# 23 Microbial Flavour Production

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## 23.1 Introduction and Scope

For thousands of years microbial processes have accompanied mankind playing the decisive but unrecognised role of producing more flavourful foods and beverages such as bread, cheese, beer, wine and soy sauce. It was in 1923 that the first scientific review on microbial flavours appeared [1]. With the dynamic development of modern analytical techniques in the middle of the twentieth century when isolation, chromatographic separation and structural identification of volatiles became routine, the basis for a more systematic elucidation of microbial flavour generation was given. Research in the last decades has led to a tremendous increase in knowledge of microbial and enzymatic flavour generation which has been frequently reviewed [2–8] and was reviewed in several multiauthor works dealing with this topic [9, 10] and one comprehensive book exclusively dedicated to aroma biotechnology published in 1995 [11].

Nowadays, biotechnological production of flavour compounds is a mature discipline in the chemical industry, with an estimated 100 molecules in the market produced by enzymatic or microbial processes [7]. The predominant driving force was and still is the fact that flavour compounds produced from natural raw materials by microbial or enzymatic methods can be labelled 'natural' in accordance with European and US legislation, thereby satisfying the unbroken consumer trend towards all 'bio' or 'natural' products in the food sector. By contrast, the involvement of chemical means leads to the less appreciated labels 'nature-identical' (EC Flavour Directive 88/388/EEC) or 'artificial' (US Code of Federal Regulations 21 CFR 101.22) for flavours not occurring in nature. This from the scientist's viewpoint rather surprising situation paved the way for 'self-sufficient' research on biocatalytic and fermentative flavour production, which started several decades ago. These research activities steadily expanded to almost all natural key flavour compounds which cannot be economically provided by classic isolation from their natural sources, e.g. by extraction or distillation, owing to too low concentrations. This happened although many of the target compounds could and still can be produced in a more efficient and less expensive way by chemical syntheses because the natural flavours achieve significantly higher market prices of up to 2 orders of magnitude. For 2005 the total worldwide flavour and fragrance market was estimated to be about US $16.0 billion, with a

growth in local currencies of about 3% in the same year (http://www.leffingwell.com/top_10.htm). In 2001 the percentage of natural flavours of all added flavours amounted to 90% (EU) and 80% (USA) in beverages, to 80% (EU and USA) in savoury foods, and to 50% (EU) and 75% (USA) in dairy foods [6].

Nevertheless, enhanced competitive pressure and a less distinguishing food labelling legislation ('natural flavouring' vs. 'flavouring' in the EU) cause companies to increasingly evaluate natural flavours by their production costs in comparison with the costs of their chemically synthesised counterparts and in most cases do not leave room for high extra charges for the naturals anymore. Instead, three characteristics of most biotechnological processes are increasingly influencing academic as well as industrial considerations: biocatalytic reactions usually (1) are highly selective (chemo, regio, stereo), (2) start from natural raw materials/renewable resources and (3) are environmentally friendly and sustainable (Table 23.1). Especially the fact that evolution has optimised biological systems on the basis of metabolism of natural organic molecules makes biotechnology an outstanding technology for the development of sustainable production processes—outdoing classic syntheses starting from petrochemicals—which will have an increasing share in the chemical industry of the twenty-first century. The nowadays much-cited discipline 'white biotechnology' as a synonym for industrial biotechnology which bundles lots of economic and ecological hopes has already blossomed into numerous examples of efficient bioprocesses in the area of microbial flavour synthesis owing to the very special situation of a virtual non-competitiveness against chemical synthesis. On the basis of the long and sound research tradition in aroma biotechnology, novel approaches combining the emerging opportunities given by modern molecular biology including '-omics' and metabolic engineering technologies, and advanced bioprocess engineering, e.g. in situ product removal strategies, will definitely lead to even more biotechnologically produced flavours in the future.

The scope of this chapter is to give a comprehensive overview of microbial processes used in industry or microbial strategies investigated in application-oriented research for the production of single flavour compounds. The chapter is subdivided by structural substance classes and exclusively focuses on compounds produced by fermentation or whole-cell biocatalysis. Biotransformation with isolated enzymes, a mature discipline of aroma biotechnology, is excluded since a separate chapter of this book is dedicated to this topic. It was the intention to primarily treat those examples within each substance class discussed which are already being industrially applied or where significant product concentrations have been reported and, thus, the research results justify the assumption of a short-term to medium-term technical realisation. Traditional non-volatile flavour compounds are included, because some of them, e.g. monosodium glutamate (MSG) or citric acid, are industrial bulk products with market volumes exceeding 1,000,000 t year$^{-1}$. These examples illustrate extremely well the beneficial impact of biotechnology on the chemical industry as commodities can be produced from renewable resources based on a sustainable technology. This chapter does not cover academic research activities in the field of biocatalytic flavour and fragrance synthesis, which are predominantly carried out on an

**Table 23.1** Driving forces to use biotechnological methods for flavour production (adapted from [270])

| 'Market pull' | 'Technical push' |
|---|---|
| Increasing consumers' demand for 'organic', 'bio', 'healthy', 'natural' | High chemo-, regio- and stereo-selectivities of biocatalytic systems |
| Industrial dependence on distant (frequently overseas) raw materials, undesired/limited raw materials | Sustainability of bioprocesses |
| Search for natural character-impact compounds | Improved biocatalysts by evolutionary and rational enzyme engineering and metabolic engineering |
| Search for natural flavour compounds with additional functionalities (e.g. antimicrobial properties) | Improved downstream processing, especially in situ product-recovery techniques |

analytical scale to elucidate sensory properties of enantiomerically pure compounds not or hardly achievable by chemical catalysis [12]. Also, genetic engineering and biochemical elucidation of microbial flavour generation pathways are only discussed if necessary for understanding or if not already covered in Chap. 26 by Schwab. Finally, as only microbially produced single-flavour molecules are discussed, traditional food fermentation processes and the impact of selecting and engineering starter cultures and/or fermentation conditions (e.g. alcoholic beverages, dairy, meat and bakery products) are not covered. The interested reader is referred to respective reviews in this field [13–16].

## 23.2 Characteristics of Microbial Flavour Production

Although for a multitude of microorganisms the metabolic potential for de novo flavour biosynthesis is immense and a wide variety of valuable products can be detected in microbial culture media or their headspaces, the concentrations found in nature are usually too low for commercial applications. Furthermore, metabolic diversity often leads to a broad product spectrum of closely related compounds. Exceptions to the rule can be found where the flavour compounds are derived from primary metabolism as is the case for some of the non-volatiles (e.g. glutamic acid, citric acid). Therefore, the biocatalytic conversion of a structurally related precursor molecule is often a superior strategy which allows the accumulation of a desired flavour product to be significantly enhanced. As a prerequisite for this strategy, the precursor must be present in nature and its isolation in sufficient amounts from the natural source must be easily feasible in an economically viable fashion. Additionally, if product and precursor are closely related with respect to their physicochemical properties, a selective product recovery during downstream processing becomes a major issue for the bioprocess development. Many of the industrially relevant microbial flavour production processes follow this 'precursor approach' (e.g. vanillin from ferulic acid or eu-

genol, 4-decanolide from ricinoleic acid, 2-phenylethanol from L-phenylalanine). Besides the problems arising from metabolic diversity, the cytotoxicity of the flavour products and often also of their precursors is another big hurdle during bioprocess development. Here, very often in situ product recovery or sequential feeding of small amounts of precursor becomes essential to improve the overall performance of a bioprocess and to render it economically viable. Owing to their hydrophobicity, flavour compounds preferentially partition to lipid structures, which makes cellular membranes the main target for product accumulation during microbial processes. The flavour molecules negatively influence the cell physiology by enhancing the membrane fluidity, eventually leading to collapsing transmembrane gradients and, consequently, to the loss of cell viability [17]. An empirical correlation was found between the $\log P_{\text{octanol/water}}$ value of an organic solvent and its corresponding partition coefficient for the cell membrane–water system ($\log P_{\text{membrane/water}}$) [18]:

$$\log P_{\text{membrane/water}} = 0.97 \log P_{\text{octanol/water}} - 0.64.$$

With this equation the actual membrane concentration of a hydrophobic compound can be estimated if its concentration in the water phase is known. For instance, limonene, a hydrophobic precursor in many biotransformations to produce monoterpenoid flavour compounds ($\log P_{\text{octanol/water}} = 4.5$), would accumulate within membranes in concentrations of up to 530 mM if it is present in the water phase at saturation concentration of only 0.1 mM [19]. This concentration would clearly not allow conventional microorganisms to survive.

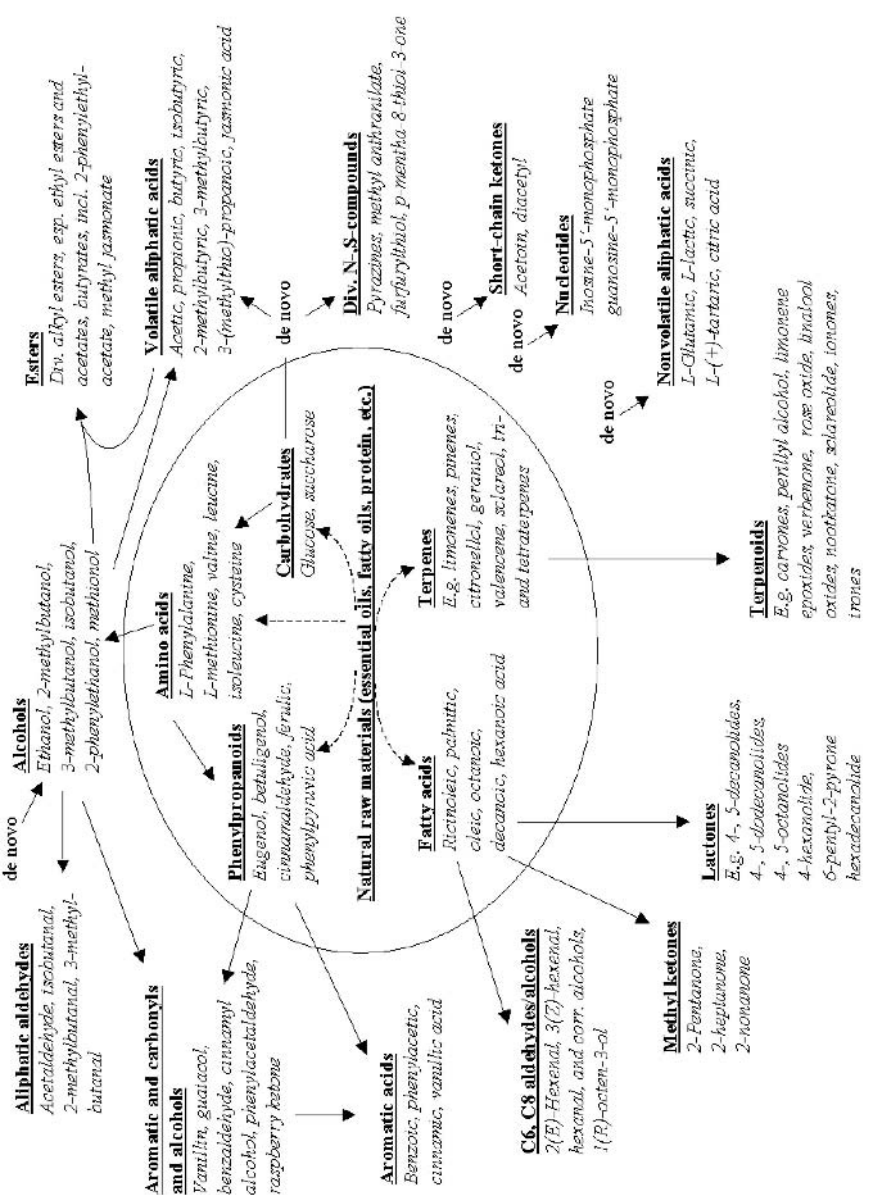
The design of recombinant microorganisms for the improved production of natural flavour molecules is being intensively investigated in academic and industrial research since, of course, it can provide the same economic benefits as in other industrial applications of modern biotechnological production processes, e.g. in the pharmaceutical industry. Although genetic engineering in food-related applications has been the subject of a controversial public discussion for quite some time, the fact that in aroma biotechnology genetically modified organisms are used as biocatalysts which are completely separated from the volatiles during the product-recovery step raises hope that this technique will also be applicable in industrial flavour production processes in the future. Further improvements will certainly be triggered by the enormous progress currently being made in the field of total genome sequencing. The time needed to determine complete microbial genomes has dramatically decreased during the last few years. Among the microorganisms already sequenced, several bacteria and fungi can be found which are valuable candidates with respect to food and flavour applications, e.g. *Bacillus subtilis*, *Brevibacterium linens*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Lactococcus lactis*, *Pseudomonas putida*, *Streptococcus thermophilus, Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Aspergillus niger*.

Table 23.2 summarises the main issues of microbial flavour production and how they may be addressed by biotechnological methods.

**Table 23.2** Main drawbacks during microbial flavour production and biotechnological strategies to address them

| Characteristics | Biotechnological strategy | Exemplary product |
|---|---|---|
| Formation of unwanted by-products owing to complex metabolic pathways | Overexpression of key genes of the synthetic pathways | 3-Methylbutyl acetate [86] (Sect. 23.4.1.4) |
| | Heterologous gene expression/use of engineered enzymes | Cinnamyl alcohol [136] (Sect. 23.4.2), verbenol [187, 188] (Sect. 23.4.3.2) |
| | Knockouts of genes involved in product degradation | Vanillin [89] (Sect. 23.4.2) |
| | 'Precursor approach' instead of de novo biosynthesis | 2-Phenylethanol [120] (Sect. 23.4.2), raspberry ketone [103, 106] (Sect. 23.4.2) |
| | Screening; enrichment cultures | Perillyl alcohol [184] (Sect. 23.4.3.2), 10-hydroxypatchoulol [207] (Sect. 23.4.3.3) |
| | Subsequent biotransformation converting a by-product to the desired product | 4-Decanolide [222] (Sect. 23.4.4) |
| Toxic properties of the flavour compounds produced | In situ product recovery by: | |
| | Adsorption, e.g. on XAD resins | 6-Pentyl-$\alpha$-pyrone [241] (4.4) |
| | Stripping and adsorption | C2–C5 alkyl esters [85] (Sect. 23.4.1.4), furfurylthiol [256] (Sect. 23.4.5) |
| | Extraction (two-phase bioprocess) | 2-Phenylethanol [120] (Sect. 23.4.2), phenylacetaldehyde [132] (Sect. 23.4.2) |
| | Membrane-based processes | |
| | Resting cells instead of growing ones | Isonovalal [181] (Sect. 23.4.3.2), 2-phenylethyl acetate [119] (Sect. 23.4.2) |
| | Product-tolerant strains | Acetaldehyde [53] (Sect. 23.4.1.2) |
| | | Vanillin [90] (Sect. 23.4.2) |
| Toxic properties of the precursor molecules | Sequential precursor feeding | 4-Octanolide [234] (Sect. 23.4.4), 3-methylbutyl acetate [48] (Sect. 23.4.1.4), carboxylic acids [39] (Sect. 23.4.1.1) |
| | On line monitoring of precursor/bioactivity | Limonene transformation products [108, 142] (Sect. 23.4.3.1) |
| | Immobilisation of microorganisms | Propanoic acid [41] (Sect. 23.4.1.1), phenylacetic acid [133] (Sect. 23.4.2) |
| | Two-phase bioprocess with an organic solvent as the precursor reservoir | 5-Decanolide [231] (Sect. 23.4.4), 4-hexanolide [236] (Sect. 23.4.4) |
| | Resting cells instead of growing ones | Carvone [170, 172] (Sect. 23.4.3.2) |
| | Precursor-tolerant (solvent-tolerant) strains | Perillic acid [156] (Sect. 23.4.3.2), carvone [171, 172] (Sect. 23.4.3.2) |
| | Fungal spores instead of mycelia | Methylketones [69, 70] (Sect. 23.4.1.3) |

In Fig. 23.1 the main biotechnological routes to flavour molecules are summarised, illustrating that a wide range of microbial (and enzymatic) processes have been developed exclusively relying on the bioconversion of natural precursors and/or the cultivation on non-fossil carbon sources, such as glucose and fatty acids. These characteristics make aroma bioprocesses a prime example of a sustainable industrial technology based on renewable resources.



Among the natural flavour molecules produced with microorganisms are some real bulk products, such as L-glutamic acid and citric acid manufactured on the million-ton scale, but the majority of the target compounds are produced for highly specific applications and thus are rather niche products with market volumes below 1 t $year^{-1}$. Here, industry avoids costly research and development effort to establish more sophisticated processes owing to the limited market volume of these products. Nevertheless some natural flavours which have a broader application are produced in amounts of around one to several tons per year, such as vanillin, 2-phenylethanol and 4-decanolide. These flavour compounds have an increasing market share owing to steadily improved bioprocesses: for instance, the price for the peach-like 4-decanolide dropped from about US $20,000 per kilogram in the 1980s to about US $300 per kilogram in 2004 [8, 20]. Table 23.3 summarises some natural flavour compounds currently being produced by microbial processes in industry. The microbial production of these and other flavour compounds is discussed in more detail in the following sections. When a flavour compound is mentioned for the first time within the respective section it is written in bold letters.

## 23.3 Non-volatile Flavour Compounds

Non-volatile flavour compounds in the sense of this chapter are defined as molecules which cause one of the sensory impressions sweet, bitter, salty, sour or umami (Fig. 23.2).

By market volume the most important flavour molecule is **L-glutamic acid**. In 2004, the worldwide annual MSG production was estimated to be amount 1,500,000 t [21]. The amino acid is extensively used as taste enhancer, frequently in conjunction with nucleotides, a flavour impression which is also referred to as 'umami', a term derived from the Japanese meaning deliciousness or a savoury or palatable taste. MSG is manufactured by aerobic cultivation of *Corynebacterium glutamicum* on starch hydrolysates or molasses media in large-scale bioreactors (up to 500 $m^3$). Production strains with modified metabolic flux profiles and highly permeable cell walls for an improved product secretion are

◄ **Fig. 23.1** Microbial routes from natural raw materials to and between natural flavour compounds (*solid arrows*). Natural raw materials are depicted within the *ellipse*. Raw material fractions are derived from their natural sources by conventional means, such as extraction and hydrolysis (*dotted arrows*). *De novo* indicates flavour compounds which arise from microbial cultures by de novo biosynthesis (e.g. on glucose or other carbon sources) and not by biotransformation of an externally added precursor. It should be noted that there are many more flavour compounds accessible by biocatalysis using free enzymes which are not described in this chapter, especially flavour esters by esterification of natural alcohols (e.g. aliphatic or terpene alcohols) with natural acids by free lipases. For the sake of completeness, the C6 aldehydes are also shown although only the formation of the corresponding alcohols involves microbial cells as catalysts. The list of flavour compounds shown is not intended to be all-embracing but focuses on the examples discussed in this chapter

**Table 23.3** Some microbially produced flavour compounds and corresponding bioprocess features

| Product | Precursor | Microorganism | Process data | Remarks | References |
|---|---|---|---|---|---|
| L-Glutamic acid | – | *Corynebacterium glutamicum* | 150 g $L^{-1}$, 60 h, 1.500,000 t $year^{-1}$ | Aerobic cultivation; up to 500-$m^3$ scale; mutants with highly permeable cell walls | [21, 22] |
| Citric acid | – | *Aspergillus niger* | >200 g $L^{-1}$ , 9–12 days, 1,000,000 t $year^{-1}$; yield >95% | Downstream processing by precipitation as calcium citrate | [21, 22, 25] |
| Acetic acid | Ethanol | *Acetobacter, Gluconobacter* | 'Vinegar' with 10 to >20 %, >190,000 t $year^{-1}$; yield ~98% | Aerobic cultivation at 100-$m^3$ scale; Frings aerator for high oxygen transfer rates | [21, 22, 38] |
| L-Lactic acid | - | *Lactobacillus* | 210 g $L^{-1}$, 140,000 t $year^{-1}$; yield >90% | More than 100-$m^3$ scale; recovery of lactic acid by salt splitting technology | [21, 22, 31] |
| Vanillin | Ferulic acid | *Amycolatopsis, Streptomyces* | Up to 18 g $L^{-1}$, 50 h, 1–10 t $year^{-1}$ | In situ product recovery by crystallisation at more than 10 g $L^{-1}$ possible | [8, 90, 91] |
| (*Z*)-3-Hexenol ('leaf alcohol') | Linolenic acid | Soy lipoxygenase + plant hydroperoxide lyase + baker's yeast | 4 g $kg^{-1}$, 5–10 t $year^{-1}$ (also by isolation from plant oils) | Addition of baker's yeast to obtain the alcohol; without yeast the aldehyde is the major product | [60, 66] |
| 4-Decanolide (γ-decalactone) | Ricinoleic acid | *Yarrowia lipolytica* | 11 g $L^{-1}$, 55 h, several tons per year | Final acidification and temperature increase effect cyclisation of all 4-hydroxydecanoic acid to the corresponding lactone | [222, 224, 228] |
| 2-Phenylethanol | L-Phenylalanine | Diverse yeasts; e.g. *Saccharomyces* and *Kluyveromyces* | >10 g $L^{-1}$, 30 h, 0.5–1 t $year^{-1}$ | Fed-batch cultivation; in situ product recovery by two-phase system with more than 25 g $L^{-1}$ in the organic phase possible | [109, 120] |
| Short-chain carboxylic acids, e.g. 2-, and 3-methylbutyrate | Fusel alcohols | *Gluconobacter, Acetobacter* | Up to 95 g $L^{-1}$, 72 h | Two-step cultivation: biomass + bioconversion period; used as flavour acids but also for ester syntheses | [39] |

used in a controlled bioprocess and up to 150 g $L^{-1}$ L-glutamic acid is obtained in 60 h [22].

**Citric acid** is another prominent biotechnological bulk product of the chemical industry. About 1,000,000 t was produced in 2004 [21]. Citric acid is used in different industrial areas: in the food sector, the odourless acid is used for its pleasant acid taste and as a preservative [22, 23]. For industrial production, submerged fed-batch cultivation of *Aspergillus niger* on starch hydrolysates or cheap sucrose sources, e.g. molasses, in large-scale stirred or tower fermenters (50–1,000 $m^3$) is preferred and final concentrations of more than 200 g $L^{-1}$ can be achieved after 9–12 days [22, 24]. Special cultivation conditions, such as maintaining a high concentration of a rapidly consumable carbon source (more than 50 g $L^{-1}$), excess aeration, suboptimal phosphate concentration, $Mn^{2+}$ limitation and a decreasing pH value falling below 3 lead to high yields of up to 95 kg of citric acid per 100 kg of supplied sugar [25]. The downstreaming includes the processing steps filtration of the cell mass, precipitation of calcium citrate by adding $Ca(OH)_2$, redissolving with sulfuric acid, filtration of $CaSO_4$ and crystallisation of citric acid.

