Biotechnology of Aroma Compounds

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Editorial

Professor A. Fiechter

Biotechnology has become one of the most promising and vital areas of science during the last twenty years. It is one of the world's future technologies and will influence all areas of daily life. Professor Armin Fiechter is one of the pioneers in biotechnology who recognized this potential in the early 1960s. From the very beginning he saw that interdisciplinary and international cooperation is a sine qua non for biotechnology. His open-minded view helped to overcome hurdles and borders limiting the rapid and dynamic growth of this new technology. He brought together experts from all over the world, infecting them with his own ingenuity and enthusiasm. His laboratories in Zürich were a major crystallization point for new ideas and trends. His active role as a teacher of young scientists led to the well-known "Fiechter School" and a worldwide network of his former students. Several generations of this "Fiechter family" have already entered high positions in industry, government and teaching.

His more then 500 publications document his research activities in different areas of biotechnology. Among others in this impressive collection are: the development of integrated bioprocesses; bioreactor developments (such as the "compact loop reactor"); process and analysis automation; metabolic control studies in yeast and bacteria; Cytochrome P-450 studies; thermophilic processes; biodegradation processes; and development of protein-free media for animal cell cultivation. In all of these projects, he was one of the driving forces to

bring together researchers from all areas of the life sciences. His creative power initiated several critical areas in biotechnology. All of these efforts are reflected in his current main activity: making biotechnology a commercial success.

All of his different interests are best reflected in the journals and series he has founded and edited for many years. What would "Advances of BiochemicalEngineering/Biotechnology" be without him? His ingenuity and creativity will be a measure for all of those who will follow as editing managers of this series – certainly not a simple task! However, I am sure that he will always be open for questions and will supply us with new ideas and enthusiasm to maintain his high standards.

Hannover, July 1996 Thomas Scheper

Preface

Biocatalysts have been used by man since ancient times to process raw food materials. Improved storability of products such as cheese or wine was an evident advantage. Modern physical and chemical treatments are now available to preserve all kinds of food, but the traditional food biotechnology has not only survived – it is blooming: It is now the aspect of sensory quality and sometimes uniqueness imparted by the microbial formation of compounds with odorous and taste properties (flavour) that is so highly estimated by the pampered consumers.

Based on accumulated knowledge, all major aroma houses have established biotechnological processes in the last decade. In both the European Union and in the United States, food legislation permits biotechnologically generated aroma compounds to be labeled "natural". This applies, if the starting materials of the process were obtained from food sources, and if isolation and purification were restricted to physical means, such as distillation or extraction. Pure aroma chemicals, flavour building blocks, or complex flavour mixtures are now biosynthetically available on a technical scale by imitating or modifying the classical "fermentations".

The present volume attempts to illustrate some of the frontiers of current research in aroma biotechnology. Enzymes, often available with tailor-made properties from other bioprocesses, offer the most convenient starting point; examples are lipases, glycosidases and other hydrolases, and isoprenoid related catalysts. If the required enzyme is not available, intact mirobial cells may deliver the necessary catalytic activity. Examples in this area refer to procaryotic pseudomonads and eucaryotic yeasts, to fungal and to plant cells. Products include oxygenated isoprenoids, ketones, pyrazines, esters, lactones and many more.

Processing principles that have proven themselves in other bioprocesses, such as precursor feeding, immobilization, and in situ recovery are now applied to increase the yields of advanced, second generation processes to commercially attractive levels. Gene transfer from microbial or plant donors to work horses,

such as *S. cerevisiae*, is feasible and has created a lot of excitement. However, significant gaps in our understanding of the competition of aroma pathways with primary metabolism, and of the partitioning of substrates and regulation of key steps are bound to impede progress. More fundamental research on the biochemistry of plant and microbial volatile metabolites is needed. The ideal bioprocess will have to beat the economy of the conventional field cultivated aroma sources, irrespective of a possibly instable legal situation. This shows that we are still at the beginnings of an integrated approach to the bioengineering of aroma compounds.

Hannover, July 1996 R. G. Berger

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Combining the Technical Push and the Business Pull for Natural Flavours

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To my fathers heroes, "the old country people" who lived and worked their lives with nature.

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This paper is an attempt to demonstrate the close interrelationship between the scientific and business aspects of biotechnology and natural flavours, and especially how the market pull and technical push dynamics can be effectively integrated. After all, it is consumers' insistent demand for 'natural flavours' and other food ingredients, and also for environmentally friendly products and "green" processes in general, that has greatly stimulated the application of enzymes and microorganisms as catalysts for flavour production. However, this is a highly fragmented and segmented industry; with very many and quite different companies involved, and a very wide range of flavour-conferring molecules, all of which can be permutated or combined creatively so as to meet the requirements of particular product requirements. Hence there are very many diverse and important factors at work which I hope to illustrate in this paper. These include manufacturing, regulatory, patenting, product labelling, raw materials supply and quality, applications and formulation factors that help make this such a challenging and fascinating area to work in. This close interrelationship of scientific and market forces is demonstrated by the following key questions that have to be answered in order for innovation to take place. Can we make the product in good quality and cost-effectively? Is it safe to use? Is there a big enough demand? Can we make and use it exclusively?

1 Introduction

The consumer-led demand for natural flavours that are consistent with customers' healthy lifestyles, 'natural' and 'bio' marketing claims and labelling and environmentally friendly 'green' production processes has stimulated considerable scientific, industrial and commercial progress in developing novel biocatalytic processes and products using microbial cells or enzymes derived from them. Production scale processes have been developed for flavour chemicals such as γ- or δ-decalactones, benzaldehyde, many esters as well as a range of savoury flavours, flavour enhances and sweeteners. These processes range from the use of single isolated enzymes, often in combination with complementary chemical recovery steps, through to the use of whole microbial cells in fermentations in which the whole or part of the microorganisms' metabolic pathways are used. In many cases commercially successful flavour chemicals are being produced that are successful because the chemical is a sufficiently powerful and desirable material to find a range of cost-effective applications when formulated into complete flavours. It should also be noted that considerable progress has been made in the related use of biocatalysts to produce other food ingredients such as polysaccharides and also ingredients for personal care products such as aroma chemicals and skin care materials.

A growing body of scientific research has been carried out relating to the bioproduction of natural flavours, starting with Omelianski in 1923 [1], who was the first to make a comprehensive survey of microbial aroma production. However the real challenge lies in developing this science into bioprocesses that can produce products to the exacting organoleptic specifications required for use in foods on a cost-effective industrial scale. Therefore this paper concentrates on examples that have either already achieved, or are capable of achieving such an industrial commercial status, together with others that are good representatives examples of generally applicable approaches.

Because the biotechnology of natural flavour production is a genuine technology that exists to meet consumer needs, it is absolutely essential to try to understand some of the characteristics of both the flavour industry and also the composition and characteristics of flavours in order to properly appreciate the role of biocatalysis-based processes in helping to supply the demand for natural flavours for use in food and beverage products.

2 Important Characteristics of Flavours

Flavours can be either pure flavour chemicals, or mixtures of different flavour chemicals. They work by interacting with receptors in the nose and tongue. These mixtures may be complete flavours in which all the flavour chemicals,

sometimes with quite different taste and aroma sensations, necessary to confer accurately a particular taste sensation, are present, for example a particular cheese or fruit taste. Alternatively this product of flavour chemicals constitutes a flavour 'block' that has a balanced mixture of the flavour chemicals which, when combined with other ingredients such as, for example, a protein hydrolysate, then gives a complete flavour sensation (Fig. 1).

Particularly important are the so-called 'character impact' flavour chemicals which are decisive in conferring the characteristic flavour and aroma of the particular food or beverage, together with the other components, often essential oils, which may contain hundreds of different chemicals, which serve to round-off and increase the complexity of the flavour but without changing its essential character. In addition to 'classical flavours' such as methylketones, esters, terpenes, etc., a number of very important non-volatile flavours are produced such as high fructose corn syrups (almost 10 million tons per annum produced), Aspartame® sweetener and monosodium glutamate flavour potentiator should not be forgotten. Some of the key and defining characteristics of the flavour and fragrance industry are as follows (Table 1).

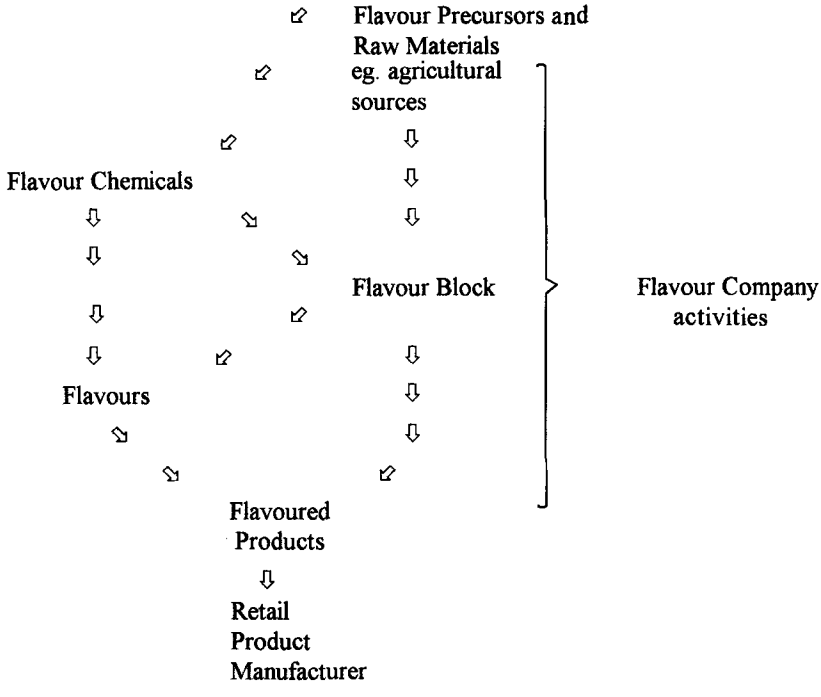


Fig. 1. Building a flavoured product. This supply chain shows the sources of raw materials and ingredients, and how they can be combined and used to help create the final product

Table 1. Characteristics of the flavour and fragrance industry

- Flavour and fragrances are used in a very wide range of consumer products. Thus the demands for flavours and fragrances is subject to a so-called 'derived demand'. That is, the demand for the flavour or fragrance is very often entirely dependent on the consumer demand for the products that contain them.
- These end-use products vary over a wide price range, from cheap, high bulk products such as washing-powders and air-fresheners, through to high-value, low-bulk products such as fine fragrances and prestige cosmetics. Consequently quite different challenges need to be overcome, from producing a cheap fragrance, stable in the presence of bleaching agents in the former cases through to simulating and even improving on complex and transient natural flavour aromas.
- The size of the flavour fragrance industry worldwide is considerable, estimated at $9.7 billion in 1994 [4]
- The industry is quite fragmented, with 50% of the business being handled by the three biggest companies. In addition there are some important food-ingredient manufacturers such as Gist-Brocades, and there is also a very large number of smaller companies that are more specialised, either in their product range, geographical area of operation, or technical area of expertise, but which constitute a very important source of more specialised service and creativity.

2.1 Some Important Characteristics of Flavours

Flavours have some very individual characteristics:–

- Very often only quite low concentrations of the key flavour molecules occur in products, whether naturally occurring or manufactured.
- Flavour and aroma molecules usually have extremely strong biological effects, exerting strong taste or smell sensations even when present at such extremely low concentrations, or very different sensations when present at different concentrations. Flavour and fragrance chemicals can be active at comparable or lower concentrations to those at which powerful drugs exert their effects. Synergy effects are often very important and minor (chemical) components are often crucially important in terms of organoleptic quality.
- However, such threshold concentrations can vary by factors of 10^6–10^7 from molecule to molecule, which is one reason why flavoured and fragranced chemicals are used at quite different concentrations in products and, as a result, need to be manufactured on scales varying from a few kilograms per annum to several thousand tonnes per annum. An important term is 'flavour value'; this is the concentration of the flavour in the product divided by its threshold for taste, i.e. the flavour value is a simple multiple of the threshold detected.
- Because of their high potency, trace impurities can have a big effect on the quality of a product, making the downstream purification and isolation steps in flavour production processes very important.
- Most flavours are chemically very complex mixtures of quite different chemicals, many of which contribute to the overall flavour sensation depending on the various flavour characteristics of the various component chemicals (See Tables 2 and 3). Compounded mixtures of different flavour chemicals

Table 2. Comparison of known and used compounds

	Number of compounds known to science	Number of compounds commonly used
Flavours	~6400	200–300
Fragrances	4–5000	200–400
Essential Oils	3000	150–200

- Only around 400 aroma chemicals are manufactured in quantities of > 1 tpa.
- Only 68 compounds have been reported to have consumption rates of more than 3000 kg per year.
- 50 to 100 flavour materials/chemicals are produced commercially by fermentation.

Adapted from [51].

Table 3. Occurrence of volatile compounds in various foods

Food product	Content (mg kg^{-1})	Number of identified compounds	Number of unidentified compounds
Meat (beef)	50	270	250
Coffee	50	468	> 500
Onion	900	96	?
Raspberry	1.7	95	120
Strawberry (see Tables 5 and 7)	10	324	?
Pineapple	11.3	59	?
Passion Fruit	12	194	50

therefore provide a more rounded and complex flavour sensation which, in extreme cases, such as whisky, caviar, fine wines etc., command luxury product prices. Therefore the most crucial test of quality is organoleptic evaluation rather than chemical analysis.

- There is a big range of chemically very different molecules that act as flavour and aroma chemicals including, for instance, ketones, acids, aldehydes, esters and many other, all with different dominant chemical functionabilities and reactive groups. This variety is illustrated by a number of flavours (Tables 2 and 3) and specically for strawberry flavour (Table 4).
- The molecules commonly used as flavours and fragrances are chemically very diverse, but are usually fairly small, with molecular weights of less than 300, because they have to be volatile to exert their effects.
- Even small changes in chemical structure usually result in very big changes in organoleptic properties in terms of intensity, flavour and fragrance quality, and the threshold of detection by the nose or mouth. Even molecules with the same basic chemical functionality, for instance, aldehyde or ketones, can often have very different organoleptic characteristics.
- Flavours and fragrances are composed of a complex mixture of different molecules. Usually, a high-quality flavour or fragrance is created by the

Table 4. Compounds present in strawberries (from [10])

Hydrocarbons	33
Alcohols	53
Aldehydes	17
Ketones	20
Acids	45
Esters	128
Lactones	10
Sulphur compounds	7
Acetals	20
Phenols	3
Furans	8
Oxides, pyrans, coumarins	5

Table 5. Major differences in the headspace composition of living and picked strawberry fruit (from [10])

Flavour chemical	Living (%)	Picked (%)
Ethyl 2-methylbutanoate	–	4.2
Isoamyl acetate	0.5	4.5
Methyl hexanoate	4.6	11.3
Hexyl butanoate	9.3	1.1
Octyl butanoate	7.1	0.3
γ-Decalactone	9.3	0.3

combined effect of a large number of different molecules, each with different taste or smell characteristics, but blended together in a complementary way by the flavourist or perfumer.

