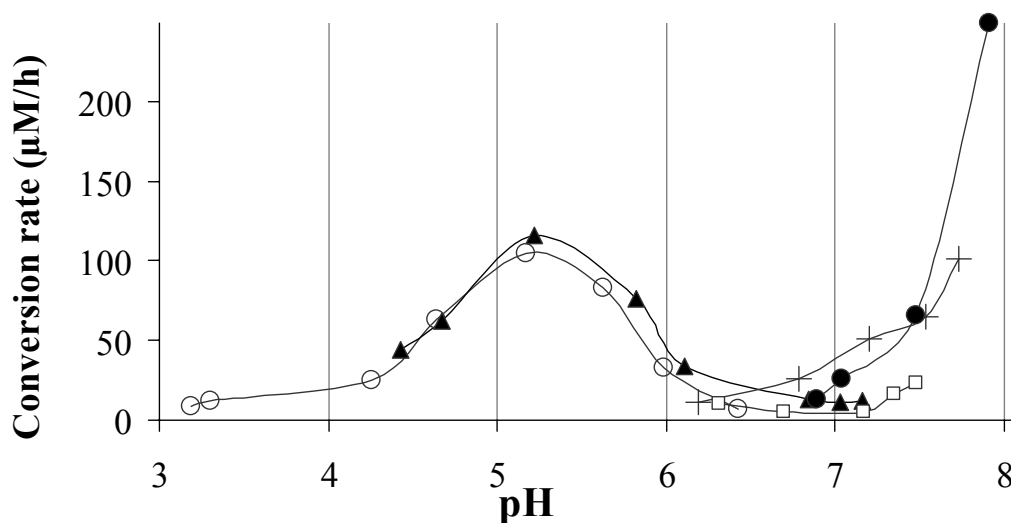
Figure 4.2. Effect of pH on the conversion rate of KaLeu to 2-methylpropanal. (10 mM KaLeu, 10 mM MnCl₂ and the following buffers: succinate (○), glycerophosphate (▲), bis-TRIS-propane (□), HEPES (+) and TRIS (●))

Varying the substrate concentration between 0.5 and 50 mM resulted in an almost linear increase of the conversion rate under the conditions tested (excess of oxygen is present in the air present in the headspace, 10 mM $MnCl_2$, pH=6.5) (data not shown). Oxygen is essential for the reaction, since very low oxygen concentrations (0.1%) in the reaction vial led to a major decrease in the conversion rate (>90%) (data not shown). Moreover oxygen was found to be consumed during the reaction (Fig. 4.3).
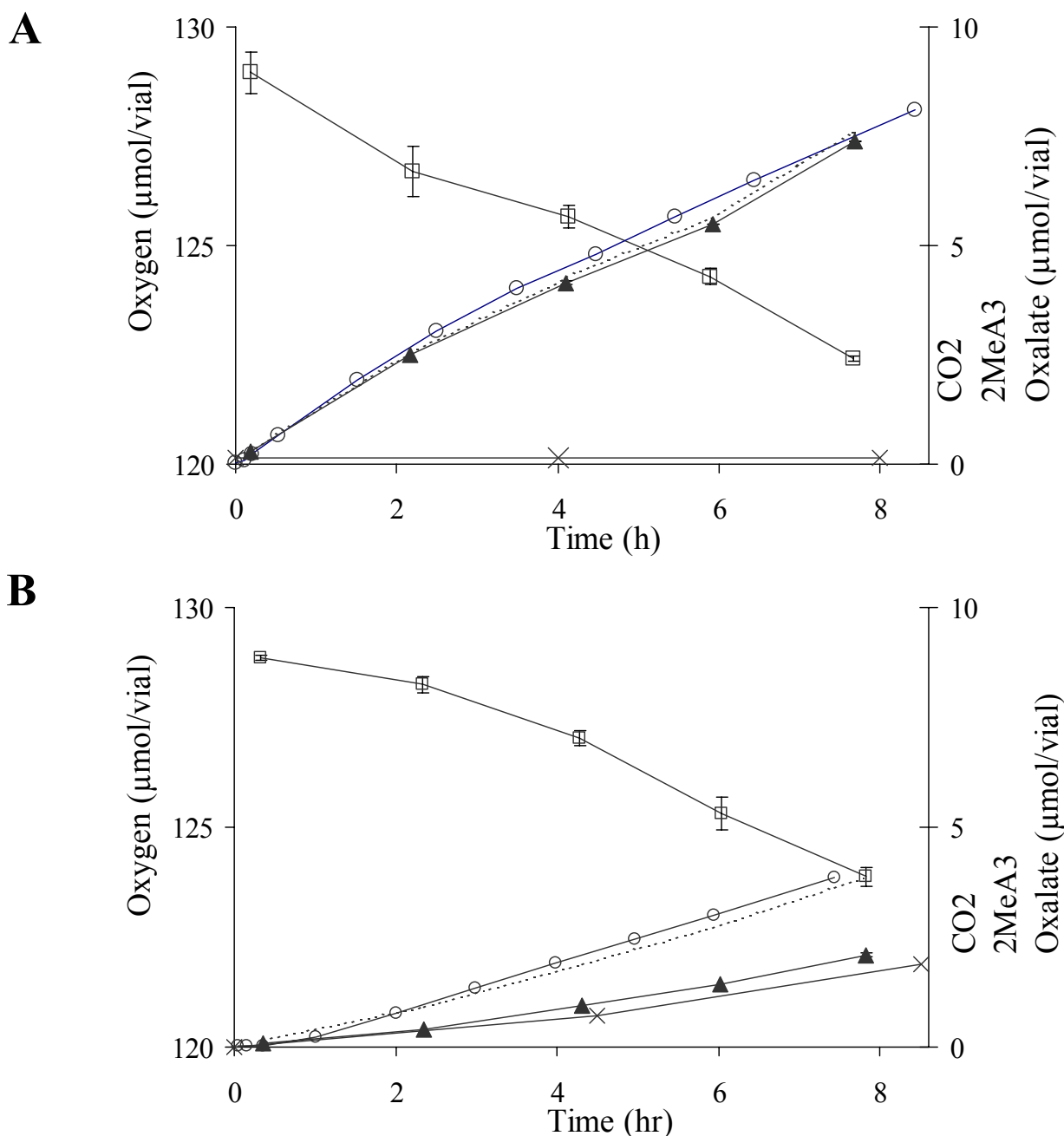

Figure 4.3. Oxygen (□) consumption and production of 2-methylpropanal (○), carbon dioxide (CO2) (▲) and Oxalate (×) during the chemical conversion of KaLeu at pH = 5.5 (panel A) and 7.5 (panel B) in the presence of 10 mM manganese. The dotted line represents the sum of oxalate and CO2.

For a better understanding of the reaction mechanism, it is important to know which products are formed. For the conversion of KaLeu to 2-methylpropanal two carbon-atoms have to be cleaved off, and the expected products are oxalic acid and/or carbon oxides (CO and CO_2), based on the homology with other reactions as described in the introduction (Fig. 4.3). The formation of these products was measured for reactions proceeding at pH=5.5 and 7.5 by using DI-MS, GC and HPLC simultaneously as described in the Materials and Methods section. The HPLC method was also optimised for detection of other di- and mono-carbonic acids, in case the two carbon atoms would split off in a different manner. Oxalate and carbon dioxide were the only products detected, but the GC columns were not suitable for measurement of CO. These two products are formed via different mechanisms. Per molecule 2-methylpropanal formed either 1 molecule oxalate or 1 molecule CO_2 were formed. In the case CO_2 was formed this must have been accompanied by CO or formiate to complete the balance of elements. Formiate was not detected by HPLC analysis, which is in agreement with the findings of the conversion of *p*-hydroxyphenylpyruvate and phenylpyruvate (Büchner *ea.*, '52; Gersmann *ea.*, '71; Jefford *ea.*, '78). The quantitative headspace data were processed, taking gas-liquid equilibriums (Henry's law (K_{HCO2}=0.034M/atm, K_{HO2}=0.0013M/atm (Sander, '99)) and acid-base equilibriums (to calculate [HCO_3^-] (K_{aHCO3-}=$2.3 \cdot 10^{-8}$M)) into account. The results are combined in Figure 4.3. The increase of 2-methylpropanal and the increase of the sum of oxalate and CO_2 clearly correlate with ratios close to 1. However, the oxygen consumption in relation to the 2-methylpropanal production is slightly larger at high pH. This could again be caused by the oxidation of manganese to MnO_2, as described earlier. No further quantitative measurements were done to confirm this. Our finding of non-enzymatic conversion of KaLeu shows that the non-enzymatic conversion of α-keto acids in fermented foods will not be limited to KaPhe and KaMet. More information on the specificity of the reaction towards several substrates was desired in order to estimate the impact of this reaction on the degradation of other α-keto acids in fermented products. Therefore, we incubated a variety of different α-keto acids and organic acids, and determined their conversion by HPLC. The substrates were chosen based on their involvement in the amino acid catabolism, or just as model substrates (linear keto acids and branched organic acids). In Table 4.2 the relative conversion rates and structure formulas of the substrates tested are shown. The results indicate that the reaction is limited to α-keto acids, and in general substrates with electron withdrawing side chains are converted faster. Also two hydrogen atoms on the ß-carbon atom seem to be preferable above one.

Table 4.2. Chemical conversion rates of several α-keto acids under standard conditions (Succinate, pH=5.5, 10 mM Mn).

Substrate	Abbreviation	Structure	Relative Conversion Rate
2-oxo-3-phenylpropanoic acid	KaPhe		~100%
3-(4-hydroxyphenyl)-2-oxopropanoic acid	KaTyr		~55%
2-oxopentanedioic acid	KaGlu		~33%
4-methylthio-2-oxobutanoic acid	KaMet		~20%
4-methyl-2-oxopentanoic acid	KaLeu		~30%
3-methyl-2-oxopentanoic acid	KaIle		~3%
3-methyl-2-oxobutanoic acid	KaVal		~5%
2-oxohexanoic acid			~45%
2-oxopentanoic acid			~52%
2-oxobutanoic acid			~37%
2-oxopropanoic acid			~8%
Isohexanoic acid			
Isopentanoic acid			
Isobutanoic acid			

Discussion

The conversion of α-ketoisocaproic acid (KaLeu) to the flavour compound 2-methylpropanal is shown to be a non-enzymatic reaction, depending on oxygen and manganese. The possible conversion mechanisms of the degradation of α-keto acids, which are discussed below, are summarised in Figure 4.4.

The reactivity of α-keto acids (A) is mainly due to the existence of their enol tautomers (B) (Pitt, '62; Carey *ea.*, '90b). This was tested by measuring the conversion of the organic acid similar to
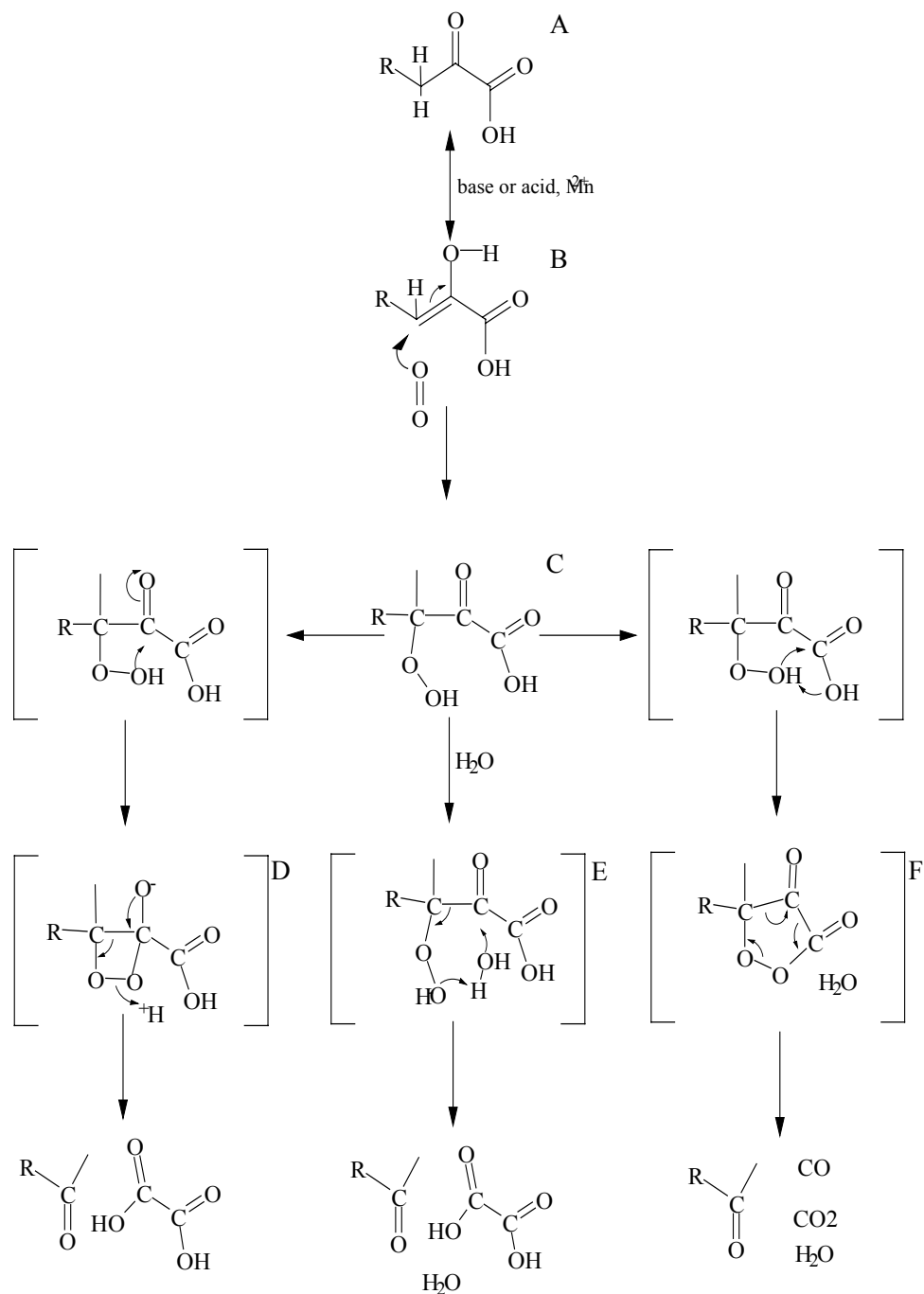
Figure 4.4. Proposed reaction mechanisms of the chemical oxidation of α-keto acids.

