J Chem Technol Biotechnol 77:1323–1332 (online: 2002)
DOI: 10.1002/jctb.722

Bioflavours and fragrances via fermentation and biocatalysis

Erick J Vandamme* and Wim Soetaert
Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty Agricultural and Applied Biological Sciences, Ghent University, Coupure links 653, B-9000 Ghent, Belgium

Abstract: A condensed overview is given of the potential offered by bacteria, fungi and yeasts to produce a wide range of bioflavours and fragrances. A few commercialised processes are also discussed. The advantages of microbial processes *versus* chemical synthesis or extraction are outlined. Both *de novo* fermentation processes as well as bioconversions based on adding specific precursors/ intermediates to microbial cells or enzymes are illustrated via typical examples. Bottlenecks, which currently hinder a wider introduction of this flavour biotechnology, are indicated and some solutions are proposed.

Keywords: biocatalysis; bioflavours; *de novo* fermentation; flavour biotechnology; fragrances

1 INTRODUCTION

Flavours and fragrances find wide application in the food, feed, cosmetic, chemical and pharmaceutical sectors. Many flavour compounds on the market still are produced via chemical synthesis or via extraction from plant and animal sources; however, a rapid switch towards the bio-production and use of flavour compounds of (micro) biological origin – bioflavours – is observed. The reasons are, among others, the facts that chemical synthesis results often in an environmentally unfriendly production process and in undesirable racemic mixture compounds. Furthermore, the consumer has developed a 'chemophobia'-attitude towards chemical or synthetic (even nature-identical) compounds, especially when related to food and products used in the home.[1–4]

Up to now, certain plant and animal sources remain an important source of bioflavours, but these bioactive compounds are often present in minor quantities, making isolation and formulation very expensive, or they are found only in exotic (plant) species.[5]

The other bio-route for flavour synthesis is based on *de novo* microbial processes (fermentation) or on bioconversions of natural precursors using microbial cells or enzymes (biocatalysis).

A summary will be given here of the current state of the art of microbial and enzymatic bioflavour synthesis, with examples of the principle of bio-synthesis and the market potential of some products cited.

2 NATURAL AND NATURE-IDENTICAL FLAVOURS

In view of the emerging concept of bioproduction of natural flavours, the term 'natural' has been clearly defined in the USA as well as in Europe (EC). In the USA a distinction is made between natural and artificial flavour compounds and according to the 'Code of Federal Regulations' (1990), the term 'natural flavour' means '… the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate of any product of roasting, heating or enzymolysis, which contains the flavouring constituents derived from a spice, fruit juice, vegetable or vegetable juice, edible yeast, herb, bud, bark, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products or fermentation products thereof, whose significant function in food is imparting flavouring rather than nutrition'.

The EC Flavour Directive (88/388/EEC) defines natural flavours as '… flavouring substances or preparations which are obtained by appropriate physical processes or enzymatic or microbiological processes from material of vegetal or animal origin'.

Both definitions state that natural flavours include products obtained through microbial or enzymatic processes as long as the precursor/raw material be natural and obtained via physical or bio-processes and that the precursor and product can be found in nature or are part of traditional foods.

Physical processes for obtaining natural flavours are extraction, distillation, concentration, crystallisation, etc, ie from animal sources (eg beef, chicken, seafood) or plant sources (eg spices, mushroom, citrus, fruits, mints).

Products that occur in nature but are produced via a chemical (a non-natural) process are called 'nature-

* Correspondence to: Erick J Vandamme, Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Faculty Agricultural and Applied Biological Sciences, Ghent University, Coupure links 653, B-9000 Ghent, Belgium
E-mail: Erick.Vandamme@rug.ac.be
(*Received 4 December 2001; revised version received 8 August 2002; accepted 28 August 2002*)

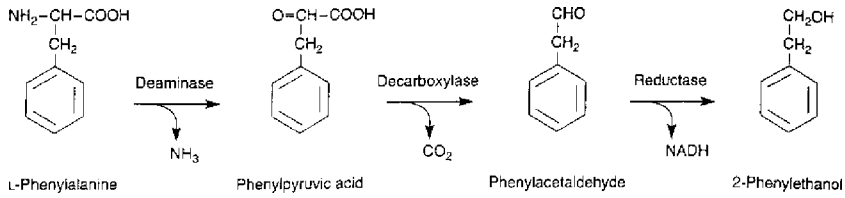

Figure 1. Bioproduction of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae* mutants.

identical'; this mode of production is no longer accepted as consumer-friendly.

3 CHEMICAL VERSUS MICROBIAL SYNTHESIS

Biotechnological processes usually involve conditions which are less damaging to the environment than chemical processes, and yield the desirable enantiomeric flavour compound. Lack of knowledge about the biochemical pathways, the enzymes, and the metabolic (de)regulation involved to obtain high yields hinders the development of biotechnological processes.

For example, 2-phenylethanol is an important flavour/fragrance component (threshold: 125 ppm) of certain fruit and beverage flavours, but also of rose fragrances. It can be produced chemically, or via extraction from roses; the naturally-obtained product is extremely expensive. During conventional yeast fermentations (*Saccharomyces cerevisiae, Kluyveromyces marxianus*), low levels of 2-phenylethanol can be recovered from the fermentation gases or broth using specific resins.[6] To improve the process, *S cerevisiae* mutants and *Aspergillus niger* strains have been selected,[7,8] which convert added L-phenylalanine via deamination, decarboxylation and subsequent reduction into 2-phenylethanol, with very little further metabolisation;[9] high yields ($>2\,g\,dm^{-3}$) are now obtained by solvent extraction of the fermentation broth (Fig 1).

Raspberry ketone (threshold: 1–10 ppb) is a characteristic flavour compound of raspberries;[10] it can be produced chemically via condensation of *p*-hydroxybenzaldehyde with acetone or extracted from raspberries; however only 3.7 mg of the ketone can be obtained from 1 kg of berries. Microbial processes can help to overcome these negative aspects. Upon hydrolysis of betuloside (found in rhododendron, birch and maple), with, for example, commercial *Aspergillus niger* β-glucosidase, into betuligenol, this intermediate can further be converted into the wanted raspberry ketone via the secondary alcohol dehydrogenase activity of the yeast *Candida boidinii* (Fig 2).[11]

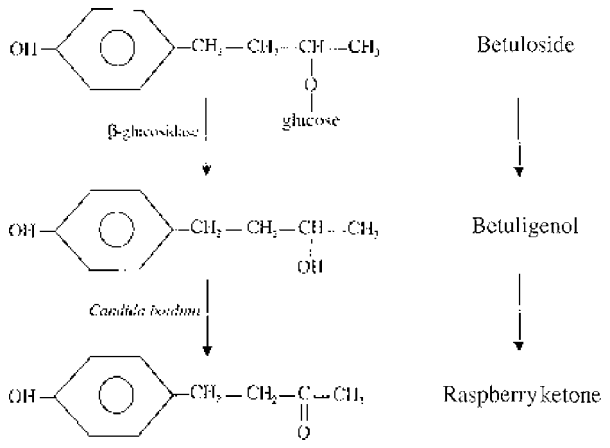

Figure 2. Bioproduction of raspberry ketone from betuloside.