- Whereas many thousands of molecules are known to have interesting tastes or aromas, only a few hundred are used regularly in flavours and fragrances, and only around 400 aroma chemicals are manufactured in quantities greater than one ton per annum.
- The composition of flavours can also be quite transitory, such as the dramatic way in which the chemical composition of strawberries changes upon picking (Table 5) so that their flavour characteristics and quality can be quite different after harvesting and storage. Particularly extreme changes occur upon processing, especially if heating is involved, causing losses of the more volatile flavour constituents and the formation of Maillard reaction products.
- Flavour balancing is often necessary when the composition of a food is changed, because of the different way flavour molecules interact with the other components of the foods, and in particular how they are released during eating. This applies not only when the modification involves materials with an intrinsic taste, such as the formulation of low salt food, but also in the formulation of reduced calorie foods, and low fat foods in which fats are replaced by carbohydrates and other fat-replacers.

- Very rarely have totally new flavours achieved sustained consumer acceptance, a rare example being cola-type beverages. Usually flavours need to imitate or provide a modified form of traditionally available tastes.
- Functional benefits, other than fragrance and flavour properties, are also important, such as preservative, deodorant and antioxidant activities, colour etc.
- Very few materials are used in large quantities and some of the most exotic are used only very infrequently. Therefore economies of scale of production and buying etc. are usually not very significant, and few materials are of sufficient value to make attractive targets for production by commodity chemical companies.
- Raw materials are derived either from cheap petrochemical sources, or from plant or animal sources that are often subject to variability in supply due to climatic or political factors.
- Flavours are complex and often dynamic mixtures of diverse molecules present in low concentrations, which, because of their high biological activity, can have a powerful taste and aroma characteristic of particular food and beverage products.
- The value of flavours and fragrances is highly dependent on their organoleptic quality and purity, i.e. sale price is very dependent on quality. In most cases, organoleptic properties are determined by the precise chemical and stereochemical structure of molecules. Therefore, biotechnology processes that are capable of highly selective regio- and stereochemical-selective reactions are at an advantage, because flavour and fragrance perception is based on the precise stereochemical properties of the molecules.
- Because of the great variety of flavour chemicals required, a correspondingly large range of methods and biocatalysts have been used to produce them (Fig. 2), using a diversity of reactions that are of considerable scientific

Traditional ⇓ ⇓ ⇓ ⇓		Chemical synthesis Extraction from botanical and other sources Reaction flavours Traditional fermentations
Current ⇩ ⇩		Fermentations Enzyme and microbial bioconversions
Emerging ↓ ↓		Genetic engineering of microorganisms Plant genetic engineering
Future		Plant cell and tissue culture

Fig. 2. Methods for flavour production, ranging from traditional processes, through current technologies, to new and possible future methods

interest and which have not been otherwise investigated, e.g. for pharmaceutical uses.

- Biocatalysts can be employed in a number of different ways varying from the use of growing cells (fermentations), through the use of nongrowing cells as the biocatalyst (bioconversions), to the use of isolated enzymes. The best mode of use will vary from application to application, depending on the particular and individual requirements (Fig. 3). Some of the important skills necessary to achieve success in developing bioprocesses and bioflavours are listed in Table 6.
- These methods should be viewed in the context of the variety of methods available to the flavour industry, only some of which can produce natural flavours (Fig. 2). For instance many natural flavours are currently produced by physical extraction from plant raw materials such as essential oils.

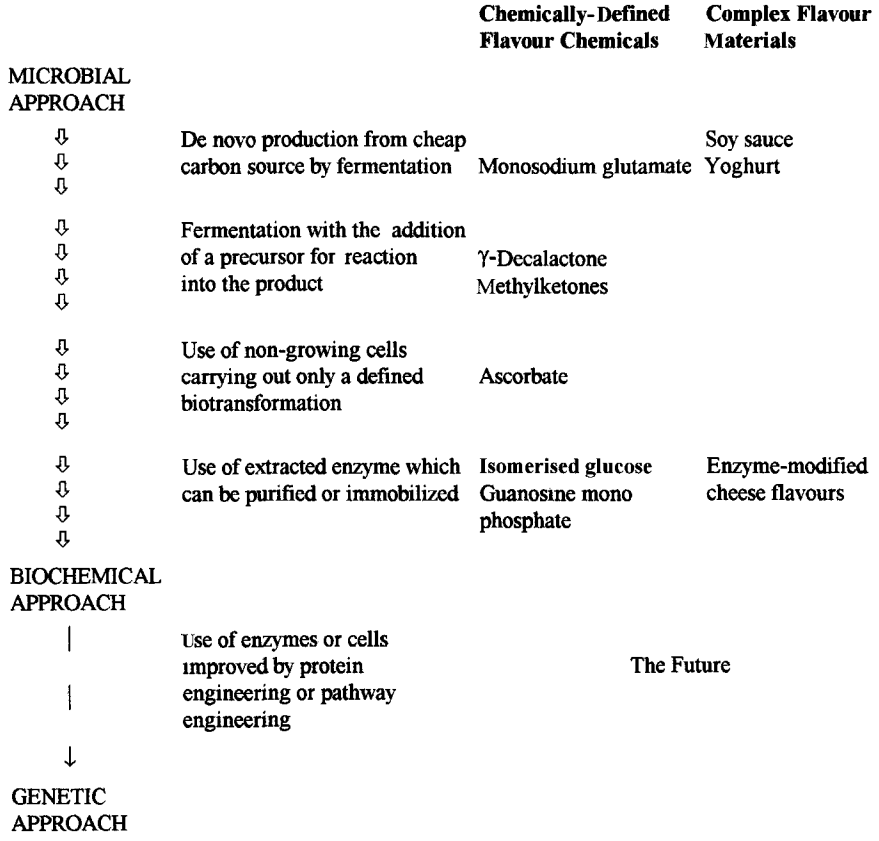


Fig. 3. Examples of the different biocatalytic methods employed for flavour production, from traditional fermentations to emerging modern methods based on genetic engineering

Table 6. Some important biological skills required in the development of biotransformation processes and products (adapted from [52])

1) *Screening for microorganisms*: to discover the bacterial, yeast and fungal strains with the required enzyme activities required to catalyze the reactions we wish to carry out. Strain selection depends critically on the great diversity of microbial life on our planet, and the development of selective isolation procedures for the identification of only those with the required enzyme acitivities and other characteristics useful in bioprocesses.

2) *Mutation and genetic engineering*: to improve the performance of microbial strains. Genetic engineering relies on our recently acquired basic knowledge of molecular genetics and protein structure. Indeed, many microbial enzymes are now produced on a commercial scale using good producer microorganisms containing the gene for the enzyme derived from another microorganism in which it occurs naturally, but which are less easy or less efficient to use on an industrial scale.

3) Efficient use of biocatalysts requires a thorough knowledge of the *biological chemistry of how enzymes work*, particularly since molecules of precise chemical structure are usually required as products.

4) The successful development of large-scale processes requires a detailed knowledge of *microbial metabolism and physiology* as well as biochemical engineering and process control engineering.

5) Expert knowledge about the physico-chemical properties and behaviour of biochemicals, especially labile molecules and biopolymers, is very important for their successful purification and isolation, so called '*down-stream processing*', which is very often low yielding and expensive compared to chemical processess.

6) Analysis, especially of biopolymers and stereoisomers.

Very many factors contribute to the successful development of a flavour product. As well as the basic research and bioprocess development these include patenting, market assessment, regulatory and safety, applications including formulation, and manufacturing including process design and costing. Important industry driving forces include both market pull (Table 7) and technical push factors (Table 8) so that ingredients are chosen on the basis of their quality, cost, availability, novelty etc. In addition, flavours are not necessary confined to human foods and beverages, as some of the biggest markets for flavoured products are in animal foods.

3 Stereochemical Considerations

It is very apparent that the particular isomeric form of the flavour molecule is often very important in dictating its flavour properties and value. For instance α-Aspartame, made from L-phenylalanine is very sweet, but β-Aspartame made from D-phenylalanine is bitter; the menthol molecule has 3 chiral centres so that 8 possible isomers are possible, but it is only the L-menthol isomer that has the very desirable mint taste and cooling sensation; D and L carvones taste of dill/caraway and spearmint respectively; L-monosodium glutamate has flavour

Table 7. Industry driving forces – 'Market Pull'

- Increasingly, consumers are demanding natural 'environmentally friendly' and 'healthy-eating' products made from renewable resources, or tested without cruelty to animals, and the use of such ingredients both in foods and personal products. This creates marketing and labelling opportunities for the manufacturer. Products manufactured by biotechnology processes are preferred, especially as they are able to produce natural or nature-identical, stereochemically pure products that are much more readily biodegradable. This is in contrast to more traditional, chemical processes, which generally produce racemic products that often contain non-nature-identical isomers that are often less easily biodegradable.
- The decline in the availability of tranditional raw materials, particularly as modern markets have increased in volume significantly. Such materials include many plant-derived materials, including essential oils. Often these materials are obtained from countries that are frequently subject to climatic and/or political instabilities, causing the supplies and prices of the materials to be variable.
- The increased size of modern markets for flavoured or fragranced products, including both the bulk, low-priced fragranced products, convenience foods and beverages, as well as medium-priced products such as mass-market fragrances.
- Changes in food processing and cooking methods such as the use of extruders to produce snack foods and the flavour loss during food processing of microwave cooking and 'cook from frozen' foods. Also the provision of more stable and easier to use ingredients, a good example being in bread making where the skill elements of baking has been significantly reduced as more easy to use ingredients have become available.
- The need to create ingredients with improved in-use performance to increase not only the taste intensity and complexity, but also the mouth-feel, appearance and stability of foods. Therefore the interactions of the flavour molecules with other food components have to be studied and optimized. Similarly, the interaction of fragrances with other ingredients is also very important.
- The industry is becoming more international in character, and thus, ingredients must be suitable for use in products that have been modified to suit individual preferences in quite different countries with very different traditions.
- A very wide range of flavours from fruits, vegetable, meats, beverages and flowers have to be simulated, including new tastes such as exotic-fruit flavours and novel fragrance materials.
- Continued and intensified pressure on costs by competitors, both from alternative ingredients and from new producer companies, must be combatted, for example, by developing cheaper and higher-yielding processes.
- Considerable efforts are continually being made to develop even cheaper, good quality-flavour materials. Consequently, a vital achievement of the fragrance industry over the past 80 years or so has been to reduce the price of most fragrances greatly by the development of much cheaper processes. This has made fragrances available to ordinary people in developed countries, and not only just to the richest members of society who, formerly, were the only people able to afford such expensive, prestige products.
- Increasingly, consumers are expressing health concerns and are demanding products with low salt, sugar and fat, and high fibre, vitamin and antioxidant contents.
- Minimal regulatory requirements, because of the time, costs and risks in gaining approval.
- Flavour and fragrance materials that also possess additional, different functionalities, such as attractive colour or antimicrobial activity, are particularly appealing. Such additional, useful properties may also include physico-chemical factors. For example, mouthfeel and texture in the case of foods, and in the case of fragrance, improved substantivity would enable the fragrance to persist and remain effective on the skin or on clothes for a longer period of time following its application.
- Any combination of the above and/or other commercial driving forces could constitute the identification of new, high-value high-profit margin and/or large-market products. In general the use of synthesised flavours is stimulated by any increases in demand for processed foods and beverages, of which they are usually an essential component. In turn research tends to focus its efforts onto the most useful 'key' molecules which, because of their value, can justify research most easily.

Table 8. Industry driving forces-technical push factors

- Improved analytical methods.
- Availability of new and/or lower cost raw materials.
- Higher efficiency, lower capital or operting cost equipment.
- Improved formulation and delivery techniques.
- The use of plants as sources of both enzyme systems and biochemicals, particularly when modified by plant breeding or genetic engineering to improve their processing properties and yields.
- The use of enzymes to modify biopolymers, thus extending the current work on the biotransformation of small molecules to much larger molecules, which do not necessarily have precisely defined chemical structures.
- Improved product isolation, purification and concentration methods (downstream processing), particularly when the number of separate operations in a process can be reduced, and/or when milder operating conditions can be used. Particularly attractive goals are in situ product recovery techniques.
- The use of genetic engineering, not only to clone enzymes into more efficient host organisms, but also to modify the metabolism of biocatalysts, and perhaps even to create significantly improved biocatalysts using, for instance, protein-engineering techniques.
- Development of a good, predictive understanding of the structure-function relationships of falvour and fragrance molecules, including how they interact with receptors in the mouth and nose. (The sense of smell can be $\sim 10^4$-fold more sensitive than the sense of taste.)

enhancing properties but D-MSG does not. Other related phenomena are also seen; the *cis*- and *trans*-2-hexenals have very different flavour thresholds. Similarly the *cis* and *trans* isomers of whiskey lactone (3-methyl-4-octanolide) have odour threshold values of 0.79 ppm and 0.067 ppm respectively. (*R*)-γ-decalactone occurs in most fruit, but the (*S*)-isomer is produced by some mangos. Yellow passion fruit contain mostly (*S*)-(+)-hydroxybutanoate, but purple passion fruit contain mostly the (*R*)-(−) isomer.

Biological reaction usually produces just one isomer, because enzymes have a three-dimensional shape specificity for their substrates molecules. By comparison, chemical processes are usually non-selective and much less environmentally friendly, particularly when reagents are used in stoichiometric amounts, or require heavy metal catalysts. Therefore, if a particular isomer is required then use of an enzyme-based process is natural, although, of course, very good asymmetric chemical syntheses have been developed such as the Niori route to L-menthol.

4 Technical Basis

The technical basis of the flavour industry lies in four main areas.

First, there is the extraction of flavours and essential oils from plants and, to a lesser extent, animal sources. For example, it requires around 5×10^6 jasmine

flowers to produce 1 kg of jasmine extract, but a good quality jasmine extract's value is comparable to that of precious metals!

Second, chemistry has played the most important role up to now. Analytical chemistry has been used to detect and identify those molecules with the most important organoleptic qualities (taste and/or smell and mouth-feel), and synthetic organic chemistry has been successful in creating efficient processes, often using cheap, petrochemically derived raw materials. This began in 1874 with the synthesis of vanillin by Haarmann, and chemically synthesised ingredients (rather than ingredients from plant and animal sources) were first used successfully in perfumery in 1922 when aliphatic nitriles were used to create 'Chanel No. 5' (which still remains a popular brand even today). For an overview of the role of chemistry in the synthesis of aroma chemicals see [2]. Chemical synthesis still remains a lower cost production technology than biotechnology; after all it is a thoroughly mature, rather than emerging technology. Where biocatalysis can have the advantage is in selectivity of reaction and the natural cachet that can apply to products made using enzymes.