Although citric acid is by far the most important fruit acid, the more expensive natural **L-(+)-tartaric acid** is also routinely used in beverage products for its milder sourness and as an antioxidant. Although different enzymatic approaches have been investigated, natural tartaric acid is still obtained by conventional purification from residues of wine fermentation and constitutes an estimated world market of 50,000–70,000 t $year^{-1}$ [26]. Two product categories exist: low-grade grape debris and yeast cell material containing 15–25% potassium tartrate (dregs, wine lees, *Weinhefegeläger*, *Weinhefe*) and high-grade material (cream of tartar, *Fassweinstein*) with 60–70% potassium tartrate. The latter can be purified by heating (140–150 °C) and neutralising with lime milk containing calcium sulfate. Pure L-(+)-tartaric acid is obtained after treating the calcium salt with sulfuric acid, filtering off the calcium sulfate, evaporation and crystallisation.

Lactic acid can be produced as a racemic mixture from lactonitrile, but this chemical synthesis has diminished since most applications require enantiopure **L-lactic acid** [27]. Up to 290,000 t $year^{-1}$ of L-lactic acid is currently produced biotechnologically of which 150,000 t is used for the production of polylactate, a biodegradable polymer, and 140,000 t is used in the fields textiles, leather and food [21]. In foods and beverages, lactic acid is used for its pleasant mild sour taste, e.g. as an additive, preservative or acidulant in fruit juices, syrups, jellies, or for the preparation of sourdoughs [22, 28]; therefore, lactic acid is discussed in this chapter although it shows a low volatility causing a slightly sour odour note [23]. Depending on the carbon source used, different *Lactobacillus* species are exploited, such as *L. delbrueckii*, *L. leichmannii*, *L. bulgaricus* and *L. lactis* [22]. In industrial bioreactors of more than 100 $m^3$, productivities of up to 3 kg $m^{-3}$ $h^{-1}$ are obtained. In (fed-)batch fermentations with homofermentative lactic acid bacteria, final concentrations of more than 200 g $L^{-1}$ are obtained and high yields of more than 90% can be achieved [29–31]. The conventional purification protocol uses lime [$Ca(OH)_2$] or chalk ($CaCO_3$) as a neutralising

agents (pH 5.0–6.8 depending on the respective strain used), causing the formation of calcium lactate during fermentation. Free lactic acid is formed by adding sulfuric acid, leading to gypsum as a by-product. Advanced technical concepts are under investigation, e.g. in situ product recovery by ion-exchange resins and organic solvents to overcome end-product inhibition caused by the undissociated form of lactic acid, or electrodialysis for selective product recovery from fermentations in cheap but complex raw materials, such as molasses and whey, which cause purification problems [32]. Significant progress in downstream processing has been made by the so-called salt-splitting technology, i.e. the lactic acid is extracted from a lactate salt concentrate by an amine-based organic solvent (forming a trialkylamine lactate in the organic phase) from which it can be recovered by re-extraction with water; another salt-splitting technology is based on a water-splitting electrodialysis with bipolar membranes [27].

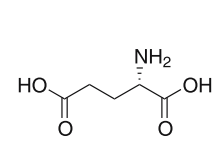
**Succinic acid** has found some use in flavour compositions to introduce tartness which is not achievable by means of conventional acids [33]. It is manufactured chemically by hydrogenation of fumaric or maleic acid [26] but its use in food applications would probably increase significantly once an economically viable biotechnological process for natural succinic acid production from glucose has been established. Currently, research activities in this field are stimulated by the fact that succinic acid can serve as an ideal platform building block for a variety of commodity and specialty chemicals, and is thus a perfect candidate for the biorefinery concept based on renewable resources, e.g. starch hydrolysates [34]. With anaerobic bacteria, especially *Actinobacillus succinogenes* and *Anaerobiospirulum succiniciproducens*, which tolerate high product concentrations, 80 to more than 100 g $L^{-1}$ succinate can be produced [34, 35]. Strains of *Actinobacillus succinogenes* with enhanced product tolerance were selected via screening on fluoroacetate media. For *Anaerobiospirulum succiniciproducens*, a continuous bioprocess was described using ultrafiltration for cell recycling and a monopolar electrodialysis unit for concentrating the permeate solution.

The most important of the aforementioned flavour-enhancing nucleotides are **inosine 5′-monophosphate** (IMP) and **guanosine 5′-monophosphate** (GMP), of which about 2,000 and 1,000 t $year^{-1}$ are produced by biotechnological processes worldwide [22] and which are used as their disodium salts. The nucleotides contribute to the flavour-enhancing effect brought into food by yeast hydrolysates. Different biotechnological strategies have been developed for the production of pure nucleotides:

1. *Candida utilis* is grown to high biomass concentrations and the extracted RNA is subsequently hydrolysed into the four 5′ nucleotides adenosine 5′-monophosphate (AMP), GMP, cytidine and uridine 5′-monophosphate by crude nuclease P1 from *Penicillium*; the desired nucleotides are isolated by ion-exchange chromatography and AMP is converted to IMP by adenyl deaminase from *Aspergillus* [22, 36].
2. The desired nucleotides are produced directly by fermentation in concentrations above 30 g $L^{-1}$: IMP or inosine, which can be chemically converted into

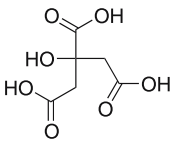
IMP, is synthesised with mutants of *Bacillus subtilis* or *Corynebacterium ammoniagenes*. Xanthosine 5′-monophosphate is produced with *Corynebacterium* or *Bacillus* and subsequently converted into GMP by *Bacillus* and other strains [6]. Alternatively, another related compound, 5′-amino-4-imidazole carboxamide-1-riboside-5′-phosphate, is produced by *Bacillus megaterium* and chemically converted into GMP [22, 36].

Certain polypeptides resulting from protease digestion of proteins contribute to the typical taste of savoury foods. The DNA sequence coding for an octapeptide known as **beefy meaty peptide** was cloned into yeast as a fusion with the yeast α factor to be secreted as free octapeptide into the medium which facilitated its recovery [37]. Alternatively, intracellular expression of tasty peptides



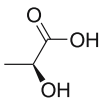
L-glutamic acid

*Aspergillus niger*



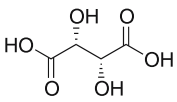
citric acid

*Corynebacterium glutamicum*



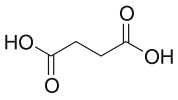
L-lactic acid

*Lactobacillus* sp.



L-(+)-tartaric acid

*Saccharomyces cerevisiae during wine fermentation*



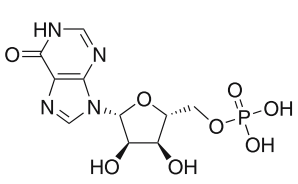
succinic acid

*Actinobacillus succinogenes*
*Anaerobiospirulum succiniciproducens*

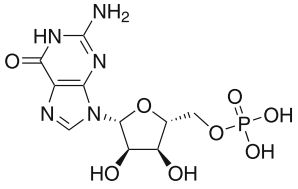
Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala

beefy meaty peptide

recombinant yeasts



inosine-5'-monophosphate (IMP)



guanosine-5'-monophosphate (GMP)

*Candida utilis*
*Bacillus subtilis*
*Corynebacterium ammoniagenes*

**Fig. 23.2** Some non-volatile flavour compounds produced with microorganisms

cloned into yeasts may lead to yeast extracts with improved flavouring characteristics.

## 23.4 Volatile Flavour Compounds

### 23.4.1 Aliphatic Compounds

#### 23.4.1.1 Carboxylic Acids

Fermentatively produced **acetic acid**, which is used as **'vinegar'** in food applications for its freshness, sourness and preservative properties, amounts to 190,000 t annually worldwide [21]. Today, the strictly aerobic cultivation of acetic acid bacteria, i.e. *Acetobacter* or *Gluconobacter* strains, in a mash containing ethanol, initial acetic acid and nutrients is mainly performed by submerged cultivation in specially designed stirred-tank reactors of about 100 $m^3$ (Frings Acetator®) [38]. A key feature is a Frings aerator, which is a self-aspirating rotor–stator system leading to high oxygen transfer rates at low aeration rates (approximately 0.1 vvm), thereby reducing the loss of the volatile ethanol and acetic acid via the exhaust air. The basic bioprocess leads to final acetic acid concentrations of approximately 10%. Repeated fed-batch processes have been developed for product concentrations of more than 15% and have to follow a carefully designed protocol to maintain optimum conditions concerning the oxygen, ethanol and acetic acid concentrations during cultivation. Final yields of up to 98% are common. In a two-stage process scheme, a portion of a first fermenter, which is replenished by new mash, is transferred into a second fermenter, where an almost complete ethanol oxidation is achieved—a final concentration of more than 20% (corresponding to a productivity of up to 50 L $m^{-3}$ $day^{-1}$ [22]) can be obtained, which is necessary for the canning industry. The biochemical principle of this primary metabolism is the stepwise oxidation of ethanol via acetaldehyde to acetic acid by the action of two pyrolloquinoline quinone dependent enzymes bound to the cytoplasmic membrane, alcohol dehydrogenase and aldehyde dehydrogenase, which feeds electrons into the respiratory chain of the organisms.

The strong oxidative capabilities of acetic acid bacteria are also harnessed for the production of other flavour acids from their corresponding alcohols, such as **propanoic acid**, **butanoic acid**, **2-methylpropanoic acid**, **2-methylbutanoic acid** and **3-methylbutanoic acid** (Scheme 23.1).

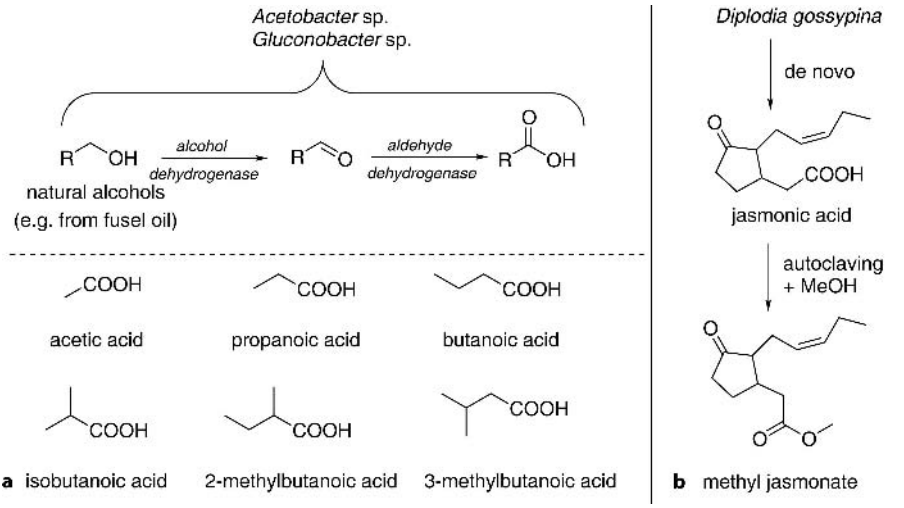
These natural acids synthesised from natural alcohols have market prices of less than €100 per kilogram and are of great importance to the flavour industry either because of their intense smell and sour taste or as substrates for enzymatic

syntheses of flavour esters [39]. The industrial process based on *Gluconobacter oxydans* DSM 12884 established by the German company Haarmann & Reimer (now Symrise) obtains molar yields above 90% and final product concentrations of more than 80 g $L^{-1}$ within 65–92 h (Table 23.3) [39]. The submerged cultivation process at 0.5 vvm aeration, 30 °C and 500 rpm usually starts with a batch fermentation period of 16–22 h for biomass growth and is followed by a bioconversion period where the alcohol is fed continuously into the reactor. By this means toxic effects of the alcohol and its loss via the exhaust air are minimised.

A microbial resolution of racemic 2-methylbutanoic acid was performed with a novel *Pseudomonas* sp. strain isolated from soil [40]. The strain was selected by screening on a medium containing racemic 2-methylbutanoic acid as the sole carbon source. The strain preferentially catabolised the fruity (*S*)-2-methylbutanoic acid, thereby yielding optically pure **(*R*)-2-methylbutanoic acid** which has a distinct odour described as being cheesy, sweaty and sharp.

It should be noted here that **propanoic** and **butanoic acid** can also be efficiently synthesised as metabolic end products of classic anaerobic fermentations on different sugars with various microorganisms, such as *Clostridium*, *Butyribacterium*, *Propiobacterium* and *Lactococcus*, which was investigated decades ago as a spin-off of acetone–butanol fermentation research [2]. Bioprocess and genetic engineering methods, e.g. in situ product removal, cell immobilisation and targeted gene inactivation, can help to significantly improve productivities and final product concentrations. Recently, immobilisation of *Propionibacterium acidipropionici* ATCC 4875 in a fibrous-bed bioreactor running under fed-batch conditions led to 72 g $L^{-1}$ propanoic acid [41]. Knocking out the *ack* gene (acetate kinase) decreased unwanted acetic acid formation by 14% [42]. The same reactor type and gene interruption strategy were successfully applied for butanoic acid production by *Clostridium tyrobutyricum* ATCC 25755 to yield about 40 g $L^{-1}$ from both xylose or glucose as the carbon source (corresponding to 0.43 g $g^{-1}$) [43, 44]. Coupling the reactor to an external hollow-fibre membrane module containing Alamine 336 in oleyl alcohol for in situ product extraction dramatically enhanced product concentration and reactor productivity [45]. The extractant was continuously regenerated by stripping with NaOH in a second membrane contactor. Thus, an impressive final butanoic acid concentration of more than 300 g $L^{-1}$ and a productivity of 7.37 g $L^{-1}$ $h^{-1}$ were achieved.

In plants the 13-hydroperoxide produced from linolenic acid by lipoxygenase (Sect. 23.4.1.2) can be converted to the allene oxide by allene oxide synthase followed by cyclisation, reduction and β-oxidation to form **jasmonic acid**, an important plant growth factor; the corresponding **methyl jasmonate** is a valuable flavour and fragrance compound that imparts a sweet-floral, jasmine-like note [46]. Recently, a patent described the use of *Diplodia gossypina* ATCC 10936 for the production of natural jasmonic acid [47]. With submerged cultures, up to 1.5 g $L^{-1}$ jasmonic acid was obtained after 11 days of incubation; the addition of 10-oxo-8-*trans*-decenoic acid, a hormone stimulating mycelial growth, proved to be advantageous; methyl jasmonate was obtained by autoclaving the




**Scheme 23.1** **a** Short-chain flavour acid production from natural alcohols by acetic acid bacteria. **b** Jasmonic acid and methyl jasmonate production with *Diploida gossypina*

jasmonic acid extracted from the fermentation broth in the presence of methanol (Scheme 23.1).

#### 23.4.1.2 Alcohols and Aldehydes

By volume, **ethanol** can be viewed as the most prominent flavour-active or flavour-enhancing compound produced by biotechnology, although in the scientific literature it is usually not categorised among the flavour compounds. With respect to the focus of this chapter on bioprocesses which aim at the synthesis of single-flavour compounds and not on traditional food and beverage fermentation processes, it should only be noted here that by traditional fermentation processes about 140,000,000 t of beer and 27,000,000 t of wine are produced worldwide annually [21]. This corresponds to roughly 5,000,000 and 2,000,000 t ethanol, respectively, making it a real 'bulk' chemical among the alcohols used in the food sector. Natural raw materials, such as starch hydrolysates or molasses, are fermented with yeasts to convert these cheap sugars to ethanol, a process which is currently gaining new public attention for its promising perspectives to provide an ecologically sound fuel from renewable resources (more than 18,500,000 t $year^{-1}$ 'bioethanol' [21]). Important flavour alcohols are derived from these ethanol-producing fermentation processes. During distillation of bioethanol or spirits, a cheap by-product of the yeast metabolism can be recovered, 'fusel oil'. This fraction usually contains **2-methylbutanol**, **3-methyl-**

**butanol** (**isoamyl alcohol**) and **2-methyl-1-propanol** as the main constituents in concentrations of 10–40 vol% [48], which are used directly in fruit flavour compositions or as starting materials for the biotechnological synthesis of natural flavour acids (Sect. 23.4.1.1), aldehydes or esters (Sect. 23.4.1.3). The fusel alcohols are products of the yeast's Ehrlich pathway, a three-enzyme cascade by which amino acids, here valine, leucine and isoleucine, are converted into their corresponding alcohols. This pathway, which is ubiquitous among yeasts, is described in more detail in Sect. 23.4.2.

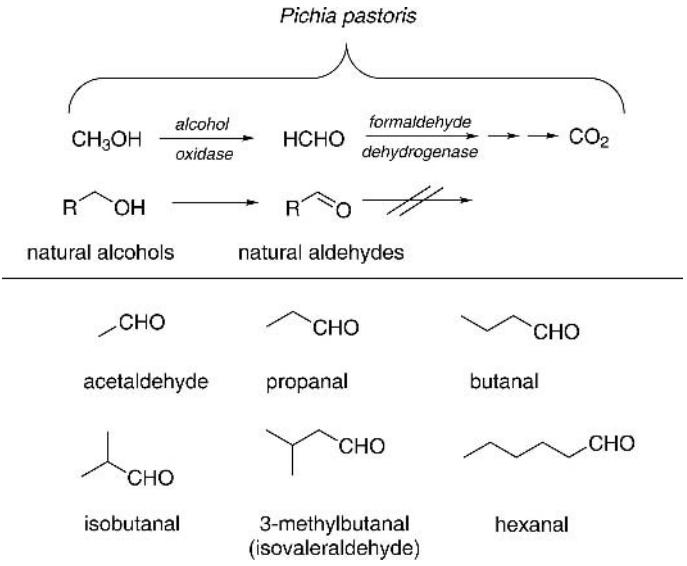
Short-chain aliphatic aldehydes, such as **acetaldehyde**, **2-methyl-1-propanal**, **2-methylbutanal** and **3-methylbutanal** (**isovaleraldehyde**), impart fruity and roast characters to flavour compositions [49]. Natural acetaldehyde is an important compound naturally occurring in a broad range of fruit flavours, essential oils and distillates; it augments fruit flavours and, for instance, it decisively contributes to the 'freshness' and 'juiciness' of foods and beverages, such as citrus juices [23, 50].

The aforementioned aldehydes can be efficiently produced by oxidation of the corresponding alcohols with alcohol oxidase (AOX) or alcohol dehydrogenase expressing microorganisms. The methylotrophic yeast *Pichia pastoris* can grow on methanol as the sole carbon and energy source using its strong alcohol oxidase (AOX) which is induced by methanol. The flavin-containing AOX naturally oxidises methanol to formaldehyde by reducing molecular oxygen to $H_2O_2$. This toxic intermediate is immediately cleaved into water and oxygen by the action of catalase, which co-acts with the AOX within special cell compartments, the peroxisomes. AOX has a low substrate specificity and also accepts alcohols other than methanol. Since the subsequent enzyme of the methanol degradation pathway, formaldehyde dehydrogenase, is highly specific, other alcohols are only converted into their corresponding aldehydes [51] (Scheme 23.2).

This makes *Pichia pastoris* an interesting biocatalyst for aldehyde production from alcohols in general. High product yields of *Pichia pastoris* catalysed oxidation of different short-chain alcohols have been described [51, 52]. In the case of acetaldehyde, a final concentration of 35 g $L^{-1}$, corresponding to an acetaldehyde productivity of 1.38 g $g_{cdw}^{-1}$ $h^{-1}$, has been reported [52], although only in small-scale analytical experiments. Resting cells were used as biocatalysts in a tris(hydroxymethyl)aminomethane (Tris)–HCl buffer to alleviate product inhibition by chelating the produced aldehyde with Tris. Performing the reaction at 5 °C instead of 30 °C and using a high Tris-HCl concentration of 3 M eliminated catabolite inactivation and product inhibition effects, respectively [53]. In a semi-continuous closed-loop pressurised bioreactor, high yields of up to 130 g $L^{-1}$ were obtained within 4 h. Stripping the volatile product via the exhaust air (where it can be recovered by cold or chemical trapping) was a simple alternative to maintain the acetaldehyde concentration below 0.2 g $L^{-1}$ in a 10-L airlift bioreactor [54]. If biphasic reaction systems are used, also more hydrophobic long-chain aliphatic, C6–C11, and aromatic alcohols, such as benzyl alcohol, 2-phenylethanol and 3-phenyl-1-propanol, are converted [51, 55] (Sect. 23. 4.2). With another methylotrophic yeast, *Candida boidinii*, an effective alcohol oxidation process on a pre-

parative scale was established [56]: induced yeast cells, grown in a methanol-fed prefermenter, were used as a biomass suspension of 33 g $L^{-1}$ in 15 L phosphate buffer pH 7.5 to convert isoamyl alcohol to isovaleraldehyde with a yield of 44% and a final concentration of about 40 g $L^{-1}$ within 7 h.

A process for aldehyde production using two bioreactors continuously operating in series was patented [57]. The first reactor was used for yeast production (e.g. *Candida boidinii, Pichia pastoris*, *Hansenula polymorpha*, *Torulopsis* sp.) on methanol, the effluent of which was directed into the second alcohol-fed reactor where the transformation to the aldehyde at a rate of 1.72 g $L^{-1}$ $h^{-1}$ occurred. Another method to produce aldehydes is alcohol dehydrogenation with acetic acid bacteria. In this case special mutants having low aldehyde dehydrogenase activities are needed to accumulate the aldehydes; otherwise overoxidation to the carboxylic acids dominates (Sect. 4.1.1). Such a mutant strain of *Gluconobacter oxydans* was isolated from beer and exploited to produce different short-chain aldehydes, such as acetaldehyde, **propanal**, **butanal** and isovaleraldehyde [58]. Especially the isoamyl alcohol oxidation worked very efficiently and showed a molar conversion of more than 90% and a productivity of about 1.5 g $L^{-1}$ $h^{-1}$ in 8 h. An integrated bioprocess with a hollow-fibre membrane contactor coupled to the bioreactor for liquid–liquid extraction with isooctane as the organic solvent was chosen to recover isovaleraldehyde continuously and thereby reduce toxic effects [59]. By this means the final product concentration was increased to 35 g $L^{-1}$ after 16 h.

