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MINI-REVIEW

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Biotechnological production of flavours and fragrances

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Abstract The biotechnological generation of natural aroma compounds is rapidly expanding. Aroma chemicals, such as vanillin, benzaldehyde (bitter almond, cherry) and 4-(*R*)-decanolide (fruity–fatty) are marketed on a scale of several thousand tons per year. Their possible production by single-step biotransformations, bioconversions and de novo synthesis using microorganisms, plant cells or isolated enzymes is shown. The perspectives of bioprocesses for the oxifunctionalisation of lower terpenes by genetically modified organisms and economic aspects are discussed.

Introduction

Food-processing operations, from premature harvesting to extended storage and physical treatments, may cause a loss of aroma (volatile flavour) that calls for subsequent supplementation. In addition, the steadily increasing market for flavours forces suppliers to search for alternative sources. The conventional routes of chemical synthesis or isolation from plants are still viable, but the biotechnological generation of aroma compounds is becoming increasingly attractive. Duplicating plant secondary metabolism in microbial systems (''fermentative processes'') leads to aroma compounds that are classified as natural by the European and US food legislation. This label represents a strong marketing advantage.

History

Since the advent of beer, wine, cheese, soy sauce, and related fermented products, microbial processes have traditionally played an integral role in the development of complex mixtures of food aromas. These very roots of modern biotechnology have evolved from artisan levels into major industries. More than 150 years ago, benzaldehyde was the first flavour compound identified (Liebig and Wöhler 1837). The isolation, identification, and synthesis of vanillin marked the beginnings of the modern flavour industry (Tiemann and Haarmann 1874; Reimer and Tiemann 1876). However, the first review of microbial flavours did not appear until 1923 (Omelianski 1923). Starting in the early 1950s the replacement of classical organic methods of analysis (Birkinshaw and Morgan 1950) by the emerging gas chromatography facilitated the separation and structural elucidation of volatile compounds. Since then, many reviews addressing the production of flavour and fragrance chemicals by microorganisms have been published (Armstrong et al. 1993; Berger 1996, 1995a; Bigelis 1992; Étiévant and Schreier 1995; Feron et al. 1996; Gabelman 1994; Gatfield 1996, 1995b; Hagedorn and Kaphammer 1994; Janssens et al. 1992; Krings et al. 1995; Maarse and van der Heij 1994; Takeoka et al. 1995; Tyrrell 1995; Winterhalter and Schreier 1993). Earlier research concentrated on screening microorganisms and the aroma compounds generated. Contemporary microbiological techniques, including genetic engineering, are now increasingly applied to enhance the efficiency of the biocatalyst.

The size of the flavour and fragrance industry worldwide is considerable, estimated at US $9.7 billion in 1994 (Somogyi 1996). Whereas about 6400 natural volatiles and about 10 000 synthetic fragrance compounds are known, only a few hundred are regularly used in flavours and fragrances, and only around 400 aroma chemicals are manufactured on a scale greater than 1 ton per annum. Thousands of tons per year of non-volatile flavours, such as sweeteners, acidulants, and savoury compounds, are produced by means of biotechnology, while bioprocesses for volatile flavours have emerged only recently (Hagedorn and Kaphammer 1994). Technical-scale processes are operating for some aliphatic alkenols and carbonyls, carboxylic and benzoic esters

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including lactones, vanillin, and certain specialities (Cheetham 1996).

Why novel biotechnology of aromas?

The growing market share of flavoured and fragranced products (convenience food, beverages, cosmetics, detergents) requires novel strategies for aroma chemicals. Nearly 80% of the flavours and fragrances used worldwide are produced chemically. However, about 70% of all food flavours used in Germany in 1990 were natural (Abraham et al. 1994b). This trend is attributed to increasing health- and nutrition-conscious lifestyles (Armstrong and Yamazaki 1986). Thus, the label ''natural'' is important for the profitability of microbiologically produced flavours. The difference in price of a natural compound and its chemically synthesised counterpart can be considerable, for example U.S. \$12 kg^{-1} for synthetic vanillin and about \$4000 kg^{-1} for vanillin extracted from vanilla pods (Feron et al. 1996).