Third, traditional fermented foods such as beer, wine, vinegar, bread, milk, cheese, yoghurt, vinegar, soy and tempeh have provided a good starting point for modern biotechnological research and are now benefiting from the advances in bioprocess engineering originally made in the production of antibiotics and in other industries. This is often in the form of improved and more reliable starter cultures. Indeed, even some food spoilage is desirable, such as the '*Botrytis cinerea*' that produces the very desirable flavour of wines such as Sauterne. The variety of chemicals that contribute to dairy flavours is illustrated in Table 11.

Finally, progress has also evolved from an increased scientific understanding of the reactions that take place during cooking and food processing. However, it must be recognised than in many cases the colour and/or taste of a material can be undesirable. Thus, for instance, riboflavin can be produced by fermentation and can be used as a yellow food colourant, but its use is rather limited by its bitter taste and slight odour.

5 Commercial Issues

Numerous issues are important in the successful translation of 'market-pull' factors into R&D strategies that can provide companies with new products and processes that have genuine competitive advantage. Some of these factors are listed in Table 7; for further details see [3].

One important commercial consideration is that flavours are ingredients that are part of a supply chain from raw materials, via ingredients, to formulated materials, through to the packaged final food or personal-care product (Fig. 4). Flavour and aroma chemicals are one of the most profitable and highest rate of growth categories of ingredients for such products.

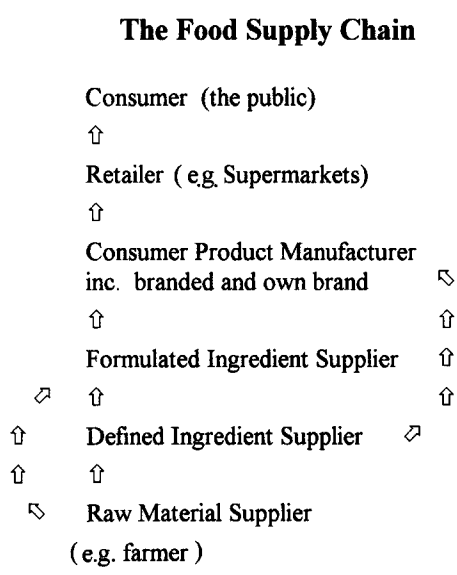


Fig. 4. The food supply chain from raw materials to consumers, illustrating the values added at each stage in the chain

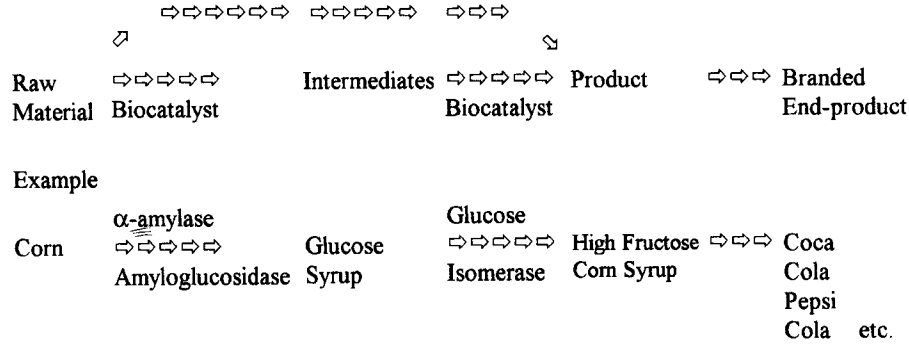


Fig. 5. The value chains created by bioprocessing, such as the conversion of corn starch into glucose syrup, and then into high fructose corn syrups, both steps using enzymes, with the high fructose syrup used as a vital ingredient of Cola type beverages and other products

This chain, in which the values of the materials increase as they move towards the final product, is well illustrated by flavours, in which a raw material is converted into a flavour chemical which is then formulated into a complete balanced flavour, and then incorporated into a final product in order to flavour it. Biocatalysis is, of course, useful in making the transitions from raw materials to defined ingredients, and/or from defined ingredients to complete flavour (Fig. 5).

Because of the long lead time required to go from the start of research through to an established product, cash-flow is a vitally important factor (Fig. 6), as substantial sums have to be spent before this investment even breaks-even. This is especially so if large capital costs are to be incurred, sometimes years in advance of first sales. Therefore, the size of the eventual profit revenues must be big enough to justify the cost of R&D, industrialisation and commercialisation together with a weighting factor to allow for the risk of failure. Therefore it is important to consider the main factors that have driven the increased demand for flavours and aroma chemicals.

Three main factors can be identified. One is the increasing affluence of consumers in 'so called' developed countries and many others that enables most people to be able to afford higher value, and indeed luxury, items. Then there is the growing industrialisation of processed food, beverage and other products that has led to a profileration of branded products containing flavour, aroma chemicals and other key ingredients. Finally there is the improved technology for creating such manufactured products, and in particular methods and the development of formulations to stabilise them, a good example being the ability to stabilise fragrances in the presence of bleaches and in washing powders.

The size of the eventual market must therefore be properly estimated, and this involves the realisation that the market for most products, including flavours and aroma chemicals, is fragmented (Fig. 7). Thus, for instance, the market for a savoury flavour will be subdivided into various applications such as soups, savoury snacks, sauces, cold meats, hot meats etc. Segmentation will

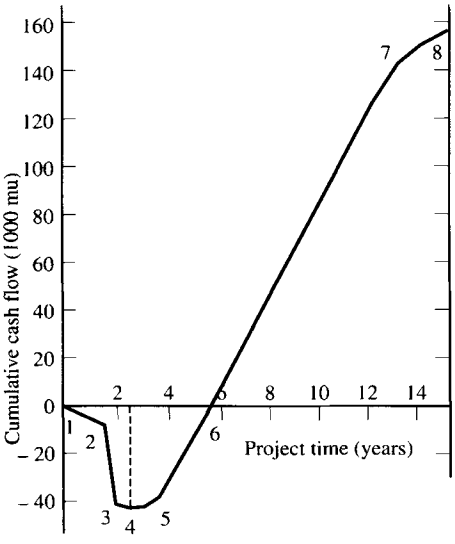


Fig. 6. An illustration of the cash-flow profile through the lifetime of a typical project. Note the large initial expenditure that has to be earned back from profits from sales, which requires successful sales for long after the product starts in order to break even on a per annum income/expenditure basis (adapted from [55])

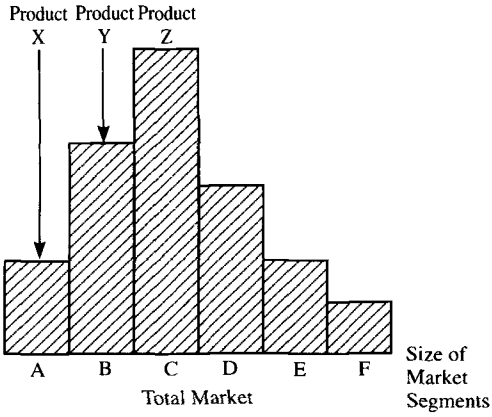


Fig. 7. An illustration of how the market for a product can usually be divided (segmented) into a number of more specific submarkets that can be targeted more specifically as regards the development of improved applications, more appropriate distribution and marketing etc. (from [56])

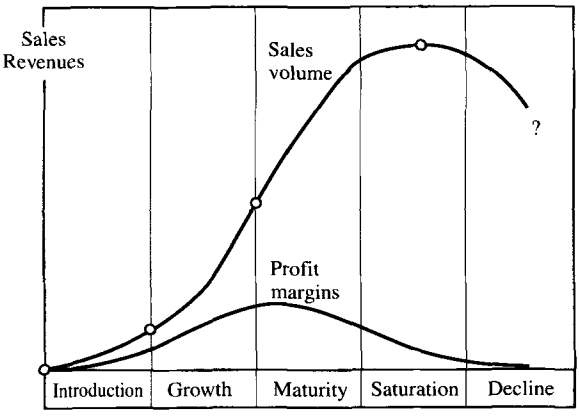


Fig. 8. The typical cycle of a product, illustrating in particular the high profit margins that can be gained early on when the product is new and has no competitors, and how new uses can greatly extend the life of the product and delay or prevent its decline into poor profitability (from [57])

also occur on a price basis and on a geographical basis, with, for instance, cold meat products such as sausages being very important in central European countries and snack products big in the UK.

Finally the product life cycle must be considered (Fig. 8). A good new product will rapidly grow in sales revenues and provide good profit margins, but unless fresh innovation occurs, for instance to find new valued added applications, its profitability will decline as competitors enter the market. This

emphasises the importance of obtaining patent protection, ideally for the product, or alternately for the process to make it, as patent protection gives the patentee the 'negative rights' to oppose in the courts any competitor who contravenes the claims of the patent during the lifetime of the patent. However, the patenting of bioinventions based on novel microbial strains presents particular risks and problems, as deposition of the strain with an accepted Culture Collection is required as a condition of patenting, which according to many, is a substantial over-disclosure of the invention that is not required in other areas of technology patenting.

6 Definitions of Natural Flavours

A special issue that must be addressed as very relevant to bioflavours is the question of definitions of 'natural'. In the USA the Code of Federal Regulations (1990) defines a 'natural' flavour as the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate of any product of roasting, heating of 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 flavourings rather than nutrition.

Thus, as long as natural raw materials are used, the reaction must be carried out using enzymes or microorganisms and the isolation of the product is similarly acceptable provided the product molecule itself occurs in nature. Thus vanillin (3-methoxy-4-hydroxy benzaldehyde) is 'natural' because it occurs in vanilla beans and other natural sources, whereas ethyl vanillin (3-ethoxy-4-hydroxy benzaldehyde) is an artificial flavour, because so far it has not been detected either in foods or in nature.

If the product molecule occurs in nature but is made using a non-natural process, such as chemical synthesis, then it is classified as 'nature-identical'. This rigorous definition of natural even extends to the product isolation procedure, so that even when benzaldehyde, which is used in cherry and other flavours, is made from an acceptable raw material using a biocatalyst, but is isolated by forming a Schiff base addition compound, then it too is classed only as 'nature-identical'. FEMA (Flavour Extract Manufacturers Association) can provide interpretation of difficult cases such as this.

Obviously the 'natural' classification has a prestige cachet and so natural flavours usually command much higher prices than native-identical or synthetic flavours. However, a complicating factor is that different countries can have varying definitions of 'natural', so that transnational marketing on the basis of a single flavour product specification is very difficult.

The superior selling prices and profit potentials of nature-identical, and especially natural flavours, emphasise the need for each and every product of research to 'earn its corn' and make a good contribution to bottom line profits. Hence the importance of financial parameters as follows.

Pay-back-time – this is simply the period of time that the product requires to earn sufficient profit to pay back all of the money spent on R&D bringing it to market.

Return on investment – return on investment (ROI), return on capital employed (ROCE) and internal rate or return (IRR) are terms all referring to the profitability of a project or product in terms of the original investment made, i.e.

$$\text{ROI} = \frac{\text{annual profits}}{\text{original investment}}.$$

Discounted cash-flow rate (DCFR) or DCFY, discounted cash-flow yield – the discounted cash-flow rate (or internal rate of return) is the discount rate at which a projects NPV equals zero. Thus in the equation

$$\text{NPV} = \sum_{t=0}^{t=n} \frac{\text{Cash-flow}}{(1+i)^n}$$

the DCFR is the value of i which the NPV is zero.

7 Flavour Sales and Companies

Sales by flavour and fragrance companies are chiefly mercantile sales to companies using flavours and fragrances as ingredients in their own products. Therefore there are only a relatively small number of large corporate customers and buying decisions are complex, involving, for instance, brand and product managers and technical specialists. Sales are concentrated in developed countries with consumers with relatively high disposable incomes.

Frequent and large changes in usage patterns of flavours and fragrances occur because of rapid changes in consumer demands, including fashion effects. The size of the flavour and fragrance industry world-wide is considerable, estimated at U.S. $ 9.7 billion in 1994 [4]. This is mostly accounted for by consumption in developed countries – U.S.A. $ 2.4 billion, Western Europe $ 3.6 billion, Japan $ 1.5 billion, with the rest of the world $ 2.1 billion. These sales break down into 14.8% aroma chemicals, 17.5% essential oils, 29.2% fragrance compositions and 38.5% flavour compositions [4]. The industry is served by a large number of diverse companies led by International Flavours and Fragrances (IFF), Givaudan-Roure and Quest International (Table 9) with the industry leader IFF having less than 14% of total sales and the top 14 companies together with only 73.4% of sales. Perhaps because of this

Table 9. Estimated flavour and fragrance product sales of leading companies, 1994 (from [4])

Company	M$[a]	% of total sales
International Flavours & Fragrances (US)	1315	13.6
Givaudan-Roure (Switzerland)	980	10.1
Quest International (UK/Netherlands)	930	9.6
Bayer (Haarman & Reimer & Florasynth) (US and Germany)	850	8.8
Firmenich (Switzerland)	708	7.3
Takasago International (Japan)	500	5.2
Bush Boake Allen (US)	375	3.9
T Hasegawa Co (Japan)	356	3.7
Dragoco Gerberding & Co (Germany)	260	2.7
Tastemaker (US)	210	2.2
Universal Flavours (US)	180	1.9
V Mane Fils (France)	160	1.7
Ogawa & Co (Japan)	158	1.6
Robertet (France)	130	1.3
Subtotal	7112	73.4
Other Companies	2575	26.6
Total	9687	100

[a] Flavour and fragrance sales only; does not include sales of non-flavour and fragrance products such as food additives, speciality chemicals, juices, ice-cream and beverage bases

fragmented industry structure, but perhaps because some of the largest flavour and fragrance companies are owned by massive multi-nationals, for instance Givaudan-Roure by Hoffmann LaRoche and Quest Int. by Unilever, there has been considerable growth by acquisition. For instance in 1995 Lautier Florasynth was purchased by Haarmann and Reimer who are owned by Bayer. Another motivation is to diversify into new but related areas, and in 1995 Quest International bought the food emulsifiers business of Eastman Chemical Company, and Haarmann and Reimer bought the acidulants and enzyme interests of Miles Laboratories. This acquistion trend also applies to smaller companies such as the acquisition of J. Flavours by Robertet, and F&C Ints fragrance interests by Technology Flavours and Fragrances. The other strategy to increase critical operating size is by merging, such as between Hercules flavour interests and Mallinkrodt to form Tastemaker, and between Rudolph Wild and McCormick [4].

