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REVIEW

Biotechnology of flavours—the next generation

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Abstract Volatile organic chemicals (flavours, aromas) are the sensory principles of many consumer products and govern their acceptance and market success. Flavours from microorganisms compete with the traditional agricultural sources. Screening for overproducers, elucidation of metabolic pathways and precursors and application of conventional bioengineering has resulted in a set of more than 100 commercial aroma chemicals derived via biotechnology. Various routes may lead to volatile metabolites: *De novo* synthesis from elementary biochemical units, degradation of larger substrates such as lipids, and functionalization of immediate flavour precursor molecules. More recently, the field was stimulated by the increasing preference of alienated consumers for products bearing the label "natural", and by the vivid discussion on healthy and "functional" food ingredients. The unmistakable call for sustainable sources and environmentally friendly production is forcing the industry to move towards a greener chemistry. Progress is expected from the toolbox of genetic engineering which is expected to help in identifying metabolic bottlenecks and in creating novel high-yielding strains. Bioengineering, in a complementary way, provides promising technical options, such as improved substrate dosage, gas-phase or two-phase reactions and in situ product recovery.

Keywords Aroma · Flavour · Fragrance · Genetic engineering · In situ product recovery · Volatiles

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Introduction

Volatile flavours generated by microorganisms have long been regarded as a laboratory curiosity. Two major discoveries changed the situation, first that an alkanophilic yeast converted castor oil to 4-hydroxydecanoic acid and further to 4-decanolide (Farbood and Willis 1983), and second that certain lipases catalyzed transesterification reactions in organic media at temperatures of up to 100°C (Zaks and Klibanov 1984) opening access to the reverse hydrolytic synthesis of carboxylic acid esters. Among the around 10,000 volatiles found in nature, both groups, the medium-sized 4- and 5-alkanolides and some carboxylic acid esters, confer pleasant organoleptic impact attributes, such as fruity, floral, spicy, creamy or nutty to food, beverages, toothpaste, fragrances, and perfumed articles. The introduction of the first microbial 4-decanolide on the European market at a

market price of around EUR 10,000 per kg sent out a strong signal. Today, the driver of research is a mixture of scientific and economic considerations:

- some valuable flavours, such as raspberry ketone occur in traces in their plant sources rendering isolation by classical extraction or distillation impossible,
- chemosynthesis offends the consumers superstition that "natural" compounds are safer; for example, 90% of all beverage flavours in Europe are "natural" (80% in the US),
- biocatalysis is highly selective (chemo-, regio-, stereo-), and
- biocatalysts preferably accept natural, that is renewable substrates, and convert them to products in environmentally favourable processes ("White Biotechnology").

In addition, flavour chemicals were shown to possess not only sensory properties, but other desirable properties such as:

- antimicrobial (vanillin, essential oil constituents),
- antifungal and antiviral (some alkanolides),
- antioxidant (eugenol, vanillin),
- somatic fat reducing (nootkatone),
- blood pressure regulating (2-[*E*]-hexenal) or
- anti-inflammatory properties (1,8-cineole).

This puts some flavours close to pharmaceuticals, a class of active agents with many well-established bioprocesses.

From a scientific point of view, the origin of a chemical cannot affect its bioactivity; however, food laws world-wide reflect the "natural/artificial" discrimination made by consumers. As a result, consumer companies wish to "capture the green advantage" (Boston Consulting Group www.bcg.com/impact_expertise/publications/files/Capturing_Green_Advantage_Consumer_Companies_Jan_2009.pdf, as by 27.04.2009). A variety of analytical tools are at hand to prove the origin of a flavour compound. Enantiodifferentiation of chiral compounds is performed by chiral capillary gas chromatography, for example on cyclodextrin phases, and achiral compounds are submitted to stable-isotope (D, ^{18}O, ^{15}N) analysis using either SNIF-NMR or IR-GC-MS. As the isotope distribution of the precursor compound remains imprinted in the isotope pattern of the flavour, the chemical source can be traced in most instances.