KaLeu, but lacking the keto-group (isohexanoic acid). This organic acid as well as other organic acids were not converted under the conditions tested. However, "linearised" KaLeu (2-ketohexanoic acid) was converted with approximately the same conversion rate as KaLeu. The organic acids are probably not able to form the reactive enol-tautomers. Therefore, we conclude that the keto group (and the formation of the enol tautomer) is most likely essential for this oxidation reaction. The amount of the enol present in the equilibrium can be stabilized by intra molecular hydrogen bonds and by conjugation of the carbon-carbon double bond with the carbonyl group (Russel *ea.*, '66; Carey *ea.*, '90b). Keto-enol tautomerisation is generally catalysed by acid or base, but also bivalent metal ions can accelerate this reaction (Büchner *ea.*, '52; Bailey *ea.*, '62; Carey *ea.*, '90b). A minimum in the conversion rates of KaLeu to 2-methylpropanal near neutral pH was confirmed in our experiments. We found a major catalytic effect of manganese ions, however we did not find effects of other divalent metal ions, as was described for KaPhe conversion (Nierop Groot *ea.*, '98). If the main catalytic effect of manganese under the conditions tested is enhancement of the tautomerism by for example increasing the conversion rate or stabilizing the enol tautomer, keto-enol tautomery most probably determines the rate of the overall conversion. This is also indicated by the slow conversion of KaIle and KaVal compared to their linear equivalent. The ß-carbon atom of KaIle and KaVal is methylated, leaving only one ß-hydrogen atom, which is essential for the tautomery, and thereby this might slow down the conversion.

Based on homology with several other ketones, the next step in the oxidation is most probably the formation of a peroxide (C) on the ß-carbon atom (Carey *ea.*, '90a). This oxidation might proceed directly or via a radical mechanism (Gersmann *ea.*, '71; Carey *ea.*, '90a). In the latter case, electron transfer of the nucleophilic enolate ion to molecular oxygen results in an α-keto radical (Bailey *ea.*, '62; Russel *ea.*, '66; House, '72). The propagation step involves the addition of molecular oxygen resulting in a hydroperoxy radical (Carey *ea.*, '90a). This radical can transfer the electron to another enol, leaving a new α-keto radical and a α-keto hydroperoxide (Bailey *ea.*, '62; House, '72).

The very reactive peroxide intermediary can react with the acid or keto carbon atom of the molecule resulting in a dioxylactone (F) or dioxethanol (D). The cyclization to the dioxethanol is not a spontaneous reaction, and only occurs under basic, non nucleophilic conditions (Jefford *ea.*, '78). The instable dioxylactone (F) will decompose into an aldehyde (RC=O) and the carbon oxides (CO_2 and CO) (Jefford *ea.*, '78). An hydrophilic solvent (R'OH) like water is also able to react with the keto function of the peroxide intermediary resulting in a non cyclic intermediary (E) (Jefford *ea.*, '78). Oxalic acid and water can be split off, leaving an aldehyde (RC=O). We identified both carbon oxides and oxalate during conversion of KaLeu. This indicates that the conversion of KaLeu most probably proceeds via both pathways described for a nucleophilic environment. We also found that the sum of oxalate and carbondioxide correlated with the 2-methylpropanal production with ratio's close to 1, and that the pathway via the non-cyclic intermediary (E) is enhanced at higher pH.

All α-keto acids, including the α-keto acids corresponding to amino acids, were converted under the mild conditions used (Table 4.2). This implies that also the products of the other amino acid derived α-keto acids are expected to be found in fermented products. In cheese and/or in the starter bacteria not all parameters are as optimal as under the conditions tested. The pH for instance, varies during the fermentation and differs between the inside and outside of the bacterial cell. During growth the pH of lactic acid bacteria is neutral, but after growth they are sometimes not able to maintain this pH, or will even lyse, which results in a decrease in the pH (Molenaar *ea.*, '91; Hugenholtz *ea.*). The pH of Gouda cheese for example decreases from 7 to below 5.4 in the first six hours of cheese production, after which it stays more or less stable (Walstra *ea.*, '93). This pH is optimal for the non-enzymatic reaction to proceed. In smear ripened and mould cheeses the pH might increase to 7.5, due to the metabolic activities like deamination (Bockelmann, '03). Some lactic acid bacteria, like *Lactobacillus casei* subsp. *casei* and *Lactobacillus plantarum* are able to accumulate manganese, leading to high local intracellular manganese concentrations (up to 50 mM) (Archibald, '86; Nierop Groot *ea.*, '99), which is also favourable for the non-enzymatic reaction. Especially when transamination is stimulated, α-keto acids accumulate in the cheese matrix (up to 17.9% of the initial leucine concentration (Yvon *ea.*, '98)), resulting in reasonable amounts of substrates present for the non-enzymatic reaction to proceed. However, the low oxygen concentration in cheese is very unfavourable, but some 2-methylpropanal was still formed at low oxygen concentrations (0.1% in headspace which equals 1.3 µM in the reaction mixture) and depending on the type of cheese, especially at the outside some oxygen is available up to several weeks (Van den Tempel *ea.*, '02). The reaction will most probably proceed much more slowly under cheese ripening conditions than under the conditions used in this study, although under simulated Cheddar cheese conditions spontaneous degradation of hydroxyphenylpyruvate to hydroxybenzaldehyde occurs (Gao *ea.*, '98a), and both benzaldehyde and hydroxybenzaldehyde were found in semi-hard cheeses (Yvon *ea.*, '98). Cheese ripening is a long process, which may take up to a year, and therefore we expect that the conversion rate will still be sufficient to change cheese flavour characteristics. In future work this will be tested in cheese model systems, pilot cheese productions using selected starter cultures containing manganese accumulating strains and transaminase over-expression mutants in combination with the addition of α-ketoglutarate in order to increase α-keto acid formation. A large difference in reaction rates between the substrates exist, the availability of the substrates in a product is very different and the flavour characteristics of the aldehydes differ largely. More research has to be done on this topic. The non-enzymatic reaction described might be used as a new control point for aroma formation and flavour diversification in several fermented food products, not only by increasing flavour formation, but also for preventing off-flavours. Increasing the oxygen concentration by using more permeable coatings, or selecting manganese-accumulating strains might in this respect be relevant control parameters.

Summarising, the non-enzymatic conversion of KaLeu and other α-keto acids shows that the non-enzymatic conversion of α-keto acids in fermented foods is not limited to the keto acids of

benzaldehyde and methionine at high pH. This implicates that almost all α-keto acids derived from amino acids can be converted into aldehydes. The impact on the flavour perception of a product, as a result of all these reactions however has still to be determined.

Chapter 5

Identification, cloning and characterisation of a branched-chain α-keto acid decarboxylase from *Lactococcus lactis* involved in flavour formation

Abstract

The biochemical pathway for formation of branched chain aldehydes, which are important flavour compounds derived from proteins in fermented dairy products, consists of a protease, peptidases, a transaminase and a branched-chain α-keto acid decarboxylase (BcKAD). Activity of the latter enzyme in the species *Lactococcus lactis* has only been found in a limited number of strains. Using a random mutagenesis approach the gene encoding the BcKAD was identified. The gene for this enzyme is highly homologous to the gene annotated as *ipd* in *L. lactis* IL1403, which gene product is probably inactive due to a deletion in the C-terminal part of the gene. A BcKAD over-expressing mutant was used for further characterisation of the decarboxylase enzyme. The molecular weight of the enzyme is 60.9 kDa, its estimated pI is 5.03, its enzyme activity was hardly affected by high salinity and optimal activity was found at pH=6.3. Moreover, of all potential substrates tested, the activity towards branched-chain α-keto acids was the highest, for which reason it is proposed to be annotated as BcKAD.

Key-words

Introduction

Flavour formation in cheese is mainly the result of catabolism of milk proteins, sugar and lipids. Degradation of proteins into amino acids followed by conversion of these amino acids by *Lactococcus* spp. is of main importance for flavour formation in semi-hard cheese types like Gouda (see for reviews Engels, '97; McSweeney *ea.*, '00; Smit *ea.*, '00; Yvon *ea.*, '01). One of the major amino acid conversion routes is initiated by transaminases. Transamination of amino acids yields the corresponding α-keto acids, which are compounds with a minor flavour impact (Engels, '97; Gao *ea.*, '97). These α-keto acids can be converted to various metabolites, such as flavour-active compounds like aldehydes, alcohols and (thio)esters. The α-keto acid of leucine for example, can enzymatically be decarboxylated by some *Lactococcus lactis* strains to 3-methylbutanal (Smit *ea.*, '03; Smit *ea.*, '04b). Subsequently, (de)hydrogenation of 3-methylbutanal results in 3-methylbutanol or 3-methylbutyric acid, which also contribute to the flavour of cheese, but their odour thresholds are higher (Maralith, '81; Sable *ea.*, '99).

An important tool for development of new starter cultures is the selection of strains with improved characteristics towards the development of important flavour compounds, like 3-methylbutanal. The rate-controlling step in this route is identified as an α-ketoisocaproic acid (KaLeu) decarboxylating enzyme (BcKAD) (Yvon *ea.*, '01; Smit *ea.*, '03). Therefore controlling the activity of this enzyme might be a good approach for the development of new starter cultures with improved flavour characteristics. Previously, we selected *L. lactis* strains, from non dairy origins, that are able to produce 3-methylbutanal, while the industrial strains tested hardly show this activity (Smit *ea.*, '04b). The 3-methylbutanal producing strain, *L. lactis* B1157, has a relatively high KaLeu decarboxylating activity (Smit *ea.*, '04b), and was therefore used in this research.

A number of TPP dependent α-keto acid decarboxylases have been identified in various organisms, e.g. pyruvate decarboxylase (EC 4.1.1.1) (Singer *ea.*, '52), phenylpyruvate decarboxylase (EC 4.1.1.43) (Asakawa *ea.*, '68; Vuralhan *ea.*, '03), branched-chain 2-oxoacid decarboxylase (BCKA)(EC 4.1.1.72) (Oku *ea.*, '88), 2-oxoglutarate decarboxylase (EC 4.1.1.71) (Shigeoka *ea.*, '91; Palaniappan *ea.*, '92) and indole-3-pyruvate decarboxylase (IPD) (EC 4.1.1.74) (Koga, '95). In *L. lactis* however, there is only one report on the partial purification of an α-keto acid decarboxylase (Amarita *ea.*, '01) and in the genome of *Lactococcus lactis* IL1403 (Bolotin *ea.*, '01) a (partial) indole-3-pyruvate decarboxylase gene and a 2-oxoglutarate decarboxylase gene have been identified, but the pyruvate decarboxylase or branched-chain keto acid decarboxylase genes lacked.

Since the decarboxylase activity is critical in flavour-generation by *L. lactis*, in this paper the identification, cloning and characterisation of the branched-chain keto acid decarboxylase responsible for this conversion by *L. lactis* B1157 is described. The gene was identified by screening a random insertion knock-out mutant library on the absence of 3-methylbutanal formation, and the activity and properties of the gene product was determined, using a nisin–controlled over-expression mutant (De Ruyter *ea.*, '96).

Materials and Methods

Strains, plasmids and media. All strains and plasmids used or constructed are listed together with a short description in Table 1. Lactococcal strains were grown in M17 broth (Difco, Detroit, USA) with lactose (LM17) or glucose (GM17) as additional carbon source added in a final concentration of 0.5% (w/v). If appropriate, chloramphenicol (Cm) or erythromycin (Ery) was added to the medium to a final concentration of 5 µg/ml. Strains were either grown at 28 or 40°C. The ability of strains to produce 3-methylbutanal was determined by static headspace gas chromatography (HS-GC) analysis after 24 hours of growth in the presence or absence of 2 mM α-ketoisocaproic acid (KaLeu) in 10 ml HS vials, containing 3 ml culture medium.

E. coli was grown aerobically at 30°C on TY medium (Difco, Detroit, MI), containing 200 µg/ml erythromycin if desired.

Table 1. Bacterial strains and plasmids used in this study

Strain or Plasmid	Description	Antibiotic resistances	Source or reference
B1157	α-Keto acid decarboxylase positive "wild" *Lactococcus lactis* strain	Tet, Cm	(Ayad *ea.*, '99)
NZ9000	MG1363; *pep*N::*nis*RK; standard host for NICE	-	(De Ruyter *ea.*, '96)
MG1363	Plasmid free, wild type strain *Lactococcus lactis*, sequenced genome	-	(Gasson, '83)
IL1403	Wild type *Lactococcus lactis*, sequenced genome	-	(Bolotin *ea.*, '01)
B2083	pGh9:ISS1 integration in the chromosome of B1157 at position 462 of the decarboxylase gene	Tet, Ery, Cm	This work
B2084	pGh9:ISS1 integration in the chromosome of B1157 at position 995 of the decarboxylase gene	Tet, Ery, Cm	This work
B2085	NZ9000:pNZ7500	Ery	This work
pGh9:ISS1	Association of the insertion sequence ISS1 with the thermosensitive replicon pG+host	Ery	(Maguin *ea.*, '92; Maguin *ea.*, '96)
pNZ8148	Standard homologous *L. lactis* vector for NICE system	Cm	(Kuipers *ea.*, '98)
pNZ7500	pNZ8148 derivate containing a 1.8 kb fragment carrying the decarboxylase gene of B1157	Cm	This work
pNZ7501	pNZ8148 derivate containing a 1.5 kb fragment carrying the partial decarboxylase gene of B1157	Cm	This work
pNZ7502	pNZ8148 derivate containing a 1.4 kb fragment carrying the decarboxylase gene of IL1403 annotated as *ipd*	Cm	This work
pNZ7503	Wild type plasmid B1157 containing lactose fermenting genes, Tet and Cm resistances	Cm, Tet	This work

DNA handling procedures. Before applying the protocols for DNA isolation, *Lactococcus lactis* cells were pre-treated for 10 minutes with 2 mg/ml lysozyme in THMS buffer at 37°C. Plasmid DNA was isolated using the Micro plasmid prep kit (Amersham/Pharmacia, Uppsala, Sweden), and total DNA was isolated using the AquaPure genomic DNA isolation kit (Bio-Rad, Veenendaal, The Netherlands) according to the manufacturers protocols. *L. lactis* cells were transformed by electroporation as described previously (Wells *ea.*, '93). Agarose gel electrophoresis was performed as described by Sambrook *et al.* ('89). DNA fragments were isolated from agarose gels by using the gel purification kit (Jetstar, Genomed, Bad Oeynhausen, Germany). Restriction enzymes, T4 ligase and other DNA modifying enzymes were purchased from Promega Corp (Leiden, The Netherlands) or Invitrogen (Carlsbad, CA) and used as recommended by the manufacturer. PCR was performed using Pwo-polymerase PCR-kit (Roche, Mannheim, Germany) and the primers listed in Table 5.2 with total DNA preparations as template. Twenty-six thermal cycles (Eppendorf Mastercycler, Hamburg, Germany) were applied on the 50μl reaction mixtures. Each cycle started with 30 seconds at 95°C followed by annealing for 30 seconds at 55°C and elongation for 3.5 minutes at 72°C. Sequencing was performed with the Thermo Sequenase Cy5.0 Dye terminator cycle sequencing kit (Amersham Pharmacia, Uppsala, Sweden), and separation was performed on an Alf sequencer (Amersham/Pharmacia).

Table 5.2. Used primers in this study. Bold are bases that will mismatch, the restriction sites used for cloning are underlined, and the start of the genes are displayed in italic.