4 NATURAL FLAVOURS VIA MICROBIAL PROCESSES

4.1 Microorganisms *versus* their enzymes as biocatalysts

Since time immemorial, man has unwittingly used microorganisms to produce flavours, especially when preparing fermented foods and drinks, the original benefit being the increased keeping quality of such products. It was only around the turn of the 20th century that the relationship between the typical desirable flavour of fermented foods and beverages, and the microorganisms involved became recognised.[12] In most cases, a typical mixed microbial flora was responsible for the formation of a desirable but complex flavour, eg in cheese, yoghurt, kefir, soy sauce, sourdough, gueuze beer, sauerkraut, kimchi, etc. Undesirable off-flavours were then caused by spoilage or contaminating microbial flora.[13]

Further analysis and optimisation of such food fermentations led to the study of pure microbial strains in regard to their capacity to produce specific single flavour molecules either *de novo* or by converting an added substrate/precursor molecule.

In a few cases, detailed research has led to the identification of the biochemical pathways involved, but much work still lies ahead here; similarly several enzymes/enzyme systems have been characterised and are now being exploited for enzymatic synthesis of flavour.

4.2 *De novo* flavour biosynthesis via fermentation

A wide range of microorganisms is known to produce flavour compounds from simple nutrients via *de novo* synthesis, eg sugars, alcohols, etc. Bulk flavour compounds belonging to this category include vinegar, monosodium glutamate, 5′-GMP and 5′-IMP-nucleotides, organic acids (eg L-lactic acid, citric acid, propionic acid), yeast extracts, etc. These will not be discussed in this paper. Here the focus will be on speciality flavours, a survey of which has been

published by the authors' group,[14] and updated several times.[3,4,15,16]

Some examples will be discussed here, including our own research data. In most cases the *de novo* fermentation processes can be boosted by supplying a limiting intermediate or precursor molecule, which is otherwise limiting the yield, to the culture.

4.2.1 Fungi

Several fungi are able to produce *de novo* odorous compounds, including floral flavours. It is well known that *Ceratocystis* species are able to produce a wide range of terpenes, with a fruity or floral odour (Fig 3).[17] The main aroma products of the fungus *Ceratocysis moniliformis* are ethyl acetate, propyl acetate, isobutyl acetate, isoamyl acetate, citronellol and gerianol. To circumvent inhibitory effects, *in situ* product-removal using pervaporation was found to decrease product concentrations in the bioreactor and increase microbial growth rates. The total yield of aroma compounds produced is higher in such an integrated bioprocess than in conventional batch cultivation. In addition, permeates obtained from pervaporation consist of highly enriched mixtures of flavours and fragrances.[18]

Trichoderma viride and *T harzianum* strains form efficiently the coconut-flavoured lactone, 6-pentyl-α-pyrone (6-PP); in an integrated fermentation process, 6-PP is continuously removed by pervaporation over a selective membrane, since it inhibits growth if it accumulates in the broth;[19] alternatively, aqueous two-phase systems can be used for *in situ* recovery of 6-PP.[20]

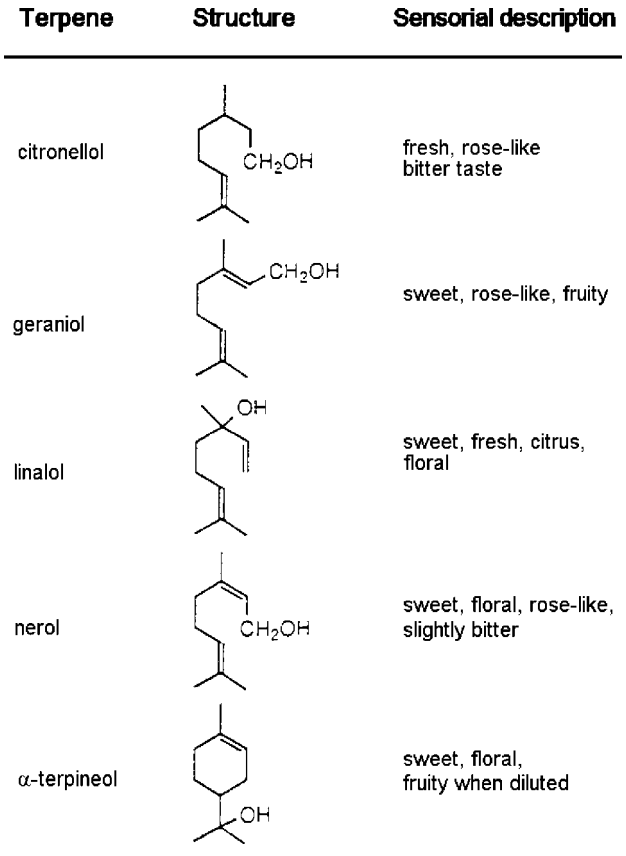

Figure 3. Terpenes produced by the fungus *Ceratocystis variospora*.

4.2.2 Yeasts

Yeasts such as *Kluyveromyces lactis* also produce *de novo* fruity, floral flavour-terpenes such as citronellol, linalol, geraniol etc.[21]

In 1972, Tahara *et al*[22] found that the yeast *Sporobolomyces odorus* (now *Sporidiobolus salmonicolor*) produces *de novo* – though in low yields – flavour-lactones, such as the peach-smelling compound γ-decalactone (4-decanolide) and 4-hydroxy-*cis*-6-dodecenoic acid-γ-lactone. The yield can be increased by supplying appropriate substrate or precursor molecules to the culture medium (see Section 4.3).

We have found several yeasts capable of producing *de novo* large amounts of fruity ester-flavours.[6,14,23,24] *Williopsis saturnus* var *mrakii* synthesises important levels of volatile branched acetates, mainly 3-methyl-butyl-acetate, the character impact compound of banana aroma. Biochemically, the amino acids valine, leucine and isoleucine are metabolised by the yeast into the intermediate branched alcohols, isobutanol, 3-methylbutanol and 2-methylbutanol, respectively. The corresponding volatile branched acetates, isobutylacetate, 3-methylbutylacetate and 2-methylbutylacetate are then formed via the action of alcoholacetyltransferases. The yeast is able to convert added branched alcohols into the corresponding fruity acetates, thereby drastically improving the yield. As a natural source of these branched alcohols, fusel oil (a cheap by-product of the rectification of fermentation alcohol) was used (Fig 4). A typical composition is given in Table 1. Due to its toxicity, fusel oil has to be added at low levels and after the active growth phase. A clear selectivity in ester formation was observed in the sense that 3-methylbutanol was esterified much faster and in higher yield (±90%) into 3-methylbutylacetate.