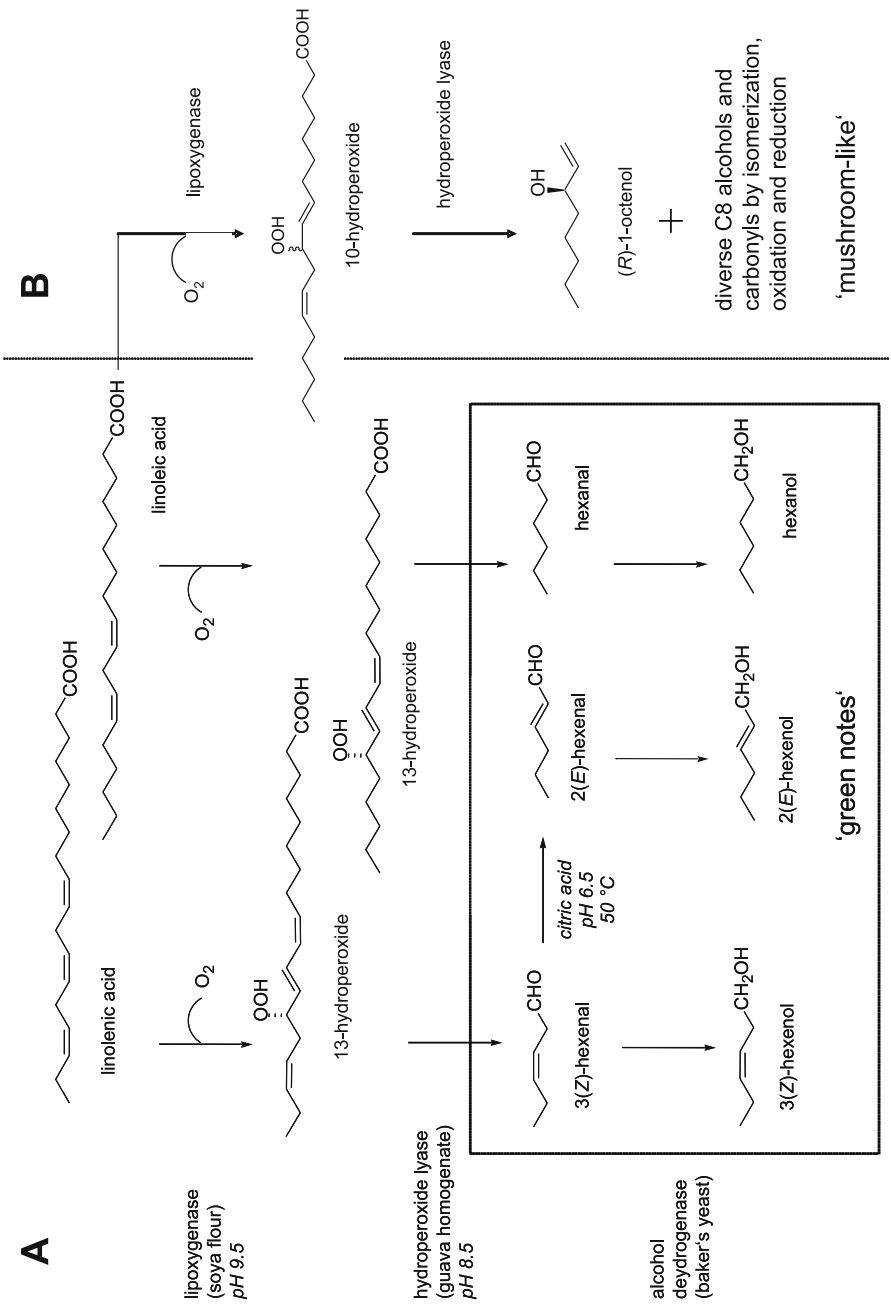



**Scheme 23.2** Production of aliphatic flavour aldehydes from natural alcohols using alcohol oxidase activity of *Pichia pastoris* cells

Among the aliphatic alcohols and aldehydes, a group of structurally related C6 compounds, comprising **(*Z*)-3-hexenal**, **(*E*)-2-hexenal**, **hexanal** and their corresponding alcohols, are of great importance to the flavour industry since they are responsible for a 'green' organoleptic sensation ('green notes'). In 1995 the market for natural green notes was estimated at 5–10 t year$^{-1}$ and US $3,000 per kilogram [60]. In nature these and also higher aliphatic aldehydes, such as C8 and C9 compounds, are derived from hydroperoxidation and cleavage of linoleic and linolenic acid by the sequential action of lipoxygenase and hydroperoxide lyase. Alcohol dehydrogenases synthesise the corresponding alcohols. The biochemistry of this reaction sequence as well as recent genetic engineering developments in this field are comprehensively described in Chap. 26. A series of quite similar biocatalytic strategies have been described based on the aforementioned biochemical principle during the last two decades [61–64]; by these methods, e.g., natural **(*Z*)-3-hexenol** is produced competitively to its isolation from peppermint oil distillation fractions [65]. A bioprocess patented by Firmenich [66] mimics the plant biochemistry starting from linoleic and linolenic acid and exploiting crude plant enzyme preparations of lipoxygenase (soya flour) and hydroperoxide lyase (e.g. guava fruit homogenate) to produce the desired aldehydes. Additionally, whole microbial cells, baker's yeast, are used as a reducing catalyst to convert the aldehydes into their corresponding alcohols, if desired (Scheme 23.3).

It is worth mentioning that the authors claimed the possibility to direct the bioprocess to each single target compound by variation of the process protocols. For instance, to obtain the desired 'leaf alcohol', (*Z*)-3-hexenol, linolenic acid is activated with lipoxygenase in the first step, but in the second step, hydroperoxide lyase and baker's yeast are added simultaneously to avoid chemical conversion of the aldehyde into its more stable isomer (*E*)-2-hexenal ('leaf aldehyde'). On the other hand, instead of adding the baker's yeast the pH is decreased to 6.5 and the temperature elevated to 50 °C to enhance leaf aldehyde formation. By this means about 4 g kg$^{-1}$ (*Z*)-3-hexenol and 1.5 g kg$^{-1}$ (*E*)-2-hexenal were obtained, indicating that the yields are still relatively low. Significant improvements of this process can be expected by heterologous expression of the respective enzymes, thereby enhancing and/or combining the activities within one host organism [37, 67] (Chap. 26).

In fungi a homologous reaction sequence leads to the formation of aliphatic C8 compounds, among which **(*R*)-1-octen-3-ol** is the most important one with an intensive mushroom-like odour (Scheme 23.3). In plants, the biosynthesis of the C6 volatiles is initiated after damage of the cells contacting the enzymes and the substrates which are located in different compartments and allowing molecular oxygen to penetrate into the tissue ('freshly cut green grass'). This principle has been transferred to a production process for natural mushroom flavour: after submerged fermentation of edible fungi, such as *Pleurotus* sp. or *Morchella* sp., the fungal mycelium suspension is fed into a homogeniser to break the cells, thereby inducing the lipoxygenase-catalysed reaction sequence followed by an agitation/aeration vessel to enable a high oxygen supply [68]. The biomass suspension is recirculated several times before it is harvested and freeze-dried to give a mushroom powder containing approximately 1.2 g kg$^{-1}$ (*R*)-1-octen-3-ol,




**Scheme 23.3** Formation of aliphatic flavour aldehydes and alcohols. **a** Biotechnological reaction sequence mimicking plant biosynthesis of C6 compounds ('green notes'). **b** Homologous reaction sequence in fungi leading to mushroom-like C8 compounds. The stoichiometric formation of *ω*-oxo-carboxylic acids during hydroperoxide lyase cleavage is not depicted

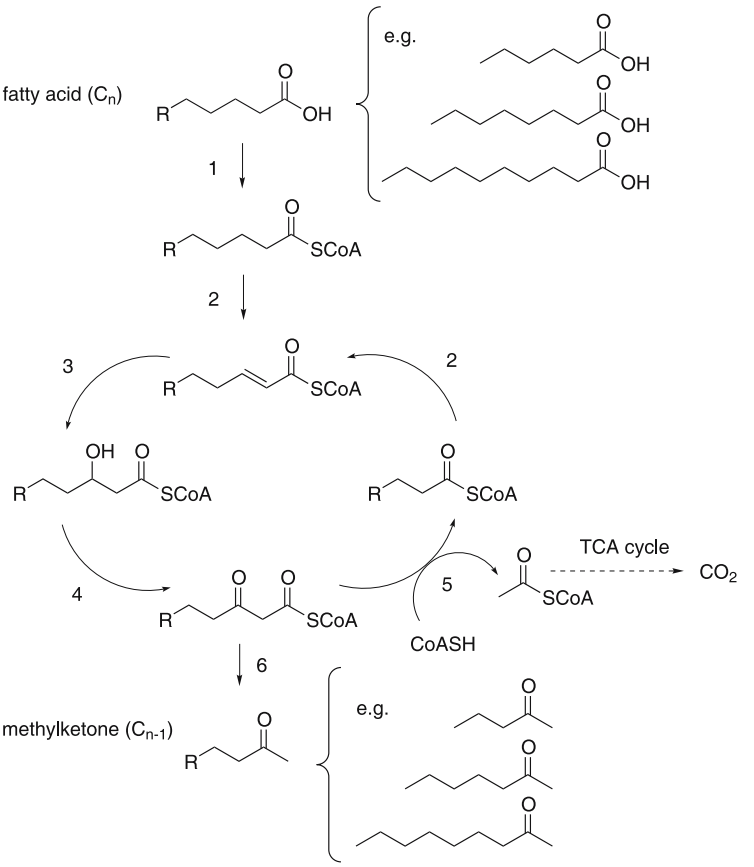
besides other C8 alcohols and carbonyls, for flavouring purposes. In another industrial-scale process wasted mushroom stems are used as enzyme-containing raw material mixed with linoleic acid as a precursor [46].

#### 23.4.1.3 Ketones

The odd-numbered **methylketones** have characteristic nutty cheese-like notes and are used in cheese flavour compositions [49]. The distinct taste of Roquefort cheese is substantially due to **2-heptanone** and **2-nonanone**. Methylketone formation is an aerobic process which is strongly favoured when the fungal growth is restricted and which does not occur with long-chain fatty acids. The fatty acid degradation pathway involves a key component, 3-oxoacylcoenzyme A (3-oxoacyl-CoA), which can be converted either into methylketone, by hydrolysis through thiohydrolase action followed by decarboxylation, or into $CO_2$, by thiolase followed by the citric acid cycle (β-oxidation of fatty acids) (Scheme 23.4). The bioformation of the methylketones results from an overflow of the β-oxidation cycle, where an excess of 3-oxoacyl-CoA ester is accumulated. One industrial process for the production of C5–C9 methylketones from the corresponding C6–C10 fatty acids uses *Penicillium roquefortii* grown by solid-state fermentation on buckwheat seeds [69, 70]. The whole sporulation medium is used for bioconversion without discarding the grains. This lowers the viscosity of the culture liquid, permitting a higher oxygen input than in a typical filamentous culture. A two-phase water/tetradecane system is used for in situ extraction of the product; there are no toxic effects of the organic solvent on the fungal spores: $\log P_{\text{octanol/water}}$(tetradecane)=7. Different product yields are reported: depending on the respective fatty acid used as the starting material 20 g $L^{-1}$ **2-pentanone**, 75 g $L^{-1}$ **2-heptanone** and 60 g $L^{-1}$ **2-nonanone** were produced.

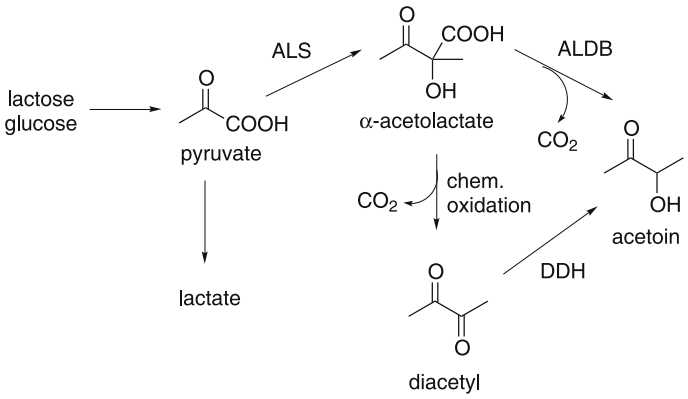
**3-Hydroxy-2-butanone** (**acetoin**) is a characteristic constituent of butter flavour used for flavouring margarine and can be obtained as a by-product of molasses-based and lactic acid fermentations [49, 71]. The closely related **2,3-butanedione** (**diacetyl**) has a much lower organoleptic threshold than acetoin and is an important strongly butter-like flavour compound in butter and other dairy products [72]; in buttermilk, for instance, the diacetyl concentration is only about 2–4 mg $L^{-1}$ [73]. α-Acetolactate (α-AL) is an intermediate of lactic acid bacteria mainly produced from pyruvate by α-acetolactate synthase. In most lactic acid bacteria, α-AL is decarboxylated to the metabolic end product acetoin by α-AL decarboxylase (ALDB) [71] (Scheme 23.5).

Special flavour-active strains, however, which do not contain ALDB, accumulate α-AL and, as a result of its chemical oxidative decarboxylation, generate high diacetyl levels in dairy products. Consequently, several processes have been patented for the production of natural diacetyl in the past few decades which usually involve a chemically enhanced conversion of α-AL into diacetyl or aim at α-AL itself as the biological product, which can serve as a less-volatile




**Scheme 23.4** Production of methylketones from fatty acids by *Penicillium roqueforti*. *1* ATP-dependent acylcoenzyme A (*acyl-CoA*) synthase; *2* flavin adenine dinucleotidedependent acyl-CoA dehydrogenase; *3* enoyl-CoA hydratase; *4* NAD-dependent 3-hydroxyacyl-CoA dehydrogenase; *5* 3-oxoacyl-CoA thiolase; *6* 3-oxoacyl-CoA thiolester hydrolase and 3-oxoacid decarboxylase. (Adapted from [46])

diacetyl precursor in food applications [74–76]. High diacetyl concentrations of up to 14 g $L^{-1}$ have been described for a patented process based on *Streptococcus cremoris* and *S. diacetylactis* in a milk or whey medium supplemented with citric acid as a precursor [70]. A characteristic feature of this process was the need for an oxidising reagent during steam distillation, e.g. ferric chloride. Even higher concentrations of acetoin plus diacetyl of 35 g $L^{-1}$ in total were described for the cultivation of *Enterobacter cloacae* ATCC 27613 in a complex nutrient broth followed by chemical conversion of the microbially produced acetoin, resulting in an overall yield of 60% diacetyl calculated on the basis of sugar consumed [77]. In recent years, detailed knowledge of the metabolism of lactic acid bac-




**Scheme 23.5** Metabolic pathways of lactic acid bacteria leading from pyruvate to α-acetolactate and acetoin and chemical diacetyl formation. *ALS* α-acetolactate synthase, *ALDB* α-acetolactate decarboxylase, *DDH* diacetyl dehydrogenase. (Adapted from [72])

teria has led to innovative strategies for engineering *Lactococcus lactis* strains to enhance α-AL diacetyl, and acetoin production [72]. With *Lactococcus lactis* overexpressing *Streptococcus mutans* NADH oxidase (to redirect the pyruvate pool from lactate production to NADH-independent pathways) and having an inactivated ALDB, no more lactic acid production was observed and the strain converted glucose into α-AL, diacetyl and acetoin with yields of 57, 16 and 5%, respectively, as well as into acetate and $CO_2$ as by-products without the need for any other precursor. Nevertheless, to improve natural diacetyl production, here 137 mg $L^{-1}$, the physicochemical reaction conditions are to be adjusted to enhance the chemical oxidative decarboxylation of α-AL, e.g. by extending the aeration time preferentially at a lower pH than used during fermentation [72].

#### 23.4.1.4 Esters

Esters are widespread in fruits and especially those with a relatively low molecular weight usually impart a characteristic fruity note to many foods, e.g. fermented beverages [49]. From the industrial viewpoint, esterases and lipases play an important role in synthetic chemistry, especially for stereoselective ester formations and kinetic resolutions of racemic alcohols [78]. These enzymes are very often easily available as cheap bulk reagents and usually remain active in organic reaction media. Therefore they are the preferred biocatalysts for the production of natural flavour esters, e.g. from short-chain aliphatic and terpenyl alcohols [7, 8], but also to provide enantiopure novel flavour and fragrance compounds for analytical and sensory evaluation purposes [12]. Enantioselectivity is an impor-

tant factor as many flavour esters often have a very different sensory profile depending on their enantioforms [79]. Since a separate chapter of this book is devoted to the use of isolated enzymes in flavour science and technology (Chap. 22 by Menzel and Schreier), the focus here is on ester formation strategies based on whole microbial cells which also yield high product concentrations.

One interesting approach takes advantage of the high esterase activity of some fungi which can be harnessed without isolating the enzymes: dried fungal mycelium, especially from *Rhizopus oryzae*, can be used as an effective ester synthesising biocatalyst in organic solvents [80–82]. After growth on different Tweens (20, 40, 60 and 80) as the main carbon source the fungus show significant carboxylesterase activity. This strategy alleviates any costly enzyme preparation; moreover, the endogenous enzyme system is stabilised by the cellular structures, such as membranes, and the lyophilised biomass can be used as a self-immobilised catalyst for efficient flavour synthesis, e.g. for the direct esterification of 2-methylbutanol, 3-methylbutanol and hexanol with acetic acid or butanoic acid [81]. With butanoate, almost quantitative conversion of 65 mM of the respective alcohol was achieved after 24 h with *Rhizopus oryzae* cells in *n*-heptane. Up to 30 g $L^{-1}$ **isopentylhexanoate** per gram of acetone-dried mycelium of *Rhizopus arrhizus* was achieved in a column reactor [83]. The esterification of a racemic mixture of **2-octanol** and **butanoic acid** proceeded with more than 97% enantiomeric excess for the *R* ester [82]. Aromatic acids are also substrates suitable for this approach using mycelium-bound carboxylesterases (Sect. 23. 3.2). High yields of short-chain fatty acid ethyl esters (C2–C8) were obtained with lyophilised *Rhizopus chinesis* cells, e.g. 98.5% for **ethylhexanoate** after 80 h using 0.6 M hexanoic acid in heptane and an acid-to-alcohol ratio of 1:1.3 [84].The initial water activity turned out to be an important parameter and $a_w$ values ranging from 0.66 to 0.97 led to higher yields. The whole-cell lipase approach contributed to a long-lasting operational stability of the biocatalyst with half lives of up to 981 h as determined by its multiple reuse in consecutive batches. In 1987 an elegant bioprocess was patented based on living microorganisms to produce C2–C5 alkyl esters useful as natural fruit-like flavours [85]. *Geotrichum fragrans* was the preferred yeast for this synthesis, which needs two kinds of precursors, C5–C6 amino acids, i.e. leucine, isoleucine or valine, and natural aliphatic alcohols, such as those described in Sect. 23.4.1.2. The key steps of this bioconversion pathway are the initial oxidative deamination of the amino acids followed by decarboxylation/CoA ester formation by $\alpha$-ketoacid dehydrogenase—a multienzyme complex—resulting in activated C4–C5 carboxylic acids (Fig. 23.3). Externally added alcohol, such as ethanol, causes interesterification of the CoA esters to the desired flavour esters. When mixtures of amino acids were used, complex fruity ester compositions were obtained. For commercially feasible yields of esters, continuous sweeping of the volatile products into the air stream was necessary. The product recovery included adsorption to activated charcoal from the fermentation exhaust air stream, extraction of the loaded charcoal by an organic solvent and distillation.

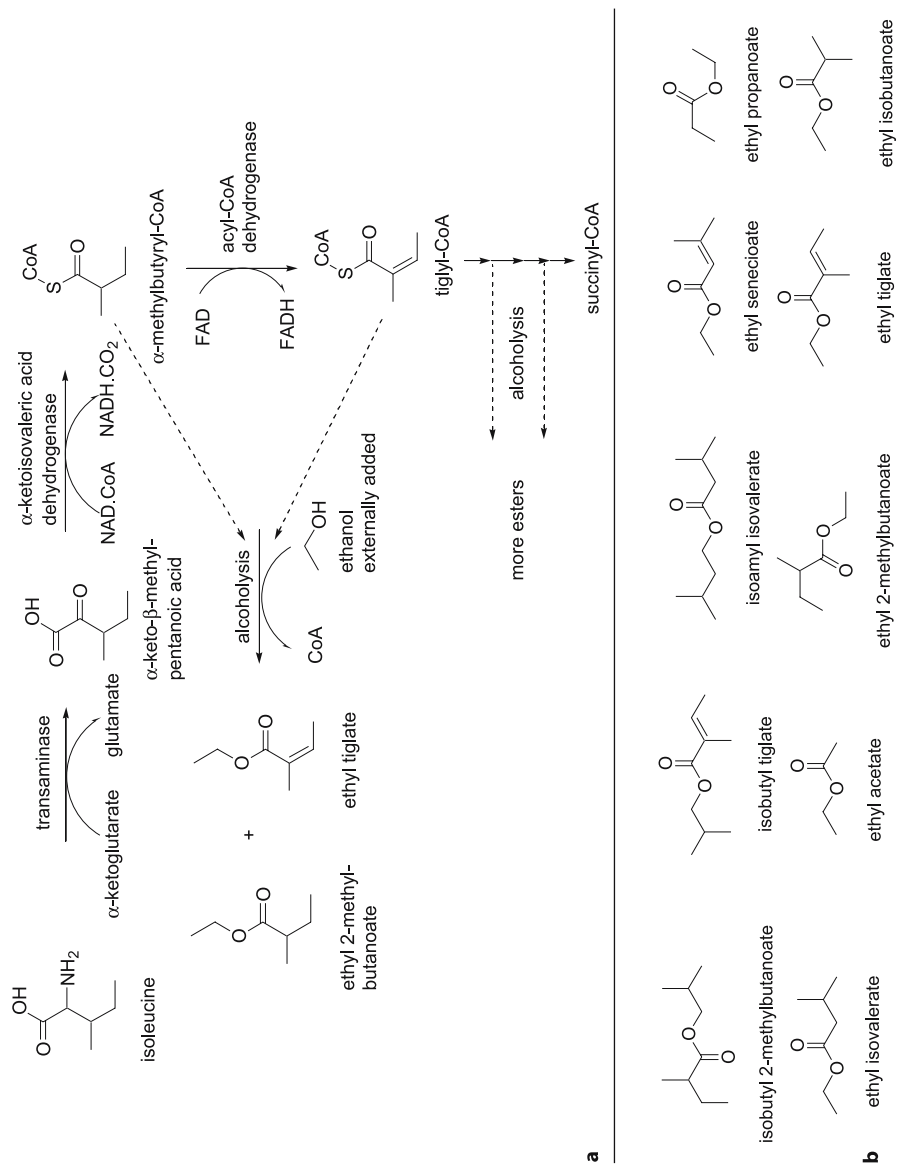



**Fig. 23.3** **a** Ester formation via alkyl-CoA alcoholysis with yeasts (preferably *Geotrichum fragrans*) according to [85], exemplarily shown for ethyl-2-methylbutanoate and ethyl tiglate. **b** Some possible flavour esters producible depending on amino acid and alcohol used as substrates

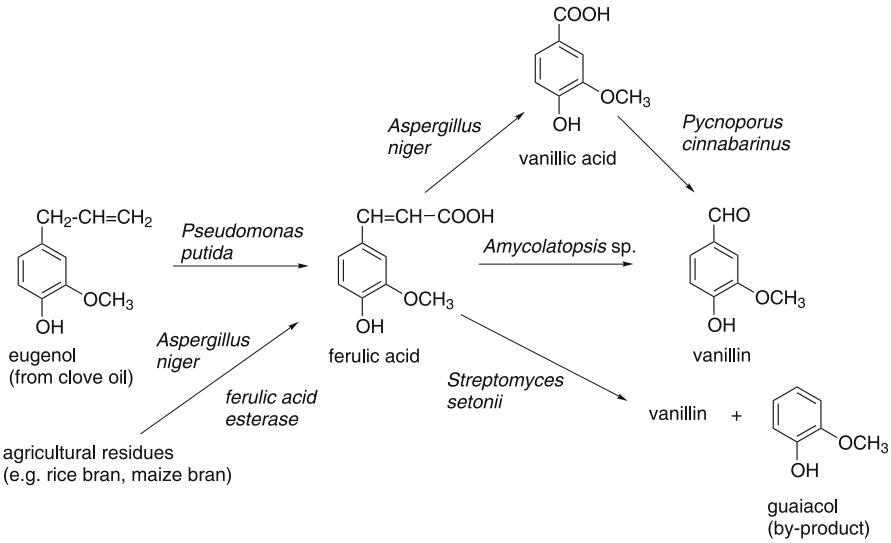
The membrane-bound alcohol acetyl transferase is the key enzyme for another yeast-based ester synthesis: natural **ethyl acetate**, the most common ester in fruits and which is used for fruit and brandy flavours [49], can be produced in high concentrations with *Candida utilis* [83]. Under iron-limiting conditions, the tricarboxylic acid cycle cycle is inhibited and the intracellular pool of acetyl-CoA increases. In a fed-batch process the concentration of ethanol produced was maintained at a high level, thereby yielding 10–15 g $L^{-1}$ ethyl acetate. Owing to the Crabtree effect ('aerobic fermentation'), *Candida utilis* converts most of the added glucose to ethanol under aerobic conditions, thus providing the substrate ethanol for the desired ester formation by alcoholysis of the acetyl-CoA. This is postulated to be a protective mechanism of the yeast against the toxic ethanol by producing the less toxic and more volatile ethyl acetate. Another yeast, *Williopsis saturnus* var. *mrakii*, shows a remarkably high de novo activity to produce fruity esters owing to its strong alcohol acetyl transferase which converts branched alcohols from amino acid metabolism into the corresponding acetates; this ester formation can be drastically enhanced by addition of the alcohols, e.g. those isolated from fusel oil, to the culture [48]. Owing to toxic effects, the fusel oil is added at low levels after the growth phase during the stationary bioconversion phase. The esters are recovered from the exhaust air by sorption on activated charcoal followed by organic solvent extraction. 3-Methylbutanol was the preferred alcohol which was converted into **3-methylbutyl acetate**, the character-impact compound of banana aroma, in a high yield (approximately 90%). Recently, a recombinant sake yeast overexpressing the *ATF1* gene coding for alcohol acetyl transferase was successfully engineered and produced up to the fivefold 3-methylbutyl acetate concentration compared with the wild type [86]; owing to a self-cloning strategy, this strain is not treated as a genetically modified organism in Japan.