Name	Sequence
Nis_292Rev	5'AATTGAACGTTTCAAGCC
Nis_115fw	5'CGGCTCTGATTAAATTCTG
IL1403-10fw	5'GGAATG<u>CC</u>*ATG***G**ATACAGTAGGAGATTACC
IL1403+1374Rev	5'AGAA<u>TCTAGA</u>TCAACTATAAGAGTTCATATGAG
B1157+1793Rev	5'GACTT<u>CTCTAG**A**</u>TTATATCTTGCTGGTT
pGh9:ISS1_ER	5'CCGTTAAATCGACTGGCG
pGh9:ISS1_EF	5'GACTTATCAGAAAACTTTGC
pGh9:ISS1_HR	5'CGGTATCTACTGAGATTAAGG
pGh9:ISS1_HF	5'GCGCGCGTAATACGACTC

Insertion mutant library was constructed based on the system developed by Maguin *et al.* ('92; '96) using the random insertion capacity of pGh9:ISS1. The pGh9:ISS1 plasmid was isolated from *E. coli*:pGh9:ISS1 and transformed to *Lactococcus lactis* B1157. Erythromycin resistant, plasmid-containing transformants were selected at the permissive temperature of 28°C. 25 ml LM17 without erythromycin was inoculated (1%) with B1157:pGh9:ISS1, and cultured at 28°C for 2.5 hours, followed by transfer of the cultures to the non-permissive temperature of 40°C for 2.5 hours to force plasmid integration. Over 8500 integrants were selected on LM17 agar containing 2 μg/ml erythromycin, and individual colonies were organized in 384-well microtiter plates containing LM17 with erythromycin and 20% glycerol, and frozen at -80°C. The frequency of integration was determined to be 2.4%, and the library was randomly validated by southern blotting and restriction analysis. Total DNA of 20 strains from the IS-library was isolated and either cut with *Eco*RI or *Hin*dIII. The obtained fragments were separated on a 0.7% agarose gel,

blotted and hybridised with *Hin*dIII cut, DIG-labelled, pGh9:ISS1 as probe (Roche, Mannheim, Germany). Eighty per cent of the colonies gave different sized fragments. Restriction analysis was also used for validation and for determination of the site of integration. The *Hin*dIII or *Eco*R1 cut total DNA fragments were ligated using T4 ligase and transformed by a heat shock to *E. coli* E10. The transformation mix was plated on TY containing 200 µg/ml Ery. The plasmids of these transformants were isolated, and used for restriction analysis with *Eco*R1 and *Hin*dIII. Plasmid sequencing was performed using the 4 pGh9:ISS1-derived primers. HR and HF primers were used for the plasmids derived from *Hin*dIII digested total DNA's and the ER and EF for the plasmids originating from the *Eco*RI digested total DNA's.

HTS screening of library was performed with a recently developed method for fast screening of volatile compounds produced in miniaturized fermentations (Smit *ea.*, '04b). Briefly, the method is based on the growth of the insertion-mutants in a 96-wells stainless steel block, containing 2 ml wells, filled with 1 ml LM17 with 2 mM KaLeu. The block is closed airtight, and after fermentation for 24 hours at 40°C, individual headspace samples of 100µl were analysed using Direct Inlet Mass Spectrometry (DI-MS).

Cloning of the decarboxylase gene. Three constructs were made: IPD gene from IL1403 (1374 bp), α-keto acid decarboxylase gene from B1157 (1644bp+166bp), and a partial α-keto acid decarboxylase gene from B1157 (1569bp, missing 75 bp)(See also Fig. 5.1). The fragments were obtained by PCR using the following two primer pairs (Table 2): IL1403-10fw / IL1403+1374R and IL1403-8fw / B1157+1793Rev on total DNA of *L. lactis* IL1403 and *L. lactis* B1157, respectively. The PCR products obtained were digested with *Nco*1-*Xba*I, and cloned in similarly digested pNZ8148, resulting in the plasmids pNZ7502 (IL1403-*ipd*) and pNZ7500 (B1157 decarboxylase gene). The PCR product obtained from B1157 was also digested with *Spe*I and *Nco*I, and cloned in to similarly digested pNZ8148, generating pNZ7501 (B1157 decarboxylase
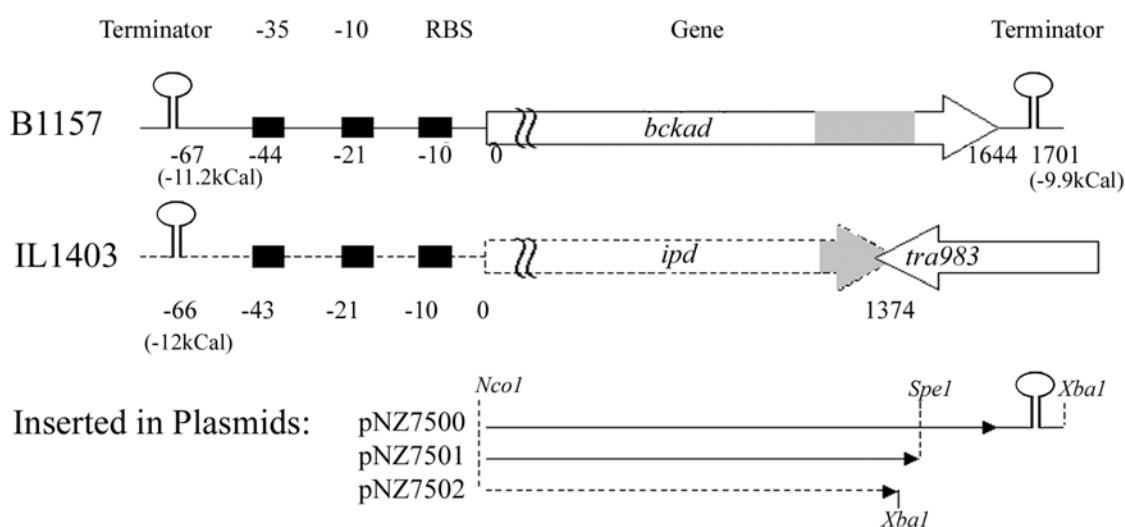

Figure 5.1. Schematic view of *bckad* gene in B1157 (straight line) compared to the *ipd* gene in IL1403 (Dotted lines). The fragments fused with pNZ8148 for over-expression in NZ9000 are shown below these schematic views. The TPP-binding site is schematically shown in grey.

gene lacking 75 bp at the 3'terminus). The identity and integrity of the inserts cloned in pNZ8148 was confirmed by sequencing. All plasmids were introduced in strain NZ9000 (Kuipers *ea.*, '98) and used for nisin-controlled over-expression of the cloned (partial) genes using established procedures (De Ruyter *ea.*, '96).

Preparation of cell free extract (CFE). Cells were harvested by centrifugation and washed twice in 25 mM sodiumcitrate buffer pH=6.5. The cells were either disrupted by the Fast Prep method (Bio101, Savant, Holbrook, NY) two times for 30 seconds or by French Pressing (SLM-instruments, NY) twice with a cell pressure of 20,000 PSI. The supernatant after centrifugation at 20.000g for 15 minutes was used as CFE. All steps were performed below 6°C.

Protein analysis. SDS Page was used to visualize the over-expression of the protein. CFE samples were cooked for 10 minutes after addition of Laemmli buffer. The samples were separated on a 12% polyacrylamide gel in a mini protean II (Bio-Rad, Veenendaal, The Netherlands). Protein concentrations were determined by the BCA Protein Assay Reagent Kit from Pierce Inc. (Rockford, IL). A 30 µl sample was mixed with 240 µl work reagent. After incubation at 37 °C for 20 minutes the OD at 562 nm was measured (Spectra max, Molecular Devices, Sunnydale, CA). Triplicate measurements were done independently.

Enzyme assays. The α-keto acid decarboxylase assay with KaLeu was performed as described previously (Smit *ea.*, '03). In essence, an enzyme sample was incubated for 3 hours at 30° C in a closed 10 ml HS vial at pH=6.3 (50 mM sodiumcitrate) with 10 mM KaLeu as substrate, and 1 mM $MgCl_2$ and 0.1 mM thiamin pyrophosphate (TPP) as cofactors. For the characterisation experiments, the pH was varied between 4 and 9 using sodium citrate, sodium phosphate and TRIS buffers; NaCl was added from 0.05 to 1.4 M; and substrate concentrations were varied from 50µM to 20 mM. After 3 hours reactions were stopped by lowering the pH below 3 using 6 N HCl. The production of 3-methylbutanal was quantified by static headspace gas chromatography (HS-GC) as described before (Smit *ea.*, '03).

The substrate specificity of the decarboxylase was determined by measuring the decrease of the substrate by RP-HPLC or ion exchange HPLC as described in previously (Smit *ea.*, '04a). The reaction proceeded in the same reaction mixture as described above. The amount of cell free extract of strain B2085 was adjusted per substrate used, aiming at a conversion of about 30% in 3 hours. The following substrates were used in a final concentration of 10 mM (all substrates were obtained from Sigma (Zwijndrecht, The Netherlands) or Fisher (Landsmeer, The Netherlands) Note: 2-oxo = α-keto, and for keto acids derived from amino acids abbreviations are given): 2-oxo-4-methyl-pentanoic acid (α-ketoisocaproic acid or KaLeu), 2-oxo-3-methylpentanoic acid (KaIle), 2-oxo-3-methylbutanoic acid (KaVal), 2-oxohexanoic acid, 2-oxopentanoic acid, 2-oxobutanoic acid, pyruvic acid, 4-methylthio-2-oxobutanoic acid (KaMet), phenylpyruvic acid (KaPhe), 3-indol-3-yl-2-oxopropanoic acid (KaTrp or Indole-3-pyruvate), 3-(4-hydroxyphenyl)-2-oxopropanoic acid (KaTyr), 2-oxopentanedioic acid (KaGlu), 4-methylpentanoic acid, 3-methylbutanoic acid and 2-methylpropanoic acid.

Results

Previous work indicated the importance of a branched-chain keto acid decarboxylase in the formation of flavour-active compounds by a number of *L. lactis* strains (Smit *ea.*, '03; Smit *ea.*, '04b). So far, no decarboxylase with activity towards branched-chain α-keto acids have been cloned in *L. lactis*, and neither did the (preliminary) genome sequences of lactic acid bacteria reveal a clear candidate gene for this activity (Bolotin *ea.*, '01; Kleerebezem *ea.*, '03, http://genome.jgi-psf.org/draft_microbes/laccr/laccr.home.html). In order to identify the decarboxylase gene an insertion sequence (IS) mutant library based on *L. lactis* B1157 was
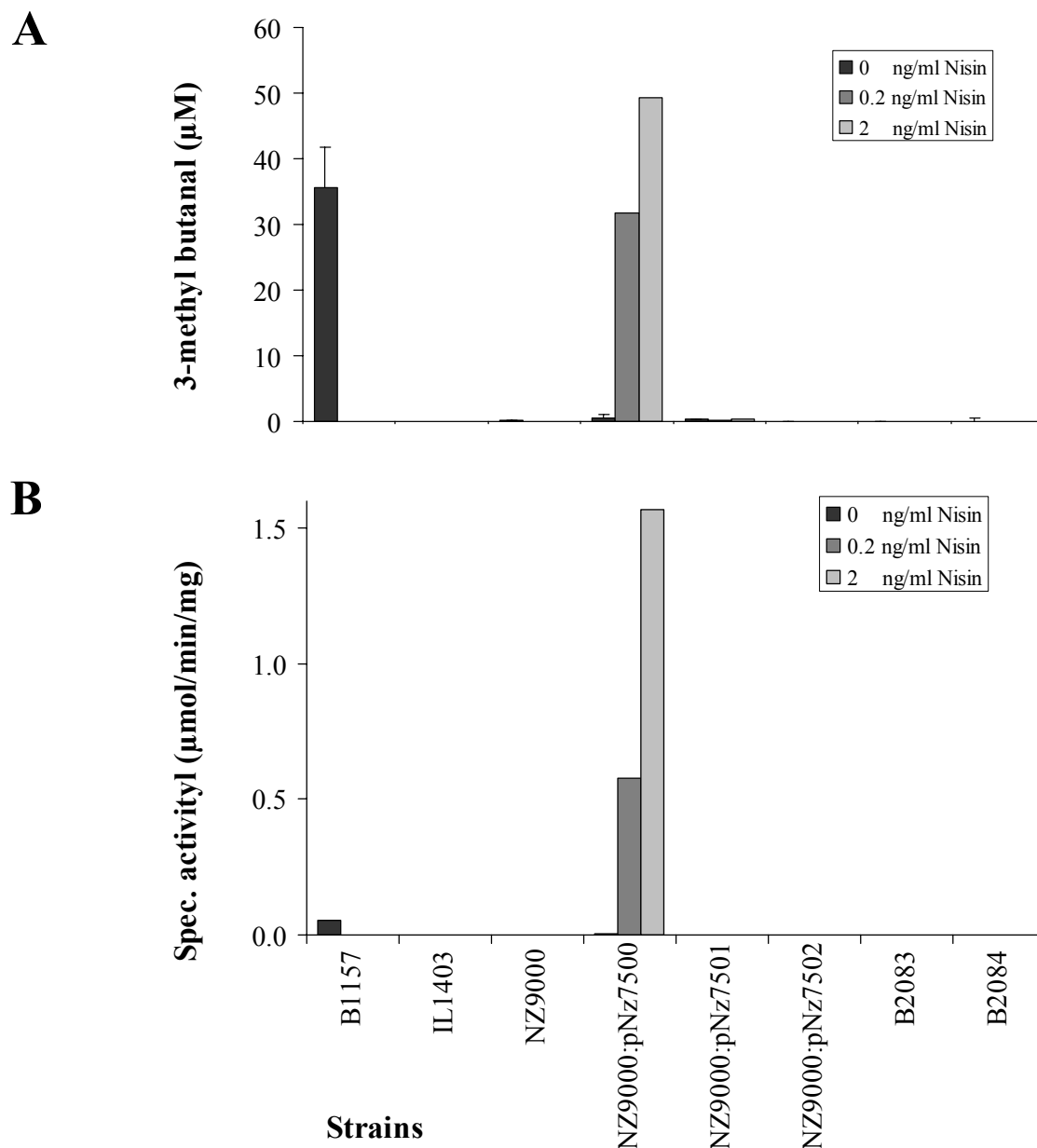
Figure 5.2. Flavour analysis of incubations in GM17 (panel A) and decarboxylase activities (panel B) of (nisin induced) decarboxylase over-expression mutants in relation to the wild type strain B1157, the cloning host NZ9000 and the knock out mutants B2083 and B2083.

constructed as described in the Materials and Methods section. The library was validated by Southern blotting and restriction analysis. Screening of the IS-mutant library using DI-MS as described in the Materials and Methods section, resulted in two 3-methylbutanal-negative strains (B2083 and B2084). The gene was recovered from these strains by cloning the chromosomal region containing the pGhost to *L. lactis* MG1363, and sequencing the obtained plasmids with primers based on pGh9:ISS1. In strain B2083 the ISS element integrated at position 453 of the newly identified gene, and in strain B2084 at position 916 of the same gene. Both strains were not able to produce 3-methylbutanal in fermentations and no decarboxylase activity towards KaLeu could be measured (Fig. 5.2). The whole gene sequence was obtained by primer walking in two directions, and consisted of 1644 bases. The region before the gene contained regions that might respectively function as terminator (-82bp, -11.2 kCal), a promoter (-44bp resp. -21bp), and a ribosome binding site (-10bp). Behind the gene also a putative terminator (-9.9 kCal) was identified starting at bp 1701 (Fig. 5.1).