The influence of fermentation parameters such as pH and aeration upon biomass formation and the

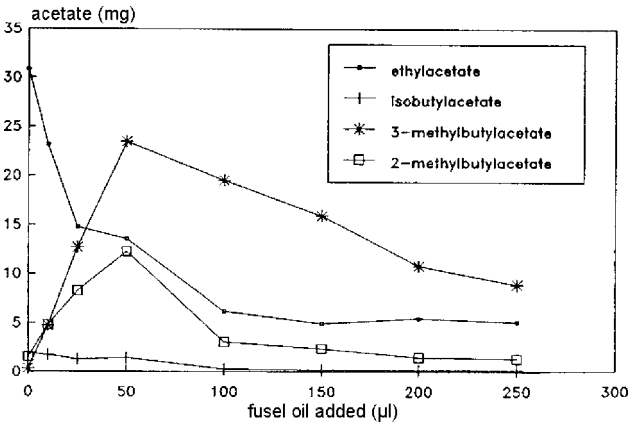

Figure 4. Influence of fusel oil addition on flavour-acetate formation by *Williopsis saturnus* var *mrakii* cultures. Addition was started only after 30 h of fermentation; the volatile acetates were collected on activated carbon between 30 and 72 h of fermentation (Janssens *et al*; unpublished results).

Table 1. Typical composition of fusel oil (vol%)

Component	*Vol %*
Propanol	1.20
Isobutanol	12.20
2-Methylbutanol	43.60
3-Methylbutanol	38.70
Butanol	0.21
Ethylacetate	0.014
3-Methylbutylacetate	0.043
2-Ethoxy-ethylacetate	0.046
2,5-Dimethylpyrimidine	0.12
2,6-Dimethylpyrazine	0.006
2-Ethyl-6-methylpyrazine	0.070
Trimethylpyrazine	0.240
3-Ethyl-2,5-dimethylpyrazine	0.120
2-Ethyl-3,5-dimethylpyrazine	0.052
3,5-Diethyl-2-methylpyrazine	0.022
Ethyloctanoate	0.061
Ethyldecanoate	0.160
Ethyldodecanoate	0.081

esterification degree were investigated on a shake flask level, up to the scale of 20 dm^3 laboratory fermenter. The process consists of an active growth phase, followed by bioconversion of the added fusel alcohols during the stationary phase. Using the aeration-air of the fermentation, the acetate-esters, which are very volatile, were stripped from the fermentation broth, adsorbed on activated coal at the exhaust of the fermenter, and subsequently recovered by solvent extraction. This process thus allows the valorisation of a cheap natural substrate such as fusel oil into a valuable flavour.[6]

Another yeast, *Geotrichum klebahnii*, produces *de novo* a broad spectrum of ethyl esters of branched carboxylic acids, generating a pleasant fruity flavour.[14,24] When supplied with isoleucine, the main product was ethyl-2-methylbutyrate.

Geotrichum fragrans partially metabolises L-leucine by oxidative deamination; addition of ethanol results in esterification, forming flavour esters such as ethylisovalerate (Fig 5), which can be recovered from the fermenter exit-air by absorption.

4.2.3 Bacteria

Acetobacter strains are able to synthesise methylbutyric acid, which is a valuable precursor of common flavour esters; as substrates, they oxidise the methylbutanols present in fusel oil; these have to be fed in the

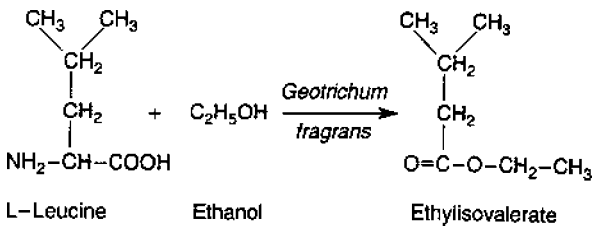

Figure 5. Bioconversion of L-leucine into the flavour ester ethylisovalerate.

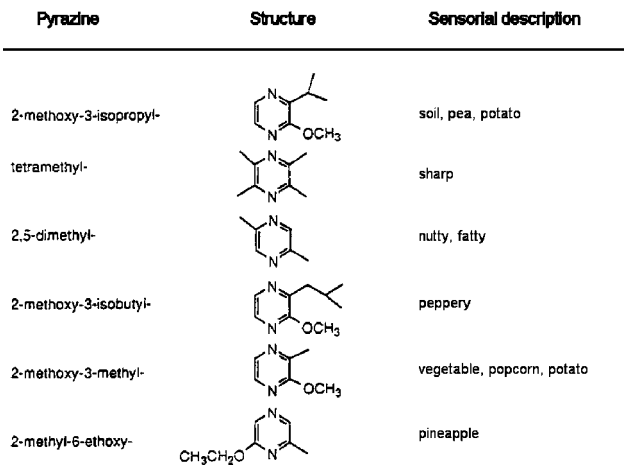

Figure 6. Formulas and flavour description of some natural pyrazines.

fermenters at low levels due to their toxic effects on cell growth. Bacteria such as *Corynebacterium glutamicum* produce *de novo* pyrazines[25] (Fig 6); these flavour compounds are responsible for the roasted, nutty flavour of heated foodstuffs such as roasted nuts, cocoa and coffee beans, baked products and meat, and are normally formed during conventional cooking/roasting of food.[26] Changes in food processing, like microwave cooking, during which no pyrazines are formed, create a demand for the addition of such natural pyrazines with a roasty flavour.