### 23.4.2 Aromatic Compounds

In this section microbially produced benzene derivatives which are important as natural flavour compounds are discussed without further subdividing this section according to the functional groups, such as alcohols, aldehydes and acids. Metabolic pathways leading to the desired targets usually start from aromatic amino acids and/or phenylpropanoids, such as cinnamic acid, ferulic acid, eugenol and phenylpyruvic acid [6]. White-rot fungi, especially basidiomycetes found on living or dead wood, are capable of degrading lignin, a polymer of substituted *p*-hydroxycinnamyl alcohols. These fungi are the preferred microorganisms for studying aromatic flavour generation owing to their versatile enzyme machinery which has emerged during evolution [11, 87]. Nevertheless, characteristic disadvantages of these filamentous fungi, e.g. slow growth, difficult technical handling of the mycelia-forming organisms, a vast number of concurrently formed flavour-active products and low yields of the target compounds, impede

their routine application in industrial processes. Thus, although the flavour compounds discussed in this section can all be produced by higher fungi, too, bacteria and yeasts are preferred, as they have a faster metabolism and, given that an appropriate production strain can be found (e.g. by enrichment cultures or mutagenesis/selection approaches), they usually result in narrower product spectra and higher yields in precursor-supplemented media. For the production of natural aromatic flavours it is of great benefit that L-phenylalanine has been made available as a natural precursor by microbial fermentation from the industrial L-aspartame process. Other phenylpropanoid precursors, such as eugenol or ferulic acid, can be found abundantly in nature.

**Vanillin** is undoubtedly the most important flavour compound with respect to both market volume and market value; a separate chapter of this book is dedicated to this flavour compound (Chap. 9 by Verpoorte and Korthout). It is the main aroma compound of the cured pods of *Vanilla* sp. [88]. Annually more than 10,000 t is produced, mainly by chemical synthesis. Whereas chemically produced vanillin is a cheap 'bulk flavour compound' available for about US $10 per kilogram, natural vanillin derived from *Vanilla* is only available in very low amounts and is therefore limited to a few select premium food applications. This opens an attractive market niche for biotechnology: natural vanillin from microbial processes currently costs up to US $1,000 per kilogram [8]. The annual world market volume of biotechnologically produced vanillin can be estimated to be 1–10 t and to be expanding steadily. This illustrates the increasing popularity of natural biotech vanillin although its discrimination from natural vanillin 'ex *Vanilla*' by isotope analysis is possible [88]. Different bioprocess strategies have been investigated based on bioconversion of ferulic acid, phenolic stilbenes, isoeugenol or eugenol and on de novo biosynthesis, applying bacteria, fungi, plant cells or engineered microorganisms [89]. The current industrial processes are based on the bioconversion of ferulic acid by different bacteria, which obviously have an outstanding tolerance against vanillin, which is cytotoxic at higher concentrations: a process patented by Haarmann & Reimer [90] uses the actinomycete *Amycolatopsis* sp. HR 167, with which a product concentration around 12 g $L^{-1}$ can be obtained in a fed-batch process (Scheme 23.6). A similar approach using *Streptomyces setonii* as a production strain was patented by Givaudan, leading to final concentrations of up to 16 g $L^{-1}$ after 50 h [91]. **Guaiacol**, missing the aldehyde group of vanillin, is a valuable by-product of this bioconversion (up to 0.4 g $L^{-1}$) because it significantly contributes to the characteristic flavour of *Vanilla* extracts and is often used together with vanillin in flavour compositions (Scheme. 23.6). In another patent, an *Amycolatopsis* mutant essentially free of by-product formation was described together with the downstream processing including precipitation of the vanillin by concentrating and cooling and further purification of the solid vanillin using a liquefied gas, preferentially $CO_2$ [92]. This strain converted 32 g $L^{-1}$ ferulic acid to almost 18 g $L^{-1}$ within approximately 50 h. The precursor currently used in all industrial biotech vanillin production processes, ferulic acid, can be obtained by microbial bioconversion of eugenol—abundantly available from the essential



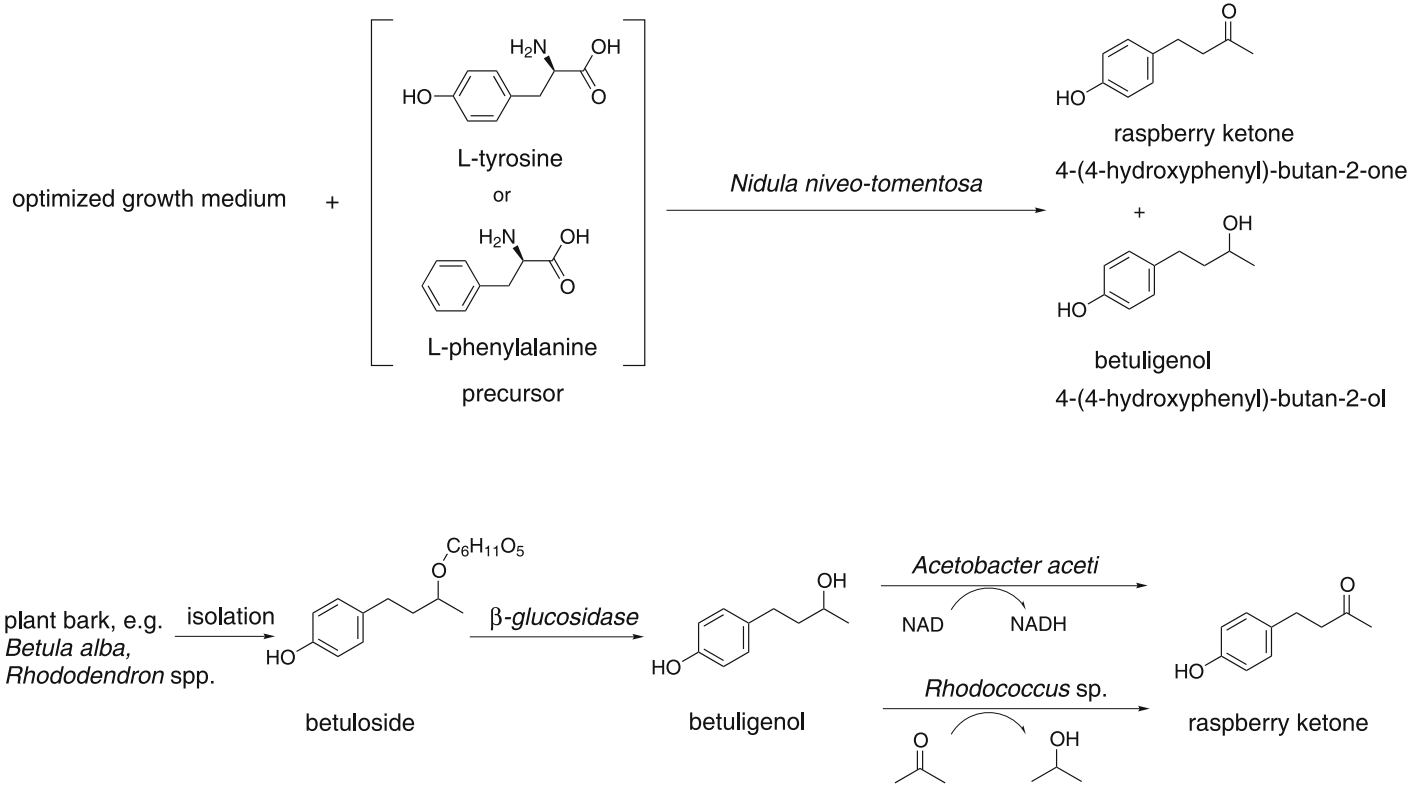

**Scheme 23.6** Microbial strategies for the production of natural vanillin

oil of the clove tree *Eugenia cariophyllus*—with a eugenol-tolerant *Pseudomonas putida* [93] under sequential precursor-feeding conditions or by direct isolation from plant materials, e.g. rice bran, using ferulic acid esterase. Genetic engineering has been successfully applied to produce vanillin by direct bioconversion of the cheaper precursor eugenol instead of ferulic acid using metabolically engineered *Pseudomonas* or *Rhodococcus* strains [89, 94]. Nevertheless this route is currently not feasible for commercial production in Europe owing to the negative public perception of any food-related use of genetically modified microorganisms. Finally, a strategy using two filamentous fungi in succession for direct bioconversion of maize bran into vanillin is worth mentioning [95]. *A. niger* is exploited to release ferulic acid from the natural raw material by its feruloyl esterase activity and to subsequently metabolically convert ferulic acid into vanillic acid, which is further transformed into vanillin by *Pycnoporus cinnabarinus*. Under theses conditions, 584 mg $L^{-1}$ vanillin was produced directly from ferulic acid containing raw material in a 'one-pot' approach (Scheme 23.6).

**Benzaldehyde**, with its bitter almond flavour, is the second-most important flavour compound, with a world market of approximately 7,000 t $year^{-1}$ [96]. Whereas by far the majority is chemically synthesised, there is, nevertheless, a growing market for the natural flavour compound, accounting for approximately 100 t $year^{-1}$ [87]. But, about 80% of this natural benzaldehyde represents a grey zone as it cannot be officially regarded as 'natural' according to EU legislation since a chemical hydrolysis is involved in its preparation from cassia oil. A biocatalytic route starting from amygdalin, a glycoside present in fruit kernels,

based on the consecutive use of $\beta$-glucosidase (to release mandelonitrile) and mandelonitrile lyase has been used in the industry but owing to safety problems associated with the generation of equimolar amounts of hydrogen cyanide alternative strategies are needed [70]. With different basidiomycetes, such as *Trametes*, *Ischnoderma*, *Polyporus* and *Bjerkandera* species, benzaldehyde concentrations in the range of several hundred milligrams per litre up to about almost 1 g $L^{-1}$ can be achieved in media supplemented with L-phenylalanine [97–100]. Benzyl alcohol was usually produced concomitantly during the same processes. In situ product recovery techniques, such as adsorption to a styrene/divinylbenzene copolymer resin selective for aromatic compounds, or organophilic pervaporation with poly(dimethylsiloxane) (PDMS) membranes have been successfully applied to improve productivities and final product concentrations [97, 101, 102] (Fig. 23.4). These effects were attributed to the circumvention of both product inhibition by benzaldehyde and its further conversion to the corresponding alcohol. Nevertheless, a disadvantage of these processes based on basidiomycetes is the long cultivation time needed for growth and bioconversion which usually amounts to more than 10 days.

Natural **raspberry ketone**, 4-(4′-hydroxyphenyl)-butan-2-one, is the character-impact compound of the aroma of raspberries. Although the chemically synthesised ketone only costs about US $10 per kilogram the flavour industry would prefer the natural ketone for many food applications. Unfortunately, its recovery from the natural source is impractical owing to the very low concentrations found in the berries (less than 4 mg $kg^{-1}$ [48]). This situation has stimulated various attempts at a biotechnological production. Nevertheless up to now no economically viable biotechnological production has been described although the target substance may achieve a market price of more than US $1,000 per kilogram as a 'natural' flavour compound. Up to now, mainly two biotechnological strategies have been proposed (Scheme 23.7). The de novo synthesis with the basidiomycete *Nidula niveo-tomentosa* can be significantly enhanced by adding natural amino acid precursors, L-tyrosine or L-phenylalanine, to the medium. In an optimised medium, this basidiomycete produced raspberry ketone and its corresponding alcohol betuligenol with a total product yield (raspberry ketone and betuligenol) of approximately 200 mg $L^{-1}$ after 22 days, a 50-fold increase compared with the non-optimised system [103]. Nevertheless, the less-flavour-active alcohol is the primary product and the overall productivity is still far too low for a commercial application. On the other hand, this approach illustrates that raspberry ketone production starting from the cheap precursor L-phenylalanine is, in principle, possible, justifying further elucidation of the respective pathway, which differs from that found in raspberry as evidenced by stable isotope labelled precursor feeding studies [104]. The second strategy to produce natural raspberry ketone is a biocatalytic two-step conversion involving the $\beta$-glucosidase-catalysed hydrolysis of the naturally occurring betuloside, a 2-glycoside of 4-(4-hydroxyphenyl)-2-butanol (glucoside, mannoside). This precursor occurs in different plants: the bark of the European white birch (*Betula alba*), rhododendron (*Rhododendron* spp.), maple (*Acer* spp.), fir (*Ab-*




**Scheme 23.7** Biotechnological strategies for the production of natural raspberry ketone

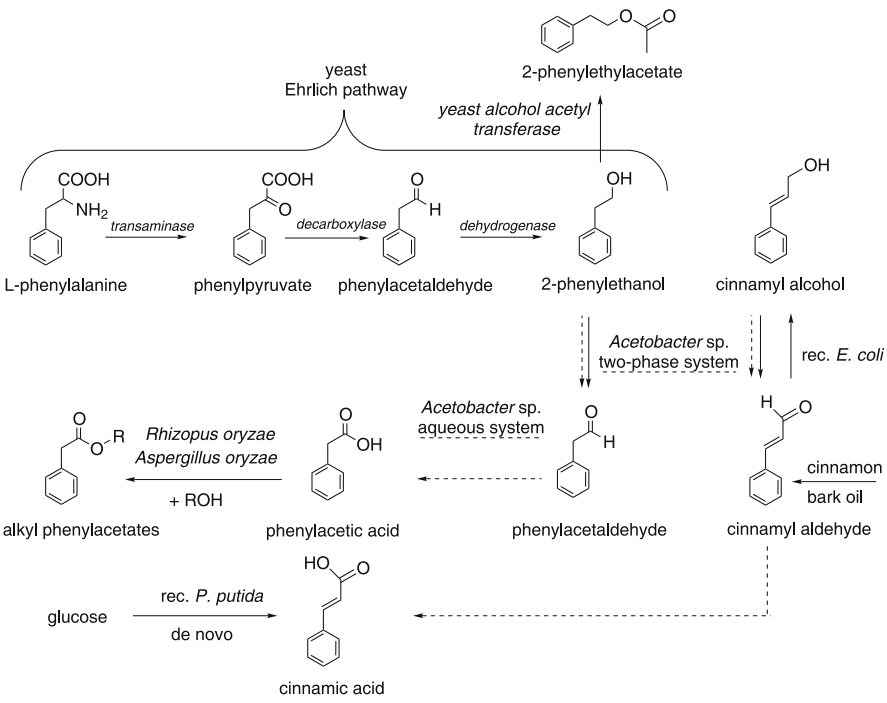
*ieta* spp.) and yews (*Taxus* spp.). By hydrolysis, betuligenol is released, and is transformed by a microorganism containing a secondary alcohol dehydrogenase such as *Acetobacter aceti* into the corresponding raspberry ketone [105]. In a recent publication, the oxidation was performed with lyophilised *Rhodococcus* cells in phosphate buffer containing 10% v/v acetone as a hydrogen acceptor [106]. This biocatalytic oxidation shows a high yield of 89% and can be performed at precursor concentrations of up to 500 g $L^{-1}$. Thus, the main bottleneck still preventing an industrial application is now obviously the lack of an economically viable supply of the natural precursor betuligenol rather than the biooxidation process itself.

**2-Phenylethanol** has a rose-like odour and makes the chemically produced compound the most used fragrance chemical in perfume and cosmetics, with a world market of about 7,000 t $year^{-1}$ [107, 108]. 2-Phenylethanol is also found in many foods as a characteristic flavour compound rounding off the overall aroma, especially in foods obtained by fermentation, such as wine, beer, cheese, tea leaves, cocoa, coffee, bread, cider and soy sauce [109]. In food applications, natural 2-phenylethanol is preferred rather than its nature-identical counterpart from chemical synthesis and it has a market volume of 0.5–1 t $year^{-1}$. This product is sold at market prices of up to US $1,000 per kiklogram and is mainly produced by yeast-based bioprocesses since its isolation from natural sources, e.g. rose oil, would be too costly [109].

Although 2-phenylethanol can be synthesised by normal microbial metabolism, the final concentrations in the culture broth of selected microorganisms generally remain very low [110, 111]; therefore, de novo synthesis cannot be a strategy for an economically viable bioprocesses. Nevertheless, the microbial production of 2-phenylethanol can be greatly increased by adding the amino acid L-phenylalanine to the medium. The commonly accepted route from L-phenylalanine to 2-phenylethanol in yeasts is by transamination of the amino acid to phenylpyruvate, decarboxylation to phenylacetaldehyde and reduction to the alcohol, first described by Ehrlich [112] and named after him (Scheme 23.8).

During the last few decades a series of microbiological and technical approaches have been published aiming at improving growth-associated 2-phenylethanol formation based on Ehrlich bioconversion [113–118]. *Kluyveromyces* and *Saccharomyces* species have been shown to be efficient biocatalysts leading to molar conversion yields of more than 90%. In situ product removal is essential for high-performance processes by alleviating product inhibition which can already significantly impair growth at a 2-phenylethanol concentration of about 0.3 g $L^{-1}$ strain-dependently [119]. Coupling an organophilic pervaporation membrane to a bioreactor cultivation of the thermotolerant yeast *Kluyveromyces marxianus* CBS 600 at 40 °C resulted in volumetric productivities of up to 5.2 mmol $L^{-1}$ $h^{-1}$ [119] (Fig. 23.4).

The flavour product from L-phenylalanine included 2-phenylethanol as the main product and **2-phenylethyl acetate** as a side product, which is also a valuable rose-like flavour compound with a more fruity note and which is formed




**Scheme 23.8** Some microbial pathways and biotransformations leading to aromatic flavour molecules

within the yeast metabolism by the action of alcohol acetyl transferase. Performing the integrated bioprocess at 45 °C yielded only a slightly lower overall product concentration but with the acetate as the major product. Recently an aqueous–organic two-liquid-phase bioprocess has been described reporting the highest 2-phenylethanol and 2-phenylethyl acetate space-time yields and final concentrations so far [120]. Again, *Kluyveromyces marxianus* CBS 600 was used in a fed-batch emulsion system with poly(propylene glycol) 1200 as a non-volatile and biocompatible organic solvent efficiently extracting the flavour compounds from the aqueous culture medium (phase ratio of approximately 1:1) within the bioreactor (Fig. 23.5). Space-time yields of 0.33 and 0.08 g $L^{-1}$ $h^{-1}$ were obtained for 2-phenylethanol and 2-phenylethyl acetate, respectively, corresponding to final concentrations of 26.5 and 6.1 g $L^{-1}$ in the organic phase after 30 h. The amino acid was provided as the sole nitrogen source in high excess (above its solubility threshold), making complicated feeding strategies unnecessary.

The elucidation of the genetics and regulations of the Ehrlich pathway leading from amino acids to alcohols and the corresponding acids and esters—a pivotal metabolic route to flavours generated by traditional food fermentation processes—has attracted much research interest in the past. More recent inves-

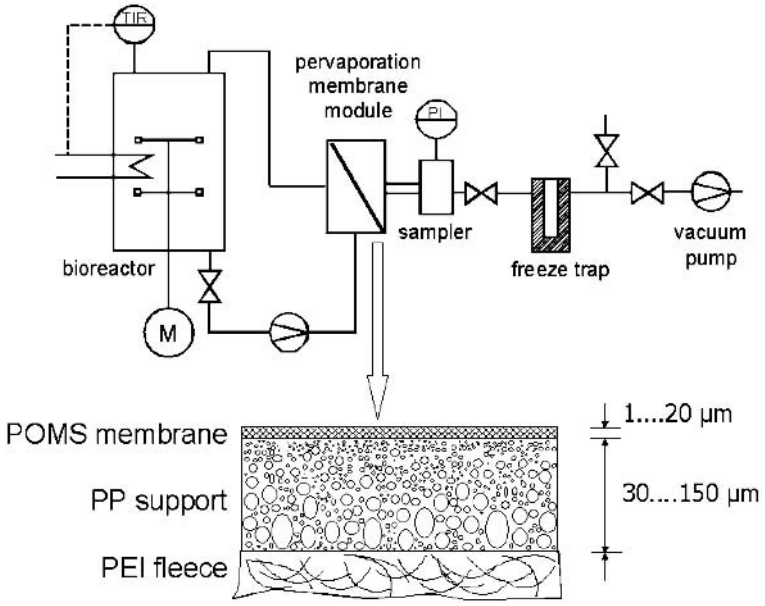



**Fig. 23.4** Organophilic pervaporation (*PV*) for in situ recovery of volatile flavour compounds from bioreactors. The principle of PV can be viewed as a vacuum distillation across a polymeric barrier (membrane) dividing the liquid feed phase from the gaseous permeate phase. A highly aroma enriched permeate is recovered by freezing the target compounds out of the gas stream. As a typical silicone membrane, an asymmetric poly(octylsiloxane) (*POMS*) membrane is exemplarily depicted. Here, the selective barrier is a thin POMS layer on a polypropylene (*PP*)/poly(ether imide) (*PEI*) support material. Several investigations of PV for the recovery of different microbially produced flavours, e.g. 2-phenylethanol [119], benzaldehyde [264], 6-pentyl-$\alpha$-pyrone [239], acetone/butanol/ethanol [265] and citronellol/geraniol/short-chain esters [266], have been published

tigations reveal a surplus of isogenes responsible for each enzymatic transformation step and indicate complex regulation principles on both transcriptional and posttranscriptional levels [121–129]. The accumulated knowledge should lead to improved production strains for this type of amino acid derived flavour compound by genetic engineering in the near future.

