

Biotechnological routes in flavour industries

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Abstract

During the past years biocatalytic production of fine chemicals has been expanding rapidly. Flavours and fragrances belong to many different structural classes and therefore represent a challenging target for academic and industrial research. Here, we present a condensed overview of the potential offered by biocatalysis for the synthesis of natural and natural-identical odorants, highlighting relevant biotransformations using microorganisms and isolated enzymes. The industrial processes based on biocatalytic methods are discussed in terms of their advantages over classical chemical synthesis and extraction from natural sources. Recent applications of the biocatalytic approach to the preparation of the most important fine odorants are comprehensively covered. Flavours and fragrances are extremely important for the food, feed, cosmetic, chemical and pharmaceutical industries. Most available flavour compounds are now produced via chemical synthesis or extraction. Drawbacks of such chemical processes are the formation of undesirable racemic mixtures and the growing aversion of the consumer towards chemicals added to his food, cosmetics and other household products. This has caused flavour companies to direct their attention towards flavour compounds of biological origin, so called natural or bio-flavours. Upto now, plants were also an important source of essential oils and flavours: however, active components are often present in minor quantities or in bound form or are only found in exotic plants, making isolation difficult and the flavour products expensive. Apart from plant cell and tissue culture techniques a directly viable alternative route for flavour synthesis is based on microbial processes, i.e. fermentation (= *denovo*) and bioconversion of appropriate precursor compounds. This review presents the current state of the art of bioflavour-synthesis, based on microorganisms (bacteria, fungi, yeasts) and their enzymes, with emphasis on currently commercialised processes. It also comments on regulatory aspects of biotechnological production of aroma-compounds. A comprehensive referenced literature survey of *de novo* fermentation and of bioconversion processes for flavour-compound synthesis concludes this review.

Key words: Hydrolytic Enzymes, Oxireductases, Transferases, Lyases, Characteristics of Microbial Flavour Production, Driving forces, Regulatory aspects and legal status, Industrial applications

Introduction

Flavours and fragrances are extremely important for the food, feed, cosmetic and pharmaceutical industries. Worldwide they

currently represent a value of almost 7 billion US\$ a year, a figure which increases each year. Financially this means 25 % of the total food additives market^{1,2}. The preparation of flavours and fragrances by isolating them

from natural resources began in ancient times. Concurrently, the production of fermented foods (beer, wine and others) allowed the generation of new aromas and formed the roots of modern biotechnology. For many centuries these were the only methods for obtaining this type of compound, albeit in complex mixtures. Although the majority of aromas products were prepared by chemical synthesis or by extraction from plants, the employment of new biotechnological processes has increased considerably in the past decades^{3,4,5,6 and 7}. Chiral flavours often occur in nature as single enantiomers. Because different enantiomers or regioisomers could show different sensorial properties, their specific synthesis is beneficial⁸. Biocatalysis represents a useful tool in this field catalysing a large number of stereo- and regioselective chemical manipulations that are not easily achieved by the less selective classical synthetic procedures. There are about 25,000 enzymes present in nature and about 400 have been commercialised mainly for stereoselective organic synthesis and also for the biotechnological production of flavour compounds. The worldwide market for enzymes is more than US \$1 billion. Furthermore, the increasing sensitivity of the ecological systems supports the choice of environmentally friendly processes and consumers have developed a preference for 'natural' or 'organic' products, thus developing a market for flavours of biotechnological origin⁹. Moreover, the increasing demand for natural flavours has led to a significant shortage of several plant resources such as peppermint and some fruit flavours such as

strawberry aroma. Many plant oils and pure isolated aroma compounds are currently only available at prices of more than 5000 \$/kg. For instance synthetic 4-decalactone, the impact flavour compound of peach, costs 150 \$/kg, while the same substance is worth 6000 \$/kg if it is extracted from a natural source. Another disadvantage of plants as a source of flavours is the strong dependence on factors which are difficult to control such as the influence of the weather and the risk of several plant diseases. Imported plant material from tropical or subtropical regions can also be the object of trade restrictions, due to socio-political instability of the region where those plants grow.

The majority of enzymes in food biotechnology comprise hydrolytic enzymes, transferases, oxidoreductases and lyases. Microbial enzymes play the greatest role in production of flavour compounds; they can also be expressed in recombinant microorganisms¹⁰.

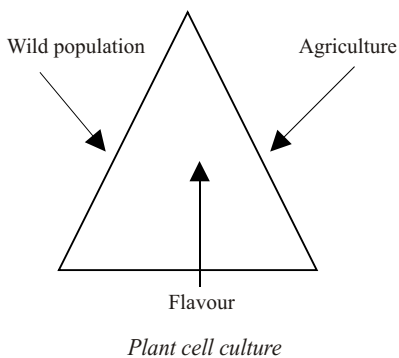


Fig.1 Three possible sources of natural flavors

In the supply of natural flavours or flavour precursors there are three options: collection from the wild plant population, agricultural cultivation, and plant tissue culture. Collection from the wild is perhaps the easiest and has been used with many flavour producing plants but overcollection has endangered the stocks in many cases. The supply can be supplemented by agricultural cultivation but in some cases the wild populations require specific growth conditions which cannot be reproduced elsewhere. Propagation may also be difficult, so agricultural cultivation may not be possible. If agriculture is not a viable option, other methods have to be found to preserve

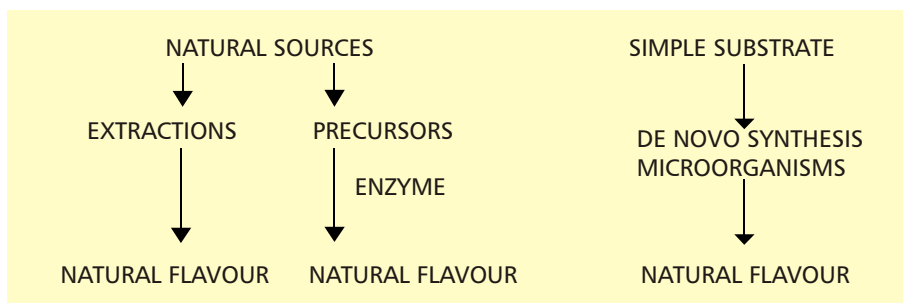


Fig.2 The three pathways for the preparation of 'natural' flavours. The first two involve the extraction of the flavour or precursors from natural sources. The last method is the de novo synthesis of the flavour by microorganisms growing on simple substrates such as glucose and sucrose.

and maintain the wild population while providing the material for flavour extraction. In this case plant tissue techniques may be suitable for the multiplication of the plant and/or its conservation. The agricultural cultivation of the plant or related species is clearly the most economic solution to the flavour supply. However, the crop may suffer from pests and diseases, and adverse climatic conditions which can affect yield and quality. In some cases political factors can also affect supplies from some countries and regions. The plant may also be difficult to propagate and may require exacting condition for growth as found with vanilla pods. Under these conditions the techniques of plant cell culture may help to alleviate the pressure on the supply of natural flavours in a sustainable manner by helping with the propagation of the particular plant or the de novo production of the flavour itself.