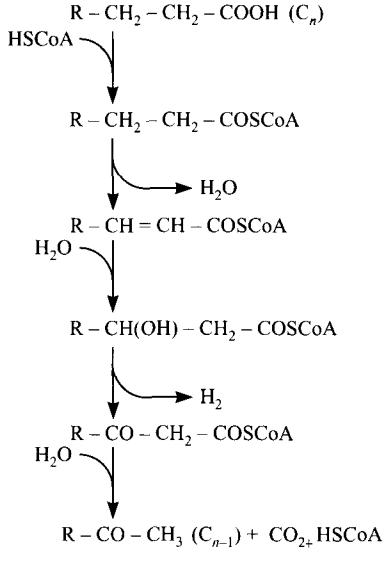
In any survey of the flavour industry it is important to note that the value of the materials used to make a flavour and fragrance are greatly enhanced upon formulation into complete flavours or perfumes. For instance the creative flavourist or perfumer achieves synergistic effects between the various individual components and gives individuality and character to the product. Flavour and fragrance companies are rarely backwards integrated into the production of specialised raw-materials such as plant-derived essential oils and are also never forwards integrated into the sale to consumers of finished products. Horizontal diversification into related areas such as food ingredients is, however, becoming more common.

8 Bioresearch

Three main opportunities can be identified.

1) Development of a new process that will compete successfully with either an already well-established chemical process, or with.
2) A well established first generation fermentation or enzyme process.
3) The development of new processes for new functional molecules or materials, in which case a major problem is the costs and time required to achieve regulatory approval.

How have new processes been developed? One way is by studying traditional products and then applying modern bioprocess techniques to them. For instance, the distinctive taste of Roquefort cheese is substantially due to methylketones produced by *Penicillium roquefortii*, which also gives this cheese its blue veins. The details of the biochemical pathway whereby the microorganism converts fatty acids into methylketones have been elucidated and have been used as the basis for a large-scale fermentation process. Commercially successful microbial processes for making methyl ketones have been used for some time by companies like Unlever and Bush Boake Allen. The way microorganisms such as *Penicillium roqueforti* produce methyl ketones from medium chain fatty acids is of considerable scientific interest as it resembles conventional β-oxidation until the final step, at which a decarboxylase produces the methyl ketone, replacing the thioesterase found in β-oxidation. Spores are more active in producing methyl ketones than mycelium, and solid state fermentations are sometimes used. For instance, Bush Boake Allen's process involves mixing



cellulose powder with coconut oil, which has a high octanoic acid content, and inoculating with *Aspergillus niger* spores. The octanoic acid is then metabolised into 2-heptanone which has a strong blue cheese flavour [5].

Another example of the use of enzymes is the production of lipolysed milk fat. This process uses selected lipases to liberate just those medium-chain fatty acids that have the best flavour qualities. An extension to this approach is the use of lipases and proteases to produce so-called enzyme-modified cheese flavours. Such a process can produce good, well balanced flavour products with 20 times the flavour of mature cheeses.

9 Flavour Production Using Intact Microorganisms

The use of enzymes and microorganisms is biotransformation has been particularly successful in the production of sweet, fruit-flavour molecules. This is because it has proved relatively easy to simulate the enzyme processes that produce such flavour chemicals in plants, by using microorganisms or isolated enzymes. Savoury flavours are less easy to produce enzymically as these are generally produced non-enzymically during cooking, for instance by Maillard reactions between sugars and amino acids. (These sugar and amino acid raw materials are however potential targets for biotechnological research.) However, even with biological processes using pure strains of microorganisms, care must be taken, as quite basic factors can also have big effects. Table 10 shows how varying the carbon and nitrogen sources available to *Ceratocystis moniliformis* can have a very big effect on the aroma produced and the aroma chemicals present.

Table 10. Effect of carbon and nitrogen source on aroma production by *Ceratocysis moniliformis* (from [53])

Carbon source	Nitrogen source	Aroma	Chemicals identified
Dextrose	Urea	Fruity, banana	Acetate esters, ethanol
Dextrose	Leucine	Fruity, over-ripe banana	Isoamyl acetate, ethanol
Galactose	Urea	Citrus, grapefruit lemon	Monoterpenes, ethanol
Corn starch	Urea	Cantaloupe, tropical flower, banana	
Dextrose	Glycine	Pineapple, lemon, sweet	
Dextrose	Methionine	Weak potato	
Glycerol	Urea	Canned pear, peach	Decalactones, ethanol

10 Traditional Processes

In order to illustrate the value of bioprocessing to the traditional flavour industry, examples from two specific flavour area have been selected.

10.1 Dairy Flavours

Dairy flavours are especially interesting because of their flavour complexity and the variety of different yoghurts, cheeses etc. that is available, and also because of the recent market changes, especially the movement away from dairy products because of the perceived undesirably high saturated fat, cholesterol, and salt contents, which has been accentuated by changes in labelling requirements that ensure more complete disclosure of the ingredients used in products (for a review see [6]).

Nearly all dairy flavours are produced by fermentation processes, involving a great variety of microorganisms and producing a range of chemically quite different flavour chemicals. These include pyrazines that occur in cheese, and that are produced by strains such as *Pseudomonas perolens* and *Ps. taetrolens* and *Corynebacterium glutamicum.* Diacetyl (2,3-butanedione) contributes to milk and other dairy flavours and is produced by *Lactobacillus lactis.* It is formed from citric acid via acetolactate and its formation is stimulated by the addition of citric acid and by acid pH, which is the optimum pH of the citrate transport system. Methanethiol and its esters are also important contributors to some cheese flavours, such as Limburger, and are made by bacteria such as *Brevibacterium linens* and *P. freudenreichii.* Other important cheese flavour chemicals include butyric acid, propionic acid, γ and δ lactones and also the methyl-ketones produced by fungal *Penicillum* species that are a characteristic of blue cheeses.

The initial cheese fermentation is carried out by *Lactobacilli*, such as *L. casei.* The subsequent ripening of the cheeses is much more idiosyncratic. Additional surface ripening by bacteria or fungi occurs in many cases, adding much of the flavour characteristics of particular cheeses. Quite often a mixed population of strains is present with successive growths of different microbial strains. For instance, with Roquefort, Camembert, Brie and others, the initial growth on the surface is of yeasts such as *Kluyveromyces lactis* or *fragilis, S. cerevisiae* or *Debaryomyces hansenii,* which deaminate amino acids and oxidise lactic acid causing an increase in pH. This increased pH encourages the growth of *Penicillium roqueforti* that gives the cheese its blue colour, especially when holes are bored into the cheese to allow the penicillium to colonise the interior of the cheese. *Penicillium roqueforti* produces lipases that liberate free fatty acids that not only contribute to the flavour of the cheese but are also the substrates for the production of methylketones that are absolutely characteristic of blue cheese flavour, and also of δ lactones. In addition, *Penicillium roqueforti* has a good

proteolytic activity so that extensive hydrolysis of the cheese proteins can take place producing large amounts of peptides and amino acids, which contribute to flavour and can be further deaminated into other flavour molecules.

Similar processes occur with different cheese varieties. For instance, Camembert cheese is surface ripened by *P. camemberti*, and Brie cheese by a mixture of *P. camemberti* and the bacterium *Brevibacterium linens*. *Brevibacterium linens* acting alone is responsible for the surface ripening of Limburger and other similar cheese varieties. It acts only after salt-tolerant yeasts have raised the pH to about 6. By contrast to *Penicillium roqueforti*, *Brevibacterium linens* does not produce lipases but proteinases, and then metabolises the resulting amino acids producing a range of flavour chemicals such as 3-methyl-1-butanol, phenylethanol, methanethiol and many others.

Table 11 lists some of the key aroma chemicals involved in producing the flavour characteristics of a wide variety of traditional dairy products. At the beginning of research about 25 years ago, the focus was on how to accelerate the ripening of cheese to make storage cost savings. Now control of flavour quality or even the creation of new flavours in order to make distinctive new branded products is the priority. The most important goal is to discover and then make those flavour compounds that are responsible for specific flavours. Here lactic acid bacteria are vital in ensuring a rapid and balanced flavour development without excessive accumulation of bitter tasting peptides.

Over the last decade more and more specially selected starter cultures have been made available for particular quality goals and product types, usually from specialised starter culture producing companies. The lactic acid bacteria (LAB) have several roles. They are crucial during the cheese manufacturing process. By metabolising lactose into lactate during growth, the LAB are responsible for the desired pH drop and thus the coagulation of the milk during manufacturing, which at the same time allows control of the moisture level in the final cheese. During the ripening process the LAB in the cheese are crucial for obtaining a well balanced proteolysis rate by releasing a set of proteolytic enzymes. These

Table 11. Flavour chemicals that contribute to natural dairy milk flavours

Molecule	Precursor	Occurrence
Dimethylsulfide	Methionine	Milk
Diacetyl, acetoin	Citric acid/lactose(pyruvate)	Butter
γ-Decalactone	Milk triglycerides	Butter
γ-Dodecalactone		
δ-Decalactone		
γ-Dodecalactone	6-dodecanoic acid from linoleic acid	Milk fat and meat defect
Acetaldehyde/lactic acid/ethanol	Lactose (pyruvate)	Yoghurt and other sour milk products
Fatty acids and others e.g. methylketones	Triglycerides	Cheese
Acetaldehyde, acetoin	Diacetyl etc.	Cottage cheese

include both proteinases located on the surface of the cells and intracellular peptidases, as only peptides smaller than about ten amino acids can be transported into the cells. The objective is a non-bitter tasting protein hydrolysate, and this is most easily obtained using LAB because they contain a variety of enzymes with different amino acid specificities. In addition, lactic acid bacteria carry out microbial preservation and stabilisation of the milk, generate specific flavours, e.g. of yoghurt, cheese, produce texturing capabilities like gel formation in yoghurt via the production of polysaccharides, and also give probiotic, and health beneficial properties, especially when specifically selected starter strains are used.

Now specially selected lactic acid bacteria have been developed that make possible shelf-stable yoghurts. This is because yoghurt results from a growth association between *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, but it is the *Lactobacillus bulgaricus* that contributes most of the flavour and creates most of the acidity, whereas *Streptococcus thermophilus* produces only a very mild and unaromatic product. They grow in milk where they ferment lactose to lactate and lower the pH from neutral to about 4.5–4.2. Upon storage of the final product at 4–12 °C for several days (in the supermarkets or at the consumer's home), the pH of the yoghurt may drop further to values below 4.0. This post-acidification leads to a gradually increasing acid and bitter taste of the yoghurt, thus degrading the initial organoleptic quality of the product. This problem has been overcome by selecting spontaneous *L. bulgaricus* mutants that lack β-galactosidase activity and so are unable to grow on milk alone, as pure cultures, without accompanying *S. thermophilus* present to provide energy source. Thus once growth of *S. thermophilus* ceases, so does growth of *L. bulgaricus*, thus limiting the drop in pH [7, 8].

10.2 Savoury Flavours

These include hydrolysed vegetable proteins and yeast proteins (Fig. 9) and yeast extracts that contain not only flavour chemicals but also flavour enhancer molecules such as monosodium glutamate, guanosine 5′-monophosphate and inosine 5′-monophosphate. These are quite remarkable products, as these cost-effective meat-like flavours are made from entirely vegetable raw materials (for a review see [9].

Their success is indicated by an annual production of MSG of 500 000 tons per annum, mostly by fermentation using specially selected strains of *Corynebacterium glutamicum*. These strains have a low α-ketoglutarate dehydrogenase activity that prevents the formation of succinate, and so instead leads to glutamate formation by reductive animation of the α-ketoglutarate. In addition, successful and high yielding fermentations depend on the use of media that have very low biotin contents. This makes the cell walls of the microorganisms leaky so that the glutamate passes out of the cell and accumulates in the medium.

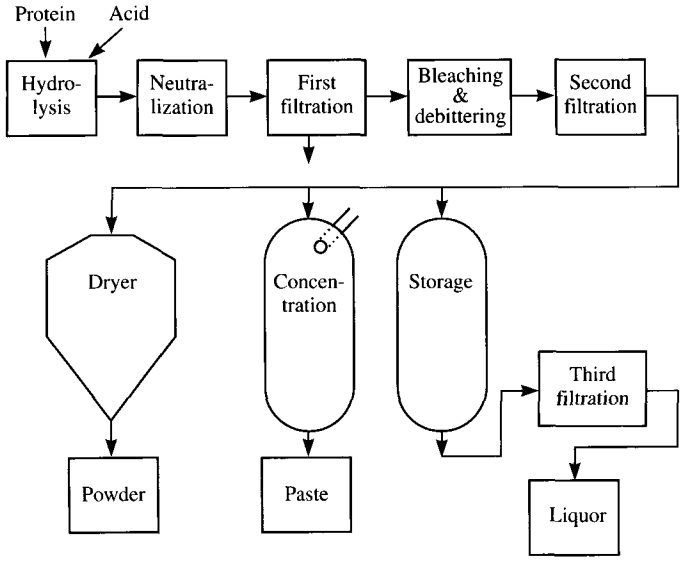


Fig. 9. Flow diagram of the manufacturing process for protein hydrolysates (from [9])

A good command of biochemical processes is also necessary for 5′-nucleotide production (Fig. 10). Strains of *S. cerevisiae* (8–11% RNA) or *Candida utilis* (10–15% RNA) are selected to have a high RNA content. RNA is released from cells and is then hydrolysed by 5′-phosphodiesterase enzymes into 5′-nucleotides. Good sources of the phosphodiesterases include *Penicillium citrinum* or malt rootlets. Of the four nucleotides formed only the 5′-guanosine MP has taste-enhancing properties, but additional flavour enhancing and financial value can be gained by the addition of adenyl deaminase. This enzyme converts 5′-adenosine MP into 5′-inosine MP, which markedly potentiates the flavour-enhancing properties of the GMP already formed.

11 Gamma Decalactone Production

The synthesis of γ-decalactone using intact microorganisms is a particularly good example of the use of biotransformations in the production of flavours because it required the development of truly innovative technology and has made a big commercial impact. The need for a natural source γ-decalactone can be illustrated by a chemical analysis of strawberries. At least 350 molecules are thought to contribute to the taste of strawberry, including γ-decalactone and over 100 esters.