Nevertheless, for the production of the flavour-active aromatic alcohol derivatives, such as the corresponding aldehydes and acids, metabolic engineering approaches have to compete with conventional oxidative biocatalysis starting from the natural alcohol as a substrate. For instance, the whole-cell oxidation system based on *Pichia pastoris* AOX already described in Sect. 23.4.1.2 can also be used to convert benzyl alcohol to benzaldehyde in aqueous media although product inhibition restricted the final product concentration to about 5 g $L^{-1}$, indicating the need for aqueous–organic two-phase reaction media [51]. **Phenylacetalde-**

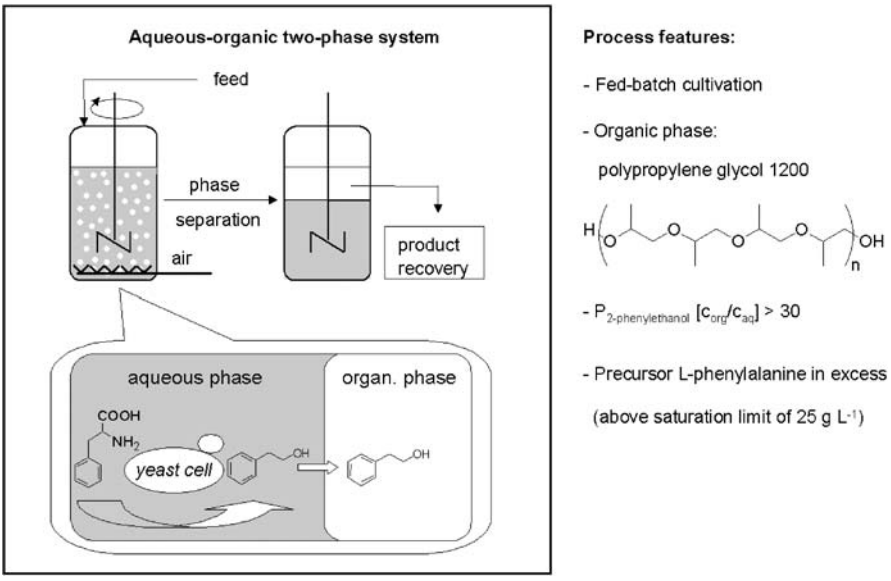



**Fig. 23.5** Aqueous–organic two-liquid-phase system for microbial production of flavour compounds. Here the formation of 2-phenylethanol from L-phenylalanine is exemplarily shown [120]. The organic solvent used for in situ extraction has to be carefully selected on the basis of multiple criteria, such as biocompatibility, non-flammability and legislative regulations. For a more detailed description of flavour production in two-phase systems, see Chap. 24 by Larroche et al.

**hyde** can be efficiently synthesised with acetic acid bacteria making use of their strong oxidative capacity provided by the dehydrogenase system (Sect. 23.4.1.2) [130]. An *Acetobacter* sp. strain immobilised in alginate beads produced 1.92 g $L^{-1}$ phenylacetaldehyde from 4 g $L^{-1}$ 2-phenylethanol and showed higher production rates than non-immobilised cells, which was explained by protection from toxic effects caused by the product and/or the precursor. In situ product recovery by a two-liquid-phase system consisting of isooctane–water (1/1 v/v) was successfully performed and yielded 9 g $L^{-1}$ phenylacetaldehyde recovered in the organic phase from 10 g $L^{-1}$ 2-phenylethanol within 4 h using another *Acetobacter* strain [131]. The composition of the medium in this biotransformation can be exploited as a control mechanism to direct the oxidation of aromatic alcohols to either the aldehyde in the presence or the acid in the absence of the organic phase [132] (Scheme 23.8). By this means 2-phenylethanol and cinnamyl alcohol were transformed to the corresponding acids, **phenylacetic acid** and **cinnamic acid**, in water with yields of more than 97% within 3 and 8 h, respectively; phenylacetaldehyde and **cinnamyl aldehyde** were produced from the alcohols within only 45 min in water–isooctane with yields of 90 and 77%, respectively. The process for the production of acids from aliphatic alcohols with *Gluconobacter oxydans* DSM 12884 described in Sect. 23.4.1.1 was also successfully applied to aromatic

alcohols: benzyl alcohol, 2-phenylethanol, and cinnamyl alcohol were converted into **benzoic acid**, phenylacetic acid and cinnamic acid [39].

Immobilisation of an *Acetobacter aceti* strain in calcium alginate resulted in improvement of the operational stability, substrate tolerance and specific activity of the cells and 23 g $L^{-1}$ phenylacetic acid was produced within 9 days of fed-batch cultivation in an airlift bioreactor [133]. Lyophilised mycelia of *Aspergillus oryzae* and *Rhizopus oryzae* have been shown to efficiently catalyse ester formation with phenylacetic acid and phenylpropanoic acid and different short-chain alkanols in organic solvent media owing to their carboxylesterase activities [134, 135] (Scheme 23.8). For instance, in *n*-heptane with 35 mM acid and 70 mM alcohol, the formation of ethyl acetate and propylphenyl acetate was less effective (60 and 65% conversion yield) than if alcohols with increased chain lengths were used (1-butanol 85%, 3-methyl-1-butanol 86%, 1-pentanol 91%, 1-hexanol 100%). This effect was explained by a higher chemical affinity of the longer-chain alcohols, which are more hydrophobic, to the solvent.

Since cinnamyl aldehyde is the main component of cassia oil (approximately 90%) and Sri Lanka cinnamon bark oil (approximately 75%) [49], it is industrially more important to generate **cinnamyl alcohol**, which is less abundantly available from nature but is important as cinnamon flavour, by biotransformation of natural cinnamyl aldehyde than vice versa. Recently, a whole-cell reduction of cinnamyl aldehyde with a conversion yield of 98% at very high precursor concentrations of up to 166 g $L^{-1}$ was described [136]. *Escherichia coli* DSM 14459 expressing a NADPH-dependent *R* alcohol dehydrogenase from *Lactobacillus kefir* and a glucose dehydrogenase from *Thermoplasma acidophilum* for intracellular cofactor regeneration was applied as the biocatalyst (Scheme 23.8).

The microbial production of significant amounts of cinnamic acid from glucose by cloning phenylalanine ammonia lyase (PAL) from the yeast *Rhodosporidium toruloides* into a solvent-tolerant *Pseudomonas putida* strain was described for the first time [137] (Scheme 23.8). Random mutagenesis and selection on the toxic antimetabolite *m*-fluorophenylalanine followed by high-throughput screening led to the isolation of a mutant strain with improved de novo phenylalanine biosynthesis and consequently a higher cinnamic acid production. In a nitrogen-limited fed-batch fermentation on glycerol, 750 mg $L^{-1}$ cinnamic acid formed within 50 h, corresponding to a conversion yield of 6.7% based on the carbon consumed. Higher productivities are being aimed at by integrating in situ product-recovery techniques owing to the inhibition of PAL by cinnamic acid and by further enhancing the intracellular phenylalanine level by proteomics and transcriptomics methodologies.

## 23.4.3 Terpenes

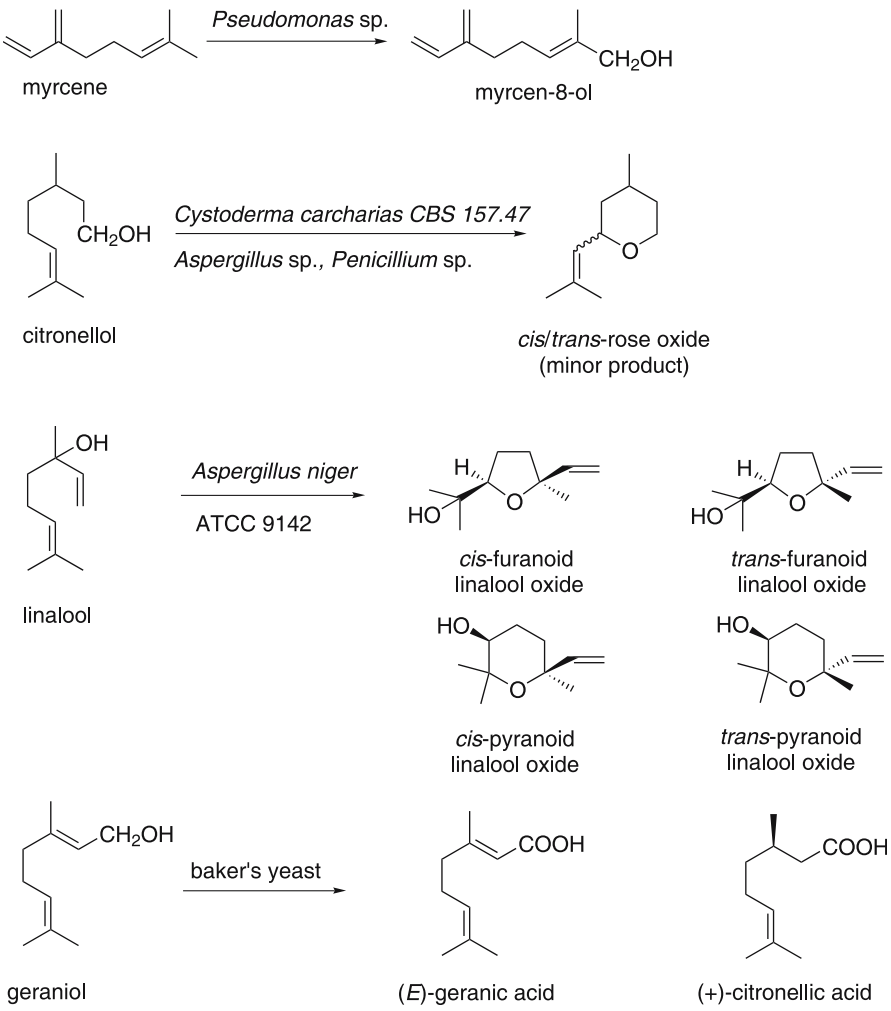
### 23.4.3.1 General Considerations

With some estimated 20,000 to more than 40,000 different molecules known, terpenes (isoprenoids) are the largest family of natural compounds in nature [138–140]. Whereas oxyfunctionalised monoterpenes and sesquiterpenes are extensively applied in industry as flavour and fragrance compounds, their precursors, the terpene hydrocarbons, are usually separated from their natural sources, essential oils, as they contribute little to flavour and fragrance and may also cause undesirable off-flavours and precipitations. The essential oil content of plants is, however, low, with concentrations of less than 0.1 to 5% and the commercial extraction of minor compounds is only in rare cases economically viable. As many terpene hydrocarbons are abundantly available in nature, e.g. (+)-limonene and the pinenes, which are the main components of citrus and turpentine oils, respectively, e.g. more than 90% (+)-limonene in orange oil, they represent an ideal starting material for biocatalytic oxyfunctionalisations leading to natural terpenoid flavour and fragrance compounds. This research area has therefore been the target of many research groups for decades focussing on individual types of bacteria that degrade terpenes, e.g. *Pseudomonas*, *Rhodococcus* and *Bacillus*, on deuteromycetes, e.g. aspergilli and penicillia, and especially on all the higher fungi of the ascomycetes and basidiomycetes, which have a marked capacity for terpene de novo biosynthesis and biotransformation. In terpene transformation a manageable number of enzyme reactions are frequently found owing to the uniform basic terpene structure which derives from the general biosynthesis principle based on five-carbon (isoprene) units [141]. Most of the monooxyfunctionalisation reactions are believed to be catalysed by cytochrome P450 monooxygenases. In contrast to most of the chemical oxidation processes, which often suffer from harsh reaction conditions and the need for hazardous reagents, e.g. toxic heavy metals, showing a low discrimination of the carbon atoms in terpene hydrocarbons, these enzymes are able to regioselectively transform multifunctional terpenoid substrates at specific sites under mild conditions. Even non-activated chemically inert carbon atoms can be functionalised by enzymatic reactions. However, only a few terpenes are produced biotechnologically on an industrial scale despite their often unique organoleptic properties, the growing demand and the unstable supply situation from the traditional (frequently overseas) sources. The main reasons stem from the physicochemical properties of terpenes, such as low water solubility, high volatility and cytotoxicity of the terpenoid precursor and the product, which impede conventional bioprocesses (Sect. 23.2). In fungal terpene biotransformations, monitoring the oxygen uptake rate [142] or the terpene concentration in the exhaust air [108] was shown to be helpful for feeding the toxic precursor in a biocompatible way; nevertheless, the engineering of terpene biotransformations definitely needs

further impetus by combining tailored process modifications, e.g controlled precursor feeding with in situ product recovery, to obtain higher product yields and to establish economically viable processes. Moreover, owing to microbial metabolic versatility, one precursor is usually converted into a variety of derivatives, partly representing undesired or not readily separable by-products which should be addressed by appropriate measures as shown in Table 23.2. Comprehensive reviews covering the research until 2001, in which the interested reader is guided through a wealth of microbial terpene biotransformation strategies, have been published [143–145]. Therefore, this chapter only gives a few examples of microbial transformations that stand out from the vast number of reported biotransformations with respect to the product concentrations achieved. Furthermore, the focus is also on more recent publications which describe the formation of highly valuable terpenoid flavour compounds (albeit usually still at low concentrations) and on approaches which may point the way ahead towards more sophisticated bioprocesses in the future by targeting modern molecular biological or biochemical engineering aspects.

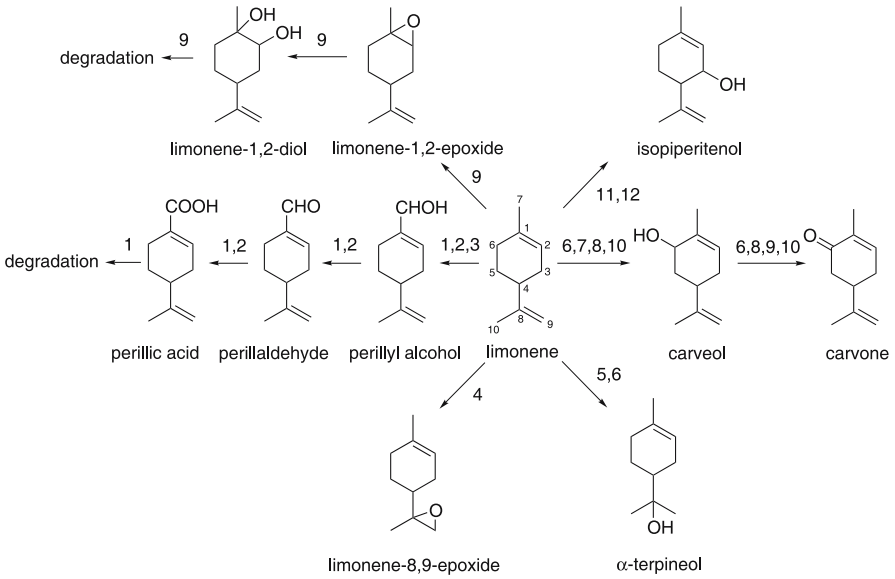
#### 23.4.3.2 Monoterpenes

The unsaturated monterpene-triene *β*-myrcene, frequently found in the terpene hydrocarbon fraction of many essential oils, has been shown to be a suitable precursor for biotransformations with basidiomycetes, although usually a multitude of metabolic derivatives in low concentrations appeared [146]. In contrast, the transformation of *β*-myrcene by a mutant obtained by transposon mutagenesis from a parental *β*-myrcene degrading *Pseudomonas* strain yielded **myrcen-8-ol** as the main product [147] (Scheme 23.9). Citronellol, an acyclic monoterpene alcohol, which can be isolated, e.g., from *Boronia*, *Eucalyptus* and geranium and rose oils in high concentrations [49] was converted by the basidiomycete *Cystoderma carcharias* to 3,7-dimethyl-1,6,7-octane-triol as the main product, but ***cis*-rose oxide/*trans*-rose oxide**, an industrially important fragrance compound, arose as one of the minor products. In a 2-L bioreactor fed-batch process, rose oxide was trapped out of the exhaust air by adsorption, albeit only in the milligram range [148]. A screening based on solid-phase microextraction revealed that sporulated surface cultures of *Aspergillus* and *Penicillium* species can also produce rose oxides from citronellol, although here again only as minor metabolic by-products [149]. Submerged shaking cultures of *Aspergillus niger* ATCC 9142 have been used to transform linalool into the furanoid and pyranoid ***cis*-linalool/*trans*-linalool oxides**, which are of interest for lavender notes in perfumery [150]. An industrial-scale bioprocess for the transformation of monoterpenes to the corresponding acids, which are important as building blocks for natural flavour esters, was patented [151]. Under aerobic conditions and at alkaline pH, geraniol was enantioselectively oxidised to **(*E*)-geranic acid** (85%) and **(+)-citronellic acid** (15%) using commercial baker's yeast. Geranic acid reached a maximum concentration of 3.6 g $L^{-1}$ after 48 h. A NADH-depen-




**Scheme 23.9** Some microbial monoterpene transformations leading to interesting flavour molecules

dent pathway from geraniol via geranial, neral and citronellal to citronellol was proposed branching off from geranial to geranic acid and from citronellal to citronellic acid. Geranic acid was shown to be the sole product formed during biotransformation of geraniol with a *Rhodococcus* sp. strain termed GR3 isolated from soil [152]. After 3 days of growth in a conventional medium containing minerals, dextrose and peptone, the bioconversion was started by adding geraniol to the medium at 1% v/v and geranic acid formed with saturation kinetics leading to a final yield of 50% after 96 h. Higher precursor concentrations caused lower conversion yields obviously owing to toxic effects of geraniol.




**Scheme 23.10** Microbial limonene transformation routes. *1* Frequently found in bacilli and pseudomonads; *2 Pseudomonas putida* DSM12264; *3 Bacillus stearothermophilus*, recombinant *Pseudomonas putida* expressing a P450 monooxygenase from *Mycobacterium* sp.; *4 Xanthobacter* sp. C20; *5 Penicillium digitatum* NRLL 1202 and DSM 62840; *6 Pseudomonas aeruginosa*; *7 Rhodococcus opacus* PWD4, *Fusarium proliferatum*; *8 Rhodococcus globerulus* PWD8; *9 Rhodococcus erythropolis* DCL14; *10 Pleurotus sapidus*; *11 Hormonema* sp. UOFS-Y-0067; *12* recombinant *Escherichia coli* expressing an evolved mutant of P450cam from *Pseudomonas putida*. For information about the stereochemistry of the biotransformations, see the text

Probably the most intensively studied monoterpene precursor is (+)-limonene, the main constituent of citrus essential oils, which accumulates in amounts of more than 50,000 t year$^{-1}$ as a cheap by-product of the citrus processing industry [153]. The current status of microbial and plant biotransformation of limonene was recently reviewed and at least six different molecule sites for initial limonene oxyfunctionalisation were reported [154]. Microbial transformations of limonene are summarised in Scheme 23.10. It is assumed that limonene-degrading *Pseudomonas* and *Bacillus* strains gain most of their carbon and energy by initially attacking the 7-position in a rather regiospecific way leading to perillyl alcohol followed by further progressive oxidation via perillaldehyde to **perillic acid**, which is mineralised by a β-oxidation-like mechanism [154]. *Pseudomonas putida* DSM 12264 growing on *p*-cymene as the sole carbon source can be used to convert limonene to perillic acid at high yields because the three enzymes, *p*-cymene monooxygenase, *p*-cumic alcohol dehydrogenase and *p*-cumic aldehyde dehydrogenase, show significant side activities towards limonene and its analogue derivatives **perillyl alcohol** and perillaldehyde [155]. Further degradation of perillic acid, the only product of limonene biotransfor-

mation, is obviously hampered by the high substrate specificity of the respective catabolic enzymes. The advantage of this *Pseudomonas* strain is its solvent tolerance, allowing for growth in the presence of high limonene concentrations (e.g. 150 mM) dramatically exceeding its saturation concentration (approximately 0.1 mM), causing a separate limonene phase finely dispersed in the aqueous phase upon stirring. In an optimised mineral medium with (+)-limonene and glycerol as the cosubstrate up to 3.0 g $L^{-1}$ (+)-perillic acid formed after 120 h. In a 3-L bioreactor under fed-batch conditions with non-limiting amounts of glycerol, ammonia and limonene, the final product concentration was increased to 11 g $L^{-1}$ (+)-perillic acid after 7 days, obviously limited by product inhibition [156]. Although these are the highest product concentrations reported for a microbial limonene oxyfunctionalisation, from the flavourist's viewpoint, the more volatile precursors of the acid, perillaldehyde and perillyl alcohol, are the industrially more interesting targets.

A *Bacillus stearothermophilus* strain with a temperature optimum near 55 °C isolated from orange peel by enrichment on (+)-limonene as the sole carbon source was able to convert the monoterpene cometabolically to perillyl alcohol as the major product (200 mg $L^{-1}$), whereas **α-terpineol** and perillaldehyde were by-products [157]. The respective (+)-limonene conversion pathways encoded on a 9.6-kb chromosomal fragment was cloned into *Escherichia coli* where the lilac-like fragrance α-terpineol became the major product [158]. By subcloning of smaller fragments, the product diversity was further narrowed and 235 mg $L^{-1}$ α-terpineol was produced after 3 days using (+)-limonene as a neat substrate phase supplying the precursor and for extractive in situ product recovery; **carvone** formed as by-product [159]. The bioconversion was carried out at 50–60 °C to repress undesired metabolic side activities of the host by processing the bioconversion at the optimum temperature of the donor strain. With use of *Penicillium digitatum* NRLL 1202, racemic limonene was converted to (+)-α-terpineol since only (+)-limonene was accepted as a substrate enantiospecifically. Bioconversion activity increased up to 12-fold after sequential substrate induction and a yield of about 3.2 g $L^{-1}$ α-terpineol was achieved after 96 h, albeit on a 5-mL analytical scale [160]. The limonene hydroxylation at the 8-position which corresponds to a hydration of the 8,9 double bond is probably catalysed by a P450 monooxygenase leading to the epoxide as an intermediate rather than by a hydratase but it has not been isolated and identified so far [160]. Immobilisation in calcium alginate and the use of organic cosolvents were proposed to improve the product yields of this system [161, 162]. Recently a fed-batch 3-L bioprocess with *Penicillium digitatum* DSM 62840 was reported yielding 0.5 g $L^{-1}$ within 7 days using a two-step approach comprising a biomass growth period followed by a biotransformation period in the same reactor [163]. A 3-L bioreactor with a closed gas loop and terpene-saturated process air was described to alleviate any loss of terpenes via the exhaust air [164]. *P. digitatum* DSM 62480 was exploited in this reactor system to produce more than 1 g $L^{-1}$ α-terpineol after about 120 h of biotransformation. In contrast, **limonene-8,9-epoxide** has been shown to be the main product of *Xanthobacter* sp. C20 catalysed conversion of both limonene

enantiomers with a pro-8*R* stereospecificity [165]. The strain was pregrown on cyclohexane as the sole carbon and energy source and used as resting cells for biocatalysis in phosphate buffer yielding up to 0.8 g $L^{-1}$ of the epoxide.