Hydrolytic Enzymes

Lipases

Lipases are serine hydrolases that catalyse the hydrolysis of lipids to fatty acids and glycerol¹¹. In contrast to esterases, they work at the lipid-water interface and show only little activity in aqueous solutions. Lipases play an important role in organic synthesis and also in flavour biotechnology. Pig pancreatic extract and especially many microbial lipases are used for ester hydrolysis, esterification (alcohol and acid), transesterification (ester and alcohol), interesterification (ester and acid) and transfer of acyl groups from esters to other nucleophiles like amines or thiols¹⁰.

Some criteria of selectivity are important for these catalysed reactions: substrate selectivity¹², regioselectivity¹³, stereoselectivity (endo/exo¹⁴ and Z/E¹⁵ differentiation), enantioselectivity¹⁶, meso differentiation¹⁷ and prochiral recognition¹⁸.

Lipolysis

Lipolysed milk fat was one of the first flavours produced with the help of enzymes. The original process was based on the controlled lipase-catalysed hydrolysis of cream¹⁹. For instance, *Mucor miehei* lipase possesses a high selectivity towards flavour-active short-chain fatty acids. The free fatty acids produced can be isolated by steam distillation and further purified. Thus, it is possible to obtain pure short-chain fatty acids like butanoic, hexanoic, octanoic and decanoic acid. Lipolysed milk fat products can serve as cream-like/butter-like flavouring agents²⁰.

Kinetic Resolution of Racemates

Stereoselectivity of lipases is often used to yield pure optically active flavour compounds from racemic precursors. This fact is important if one isomer of a molecule has more desirable properties than the other one.

For instance, (-)-menthol is one of the most important flavouring agents and is the major compound in natural peppermint oil. The characteristic peppermint odour and the typical cooling effect is limited to (-)-menthol. The other isomers do not show this refreshing effect. There are several biochemical and chemical processes for the

resolution of a racemic mixture of menthol. Many microbiological lipases hydrolyse menthyl esters and prefer the (-)-menthyl esters, whereas (+)-menthyl esters are not hydrolysed at all. This asymmetric hydrolysis of menthyl esters can be performed with lipases from *Penicillium*, *Rhizopus*, *Trichoderma* and various bacteria²¹.

The enantioselective hydrolysis of racemic menthyl benzoate (industrially key compound) by recombinant *Candida rugosa* lipase LIP1 leads to optically pure 1(-)-menthol; ~>99%²².

The resolution of the commercially available racemic trans-jasmonate to (-)-trans-jasmonate by microbial lipase has been described by Serra *et al.*²³.

Nozaki *et al.*²⁴ characterised the production of (+)-mesifuran [2,5-dimethyl-4-methoxy-3(2H)-furanone], an important flavour compound in arctic bramble, but which also occurs in strawberry and pineapple. After lipase-catalysed (*Candida antarctica*) enantioface-differentiating hydrolysis of the enol acetate, the pure optically active (+)-mesifuran could be obtained.

Kinetic resolution of branched-chain fatty acids has been reported recently by Franssen *et al.*²⁵. With the help of immobilised *Candida antarctica* lipase B, racemic 4-methyloctanoic acid (responsible for sheep-like and goat-like flavours in sheep and goat milk and cheese, respectively) was esterified with ethanol. Only the R ester could be obtained, whereas (S)-4-methyloctanoic acid was not converted fig.3.

Catalysis in Organic Media

Lipase-catalysed esterification and transesterification reactions have a wide range of applications in the synthesis of aroma compounds. The reaction conditions have a great influence on the enzyme-catalysed reactions in organic media and determine the reaction's yield and selectivity. Enzymes require only a monomolecular water phase for their activity in organic solvents²⁶; the pH of the water phase²⁷, temperature²⁸, type of solvent²⁹ and immobilisation techniques³⁰ will influence the reaction too.

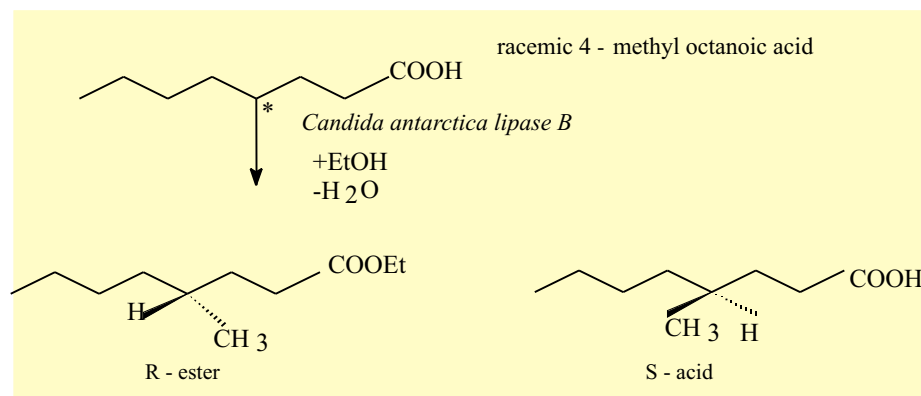


Fig.3. Kinetic resolution of racemic 4-methyl octanoic acid with *Candida antarctica* lipase B

The biotechnological production of flavour compounds is particularly focused on esters and lactones. Lipase from *Mucor miehei* is the most widely studied fungal lipase³¹⁻³⁶. Esters of acids from acetic acid to hexanoic acid and alcohols from methanol to hexanol, geraniol and citronellol have been synthesized using lipases from *Mucor miehei*, *Aspergillus sp.*, *Candida rugosa*, *Rhizopus arrhizus* and *Trichosporum fermentans*³³⁻³⁸.