State-of-the-art

Although still overlooked by most textbooks, flavours from biotechnology have conquered the marketplace in recent years. Many pure flavour compounds are offered with the label "natural" (Table 1). The European regulation on flavours (EEC No 1334/2008) defines in article 3 (2) c): "*Natural flavouring substance shall mean a flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes*". Under the terms of the US Food regulation, flavours fall under the category of food additives. The definition of "natural" is similar, and an expert panel examines new flavours which, upon positive evaluation, are entered into the GRAS list (generally recognised as safe). It is the producer's decision to declare the source that was actually used. Backed by published work, one may speculate on those chemicals for which, in the meantime, a microbial process has replaced the traditional plant source.

Recent reviews have presented the advantages of biocatalysis over classical chemosynthesis or extraction (Serra et al. 2005; Borges et al. 2009), and have discussed the most suitable biosystem for the target (Schrader 2007). It is obvious that isolated enzymes are the preferred biocatalysts if a single step reaction, such as (reverse) hydrolysis or transglycosidation is aimed at. Prokaryotic organisms produce mainly simple alcohols, carboxylic acids and other constituents of fermentation flavours. *Saccharomyces* and wild yeasts add a pronounced esterification capability and also possess carbon–carbon coupling activity. The most plant-like flavour metabolism is located in higher fungi, particularly in the basidiomycetes.

Flavour enzymes

Volatile flavours, like most bioactive chiral agents, depend on the correct stereochemistry to exert the desired physiological action. Thus, kinetic resolution of racemates using members of the lipase/esterase family was applied to many esters and alkanolides. A mathematical model simulated the kinetics of this equilibrium reaction in a fixed bed reactor

Table 1 Commercial natural aroma chemicals (LiAxx Biotech, www.axxence.de/, product catalogue 2007; Schrader 2007)

Acetaldehyde[a]	Citronellyl esters[b]	2-Heptanone[a]	2-Octanone
Acetoin[a]	*n*-Decanal[a]	*n*-Hexanal	4-Octanolide[a]
Acetophenone	4-Decanolide[a]	2-(*E*)-Hexenal/ol	1-Octen-3-ol[a]
Anethol	5-Decanolide[a]	3-(*Z*)-Hexenol/acetate[b]	2-Pentanone[a]
Anisyl acetate	2-Decenolactone[a]	4-Hexanolide[a]	*i*-Pentyl alcohol[a]
Benzaldehyde[a]	Dimethyl pyrazines	4-Hydroxy-2,5-dimethyl-3(2H)-furanone	*i*-Pentyl esters[b]
Benzyl butanoate[a]	4-Dodecanolide[a]	Indole	Phenylacetaldehyde[a]
n-Butyl esters[b]	5-Dodecanolide[a]	*β*-Ionone[a]	1-Phenylethyl acetate[a]
n-Butanol[a]	Ethyl acetate[a]	Maltol	*n*-Propanol[a]
i-Butyl alcohol[a]	Ethyl benzoate[b]	Methional[a]	2-Propenyl hexanoate[c]
i-Butyl esters[b]	Ethyl butanoate[a]	Methyl anthranilate[a]	*n*-Propyl esters[b]
i-Butanal[a]	Ethyl 2,4-(*E,Z*)-decadienoate[b]	2-Methylbutanoic acid/esters[a]	Raspberry ketone
d-Carvone	Ethyl 2-methylbutanoate[a]	3-Methylbutanal[a]	Sclareolide[a]
ß-Caryophyllene	Ethyl phenylacetate[a]	Methyl salycilate	4-Undecalactone[a]
Cinnamic acid[a]	Farnesol	*n*-Nonanal/ol[a]	2-Undecanone[a]
Cinnamic alcohol[a]	Fenchol	2-Nonanone[a]	Vanillin[a]
Cinnamaldehyde[a]	Furfuryl thiol[a]	Nootkatone[a]	
Cinnamyl esters[b]	Geranyl acetate[b]	*n*-Octanal	
Citronellol	*n*-Heptanal	3-Octanol	