Carvone is an important monoterpene ketone, of which the (+)-isomer represents the character-impact compound of caraway flavour (up to 60% in caraway oil), whereas the (-)-isomer has a typical spearmint note (70–80% in spearmint oil). A *Pseudomonas aeruginosa* strain was described that was capable of converting (+)-limonene into carvone and $\alpha$-terpineol as the main products at 37 °C in 200-mL shake flasks after 13 days, and final concentrations of up to 0.63 and 0.24 g $L^{-1}$, respectively, were achieved [166]. The toluene-degrading strain *Rhodococcus opacus* PWD4 was found to oxyfunctionalise (+)-limonene exclusively at the 6-position, yielding enantiomerically pure ***trans*-(+)-carveol**, whereas **(+)-carvone** formed as a by-product (1.3% of the amount of ***trans*-carveol**) [167]. The initial specific activity for carveol formation was 14.7 U $g_{cdw}^{-1}$ accompanied by a molar yield of 94–97%. One of the enzymes from the toluene-degradation pathway has to be responsible for the (+)-limonene hydroxylation since, on the one hand, cells pregrown on glucose did not convert limonene at all and, on the other hand, toluene proved to be a strong competitive inhibitor. Another toluene degrader, *Rhodococcus globerulus* PWD8, showed a lower specific activity of 3 U $g_{cdw}^{-1}$ and slowly overoxidised most *trans*-(+)-carveol to 0.29 mM (+)-carvone, the more valuable terpene ketone, from 1.2 mM (+)-limonene after 27 h under small-scale (2.5-mL) assay conditions. *Rhodococcus erythropolis* DCL14, which grows on limonene as the sole carbon source, starts metabolising limonene by a rather uncommon epoxidation at the 1,2 double bond, forming **limonene-1,2-epoxide** [168], while further mineralisation proceeds via limonene-1,2-diol and 1-hydroxy-2-oxolimonene, pointing to a β-oxidation degradation. The same strain also contains several carveol dehydrogenases enabling it to convert carveol stereospecifically to carvone [169]. This enzyme activity was exploited to produce **(-)-carvone** from *cis*-(-)-carveol/*trans*-(-)-carveol corresponding to a diastereomeric excess of more than 98% and a yield of 0.68% w/w [170]. The cells were used in an aqueous–organic two-liquid-phase air-driven column reactor containing *n*-dodecane as a protecting organic phase, but product inhibition above 50 mM carvone impeded higher product concentrations. These limitations were overcome by adapting *Rhodococcus erythropolis* cells in mineral medium to carveol and carvone dissolved in *n*-dodecane prior to biotransformation [171]. The air-driven column reactor was used after an adaptation period of 197 h and an 8.3-fold increase in carvone production rate compared with non-adapted cells was achieved. The highest final concentration was achieved with cells adapted for 268 h which produced 1.03 M carvone after 167 h at room temperature. The cellular adaptation mechanism was explained by a dose-dependent increase in the degree of saturation of the membrane phospholipids [172]. The basidiomycete *Pleurotus sapidus* was shown to regiospecifically oxyfunctionalise the same limonene 6-position: by means of precultivation in the presence of small amounts of the precursor fed via the gas phase, the concentrations of ***cis*-carveol**/*trans*-carveol and carvone increased to yield

a total product concentration of more than 0.1 g $L^{-1}$ [173]. In contrast to *Pleurotus sapidus*, the ascomycete *Fusarium proliferatum* did not form measurable amounts of carvone, but converted both limonene enantiomers: (+)-limonene to *cis*-(+)-carveol, and (-)-limonene predominantly to *trans*-(-)-carveol, which could be further oxidised to (-)-carvone, again by *Pleurotus sapidus* [163]. These examples illustrate that by combining two microbial strains with hydroxylase and dehydrogenase activity a biotechnological stereospecific production of both carvone enantiomers from (+)-limonene and (-)-limonene may become possible in the future.

The black yeast *Hormonema* sp. UOFS Y-0067 isolated from monoterpene-rich environments transformed (+)-limonene into ***trans*-isopiperitenol** by regioselectively attacking the 3-position, a biotransformation reported for the first time [174]. A product concentration of 0.5 g $L^{-1}$ was achieved after 12 h of incubation in shake flasks (31% molar conversion), obviously limited by product and/or precursor toxicity. Unfortunately, the product concentration of *trans*-isopiperitenol, which requires only a catalytic hydrogenation step to yield the desired (-)-menthol, was not always reproducible owing to morphological mutability and, thus, this microorganism was obviously not well suited for further process development.

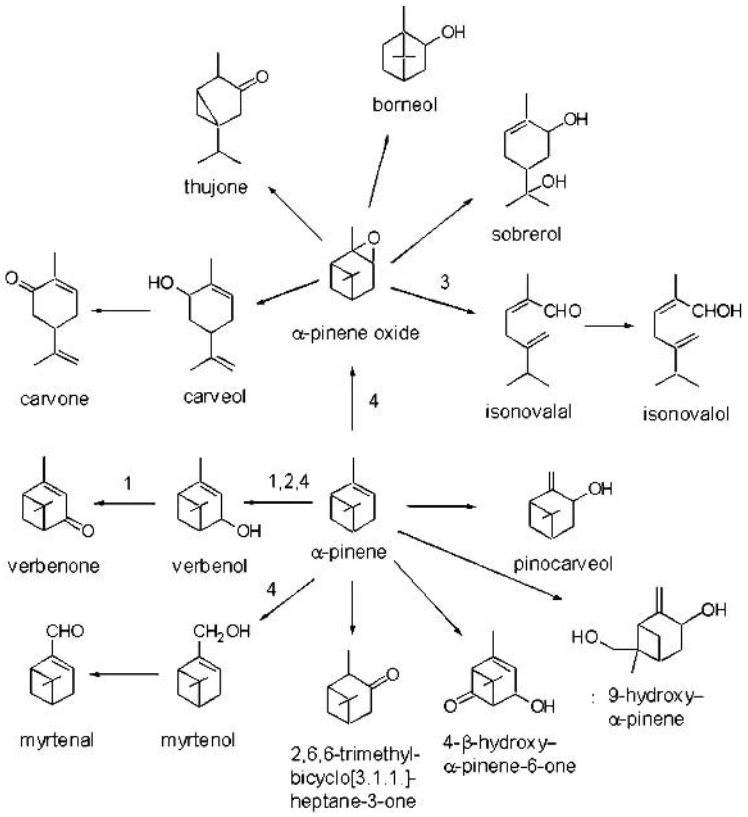
The pinenes are a cheap natural starting material abundantly available as main constituents of turpentine oil (up to 75–90%) with up to 160,000 t $\alpha$-pinene and 26,000 t $\beta$-pinene per year and are also found in relevant amounts in the essential oils of non-coniferous plants, e.g. up to 12% in citrus oils [175]. As for most terpenes, the microbial metabolism of the pinenes often leads to diverse degradation pathways and therefore to a wide variety of products [176]. This effect is even more marked in the case of the structurally more complex bicyclic monoterpenes compared with the aforementioned acyclic and monocyclic monoterpenes [177]. Some interesting terpenoid compounds accessible by microbial conversion of $\alpha$-pinene are illustrated in Scheme 23.11. From the biochemical engineer's viewpoint, microbial pinene transformations yielding only one or a few main products are of special interest as they are close to commercialisation or may serve as the starting point for further improvements by process and/or genetic engineering approaches. **Verbenone**, an impact compound of rosemary oil, and its precursor ***trans*-verbenol** were described to be the main products of an $\alpha$-pinene conversion using the self-isolated black yeast *Hormonema* sp., which has already been mentioned for the limonene transformations. Although yielding extraordinarily high concentrations with respect to pinene bioconversions (0.3 g $L^{-1}$ verbenone and 0.4 g $L^{-1}$ *trans*-verbenol after 96 h), the unwanted morphological characteristics of the microorganism restricted further process development, as mentioned above. Verbenone was also produced as the main product using *Aspergillus niger* in a two-step approach [178]: with resting cells, pregrown until the late-exponential phase in potato–dextrose broth with 6% (w/v) glucose, 200 mg $L^{-1}$ $\alpha$-pinene was converted into 32.8 mg $L^{-1}$ verbenone after 6 h of incubation. The yield of **verbenol**, itself a valuable flavour compound with a fresh pine, ozone odour, was improved compared with that of UV mutant strains described earlier [179] by generating an intergeneric hybrid strain

from an *Aspergillus niger* strain showing high product yields and *Penicillium digitatum* showing high biomass yields [180]. By this means (-)-*cis*-α-pinene was transformed into **(-)-*cis*-verbenol** at a yield of 60%; nevertheless, the corresponding product concentration of 1.08 mg $g^{-1}$ biomass is obviously still too low for industrial application. One example where an outstanding productivity and product concentration was achieved is the formation of **(*Z*)-2-methyl-5-isopropyl-2,5-hexadienal** (**isonovalal**), a non-plant fragrance compound with a citrus-like note for potential use in perfume formulations. It was produced from **α-pinene oxide** in concentrations of up to 400 g $L^{-1}$ within 2.5 h using 25 g $L^{-1}$ precultivated *Pseudomonas rhodesiae* CIP 107491 biomass; the cells had been permeabilised by freeze-thawing and organic solvent treatment prior to use. The bioprocess was performed with in situ product recovery using hexadecane in a biphasic medium and by sequential feeding of biomass and precursor to compensate the irreversible biocatalyst inactivation by the product. Recently, a bioreactor coupled to an external membrane module for in situ product removal was reported for the same biotransformation reaction with *Pseudomonas fluorescens* NCIMB 11671 [181]. A dense silicone membrane comprising 70% PDMS and 30% fumed silica coiled into a hexadecane reservoir was used to separate the aqueous fermentation broth, which was recirculated inside the membrane tubing from the organic phase. With an optimised continuous feeding of the precursor α-pinene oxide directly into the fermentation broth containing about 60 g $L^{-1}$ biomass, a stable process for over 400 h was achieved and more than 100 g $L^{-1}$ isonovalal in the organic phase was produced despite the limitations of the membrane area used.

The general concept of heterologous expression of terpene functionalising enzymes in tailored host microorganisms has been extensively pursued in recent years not only for the purpose of biochemical characterisation of novel enzymes, e.g. those from plant terpene biosynthesis (which is not the focus of this review; for examples see [182, 183]), but also for designing more efficient whole-cell biocatalysts. A recent example is the production of **perillyl alcohol** from limonene by using a recombinant cytochrome P450 alkane hydroxylase expressed in *Pseudomonas putida* [184]. The monooxygenase together with a ferredoxin reductase and a ferredoxin—a typical bacterial class 1 cytochrome P450 system—was encoded by an operon found in a novel *Mycobacterium* sp. strain termed HXN-1500. This strain had been selected from 1,800 mainly hydrocarbon degrading bacteria screened for their ability to hydroxylate limonene at the 7-position. With a recombinant *Pseudomonas putida* host strain which allowed selection for growth on alkanes if alkane hydroxylase is functionally expressed, a two-liquid-phase biotransformation in a 2-L bioreactor was performed after the cells had been pregrown to a cell density of approximately 10 g $L^{-1}$ on *n*-octane. Bis(2-ethylhexyl)phthalate served as the organic phase for in situ precursor supply and product recovery from the aqueous phase, and 2.3 g $L^{-1}$ perillyl alcohol was produced after 75 h under fed-batch conditions (feeding of *n*-octane as the carbon source), calculated for the total liquid content of the reactor. Although still showing a threefold lower enzyme activity than the wild-type *Mycobacterium* strain, which is categorised as safety class 2, this recombinant

*Pseudomonas putida* strain is a safety class 1 microorganism and, furthermore, it still harbours great potential for optimisation (e.g. substrate uptake, product export). Recently, with P450cam-catalysed camphor to 5-*exo*-hydroxycamphor transformation as a model reaction, a tenfold increase of the activity was generated by coexpression of the *Pseudomonas* P450cam system and glycerol dehydrogenase for enhanced cofactor (NADH) regeneration within recombinant *E. coli* cells [185].

The active site of P450cam was remodelled by site-directed mutagenesis and the most active double mutant Y96F-V247L showed completely different substrate and product spectra [186]: (-)-limonene and (+)-α-pinene were transformed with high regioselectivities and stereoselectivities to **(-)-*trans*-isopiperitenol** and **(+)-*cis*-verbenol** as main products (about 70% of all products formed), respectively. The triple mutant F87W-Y96F-V247L was less active but even more



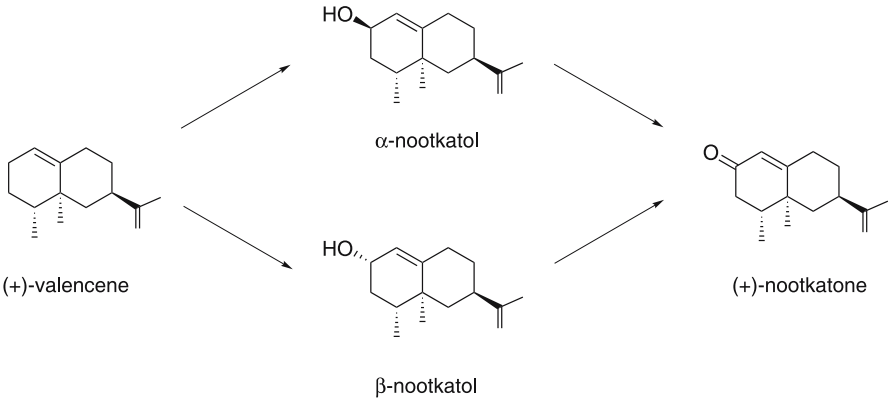

**Scheme 23.11** Microbial transformations of α-pinene [176, 267–269]. *1 Hormonema* sp. UOFS-Y-0067, *Aspergillus niger*, recombinant *Escherichia coli* expressing an evolved mutant of P450cam from *Pseudomonas putida*; *2 Aspergillus niger* + *Penicillium digitatum* fusant strain; *3 Pseudomonas rhodesiae* CIP107491, *Pseudomonas fluorescens* NCIMB11671; *4* recombinant *Escherichia coli* expressing an evolved mutant of P450 BM3 from *Bacillus megaterium*

selective giving 85% (+)-*cis*-verbenol. By crystal-structure-based engineering of the active site another selective triple mutant (F87W-Y96F-L244A) was created which gave 86% (+)-*cis*-verbenol and 5% **(+)-verbenone**, while Y96F-L244A-V247L gave 55 and 32%, respectively [187, 188]. A triple mutant of P450 BM3 from *Bacillus megaterium* (F87V-L188Q-A74G) designed by rational evolution [189] capable of oxyfunctionalising hydrophobic aliphatic and aromatic substrates was exploited to convert $\alpha$-pinene [164]. A recombinant *E. coli* strain was used as whole-cell biocatalyst in an aqueous–organic two-liquid-phase bioprocess to produce verbenol, **myrtenol** and $\alpha$-pinene oxide in a total concentration of several hundreds of milligrams per litre after about 8 h of bioconversion.

#### 23.4.3.3 Sesquiterpenes and Diterpenes, Norisoprenoids

Sesquiterpenes, biosynthetically derived from the trimeric precursor farnesyl diphosphate, constitute the structurally most diverse class of terpenoids and play key roles in food flavours and fragrances as well as pharmacologically active compounds. Their difficult total synthesis coupled with the abundance of non-functionalised, economically less important sesquiterpene hydrocarbons in many essential oils have stimulated much research during the last few decades dealing with sesquiterpene substrates from the over 70 subclasses [190]. The following discussion will be limited to only a few examples involving biotechnology which are of industrial relevance either because of the key character of the target flavour compounds or because of the progress in process development which has already been made.

The biotransformation of (+)-valencene, a sesquiterpene hydrocarbon found in orange oil, to **(+)-nootkatone** has attracted much research activity during the last few decades. (+)-Nootkatone possesses a citrus/grapefruit-like aroma and a bitter taste; it has a very low odour threshold (1 ppb) [175] and as a character-impact constituent of citrus aromas it is a very sought after natural flavour compound for foods and beverages. Recently, it has also been described to lower the somatic fat ratio, making it a natural product demanded by the cosmetic and fibre industries [191]. Although enzymatic cooxidation in the presence of lipoxygenase or laccase [192, 193] and bacterial valencene biotransformation with a *Rhodoccocus* strain [194] have been patented, it is doubtful that these processes will ever be applied owing to low specificities and/or activities. Recently, a relatively high selectivity was described for *Gynostemma pentaphyllum* cell cultures which converted valencene to nootkatone with 72% conversion yield corresponding to 650 mg $L^{-1}$ nootkatone after 20 days [195]. ***α*-Nootkatol** (11%) and ***β*-nootkatol** (5%) were minor products; they are the direct metabolic precursors of nootkatone produced by an initial hydroxylation of valencene which, upon dehydrogenase-catalysed oxidation, are transformed into the target product (Scheme 23.12). Although this plant cell culture is obviously still too inefficient and too costly for commercial application, it is rather productive compared with other plant cell culture based biotransformations. Microsomal




**Scheme 23.12** Biotransformation of (+)-valencene to (+)-nootkatone via *α*-nootkatol and/or *β*-nootkatol

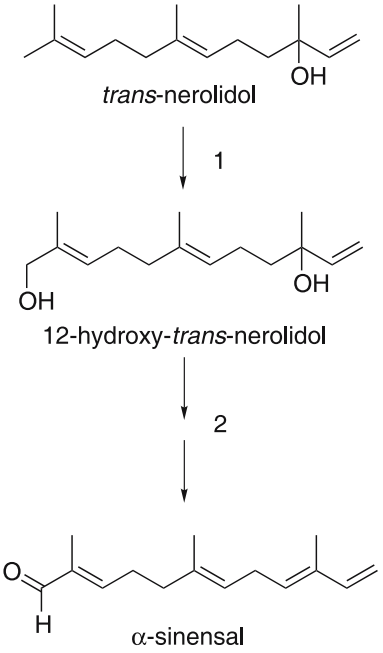
enzyme preparations from chicory (*Cichorium intybus* L.) roots have also been shown to catalyse the reaction from valencene to nootkatone as the main product with only negligible by-product formation [196]. Here, *β*-nootkatol turned out to be the only intermediate. Different higher fungi, such as *Mucor* species, *Botryosphaeria dothidea* and *Botryodiplodia theobromae*, and, interestingly, also green algae *Chlorella* species are also promising valencene-to-nootkatone biocatalysts [191]. For instance, *Chlorella pyrenoidosa* converted 89% of (+)-valencene added to the culture after 7 days of precultivation (20 mg in 50 mL) into (+)-nootkatone within a further 12 days, while *Chlorella vulgaris* even showed a conversion yield of 100% under the same conditions; with the fungus *Mucor* sp. a comparable yield of 82% was obtained after 7 days of precultivation followed by 7 days of biotransformation. During investigations with submerged cultures of the ascomycete *Chaetomium globosum*, it was found that the biotransformation proceeded via *α*-nootkatol as the intermediate and that major parts of the valencene and its monooxyfunctionalisation products accumulated within the cells, while dioxygenated and polyoxygenated products were found in the medium [197]. The bioprocessing limitations associated with the hindered mass transfer of terpenes across microbial cell membranes, especially the inefficient export of the transformation products out of the cells, may be overcome by an alternative cell preparation which has been described in a patent application very recently [198]. It is claimed to treat filamentous fungi known for their versatile terpene catabolism by lyophilising the mycelia prior to biotransformation which was preferentially carried out in an aqueous–organic two-phase system with *n*-decane as the organic phase. The authors claimed a better availability of the terpenes to the membrane enzymes after lyophilisation, leading to a more efficient biotransformation system; nevertheless, no yields have been reported.

Recently, an industrial process development for nootkatone production from valencene by microbial transformation (bacteria, fungi) was mentioned [199, 200]. Although no details were given, the author claimed the development of an in situ product-removal technique by which an extremely selective recovery of nootkatone from the reaction mixture and the excess precursor during the proceeding production was achieved and which was said to be essential for an economically viable bioprocess.

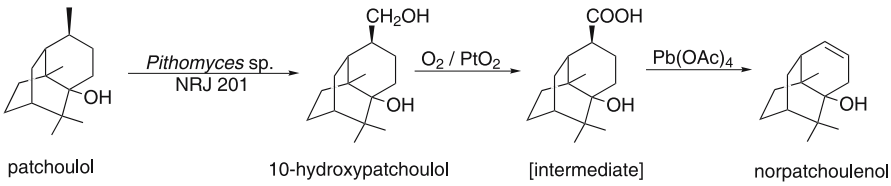
The same rational P450cam mutants which have already been described for limonene and pinene oxyfunctionalisations were also successfully applied to valencene. In whole-cell biotransformations $\beta$-nootkatol and **nootkatone** formed as main products with up to 25% overall yield, corresponding to activities of up to 9.9 nmol (nmol P450)$^{-1}$ min$^{-1}$ [201]. Higher activities (up to 43 min$^{-1}$) but lower selectivities than those with P450cam were obtained with mutants derived from *Bacillus megaterium* P450 BM3.

The sesquiterpene aldehydes **$\alpha$-sinensal** and **$\beta$-sinensal** contribute particularly to the special sweet orange aroma and also occur in other citrus oils; the former has a very low odour threshold of 0.05 ppb [175]. The sesquiterpene hydrocarbon farnesene may serve as closely related starting material and, consequently, farnesene isomers were used in biotransformations with *Arthrobacter*, *Bacillus*, *Nocardia*, and *Pseudomonas* with the aim to produce precursors of sinensal, but only little conversion was achieved when using the more stable farnesene sulfones [202]. Another strategy to produce $\alpha$-sinensal starts from *trans*-nerolidol and aims at microbial ω-hydroxylation with fungi or bacteria, such as *Aspergillus* and *Rhodococcus* species, to produce 12-hydroxy-*trans*-nerolidol, which itself serves as precursor for the chemical conversion to the desired product [203–205] (Scheme 23.13). Certain self-isolated *Aspergillus* strains were shown to be very regioselective (74% of total product formed) [204]. The physiological state of an *Aspergillus* culture before nerolidol addition—monitored by on-line quantification of titrant addition in pH control—had a major impact on the biotransformation efficiency [205]. The maximal conversion yield of 8–9% was obtained by addition of a (±)-*cis*-nerolidol/(±)-*trans*-nerolidol mixture to the culture in the postexponential phase at high dissolved oxygen pressure (above 50% air saturation) in minimal and complex media after 25 and 14 h, respectively.