Methyl butanoates and methyl butyl esters are essential flavour compounds in fruit flavours; they can be produced biotechnologically as mentioned before. Chowdary *et al.*³³ have described the production of a fruit-like flavour: isoamyl isovalerate by direct esterification of isoamyl alcohol and isovaleric acid in hexane with the help of *Mucor miehei* lipase immobilised on a weak anion exchange resin.

Synthesis of short-chain geranyl esters catalysed by esterase from *Fusarium oxysporum* in an organic solvent was reported by Stamatis *et al.*⁴⁰.

Large-scale synthesis of (Z)-3-hexenyl acetate in hexane with lipase, (Z)-3-hexenyl and acetic acid was described by several authors⁴¹⁻⁴³. (Z)-3-Hexenyl acetate has a fruity odour and shows a significant green note flavour. It can be produced using lipase from *Candida antarctica* immobilised on an acrylic resin^{41, 42} or using immobilised lipase from *Mucor miehei*⁴³. The conversion was reported to be about 90%.

An optimised enzymatic synthesis of methyl benzoate in an organic medium was reported by Leszczak and Tran-Minh⁴⁴. Methyl benzoate is part of the aroma of some exotic

racemic 4 - methyl octanoic acid

Candida antarctica lipase B

+EtOH
-H₂O

R - ester

S - acid

fruits and berries. The ester has been produced by direct esterification of benzoic acid with methanol in hexane/toluene catalysed by lipase from *Candida rugosa*.

Gatfield *et al.*⁴⁵ reported in 2001 a method to produce natural ethyl (E,Z)-2,4-decadienoate, the impact compound of pear. Immobilised lipase from *Candida antarctica* is capable of transesterifying *Stillingia* oil in the presence of ethanol.

In 2004, Ley *et al.*⁴⁶ showed a stereoselective enzymatic synthesis of cispellitorine [N-isobutyldeca-(2E,4Z)-dienamide], a taste-active alkamide naturally occurring in tarragon. The reactants were ethyl (E,Z)-2,4-decadienoate the pear ester described before and isobutyl amine. The reaction is catalysed by lipase type B from *Candida antarctica* (commercially available), which shows a remarkable selectivity towards the 2E,4Z ester. The yield was about 80%.

The biotechnological synthesis of lactones has reached a high standard. Besides microbial production, lactones can also be enzymatically produced. For instance, a lipase-catalysed intramolecular transesterification of 4-hydroxycarboxylic esters leads enantioselectively (>80%) to (S)-lactones; the chain length may vary from C5 to C11¹⁶. -Butyrolactone can be produced in that way with lipase from *Mucor miehei*³¹. The preparation of optically active -lactones is more difficult because of the lack of selectivity of most lipases.

Glycosidases

It is well-known that in plant tissues certain amounts of flavour compounds are bound as

non-volatile sugar conjugates. Most of these glycosides are β -glucosides, but there are other glycones like pentoses, hexoses, disaccharides and trisaccharides too⁴⁷. Acylated glycosides and phosphate esters have also been reported^{48,49}.

During winemaking, the grape's β -glucosidase is rapidly inactivated. Glucosidases from *Saccharomyces cerevisiae* and *Candida molischiana* have been suggested to solve this problem⁵⁰.

Sensory quality of food can be improved by synergistic action of monoglycanases, oligoglycanases and polyglycanases. A process for the production of vanilla extracts involving the treatment of crushed green vanilla beans with enzymatic preparations that degrade plant cell walls and the glucosidic precursor together has been patented⁵¹.

Raspberry ketone [4-(4'-hydroxyphenyl)-butan-2-one], the impact compound found in raspberries, can be obtained by enzymatic reactions: The first step is the β -glucosidase-catalysed hydrolysis of the naturally occurring betuloside to betuligenol. The latter can be transformed into raspberry ketone by microbial alcohol dehydrogenase fig.4⁵².

As the chemical synthesis of glycosides is cumbersome, biotechnological transglycosidation using glycosidases is attracting more and more attention⁵³.

Flavorzyme®

Flavorzyme® is a commercially available proteolytic enzyme preparation by Novo Nordisk Bioindustrials. It can be used to obtain a meat-like process flavouring from defatted soybean meal. With the help of aroma extract dilution analysis, Wu and Cadwallader⁵⁴ showed in their study of 2002 the presence of key aroma compounds of roasty, meat-like aroma in the enzymatically hydrolysed and heated hydrolysed protein, e.g. maltol, furaneol, methanethiol and furanethiol derivatives.

Oxireductases

Many enzyme-catalysed redox processes include the transfer of the equivalent of two

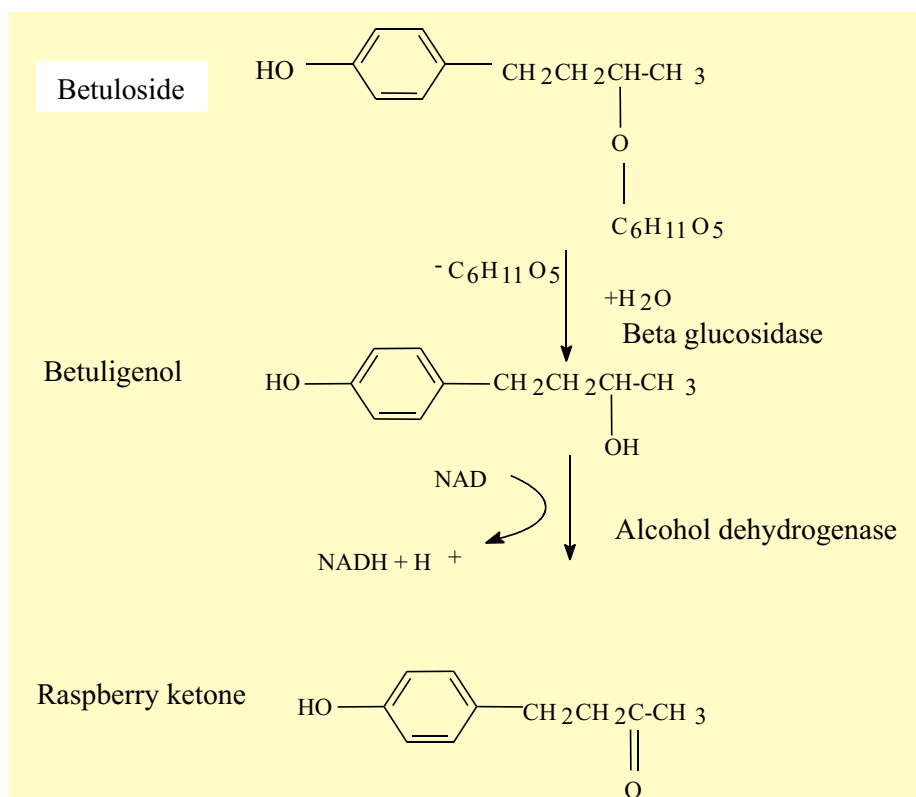


Fig.4. Enzymatic production of raspberry ketone from betuloside.

electrons by one two-electron step or two one-electron steps. The latter is considered as a radical process involving the use of co factors like flavin, quinoid coenzymes or transition metals. The two-electron process is either a hydride transfer or a proton abstraction followed by two-electron transfer.