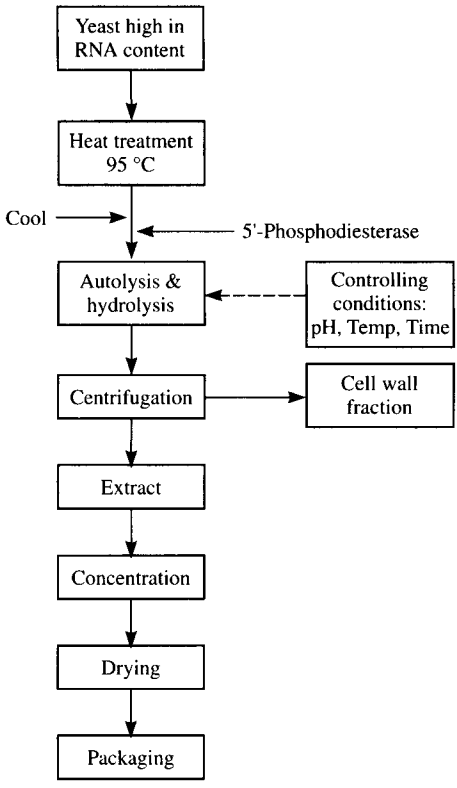


Fig. 10. Flow diagram of the manufacturing process for 5'nucleotide-rich yeast extract as a flavour exhancer for foods (from [9])

This molecule is also an important component of many fruit flavours and is an especial key element of peach and apricot flavours. However, head-space analysis of the chemicals released by strawberries shows that γ-decalactone is rapidly lost once the fruit has been picked, presumably due to metabolism by enzymes activated by the trauma of harvesting. This 30-fold reduction is an important cause of the loss of strawberry-flavour quality post-harvest. In contrast, a similar head-space analysis of the aroma chemicals released by growing and harvested ripe peaches showed a 16-fold rise in γ-decalactone in the harvested fruit [10]. Similar changes (both increases and decreases) have been found in the amounts of aroma chemicals produced by flowers when the air surrounding growing and picked flowers is sampled and analysed.

Thus, γ-decalactone is important in creating high quality fruit flavours. Although γ-decalactone can be synthesised easily by conventional chemistry, the consumer demand for 'natural' flavours created a need for a biotechnologically produced material. In addition, γ-decalactone is a chiral molecule, and

molecules extracted from fruit have a defined stereospecificity. The (*R*)-isomer predominates in peaches and most other fruit, but appreciable amounts of the (*S*)-isomer are found in some varieties of mango. Chemical synthesis of γ-decalactone only yields a racemic mixture. Therefore, a microorganism capable of forming optically active γ-decalactone was sought by a number of companies by screening naturally occurring microorganisms. This search was aided greatly by the earlier observation by Japanese workers that an unlikely source, castor oil, provided a very good precursor molecule [11].

Castor oil is predominantly an ester of ricinoleic acid, an unsaturated, C18, 12-hydroxy fatty acid, which contains a C10, 4-hydroxydecanoic acid substructure. Heating 4-hydroxydecanoic acid under conditions of acid pH results in the formation of γ-decalactone in almost quantitative yields from the ricinoleic acid that is metabolised. A variety of microorganisms, chiefly yeast's was found that has lipase activity to hydrolyse the castor oil, that was able to tolerate the fatty acids produced, and, most importantly, could carry out partial β-oxidation of ricinoleic acid to form 4-hydroxydecanoic acid. As a result, a process involving the growth of a specially selected yeast strain in a medium containing peptone, yeast extract and ricinoleic acid, followed by treatment of the fermentation broth by pasteurisation, acidification, clarification by centrifugation or microfiltration extraction with butylacetone, separation, solvent extraction, distillation and fractionation has been developed. This approach has been patented, scaled up to yield up to 10 g product per litre and, for several years has successfully produced γ-decalactone for a wide range of commercial uses (Fig. 11) [12–16].

Subsequently, this method has been extended and improved. Esters of ricinoleic acid, prepared by transesterification, are better precursors because they form less foam and emulsion in the fermenter than castor oil. Mixtures of γ-decalactone and unsaturated lactones with new organoleptic qualities have also been made. Addition of glycerol also stimulated γ-decalactone formation. Initially this was thought to be an effect of water activity, but in fact the glycerol was acting as a metabolic precursor of lactone formation. Quite different approaches have also been explored, such as the preparation of 5-decanolide and 5-dodecanolide by the use of *S. cerevisiae* to saturate unsaturated lactones that can be extracted from Massoi bark oil [17].

Using the hydroxyacid approach, yeasts have been used to produce δ-lactones from 11-hydroxypalmitic acid obtained from sweet potatoes or jalap resin (Fig. 11). In this process, the yeast has an absolute requirement for an odd number of carbon atoms between the carboxyl group of the fatty acid and its hydroxyl group [18]. An alternative approach for biolactone production is to produce the hydroxy fatty acid intermediate by microbial hydroxylation of a fatty acid. *Mucor* species can produce 11-hydroxdodecalactone from dodecanoic acid or its esters, rather than having to rely on a preformed source of the hydroxy fatty acid intermediate. Similarly, octalactones can be formed by the fermentation of coconut oil caprylic acid (*n*-octanoic acid) by *Mortierella* species to form the readily lactonised γ-hydroxyoctanoic acid or ester [19].

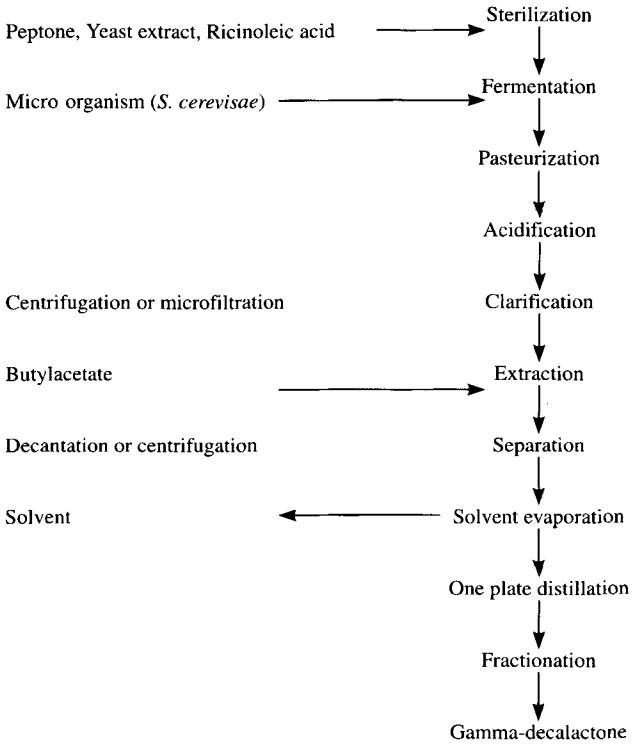
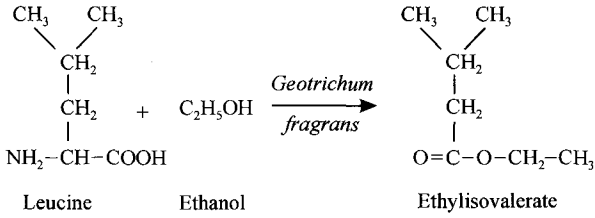


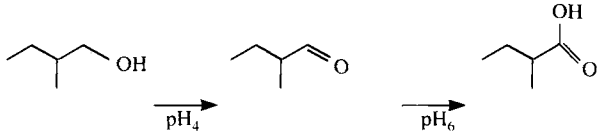
Fig. 11. Flow diagram of the manufacturing process for the natural flavour chemical γ-decalactone (from [12])

12 Other Fruit and Related Flavours

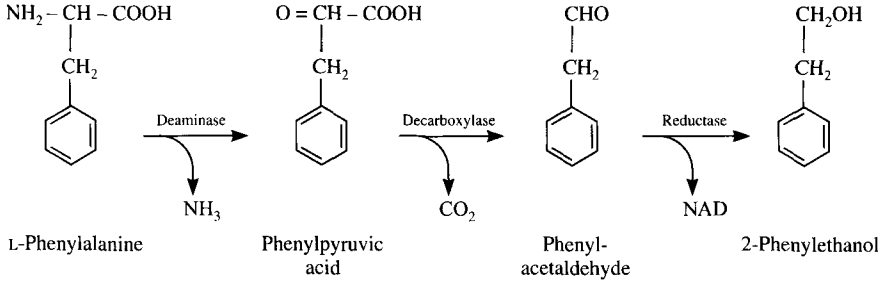
A similar approach has been successful for producing 2-phenylethanol, an important component of some fruit and beverage flavours and rose fragrances. A yeast was used that partially metabolises L-phenylalanine by deamination, decarboxylation, and then reduction, but carries out very little subsequent metabolism, enabling high yields of 2-phenylethanol to be obtained by solvent extraction of the fermentation broth. Yeast strains with improved abilities to make 2-phenylethanol were obtained by selecting strains of yeast that are resistant to the phenylalanine analogues or *p*-fluorophenylalanine [20]. This process is aided by plentiful cheap supplies of L-phenylalanine which is now produced extensively as a precursor of the high-intensity sweetener, Aspartame. More recently Mazoni et al. have used *A. aceti* to convert 2-phenylethanol into phenylacetaldehyde in yields of greater than 50% [21].



Esters are very important flavour chemicals. Gatfield and co-workers have pioneered the use of lipases and esterases 'in reverse' to synthesise esters. Strains of *Acetobacter* are used to synthesise methylbutanoic acids, which are valuable precursors of the esters that are common components of many flavours. The microorganisms oxidise methyl butanols obtained from fusel oils, which are a by-product of the distillation of alcoholic fermentations. The process operates as a fed-batch system to prevent the toxic effects of the methyl-butanols (Fig. 12) [12]. A double oxidation process is involved operating via the aldehyde intermediate, with the enzymes maximally active at different pHs. For instance:



Another bioprocess for synthesising esters uses *Geotricium fragans.* This microorganism partially metabolises leucine or related amino acids such as isoleucine by oxidative deamination. The addition of ethanol to the reactor results in esterification forming flavour esters such as ethylisovalerate in a one-step reaction. The product can be recovered easily from the exit gases by adsorption onto activated charcoal.



Other key flavour chemicals produced by microorganisms include *O*-methyl-anthranilate which is a key flavour character impact molecule of concord/lambrusco grapes and is also used in perfumes. Selective microbial *N*-de-methylation of *N*-methyl-methylanthranilate, which is readily available from

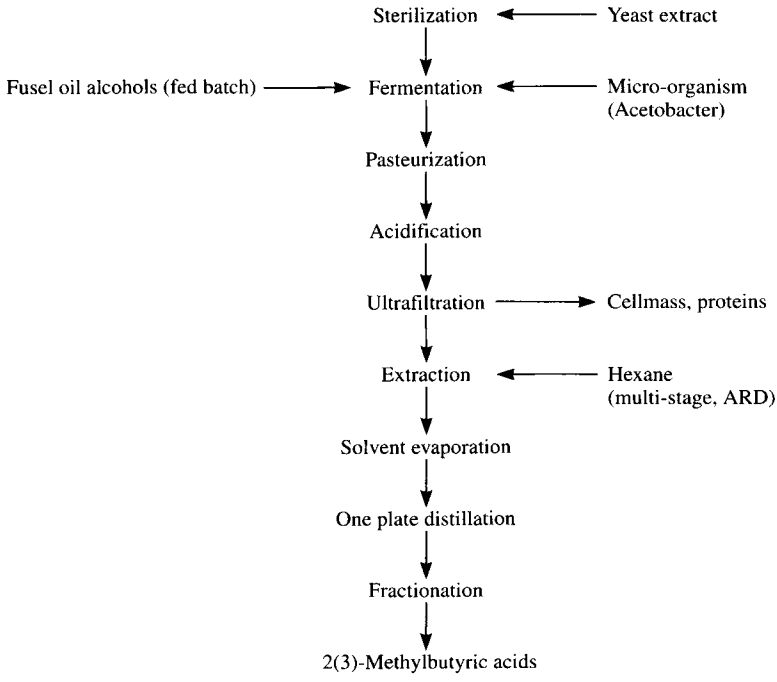
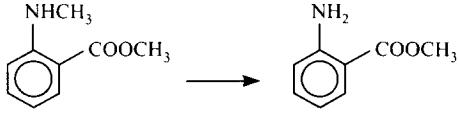
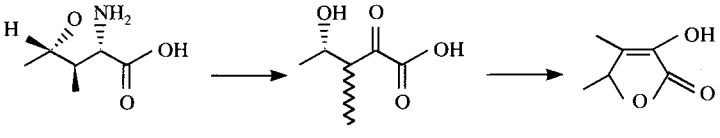


Fig. 12. Flow diagram of the manufacturing process for the natural 2 and 3-methyl butyric acids used to make a range of ester natural flavour chemicals (from [12])

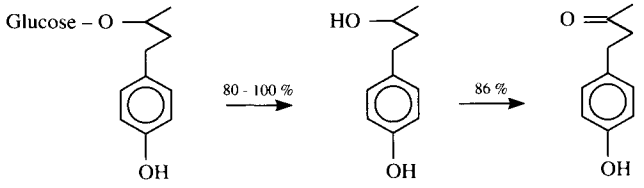
petitgrain mandarin leaf oil; without significant *O*-demethylation has been reported using *Trametes versicolor* and related microorganisms [22].



More recently discovered processes include those for sotolone which occurs in lovage, fenugreek, sherry and raw sugar. This involves a deamination of the precursor, 4-hydroxy isoleucine, that is present in fenugreek seed, into 3-keto-3-methyl-4-hydroxy valeric acid, followed by cyclisation into the sotolone (4,5-dimethyl-3-hydroxy-2(5*H*) furanone).

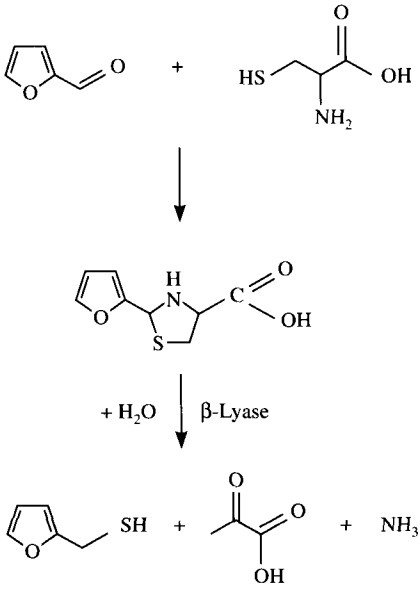


Raspberry ketone can be made from a glucoside (betuloside or rhododendrine) present in white birch bark by treatment with β-glucosidase to release the phenol and then selective oxidation of the secondary group o-hydroxy group on the side chain using *A. aceti* to form the product 1-(4-hydroxyphenyl)-3-butanone.



A good example of a microbial oxidation process is the biosynthesis of the grapefruit flavour characteristic sesquiterpene nootkatone from the much cheaper and more abundant valencene by a double oxidation process involving regio-selective hydroxylation and then oxidation to the ketone (nootkatone) [23].

Finally a very interesting example is the use of β-lyase possessed by *Enterobacter cloacae* to produce furfurythiol by cleavage of both C–N and C–S bonds [17]. Product inhibition was marked, but by using continuous removal of the volatile product, yields greater than 50% were obtained.



13 Organic Acids

Organic acids are frequently key components of flavours; for instance the role of lactic acid produced by *Lactobacilli* in dairy products and acetic acid produced by *Acetobacter aceti* in vinegar-based products are very well known.