The biotechnological approach implies additional advantages. Flavours are bioactive compounds, and the known effects of chirality on odour perception suggest the use of biocatalysts (Fig. 1). Further advantages associated with the biotechnological principle are:

Independence from agriculture and possible shortages caused by local conditions of production (climate, diseases, pesticides, fertilisers, trade restrictions, socio-political instabilities)
Ability for scaled-up and industrial-scale production using engineered pathways, up-regulated metabolisms, and gentle product recovery to create an inexhaustible source of homogenous, well-defined product
Responsible care of natural resources in developing countries.

Metabolic pathways to target aroma compounds

Essential oils of higher plants, fruit juices, vegetable extracts, and a very few products of animal origin (amber, musk, zibet) were, for a long time, the sole sources of natural flavours. Biotechnological options comprise single-step biotransformations, bioconversions and de novo synthesis with microorganisms, plant cells and enzymes. Whole cells should be used for complex targets or product mixtures, whereas isolated enzymes are able to carry out single-step processes. Among microorganisms, the genuine volatile spectrum of fungi, especially of basidiomycetes, is closest to the fascinating diversity of plant volatiles. Meanwhile, many of the fungal volatiles have been identified and are structurally identical to the character-impact components of higher plant flavours (Table 1).

De novo synthesis

Whole cells catabolise carbohydrates, fats and proteins, and further convert the breakdown products to more complex flavour molecules, a property that is traditionally used during the production of fermented foods with their amazing number of aroma chemicals (Engels and Visser 1994; Imhof and Bosset 1994; Jeon 1994; Hamada et al. 1991; Maarse 1991; Pinches 1994). Common starter cultures produce primary metabolites in considerable amounts, but only traces of more complex aroma chemicals. For example, very efficient lactic acid producers contribute to dairy flavours. Small amounts of chemically quite different volatile flavours, such as short-chain alcohols, aldehydes, ketones, methyl ketones and acids as well as pyrazines, lactones and thiols are formed concurrently (Cogan 1995; Imhof and Bosset 1994). Rapid and continuous lactic acid formation should now be taken for granted, and more attention should be paid to starter cultures with enhanced flavour potential. However, an immediate improvement is often prevented by a lack of metabolic knowledge.

Biotransformation/bioconversion

Inexpensive, readily available and renewable natural precursors, such as fatty or amino acids, can be converted to more highly valued flavours. Biocatalysis

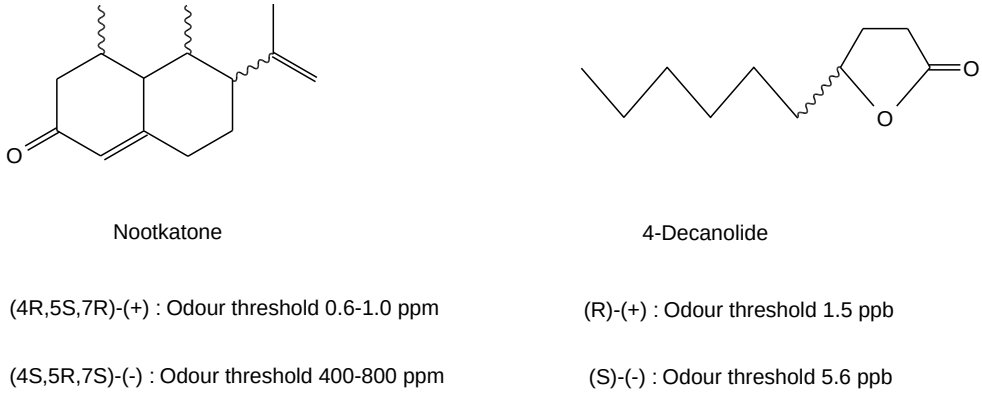

Fig. 1 Odour threshold differences of enantiomers (Bernreuther et al. 1997)