To prove the activity of the B1157 BcKAD gene product towards KaLeu and the absence of activity of the IL1403 (partial) gene product, strains which over-express the gene (fragments) were constructed as described in the Materials and Methods section and shown in Figure 5.1. All mutant strains and strains B2083, B2084, B1157, IL1403 and NZ9000 were analysed on their protein profile by SDS-page, their ability to produce 3-methylbutanal and on decarboxylase
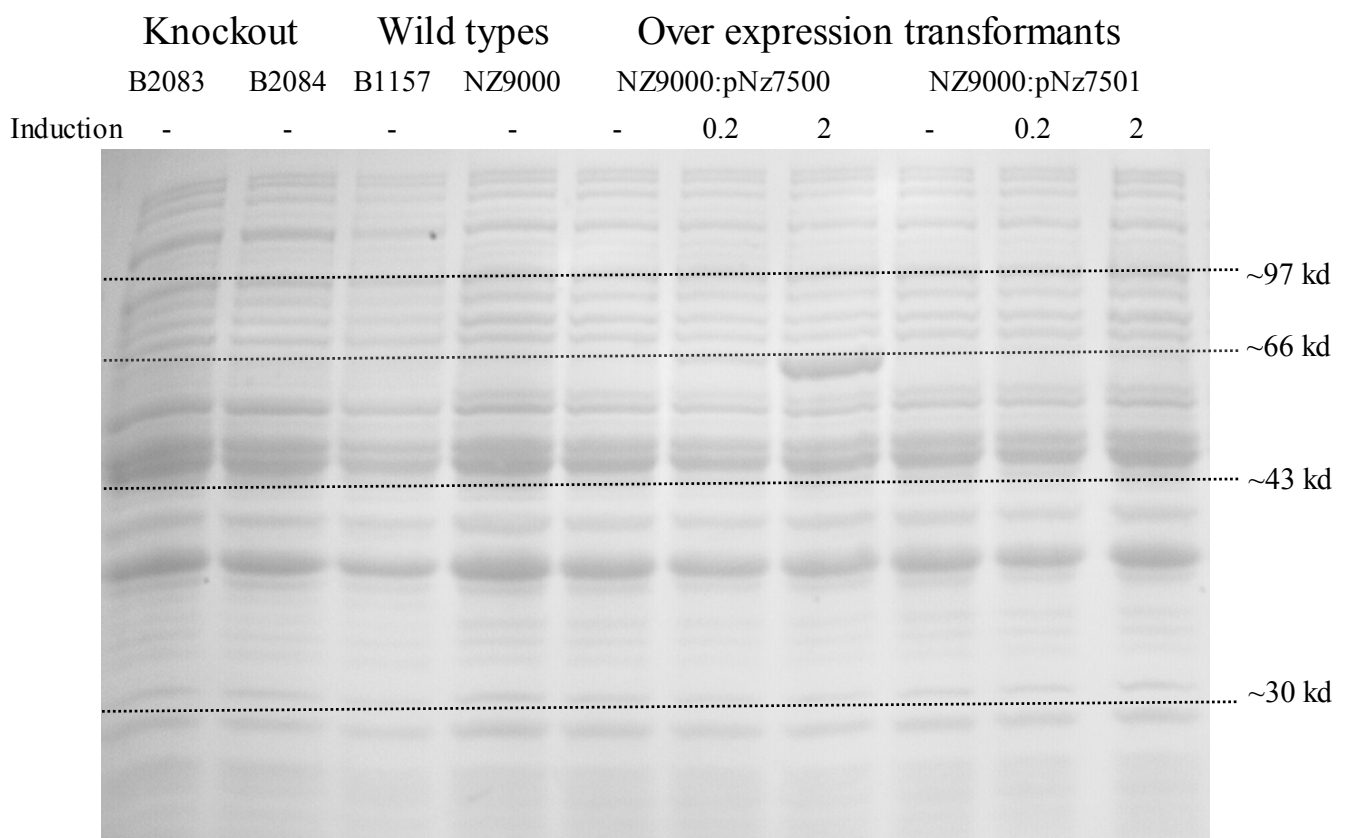
Figure 5.3. SDS Page of knock-out mutants, wild types and decarboxylase over-expression transformants. The over-expression mutants were induced with 0, 0.2 and 2 ng/ml nisin A.

activity towards KaLeu. The results are shown in Figures 5.2 and 5.3. Depending on the nisin concentration, strain NZ9000:pNZ7500 clearly produces an extra protein. By SDS gel electrophoresis the apparent molecular mass was estimated to be 62 kDa, which is in agreement with the predicted mass from sequence (60.9 kDa). The decarboxylase activity in CFE of this induced strain is more than 30 times higher than the wild type, B1157. Although plasmid construction and transformation of the two partial genes (pNZ7501 and pNZ7502) was successful, based on the sequence, no clear over-expression of the gene was obtained with these mutants.

The pH optimum, salt tolerance and substrate specificity are important parameters of enzymes with respect to their potential activity under cheese ripening conditions. As shown in Figure 5.4, the optimal pH was 6.3 with a broad pH activity profile, which corresponds well with known pyruvate and indole-3-pyruvate decarboxylases, described in other micro-organisms. The enzyme activity was not negatively affected, in fact slightly increased at high salinity (tested up to 1.3 M NaCl, data not shown) and the main activity of the decarboxylase was on branched chain α-keto acids, derived from valine, leucine and isoleucine, but also linear α-keto acids with 4 and 6 carbon atoms and the α-keto acids derived from methionine, phenylalanine and tryptophan can be converted by the enzyme. Since the sequence of the identified decarboxylase gene of B1157 shows high similarity with the gene annotated as indole-3-pyruvate decarboxylase in strain IL1403, we were particularly interested in the activity on indole-3-pyruvate (KaTrp), because this is the major substrate for IPD (EC 4.1.1.74). Based on the results shown in Table 5.3 it is clear that the identified enzyme has the highest activity towards branched-chain α-keto acids. α-Ketoglutarate, the substrate of α-ketoglutarate decarboxylase (EC 4.1.1.71) is not converted at all under the conditions tested. The same counts for the branched organic acids, indicating that the
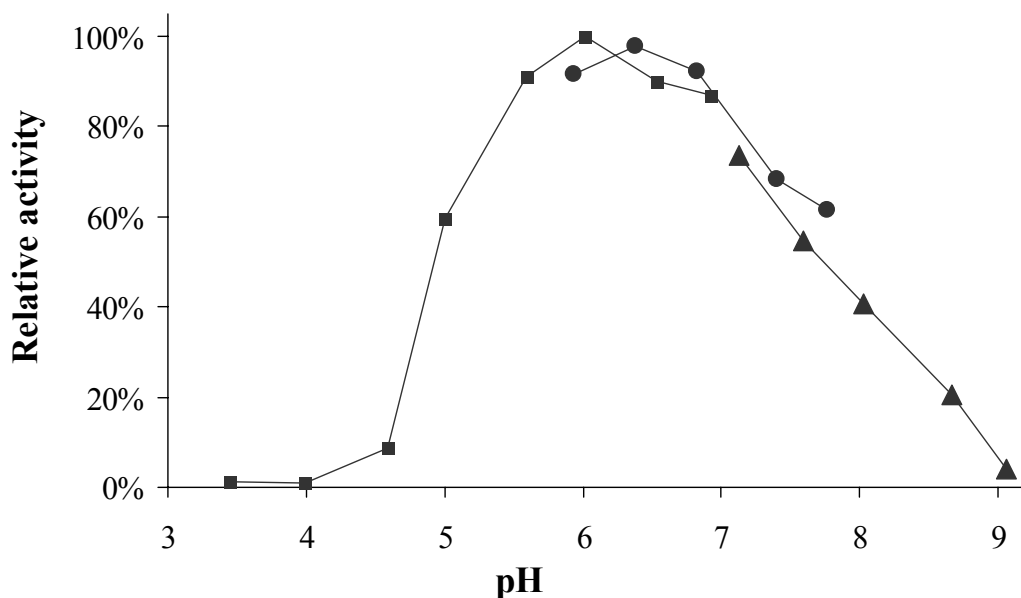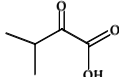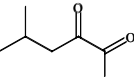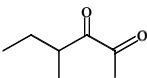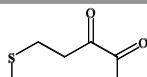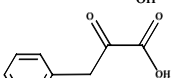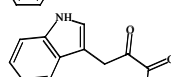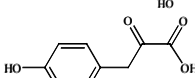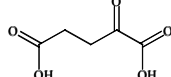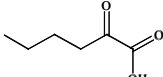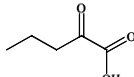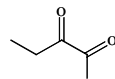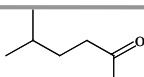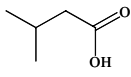

Figure 5.4. Effect of pH on the activity of the BcKAD, measured in 50 mM sodium citrate (■), sodium phosphate (●) or TRIS (▲) buffer with α-ketoisovaleric acid as substrate.

activity of the enzyme is limited to the decarboxylation of certain α-keto acids. Taken together, the identified enzyme has characteristics that might be relevant under cheese ripening conditions and has the highest affinity towards branched-chain α-keto acids.

Table 5.3. Substrate specificity of the B1157 BcKAD

Substrate	Abbreviation	Structure	Relative Conversion Rate
3-methyl-2-oxobutanoic acid	KaVal		~100%
4-methyl-2-oxopentanoic acid	KaLeu		~30%
3-methyl-2-oxopentanoic acid	KaIle		~28%
4-methylthio-2-oxobutanoic acid	KaMet		~8%
2-oxo-3-phenylpropanoic acid	KaPhe		~7%
3-indol-3-yl-2-oxopropanoic acid	KaTrp		~3%
3-(4-hydroxyphenyl)-2-oxopropanoic acid	KaTyr		~1%
2-oxopentanedioic acid	KaGlu		0%
2-oxohexanoic acid			~30%
2-oxopentanoic acid			~25%
2-oxobutanoic acid			~10%
2-oxopropanoic acid			~2%
Isohexanoic acid			0%
Isopentanoic acid			0%
Isobutanoic acid			0%

Discussion

The newly identified gene codes for a branched-chain keto acid decarboxylase (BcKAD), since knocking out the gene in B1157, resulted in non-3-methylbutanal producing mutants, and over-expressing the gene in a non-3-methylbutanal producing strain resulted in a thirty times increase of this activity compared to the wildtype, B1157. Of all substrates tested, the decarboxylase had the highest relative activity towards branched-chain α-keto acids, and can therefore best be named as branched-chain α-keto acid decarboxylase. In respect to the enzyme specificity, the enzyme resembles the earlier described α-keto acid decarboxylase reported by Amarita *et al*. ('01). The protein sequence and SDS gel electrophoresis of the enzyme studied however revealed a molecular mass of about 61 kDa, which is roughly twice the mass reported by Amarita *et al*.

Although the molecular mass and sequence of the gene and protein differed largely from the known branched-chain α-keto acid decarboxylase from *Bacillus subtilis* (EC 4.1.1.72) (Oku *ea.*, '88), comparison of the BcKAD protein sequence with several non lactic acid bacterial IPD-sequences, sequences annotated as IPD, and PDC-sequences by aligning their sequences in Clustal revealed very high global similarity. However these enzymes differ largely in substrate specificity (Koga *ea.*, '92). A blastN and tBlastX search of the newly identified BcKAD gene against the (preliminary) genome sequences of lactic acid bacteria (www.jgi.doe.gov and www.ncbi.nlm.nih.gov) only revealed very high similarity with the *ipd*-annotated open reading frame of *L. lactis* IL1403, while the other lactic acid bacteria seemed to lack the gene. From previous work it is known that only a limited number of lactococci do possess this decarboxylase activity (Smit *ea.*, '03; '04b). Preliminary results using colony PCR on 45 strains, which were also used in earlier studies (Smit *ea.*, '04b), using four primers based on the *bckad* of B1157 indicated a clear correlation between the presence or absence of 3-methylbutanal production of these strains, and the presence or absence of the PCR products (data not shown). Taken together one might speculate that many lactic acid bacteria lack decarboxylase activity due to the lack of the gene. Within the first 1300 bases, the BcKAD gene and IL1403-*ipd* only differs marginally, but 74 bases further in the sequence of IL1403-*ipd* a stop codon and transposable element have been identified (Fig.5.1). The stop codon was found within the TPP binding site, and results in a shorter open reading frame compared to *ipd* genes in other micro-organisms and the *bckad* gene of *L. lactis* B1157. Therefore it is quite logical that expression of the *ipd* gene from IL1403 leads to an inactive decarboxylase, which is in line with the absence of 3-methylbutanal production of cultures of strain IL1403 (Fig. 5.2). Taken together we propose to annotate this gene in B1157 as *bckad* and consequently the *ipd* gene in IL1403 might be considered as a truncated *bckad* gene.

Since the decarboxylase is active at pH=5.3 and at high salinity, which are typical conditions for Gouda cheese ripening, the enzyme has the potential to play an important role in cheese flavour formation. This is also illustrated by the malty flavour of cheese made with strain B1157 (Ayad

ea., '00). Over-expressing the decarboxylase resulted in increased 3-methylbutanal production in fermentations (Fig.5.2). This increase was much less than expected based on the level of over-expression, but this is most probably caused by a lack of the substrate, KaLeu. The formation of KaLeu by transamination has previously been shown to be sub-optimal for the production of Leu-derived flavour compounds under cheese ripening conditions (Yvon *ea.*, '98; Banks *ea.*, '01). A combination of transaminase stimulation and the decarboxylase over-expression mutant might result in a major increase in 3-methylbutanal formation, but also in higher 2-methylpropanal and 2-methylbutanal which are derived from valine and isoleucine, respectively. Future research will focus on the impact of application of these kind of strains in model systems and cheese making, in order to control flavour formation better.

Chapter 6

Discussion and concluding remarks

Abstract

The results described in this thesis add to the understanding of the flavour formation in fermented products like cheese. The main focus is on the existence and relevance of some of the amino acid converting pathways. The leucine degradation pathway has been analysed in detail, and an enzymatic and a non-enzymatic conversion reaction of the keto acid of leucine have been characterised using a newly developed technique for analysing volatile flavour compounds. Also, the α-keto acids of other amino acids appear to be prone to similar enzymatic and non-enzymatic conversions. In order to actually control the fermentation process more than only the presence of a pathway is needed. On the one hand all elements of a pathway may be present, but the actual conversion rate is influenced by a range of regulatory factors, such as environmental conditions, availability of substrates and, enzyme (catalyst) concentration. On the other hand, the absence of a complete pathway does not automatically cause the absence of its end product(s), since also complementary processes have been found in several cases. Studying the regulatory and complementary effects would be promising for understanding the control flavour formation in fermented products.

Key-words

Leucine, valine, methionine, phenylalanine, flavour pathway, Control of flavour formation.