[a] Biotechnological origin probable

[b] One of the moieties probably from plant

[c] Not found in nature

(Berendsen et al. 2006). An industrial research group used a *Pseudomonas* strain to generate pure (*R*)-2-methylbutyric acid, a flavour compound and an acyl moiety for the preparation of the respective flavour esters (Table 1) (Tachihara et al. 2006). The reverse reaction was carried out using carboxylesterases of lyophilised fungal mycelia in solvents such as *n*-heptane. This may perform better than using purified acyl transferases of living yeast. For example, the acylation of ethanol with phenylacetic acid, a metabolite of L-phenylalanine, was achieved using various *Aspergillus* and *Rhizopus* strains (Converti et al. 2005).

Another central motif of aroma biotechnology is oxyfunctionalisation. Thousands of tons of terpene hydrocarbons, such as (+)-limonene and pinenes, are separated each year from plant essential oils and discarded because of their low aroma value and chemical instability, while many of their oxyfunctionalised metabolites represent flavour impact constituents (Marostica et al. 2007). Enzymes, cell extracts and all kinds of intact cells were used to perform regio- and stereoselective transformations of terpene substrates (de Carvalho and da Fonseca 2006). Typical transformation products of the (+)/(−)-limonenes were carveols, carvone (Table 1), dihydrocarveol and α-terpineol, while the pinenes yielded pinene oxide, verbenols, verbenone and myrtenol (Divyashree et al. 2006; Bicas et al. 2008). Linalool was the substrate in extended screenings that identified fungi producing linalool oxides, 8-hydroxylinalool and lilac aldehyde plus alcohol (Mirata et al. 2008). Resting cells were often used to overcome the cytotoxicity of the hydrocarbon substrates. Monoalcohols, such as linalool, are less of a problem because of their lower solubility in the membrane of the cells. Some pathways were inducible, but a general mechanistic understanding of these detoxification reactions is missing.

Cytochrome P450 isoforms have frequently been suspected to be the key catalysts. Their general preference for lipophilic substrates, broad reaction specificity, rapid inactivation and wide-spread occurrence (especially in plants) suggested an important role (Bernhardt 2006). However, only a few terpene-transforming P450 proteins were purified and

characterized, although more than 5,000 presumptive cytochrome genes are known. Among the few exceptions are P450cam from *Pseudomonas putida* and P450BM-3 from *Bacillus megaterium* (Sowden et al. 2005). These enzymes transformed the sesquiterpene (+)-valencene selectively (P450cam mutants) or non-selectively (P450BM-3) to metabolites of the nootkatone family (Table 1). There are, however, many more enzymes capable of introducing oxygen selectively, for example, non-haem iron mono-oxygenases, haloperoxidases and the Bayer-Villiger-type mono-oxygenases (Leak et al. 2009). The potential of these enzyme classes for flavour production has not yet been systematically exploited. A first example of a non-P450 catalysed oxyfunctionalisation may be the transformation of (+)-valencene to the α- and β-nootkatols and nootkatone by a purified enzyme of *Pleurotus sapidus* (Fraatz et al. 2009). The isolated enzyme did not show haem absorption nor was it inhibited by carbon monoxide, but showed homologies of ~50% to putative lipoxygenases from *Aspergillus fumigatus* and *Laccaria bicolor* on the protein level.

Carotenoids protect cells from oxidative damage and serve as precursors of vitamins upon symmetric cleavage. Asymmetric cleavage should result in the formation of C8-, C10- and C13-norisoprenoids (Fig. 1), volatiles which possess low olfactory perception thresholds and which are abundantly found in nature, for example in tea, grape, tobacco, and wine flavour (Uenojo et al. 2007). While the fortification of wine flavour by enzymatic hydrolysis of the respective glycosides of norisoprenoids has been proven (Mendes-Pinto 2009), the concentration of these volatiles in the natural sources is often too low to render an industrial extractive isolation economically attractive (Rodriguez-Bustamante and Sanchez 2007).