Table 1 Aroma compounds with impact character generated by microorganisms

Character impact compound	Species (selection)	References
Fungi		
Vanillin	*Pycnoporous cinnabarinus*	Falconnier et al. 1994
Benzaldehyde	*Ischnoderma benzoinum*	Fabre et al. 1996
4-Methoxybenzaldehyde	*Ischnoderma benzoinum*	Fabre et al. 1996
Methyl anthranilate	*Pycnoporous cinnabarinus*	Falconnier et al. 1994
	Trametes sp.	Page et al. 1989
4-(4-Hydroxyphenyl)-2-butanone	*Nidula niveo-tomentosa*	Ayer and Singer 1980
Methyl salicylate	*Phellinus* sp.	Welsh 1994; Manley 1994
Methyl benzoate, ethyl benzoate	*Polyporus tuberaster*, *Phellinus* sp.	Kawabe and Morita 1993
2-Phenylethanol	*Ascoidea hylecoeti*	Berger 1995a
Oakmoss volatiles	*Polyporus* sp.	Abraham et al. 1994a
Lenthionin	*Lentinus edodes*	Yasumoto et al. 1974
1-Octen-3-ol, 1-octen-3-one	*Lentinus edodes*, *Grifola frondosa*, *Pleurotus pulmonarius*	Amstrong and Brown 1994; Assaf et al. 1995
Citronellol	*Mycena pura*	Krings et al. 1995
Linalool	*Wolfiporia cocos*	Krings et al. 1995
Coumarins	*Pleurotus euosmus*	Berger 1995a
Methyl ketones	*Aspergillus niger*, *Penicillium* sp., *Aureobasidium pullulans*	Hagedorn and Kaphammer 1994; Armstrong and Brown 1994
Pyrazines	*Aspergillus* sp.	Seitz 1994
Lactones	See Fig. 3	
Long-chain fatty acids esters	*Rhizopus arrhizus*	Armstrong and Brown 1994
Jasmonates	*Botryodipoldia theobromae*, *Gibberella fujikurio*	Miersch et al. 1993; Broadbent et al. 1968
Sulphur-containing volatiles	*Marasmius alliaceus*	Rapior et al. 1997
Yeast		
Furaneol	*Zygosaccharomyces rouxii*	Hecquet et al. 1996
Lactones	See Fig. 3	
Macrolytic lactones	*Torulopsis bombicola*	Jeffcoat and Willis 1988
Phenylethanol and esters	*Kluyveromyces* sp.	Welsh 1994
Citronellol, geraniol, linalool	*Kluyveromyces lactis*	Welsh 1994
Bacteria		
Diacetyl	*Lactobacillus lactis*	Cheetham 1996
Short-chain fatty acids	*Acetobacter aceti*, *Gluconobacter oxydans*, *Propionibacterium* sp., *Clostridium* sp., *Fusarium* sp.	Sharpell and Stemann 1979
Methyl ketones	*Pseudomonas oleovorans*	Armstrong and Brown 1994
Geosmin	*Streptomyces citreus*	Pollak and Berger 1996
Pyrazines	*Bacillus* sp., *Penicillium* sp., *Pseudomonas* sp.	Manley 1994
2-Acetyl-1-pyrroline	*Bacillus cereus*	Romanczyk et al. 1995
Nootkatone	Soil bacteria	Latrasse et al. 1985
	Enterobacteriaceae	Janssens et al. 1992
Borneol, isoborneol	*Pseudomonas pseudomallei*	Janssens et al. 1992
β-Ionone	Xanthine oxidase	Bosser and Belin 1994

competes best with chemical catalysis in the following types of reactions:

Introduction of chirality
Functionalisation of chemically inert carbons
Selective modifications of one functional group in multifunctional molecules
Resolution of racemates.