Biotechnological routes to other organic acids have also been devised. Propanoic acid fermentation is important for both the flavour and characteristic holes of Emmenthaler cheese. The acid can be produced in batch fermentation by *Propionibacterium* species from lactic acid, producing 6.3 g l^{-1} of propanoic acid and 2.8 g l^{-1} of acetic acid from 18 g l^{-1} of sodium lactate after 5 days incubation [24]. Similarly, succinic acid is important for both savoury, dairy and fermented beverage flavours because of its characteristic sour-salt-bitter taste. Kaneuchi et al. [25] have found that *Lactobacillus reuteri* strains isolated from fermented cane molasses can give up to a 70% molar yield of succinic acid from diammonium citrate.

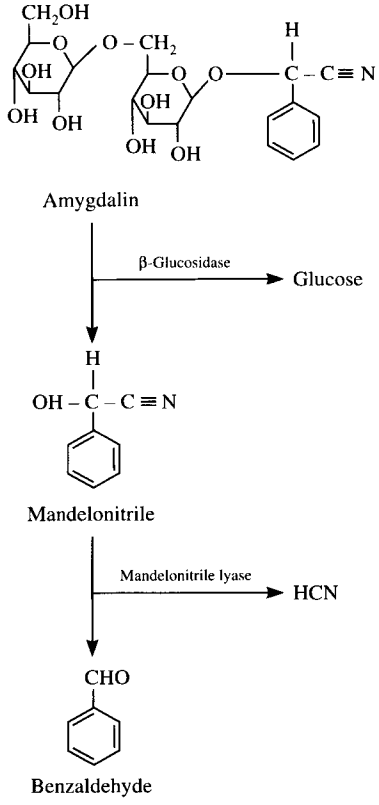
The key problems that had to be solved in developing the processes described above were as follows.

1) Screening to find microbial strains that had the required biotransformation activities and were productive and stable under the conditions of use.
2) The development of cost effective defined media and sources of precursor.
3) The development of cost effective product isolation and purification methods.

Thus, a typical process involves extraction and purification of the precursors, the biotransformation reaction itself and downstream processing of the product into an organoleptically satisfactory form, all carried out at an acceptable cost.

14 Use of Isolated Enzymes

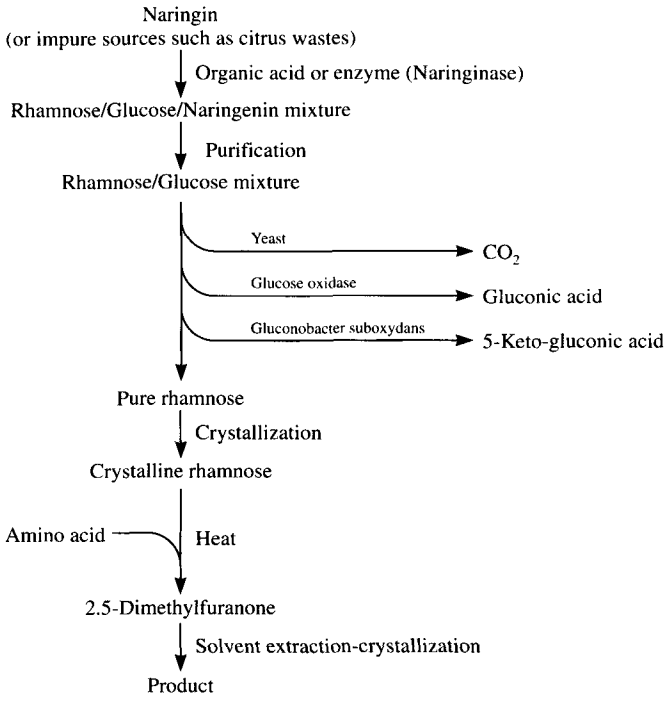
A complementary approach is the use of isolated enzymes. Benzaldehyde can be produced from the cyanogenic glycoside amygdalin, which can be obtained from cherry stones, using β-glucosidase and mandelonitrile lyase. Both these enzymes are present in crude almond meal (emulsin). Similarly, rhamnosidases, which are present in commercial preparations of pectinases, can be used to liberate the 6-deoxysugar rhamnose from the bitter citrus-plant glycosides, naringin and hesperidin. The rhamnose is used as a precursor of the very widely used flavour, 2,5-dimethyl-4-hydroxy-2,3-dihydrofuran-3-one (Furaneol)®, which is found in pineapple and other foodstuff. In this process it is very important that the rhamnose is pure as other sugars, such as glucose, which are liberated by any nonselective hydrolysis of naringin greatly reduce the yield of the furan product. Therefore not only is it important to use rhamnosidases that are as selective as



possible, but also to remove any remaining glucose either by fermentation using immobilised yeast cells or by conversion to gluconic acid using glucose oxidase. The glucose can even be converted by *Gluconobacter suboxydans* into 5-keto-gluconic acid, a good precursor of a savoury, meat-flavour molecule, 4-hydroxy-5-methyl-3(2H)-furanone [26].

Another very good example is the use of enzymes in the production of flavour nucleotides. Thus 5′-phosphodiesterases are used to hydrolyse yeast source RNA (see Fig. 10), and then deaminase is used to convert guanosine monophosphate into inosine monophosphate by removal of the amine group on the aromatic ring, because the IMP has a much stronger flavour-enhancing activity [27].

Alcohol oxidases from yeasts such as *Pichia pastoris* or *Hansenula morphosa* have been found to convert a wide range of alcohols into their corresponding aldehydes in high yield and produce very high final concentrations of the product aldehydes, sometimes higher than 100 g l^{-1}. For instance, conversion of hexanol into hexanal proceeded with a 96% yield. Larger alcohols such as



benzyl alcohol were less reactive in aqueous reactions, but when the reaction was carried out in a biphasic mixture with most of the solvent being hexane, very high productivities were then obtained [28].

Enzymes are used to enhance fruit juice taste. This is possible because many fruit flavour molecules are present as tasteless glycosides in the juice, and can be treated with β-glucosidase to liberate the aglycone and thereby enhance the flavour intensity of the juice (but perhaps also with a change of flavour profile and balance). Since fruit juices have acid pHs a β-glucosidase with an acid pH optimum is required, such as from *A. oryzae.*

15 Vanillin Synthesis

Effective bioprocesses for producing some of the most important flavour molecules have not yet been developed. These include vanillin, which is, organoleptically, the most important component of vanilla. The metabolic pathway that synthesises vanillin in the vanilla bean is still not completely understood. Nevertheless a promising start has been made in developing microbial methods for the production of vanillin. For example eugenol extracted from oil of cloves has been converted into vanillin using microorganisms such as

a mutant strain of *Arthrobacter globiformis* that is deficient in aldehyde dehydrogenase. This transforms eugenol into ferulaldehyde, which is then converted into vanillin by treatment with mild base (Fig. 13) [29]. Another approach has been to synthesise *m*-methoxytyrosine, deaminate it using *Proteus vulgaris*, and then form the aldehyde group of vanillin by treatment with mild base (Fig. 13) [30].

A quite different approach is to use cultured vanilla plant cell, or plant callus tissue. For instance, vanilla plant cells specially selected for high productivity and secretion of vanillin have been grown in tissue-culture medium, and vanillin and other metabolites were produced. However, in comparison with microorganisms, plant cells grow quite slowly and are also very prone to microbial contamination. Thus, this is likely to be a process that will be difficult to be commercialised on a large scale [31].

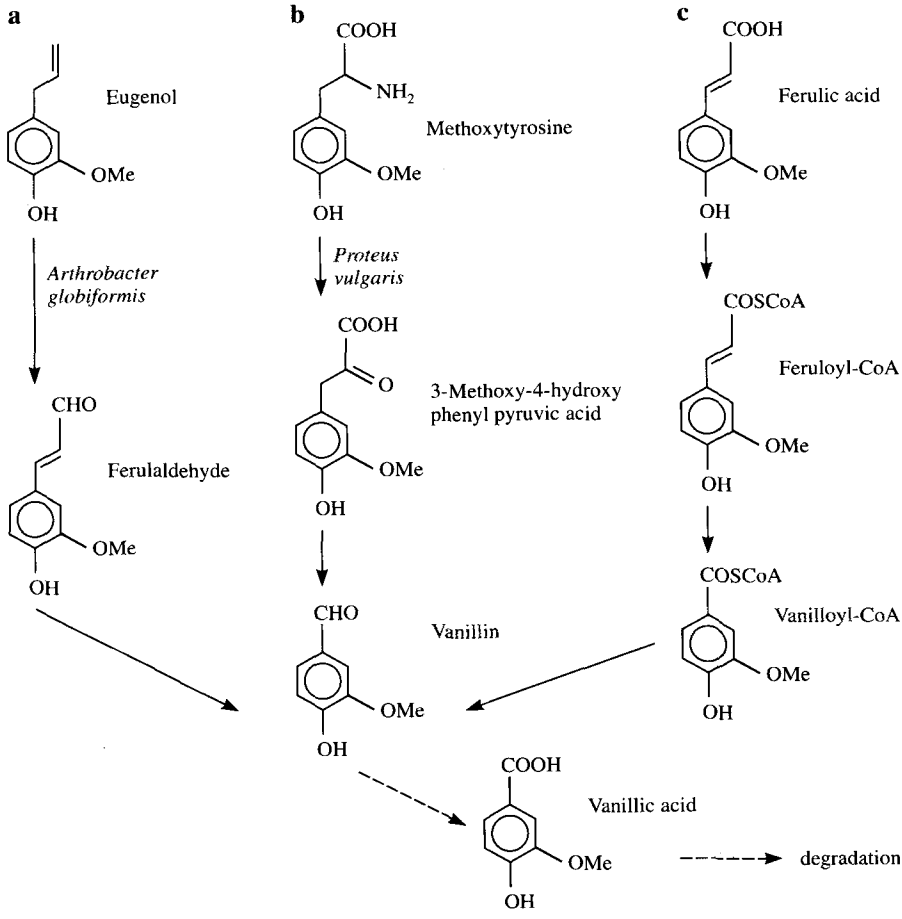


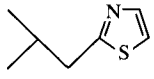
Fig. 13. Various biosynthetic routes that have been used to make vanillin, the key flavour-conferring component of vanilla (from [54])

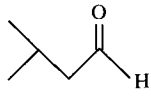
An alternative is to use organised vanilla-plant tissue as the biocatalyst. This is possible because the vanilla plant is a climbing orchid that forms many aerial roots. When young, these roots are covered by numerous fine hairs which give them a high absorptive capacity. It was found that when the aerial roots were incubated with ferulic acid it was converted into vanillin (Fig. 13), which could be recovered from the medium by adsorption onto activated charcoal. The root biocatalyst could be reused by resuspension in fresh ferulic acid solution. Vanillin productivities of 400 mg kg^{-1} (dry weight) tissue per day, and concentrations of 7 g kg^{-1} root tissue were regularly obtained [32].

16 Vegetable Flavours

Some of the most difficult flavour molecules to make are those that give vegetables their characteristic tastes. These are usually examples of 'endogenous biotransformations'. This is because of the following.

- Most vegetable flavours are secondary flavours, i.e. produced from precursors after the plant material has been cut up, heated or fermented; they do not pre-exist in the plant tissue and are not produced per se by the plant metabolism. Exceptions are 2-methoxy-3-isobutyl pyrazine (bell peppers), 2-isobutylthiazol (tomato) and 1,2-dithiolan-4-carboxylic acid (asparagus).
- Most flavours are true secondary products formed from the further metabolism of primary metabolites such as amino acids, carbohydrates and fatty acids, such as



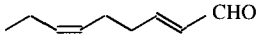
Formed from 

+ $NH_2-CH_2-CH_2-SH$

Isovaleraldehyde

Cysteamine (by decarboxylation of cysteine) which is formed

It is of interest that 2,6-nonadienal, identified as being responsible for cucumber aroma, which is produced from linoleic acid by lipoxygenase, hydroperoxide



lyase and then isomerase, and 1-octen-3-one has now been found to be the key mushroom flavour chemical with a threshold 100 fold lower than 1-octen-3-ol.

It is also a flavour component of other edible fungi and is produced from linoleic acid via lipoxygenase and hydroperoxide lyase.

17 Complex Flavours

In addition to the use of biocatalyst to produce individual flavour molecules, bioprocesses have also been developed to produce chemically complex flavours or flavour blocks, such as those that give soy sauce its distinctive flavour. The traditional process for making soy sauce takes several months. Koji, produced by the solid-state fermentation of soy beans and wheat by *Aspergillus sojae* and *A. oryzae*, is mixed with brine. Enzymes from the koji, including peptidases, proteases and amylases, hydrolyse the biopolymers to amino acids, peptides and sugars, which are then fermented by salt-tolerant microorganisms into a variety of flavour compounds including the taste-enhancer glutamic acid. Lactic acid is produced by *Pediococcus halopilus*, ethanol is produced by *Zygosaccharomyces rouxii* and 4-ethyguaiacol and 4-ethylphenol are often produced by *Candida* species. Recently an improved process has been developed that uses columns of immobilised *P. halophilus*, *Z. rouxii* and Candida strains in sequence to simulate the traditional process. As a result soy sauce of equivalent flavour quality can be produced in around 10% of the time required for the traditional process [33].

Another interesting process has been developed by Unilever to produce savoury flavours from yeast. The yeast is heat activated, proteases, amylases, and RNAases added to degrade the yeast biopolymers, and then a second stage fermentation carried out to produce organic acids, flavour potentiator nucleotides etc. This is then heated to produce reaction flavours. This second stage is of particular interest because, depending on the source of yeast used and the microbial strains used to carry out the fermentation, the characteristics of the flavour can be varied considerably.

18 Fragrances

18.1 Musks

Compared to flavour chemicals, rather less progress has been made in the development of bioprocesses for the manufacture of fragrance materials. Musks are one of the most important ingredients of fragrances, but virtually all the musks used are polycyclic aromatics still produced by synthetic chemistry from

petrochemically derived raw materials. Naturally occurring musks include the macrocyclic lactones found in some plants such as ambrette seed oil and galbanum, and the keto musks produced by some animals such as musk deet and civet cats which tend to be difficult or unacceptable raw material sources.

One major attempt to produce a musk using biotransformation involves the bioconversion of palmitic acid, which is readily available from renewable palm tree sources into ω-hydroxypalmitic acid. The yeast *Torulopsis bombicola* which has been mutated to minimise β-oxidation and ω-1-hydroxylation of palmitic acid was able to form ω-hydroxypalmitic acids in concentrations of greater than 100 $g l^{-1}$ [34]. However the subsequent cyclization of this precursor into hexadecanolide lactone musk was very difficult, principally because when reasonable high concentrations of the ω-hydroxypalmitic acid were used, intermolecular reactions to form the polymer occurred in preference to the required intramolecular cyclization reaction. A second approach involves the bioconversion reaction of C10–C18 alkanes into dicarboxylic acids for instance by *Candida tropicalis*. Productivities of 140 $g l^{-1}$ of the C13 dicarboxylic acid per litre and production on a scale of 150 tons per annum have been reported, but again the cyclization of the precursor into muscone ((−)3-methylcyclopentadecan-1-one) was difficult to achieve and gave only very low yields of the musk [35].