Horse Liver Alcohol Dehydrogenase

Horse liver alcohol dehydrogenase is able to oxidise primary alcohols except methanol and to reduce a large number of aldehydes. Aqueous solution or organic solvents can be used⁵⁵.

Lipoxygenase

Lipoxygenase (LOX) is a non-haem, iron-containing dioxygenase that catalyses the regioselective and enantioselective dioxygenation of unsaturated fatty acids containing at least one (Z,Z)-1,4-pentadienoic system. LOX is an important factor in the large-scale use of plant enzymes for the production of natural "green note" aroma compounds, a group of isomeric C6 aldehydes and alcohols⁵⁶.

A patented process for the production of green notes applying baker's yeast for in situ reduction of enzymatically produced aldehydes^{57,58} has been called into question regarding the effective production of (Z)-3-hexenol. According to Gatfield's report⁵⁹ the isomerisation of (Z)-3-hexenol to (E)-2-hexenal is a very fast process. The latter undergoes facile conversion to hexanol. Beside this, baker's yeast can add activated acetaldehyde to (E)-2-hexenal, forming 4-octen-2,3-diol.

At present, there are some patents concerning the production of green notes by recombinant guava 13-hydroperoxide lyase expressed in *Escherichia coli*^{60,61} and *Cucumis melo* hydroperoxide lyase; the latter yields a mixture of C6 and C9 compounds⁶². A pathway for the production of β -ionone and γ -ionone by LOX-catalysed cooxidation of carotenes has been described⁶³.

Peroxidases

Soybean Peroxidase

The production of methyl anthranilate, which has a fruity odour, by enzymatic N-

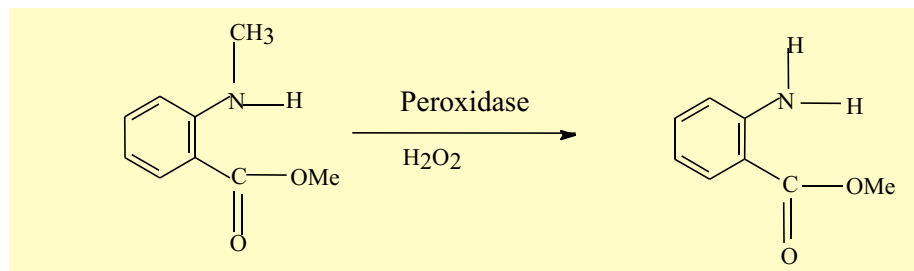


Fig.5. Production of methyl anthranilate by enzymatic N-demethylation of methyl N-methyl anthranilate.

demethylation of methyl N-methyl anthranilate fig.5 has been reported by van Haandel *et al.*⁶⁴. An alternative method for the production of methyl anthranilate with the help of *Bacillus megaterium* was recently reported by Taupp *et al.*⁶⁵; the latter pathway resulted in higher yields of methyl anthranilate.

Lepista irina Peroxidase

In 2003, Zorn *et al.*⁶⁶ discovered a fungal peroxidase from *Lepista irina* valued edible fungus that cleaved β -carotene to flavour-active compounds. According to the authors, the cleavage of β -carotene to aroma compounds by a fungal peroxidase had not been reported before. It was found that extracellular liquid of the fungus can degrade β -carotene to β -cyclocitral,

dihydroactinidiolide, 2-hydroxy-2,6,6-trimethylcyclohexanone, β -apo-10'-carotenal and β -ionone; the last two compounds are the main products fig.6. The key enzyme catalysing the oxidative cleavage was isolated and characterised. As there is great interest from the detergent, food and perfume industry in the potent aroma compounds formed by carotenoid breakdown, and as the β -ionone obtained can be labelled as natural aroma if natural carotenoids are used this cleavage reaction might have a high potential.

Laccase Germacrene A Hydroxylase

Laccase, a group of multi-copper proteins of low specificity, acting on both o-quinols and p-quinols and often on aminophenols and

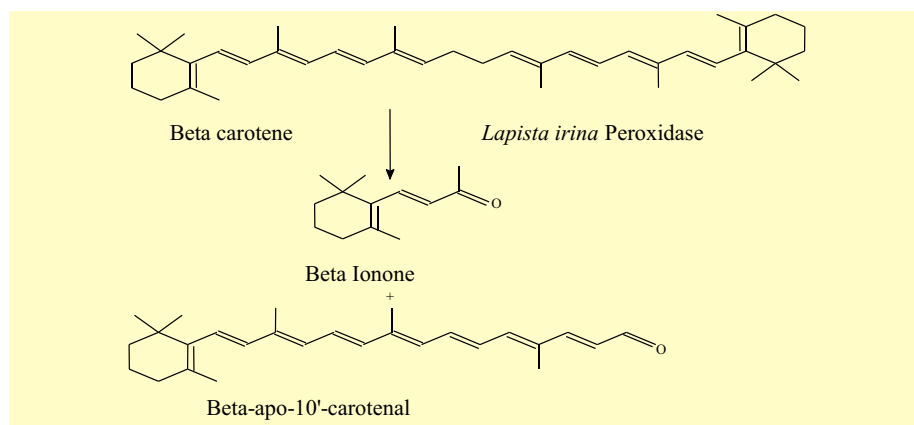


Fig.6. Cleavage of Beta carotene by *Lepista irina* Peroxidase

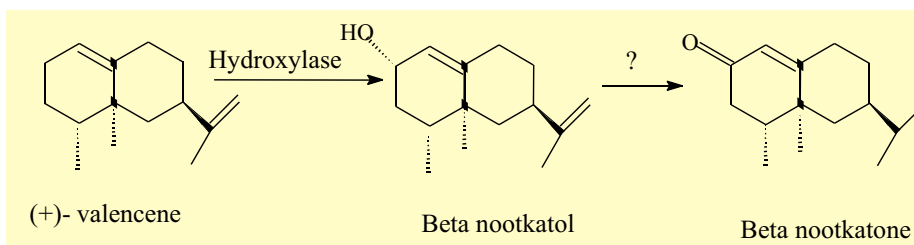


Fig.7. Production of nootkatone from valencene catalysed (+)-germacrene hydroxylase

phenylenediamine, is used for the biotechnological production of nootkatone, the impact compound of grapefruit. Huang *et al.*⁶⁷ described a process for the laccase-catalysed oxidation of valencene to nootkatone; they used whole microorganisms with laccase activity such as from *Botrytis cinerea* but they reported a process with isolated laccase too.