Introduction

The production of volatile aldehydes in fermented products like cheese is very important for the development of the flavour of the product. The formation of these aldehydes depends on the presence of suitable precursors in the product, the presence of the enzymes required in the fermenting organism and the appropriate regulation of their biosynthesis. This thesis mainly dealt with the presence and activity of amino acid catabolism of *Lactococcus lactis*, with the main focus on the conversion of leucine to 3-methylbutanal and 2-methylpropanal. For studying these pathways new methods for the quantitative analysis of these volatile aldehydes had to be developed. These methods appeared to have a much wider applicability than they were originally designed for. The integration of the obtained knowledge on the biochemical route of leucine is described in the next section. An updated (bio-)chemical pathway will be presented, and few additional experiments will be discussed. Increasing only the conversion of leucine to 3-methylbutanal will most probably not be desired for application in fermented products, since it leads to an unbalanced flavour of the end product. Therefore in chapters 4 and 5 also the occurrence of conversions of other substrates was tested; firstly that of branched-chain keto acids, and secondly that of the keto acids of other for flavour development important keto acids like the keto acids of methionine (KaMet) and phenylalanine (KaPhe). The extrapolation of the data obtained on the conversion of these keto acids will be discussed in the third section of this chapter. Knowing that all these pathways exist, and proceed under certain conditions, several starting-points for further research on regulation of these flavour forming pathways are discussed in the last section.

Branched-chain amino acid conversion

The general conversion pathways for amino acids were reviewed in Chapter 1. In addition to Figure 1.4, the knowledge obtained in this research on the conversion of leucine and valine will be presented in this section in a new figure (Fig. 6.1). Both the chemical and enzymatic conversion lead to a branched-chain aldehyde, but the characteristics of these reactions are different. Both the non-enzymatic conversion of KaLeu and the enzymatic conversion of KaVal lead to the same aldehyde, 2-methylpropanal. In this section the characteristics and impact of these conversions are placed in a larger perspective, to be able to speculate in the next section on the conversion of methionine and phenylalanine, and discuss and recommend approaches for applying this knowledge in controlling cheese flavour formation.
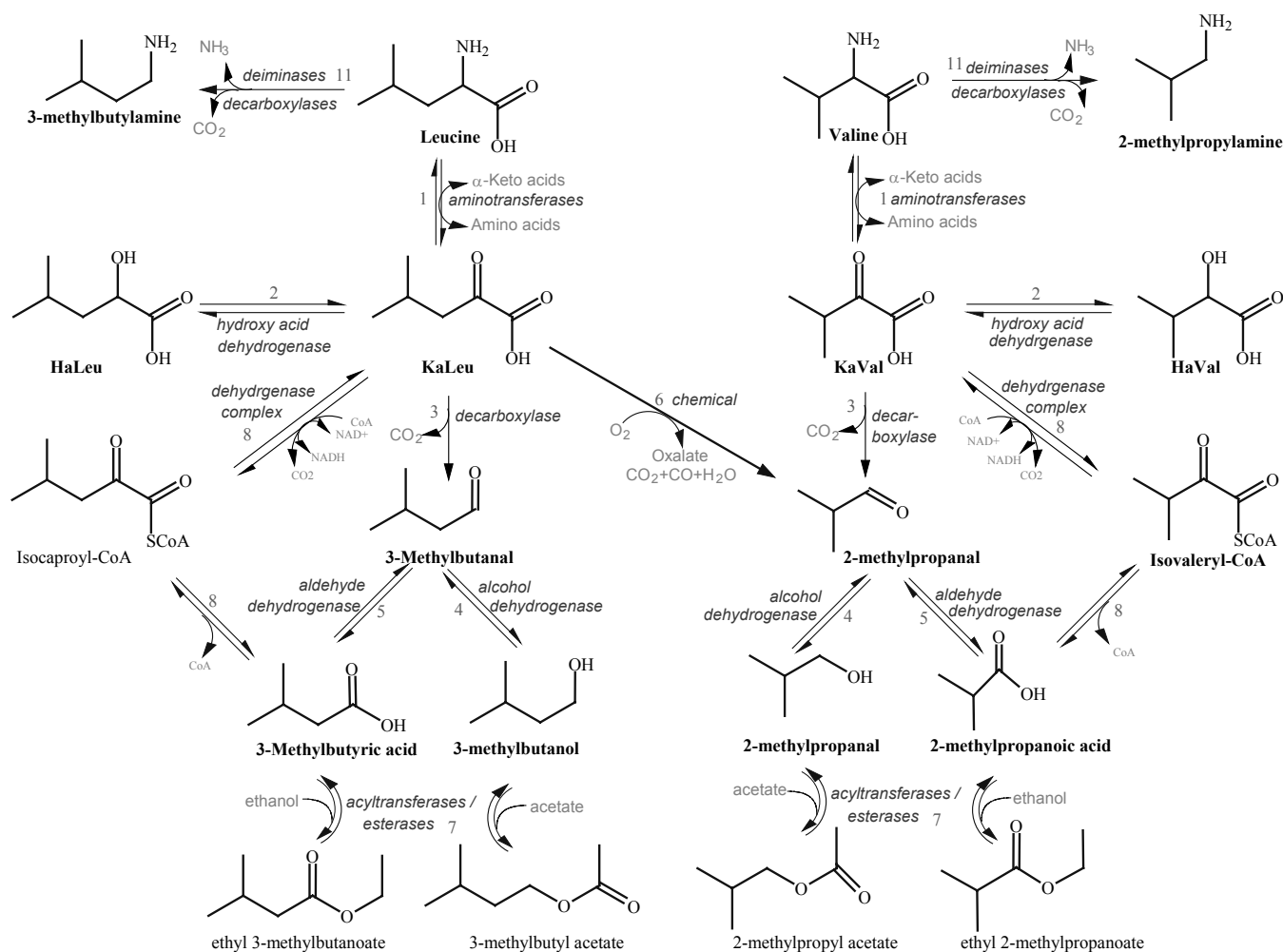
Figure 6.1 Conversion routes of leucine and valine leading to flavour compounds. Flavour compounds derived from these pathways are also mentioned in Table 1.6. Hydroxy and keto acids are abbreviated with Ha and Ka + abbreviation of their corresponding amino acid.

It was generally accepted that amino acids are converted mainly enzymatically, but the characterisation of a chemical conversion mechanism of α-keto acids (Chapter 4, [6] in Fig. 6.1) indicated that this reaction has the potency of influencing the flavour development in cheese. The essence of the reaction mechanism is most probably the formation of a reactive peroxide as a result of a reaction between oxygen and the enol tautomer of the α-keto acid (Chapter 4). The reaction is strongly influenced by the availability of substrates (α-keto acid and oxygen), manganese and the pH (Chapter 4). Although the pH of cheese ripening seems to be rather optimal, the manganese, oxygen and α-keto acid concentration are most probably not. Increasing these concentrations (locally) might therefore be an obvious way for controlling this chemical reaction. High local manganese concentrations might be obtained by selecting strains, which are able to accumulate manganese and lyse later in the ripening process (Archibald, '86; Meijer $ea.$, '98). Since we only observed the catalytic effect of Mn^{2+}, it would be relevant to know in which form the manganese in these bacteria will be present, and what happens with the manganese if

these bacteria lyse. Oxygen is consumed quickly in the fermentation process in semi-hard cheeses, thereafter being almost absent, which is a major difference with the laboratory experiments described in this thesis. Only low concentrations of oxygen were needed for the non-enzymatic conversion to proceed, and the oxygen concentration at the edge of cheese might be increased marginally, by selection of specific coatings. On the other hand, on the surface of smear-type cheeses and fungal-ripened cheeses the situation might be totally different. Nevertheless, in the cheese the oxygen concentration is likely to be rate-limiting, and it would therefore also be interesting to test whether other oxidising agents can trigger this reaction. The formation of the α-keto acid can be increased by increasing the conversion by the transaminase [1], or decreasing the conversion by other α-keto acid converting enzymes. The hydroxy acid dehydrogenase [2] is the most obvious target for this approach, since the conversion of α-keto acid by this enzyme is relatively high (Yvon *ea.*, '98). Approaches for increasing the transaminase activity have been reviewed in section 1.5 of the general introduction of this thesis. All the variations described could easily be tested in model systems, like cheese paste. As more knowledge becomes available, the reaction might turn out to be (more) relevant for other fermented products as well, e.g., sourdough bread, dry sausages, and some alcoholic beverages.

The enzymatic decarboxylation (Fig. 6.1 [3]) of KaLeu leads to 3-methylbutanal instead of 2-methylpropanal. Studying this pathway in several dairy related micro-organisms revealed that the aldehyde, 3-methylbutanal, was only formed in considerable amounts in the few strains possessing α-keto acid decarboxylase activity (Chapter 2 and 3). In contrast, the flavour compound 3-methylbutyric acid, which can be produced by subsequent oxidation of the aldehyde, has been found in many LAB. The lack of aldehyde, but presence of organic acid, was most probably not caused by the rapid conversion of the aldehyde into the organic acid, but by a supplementary enzyme (complex) [8] (Yvon *ea.*, '01; Thierry *ea.*, '02b). Selecting strains with high decarboxylase activity, and subsequent application of such strains, might therefore be a good approach for increasing 3-methylbutanal production levels. In addition to this, knowing the characteristics of the decarboxylating enzyme will give information about the substrate specificity, and will offer opportunities to regulate the activity of this enzyme in these strains. In order to identify the gene, several approaches can be used, among them enzyme purification and identification of the corresponding gene, followed by over-expressing this gene. Enzyme purification did in our case not result in a protein sample, pure enough for determining the terminal protein sequences, and the Michalis-Menten parameters, K_m and V_{max} (Data not shown). Genetic analysis of the 3-methylbutanal producing *L. lactis* B1157 revealed a large plasmid of roughly 23 KB, which is not present in decarboxylase-negative strains. Since plasmids often carry additional characteristics of one strain compared to another, the plasmid of B1157 was partially characterised by expressing it in MG1363. This plasmid possessed lactose fermenting genes, tetracycline and chloramphenicol resistances, but no decarboxylase activity could be detected in this MG1363 mutant. Finally the enzyme was identified using mutant libraries, as discussed in chapter 5. Three over-expression mutants were created in the decarboxylase-negative

L. lactis NZ9000: (i) the *L. lactis* B1157 gene; (ii) the B1157 gene, lacking a C-terminal region; and (iii) the gene annotated in *L. lactis* IL1403 as *ipd*. Only the first mutant resulted in an increased decarboxylase activity (thirty times). The partial gene products were inactive. The data show that the enzyme is the major α-keto acid decarboxylating enzyme in *L. lactis* B1157, and that absence of decarboxylase activity in IL1403 is most probably due to an incomplete gene. Initial experiments with the over-expression mutant showed an increased 3-methylbutanal production (Chapter 5), but this increase (1.5x) was much lower than the increased specific enzyme activity (30x) which was measured in cell free extract of this mutant. This suggests a second rate-limiting step in the route, which is most probably the availability of the substrate, α-keto acid (Yvon *ea.*, '98; Banks *ea.*, '01). See again section 1.5 for a description of ways to increase the α-keto acid concentrations.

In Chapter 5 the partial characterisation of the enzyme, based on the over-expression mutant was, described. The optimal pH was 6.3, the enzyme was well resistant to high salinity and reasonably stable during repeated freeze-thaw cycles (about 80% of the activity was retained after 3 cycles in 50 mM citrate buffer pH=6.5). The highest activity was measured on branched-, and straight-chain α-keto acids with 4-6 carbon atoms, but also the keto acids of methionine, phenylalanine and tryptophan were converted with a relative conversion rate of about 10% compared to the conversion of KaVal under the conditions tested. The very high activity on the α-keto acid of valine and other branched-chain α-keto acids, suggests that the enzyme is best described as a branched-chain α-keto acid decarboxylase (BcKAD). Comparison of this enzyme with other enzymes revealed that the sequence and molecular mass are very similar to IPD and PDC, but not to the branched-chain α-keto acid decarboxylase described by Oku ('88) and the molecular mass reported by Amarita ('01).

Surprisingly, searching the genomes of other LAB did not result in any genes for similar decarboxylating enzymes, suggesting that this gene is absent in these genomes (Chapter 5). To get a better impression on the occurrence of this specific gene in *Lactococcus*, colony PCR was performed on 45 strains. Four primers were used, one located at the end of the gene, the part strain IL1403 lacked. Four PCR products were therefore expected in strains containing the decarboxylase, two PCR products were expected in IL1403 and similar strains lacking the end of the gene, and no products were expected in strains lacking the decarboxylase gene. About 70% of the non-3-methylbutanal producing strains lacked all four PCR products, and 28% lacked the end of the gene. PCR on twelve 3-methylbutanal producing strains resulted in three or four bands in respectively three and six of the strains. Summarising, the vast majority of the PCR results (80%) corresponded with the flavour forming abilities of the strains, but 20% of the strains seemed to lack one, sometimes two, PCR bands. This might be due to biological diversity or a PCR error. Because this experiment does not deliver the proof for the absence of a KaLeu decarboxylating gene, further research, by for example Southern blotting, is desired.

The *bckad* gene sequence was highly similar to the sequences of known *pdc* and *ipd*. The crystal structures of these enzymes are available (Koga, '95; Dobritzsch *ea.*, '98; Lu *ea.*, '00; Schutz *ea.*, '03), and the PDC complexed with pyruvamide of *Saccharomyces* has been used to model the B1157 *bckad* gene and the gene of IL1403 annotated as *ipd*. The BcKAD could easily be folded similar to the PDC. In contrast to what one might expect based on the difference in substrate specificity, the substrate binding site of this model was very similar to the substrate binding site of PDC. All essential residues were equal, and occupied the same position in space. Therefore the preference for larger substrates could not be explained with this model. The IL1403 lacked the end of the gene, which contains Glu477. This amino acid is important for catalysis, which might also explain the unsuccessful over-expression of this gene in NZ9000.

Taken together, the research described in this thesis has shown that some lactococci do posses the decarboxylase, which can highly increase the flavour formation from α-keto acids derived from branched-chain amino acids, as shown in Figure 6.1, reaction [3]. Furthermore, the chemical reaction as described for the conversion of phenylalanine [6], has been proved to be relevant for a whole range of α-keto acids. The conditions for optimal conversion differ largely, which offers opportunities for regulation. The chemical reaction needs the reactant oxygen and the catalyst manganese. It proceeds maximally at pH =5.5 and 8, while the enzymatic reaction depends on a TPP and magnesium dependent enzyme which works optimal at pH=6. Both reactions seem rather insensitive for high ionic strength (1.5 M NaCl). Based on these characteristics, it might be expected that both conversions could be stimulated in the ripening process, and future research should focus upon this. However, these reactions will proceed at much lower rates in cheese. Especially the lack of oxygen, and the state of the manganese ions, will have a limiting effect on the non-enzymatic conversion, while the enzymatic reaction appears to be limited in the enzyme and/or substrate concentration. In the next section extrapolation of this knowledge to the conversion of other amino acids is discussed.