One interesting use of biocatalysts is the use of strains of yeast of *E. coli* to convert trytophan into indole, which is useful as an ingredient for flavours and especially perfumes. This new method eliminates the traditional source in indole, from coal tar, which is well known to contain carcinogenic materials [36].

18.2 Terpenes (for a Review see [37])

Terpenes are responsible for very many of the varied and valuable plant-derived fragrance and flavour materials, often used in the form of essential oils. The application of biocatalyst technology to terpenes has been slow, perhaps because of their poor water solubility and biocidal properties, but also because of the easy availability of many terpenes by simple extraction from plant sources. Nevertheless some good examples exist. These include a high yielding biotransformation of limonene into α-terpineol by selective hydration [38]. Also of interest is the conversion of geraniol into 6-methyl-5-hepten-2-one which is an important component of mushroom flavour, and a more complex bioconversion of pulegone into 8-mercapto-*p*-menthan-3-one, which is a very low threshold flavour component of blackcurrant and buchu leaf etc., by means of cysteine addition across the double bond of the pulegone followed by the use of specially selected β-lyase to cleave the S–C bond to produce the sulphur-containing terpene [17].

However, the best example of a terpene biotransformation is with the sesquiterpene, sclareol.

18.3 Ambergris Aroma Chemicals

A scientifically very interesting bioprocess for an ingredient for fragrances produces a terpene furan called Ambrox, which has an annual usage worth several million US$ per annum. Ambrox is one of the most important aroma components of ambergris, a naturally occurring excretion product of sperm whales. Ambergris has, traditionally, been a prized component of many fine fragrances; however, as a result of first the decline, and now the protection, of whale populations, it is effectively unavailable, creating a problem for the perfumer.

A chemical process for producing Ambrox uses sclareol which can be extracted from clary sage (*Salvia sclarea*). Sclareol is oxidized by chromium or manganese into sclareol, followed by reduction into a diol and then cyclization to form Ambrox (Fig. 14). However, the oxidation step is not high yielding and creates problems of heavy metal pollution. Therefore a microbial method of producing sclareolide was sought by screening microorganisms for the ability to use sclareol as the sole carbon source. A novel microorganism was isolated that was able to form the diol intermediate in one step via a number of reactions in high yields of $\sim 80\%$, but only after a long fermentation (Fig. 13). This organism proved to be a dimorphic yeast that belonged to a completely new genus, *Hyphozyma roseoniger*, that can exist in both yeast-like and filamentous forms. Subsequently other suitable microbial strains have been found by continued screening; for example, *Cryptococcus* strains that are able to metabolise the sclareol even further, producing a ketone lactone (sclareol) in high yields of over 100 g l^{-1}. The sclareol is then chemically converted back to the diol and thence to Ambrox (Fig. 14). The complex metabolic pathway required for this bioconversion has been investigated and appears to involve allylic rearrangement, Baeyer-Villiger oxidation and other steps. In addition, sclareolide has subsequently been found to be useful in its own right as a salt-enhancer and a contributor to fat-replacer mouthfeel in foods [39, 40].

19 'Negative' Biotechnology

In some cases the actions of microorganisms have undesirable effects. For instance trichloroanisole is a microbial metabolite that causes the 'Rio' off-flavour of coffee. However, in other instances the use of enzymes or microorganisms can ameliorate such flavour defects. Sulphydryl oxidase has been used to reduce the 'cooked' off-flavour of UHT-treated milk. Naringinase treatment of orange juice reduces the bitterness caused by flavanoid glycosides and the bitter taste of grapefruit can be eliminated by using *Arthrobacter globiformis*, by opening the lactone ring of the bitter tasting terpenes nomilin and limonin [41].

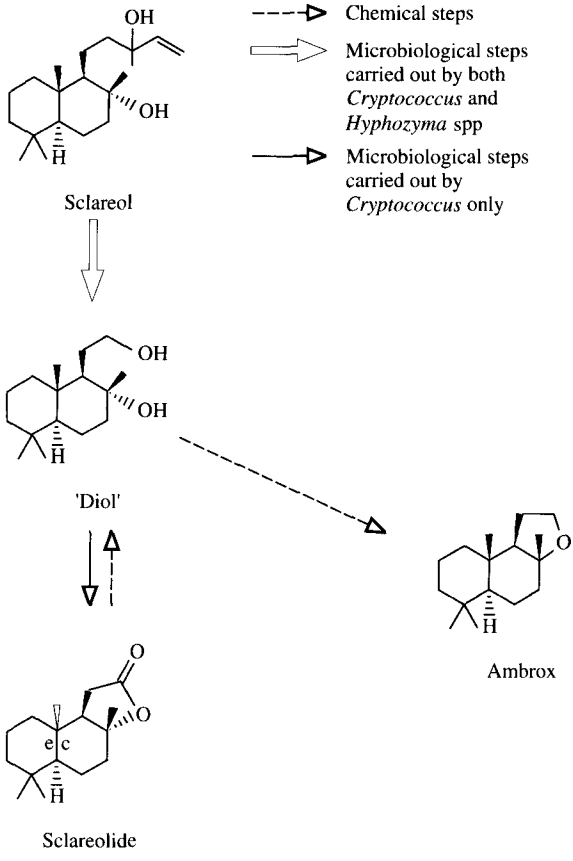


Fig. 14. Microbial methods for the production of sclareolide and Ambrox from the sesquiterpene sclareol (from [54])

20 Newer Emerging Technologies

20.1 Genetic Engineering

Compared to higher value pharmaceutical applications, advances in genetic engineering have been rather slow in being applied to food formulations. Indeed the use of genetic engineering to crop plants such as tomatoes is well advanced, promising improved flavour, improved pest and frost resistance and eventually perhaps even nitrogen fixation.

Technical requirements for successful genetic engineering include the use of microbial strains whose basic genetics are relatively well understood and are easy to work with, which tends to rule out many food microorganisms, at least

until rather lengthy and expensive basic research has been carried out. Successful genetic engineering also requires a well-defined target, such as the elimination of an enzyme responsible for the formation of an off-flavour. This creates a problem because usually most of the more valuable flavours and colours are chemically complex mixtures and these molecules require entire metabolic pathways for the production of a valuable product; e.g. vanilla flavour requires the co-ordinated modification of quite a number of genes. Genetic engineering has, so far, had little impact on producing materials with substantially improved functional characteristics; for instance, natural colours tend to be rather weak in intensity and unstable, and there is no obvious way in which genetic engineering could be used to overcome this inherent and generic problem.

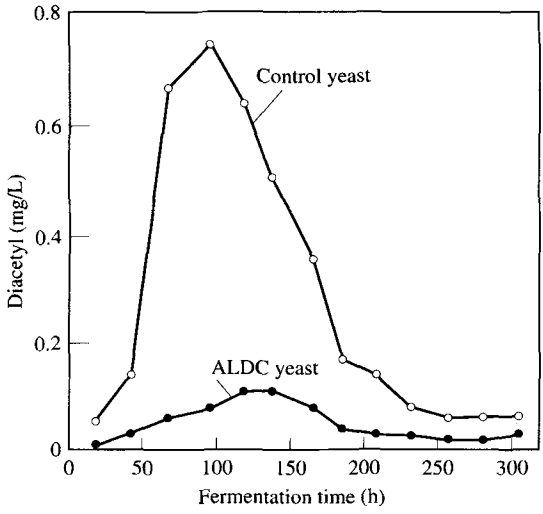
Many industrial enzymes widely used in the food industry are now produced using cloned producer strains in which the gene for the enzyme has been cloned into a host organism for easy and cost-effective large scale production by fermentation. Such enzymes include lipases, esterases and proteinases used in flavour production.

So far the use of protein engineering to modify the basic amino acid structure of enzymes so as to produce enzymes with, for instance, a modified substrate specificity or more pH or thermostable operating characteristics has not yet found widespread applications for food ingredients.

Cloning of genes for new enzymes is proving successful. These include the cloning of genes for various enzymes such as β-galactosidase and proteinases into lactobacilli so as to increase their metabolic versatility and range of applications in the food industry. Similarly, rather than a new activity, higher activity may just be required as in the case of microbial strains producing L-phenylalanine as a precursor of the high intensity sweetener Aspartame, where the number of gene copies has been increased so as to increase microbial productivity and reduced manufacturing costs.

One very effective use of genetic engineering is to remove the off-flavour caused in beers by the accumulation of diacetyl, and thereby reduce the beer maturation time required for the slow metabolism of the diacetyl by the maturing yeast which converts it into acetoin. The diacetyl is formed from α-acetolactate, and its formation can either be prevented or it can be rapidly removed before it can be converted into diacetyl. In order to prevent its formation, acetolactate decarboxylase (ALDC) can be cloned into the yeast. Thus yeasts transformed with the *Acetobacter aceti* ssp. *xylinum* ALDC gene have been used for beer production at the pilot scale and produce much less diacetyl during fermentation than their untransformed parents, resulting in a significant saving in maturation time and therefore production costs [42] (Fig 15).

Diacetyl formation can be reduced by modifying genes in the amino acid biosynthetic pathway that leads to α-acetolactate, especially the IL V2 gene that codes for acetolactate synthetase, followed by mating of these strains to produce hybrids that both produce little diacetyl and good quality beer [43]. Alternatively, metabolism of α-acetolactate can be increased by increasing the turnover of the biosynthetic pathway that leads to its formation, for instance by



Diacetyl formation during fermentation by a brewing yeast strain transformed with the ALDC gene from *Acetobacter aceti* ssp. *xylinum*.

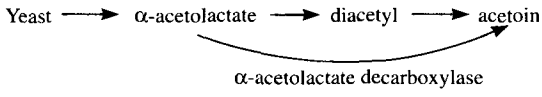


Fig. 15. The use of yeast containing cloned α-acetolactate decarboxylase to reduce the concentration of diacetyl during the brewing of beer, thereby improving its flavour (from [42])

constructing strains with extra copies of the IL V5 gene so as to have enhanced acetolactate reductoisomerase activity [44].

One successful genetic engineering effort has been to develop inosine overproducing strains of *Bacillus subtilis*. This is of great value because inosine is an important flavour enhancer. Enhanced inosine production was achieved by constructing an IMP-dehydrogenase deficient mutant strain by in vitro mutation and recombination. This modification works because this enzyme is responsible for channelling IMP into guanosine synthesis. Similarly, guanosine production can be enhanced by increasing the number of gene copies of the IMP dehydrogenase [45]. More conventional technologies can also be used; for instance, Yasuyuki et al. [46] enhanced the glutamine content of the koji-derived Huki seasonings by treating them with immobilised glutaminase. Also Noguchi et al. [27] produced 5^1-IMP from 5^1-AMP derived from ribonuclease-treated yeast using deaminase.

Uses of genetic engineering also extends to useful characteristics that are only indirectly related to flavour production, such as increasing the phage resistance of lactobacillii or eliminating β-glucan production. However, what is clear is that the food industry does not really want genetic engineering per se,

and in fact it would prefer not to have to address the consumer perception and other issues that rise from genetic engineering. Genetic engineering is also subject to hard competition from other technologies in providing solutions to the food industry's needs. Genetic engineering can however be an attractive option when it can genuinely deliver products at substantially lower cost and with significantly increase functional performance. The technical challenge is therefore how to bring genetic engineering technology costs and time scales down to those required for effective application to food industry targets.

20.2 Plant Cell Culture

Plant cell tissue culture is also a promising new technique but, despite its obvious potential, it has not yet been successfully used to make flavours or other plant products on a production scale. Intrinsic difficulties include the low productivity and mechanical fragility of plant cells; cultures are very prone to contamination, especially considering the very long fermentations required. However, chilli pepper cells (*Capsicum frutescens*) immobilised in polyurethane particles have produced 20–30 times the yields of capsaicin that is made by freely suspended cells. Supplementation of the growth media with precursors such as phenylalanine and isohexanoic acid enhanced productivity further and columns of these immobilised cells could be maintained active for at least six months. When simultaneous oil extraction of capsaicin was carried out a specific production rate of 0.1 $mg\,g^{-1}$ per day was obtained, that is about 20% of the rate of synthesis observed in ripening peppers [47, 48].

21 Future Prospects – R&D Issues

The objective of R&D in the flavour and fragrance industry is to produce the best quality product at minimum production costs. This is achieved by developing efficient processes which produce the biologically active product in high yield and quality. The use of biotransformations for producing flavours and fragrances is characterised by the need to use a very wide range of different enzyme activities including not only simple hydrolases and isomerases but also lyases, deaminases, oxygenases, and oxidases, hydroxylases and decarboxylases, as well as whole or partial metabolic pathways involving such enzymes. Thus the use of many new enzymes and biocatalyst systems is being pioneered by applications in flavours and fragrance production rather than in pharmaceutical processes which at first sight might be presumed to be the leading edge of biotransformations technology.

The following points are vital if the range of biocatalysts currently in use is to be expanded.

- The importance of screening procedures to obtain strains of microorganisms from nature that have the required enzyme activities to produce flavour materials in good yield and at low cost. Sophisticated and clever screening strategies and techniques are often required so as to achieve the selectivity required in isolation. Many examples of the results of such screening projects have been given in this paper, but as an indication of the subtleties required refer, as an example, to [49] who have developed a dynamic screening method that can accurately monitor the dynamics of flavour chemical formation by microorganisms during the fermentation. Possible enzymes that may be exploited in the future could include the cyclases that are involved in the synthesis of some terpenes by plants [50].
- It is important that the microorganisms and enzymes used are true biocatalysts, very suitable for use on a process scale. Thus, they need to be high yielding and so need to be resistant to inhibition by substrates and products and resistant to inactivation by proteases. In addition they may need to be thermostable and stable to organic solvents when required. The bioprocess must also use microorganisms that are safe and acceptable to the regulatory authorities, that can be grown easily and are stable under the conditions of use. An ideal biocatalyst is manufactured easily and is stable under the conditions of use. It should have a high stereospecificity, but a broad substrate-specificity so that it can be potentially useful for a range of related reactions. A list of some of the different enzyme activities used is given in Table 12.
- A well integrated use of microbiology, biochemistry, organic and analytical chemistry and biochemical engineering is essential. This should include the use of enzymes and microbial steps, in conjunction with complementary chemical steps. Input from marketing, sales, patent, and regulatory experts is essential if the process is to be commercialised successfully.
- Also very important is a good appreciation of the process requirements for the successful isolation, concentration and purification of the products of bioreactions (down-stream processing). Some of the engineering considerations related to specific flavour producing processes are given in Table 13.
- Similarly, easy regulatory approval for bioprocesses and products is crucially important.