Monoterpenes

Monoterpenes, widely distributed in nature (more than 400 structures), constitute suitable precursor substrates. Soil bacteria and filamentous fungi transform acyclic, monocyclic, and bicyclic monoterpenoids. Reviews of isoprenoid biosynthesis, de novo generation and opportunities for microbial biotransformation were published recently (Breheret et al. 1997; McCaskill and Croteau 1996; Seitz 1994; Van der Werf et al. 1996). Most of the monoterpene biotransformation studies described so far have been of more academic than practical value, and no monoterpene biotransformation process has been commercialised yet. Major problems encountered are:

Chemically instability of both precursor (monoterpene) and product (terpenoid)
Low water solubility of the monoterpene precursors
High volatility of both precursor and product
High cytotoxicity of both precursor and product
Low transformation rate.

Higher terpenes/terpenoids

The cytotoxicity of the terpene precursor was a minor problem in some higher terpenoid biotransformations, and transformation rates and product yields increased accordingly. For example, patchouli alcohol was regioselectively hydroxylated by a soil isolate to 10-hydroxypatchoulol with a product yield of less than 1.2 g l^{-1} in a 5-l fermentation. 10-Hydroxypatchoulol was then converted chemically to norpatchoulenol, the impact component of patchouli essential oil (Suhara et al. 1981). The bioconversion of β-ionone by several fungi yielded tobacco flavourings, and sclareolide and ambrox were generated with *Cryptococcus* for perfumery applications using sclareol as the precursor (Cheetham 1993; Farbood et al. 1990a). The latter examples demonstrate the usefulness of biotransformations in the field of perfumery and tobacco flavourings (Cheetham 1996; Berger 1995a; Seitz 1994; Kieslich et al. 1985).

Vanillin

Vanillin, the most universally appreciated aroma chemical, occurs in the bean of *Vanilla planifolia* at a level of about 2% by weight. At present, only 0.2% of the world flavour market (20 t $year^{-1}$ out of 12 000 t $year^{-1}$ worldwide) are extracted from the botanical source, whereas the remainder is of synthetic origin (Berger 1995a). Limited supply and the high price of the phytochemical stimulated research for a biotechnological substitution (Audras and More 1996; Cooper 1987; Gross et al. 1991; Sahai and Knuth 1985; Labuda et al. 1994, 1992; Lesage-Meessen et al. 1996; Rabenhorst 1991). Neither de novo routes in plant cell cultures of *Vanilla* nor those in bacteria or fungi afford anything like acceptable yields. The precursor approach holds more promise. Several starting materials appear to be suitable including lignin, eugenol, ferulic acid, curcumin and benzoe siam resin (Benz and Muheim 1996). Turnover rates of less than 30% and production levels below 1 g l^{-1} have been reported. Again the toxicity of both the precursor and the product, as well as product degradation in the course of fermentation, prevented a better yield.

Benzaldehyde

In quantity, benzaldehyde is the second most important flavour molecule after vanillin. Natural benzaldehyde is usually liberated from amygdalin, a cyanogenic glycoside present in fruit kernels, and is used as a key ingredient in cherry and other natural fruit flavours. The concurrent generation of equimolar amounts of hydrocyanic acid causes major safety problems. The microbial degradation of natural phenylalanine offers an alternative. This process is aided by a plentiful cheap supply of natural L-phenylalanine, which has become available as an intermediate of the synthesis of the high-intensity sweetener, aspartame (Cheetham 1996).

Research on the microbial metabolism of L-phenylalanine to avoid side-reactions would increase the bioconversion efficiency. The metabolic pathways of submerged cultured *Ischnoderma benzoinum*, a basidiomycete, were elucidate using ring-labelled deutero-L-phenylalanine (Krings et al. 1996). In this study phenylalanine was almost completely converted to the flavour compounds benzaldehyde and 3-phenylpropanol (flowery, rose-like) following two different degradation pathways (Fig. 2). The oxidative degradation pathway to benzaldehyde was also found in bacteria and subsequently patented (Geusz and Anderson 1991).