4.3 Flavour and fragrance biosynthesis via bioconversion of specific substrates

4.3.1 Microorganisms as biocatalysts

The principle here is that specific substrates, added to the fermentation medium, can be transformed into the desirable flavour compound(s). A survey of these aspects has been published by the authors[14] and several examples have been reported since then; few have reached economic relevance, however.[9]

4.3.1.1 Aromatic phenols as substrate for formation of vanillin flavour. A typical example here is the biosynthesis of vanillin (Fig 7). Vanillin is now produced by chemical synthesis from guaiacol (at 12 000 tonnes per year and 13.5 US$ kg^{-1}) or via extraction from vanilla beans (natural vanilla content 2% w/w; at 20 t per year and 3200 US$ kg^{-1}). The high price of natural vanillin and the trends towards natural flavours has driven the search for natural vanillin production using microbial processes. This requires a natural precursor such as ferulic acid, which is present in plant cell walls (eg sugar beet pulp etc). White rot fungi and bacteria are able to metabolise ferulic acid into vanillic acid and/or vanillin. A two-step fungal process has been developed, whereby *Aspergillus niger* transforms ferulic acid into vanillic acid, which basidiomycetes such as *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium* can further convert into vanillin (500 mg dm^{-3}). The optimisation of the fermentation process resulted in levels of over 1 g dm^{-3}. Above this level, vanillin is

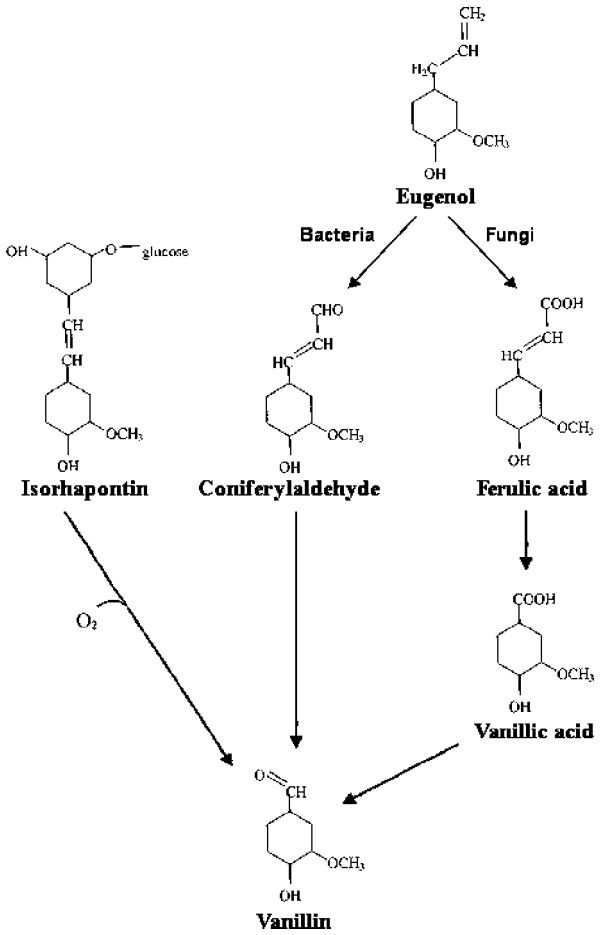

Figure 7. Routes for the bioproduction of vanillin.

highly toxic for the producer cells; *in situ* adsorption on Amberlite XAD-2 resins reduced the toxicity by entrapping the produced vanillin and further increased the yield up to 1.57 g dm^{-3}.[27] Bacteria seem to perform this biosynthesis better than fungi; indeed, it was found that a *Pseudomonas putida* strain was able to convert ferulic acid very efficiently into vanillic acid and that a *Streptomyces setonii* strain transformed ferulic acid into vanillin at levels of up to 6.4 g dm^{-3}.[28]

A second possible route is bioconversion of eugenol via ferulic acid, ferulaldehyde or coniferylaldehyde by *Arthrobacter, Corynebacterium* or *Pseudomonas* strains; eugenol is commercially available as the main constituent of clove oil. Soy lipoxygenase is claimed to be able to convert eugenol and coniferylaldehyde directly into vanillin. However, the yield is still poor, mainly because the metabolic pathway involved has not yet been resolved.[16]

A third route consists of the oxidation of the phenolic natural stilbene, isorhapontin, found in spruce bark; the responsible enzyme is a stilbene dioxygenase, present in certain *Pseudomonas* strains. Other approaches which are being explored are the use of cultured vanilla plant cells or aerial roots as biocatalysts.

4.3.1.2 Fatty acids as substrate for flavour formation. Many valuable flavours and fragrances can be produced by microorganisms from fatty acids added as precursors, including compounds that provide green notes, mushroom flavours, specific lactones and methylketones. Commercial sources of such precursor fatty acids[19] are given in Table 2.

Methylketones (2-alkanones) are derived from medium length fatty acids, and confer strong cheese-associated flavours; they are the basis for flavour development in cheeses such as Roquefort, Camembert and Stilton. Methylketones are formed by the cheese fungi (*Penicillium roquefortii*, etc) via an incomplete β-oxidation route and specific enzymes such as 3-ketoacyl CoA-thioester hydrolase; these methylketones are one carbon shorter than their precursor 6- to 12-carbon fatty acid, such as 2-pentanone, 2-heptanone, 2-nonanone and 2-undecanone (Fig 8). A large-scale process uses the conidiospores of *Penicillium roquefortii* as the enzyme source and lipase-treated milk fats as the fatty acid source.

Starting from α-linolenic acid (C18:3; found in linseed oil), the fungal plant pathogen *Botryodiplodia theobromae* can form jasmonic acid. This can then be esterified, using commercial lipases, to obtain the final

Precursor fatty acids (chain length: unsaturation)	*Typical commercial source*
Saturated fatty acids	
C_4–C_{12}	Milk fat
C_8–C_{12}	Coconut oil fractions
C_{10}–C_{18}	Butter fat
Unsaturated fatty acids	
C_{18}:1 oleic acid	Plant oils, animal fats
C_{18}:2 linoleic acid	Most plant oils
C_{18}:3 α-linolenic acid	Linseed oil
C_{18}:4 arachidonic acid	Animal fats, fungi
C_{20}:5 eicosapentaenoic acid (EPA)	Fish oil, fungi
C_{22}:6 docosahexaenoic acid (DHA)	Fish oil, fungi
Hydroxylated fatty acids	
C_{16}:0 —OH 11-hydroxypalmitic acid	Jalap root (sweet potato)
C_{18}:1 —OH ricinoleic acid	Castor oil (seeds of *Ricinus communis*)

Table 2. Sources of precursor fatty acids

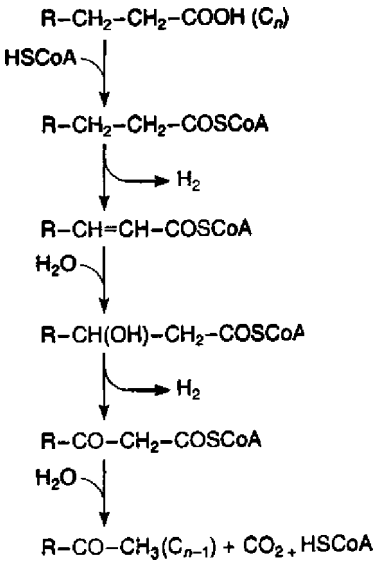

Figure 8. Cheese-associated flavours (methylketones) formed by *Penicillium roquefortii* from medium length fatty acids.

flavour product, methyl (+)-7-isojasmonic acid, which displays a sweet floral, jasmine-like odour. A complex pathway – which occurs normally in plants – involves lipoxygenase (a dioxygenase that acts on *cis-cis*-pentadiene units of PUFAs), allene oxide synthase, and cyclase enzymes, followed by β-oxidation steps and double bond reduction[19] (Fig 9).