Franssen *et al.*²⁵ pointed out an alternative method of production of nootkatone from valencene catalysed by (+)-germacrene A hydroxylase, an enzyme of the cytochrome P450 monooxygenase type that was isolated from chicory roots..

Microbial Amine Oxidases

Amine oxidase from *Aspergillus niger* and monoamine oxidase from *Escherichia coli* can be used for the oxidative deamination of amines, forming the corresponding aldehydes, hydrogen peroxide and ammonia. Using these enzymes, Yoshida *et al.*⁶⁸ described a pathway for the production of vanillin.

Vanillylamine is the substrate of choice for the formation of vanillin with the help of amine oxidase. It can be obtained by cleavage of capsaicin isolated from pepper and capsicum⁶⁹. As natural vanillin extracted from beans of *Vanilla planifolia* is rare and extremely expensive, this pathway for the production of natural vanillin is regarded to have a great potential.

Vanillyl Alcohol Oxidase

Vanillyl alcohol oxidase (VAO) is a flavoenzyme from the ascomycete *Penicillium simplicissimum* that converts a broad range of 4-hydroxybenzyl alcohols and 4-hydroxybenzylamines into the corresponding aldehydes. This large substrate specificity makes it possible to obtain vanillin from two major pathways. As VAO is able to perform an oxidative deamination of capsaicin-derived vanillyl amine, vanillin can be produced by the pathway described in the previous subsection. Van den Heuvel *et al.*⁶⁹ pointed out this biocatalytic route of synthesis in 2001 using penicillin G acylase to obtain vanillyl alcohol from natural capsaicin fig.8.

As the vanillin obtained can be labelled as natural, the enzymes used do not require

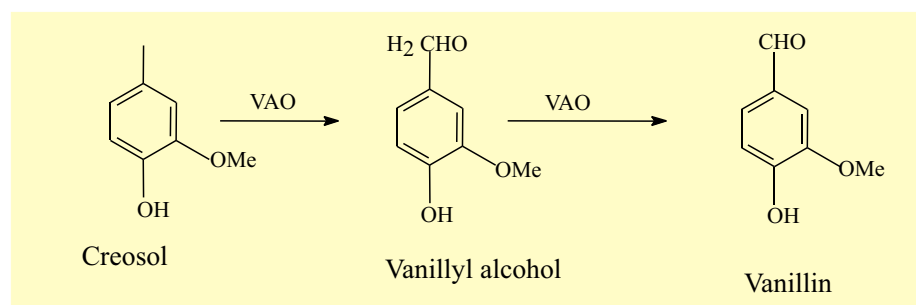


Fig.8. Production of vanillin from creosol by two enzymatic reactions

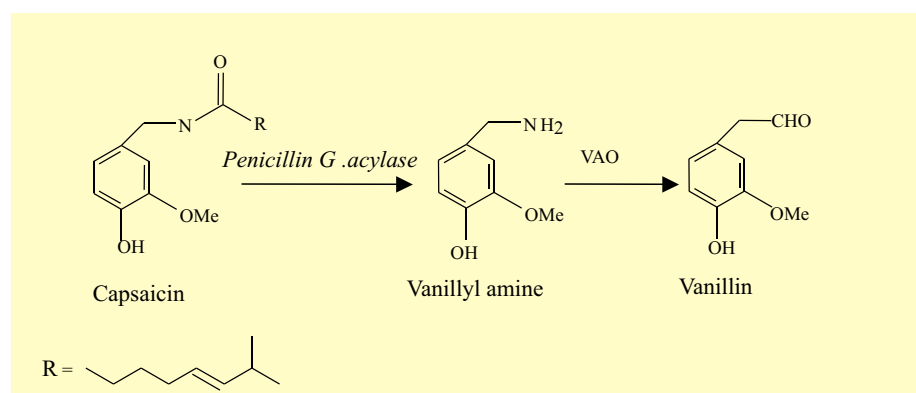


Fig.9. Oxidative deamination of capsaicin-derived vanillyl amine and formation of vanillin VAO vanillyl alcohol oxidase.

expensive cofactors and the enzymes can be produced on a large scale, this bi-enzymatic process could be promising. The second pathway using VAO reported by van den Heuvel *et al.*⁶⁹ is the VAO-catalysed oxidation of vanillyl alcohol to vanillin. Vanillyl alcohol is not very abundant in nature but can be generated by VAO-catalysed conversion of creosol (2-methoxy-p-cresol). As creosol can be found in creosote obtained from heating wood or coal tar, the feedstock for this pathway is very abundant. The process comprises two steps: the conversion of creosol to vanillyl alcohol and the oxidation of the alcohol to vanillin fig.9. Interestingly, these two steps are catalysed by the same enzyme, i.e. VAO. In 2004, van den Heuvel *et al.*⁷⁰ described in another study the characteristics of VAO and pointed out details of the reaction's mechanism.

Transferases

Cyclodextrin Glucanotransferase

In 2002, Do *et al.*⁷¹ proposed a pathway for the enzymatic synthesis of (-)-menthyl α -maltoside and α -maltooligosides from (-)-menthyl α -glucoside using cyclodextrin

glucanotransferase obtained from *Bacillus macerans*. The reaction can be performed in a reactor containing (-)-menthyl α -glucoside, the enzyme and soluble starch; the yield was about 80%:15% (-)-menthyl α -maltoside and 65% (-)-menthyl α -maltooligosides, respectively. Treatment of the starch with α -amylase can raise the proportion of (-)-menthyl α -maltoside. At first, (-)-menthyl α -maltoside has a bitter and sweet taste, but after a few minutes, the refreshing flavour occurs. It has the potential to become a slow-release aroma compound in foods or cigarettes because it possesses higher solubility in water and has a lower tendency to sublimate.