Patchouli alcohol (patchoulol) is a major constituent (30–45%) in steam distillates of dried leaves of *Pogostemon cablin* (Blanco) Benth; around 1,000 t of essential oil is produced worldwide per annum, primarily in Indonesia [49, 206]. Patchouli oil is very tenacious and is used in perfumery for oriental and masculine notes. The primary fragrance molecule in the essential oil is the sesquiterpene alcohol **norpatchoulenol**, which is present at 0.35–1.0% or less. In 1981, a combined biocatalytic and chemocatalytic method for the preparation of norpatchoulenol from patchoulol was published [207] (Scheme 23.14). The first step involved a microbial process to convert patchoulol to **10-hydroxypatchoulol**. *Pithomyces* species, filamentous fungi isolated from soil samples by enrichment on patchoulol as sole the carbon source, catalysed the regioselective




**Scheme 23.13** Biocatalytic–chemocatalytic reaction sequence to produce α-sinensal from *trans*-nerolidol. *1 Aspergillus niger* sp., *Aspergillus niger* ATCC 9142, *Rhodococcus rubropertinctus* DSM 43197; *2* chemical conversion steps

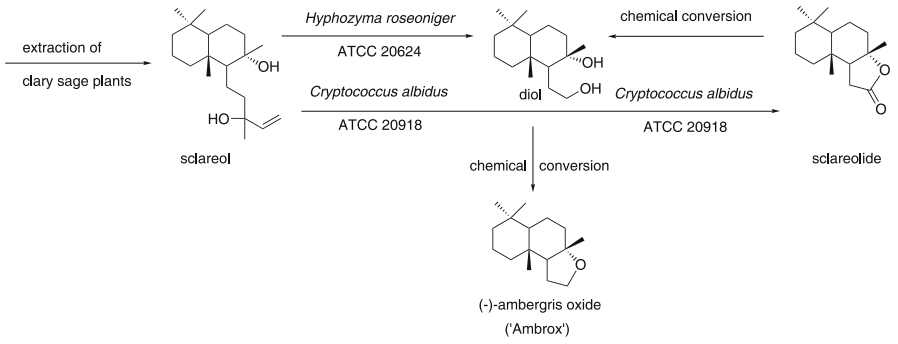



**Scheme 23.14** Regioselective biohydroxylation of patchoulol and the following chemical steps to produce norpatchoulenol according to [207]

hydroxylation reaction with yields of up to 45%, corresponding to a maximum product concentration of 1.1 g $L^{-1}$. Maximum yields were achieved after biotransformation periods of 3–7 days which were carried out in 1–5-L bioreactors with fungal cultures pregrown in complex media for about 3 days. The 10-hydroxy compound was subsequently converted chemically via a two-step process to norpatchoulenol.

**(-)-Ambergris oxide** (Ambrox®) is one of the most important ambergris fragrance compounds and is a key compound of ambra, a secretion product of the

sperm or cachalot whale, possibly resulting from pathological conditions [23, 49]. A novel microorganism, classified as *Hyphozyma roseoniger* CBC 214.83 (ATCC 20624), which can exist in both yeast-like and filamentous forms, was isolated and was capable of forming a diol from the diterpene alcohol sclareol found in the leaf oil from *Salvia sclarea* L.; the conversion proceeded in one microbial step via a cascade of reactions in high yields of more than 75%, but only after around 12 days of incubation [208]. Subsequently, other suitable microbial strains have been found by continued screening; e.g. the yeast *Cryptococcus albidus* ATCC 20918 which can metabolise sclareol even further, producing the ketone lactone sclareolide at high yields of more than 100 g $L^{-1}$ [209]. The sclareolide is then chemically converted back to the diol and further to ambergris oxide (Ambrox®) (Scheme 23.15).

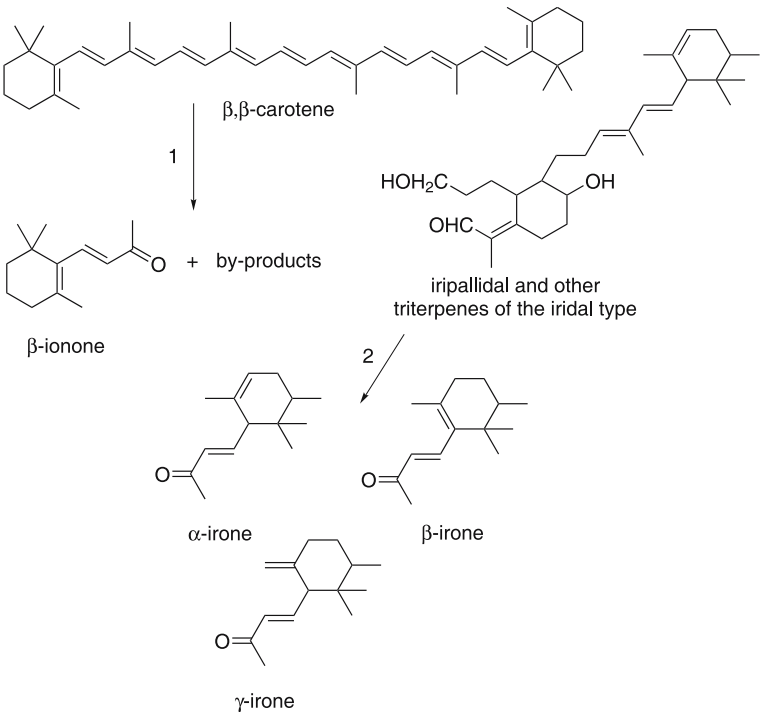



**Scheme 23.15** Biocatalytic–chemocatalytic synthesis of Ambrox® (adapted from [270])

Although certain microorganisms, especially higher fungi, show a remarkable capability for de novo biosynthesis of terpenoid flavours, product titers of single terpenoid flavour molecules rarely exceed 100 mg $L^{-1}$ and are, thus, too low for commercial processes. This situation may change dramatically in the near future owing to the great progress currently being made by metabolic engineering of microbial terpene biosynthesis and by heterologous expression of key enyzmes catalysing plant terpene functionalisation reactions in tailored host microorganisms. Recently, the total biosynthesis of terpenoids by engineering the mevalonate-dependent isoprenoid (MEV) pathway from *Saccharomyces cerevisiae* in *Escherichia coli* thereby alleviating the bacterial 1-deoxy-d-xylulose-5-phosphate (DXP) pathway has been reported [210]. By this means, the sesquiterpene amorphadiene, a precursor of the antimalaria drug artemisinin, was produced by successfully cloning a sequence comprising nine genes leading from acetyl-CoA via the universal C5 units isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) to the target compound. Because IPP and DMAPP are the ultimate precursors for all terpenoids, such a strain, after further enhancing its metabolic terpene flux, may serve as a platform cell

factory for de novo biosynthesis of any terpenoid for which the biosynthetic genes are available, i.e. flavour and fragrance compounds included. The same group also showed that engineering both *Escherichia coli*'s DXP pathway as well as *Saccharomyces cerevisiae*'s MEV pathway in the respective native hosts can also serve as a promising alternative strategy to design high-performing terpenoid producer strains [211, 212].

Closely related to terpenes and thus generally considered a subclass are the C13 norisoprenoids, e.g. ***α*-ionone and *β*-ionone**, volatile ketones generated by oxidative degradation of carotenoids, and **irones**, e.g. ***α*-irone and *γ*-irone**, C14 ketones derived from the triterpenes of the iridal type (Scheme 23.16). The best biotechnological strategy for the production of the most important natural ionone, *β*-ionone, a violet-like flavour and fragrance compound (threshold in water 0.007 ppb), still relies on the cooxidative cleavage of carotenoid-rich raw materials using enzymatic oxidation systems, e.g. lipoxygenase or xanthine oxidase in the presence of unsaturated fatty acids. These enzymes initially oxidise the unsaturated fatty acids, e.g. linoleic or linolenic acid, to free-radical species which themselves attack the conjugated double bonds of carotenoids, resulting in a non-specific cleavage pattern and thus broad product spectra [61, 213].




**Scheme 23.16** Microbial pathways from triterpene and tetraterpene (carotenoid) precursors to valuable flavour and fragrance compounds. *1* Carotenoid-cleaving peroxidase-containing supernatant of certain fungal cultures, e.g. *Lepista irina*; *2 Serratia liquefaciens*, *Botrytis* sp.

A direct cleavage mechanism has been proposed for novel carotenoid-cleaving peroxidases found in the basidiomycete *Lepista irina* [214] and other fungi [215]. Whereas submerged cultures did not accumulate significant amounts of volatile degradation products (probably owing to total catabolism), mycelium-free supernatants yielded $\beta$-ionone and further degradation products. Microbial whole-cell approaches are not yet used in industry for the production of natural ionones but, here as well, metabolic engineering might grant access to the desired target compounds in a superior way, i.e. highly regioselective reaction, in the future. On the one hand, carotenoid-producing *E. coli* strains have successfully been designed during the past decade [216] and, on the other hand, the native plant enzymes responsible for the regioselective cleavage of carotenoids to produce C13 norisoprenoids and other highly desired flavour-active apocarotenoids, the carotenoid-cleaving dioxygenases, have recently been identified and functionally expressed in *E. coli* for the first time [217]. If the technical potential of such engineered organisms is evaluated, in situ product recovery strategies will certainly be needed to circumvent catabolism of the volatiles by their rapid removal from the cells.

Conventional approaches to produce irones, also valuable fragrance compounds with a typical violet-like (orris) odour, were published in the 1980s. It is known that during storage of *Iris* rhizomes the content of the desired irones increases slowly, probably by chemical oxidative degradation of the triterpenes initially present in the rhizomes; therefore, prior to the production of the orris root oil by steam distillation the rhizomes are stored for several years [49]. Bacteria, especially *Serratia liquefaciens*, isolated from *Iris palladia* rhizomes, were used to convert rhizome preparations naturally containing the triterpenoid iridales to the desired target compounds [218, 219]. By this means, the four natural isomeric irones, ***trans*-$\alpha$-irone, *cis*-$\alpha$-irone, *cis*-$\gamma$-irone** and **$\beta$-irone** formed in similar proportions as in the traditionally processed rhizomes and a maximum irone content of 1.2 g $kg^{-1}$ was obtained after 3–4 days of cultivation. Owing to an early discontinuance of microbial growth which already occurred at day 1 (probably because of toxic effects of the products), the irone formation was supposed to be a combined biocatalytic–chemocatalytic reaction sequence initialised by microbial activity. *Botrytis* species were also claimed as biocatalysts for the same purpose [220]. Dried organic solvent extracts of the rhizomes were added as an emulsion in water–acetone–Tween 80 after 2 days to the fungal culture in a corn steep liquor medium. Up to 2.3 g $kg^{-1}$ irones was produced after a further 48 h and the irones were isolated by steam distillation.

### 23.4.4 Lactones

Saturated and unsaturated $\gamma$-lactones and $\delta$-lactones which are synthesised from the corresponding acyclic hydroxy fatty acids by intramolecular esterification are important flavour compounds found ubiquitously in fruits and also in milk and fermentation products in parts-per-million concentrations. The natural lac-

tones belong to the most desired targets for aroma biotechnology. It seems as if almost every big flavour company has claimed a preparation method starting from natural fatty acids, hydroxy fatty acids or unsaturated lactones as precursors during the last two decades. Scheme 4.17 summarises different strategies for the production of natural *γ*-lactones and *δ*-lactones.

**4-Decanolide** (**γ-decalactone**), which imparts a powerful fruity, especially peach-like aroma has a market volume of several hundred tons per year. In the early 1980s, natural 4-decanolide was an extremely expensive, rare natural flavour (price in excess of US $10,000 per kilogram). The subsequent introduction and optimisation of its biotechnological production has resulted in a steady decrease of the price to approximately US $300 per kilogram and an increase of the market volume to several tons per year [8].

Most of the commercial processes for the formation of 4-decanolide are based on the natural hydroxy fatty acid ricinoleic acid [(*R*)-12-hydroxy-(*Z*)-9-octadecenoic acid], the main fatty acid of castor oil, or esters thereof as substrates and fatty acid degrading yeasts or higher fungi as biocatalysts [221]. Ricinoleic acid is degraded by four cycles of β-oxidation and one double-bond hydrogenation into 4-hydroxydecanoic acid, which lactonises at lower pH to 4-decanolide, resulting in the same *R* configuration of the lactone as is found in peaches and other fruits [222]. Many processes for which high product concentrations have been reported are based on strains of *Yarrowia lipolytica*, a yeast which is particularly well adapted to hydrophobic environments and which was patented for 4-decanolide production for the first time in 1983 [223].

In a process established by Haarmann & Reimer, up to 11 g $L^{-1}$ 4-decanolide was obtained in 55 h with a wild-type strain and with raw castor oil as the substrate [224]. Metabolic engineering of *Yarrowia lipolytica* aims at optimising the flux along the complex β-oxidation pathway and decreasing the formation of unwanted by-products, such as 3-hydroxy-4-decanolide, dec-2-en-4-olide and dec-3-en-4-olide [225, 226]. More genetic engineering approaches with *Yarrowia lipolytica* can be expected in the future since its total genome has been sequenced recently [227]. An elegant conventional method to improve the overall yield of 4-decanolide uses baker's yeast for reduction of the double bond of the decenolides produced as by-products [70, 222].

Another process patented by Givaudan uses *Mucor circinelloides* as a biocatalyst for the production of 4-decanolide [228]. Here the natural substrate is the ethyl ester of decanoic acid which is isolated from coconut oil. The key microbial activity harnessed in this process is the stereoselective and regioselective hydroxylation of the fatty acid in the γ-position, which is followed by spontaneous lactonisation of the hydroxy fatty acid under acidic conditions and results in yields of up to 10.5 g $L^{-1}$ 4-decanolide after 60 h.

The closely related **5-decanolide (*δ*-decalactone)**, not only found in many fruits but also found in dairy products, exhibits a creamy-coconut, peach-like aroma [49] and can be synthesised from the corresponding α,β-unsaturated lactone 2-decen-5-olide found in concentrations of up to 80% in *Massoi* bark oil using basidiomycetes or baker's yeast [229]. After about 16 h of fermentation, 1.2 g $L^{-1}$ 5-decanolide was obtained. At the same time, the minor lactone in

*Massoi* bark oil, 2-dodecen-5-olide (7%), is converted to **5-dodecanolide**, which is also a desired fruity lactone. Different bacteria were used for the same *Massoi* lactone conversion in a medium containing natural oils as cosolvents for dissolving the precursor [230]. From 30-L culture volume, 195 g 5-decanolide was isolated after 48-h aerobic biotransformation with *Pseudomonas putida* ATCC 33015, corresponding to a conversion yield of 99.1%. More recently, growing *Saccharomyces cerevisiae* cells were claimed to be used in a two-phase bioprocess with triglycerides or high molecular weight hydrocarbons, e.g. Neobee® (C8–C10 fatty acid triglyceride), olive oil or hexadecane, as the organic phase containing the *Massoi* lactones as precursors [231]. With use of this two-phase system, toxic effects of the precursors and the products on the cells were avoided and further downstream processing was facilitated. Feeding dextrose to adjust a low operational concentration (preferably at 0.03–0.07 g $L^{-1}$) and maintaining a sufficiently high oxygen supply (more than 10% dissolved oxygen pressure) yielded maximum 5-decanolide and 5-dodecanolide concentrations of up to 7.45 and 1.7 g $L^{-1}$ after 60–70 h, respectively. Other strategies for 5-decanolide production start from other natural precursors, such as 11-hydroxypalmitic acid (sweet potato, Jalap resin) and coriolic acid (13-hydroxyoctadeca-9,11-dienoic acid) (*Coriana nepalensis* seed oil) and use *Saccharomyces cerevisiae* and *Cladosporium suaveolens* as biocatalysts [222].

Oxidation of oleic acid to 10-hydroxyoctadecanoic acid by a gram-positive bacterium was described with a transformation yield of 65% at a concentration of 50 g $L^{-1}$ oleic acid after 72 h in a medium containing Tween 80 [232]. The hydroxy fatty acid can be converted to **4-dodecanolide**, an important coconut-fruity like lactone, by β-oxidation with yeasts, affording a total lactone yield of about 20% from oleic acid [222, 232].

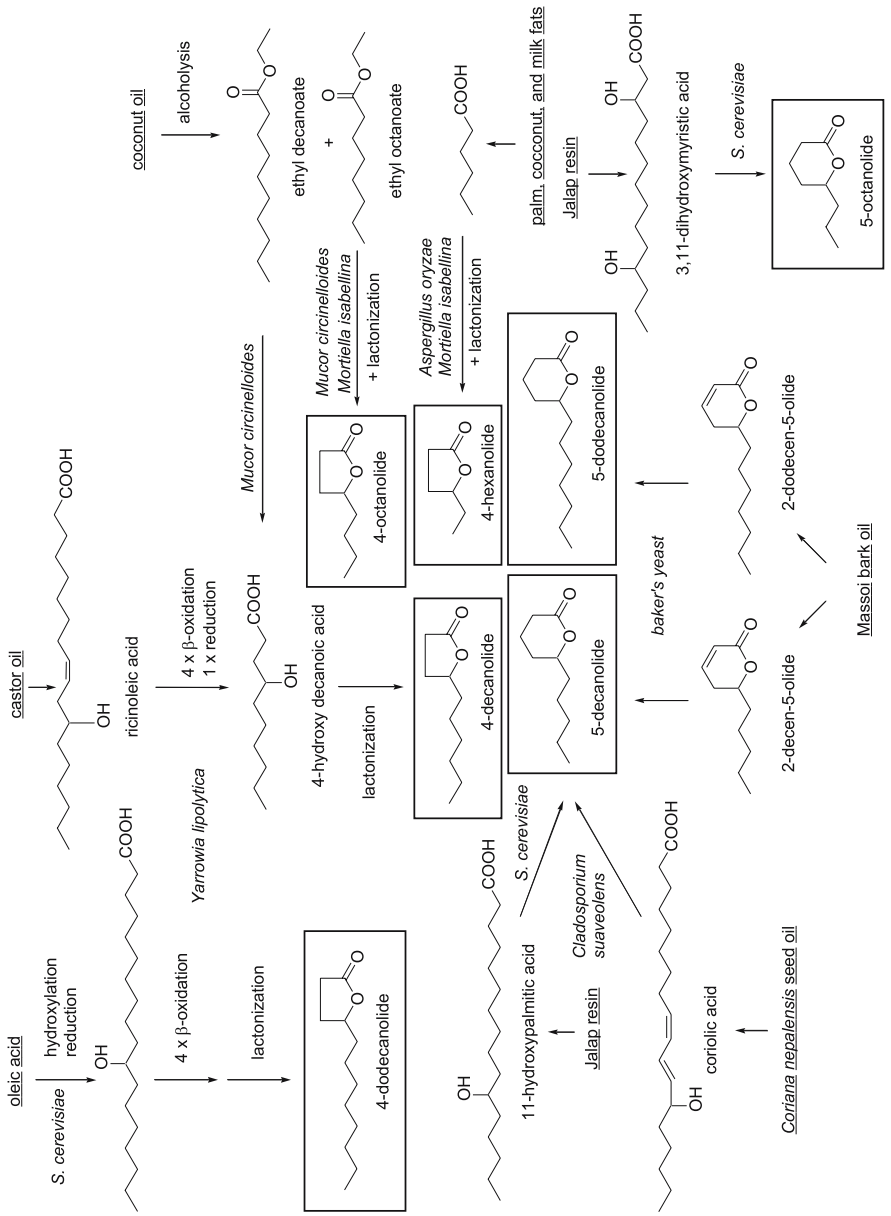
The bioconversion of native oils, e.g. sunflower, castor oils and especially coconut oil, which is rich in octanoic acid, with fungal catalysts, such as *Cladosporium suaveolens*, *Aspergillus niger* or *Pichia etchellsii*, yields about 1 g $L^{-1}$ **4-octanolide**, which is also a desired lactone-type flavour compound with a sweet herbaceous coconut-like odour [233]. Even higher concentrations of up to 7.56 g $L^{-1}$ were obtained in a bioreactor with octanoic acid or its ethyl ester as a substrate and *Mortiella isabellina* as a biocatalyst [234]. The bioconversion was carried out in a complex nutrient broth with 0.05% Tween 80 as cosolvent and 0.5 vvm aeration at pH 6 and 27 °C. After 5 h the bioconversion was started by feeding ethyl octanoate (or octanoic acid) and after 77 h the reaction was completed by acidifying the culture broth to pH 2–3 and heating it to 121 °C for 15 min (lactonisation). The same precursor was converted to 11.2 g $L^{-1}$ 4-octanolide *by Mucor circinelloides* within 47 h [235]. **4-Hexanolide** can be produced by a homologous strategy from natural hexanoic acid found in palm, milk and coconut fats using *Aspergillus oryzae* or *Mortiella isabellina* as biocatalysts in a two-phase system (e.g. Primol® as an organic phase containing hexanoic acid) with sufficient oxygen supply [236]; final product concentrations of up to 19.4 g $L^{-1}$ 4-hexanolide were obtained, while more than 16 g $L^{-1}$ **2-pentanone** formed as an additional, valuable flavour-active product during the same cultivation. The methylketone was recovered from the ex-

haust air by trapping it on charcoal. **5-Octanolide**, naturally found in meat, cheese, fermented beverages and fruits, can be produced biotechnologically as a by-product besides 5-decanolide when a mixture of 11-hydroxypalmitic acid and 3,11-dihydroxymyristic acid from Jalap resin is converted by *Saccharomyces cerevisiae* [237].