Other fungi can oxidise linoleic acid (C18:2) via lipoxygenase action into 1-octene-3-ol, the top note in the typical mushroom aroma; this flavour molecule is now produced from waste mushroom stems by adding linoleic acid, present in many plant oils, as a precursor to the fermentation.

4.3.1.3 Fatty acids and alkanes as substrate for musk fragrances. Apart from 2-phenylethanol (Fig 1), few other fragrances are produced based on microbial processes.[8]

Musk and civet components evaporate very slowly and fix other odours. In this respect, musks are important components of many fragrances; most are of a polycyclic aromatic nature and are produced via petrochemical synthesis. Naturally occurring macrocyclic lactone musks are found in some plants (eg ambrette seed oil, galbanum), while keto-musks are produced by musk deer and civet cats, now very expensive and unethical sources.

Mutants of the yeast *Torulopsis bombicola* have been obtained which are able to convert palmitic acid into ω-hydroxypalmitic acid ester, which can then be cyclised into hexadecanolide lactone musk, albeit with a low yield.[1]

Another process, based on the yeast *Candida tropicalis*, converts C_{10}–C_{18} alkanes, into α, ω-dicarboxylic acids, which can then be polymerised and cyclised into macrocyclic musks: for example, brassylic acid is prepared from tridecane and then converted into ethylene brassylate (Fig 10).

4.3.1.4 Terpenes as substrate for Ambrox®-fragrance. Another bioprocess has been described to produce the fragrance ingredient called Ambrox®, a terpene furan. It is one of the important components of ambergris, an excretion product of sperm whales. It is, just like musk, a fixative for other fragrances. A chemical process starts from the terpene sclareol, extracted from the *Salvia sclarea* plant, which is converted via sclareolide into ambrox.[2] Also, fungal and yeast strains (*Hyphozyma roseoniger, Cryptococcus* sp) have been screened for their ability to use sclareol as sole carbon source and to accumulate sclareolide, which is then chemically converted via its diol into Ambrox® (Fig 11).

4.3.1.5 Hydroxy fatty acids and unsaturated lactones as substrates for flavour-lactones. A well studied and industrial example here is the microbial conversion of ricinoleic acid (12-hydroxy-C_{18}:1, the main constituent of castor or ricinus oil) via partial β-oxidation into the peach-like lactone aroma, γ-decalactone (which is a chiral molecule) by yeasts such as *Sporidiobolus salmonicolor* and *Yarrowia lipolytica* (Fig 12). Yields greater than $10\,g\,dm^{-3}$ have been reported.[29] The *R*-enantiomer occurs in peaches and most other fruits, the *S*-enantiomer in mango-varieties. Chemical synthesis yields the undesirable racemic mixture. The annual potential market for natural γ-decalactone is estimated at 10 tonnes; this corresponds to a fermentation capacity need of about $2.500 m^3$ at current yields.

Similarly, coriolic acid is the precursor hydroxylated

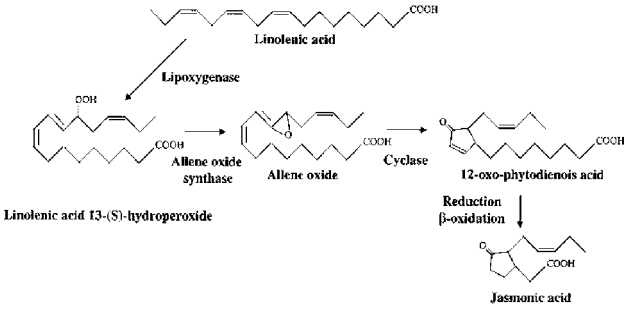

Figure 9. Bioconversion of α-linolenic acid into jasmonic acid by the fungus *Botryodiplodia theobromae*.

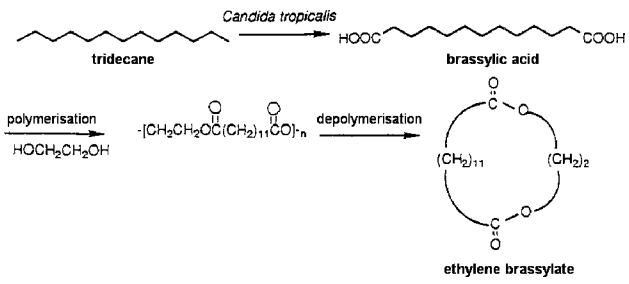

Figure 10. Process for the production of the macrocyclic musk compound, ethylene brassylate.

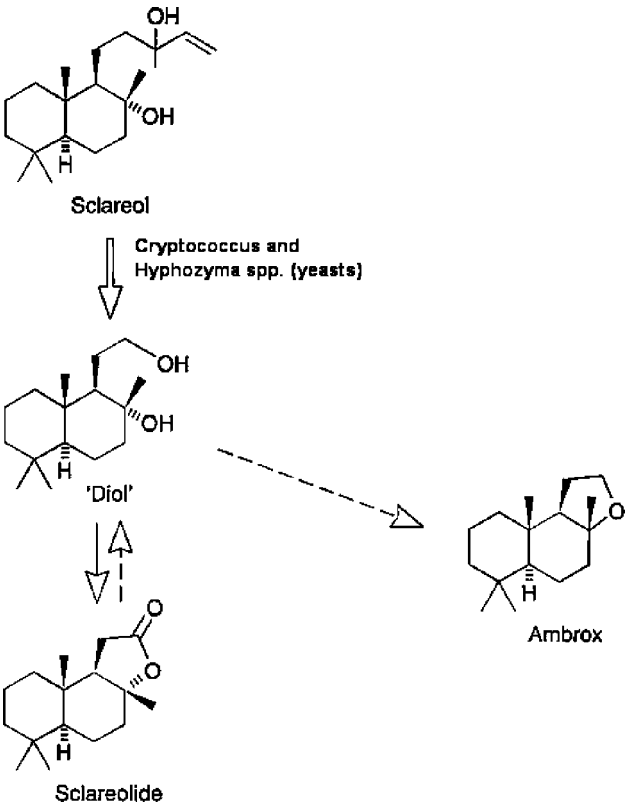

Figure 11. Biotransformation of the plant terpene sclareol into scalareolide and chemical conversion into Ambrox®.