Conversion of other amino acids to flavour compounds

Similar to transamination, both the chemical conversion and enzymatic decarboxylation described are not limited to branched chain amino acids (see Tables 4.2, 5.3 and Chapter 1.4). Therefore a general pathway was presented in the introduction (Figure 1.6). Depending on the characteristics of the substrate, (flavour) characteristics of the end products, and the conversion rates might differ largely. Therefore also the specific pathways for phenylalanine and methionine degradation are shown here in more detail in (Fig. 6.2 and 6.3). It has been shown that the lyase pathway can greatly enhance flavour formation from methionine, but also other pathways have to be present, since knocking out the lyases did not result in absence of methionine-derived flavour compounds [15](Fernandez *ea.*, '00a). In Chapter 4 we have shown that KaMet is chemically

converted under the same conditions as KaLeu, and recently methylthioacetaldehyde has been identified as the product [6](Yvon *ea.*, '02). The subsequent conversions have not yet been studied in detail, but are believed to proceed readily. In Chapter 5 we also showed the enzymatic decarboxylation of methionine [3]. The most obvious product of this reaction is methional, a flavour compound that is found in many fermented dairy products (Table 1.2). Although the enzyme is active under cheese-like conditions, the actual conversion in cheese has not been shown yet. The conversion from methional to methanethiol has also been suggested (Engels, '97) and resembles the reaction of methylthioacetaldehyde to methanethiol [16 in Fig. 6.2], but this reaction has not been characterised in relation to fermented products yet.
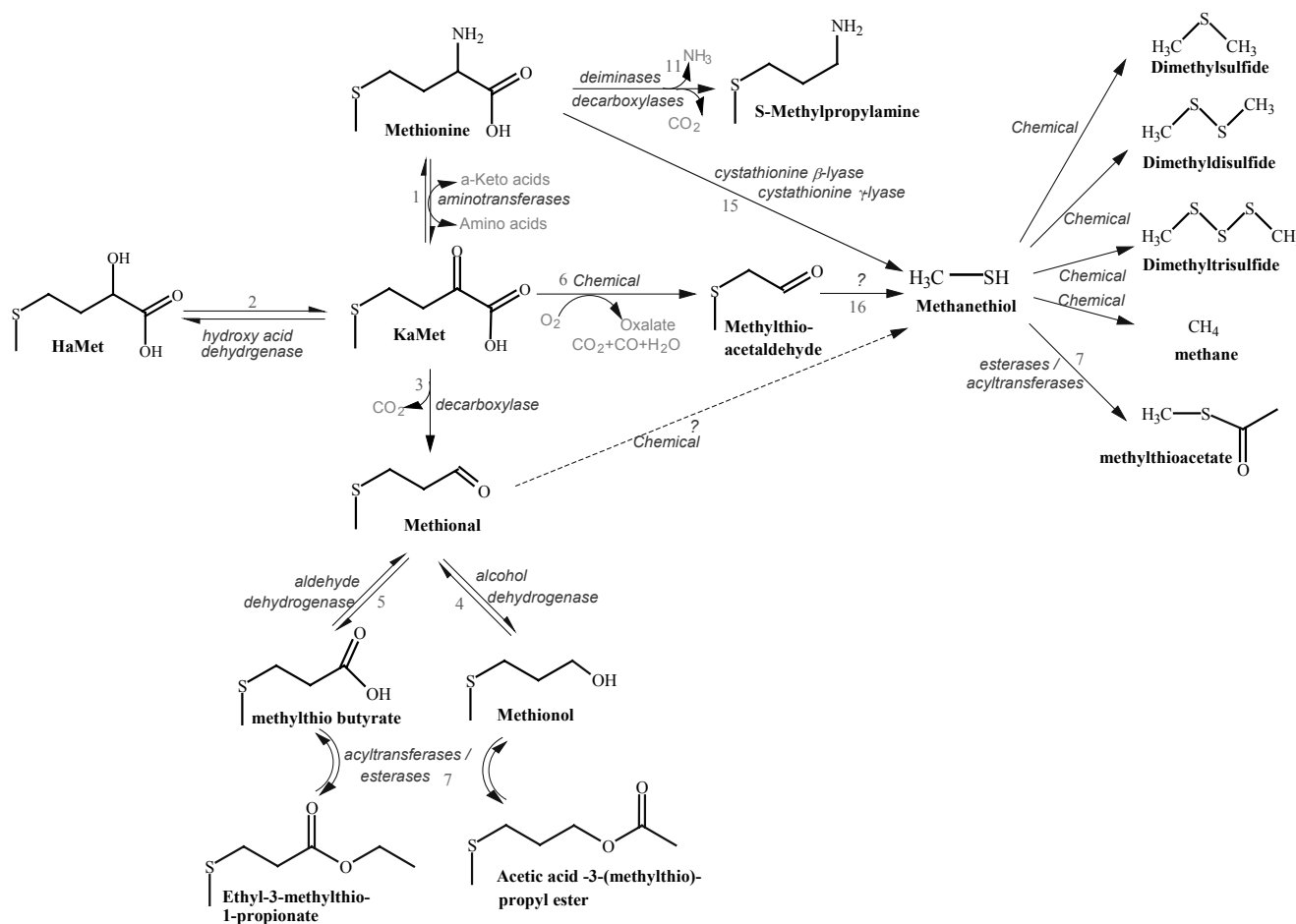
Figure 6.2 Possible conversion routes of methionine, mainly leading to flavour compounds

In the research described in this thesis, the chemical conversion of phenylpyruvic acid (KaPhe) to benzaldehyde was confirmed, and characteristics and by-products of this reaction were identified [6]. We also found that benzaldehyde was the main product (data not shown). Besides benzaldehyde, also the formation of phenyl acetic acid has been reported to proceeded chemically, and the formation of phenylglyoxylic and mandelic acid has been supposed to be chemical as well(Nierop Groot *ea.*, '98). No details on the conversions between these compounds

were found in literature, but it can be assumed that the rather unspecific hydroxyacid dehydrogenase [2] is also able to inter-convert phenylglyoxilic acid and mandelic acid.

Production of the flavour compound acetophenon in smear ripened cheeses has been described as deamination of phenylalanine to ß-phenylpropionic acid followed by few unspecified steps to acetophenon and benzoic acid (Bosset *ea.*, '90). In addition to the non-enzymatic conversion, also the enzymatic decarboxylation of KaPhe [3] has been reported in this thesis (Table 5.3). Enzymatic decarboxylation of KaPhe proceeded with a similar relative conversion rate as KaMet (see Table 5.3). In this case the most obvious product would be phenylacetaldehyde. This important flavour product can be converted to phenylethanol, or phenylacetic acid by dehydrogenases [4,5].

The relative conversion rates of the chemical and enzymatic conversion of the individual
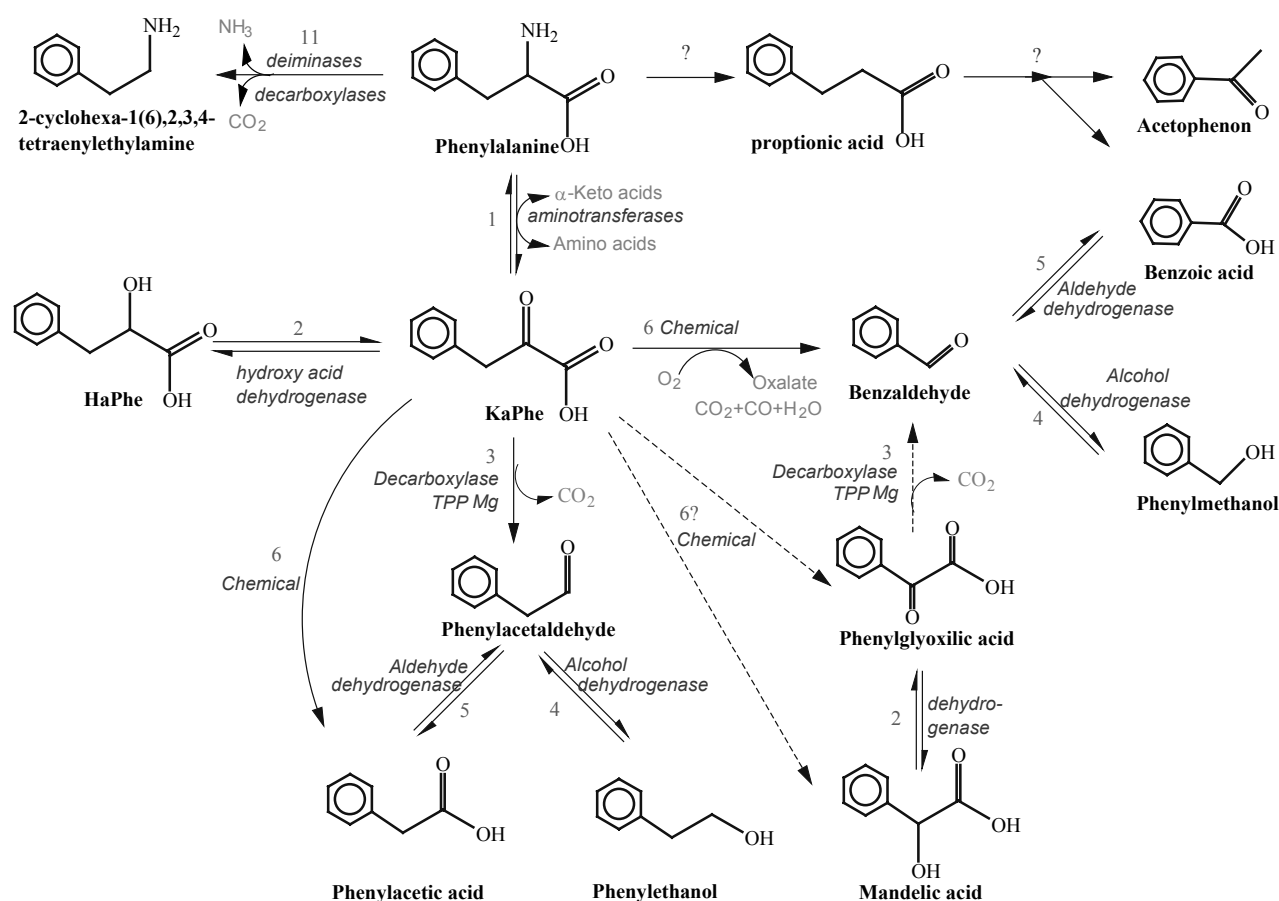
Figure 6.3 Possible conversion routes of phenylalanine, mainly leading to flavour compounds

substrates differ largely (Tables 4.2 and 5.3). While the aromatic KaPhe (100%) and KaTyr (54%) are converted very well non-enzymatically, enzymatically they are only converted with about 10% of the conversion rate of KaVal (100%). This is different for the branched-chain α-keto acids. Enzymatically they are the preferred substrates, but non-enzymatically, KaLeu is converted (29%), while KaVal and KaIle are hardly converted (2 and 3%). The latter difference

can be explained by the methylated ß-carbon atom in KaVal and KaIle, which participates in the chemical conversion. If both the chemical and enzymatic conversion proceed considerably in cheese, these differences in relative reaction rates can be relevant for deciding how to direct an adjustment in the flavour development of cheese.

Controlling flavour formation

The research described in this thesis is a part of research towards controlling flavour formation in fermented products. Especially the presence and characteristics of (elements in) biochemical pathways were studied. Although this information is important, it is as such not enough to control the flavour formation. Therefore, this section contains some relevant aspects with regard to potential control points are discussed.

On the one hand, knowing that a certain pathway is present does not automatically result in desired conversion(rates). The actual conversion by (bio-)chemical pathways is influenced at several levels, amnoung them (i) environmental factors such as substrate and cofactor availability, pH, redox potential, hydrophobicity, ionic strength, etcetera., and (ii) apparent catalyst concentration, which in the case of enzymes is influenced by several types of regulation. On the other hand the absence of a complete route does not automatically cause the absence of its end product(s). An incomplete pathway can for example be complemented by the presence of additional pathways, presence of side activities of enzymes, or occurrence of chemical conversions. For example, the existence of the branched chain dehydrogenase complex in LAB indicates that the branched-chain keto acid decarboxylase is not essential for the formation of branched-chain fatty acids (this thesis and Yvon *ea.*, '01). Another example, discussed in this thesis, is the appearance of non-enzymatic conversion of α-keto acids, that like the enzymatic decarboxylation results in branched chain aldehydes.

Changing process parameters is an important approach for influencing (bio-)chemical conversions, and this can influence several environmental factors, which might of course also influence the apparent catalyst concentrations. The cystationine lyase and peptidase expression levels are for example often strongly regulated by components in the medium (Guedon *ea.*, '01a; Guedon *ea.*, '01b; Fernandez *ea.*, '02). Changing the (mix of) fermenting micro-organisms is another approach for influencing the conversion rates. This approach has been mentioned at several places in this thesis, but since this approach can be targeted in many ways, some examples will now briefly be addressed.
(i) Classical mutagenesis aims at the selection of desired properties from a more or less randomly genetically altered culture. However, this approach is often labour intensive, and rather aspecific.

This process can be speed up by the newly developed high throughput screening methodologies such as the method described in this thesis.

(ii) Co-operation between various strains in a defined starter culture can result in complementation of biochemical pathways. An example is the production of 3-methylbutanal by a mixed culture consisting of a protease-positive, keto acid decarboxylase-negative strain and a protease-negative, keto acid decarboxylase-positive strain (Ayad *ea.*, '01; Smit *ea.*, '02a). The aspect of co-operation for specific enhancement of flavour compounds is rather new, and has the advantage that the properties of the strains do not have to be altered. This approach depends however on the availability of culture collections and, again, fast screenings techniques.

(iii) Lysis of bacterial cells leads to the liberation of the cell contents in to the cheese matrix. The environment in the cheese matrix differs largely from the intracellular environment, with respect to pH, presence of energy sources like ATP, substance concentrations, etcetera. Therefore, many conversions will be influenced by lysis of the strains. This has especially been studied in relation to proteolysis and peptidolysis. Lysis of cells can be triggered by several factors, e.g. changes in the environment (pH, T), growth stage of the organism and presence and sensitivity to bacterial phages. Several systems have been developed for induced and controlled lysis of bacterial cells. Proteolysis is in cheese generally enhance by lysis of starter cells (Visser, '93; De Ruyter *ea.*, '97; Meijer *ea.*, '98). However, it will depend on the type of enzyme(system) whether lysis will improve the activity or not. For instance, enzymes which require cofactors or co substrates (e.g., PLP, NAD, NADP) might be negatively affected by lysis of the cells. Studying the effect of lysis on relevant conversions would be an obvious approach to continue this research on the control of flavour formation.

(iv) Modern biotechnology offers the possibility to introduce very specific changes in the properties of a micro-organism, but is not yet allowed in food applications in various countries. Because this approach is very targeted, and fast, it is mainly used to study certain properties before applying other (non-genetic) approaches to obtain a desired product modification.

Controlling flavour formation by enhancing specific (bio)-chemical routes might lead to an unbalanced flavour profile. To some extend this could be desirable in developing new varieties, but when aiming primarily at accelerated ripening, this unbalance is undesired. In this thesis, much attention was paid to the specificity of reactions, and diversity of routes. Stimulating a pathway will mostly result in the increase of several flavour compounds. Therefore, to be able to actually control flavour formation, the effects of changing control points (such as the ones mentioned in this section) should be studied in more detail, especially in relation to the application.