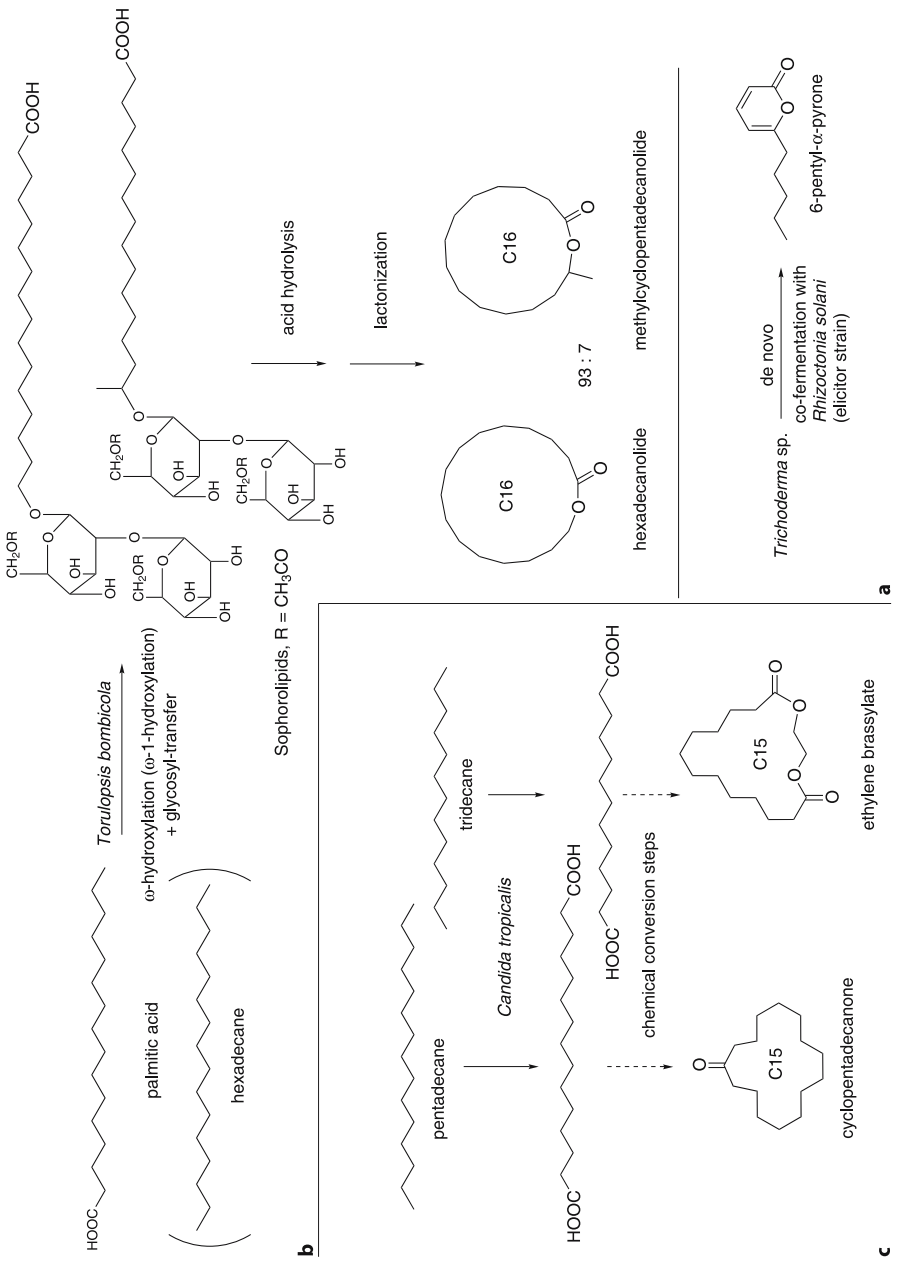
The twofold unsaturated short-chain lactone **6-pentyl-α-pyrone** imparts a strong coconut-like odour and, interestingly, it was found to be the major volatile product from de novo biosynthesis of the fungus *Trichoderma*, with concentrations of up to 200 mg $L^{-1}$, which was described in the early 1970s [238] (Scheme 4.18). After an extended cultivation of 27 days, the harvested fermentation broth was processed by organophilic pervaporation and about 1 g $L^{-1}$ calculated on the basis of culture volume was recovered; the efficiency of coupling organophilic pervaporation to the bioreactor for continuous product removal was limited by too low feed concentrations of the aroma compound [239]. Other in situ product-removal techniques, such as adsorption to XAD resins and aqueous–organic two-liquid-phase fermentation [240, 241], have also been tried to enhance overall yields by circumventing product inhibition effects which already occur at low 6-pentyl-α-pyrone concentrations (100 mg $L^{-1}$). The combination of in situ product removal by extractive bioconversion and cofermenting *Rhizoctonia solani* as an elicitor strain showed a significantly positive effect on 6-pentyl-α-pyrone production with *Trichoderma harzianum* [242]. The presence of non-viable mycelium of the phytopathogenic fungus *Rhizoctonia solani* led to an increase of product concentration from 147 to 474 mg $L^{-1}$ and a decrease of process time from 192 to 96 h. A surface culture of *Trichoderma harzianum* was shown to be superior to a submerged culture which produced 455 mg $L^{-1}$ 6-pentyl-α-pyrone after 96 h and 167 mg $L^{-1}$ after 48 h, respectively, under the same bioreactor conditions [243].

Musks are important ingredients of fragrance formulations, but almost all the musks used are polycyclic aromatics produced chemically from petrochemically derived raw materials. Naturally occurring musks include the macrocyclic lactones found in some plants, such as ambrette seedoil and galbanum, and the keto musks produced by some animals, such as musk deer and civet cats.

The macrocyclic lactones are preferred to traditionally synthesised nitromusk compounds owing to their better skin compatibility and natural degradation [222]. **Hexadecanolide** is efficiently produced by a combined biosynthetic and chemosynthetic reaction sequence: the yeast *Torulopsis bombicola* converts palmitic acid, its ester (or even hexadecane), by ω-hydroxylation and ω-1-hydroxylation in very high yields of up to 40% [244] (Scheme 23.18). Owing to a concurrent glycosyl transfer, up to 300 g $L^{-1}$ sophorolipids can be produced by this fermentative approach. Subsequent acid hydrolysis and lactonisation yielded hexadecanolide and **methylcyclopentadecanolide** in a 93:7 mixture, a difficult reaction as at high concentrations ω-hydroxypalmitic acid tends to polymerise. In a comparable process patented by a Japanese company, tridecane and pentadecane were converted by *Candida tropicalis* into the corresponding terminal dicarboxylic acids, which, upon chemical conversion and polymerisation steps, yielded the musk fragrance macrocycles **ethylene brassylate** and **cy-**




**Scheme 23.17** Microbial processes for the production of natural flavour-active lactones




**Scheme 23.18** **a** De novo biosynthesis of coconut-like 6-pentyl-α-pyrone by *Trichoderma* sp. **b** Production of macrocyclic musk-like lactones by a combination of microbial ω-hydroxylations and ω-1-hydroxylations and subsequent chemical conversion steps. **c** Production of macrocyclic musk fragrances initiated by terminal oxidation of hydrocarbons with *Candida tropicalis*

**clopentadecanone**, respectively [3] (Scheme 23.18). Final concentrations of up to 120 g $L^{-1}$ and a final product purity of 94% at 20-$m^3$ scale were reported.

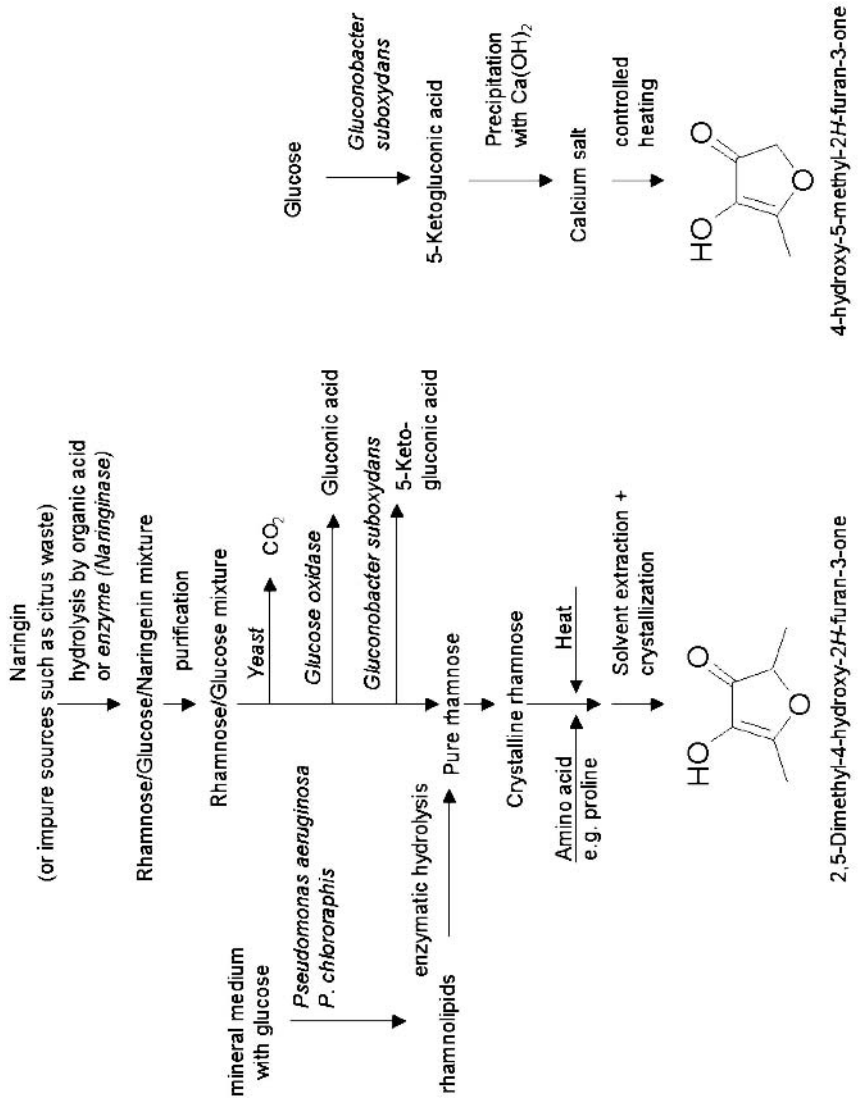
### 23.4.5 O-Heterocycles, S- and N-Containing Compounds

Besides the aforementioned lactones, **Furaneol®, 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one** (DMHF), is another very important O-heterocyclic compound where biotechnology is involved in its manufacture. It is a key-impact compound of pineapple and strawberry aroma and is also found in savoury foods. It exhibits a pineapple, strawberry-like odour in dilute solutions and a caramel-like one in concentrates and is synthesised by heating rhamnose with an amine source, preferentially proline.

Biocatalysis can be used for the generation of rhamnose (6-deoxymannose) by the selective enzymatic hydrolysis of plant-derived flavanoid glycosides containing rhamnose in the terminal position, such as naringin or rutin (Scheme 23.19). The yield of the subsequent flavour-development step by heating is reduced by even small traces of glucose, which cause an off-taste. This problem can also be solved biocatalytically by eliminating the glucose via selective conversion of the glucose using immobilised *Saccharomyces cerevisiae* (to ethanol and $CO_2$) or using *Gluconobacter suboxydans* to (to 5-ketogluconic acid, which is precipitated as the calciumsalt) [70]. Pure rhamnose for DMHF production may also be produced by cultivating *Pseudomonas aeruginosa*, which synthesises large amounts of rhamnolipids in oil-containing media (50 to more than 130 g $L^{-1}$) [245, 246], and subsequent selective rhamnolipid hydrolysis and purification [247]. Recently, a nonpathogenic species, *Pseudomonas chlororaphis*, was described to produce about 1 g $L^{-1}$ rhamnolipids on glucose, an amount comparable to the levels reported for the pathogenic *Pseudomonas aeruginosa* under the same conditions, which might give access to a food-grade strategy in the future [248].

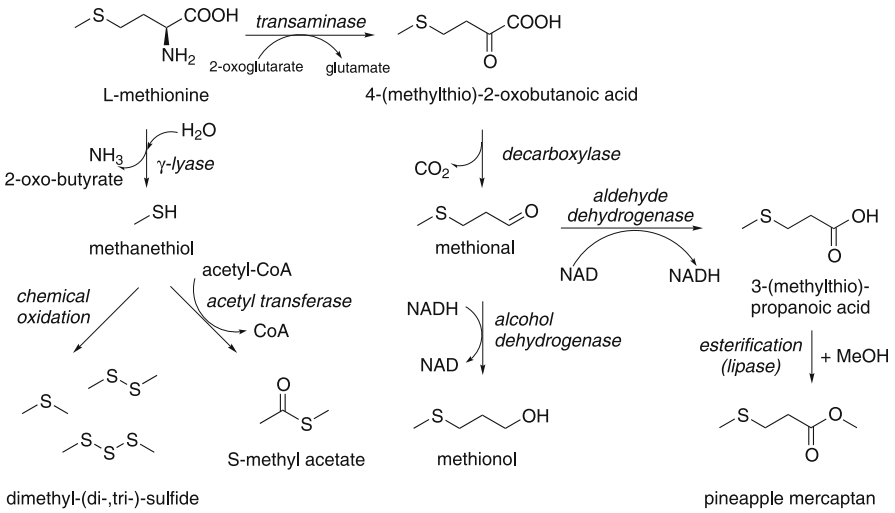
The production of a closely related furanone starts with natural 5-oxo-gluconic acid production from glucose with *Gluconobacter suboxydans;* the acid is recovered by precipitation as the calcium salt; for flavour applications, it is converted by heating to **4-hydroxy-5-methyl-2*H*-furan-3-one**, a typical savoury reaction flavour with a meat-like taste [70] (Scheme 23.19).

Owing to very low thresholds, volatile sulfur compounds (VSCs) usually have prime impact on food aromas; they are found in lots of natural sources, including fermented foods (e.g. wine, beer, cheese), and act as both flavours and off-flavours [249, 250]. Although their biogenetic formation has been elucidated in detail, only few biotechnological processes with potential for commercial application have been reported. The sulfur-containing amino acids L-methionine and L-cysteine are the natural precursors of a wide variety of VSCs. **Methanethiol** is the most frequently found VSC in cheese and can be readily oxidised to other VSCs, such as **dimethyl sulfide** and **dimethyl disulfide**, or




**Scheme 23.19** Furanone production schemes involving biocatalytic steps (*italicised*) (adapted from [270, 271])

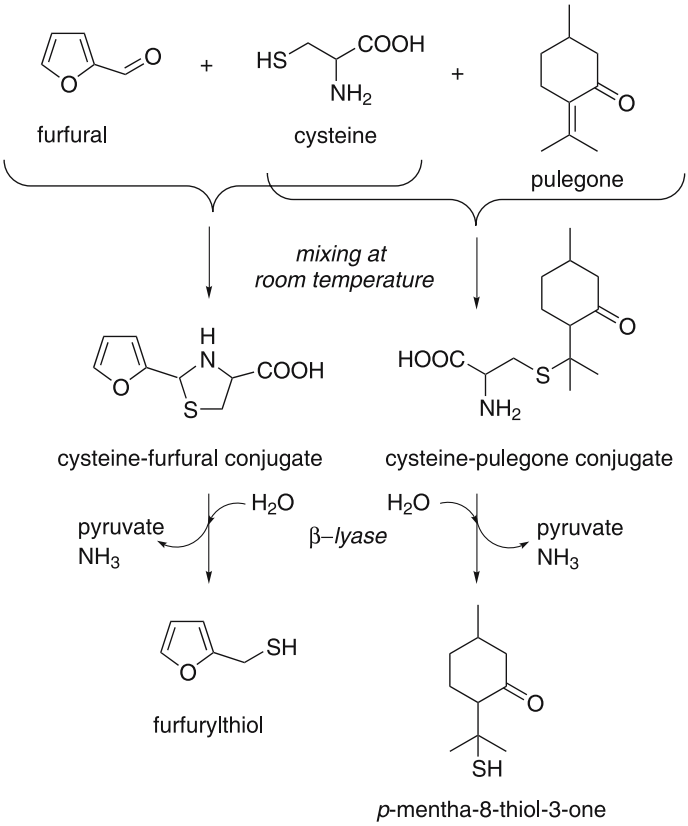
esterified to S-methylthioesters, e.g. ***S*-methyl acetate** [249] (Scheme 23.20). For instance, the cheese-ripening yeast *Geotrichum candidum* produces methanethiol and *S*-methyl acetate in the lower parts-per-million range [249, 251] . Recently, the gene encoding L-methionine-$\gamma$-lyase (MGL) was cloned from the cheese-ripening bacterium *Brevibacterium linens* [252]. MGL converts L-methionine to methanethiol, $\alpha$-ketobutyrate and ammonia. The potato-like **me-**




**Scheme 23.20** Some sulfur-containing flavour compounds generated from L-methionine by microbial metabolism plus chemical or enzymatic transformations

**thylthiopropanal** (**methional**) was shown to be produced from L-methionine in concentrations of up to 62 mg $L^{-1}$ by *Lactococcus lactis* under assay conditions [253]. The corresponding alcohol, **3-(methylthio)propan-1-ol**, known as **methionol**, which has also a potato-like odour, can be formed by yeast-based bioconversion of L-methionine following the Ehrlich pathway as already described for the other flavour-active alcohols (Sect. 23.4.2). Depending on the redox status of yeast cells, methional is reduced by alcohol dehydrogenase to methionol or is oxidised by aldehyde dehydrogenase to the corresponding acid, **3-(methylthio)propanoic acid** [129] (Scheme 23.20). Both products were obtained in total yields of up to 55% and concentrations of up to 11.2 g $L^{-1}$ with different yeasts, especially with *Saccharomyces* and *Hansenula*, in a fermentation with a high biomass loading and glucose and precursor feeding [105, 254]. The acid can also be synthesised from L-methionine by oxidation with acetic acid bacteria as described for aliphatic acids in Sect. 23.4.1.1 [39]. The methyl ester of the acid, the important flavour compound **pineapple mercaptan**, can be obtained by subsequent lipase-catalysed esterification. Nevertheless, for labelling the products as 'natural', a natural source of L-methionine must be available, which is not the case so far, although fermentative L-methionine production has been improved during the last few years [255].

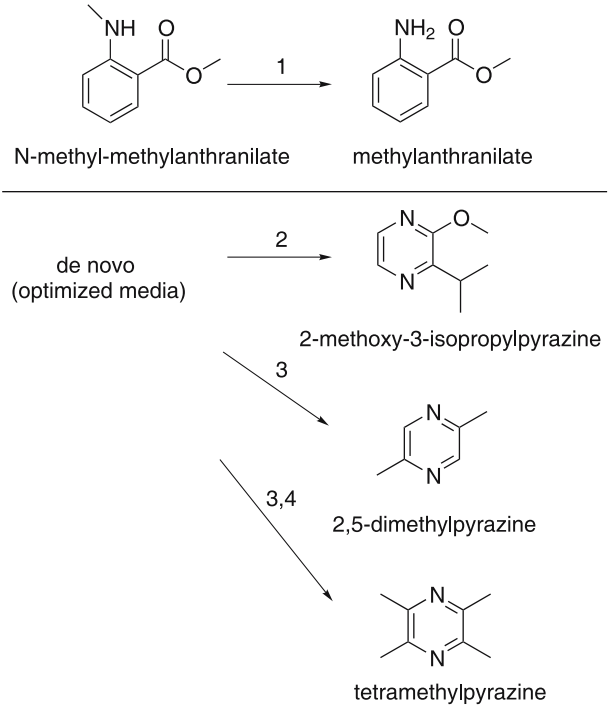
**Furfurylthiol** is a key flavour especially for coffee, beef and roast-like food aromas. It was synthesised in concentrations of up to 1 g $L^{-1}$ using $\beta$-lyase activity of whole bacterial cells, e.g. *Enterobacter cloacae* or *Eubacterium limosum* [256] (Scheme 23.21). Resting cells were used to cleave the sulfur–carbon bond of a furfural–cysteine conjugate and an XAD-4 resin connected to the gas outlet




**Scheme 23.21** Syntheses of valuable sulfur-containing flavour compounds involving $\beta$-lyase activity of *Enterobacter cloacae* or *Eubacterium limosum* cells

served for in situ product removal by periodical nitrogen flushing. The same $\beta$-lyase based strategy was pursued earlier to produce ***p*-mentha-8-thiol-3-one**, a very potent, extremely low-threshold blackcurrant flavour compound, from a pulegone–cysteine conjugate using *Eubacterium limosum* ATCC 10825 [257] (Scheme 23.21). After synthesis of the *S*-cysteinyl-pulegone simply by mixing at room temperature and allowing for precipitation over 3 days, the filtered conjugate was converted by resting cells, pregrown anaerobically on a complex medium, in a buffer system.

As for S-containing heterocycles, many N-containing heterocycles are also found in heat-treated foods as secondary flavours as a result of Maillard-type reactions between reducing sugars and amino acids. Pyrazines are N-heterocycles important contributors to the taste and aroma of roasted and toasted foods as well as vegetables and fermented foodstuffs. In cultures of *Pseudomonas perolens* ATCC 10757, amino acids such as valine, glycine and methionine were shown to




**Scheme 23.22** Some nitrogen-containing flavour compounds produced by microorganisms. **a** Methylanthranilate formation from *N*-methyl methylanthranilate: *1 Trametes* sp., *Polyporus* sp. **b** Different pyrazines produced with microorganisms in optimised media: *2* mutant strain from *Pseudomonas perolens* ATCC 10757; *3 Bacillus subtilis*, *Brevibacterium linens*; *4* mutant strain of *Corynebacterium glutamicum*

be precursors for the production of the musty, potato-like **2-methoxy-3-isopropylpyrazine** [258], with lactate and pyruvate being important components to enhance pyrazine formation [259] (Scheme 23.22). Nevertheless, the final product concentrations remained low although medium optimisation and selection of a mutant strain auxotrophic for leucine increased 2-methoxy-3-isopropylpyrazine production from around 20 μg $L^{-1}$ to 15.7 mg $L^{-1}$. *Bacillus subtilis* and *Brevibacterium linens* were shown to naturally produce pyrazines under solid substrate conditions from ground soy beans or soy flour enriched with L-threonine, acetoin and ammonia as precursors fed separately to the medium [69, 70, 260]. By this means, up to 4 g $L^{-1}$ **2,5-dimethylpyrazine** and 1 g $L^{-1}$ **tetramethylpyrazine** were produced. A mutant of *Corynebacterium glutamicum* deficient in a single enzyme of the isoleucine–valine pathway was described to produce 3 g $L^{-1}$ tetramethylpyrazine after 5 days, which crystallised upon cooling of the culture broth; thiamine was found to be essential for product formation [261].

**Methylanthranilate**, which occurs in small quantities (0.5–3 mg $L^{-1}$) [87] in a large number of blossom essential oils, grapes and citrus oils is mainly used

as a fragrance compound but also finds application as a flavour compound in grape and citrus compositions [49]. A patent describes the formation of the natural compound by microbial N-demethylation of *N*-methyl methylanthranilate that is abundant in 'petit grain' mandarin leaf oil [262] (Scheme 23.22). Higher fungi, preferentially *Trametes* and *Polyporus* strains, tolerating elevated concentrations of the toxic precursor were used; for instance, after biomass growth *T. versicolor* was supplemented with 0.2% (w/w) *N*-methyl methylanthranilate and converted it into methylanthranilate with a yield of about 30% after 3 days of incubation. *N*-formyl methylanthranilate formed as an undesired by-product but could be transformed to the target compound by heat or base-catalysed decarboxylation. Nevertheless, compared with this microbial demethylation approach, the use of free lipase to esterify natural anthranilate, found in protein hydrolysates from food processing, with methanol in biphasic media may be the more efficient strategy to yield the natural flavour compound [263].

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