Lyases

D-Fructose-1,6-biphosphate Aldolase

The formation of C-C bonds by aldol condensation is a very useful method in synthesis. Besides the chemical synthesis, aldolases can be used to perform this reaction. The reaction yields a stereoselective condensation of an aldehyde with a ketone donor. In nature, four complementary aldolases can be found in the carbohydrate

metabolism. They show different stereoselectivity and this broad range of enzymes makes it possible to fulfil a large variety of synthetic tasks. In biotechnology, Furaneol® (2,5-dimethyl-4-hydroxy-2H-furan-3-one) can be produced from fructose-1,6-biphosphate with the help of a three-step enzymatic process involving fructose-1,6-bisphosphate aldolase (rabbit muscle aldolase). The first step is the aldolase-catalysed cleavage of the sugar biphosphate to dihydroxyacetone phosphate and glyceraldehyde phosphate. The latter is isomerised by a coimmobilised triose phosphate isomerase to obtain dihydroxyacetone phosphate, which is the substrate for the aldolase-catalysed aldol condensation with d-lactaldehyde. The condensation's product, 6-deoxyfructose phosphate, can be easily converted to Furaneol®⁷². In spite of the intensive effort regarding the biosynthesis of Furaneol® (including the detection of some important enzymes), the biosynthesis in plants is still not fully understood⁷³.

Sesquiterpene Synthase

In the last few years, sesquiterpene synthase from different plants has raised attention. In 2004, Schalk and Clark⁷⁴ described a process (patented by Firmenich, Switzerland) that makes it possible to obtain sesquiterpene synthase and to produce various aliphatic and oxygenated sesquiterpenes from farnesyl diphosphate. For instance, valencene can be obtained in this way. One year later, Schalk⁷⁵ described a process for cloning sesquiterpene synthases from patchouli plants (*Pogostemon cablin*) and the enzyme-catalysed terpenoid production. Various sesquiterpenes can be obtained by this method, for instance patchoulol and other germacrene-type sesquiterpenes.

Characteristics of Microbial Flavour Production

Although for a multitude of microorganisms the metabolic potential for de novo flavour biosynthesis is immense and a wide variety of valuable products can be detected in microbial culture media or their headspaces, the concentrations found in nature are usually too low for commercial applications.

Furthermore, metabolic diversity often leads to a broad product spectrum of closely related compounds. Exceptions to the rule can be found where the flavour compounds are derived from primary metabolism as is the case for some of the non-volatiles (e.g. glutamic acid, citric acid). Therefore, the biocatalytic conversion of a structurally related precursor molecule is often a superior strategy which allows the accumulation of a desired flavour product to be significantly enhanced. As a prerequisite for this strategy, the precursor must be present in nature and its isolation in sufficient amounts from the natural source must be easily feasible in an economically viable fashion. Additionally, if product and precursor are closely related with respect to their physicochemical properties, a selective product recovery during downstream processing becomes a major issue for the bioprocess development. Many

of the industrially relevant microbial flavour production processes follow this 'precursor approach' (e.g. vanillin from ferulic acid or eugenol, 4-decanolide from ricinoleic acid, 2-phenylethanol from l-phenylalanine). Besides the problems arising from metabolic diversity, the cytotoxicity of the flavour products and often also of their precursors is another big hurdle during bioprocess development. Here, very often in situ product recovery or sequential feeding of small amounts of precursor becomes essential to improve the overall performance of a bioprocess and to render it economically viable. Owing to their hydrophobicity, flavour compounds preferentially partition to lipid structures, which makes cellular membranes the main target for product accumulation during microbial processes. The flavour molecules negatively influence the cell physiology by enhancing the

membrane fluidity, eventually leading to collapsing transmembrane gradients and, consequently, to the loss of cell viability⁷⁶. Although genetic engineering in food-related applications has been the subject of a controversial public discussion for quite some time, the fact that in aroma biotechnology genetically modified organisms are used as biocatalysts which are completely separated from the volatiles during the product-recovery step raises hope that this technique will also be applicable in industrial flavour production processes in the future. Further improvements will certainly be triggered by the enormous progress currently being made in the field of total genome sequencing. The time needed to determine complete microbial genomes has dramatically decreased during the last few years. Among the microorganisms already sequenced, several bacteria and fungi can be found which are

Table 1. Main drawbacks during microbial flavour production and biotechnological strategies

Characteristics	Biotechnological strategy	Exemplary product
Formation of unwanted byproducts owing to complex metabolic pathways	Over expression of key genes of the synthetic pathways Heterologous gene expression/use of engineered enzymes Knockouts of genes involved in product degradation 'Precursor approach' instead of de novo biosynthesis Screening; enrichment cultures Subsequent biotransformation converting a by-product to the desired product	3-Methylbutyl acetate ⁷⁷ Cinnamyl alcohol ⁷⁸ Verbenol ^{79,80} Vanillin ⁸¹ 2-Phenylethanol ⁸² raspberry ketone ^{83,84} Perillyl alcohol ⁸⁵ , 10-hydroxy patchouli ⁸⁶ 4-Decanolide ⁸⁷
Toxic properties of the flavour compounds produced	In situ product recovery by: Adsorption, e.g. on XAD resins Stripping and adsorption Extraction (two-phase bioprocess) Membrane-based processes Resting cells instead of growing ones Product-tolerant strains	6-Pentyl- δ -pyrone ⁸⁸ C2C5 alkyl esters ⁸⁹ (furfurylthiol ⁹⁰) 2-Phenylethanol ⁸² phenylacetaldehyde ⁹¹ sonovalal ^{92, 2-} phenylethyl acetate ⁹³ Acetaldehyde ⁹⁴ Vanillin ⁹⁵
Toxic properties of the precursor molecules	Sequential precursor feeding On line monitoring of precursor/bioactivity Immobilisation of microorganisms Two-phase bioprocess with an organic solvent as the precursor reservoir Resting cells instead of growing ones Precursor-tolerant (solvent-tolerant) strains Fungal spores instead of mycelia	4-Octanolide ⁹⁶ 3-methylbutyl acetate ⁹⁷ carboxylic acids ⁹⁸ Limonene transformation products ^{99, 100} Propanoic acid ¹⁰¹ phenylacetic acid ¹⁰² 5-Decanolide ¹⁰³ 4-hexanolide ¹⁰⁴ Carvone ^{105, 106} Perillic acid ¹⁰⁷ carvone ^{108, 106} Methylketones ^{109, 110}

valuable candidates with respect to food and flavour applications, e.g. *Bacillus subtilis*, *Brevibacterium linens*, *Clostridium acetobutylicum*, *Corynebacterium glutamicum*, *Gluconobacter oxydans*, *Lactococcus lactis*, *Pseudomonas putida*, *Streptococcus thermophilus*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Aspergillus niger*. Table 1 summarises the main issues of microbial flavour production and how they may be addressed by biotechnological methods.