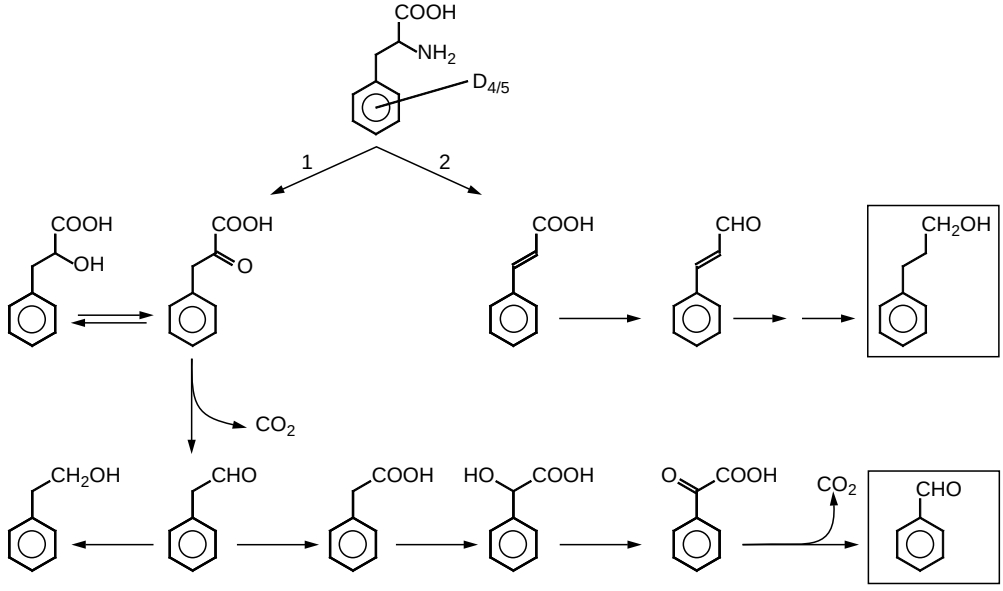

Fig. 2 Degradation pathway of L-phenylalanine by *I. benzoinum* derived from the identification of labelled degradation products (modified from Krings et al. 1996)

Pathways to 4-decanolide

Lactones are ubiquitous volatile flavours. The importance of aliphatic 4- and 5-alkanolides as food flavours is based on their characteristic sensory properties (Gatfield 1996). 4-Decanolide is an impact component in a number of fruits, such as strawberries, peaches and apricots, and also in milk products and some fermented foods. It is produced in plants in minute amounts and therefore one of the prime research targets (Fig. 3). After a bioprocess was established in the early 1980s, the price for natural 4-decanolide decreased from U.S. \$20 000 kg^{-1} to \$1200 kg^{-1} (Feron et al. 1996). Generally, microbial lactones are produced by the β-oxidation of hydroxy fatty acids. The position and stereochemical orientation of the hydroxy group in the natural precursor determine which particular lactone will be produced. This type of process can typically result in product concentrations of above 5 g l^{-1} (Cheetham 1993; Meyer 1993; Nozaki and Yamaguchi 1994).

Isolated enzymes

Upward of 3000 enzymes have been described in the literature, but there are probably a few hundred only that are commercially available, and only 20 are available in amounts suitable for use in commercial processes (Armstrong and Brown 1994). Lipases, esterases, proteases, nucleases and various glycosidases aid flavour-extraction processes, and directly hydrolyse flavour molecules from larger progenitors. A good example of reversed lipolysis is the esterification reaction in non-aqueous systems using lipases (Gatfield 1992; Gillies et al. 1987; Langrand et al. 1990). These cofactor-independent enzymes have shown potential for use in stereo- and regiospecific hydrolyses and transesterifications to yield optically pure aliphatic and aromatic esters and lactones (Armstrong and Brown 1994; Gatfield 1996). Lipoxygenases are essential components of the oxylipin pathway, converting unsaturated fatty acids, among others, into flavours, such as (*Z*)-3-hexenol and (*E*)-2-hexenal (Hatanaka 1993; Hsieh 1994; Nishiba et al. 1995; Whitehead et al. 1995). Soy lipoxygenase oxidises unsaturated fatty acids to the corresponding hydroperoxides, which can then be reduced to hydroxy fatty acids; subsequent microbial chain-shortening converts the latter into lactones (Cardillo et al. 1991).