Summary

Fermentation of food can contribute greatly to longer and safer use, to the nutritional value, perception and texture of a product. Especially nutritious foods, such as milk, are often fermented to a wide variety of products by various micro-organisms. Lactic acid bacteria like *Lactococcus lactis* are often used for the fermentation of milk into various cheeses. Most lactic acid bacteria convert lactose into lactic acid, thereby lowering the pH. This decrease in pH, together with the addition of salt and the removal of water results in a stable product. Besides production of acid, micro-organisms like lactic acid bacteria often produce other metabolites from milk sugar, protein and fat. These reactions primary take place for own maintenance and growth, but the resulting low molecular compounds sometimes have strong flavour characteristics. The most dominant flavour compounds (key-flavours) can be isolated and identified. Their subsequent recombination usually results in a good impression of the original product-flavour. Many key flavours in semi-hard cheeses, such as Gouda, are derived from protein degradation. The biochemical conversion from protein to flavour compounds starts with proteolysis and peptidolysis, which results in free amino acids. These amino acids can be converted in several ways, leading to a large variety of (flavour) compounds. This thesis focuses on the production of one class of these compounds as a model system: aldehydes, in particular the key-flavour 3-methylbutanal, which is derived from the amino acid leucine. The aim of this research is to gain insight in the formation pathway of this kind of flavour compounds, in order to improve the control of flavour development during fermentation.

Various micro-organisms, belonging to the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Bifidobacterium*, *Propionibacterium*, *Brevibacterium*, *Corynebacterium* and *Arthrobacter*, used in dairy fermentations such as cheese making, were analysed for their potential to convert leucine into flavour compounds, most notably 3-methylbutanal. A large variation between and within species was observed for various enzyme activities involved in the conversion pathway, e.g. transaminases, α-hydroxy acid dehydrogenase and α-keto acid decarboxylase. In particular, α-keto acid decarboxylase activity, leading to 3-methylbutanal, was found to be present in only two of the strains tested, one of them being *Lactococcus lactis* B1157. Therefore, it is proposed that this activity is rate-controlling in the conversion pathway leading to the flavour compound 3-methylbutanal.

Identification and characterisation of this α-keto acid decarboxylase should provide more information which can be used in controlling the pathway towards 3-methylbutanal. Enzyme purification and bio-informatic tools did not lead to the proper identification of the enzyme, and

therefore mutant libraries were created, consisting over 8500 mutants of *L. lactis* B1157 with a possible deletion in 3-methylbutanal formation. In order to screen these libraries a fast and reliable method was needed for screening miniaturised fermentations on the production of 3-methylbutanal. Such a high throughput screening method could also be used for screening large culture collections, in order to select strains with certain properties for application in cheese, and, in general, to study the biodiversity of micro-organisms on the level of metabolic routes. A method was developed based on measuring volatile metabolites by direct-inlet mass spectroscopy (DI-MS). Small headspace samples (100µl) from culture vials in a 96-well format were directly injected into the MS. In one day over 1500 samples could be analysed. The coefficient of variation for the response was less than 5%. The effectiveness of the method was illustrated by screening 72 strains belonging to the genus *Lactococcus* in quadruple on the production of the key-flavour compound 3-methylbutanal. Furthermore, knowledge of the biochemistry and physiology of 3-methylbutanal formation was used to optimise the composition of the growth medium to enhance 3-methylbutanal production, and thereby improve the screening.

Screening the mutant libraries grown in medium optimised for 3-methylbutanal production, resulted in two independent knock-outs, which did not produce the aldehyde. From these knock-out mutants the gene encoding for the decarboxylase was identified. The gene is highly homologous to the gene annotated as *ipd* in *L. lactis* IL1403, which gene product is probably inactive due to a deletion in the C-terminal part of the gene. A decarboxylase over-expressing mutant was used for further characterisation of the decarboxylase enzyme. The molecular weight of the enzyme is 60.9 kDa, its estimated pI is 5.03, its enzyme activity was hardly affected by high salinity and optimal activity was found at pH=6.3. Moreover, of all potential substrates tested, the activity towards branched-chain α-keto acids was the highest, for which reason it is proposed to be annotated as BcKAD.

Although the formation of flavour compounds from branched-chain α-keto acids in fermented foods like cheese is believed to be mainly an enzymatic process, the conversion of phenyl pyruvic acid, which is derived from phenylalanine, proceeds chemically. α-Ketoisocaproic acid is converted in a similar reaction to the aldehyde 2-methylpropanal. A manganese-catalysed reaction mechanism is suggested for this conversion. The mechanism involves keto-enol tautomery enabling molecular oxygen to react with the ß-carbon atom of the α-keto acid, resulting in a peroxide. This peroxide can react in several ways leading to instable dioxylactone or tetrahedral intermediaries. These intermediary compounds will break down into an aldehyde and oxalate or carbon oxides. All the α-keto acids tested were converted at pH=5.5 in the presence of manganese, nevertheless their conversion rates were rather diverse. This chemical reaction might open new ways for controlling cheese flavour formation with the aim of acceleration of the ripening process, or diversification of the flavour characteristics.

The results described in this thesis add to the understanding of the flavour formation in fermented products like cheese, but focuses mainly on the existence and relevance of some of the amino acid converting pathways of certain bacterial strains. The leucine biodegradation pathway has been analysed in detail, and a chemical conversion reaction and a decarboxylating enzyme have been identified, using a newly developed technique for analysing volatiles. Both the enzymatic and chemical conversions were not limited to one substrate, but have the potency for converting the α-keto acids of other amino acids too. The rate-limiting factors for these conversions are totally different. The decarboxylase activity depends mainly in its presence, and the presence of the substrates, and the chemical conversion in cheese will probably be limited by the availability of oxygen, and possibly manganese. Besides the existence of the reaction, also the actual conversion rates are very important, and these are influenced by a set of factors, such as several types of regulation, environmental parameters (substrate and cofactor availability), and processes like cell lysis. Studying these areas would be very useful for increasing the possibilities for controlling the ripening process. Besides these regulatory effects, also complementary effects are very interesting for new ways for process controls. In this respect one might think of the presence of alternative (chemical) routes, or co-operation between several organisms. Both the regulatory and co-operative processes have only been studied marginally in regard to flavour formation in fermented products, and are therefore recommended for further research.

Samenvatting

Door fermentatie kunnen voedingsrijke grondstoffen omgezet worden in voor de consument aantrekkelijke producten met een meerwaarde wat betreft houdbaarheid, consistentie, smaak en een verhoogde beschikbaarheid van micronutriënten zoals vitaminen. Melk is zo'n grondstof die door fermentatie met verscheidene micro-organismen en enzymen wordt omgezet in een grote verscheidenheid aan gefermenteerde producten, zoals kaas en yoghurt. Bij dergelijke fermentaties is een belangrijke rol weg gelegd voor melkzuurbacteriën, zoals *Lactococcus lactis*, die de melk verzuren. Verzuring leidt tot remming van de groei van bederf-veroorzakende micro-organismen, hetgeen de houdbaarheid van het product vergroot. Door omzetting van de melkbestanddelen, melksuiker (lactose), vet en eiwit (caseïne), kunnen de melkzuurbacteriën bovendien belangrijke smaak- en aromacomponenten vormen. Dit onderzoek was gericht op de moleculaire mechanismen van de vorming van enkele in kaas belangrijke geur-/smaakcomponenten.

De belangrijkste vluchtige aromaverbindingen kunnen worden bepaald door analytische identificering te koppelen aan de menselijke perceptie. Dit kan bijvoorbeeld door gebruik te maken van gaschromatografie in combinatie met olfactometrie. Bij alle soorten kaas blijkt een aantal uit melkeiwit gevormde smaak componenten een rol te spelen. Eiwitafbraak start buiten de bacteriële cel, door proteases van de startercultuur, het stremsel, enzymen uit de melk zelf en soms verontreinigingen of activiteiten van niet-starter micro-organismen. Het resultaat van deze eiwitafbraak zijn peptiden die vervolgens zowel intra- als extracellulair verder afgebroken kunnen worden tot vrije aminozuren (peptidolyse). Hoewel deze aminozuren zelf vaak een karakteristieke smaak hebben, zijn ze vooral belangrijk als substraat voor verscheidene vervolgreacties die in de cel kunnen plaatsvinden. De belangrijkste aminozuurafbrekende route wordt geïnitieerd door transaminases, die aminozuren omzetten in de corresponderende α-ketozuren. Deze ketozuren zijn belangrijke componenten in de aminozuurafbraak en worden door verschillende enzymen verder afgebroken (zie Fig. 1.4). Reductie van α-ketozuren leidt tot de corresponderende α-hydroxyzuren, welke geen aromatische eigenschappen hebben en niet verder omgezet worden. Oxydatieve decarboxylering door een dehydrogenase complex leidt tot een organisch zuur. Dit zuur kan echter ook gevormd worden door het ketozuur eerst te decarboxyleren tot het corresponderende aldehyde en vervolgens dit aldehyde te oxideren tot het organische zuur. Het aldehyde kan in deze route echter ook gereduceerd worden naar het corresponderende alcohol. Zowel het aldehyde, alcohol als het organische zuur zijn belangrijke aromacomponenten, waarbij het aldehyde verreweg de laagste smaakdrempel heeft (Tabel. 1.6). In dit onderzoek is de vorming van aldehyden uit aminozuren bestudeerd, en met name is naar de limiterende stap in deze vorming gezocht. Het doel hierbij was het genereren van kennis, die kan leiden tot het stuurbaar maken van de aromavorming door de micro-organismen. Er is gekozen

voor onderzoek naar de productie van 3-methylbutanal uit het vertakte aminozuur leucine, om het vervolgens als modelsysteem voor soortgelijke omzettingen te gebruiken. 3-Methylbutanal heeft een dominant moutig/chocolade achtig aroma, die in te grote hoeveelheden zelfs leidt tot een zogenaamde "off-flavour". Daarnaast wordt de precursor voor dit aldehyde, leucine, in relatief grote hoeveelheden vrijgemaakt uit het melkeiwit (tabel 1.5). Verder zijn er twee andere vertakte aldehyden die via een vergelijkbare route worden gevormd en die qua smaakgewaarwording op 3-methylbutanal lijken. Tot slot beschikten we ook over een stam, waarvan bekend was dat hij in tegenstelling tot veel andere stammen in staat is aanzienlijke hoeveelheden 3-methylbutanal te produceren. Deze stam heet *Lactococcus lactis* B1157.

In hoofdstuk 2 wordt beschreven hoe onderzocht is welke stap in de route, die leidt tot 3-methylbutanal, beperkend is voor hoge productieniveaus. Proteolyse en peptidolyse zijn niet limiterend, omdat de concentratie aan vrije aminozuren en kleine peptiden in kaas toereikend is voor vervolgreacties (Tabel 1.5), en daarnaast omdat de toevoeging van vrije aminozuren aan de kaas niet of nauwelijks leidt tot toename in de vorming van aromacomponenten zoals 3-methylbutanal. Transaminering van leucine (Leu) naar het ketozuur α-ketoiscoproate (KaLeu), is een limiterende stap in de productie van het organische zuur 3-methylboterzuur. Deze stap kan in kaas aanzienlijk versneld worden door toevoeging van het co-substraat α-ketoglutarate. Dit gegeven is belangrijk, omdat transaminering ook de eerste stap is in de vorming van 3-methylbutanal. Stimulering van de transaminering leidt tot een aanzienlijk verhoogde afbraak van leucine en een toename in de vorming van voornamelijk KaLeu, het corresponderende hydroxyzuur (HaLeu), maar ook 3-methylboterzuur. De ophoping van KaLeu impliceert dat vervolgreacties tot aroma componenten vanuit dit intermediair niet snel genoeg verlopen. De enige stap die nodig is voor de omzetting van het KaLeu naar 3-methylbutanal is een decarboxylering. Hieruit kan geconcludeerd worden dat deze stap slecht verloopt onder de geteste condities bij het merendeel van de onderzochte stammen. Om dit nader te onderzoeken zijn 23 zuivelgerelateerde stammen onderzocht op hun vermogen om 3-methylbutanal en/of 3-methylbutanol te maken en hun maximale omzettingscapaciteit voor relevante stappen in dit reactie schema. De resultaten lieten zien dat er een grote biodiversiteit bestaat tussen de stammen met betrekking tot aromavorming en de capaciteit om individuele stappen in de route te katalyseren. Dit impliceert dat er stammen kunnen worden geselecteerd die bepaalde stappen in de route al dan niet kunnen uitvoeren. Slechts 2 van de 23 stammen waren in staat aanzienlijke hoeveelheden 3-methylbutanal en 3-methylbutanol te produceren. Juist deze stammen, waaronder ook *L. lactis* B1157 beschikten over een ketozuur decarboxylase, terwijl geen activiteit in de andere stammen waargenomen werd. De maximale capaciteit van dit decarboxylase was echter heel laag, terwijl de capaciteit voor de vorming van HaLeu juist heel hoog was. Omdat beide reacties concurreren om hetzelfde substraat, zijn *in vitro* twee metabole situaties nagebootst: overmaat aan NADH en een tekort aan NADH. In het geval van NADH overmaat werd alle KaLeu direct omgezet in HaLeu, en werd er slechts een kleine hoeveelheid 3-methylbutanal gevormd. Bij de afwezigheid van NADH werd het ketozuur nauwelijks afgebroken, maar werd er

duidelijk meer 3-methylbutanal gevormd dan in de situatie met een overmaat aan NADH. De vorming van 3-methylbutanal bleef echter relatief laag, hetgeen overeenkwam met grote dehydrogenase capaciteit ten opzichte van decarboxylase capaciteit.

Concluderend kan gesteld worden dat de aan- of afwezigheid van het decarboxylase bepaalt of er aanzienlijke hoeveelheden 3-methylbutanal worden gevormd door de culturen. Daarnaast is in het algemeen de decarboxylase activiteit dermate laag dat concurrentie met andere ketozuur-consumerende reacties ongunstig uitvalt voor het decarboxylase. Verhoging van de decarboxylase-activiteit is daarom een zeer voor de hand liggende aanpak voor het verhogen van 3-methylbutanalproductie, eventueel ook in kaas. Om dit te kunnen bereiken is meer kennis van het decarboxylase nodig, alsmede technieken om culturecollecties te kunnen screenen op 3-methylbutanal vorming.

Er waren nog geen geschikte technieken voor handen om grote aantallen monsters te screenen op voorkomen van vluchtige verbindingen als 3-methylbutanal. Daartoe zijn een nieuwe methode en een monsterhouder in combinatie met een gebruiks protocol ontwikkeld (Hoofdstuk 3). De eisen waaraan moest worden voldaan, waren: snelle analyse, kleine volumina, standaard afmetingen van monsterpotjes, weinig "hands on" tijd, geautomatiseerd en robuust. Gekozen is voor de directe injectie (DI) van een gas-monster in een massa spectrometer (MS). Dit concept (DI-MS) is uitgewerkt en de respons van de MS bij een specifieke massa bleek, zoals verwacht, representatief te zijn voor de concentratie van een bepaalde vluchtige component zoals 3-methylbutanal. Na optimalisatie konden maximaal 1500 monsters per dag met 3-methylbutanal concentraties tussen de 5 en 1000 μM gemeten worden. De variatiecoefficient was 5%. Om een zo kort mogelijke monster voorbereidingstijd te realiseren werd een roestvrijstalen (RVS) blok ontwikkeld met de dimensies van een 96-deep-well plaat. De belangrijkste voordelen van een dergelijk blok zijn de mogelijkheid om standaard high-throughput-apparatuur te gebruiken, de mogelijkheid het blok zowel te verwarmen als te steriliseren, en de relatieve inertie van het materiaal. Voor toepassingen waarvoor RVS niet inert genoeg is, kan het blok gevuld worden met glazen inserts. Het blok kan gasdicht afgesloten worden met een steriele teflon laag, een siliconen laag, en een RVS deksel, waarin 96 gaten zitten voor monstername.