Among the natural flavour molecules produced with microorganisms are some real bulk products, such as L-glutamic acid and citric acid manufactured on the million-ton scale, but the majority of the target compounds are produced for highly specific applications and thus are rather niche products with market volumes below 1 t year⁻¹. Here, industry avoids costly research and development effort to establish more sophisticated processes owing to the limited market volume of these products.

Nevertheless some natural flavours which have a broader application are produced in amounts of around one to several tons per year, such as vanillin, 2-phenylethanol and 4-decanolide. These flavour compounds have an increasing market share owing to steadily improved bioprocesses: for instance, the price for the peach-like 4-decanolide dropped from about US \$20,000 per kilogram in the 1980s to about US \$300 per kilogram in 2004^{111,112}.

Table 2 summarises some natural flavour

Table 2 Some Microbially produced flavour compounds and corresponding bioprocess feature.

Product	Precursor	Microorganism	Process data	Remarks	References
L-Glutamic acid	-	<i>Corynebacterium glutamicum</i>	150 g L ⁻¹ , 60 h, 1.500,000 t	year-1 Aerobic cultivation; up to 500-m ³ scale; mutants with highly permeable cell walls	113,114
Citric acid	-	<i>Aspergillus niger</i>	>200 g L ⁻¹ , 912 days, 1,000,000 t year ⁻¹ ; yield >95%	Downstream processing by precipitation as calcium citrate	113,114 115.
Acetic acid	Ethanol	<i>Gluconobacter Acetobacter</i> ,	'Vinegar' with 10 to >20 %, >190,000 t year ⁻¹ ; yield ~98%	Aerobic cultivation at 100-m ³ scale; Frings aerator for high oxygen transfer rates	113,114, 116
L-Lactic acid	-	<i>Lactobacillus</i>	210 g L ⁻¹ , 140,000 t year ⁻¹ ; yield >90%	More than 100-m ³ scale; recovery of lactic acid by salt splitting technology	113,114, 117.
Vanillin	Ferulic acid Amycola topsis,	<i>Streptomyces</i>	Up to 18 g L ⁻¹ , 50 h, 110 t year ⁻¹	In situ product recovery by crystallisation at more than 10 g L ⁻¹ possible	118,119, 120.
(Z)-3-Hexenol ('leaf alcohol')	Linolenic acid	Soy lipoxygenase + plant hydroperoxide lyase + baker's yeast	4 g kg ⁻¹ , 510 t year ⁻¹ (also by isolation from plant oils)	Addition of baker's yeast to obtain the alcohol; without yeast the aldehyde is the major product	121,122.
4-Decanolide (-decalactone)	Ricinoleic acid	<i>Yarrowia lipolytica</i>	11 g L ⁻¹ , 55 h, several tons per year	Final acidification and temperature increase effect cyclisation of all 4-hydroxydecanoic acid to the corresponding lactone	123,124, 125.
2-Phenylethanol	1-Phenylalanine	Diverse yeasts; e.g. <i>Saccharomyces</i> and <i>Kluyveromyces</i>	>10 g L ⁻¹ , 30 h, 0.51 t year ⁻¹	Fed-batch cultivation; in situ product recovery by two-phase system with more than 25 g L ⁻¹ in the organic phase possible	126,127.
Short-chain carboxylic acids, e.g. 2-, and 3-methylbutyrate	Fusel alcohols	<i>Gluconobacter</i> , <i>Acetobacter</i>	Up to 95 g L ⁻¹ , 72 h	Two-step cultivation: biomass + bioconversion period; used as flavour acids but also for ester syntheses	128.

compounds currently being produced by microbial processes in industry.

Driving forces

For thousands of years microbial processes have accompanied mankind playing the decisive but unrecognised role of producing more flavourful foods and beverages such as bread, cheese, beer, wine and soy sauce¹²⁹. With the dynamic development of modern analytical techniques in the middle of the twentieth century when isolation, chromatographic separation and structural identification of volatiles became routine, the basis for a more systematic elucidation of microbial flavour generation was given. Research in the last decades has led to a tremendous increase in knowledge of microbial and enzymatic flavour generation. Nowadays, biotechnological production of flavour compounds is a mature discipline in the chemical industry, with an estimated more than 100 molecules in the market produced by enzymatic or microbial processes¹³⁰. The predominant driving force was and still is the fact that flavour compounds produced from natural raw materials by microbial or enzymatic methods can be labelled 'natural' in accordance with European and US legislation, thereby satisfying the unbroken consumer trend towards all 'bio' or 'natural' products in the food sector. By contrast, the involvement of chemical means leads to the less appreciated labels 'nature identical' (EC Flavour Directive 88/388/EEC) or 'artificial' (US Code of Federal Regulations 21 CFR 101.22) for flavours not occurring in nature. This from the scientist's view point rather surprising situation paved the way for 'self-sufficient' research on biocatalytic and fermentative flavour production, which started several decades ago. These research activities steadily expanded to almost all natural key flavour compounds which cannot be economically provided by classic isolation from their natural sources, e.g. by extraction or distillation, owing to too low concentrations. This happened although many of the target compounds could and still can be produced in a more efficient and less expensive way by chemical syntheses because the natural flavours achieve significantly higher market prices of up to 2 orders of magnitude. For 2005 the total worldwide flavour and fragrance market was

Table 3. Driving forces to use biotechnological methods for flavour production.¹³²

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

estimated to be about US \$16.0 billion, with a growth in local currencies of about 3% in the same year. In 2001 the percentage of natural flavours of all added flavours amounted to 90% (EU) and 80% (USA) in beverages, to 80% (EU and USA) in savoury foods, and to 50% (EU) and 75% (USA) in dairy foods¹³¹.