Previous research focussed on plant dioxygenases and showed that carotenoid levels correlated with type and amount of norisprenoids in the tissue; in tomato, for example, decreased lycopene level led to decreased concentrations of 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol, volatiles derived from 5,6 (5′,6′) bond cleavage (Gao et al. 2008). This carotenoid-cleavaging dioxygenase also occurred in maize, *Arabidopsis* (Vogel et al. 2008) and in rice (Ilg et al. 2009) and showed low substrate specificity, acting on cyclic and acyclic carotenoids. The enzymes also mediated the formation of geranial and ionones through a 7,8 (7′,8′) and a 9,10 (9′,10′) bond cleavage, respectively. Similar enzymes from basidiomycetes, a versatile peroxidase of *Lepista irina* and peroxidases of *Marasmius scorodonius*, were patented recently (Zorn et al. 2004). These stable extracellular enzymes efficiently degraded ß-carotene to ß-ionone and a few minor volatiles (Scheibner et al. 2008). Heterologous expression to study the reactions in more detail will be required.

The concept of degrading complex precursor substrates which contain a small flavour target preformed in their structure may not sound chemically convincing. It is, however, the route along which nature itself proceeds in ripening fruits or other senescing tissues. Hydrolysis of acyl glycerols yields fatty acids, aldehydes, alcohols and esters, hydrolysis of proteins yields the precursors of biogenic amines and fusel oil constituents (Table 1). The degradation of carotenoids requires redox enzymes and is more challenging. To maintain a co-factor dependent enzymatic redox reaction is even more difficult, as a continuous supply of co-factor is prohibited by cost reasons. An option that has become popular is coupling of the enzyme catalysing the desired reaction with a complementary second one. An elegant example was coupling

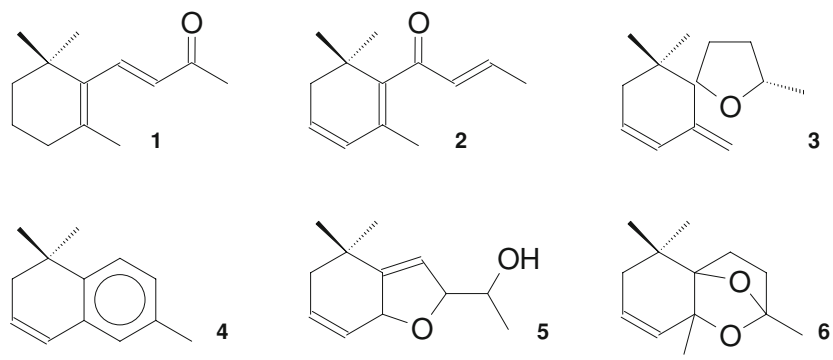

Fig. 1 Volatile norisoprenoids with important sensory properties deducible from the degradation of carotenoids: β-ionone **1**, β-damascenone **2**, vitispirane **3**, TDN (1,1,6-trimethyl-1,2-dihydronaphthalene) **4**, actinidol **5**, riesling acetal **6** (Mendes-Pinto 2009)

P450cam with an alcohol dehydrogenase from yeast to regenerate NADH in a two-phase system (Ryan and Clark 2008). Both the oxidized and the reduced product of the reaction may be useful. The conversion of easily available cinnamaldehyde to both cinnamyl alcohol (hyacinth odour) and to cinnamic acid (as the acid moiety of tropical fruit esters) using two enzymes sharing a common electron shuttle is among the alluring examples.

Cell based processes

It is the traditional food biotechnologies which are the roots of modern flavour biotechnology. Intact cells provide enzyme and co-factor regeneration and active transporters; moreover, the typical food strains command the "generally recognized as safe" (GRAS) status.