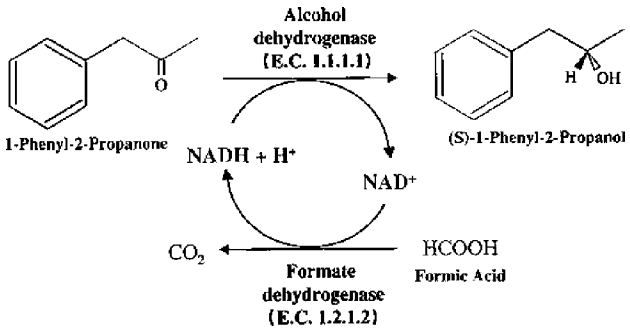

Figure 14. Enzymatic synthesis of (*S*)-l-phenyl-2-propanol with cofactor regeneration via the coupled conversion of formic acid into CO_2.

fatty acid for the production of δ-decalactone with *Cladosporium suaveolens*. This lactone and δ-octalactone can also be obtained via yeast fermentation of natural hydroxylated fatty acids, such as 11-hydroxypalmitic acid, present in Jalap root (sweet potato). The α, β-unsaturated lactone, 2-decen-5-olide, a main component of Massoi bark oil, can also be efficiently converted by baker's yeast into δ-decalactone[1,2] (Fig 13).

Even non-conjugated carbon double bonds in lactone rings can be enantioselectively converted; thus 3-decen-4-olide is efficiently reduced into $R(+)$-γ-decalactone by baker's yeast.

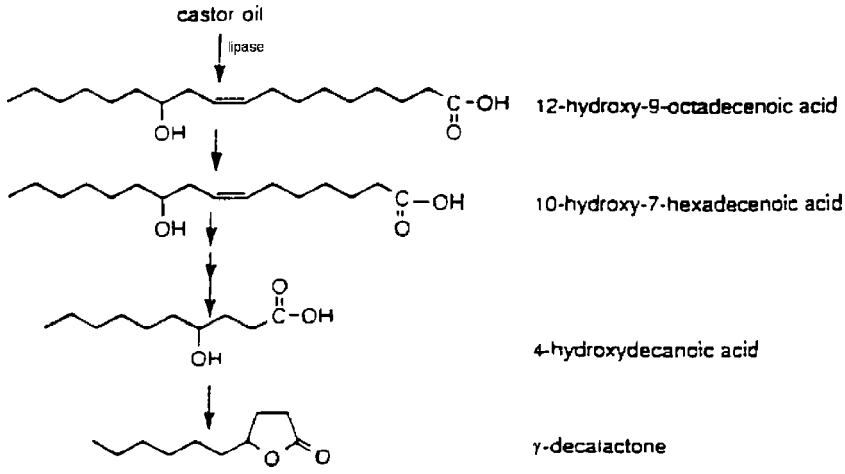

Figure 12. Production of 4-decalactone through bioconversion of castor oil.

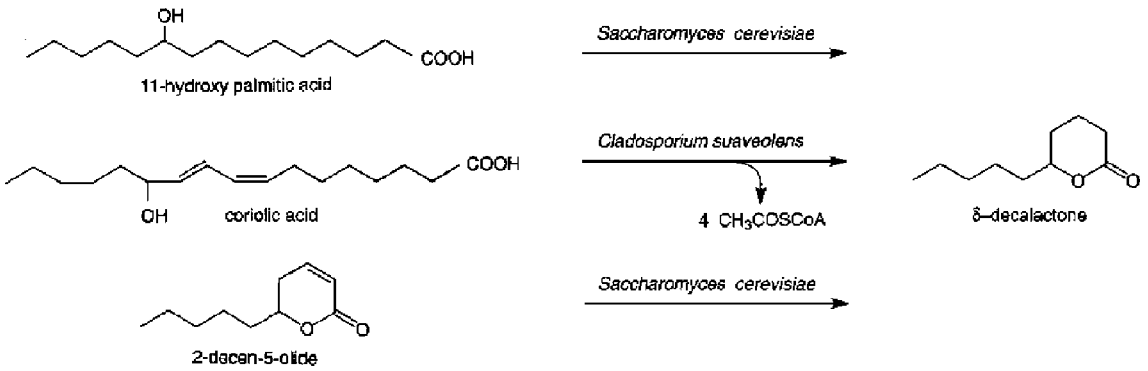

Figure 13. δ-Decalactone formation by fungi and yeasts.

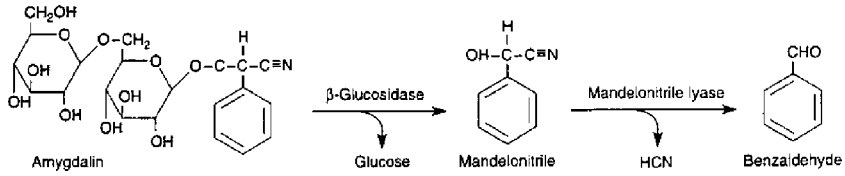

Figure 15. Enzymatic route to benzaldehyde.

4.3.2 Enzymes as biocatalysts

Despite the higher costs involved, microbial enzymes – rather than cells – can offer high stereo- and enantioselectivity towards substrate conversion.[30]

Techniques such as enzyme immobilisation and eventually coenzyme regeneration might result in highly efficient and specific biocatalytic processes for flavour synthesis.[31] A sophisticated example here is the conversion of 1-phenyl-2-propanone with NADH+ H^+-dependent yeast alcohol dehydrogenase into (*S*)-1-phenyl-2-propanol; NADH+H^+ regeneration is obtained via coupling with a reaction, whereby formic acid is converted into gaseous CO_2 by formate dehydrogenase (Fig 14).

The cherry- and almond-tasting benzaldehyde can be produced from the cyanogenic glucoside amygdalin, present in cherry kernels and almond meal, using β-glucosidase and mandelonitrile lyase enzymes (Fig 15).

An industrial example is the production of L-menthol, the major constituent of peppermint oil. Microbial lipases have been found which preferentially hydrolyse L-menthylesters (from the D,L-racemate mixture) into L-menthol, leaving the D-menthylesters intact[30] (Fig 16).