Nevertheless, enhanced competitive pressure and a less distinguishing food labelling legislation ('natural flavouring' vs. 'flavouring' in the EU) cause companies to increasingly evaluate natural flavours by their production costs in comparison with the costs of their chemically synthesised counterparts and in most cases do not leave room for high extra charges for the naturals anymore. Instead, three characteristics of most biotechnological processes are increasingly influencing academic as well as industrial considerations: biocatalytic reactions usually (1) are highly selective (chemo, regio, stereo), (2) start from natural raw materials/renewable resources and (3) are environmentally friendly and sustainable.

On the basis of the long and sound research tradition in aroma biotechnology, novel approaches combining the emerging opportunities given by modern molecular biology including '-omics' and metabolic engineering technologies, and advanced bioprocess engineering, e.g. in situ product removal strategies, will definitely lead to even more biotechnologically produced flavours in the future.

Traditional non-volatile flavour compounds are included, because some of them, e.g.

monosodium glutamate (MSG) or citric acid, are industrial bulk products with market volumes exceeding 1,000,000 tonnes/year. These examples illustrate extremely well the beneficial impact of biotechnology on the chemical industry as commodities can be produced from renewable resources based on a sustainable technology.

Regulatory aspects and legal status

It is important to establish whether or not the biotechnologically produced aromas can be considered to be natural. With the exception of traditional applications such as cheese and beer, the use of biotechnological methods for the production of food ingredients is fairly recent. It is regrettable that in many countries the legislative authorities lag behind in regulating new developments. At the European level, attempts are now being made by the European Commission to work out a common legislation. In the USA two classes of flavour chemicals exist: natural and artificial. The Code of Federal Regulations defines a natural flavour as follows:¹³³ the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or 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 flavouring

rather than nutrition. This definition of natural flavours thus comprises products which are converted by living cells or parts thereof, including enzymes. A third classification, namely the nature-identical flavours, exists in most of the European countries. These compounds are synthesized via chemical processes, but are in all chemical aspects identical to aromas identified in nature. The distinction between natural and synthetic flavours is analytically possible via GC/MS, in particular by determining isotope ratios¹³⁴. In this respect then, flavours produced by microorganisms are natural if the precursor material is also of natural origin. There is however a great distrust of biotechnological products with applications in food, especially when genetic manipulation is also involved. This results in a supplementary regulation. In the USA, admissions for such new products are given by the Food and Drug Administration (FDA). Compounds with a GRAS-status (Generally Recognized As Safe) would also remain classified as GRAS, even if they are produced by microbial or enzymatic processes.¹³⁵ This GRAS-label is important because these compounds are not considered as additives. Notwithstanding these general principles, in practice each inquiry in the USA for the application of biotechnological flavours is investigated separately. The applicant has to prove the safety of his new product with an extensive dossier.¹³⁶

Industrial applications

Although many microbial processes have been described able to produce interesting flavours, the number of industrial applications are limited. A reason for this in most cases is the low yield. The microbial flavours are often present only in low concentrations in the fermentation broths, resulting in high costs for down-stream processing. This is compensated by the fact that the market price of natural aromas is 1 & 100 times higher than that of synthetic aromas. This means that, in order to be competitive, the price of microbial flavours has to range between 200 and 2000 US \$/kg^{137,138}. Other problems hampering industrial applications have a technological character in that the volatility and low solubility in water of many

flavours makes their recovery often difficult to perform. On the other hand, it is often necessary to keep the concentration of the end-product in the fermentation broth below a certain level because of end-product inhibition and toxicity towards the microorganisms themselves. Another method for the continuous removal of an apolar end product is the use of apolar resins such as Amberlite XAD. A German patent describes the use of Amberlite XAD2 for the recovery of terpenes produced by *Ceratocystis sp.*^{139,140}. Based on this technology, yields of 2 g of terpenes per litre became possible. In order to recover volatile compounds, adequate adsorbents can be considered and volatiles can also be removed continuously by gas stripping or vacuum fermentation. The development of specific fermentation techniques and recovery methods is an important challenge for researchers in this field. Another obstacle to commercialization is the framework of legal regulations mentioned above. New products have to be examined by these authorities before the status 'natural' is awarded. The procedure required can take years and bring about high costs for biotechnological products. Notwithstanding these difficulties, a number of flavour compounds are already produced by micro-organisms on an industrial scale. Nearly all important flavour companies declare indeed that they regularly use fermentation techniques for the production of aroma compounds, yet only a few of them mention the specific products.^{141,142} This secrecy is typical for a sector with high competition and where research and development of new products is extremely important. Recently BASF (Germany) started the microbial production of 4-decalactone, a peach aroma which is distributed by its subsidiary company Fritzsche, Dodge & Olcott. The process involves the bio-conversion by *Yarrowia lipolytica* of castor oil, an oil that is pressed from the seeds of *Ricinus communis* and is composed of 80 % of a triglyceride of 12-hydroxy-9-octadecene acid, also known as ricinoleic acid¹⁴³. In the UK, (R)-S-dodecanolide is prepared by Unilever on a commercial scale using baker's yeast and starting from 5-ketododecanoic acid.³ This process takes place in a 30000 litre fermentor and the lactone produced can

be applied as a butter flavour in margarines. Butyric acid and ethyl butyrate are produced microbiologically by the American company Hercules Inc.^{142,144}. *Clostridium butyricum* converts glucose under anaerobic conditions into butyric acid, the concentration of which can reach 1.2 % in the fermentation broth. Butyric acid, a component naturally present in butter and some cheeses, can be applied for instance as a natural cheese aroma¹⁴⁵.

Esterification with ethanol gives rise to ethyl butyrate, an important fruity flavour with a low odour threshold. The cost of biotechnologically produced ethyl butyrate amounts to about 180 \$/kg. There is also a possibility to collect 'natural' ethyl butyrate during the concentration of fruit juices: the price of this product however comes to about 5000 \$/kg. Synthetically produced ethyl butyrate costs only 4 \$/kg. Several processes using *Penicillium roquefortii* for the production of methylketones, which are important flavours of blue-veined cheeses, were patented in the early 1960^{146,147}.

Because of the growing demand from the consumers side for 'natural' additives for food, feed and cosmetics, the commercial importance of biotechnologically produced flavours will certainly grow further in the near future.

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