Volatile sulfur compounds with their often low odour detection thresholds determine the flavour of certain cheese varieties, but are also of interest for meat, potato, and fruit flavours. A carbon–sulphur lyase of a *Lactococcus lactis* was identified on the genetic level, cloned in *E. coli* and over-expressed for substrate testing (Martinez-Cuesta et al. 2006). The enzyme showed α, γ-elimination activity for L-methionine and was thought to play a significant role in the development of a number of cheese impact flavours. A similar study on *Oenococcus oeni* isolated from wine confirmed the central role of methionine as a common precursor of sulfur volatiles in food: Methanethiol, dimethyl disulfide, 3-(methylthio)-1-propanol, 3-(methylthio)-1-propanal (methional) (Table 1), and 3-(methylthio)propionic acid were detected (Vallet et al. 2008). Another precursor, 2-oxo-4-(methylthio)butanoic acid, seemed to play a role.

The excess of reduction equivalents in fermenting yeast directed methionine degradation to a series of alcohols (Etschmann et al. 2008). The formation of the most abundant volatiles, methionol and 3-(methylthio)-propylacetate, can be explained by the Ehrlich pathway. To aid the yeast cell in coping with the inhibitory methionol, an alcohol acetyl transferase gene was incorporated. This increased the concentration of the acetate at the expense of the alcohol. The *Ehrlich* pathway is crucial for the production of volatiles by *Saccharomyces cerevisiae*. Amino acids,

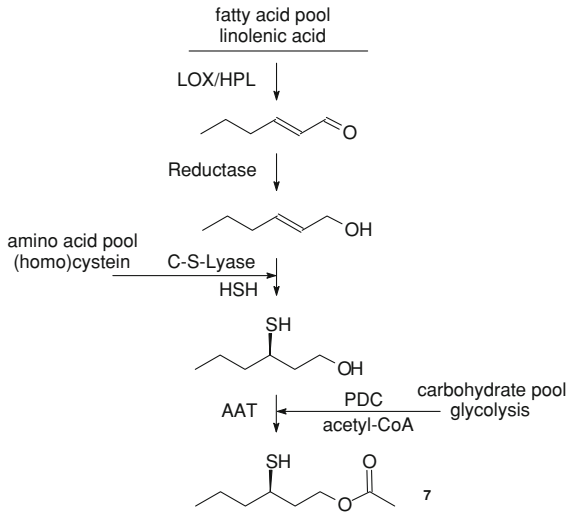

Fig. 2 3-(*R*)-Thiohexyl acetate **7** has attractive exotic-fruit notes and is deducible from precursors of different metabolic pools. *LOX* lipoxygenase, *HPL* hydroperoxyde lyase, *AAT* alcohol-O-acyltransferase, *PDC* pyruvate decarboxylase)

liberated during mashing, are transaminated, decarboxylated and the resulting aldehydes reduced to alcohols which in turn are esterification substrates for alcohol acetyl transferases (Hazelwood et al. 2008). Volatile thiols, such as 4-mercapto-4-methylpentan-2-one, 3-mercapto-hexanol and 3-mercapto-hexyl acetate affect the "tropical-fruit" character of *sauvignon blanc* wines (Swiegers et al. 2009). At this metabolic crossroad, a sulfur compound, a fatty acid degradation product (2-(*E*)-hexenal; Table 1) and the transacylation reaction merge (Fig. 2) These metabolic traits occur in a highly strain-dependent way. Classical screening and selection was thus suggested to create fermentation flavours according to consumer expectation.

The shikimate pathway generates, *via* phenylalanine and cinnamic acid, precursors of phenylpropanoid volatiles. Compounds such as eugenol, isoeugenol or vanillin (Table 1) still bear the ring substitution pattern of coniferyl alcohol. Anethol, estragol, and anisaldehyde (Table 1) are related 4-methoxy compounds. As well, water-soluble substrates, such as ferulic acid, are easily available and many papers have appeared which deal with microbial conversion reactions (Xu et al. 2007).