Furaneol®, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) is an aroma which exhibits strawberry flavour in dilute solutions and caramel-like flavour in concentrates (Fig 17). Furanones occur in many fruits (eg pineapple, strawberries, mangoes, raspberries), but also in certain microbial cultures and in soy sauce. They are also formed by the chemical reaction of sugars with amines during the Maillard reaction, and can be chemically prepared, starting from speciality sugars such as L-rhamnose, L-fucose, etc. The deoxy-sugar L-rhamnose can be liberated from the bitter citrusglycosides, naringin and hesperidin, via rhamnosidase, an activity present in commercial *Aspergillus* pectinase preparations. Since pure L-rhamnose is needed for chemical conversion, other liberated sugars (glucose) can be selectively removed by fermentation or bioconversion into gluconic acid.

L-Fucose is a major constituent deoxy-sugar moiety of the capsular exopolysaccharide (EPS) of certain *Clavibacter* and *Klebsiella* strains. High yielding *Clavibacter*-EPS fermentation processes have been developed by the authors; the purified EPS, called clavan, can then be chemically or enzymatically hydrolysed to deliver free L-fucose.[32]

Recently, it has been shown that the soy sauce yeast *Zygosaccharomyces rouxii* is able to form DMHF, especially when the medium is supplied with D-fructose-1,6-biphosphate (FBP) and glucose;[33] the cheap availability of FBP could help here to develop a microbial rather than a chemical process.

Glucose or gluconic acid can be converted by *Gluconobacter suboxydans* into 5-ketogluconic acid, which is a good precursor for the chemical synthesis of the savoury flavour monomethyl furanone.

5 OPPORTUNITIES AND HURDLES

Numerous microbial strains are capable of synthesising potentially valuable flavour compounds; however, yields are quite often disappointingly low, rarely above 100 mg dm^{-3}, making these processes economically unattractive.

Better understanding of the microbial biochemistry and enzymes involved, metabolic regulation and genetic modification are the basic requirements to improve yields, in addition to the application of novel fermentation technology and flavour recovery.

Screening should be intensified for new microbial strains, producing more efficiently – for example – vanillin from eugenol, or capable of forming β-ionon from β-carotene, or green notes from linolenic acid, etc. The 'green notes' in damaged green tissue (eg cut grass) and in aromas of many fruits and vegetables are

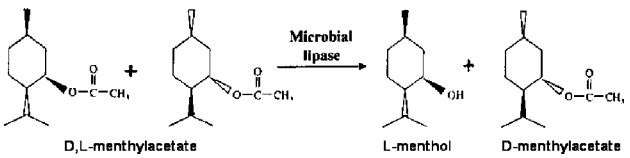

Figure 16. Stereoselective bioconversion of D,L-menthylacetate into L-menthol.

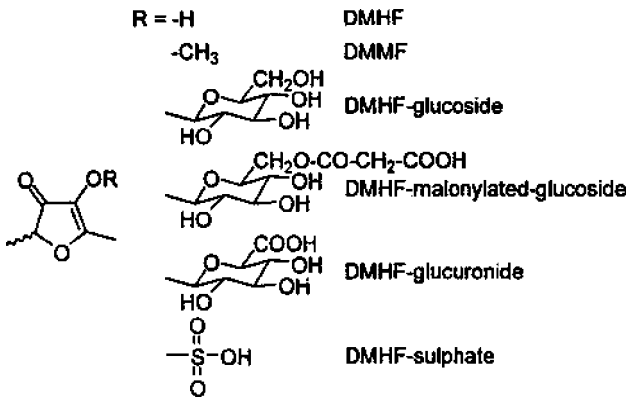

Figure 17. Chemical structures of naturally occurring 2,5-dimethyl-4-hydroxy-3(2H)-furanones (DMHF).

formed via lipoxygenases, which degrade plant PUFAs (linolenic acid) and form hydroperoxides, which in turn are cleaved by hydroperoxide lyases to form the volatile aliphatic 6- to 9-carbon *cis*-3-hexenol (leaf alcohol) and *trans*-2-hexenal (leaf aldehyde).[34] These enzymes have not been detected so far in bacteria or fungi; and green notes are now obtained as byproducts of the essential oil industry. Alternatively, cloning and expression of plant genes, coding for lipoxygenase and lyase in yeast or bacterial cells, could allow formation of the green note flavour by fermentation directly from added linolenic acid.[35,36] Recently, the almond seed lipoxygenase has been cloned and expressed in recombinant *E coli* strains.[37]

Another challenge is the fact that many flavour compounds, or their added precursors (especially fatty acids, ricinoleic acid, fusel oil) are inhibitory or even toxic (at higher levels) to the producer strains, although yeasts can handle them easier.

In this respect, slow continuous feeding of low precursor levels (fed batch fermentation),[28] cell protection via immobilisation[29] or *in situ* flavour extraction (via membranes, solvents, etc)[18,19,20] are fermentation technologies which could help to circumvent these limitations.

The cost of the raw materials, precursors and the formation and elimination of unwanted side products (even when present at traces) also add up to the economic viability of the bioflavour process. A trick, which sometimes can be applied, is to metabolise away the undesirable side product or intermediate from the fermentation or extraction liquid with another microorganism. For example, during the γ-decalactone process from ricinoleic acid, a side product 3-hydroxy-γ-decalactone is formed, which is co-extracted; during subsequent distillation, the side product is converted into 3,4 unsaturated γ-decalactone, and then stereoselectively reduced into the desirable γ-decalactone by *Saccharomyces Cerevisiae* yeast.

Also the detection, identification and characterisation of novel strains and their flavour compounds (mixture or top notes) needs to be further optimised. Due to their low threshold values (ppm to ppb) – though still easily detectable by the human nose – sophisticated sensitive, quantitative and continuous detection systems for such volatile substances (headspace analysis) have to be further developed.

It is clear from the above statements and examples that intensive collaboration between microbiologists, biochemists, organic chemists and bioprocess engineers is a prerequisite to develop interesting laboratory findings into industrial processes for bioflavour production.

REFERENCES

1 Cheetham PSJ, The use of biotransformation for the production of flavours and fragrances. *Trends in Biotechnology* **11**:478–488 (1993).

2 Cheetham PSJ, Combining the technical push and the business pull for natural flavours. *Adv Biochem Eng Biotech* **55**:1–49 (1997).

3 Berger RG (Ed), *Aroma Biotechnology*, Springer-Verlag, Berlin (1995).

4 Gatfield IL, Enzymatic and microbial generation of flavours. *Agro-Food-Industry Hi-Tech* **8**(3):35–38 (1997).

5 Whitaker RJ and Evans DA, Plant biotechnology and the production of flavour compounds. *Food Technology* **41**:86–101 (1987).

6 Janssens L, Microbiële productie van fruitaromas, *PhD thesis*, University of Gent, Belgium (1991).

7 Atika O, Ida T, Obata T and Hara S, Mutants of *Saccharomyces cerevisiae* producing a large quantity of β-phenetyl alcohol and β-phenetyl acetate. *J Fermentation and Bioengineering* **69**:125–128 (1990).