Perspectives of biotechnological processes for aroma compounds

A current research project of the European Community (BIO4-CT950049) applies genetic engineering to transform monoterpene hydrocarbons to oxifunctionalised products with stronger odour/bioactivity. A *Pseudomonas putida* wild strain was chosen as a host for the introduction of genes encoding terpene-converting enzymes (Van der Werf et al. 1996).

Another envisaged use of genetic modification is the removal of diacetyl (buttery off-flavour) from beer. Supplementing yeast with the gene encoding α-acetolactate decarboxylase would eliminate the formation of the immediate precursor of diacetyl, and the time-consuming post-fermentation (''lagering'') would no longer be required (Gabelman 1994). A similar ''single-gene'' approach aims to supplement wine yeast with the malolactic enzyme. This would decrease the acidity of the wine and could contribute to an improvement in the

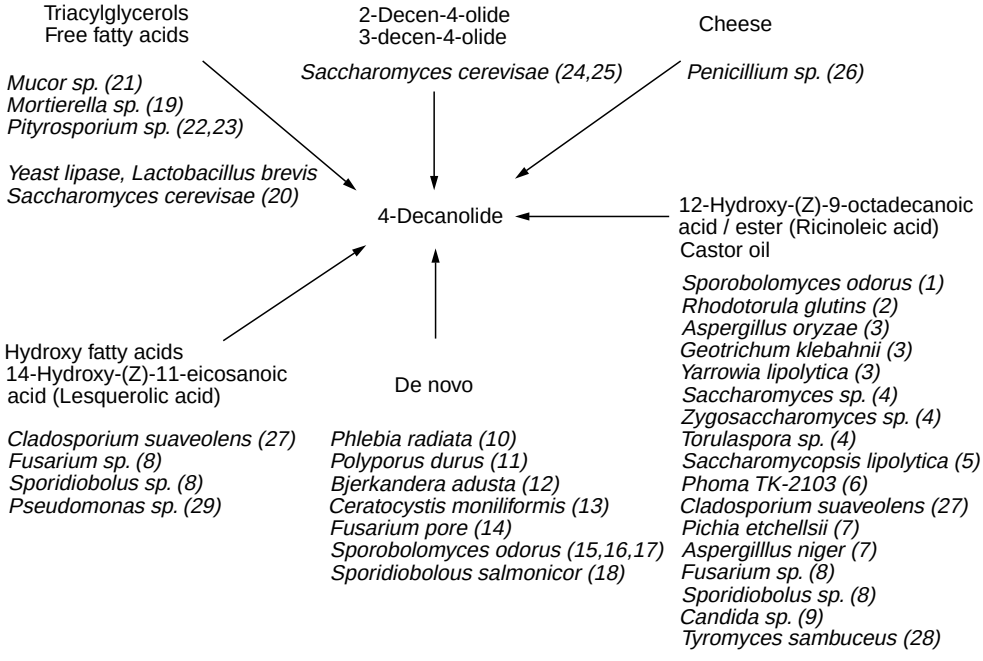

Fig. 3 Metabolic pathways to 4-decanolide. References: *1* Lee and Chou 1994, *2* Cheetham et al. 1988, *3* Farbood and Willis 1985, *4* Boog et al. 1990, *5* Farbood and Willis 1983, *6* Nozaki and Yamaguchi 1994, *7* Cardillo et al. 1990, *8* Spinnler et al. 1994, *9* Farbood et al. 1990b, *10* Gross et al. 1989, *11* Berger et al. 1986, *12* Kapfer et al. 1989, *13* Lanza et al. 1976, *14* Sarris and Latrasse 1985, *15* Manlay 1994, *16* Haffner and Tressl 1996, *17* Tahara et al. 1973, *18* Gervais and Battut 1989, *19* Han and Han 1995, *20* Hosoi and Ookawa 1995, *21* Page and Eilerman 1989, *22* Labows et al. 1983, *23* Labows et al. 1985, *24* Gatfield 1996, *25* Gatfield 1995a, *26* Cheetham 1996, *27* Cardillo et al. 1991, *28* Freeman 1995, *29* Feron et al. 1996

composition of the volatile fraction (Berger 1995b). The transfer of a highly stereoselective lipase from a non-characterised microorganism through gene transfer to a suitable food-grade host organism is another application (Riisgaard 1990). There are technical limitations if entire metabolic pathways for the production of aroma compounds are to be transferred (Cheetham 1996).

As for every bioprocess, the screening of suitable microorganisms, the adjustment of a full set of chemical and physical parameters, the design of the reactor, and a reliable on-line monitoring must be considered, and there are additional specific points if the target molecule is a volatile flavour:

Precursor screening: time and mode of feeding.
In situ product recovery: protection of product, shift of metabolic equilibria, prevention of feedback inhibition or even cytotoxic product concentrations.
Continuous fermentation: product generation may coincide with growth; many volatile metabolites are not secondary in terms of a preferred accumulation in the stationary phase.