A prime target of flavour biotechnology is vanillin, the world's most popular single flavour. While the synthetic material is traded well below €10 per kg,

the pure natural vanillin *ex Vanilla* pod may reach €15,000 per kg; the biotech "natural" vanillins are sold at intermediate prices. In view of the commercial significance, efforts were made to elucidate the pathway of formation. A labelling study using deuterated ferulic acid and *Pycnoporus cinnabarinus* showed that, by analogy to fatty acid degradation, the mechanism comprised the hydration of the double bond of feruloyl-CoA and then the cleavage of the resultant β-hydroxy thioester by a retro-aldol reaction (Krings et al. 2001). Recently, the crystal structure of a substrate bound hydroxycinnamoyl-CoA hydratase-lyase was reported (Bennett et al. 2008). Only a few flavour formation pathways have received this high degree of attention.

Engineering the catalyst

While the persistent public concern about food applications of genetic engineering continues to discourage agronomy and industry, research on flavour coding genes prepares the grounds for the next generation of bioprocesses. Most attention is again paid to the "impact" flavours, such as the above volatile sulfur compounds. Genome databases are now available for species, such as *Lactobacillus casei* which enables the search for putative homologues of known flavour associated genes. Two L-cystathione-β-lyase genes were found, the enzymes cloned and substrate specificity and other parameters were determined (Irmler et al. 2008). Conversely, disruption of a gene coding for L-methionine-γ-lyase in *Brevibacterium linens* resulted in an almost complete loss of sulfur volatiles (Yvon et al. 2006). A similar bridge from genetic structure to metabolic trait was built for fruity odour notes of wine. Common *S. cerevisiae* leave the respective precursors in must (cysteine, cystathionine and methionine) almost untouched. But when a lyase gene, obtained from *E. Coli,* was incorporated under the control of a promoter of a housekeeping activity (yeast phosphoglycerate kinase), the recombinant yeast produced an additional tropical fruit note (Swiegers et al. 2007).

"Biotech vanillin" is currently produced using vanillin tolerant relatives of *Pseudomonas* and ferulic acid as a substrate. A recently developed triple mutant (*Rhodococcus opacus*) converted eugenol to ferulic acid suggesting broader substrate options for the process (Plaggenborg et al. 2006). Response surface methodology was described for a recombinant *E. coli* supplemented with ferulic acid degrading genes from *Pseudomonas fluorescens* (Barghini et al. 2007). Resting cells harbouring a low-copy number vector gave a final product concentration of 2.5 g vanillin l^{-1}. The industrial strain was claimed to yield more than 10 g l^{-1} peak concentration.

Terpene synthases convert the acyclic precursors delivered along the mevalonate or the DXP/MEP (1-deoxy-D-xylulose/2-C-methyl-D-erythritol-4-phosphate) pathway to a vast diversity of mono- and sesquiterpenoid structures, as they occur in essential oils and many flavours and fragrances. Conserved sequences of this large gene family allow the design of degenerate primers for direct amplification of plant cDNA; genome data are no longer required because of the progress of knowledge on the gene sequences. For example, synthases cloned from lavender catalyzed the formation of (*R*)-(−)-linalool, the main component of lavender essential oil, and of (*R*)-(+)-limonene, terpinolene (1*R*,5*S*)-(+)-camphene (1*R*,5*R*)-(+)-α-pinene, β-myrcene and traces of α-phellandrene (Landmann et al. 2007). Santalols and santalenes are sesquiterpenes which impart the unique odour of sandalwood. Genomic fragments were identified and translated into protein sequences, and a high homology to known terpene synthases of *Vitis vinifera* was found (Jones et al. 2008). Although the heterologously expressed proteins did not yield the expected sandalwood impact constituents, this kind of work will obviously pave the way to a biotechnological exploitation of similar catalytic properties in heterologous hosts. Advanced strain engineering has been reported for *E. coli* expressing a quintuple mutant CYP450 activity. α-Pinene was transformed to α-pinene oxide, verbenol, and myrtenol in an NADPH-dependent reaction. Integrating a recombinant intracellular NADPH regeneration system through co-expression of a glucose facilitator from *Zymomonas mobilis* and a $NADP^+$-dependent glucose dehydrogenase from *Bacillus megaterium* provided a functioning cofactor regeneration system and demonstrated the feasibility of such coupled reactions in an engineered host (Schewe et al. 2008). Intermediate radical species, shown to occur, for example, during the biotransformation of α-farnesene (Krings et al. 2008a, b) and fatty acid hydroperoxides (Santiago-Gomez et al. 2007) ("green" 2-(*E*)-hexenal; Table 1), are supposed to