Om de methode en de blokken te testen zijn 72 willekeurige stammen behorende tot het geslacht *Lactococcus* in deze blokken gekweekt en gescreend op 3-methylbutanal productie. Elke stam was in viervoud verspreid over het blok, om daarmee inzicht te verkrijgen in de reproduceerbaarheid. Daarnaast zijn 24 stammen in viervoud op verschillende dagen gemeten, om inzicht te verkrijgen in de betrouwbaarheid van de methode. De methode bleek zeer geschikt voor de bedoelde screening. Uit de resultaten van de screening bleek allereerst dat de keuze van het kweekmedium een zeer grote invloed had op het oplossend vermogen van de methode. Met andere woorden, de verschillen in productieniveaus werden bij de keuze van een optimaal medium groter en dus beter meetbaar. Er bleek dat slechts een klein percentage van de stammen in staat was 3-methylbutanal te produceren. Juist deze stammen bleken bijna allemaal geen geschiedenis te hebben in industriële fermentaties; het waren de zogenaamde "wilde" stammen.

Toepassing van dit soort geselecteerde stammen in kaas is relevant voor mogelijke sturing van de aromaproductie in kaas.

Naast het screenen van culture collecties, bleek de nieuwe DI-MS methode ook geschikt voor het volgen van (bio)chemische reacties in de tijd, omdat de tijd tussen twee monsternames relatief kort is. Tijdens de identificatiefase van de biochemische afbraakroute van leucine, werd naast 3-methylbutanal ook de aroma component 2-methylpropanal geïdentificeerd. Deze aromacomponent is bekend van de enzymatische afbraak van valine. In onze experimenten was echter geen valine beschikbaar. Uiteindelijk bleek de vorming het resultaat van een chemische omzetting van KaLeu. Deze reactie vertoont homologie met de chemische afbraak van het ketozuur van phenylalanine (KaPhe) en methionine (KaMet), maar slechts enkele karakteristieken van dit soort reacties waren bekend en een reactiemechanisme was nog nooit voorgesteld. Onze experimenten en een literatuurstudie, beschreven in hoofdstuk 4, hebben geleid tot een voorstel voor het globale reactie mechanisme. Zuur of base gekatalyseerde, eventueel Mn^{2+} gestabiliseerde keto-enol tautomerie leidt tot de reactieve enol vorm van KaLeu. Moleculaire zuurstof kan vervolgens aanvallen op het licht zure ß-waterstof atoom, waardoor een peroxide ontstaat. Dit peroxide kan, afhankelijk van de omgeving, op verschillende manieren snel verder reageren. In water kunnen hoogstwaarschijnlijk zowel een circulair (dioxylacton) als een niet-circulair intermediair gevormd worden, welke in het geval van het lacton uiteenvalt in het aldehyde 2-methylpropanal en koolstofoxides, of 2-methylpropanal en oxaalzuur in het andere geval. Bij een hogere pH wordt het niet-circulaire intermediair meer gevormd waardoor hogere oxaalzuur concentraties gemeten worden. De reactie is inderdaad zuur en base gekatalyseerd, bij pH < 5 bleek echter de omzettingssnelheid af te nemen. Bij pH>8 konden geen nauwkeurige metingen verricht worden ten gevolge van het neerslaan van mangaan. In het zure gebied verliep de reactie maximaal bij een pH van ongeveer 5.3, welke ongeveer overeenkomt met de pH van kaas. In eerder onderzoek naar de conversie van KaPhe werd gevonden dat meerdere divalente metaalionen de reactie kunnen katalyseren. KaLeu degradatie lijkt echter alleen mangaan gekatalyseerd. Naast KaLeu, KaPhe en KaMet, worden ook andere ketozuren met meer dan 4 koolstofatomen onder deze omstandigheden afgebroken. De ketozuren van valine en isoleucine worden realief langzaam afgebroken, waarschijnlijk omdat ze slechts 1 ß-waterstof atoom hebben. De impact van deze reactie voor de vorming van aroma componenten onder kaascondities is nog niet onderzocht. Hierover kan daarom alleen gespeculeerd worden. Kaas heeft een lage redox potentiaal en binnen enkele uren is er nauwelijks nog zuurstof beschikbaar. Daarnaast zijn mangaan concentraties in kaas niet zo hoog als in de experimenten en komt mangaan wellicht in een niet-katalytisch geschikte vorm voor. Aan de andere kant wordt de vorming van benzaldehyde (chemisch omzettingsproduct van KaPhe) in gefermenteerde zuivel producten wel waargenomen en is de rijping van kaas een langdurig proces, waardoor ook bij lage omzettingssnelheden toch significante hoeveelheden 2-methylpropanal gevormd zouden kunnen worden. In de *in vitro* experimenten werd per uur ca. 100 µM 2-methylpropanal gevormd, terwijl de smaakdrempel van deze component in zuivel producten lager is dan één µM. Het loont

daarom waarschijnlijk de moeite om het al dan niet optreden van deze reactie in een kaas modelsysteem te bepalen.

In hoofdstuk 2 is gebleken dat in de vormingsroute van 3-methylbutanal het ketozuur decarboxylase een belangrijke limiterende factor was. Om deze informatie te kunnen gebruiken voor sturing van de aromavorming in kaas is meer informatie over dit enzym gewenst. De meest voor de hand liggende aanpak is het zuiveren van het enzym, maar de gezuiverde fracties bleken niet zuiver genoeg voor de bepaling van de aminozuursequenties aan de uiteinden van het eiwit. Daarnaast werd het zuiveren in eerste instantie bemoeilijkt door het ontbreken van een snelle analysemethode voor 3-methylbutanal. Daarom werd besloten eerst het gen te identificeren, en vervolgens het bijbehorende enzym te karakteriseren (Hoofdstuk 5). *Lactococcus lactis* B1157 is in dit onderzoek gebruikt als modelorganisme, omdat deze stam grote hoeveelheden 3-methylbutanal bleek te kunnen maken. Omdat het gen, coderend voor het decarboxylerende enzym, niet bleek te liggen op het grote plasmide waarover deze stam beschikt, is een totaal-DNA knock-out mutantenbank (>8500 kolonies) gemaakt, en gescreend met de nieuw ontwikkelde DI-MS high throughput screening methode op de afwezigheid van 3-methylbutanal boven geminiatururiseerde fermentaties. Deze screening leverde 2 knock-out mutanten op die elk in een ander gedeelte van hetzelfde gen gemuteerd bleken te zijn. De sequentie van dit gen was nagenoeg identiek aan een gen dat in het genoom van *L. lactis* IL1403 geannoteerd was als *ipd* (indol-3-pyruvaat dearboxylase). Dit gen in IL1403 bleek echter incompleet. Op basis van de gen-sequentie is gekeken naar het voorkomen van dit gen in de voorlopige genomen van melkzuurbacteriën en in de stammen die ook gebruikt zijn voor de screening die is beschreven in hoofdstuk 3. Voorlopige resultaten toonden een goede correlatie tussen de vorming van 3-methylbutanal en de aan- of afwezigheid van het gen. Overexpressie van het gen, en karakterisatie van het genproduct leidde tot de conclusie dat het een "branched-chain keto acid decarboxylase" is en geen IPD of PDC (pyruvaat decarboxylase), omdat de relatieve activiteit op indol-3-pyruvaat of pyruvaat minder dan 10% was ten opzichte van de omzetting van het vertakte α-ketozuur van valine. Het enzym bleek een brede substraatspecificiteit te hebben en bij pH=6.3 en bij een hoog zoutgehalte de maximale activiteit te hebben. De cofactoren TPP en Mg^{2+} zijn hoogstwaarschijnlijk sterk gebonden zoals bij de vergelijkbare enzymen pyruvaat decarboxylase en indol-3-pyruvaat decarboxylase het geval is, omdat toevoeging van TPP en Mg^{2+} aan de assay niet altijd essentieel is. Dit duidt erop dat dit enzym hoogst waarschijnlijk zowel intracellulair als extracellulair actief kan zijn gedurende de kaasrijping. Bevestiging van deze suggestie door applicatieve proeven is echter nog niet gebeurd. De manier waarop eventuele regulatie plaats vindt, is nog niet geïdentificeerd, maar vermoedelijk leiden hogere enzymniveaus direct tot hogere aldehydeproductie. In dat geval zou de toepassing van stammen met een hoge decarboxylaseactiviteit een goede mogelijkheid zijn voor het verhogen van de aldehydeconcentraties in kaas.

In het Hoofdstuk 6 worden onder andere de omzettingssnelheden van andere substraten door de reacties in relatie tot de aromavorming bediscussieerd. Met het oog op versnelling van kaasrijping is het gunstig als de gekarakteriseerde reacties weinig selectief zijn, zodat stimulering van deze route in dat geval zal leiden tot een verhoogde productie van meerdere aromaverbindingen. Dit zal minder snel leiden tot een onbalans in de smaak van het product, dan wanneer slechts de concentratie van een enkele aromaverbinding verhoogd wordt. Dit laatste zou wel wenselijk kunnen zijn in het geval er gezocht wordt naar diversificatie van smaak, dus naar nieuwe kaas variëteiten.

In dit onderzoek is veel gebruik gemaakt van moleculair biologische technieken voor identificatie van het decarboxylase. Op verscheidene momenten is ook aangegeven dat dit niet hoeft te leiden tot toepassing van GMO's in het uiteindelijke product. Gerichte selectie van natuurlijke stammen is met de nieuwe kennis en methoden mogelijk. Enerzijds kan er gerichter gezocht worden naar mogelijke regulerende factoren, maar anderzijds is aanvulling van reeds deels aanwezige biochemische routes (complementatie) ook mogelijk. Door het samenbrengen van twee stammen die elk een deel van een route goed kunnen uitvoeren, zal op een elegante wijze de aromaproductie in kaas gestuurd kunnen worden.

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Nawoord

Na enige wispelturingheid, werd het tijdens mijn studies al snel duidelijk, dat mijn interesses het beste te vangen waren in de term, Industriële Microbiologie. Naast een enorme fascinatie voor eten, en alles wat daar mee te maken heeft, houden de mogelijkheden die wij als mens hebben om micro-organismen in te zetten voor ons nut me enorm bezig. Sturing van deze organismen kan op veel verschillende niveaus, waarbij veel verschillende disciplines komen kijken, van proceskunde, via de microbiologie tot biochemie en moleculaire biologie. Pas aan de sollicitatietafel drong het tot mij door dat veel van deze facetten terug kwamen in het project, dat uiteindelijk heeft geleid tot dit proefschrift.

Het project bleek veel verschillende kanten te hebben. Zoals vaker in een promotie onderzoek bleken sommige stappen in het onderzoek eigenlijk niet uitvoerbaar, en zijn er onderweg veel verleidingen geweest tot diversificatie en versnippering van aandacht. Soms heb ik me er toe laten verleiden er aandacht aan te besteden, zoals bijvoorbeeld aan de interactie tussen alcoholen en aldehyden, met betrekking tot verminderde smaakbeleving van mengsels van deze componenten. Suzanna heeft in haar afstudeerproject goed onderzoek gedaan met betrekking tot deze interacties, maar uiteindelijk is er geen tijd geweest om dit onderwerp verder uit te werken en in dit proefschrift op te nemen. Een andere verleiding heeft wel geleid tot een hoofdstuk, waarin de niet-enzymatische afbraak van α-ketozuren beschreven is. Vooral Gijs en Erwin hebben me in dit onderdeel bijgestaan. De bulk van de onderzoekstijd is besteed aan de opheldering van de enzymatische conversie van het aminozuur leucine, naar het aldehyde 3-methylbutanal. Bijgestaan door Laura en Erik is het knelpunt (een enzym) in de aromavormende route opgehelderd, geïdentificeerd en gekarakteriseerd. Bernadet en Roland van het CMBI hebben vervolgens een 3D model gemaakt van dit enzym. Door de zijstapjes en de tegenslagen in de identificatie van het knelpunt, is relatief weinig aandacht besteed aan het sturen van de aromavorming, hetgeen toch een van de meest uitdagende en applicatieve onderdelen van het onderzoek beloofde te worden. Vervolgonderzoek op dit punt lijkt me daarom ook zeer relevant. Bij deze wil ik, zonder in enorme opsommingen te vervallen, alle mensen die direct of indirect bij al deze onderwerpen betrokken zijn geweest bedanken, hetgeen neer komt op bijna alle NIZO-medewerkers, de afstudeer-studenten en hun begeleiders. Een aantal mensen heb ik toch wel heel vaak "aan hun lurven getrokken", en daarom speciale dank aan: Annereinou, Charles, Esther F., Gerben, Henk, Iris, Jan v. R. Jildert, Johan, Marke, Peter, Rita en Roelie.

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Bart Smit

Helsinki, februari 2004.

List of publications

Articles

Smit, B.A., L. Meijer, W.J.M. Engels, J.T.M. Wouters, and G. Smit. Identification, cloning and characterization of a branched-chain α-keto acid decarboxylase from *Lactococcus lactis*, involved in flavor formation. Submitted.

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Curriculum vitae

Bart Smit werd geboren op 14 april 1976 te Heerlen. Na het behalen van zijn Atheneum diploma aan het Eijkhagen College te Landgraaf in 1994, werd in hetzelfde jaar begonnen met de studie Levensmiddelentechnologie aan de toenmalige Landbouw Universiteit Wageningen. In 1997 besloot hij tevens te starten met een tweede studie, Bioproces Technologie aan dezelfde universiteit. Zijn eerste afstudeerproject werd uitmuntend uitgevoerd aan de vakgroepen Industriële Microbiologie en Proceskunde, en betrof het karakteriseren en beïnvloeden van biochemische routes in een halotolerante gist (Dr. C. van der Sluis, Dr. S. Hartmans, Dr. R.Wijffels en Prof. H. Tramper). Vervolg projecten brachten hem naar het IJslands Technologisch Instituut, alwaar aan de fylogenie in relatie tot fysiologie van thermofiele bacteriën werd gewerkt (Dr. G. Oli en Prof. J. Kristjansson) en naar Unilever Research in Vlaardingen, alwaar gewerkt werd aan de modellering van schimmelslurrie-fermentaties (Dr. Cui Yi-Qing, Dr. A. Rinzema). In 1999 werden beide studies succesvol afgerond waarbij de term Industriële Microbiologie zijn interesses en vaardigheden goed samenvat.

Geheel in lijn met zijn interesses op het vlak van microbiëel metabolisme in een breed perspectief, begon hij in september 1999 aan een promotieonderzoek onder leiding van Dr. W. Engels, Prof.. G. Smit en Prof. J. Wouters, met dit proefschrift getiteld "Formation of Amino Acid Derived Cheese Flavour Compounds" als resultaat.

In februari 2004 is Bart begonnen als "research scientist" bij VTT biotechnology, Espoo, Finland. In dit instituut zal hij gaan werken aan de fysiologie van en de enzym productie door de schimmel *Trichoderma reesei* in vloeistoffermentaties. Om dit veel belovende project uit te kunnen breiden, heeft hij een twee-jarige beurs aan gevraagd bij de Europese unie.