8 Lomascolo A, Lesage-Meesen L, Haon M, Navarro D, Antona C, Faulds C and Marcel A, Evaluation of the potential of *Aspergillus niger* species for the bioconversion of L-phenylalanine into 2-phenylethanol. *World J Microbiol Biotechnol* **17**(1):99–102 (2001).

9 Feron G, Bonnarme P and Durand A, Prospects in the microbial production of food flavours. *Trends in Food Science & Technology* 7:285–293 (1996).

10 Larsen M and Poll L, Odour thresholds of some important aroma compounds in raspberries. *Zeitschrift für Lebensmittel-Untersuch und-Forschung* **191**:129–131 (1990).

11 Huguency P, Dumont B, Ropert F and Belin JM, The raspberry ketone, a biotechnological way for its production, in *Proceedings, Bioflavour 95*, Dijon, France. pp 269–273 (1995).

12 Omelianski VL, Aroma-producing micro-organisms. *J Bacteriology* **8**:393–419 (1923).

13 Verachtert H, General properties of mixed microbial cultures, in *Microbial Comtaminants*, Ed by Korhola M and Backström V, Foundation for Biotechnical and Industrial Fermentation Research, Helsinki, Finland. Vol 7, pp 159–184 (1992).

14 Janssens L, Depooter HL, Schamp NM and Vandamme EJ, Production of flavours by microorganisms. *Process Biochemistry* **27**:195–215 (1992).

15 Hagedorn S and Kaphammer B, Microbial biocatalysis in the generation of flavour and fragrance chemicals. *Ann Rev Microbiol* **48**:773–800 (1994).

16 Van Den Bremt K, Gasarasi G, Delvaux F, Derdelinckx G and Verachtert H, Bioflavouring by refermentation: I. General introduction: natural flavours for food and beverages. *Cerevisia: Belgian J Brewing & Biotech* **24**(4):31–39 (1999).

17 Collins RP, Terpenes and odoriferous materials from microorganisms. *Lloydia* **39**:20–24 (1976).

18 Bluemke W and Schrader J, Integrated bioprocess for enhanced production of natural flavors and fragrances by *Ceratocystis moniliformis*. *Biomolecular Engineering* **17**(4–5):137–142 (2001).

19 Häusler A and Munch T, Microbial production of natural flavours. *ASM-News* **63**(10):551–559 (1997).

20 Palomares MR, Negrete A, Miranda L, Flores C, Galindo E and Serrano-Carreon L, The potential application of aqueous two-phase systems for in situ recovery of 6-pentyl-α-pyrone produced by *Trichoderma harzianum*. *Enzyme Microb Technology* **28**:625–631 (2001).

21 Drawert F and Barton H, Biosynthesis of flavour compounds by micro-organisms. 3. Production of monoterpenes by the yeast *Kluyveromyces lactis*. *J Agric Food Chemistry* **26**:765–766 (1978).

22 Tahara S, Fujiwara K, Ishizaka H, Mizutani J and Obata Y, γ-Decalactone—one of constituents of volatiles in cultured broth of *Sporobolomyces odorus*. *Agricultural and Biological Chemistry* **36**:2585–2587 (1972).

23 Janssens L, Depooter HL, Vandamme EJ and Schamp NM, Production of flavours by the yeast *Hansenula mrakii*. *Med Fac Landbouwwet*, Rijksuniv Gent **32**:1907–1913 (1987).

24 Janssens L, Depooter HL, De Mey L, Vandamme EJ and Schamp NM, Fusel oil as a precursor for the microbial

production of fruity flavours. *Med Fac Landbouwwet, Rijksuniv Gent* **54**(4a):1387–1391 (1989).
25 Demain AL, Jackson M and Trenner NR, Thiamine-dependent accumulation of tetramethylpyrazine accompanying a mutation in the isoleucine–valine pathway. *J Bacteriol* **94**:323–326 (1967).
26 Maga JA, Pyrazines in foods: an update. *Food Science and Nutrition* **16**:1–48 (1982).
27 Stentelaire C, Lesage-Meessen L, Oddou J, Bernard O, Bastin G, Collonna-Ceccaldi B and Asther M, Design of a fungal bioprocess for vanillin production from vanillic acid at scalable level by *Pycnoporus cinnabarinus*. *J Bioscience Bioeng* **89**:223–230 (2000).
28 Muheim A and Lerch K, Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl Microbiol Biotechnol* **51**(4):456–461 (1999).
29 Lee SL, Cheng H-Y, Chen W-C and Chou C-C, Production of γ-decalactone from ricinoleic acid by immobilized cells of *Sporidiobolus salmonicolor*. *Process Biochemistry* **33**(4):453–459 (1998).
30 Schreier P, Enzymes and flavour biotechnology. *Adv biochem eng biotech* **55**:51–72 (1997).
31 Kragl U, Kruse W, Hummel W and Wandrey C, Enzyme engineering aspects of biocatalysis: cofactor regeneration as example. *Biotech Bioeng* **52**:309–319 (1996).
32 Vanhooren P and Vandamme EJ, L-Fucose: occurrence, biological role, chemical and microbial synthesis. *J Chem Technol Biotechnol* **74**:479–497 (1999).
33 Dahlen T, Hauck T, Wein M and Schwab W, 2,5-Dimethyl-4-hydroxy-3(2H)-furanone as a secondary metabolite from D-fructose-1,6-diphosphate metabolism by *Zygosacharomyces rouxii*. *J Bioscience Bioeng* **91**:352–358 (2001).
34 Hatanaka A, The biogeneration of green odour by green leaves. *Phytochem* **34**:1201–1218 (1993).
35 Hughes RK, Wu Z, Hornostaj AR, Robinson DS, Hardy D, West SI, Owusu-Apenten RK, Fairhurst SA, Chan M, Lawson DM and Casey R, Recombinant pea and potato lipoxygenases: versatile biocatalysts for industry. *Agro-Food-Industry Hi-Tech* **10**(3):3–6 (1999).
36 Mita G, Gallo A, Zasiura C, Casey R, Zacheo G and Santino A, Molecular cloning and biochemical characterization of a lipoxygenase in almond (*Prunus dulcis*) seed. *Eur J Biochem* **268**:1500–1507 (2001).
37 Gallo A, Mita G, Zasiura C, Casey R, Santino A and Zacheo G, Recombinant lipoxygenases as biocatalysis for natural flavour production. *Med Fac Landbouw, Gent Universiteit* **66**(3a):267–272 (2001).