account for the frequently observed multitude of reaction products.

Engineering the process

Biotechnology of flavours shows some peculiarities. Substrates as well as products are often chemically unstable, poorly water soluble, and bound to get lost through the waste air stream of the reactor. Both hydrophobic precursor substrates and flavour products may affect the viability of the biocatalyst. Sometimes simple measures like the change of temperature, pressure, or pH have significantly improved productivity. Response surface methods help in cutting down the number of experiments required in multi-factorial systems (Barghini et al. 2007). Among the more concerted measures to overcome some of the problems are:

- in situ recovery of product, for example by gas stripping (Krings and Berger 2008),
- two-phase systems to separate non-polar conversion chemistry from biology (Morrish et al. 2008),
- fed-batch of substrate to avoid cytotoxic concentrations of substrate and product (Etschmann and Schrader 2006),
- specific reactor construction, such as membrane (Boontawan and Stuckey 2006), solid state (Longo and Sanroman 2006) or closed loop reactors, the latter to prevent volatile substrate from loss through the exhaust stream (Pescheck et al. 2009), or
- use of non-conventional media, such as organic solvents, ionic liquids or supercritical fluids (Cantone et al. 2007).

It appears a particularly attractive idea to perform the production of volatiles using functional (hydrated) enzymes in the gas phase (Mikolajek et al. 2007). The concept was applied to reverse hydrolyses, but also to carboligation. Little systematic work has been devoted to this intriguing technique so far.

Conclusion

Flavour biotechnology could be defiend as the overexpression of microbial genes in food grade and other microorganisms, or the transfer of plant flavour pathways into suitable microbial hosts. Much knowledge has been accumulated about diverging genes and converging evolution (Pichersky et al. 2006), flavour genes and enzymes (Dherbecourt et al. 2008). The toolbox of molecular biology and the volume of sequence databases is ever-increasing (Matsuta et al. 2009). Progress could be faster, however, it the investigation of metabolic traits was given priority, followed by investigation of the genetic and enzymatic background. A few recent metabolic studies have used labelled precursors and have developed well-founded biosynthetic schemes (Hampel et al. 2006; Matich and Rowan 2007; Krings et al. 2008a, b). Likewise, sound studies on the regulation of flavour genes are scarce. As long as we cannot explain the activation of a flavour pathway by UV-light in a non-phototrophic microorganism (Taupp et al. 2008), we are far away from a thorough metabolic understanding which, in turn, would appear to be the first prerequisite for a more rational application of biological producer systems (Schwab et al. 2008).

The world market of flavours and fragrances has a current volume of $20 billion. Still <10% of the supply is derived from bioprocesses. Examples, such as the Bartlett pear impact compound, ethyl 2,4-(*E*,*Z*)-decadienoate (Table 1), which is cheaper to produce using enzyme catalysis than chemosynthesis, should encourage further research. Looking at the rapid progress in so many areas of the life sciences and considering the decreasing reliability of traditional sources, it is easy to predict that the share of biotechnology will grow in the future.

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