Cost considerations

Although many microbial processes have been described to yield attractive flavours, the number of industrial applications is limited. Even if patent protection has been obtained and a product appears on the market, it may be impossible to assess whether "bio-flavour" or a conventional distillate or extract is offered. Feeding of *Ischnoderma benzoinum* with L-phenylalanine, for example, yielded concentrations of benzaldehyde above 300 mg l^{-1}. In situ recovery increased the yield to 1 g benzaldehyde l^{-1} (Krings 1994). Techniques used were:

1. Stripping of the benzaldehyde from the bioprocess medium by an inert gas (Berger et al. 1990)
2. Adsorptive recovery from the medium using macroporous resins (Krings 1994)
3. Removal of benzaldehyde via pervaporation (Souchon et al. 1995)

While more "natural" benzaldehyde is sold than is distilled from plants, it is unclear whether the *Ischnoderma* process operates on an industrial scale. Retroaldol cleavage of abundant cinnamaldehyde opens a "grey-zone chemistry" route to so-called natural benzaldehyde. Methods of chiral analysis and isotope-distribution analysis of flavours have reached a high standard and are now applied by specialised laboratories to prove authenticity (Mosandl 1995).

The scientifically unfounded opinion of the average consumer that natural chemicals are somehow healthier than synthetics is reflected by food laws that discriminate between natural and synthetic (nature-identical) flavours. Natural flavour chemicals often command a premium price, but this appears to be over-compensated by the marketing benefits (Gatfield 1996).

The price of a microbial flavour should range between U.S. \$200 and \$2000 kg^{-1} to be competitive (Janssens et al. 1992). The major factors in production costs are, in order of decreasing importance, raw materials, man power and energy (Delest 1995). The forces that will have a growing impact on the biotechnological production of aroma chemicals not only include technical aspects, but also market developments, regulatory considerations, economics and, more recently, an increasing care for the environment in chemical processing (Armstrong and Brown 1994). A break-even analysis has been developed for the microbial production of a hypothetical flavour or fragrance with potential sales of either 1000 kg, 10 000 kg or 100 000 kg/year. A production level of 1 g l^{-1} was assumed and annual depreciation was estimated using a 5-year amortisation period and no discount rate. If this is taken into account, the break-even price for a hypothetical flavour decreases from \$1240 (1000 kg $year^{-1}$) to over \$300 (10 000 kg $year^{-1}$) to \$202 kg^{-1}, if 100 000 kg $year^{-1}$ were sold (Welsh 1994).

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