Overall, what is certain is that technological innovation and successful development of new processes and products are not simple: For instance how can the complex mixtures of specific flavour chemicals be created using food grade biocatalysts so as to produce authentic tasting tastes, without having to adopt often lengthy, variable and expensive traditional processes? What is needed is a continued input of fundamental 'step jumps' in science, such as practicable and cost-effective means of using plant derived cells and tissues as biocatalysts for flavour production, and an accurate and predictive understanding of

Table 12. The use of biocatalysts in the production of flavour enhancers (from [54])

Flavour chemical	Taste	Microorganism/enzyme used for production
Methylketones	Dairy	*Penicillium roquefortii*
γ-Decalactone	Fruit, especially peach, dairy	Yeasts; β-oxidation and lipase
δ-Lactones	Various	Yeasts; β-oxidation and lipase
Diactetyl	Dairy	*Steptococcus diacetylactis*
1-Octen-3-ol	Mushroom	Homogenized mushroom
cis-3-Hexenol/*cis*-3-hexen -l-al	Fruit and vegetables, 'fresh taste'	Plant lipoxygenase, hydroperoxide lyase and dehydrogenase, e.g crushed strawberry leaves
Meat hydrolysates	Species-specific meat tastes	Proteases
Benzaldehyde	Cherry and almond	β-Glucosidase and nitrile lyase
Aspartame	High-intensity sweetener	Metalloprotease (thermolysin)
Enzyme-modified cheeses	Cheese (various types)	Lipases and proteases
Esters	Various, including fruit	Lipases and esterases
Methylbutanoic acids	(For esterification)	*Acetobacter acetii*, dehydrogenases
Ethyl esters, e.g. ethyl isovalerate	Various	*Geotrichum fragrans*; oxidative deamination
Methylanthranilate	Lambrusco grape	*Polyporous versicolor*, *N*-demethylation
2-Phenylethanol	Fruit and beverage	Yeast; deaminase, decarboxylase and reductase
Furaneol®	Fruit, especially strawberry	Rhamnosidases to liberate L-rhamnose precursor
Naringin degradation	Debittering of citrus, especially oranges	Naringinase (β-glucosidase and rhamnosidase)
Limonin/limonoate degradation	Debittering of citrus, especially grapefruit	*Arthrobacter globiformis*, limonoate dehydrogenase
Diallylthiosulphonate	Garlic	Alliinase
Isothiocyanates	Mustard	Myrosinase
Monosodium glutamate	Taste-enhancer	*Corynebacterium glutamicium*
Guanosine monophosphate	Taste-enhancer	Phosphodiesterase,e.g. from *Penicillium citrinium*
Inosine monophosphate	Taste-enhancer	Adenyldeaminase, e.g. from *Brevibacterium ammoniagenes*

Table 13. Bioengineering aspects of bioflavour production

γ-Decalactone	The precursor is an oil and so water-miscibility issues had to be solved.
Benzaldehyde precursors	Toxic HCN is produced when using cyanogenic nitrile glycoside.
Vanillin	A complex pathway is involved. When eugenol is used as the precursor then precursor toxicity to the cells has to be dealt with.
Soy sauce	Several strains of microorganism have been used, separately immobilised and operated in series.
Milk souring	Microbial mixed cultures are used.
Hexanal	Use of a alcohol oxidase in two-phase water-hexanol reaction has been demonstrated to be high yielding
Ethyl isovalerate	Recovery of product from the exit gases from the reactor has been reported as successful.
Monosodium glutamate	Rod-shaped β-crystals of monosodium glutamate are easier to filter and wash, but the α-form crystalises preferentially and so they are redissolved and then recrystalised in the β-form.

structure-function relationships for how flavour molecules interact with receptors in the mouth and nose and thereby trigger taste and smell sensations.

22 Note Added in Proof

This addition gives details of publications that have only recently come to my attention, also to illustrate further the rapid rate of progress being made in the field of bioflavours by a growing range of expert research groups, and finally to include some papers that I just plain forgot to include.

The identification of microbial strains that produce flavour molecules and the elucidation of the enzymatic mechanisms whereby plants produce flavour molecules are both proceeding apace. Christen et al. (1995) have described the production of fruit flavours, especially those characteristic of bananas by *Ceratocystis fimbriata*. Chassagne et al. (1995) have investigated the role of a number of glycosidases in the release of flavour molecules such as terpenols, aliphatic and aromatic alcohols etc. from glycosidically bound precursors present in passion fruit.

The precise biochemical mechanisms whereby flavour chemicals are made by enzymes is increasingly coming under rigorous scrutiny. Fronza et al. (1996) have demonstrated the stereochemistry of the double bond saturation step involved in the formation of raspberry ketone (4-(4-hydroxyphenol)-2-butanone by bakers yeast. Haffner and Tressl (1996) have investigated by the biosynthesis of (R)-γ-decalactone by *Sporobolomyces odorus* involving a strictly enantioselective (R)-12-hydroxylation of oleic acid, followed by β-oxidation and then lactonisation. Also, Gatfield (1995) has mentioned that in the conversion of the 2-methyl butanol present in fusil oil by *Acetobacter aceti*, into the corresponding 2-methylbutannoic acid, very little racemisation takes place, which is surprising as it is well known that this bioconversion is a two-step process which proceeds via the intermediate aldehyde, which is well known to undergo facile tautomerisation.

As always increasingly sophisticated analysis of natural products, especially of foods and beverages, is a key source of new leads for useful flavour chemicals. Continuing this field of study are Benn and Peppard (1996) who analysed Tequila and found the most powerful odorants to be isovaleraldehyde, isoamylalcohol, β-damascenone, 2-phenylethanol and vanillin. However, attempts to reconstitute a tequila flavour from its constituents was unsuccessful indicating that further constituents remain to be identified. Semmelroch and Grosch (1996) analysed coffees and found 2-furfurylthiol, 3-mercapto-3-methylbutyl formate, methanethiol, β-damascenone, methylpropanal and 3-methylbutanal as the most potent odorants. However, odour activity rankings differed between different coffee types, which may be because the extraction of non-polar molecules was much higher yielding than for the polar molecules.

Even new molecules are still being found to be involved in flavour. Dugelay et al. (1995) have found that the off-flavour due to excess vinylphenols in white wines actually decreases during storage due to their slow reaction with ethanol to form 4-(1-ethoxyethyl)-phenol and 4-(1-ethoxyethyl)-guaiacol, whose flavour threshold levels indicate that they have no influence on the flavour of the wines.

Methods for isolating and purifying flavours are of vital importance both in terms of the cost of processes and also because of their influence on the flavour quality of the flavours and flavour chemicals produced. Lamer et al. (1996) have investigated the use of pervapouration to extract benzaldehyde produced by fermentation using *Bjerkandera adusta*. Pervapouration is the selective vapourisation of a liquid across a membrane, involving the dissolution of solute molecules in the membrane and then their evaporation into the gas phase on the other side of the membrane. In this particular case the system was optimised to a point at which the productivity of the *B. adusta* fungus had become the limiting factor in the overall process.

Perhaps the most important area of research is on the bioproduction of the key high-value flavour chemicals that give the impact and characteristics to flavours. One of the most important flavour chemicals is 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF). Now, Hecquet et al. (1996) claim to have demonstrated the first microbial production of HDMF using *Zygosaccharomyces rouxii*. Addition of the precursor D-fructose 1,6-bisphophate stimulated production and concentrations of HDMF as high as 100-ppm could be obtained. An alternative strategy for the production of HDMF using strawberry plant tissue culture has been pursued by Zabetakis and Holden (1995) which involves feeding precursors such as 6-deoxyfructose and the use of elicitors. Ionones are another high-impact flavour and aroma chemical present in many essential oils. Bosser and Belin (1994) have demonstrated the enzymatic co-oxidation of β-carotene by xanthine oxidase to produce β-ionone and derivatives such as epoxy-β-ionone, dihydroactinidiolide etc. This reaction was demonstrated to be due to free radical formation and was dependent on the use of aldehydes such as acetaldehyde as the cosubstrate.

One of the most tempting of flavours for bioresearch is vanillin. Two interesting approaches have been reported recently. Researchers at Wageningen have found that *Penicillium simplicissimum* carries out two exceptional reactions. Firstly, it has an enzyme that converts eugenol into conniferyl alcohol (van Berkel et al., 1995) and secondly, it has an aromatic alcohol oxidase that will convert vanillyl alcohol into vanillin (Fraaije et al., 1995). The other work has been carried out at INRA (France). Again useful progress towards developing a bioprocess for vanillin has been made by finding an *A. niger* strain that will convert ferulic acid into vanillic acid; and then more importantly a strain of *Phanerochaete chrysosporium* that will convert vanillic acid into vanillin (Lesage Meessen et al., 1996). This second reaction could also be of wider biotechnological significance because very few examples of the enzyme-catalysed reduction of acids into aldehydes have been demonstrated.

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Enzymes and Flavour Biotechnology

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The main impetus for the use of enzymes in flavour biotechnology is the continuous demand for "natural" flavour compounds. Besides microbial fermentation, the use of enzymes is an alternative for their biotechnological production. A survey of the actual use of enzymes in organic synthesis shows that 65% of all reported applications fall into the classes of hydrolytic and dehydrogenase reactions. However, dehydrogenase reactions are mostly performed by use of whole microorganisms and less by isolated enzymes. Next in importance are oxygenase-mediated reactions; only a few reports are available about enzymatic carbon–carbon synthesis. From these main groups, the most important reaction types for biotechnological flavour production are represented, i.e. lipases and glycosidases, lipoxygenases and peroxidases, as well as aldolases and a number of miscellaneous enzymes are treated for enzyme-catalyzed hydrolyses and esterifications, oxidoreductions and carbon–carbon bond formation.

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1 Introduction

Of the estimated 25 000 enzymes present in nature, about 2800 have been classified, and about 400, mainly hydrolases, transferases and oxidoreductases have been commercialized. On a larger industrial scale, in particular, for detergents and food processing, only approximately 50 enzymes are used. In 1993, the worldwide market for enzymes was US $1 billion. The majority of enzymes in food processing comprises hydrolases, such as amylases, proteases, pectinases, cellulases, hemicellulases, invertase and lactase. Since microbial enzymes have become an integrated part of food processing, it is attractive to propagate these biocatalysts for the generation of flavour compounds [1–3].

2 General Aspects

Enzymes are proteins; they catalyze biological reactions in vivo and also catalyze reactions involving both natural and unnatural substrates in vitro [4–14]. As catalysts, enzymes show the following properties: (i) they operate under mild conditions at narrow temperature (commonly between 20 and 40 °C) and pH ranges; (ii) they can be highly selective for substrates and stereoselective in the catalyzed reactions, although selectivity can range from very narrow to very broad; (iii) the catalytic activity may be strongly influenced by the concentrations of substrates, products or other species present; (iv) they are generally unstable; (v) in cofactor-dependent reactions, some cofactors may be altered during a catalytic cycle, requiring recycling to the active form.

Over a long period, the properties of instability, high cost and narrow substrate selectivity have been regarded as the most serious drawbacks of enzymes for use as catalysts in synthetic chemistry. Owing to new developments in chemistry and biology and new industrial requirements, however, this perception has changed. There are various reasons for this. (i) Several enzymatic reactions have been shown to transform natural or unnatural substrates stereoselectively to useful synthetic products [4–14]. In Table 1 the enzymes commonly used in synthetic chemistry are listed. (ii) New techniques have been developed for enzyme immobilization and stabilization [11, 5–17], as well as scaling-up of processes [3, 18–21]. (iii) Recent advances in molecular and cell biology, computation, and analytical chemistry have created new tools for the manipulation of genetic materials to construct genes for expression of desired proteins [22, 23]. (iv) Recombinant DNA technology has opened the way for low-cost production of proteins including enzymes and the rational alteration of their properties [24, 25]. (v) Enzyme catalysis is further stimulated by the discovery of catalytically active antibodies ("abzymes") [26, 27].

Table 1. Enzymes commonly used for organic synthesis [13]

Not requiring cofactors	Not requiring added cofactors	Cofactor requiring[a]
1. Hydrolytic Enzymes:	1. Flavoenzymes:	1. Kinases – ATP
		2. Oxidoreductases – NAD(P)
Esterases	Glucose oxidase	3. Methyl-transferases – SAM
Lipases	Amino acid oxidases	
Amidases	Diaphorase	
Phospholipases	Pyridoxal phosphate	
	2. Enzymes:	
Epoxide hydrolases		
Nucleoside phosphorylase	Transaminases	
2. Isomerases and Lyases:	Tyrosinase	
	3. Metalloenzymes:	
Glucose isomerase		
Phenylalanine ammonia lyase	Galactose oxidase	
Fumarase	Monooxygenases	
Cyanohydrin synthethase	Dioxygenases	
3. Aldolases	Peroxidases	
4. Glycosyl transferases	Hydrogenases	
5. Glycosidases	Enoate reductases	
6. Oxynitrilase	Aldolases	
	Nitrile hydrase	
	4. Thiamine pyrophosphate dependent enzymes:	
	Transketolases	
	Decarboxylases	

[a] ATP, adenosine triphosphate; NAD(P), nicotinamide adenine dinucleotide (2′-phosphate); SAM, (−)-(*S*)-adenosyl-L-methionine

Thus, a number of organic reactions can be performed with the use of enzymes, i.e. synthesis of chiral intermediates; transformation of sugars, nucleotides, and related species; synthesis of physiologically active compounds such as amino acids, sugars and their phosphates; transformations of peptides and proteins; and other transformations in which classical chemical methodology is constrained [4–14]. A survey of the actual use of enzymes in synthetic organic chemistry shows that 65% of all reported applications fall into the classes of hydrolytic reactions (40%) and dehydrogenase reactions (25%). However, dehydrogenase applications are mostly performed by use of whole microorganisms and less by isolated enzymes. Next in importance are oxygenase-mediated reactions (24%); only a few reports about enzymatic carbon–carbon synthesis are available (4%). All other reaction types comprise about 7% of the total.

The main impetus for the use of enzymes is the continuous growth in the demand for enantiomerically pure compounds. This trend plays an important role in the synthesis of chiral drugs [28, 29], but is also essential in the flavour industry where, besides microbial fermentation, enzyme technology is an alternative for the biotechnological production of flavour materials [30, 31]. In the following, the state-of-the art in the field of enzymatic flavour production is reviewed by means of the classes of biocatalysts used, i.e. lipases and

glycosidases, lipoxygenases and peroxidases, aldolases, and a number of miscellaneous enzymes are discussed for enzyme-catalyzed hydrolyses and esterifications, oxidoreductions and carbon–carbon bond formation.

3 Hydrolytic Enzymes

3.1 Lipases

Lipases are serine hydrolases that catalyze the hydrolysis of lipids to fatty acids and glycerol at the lipid-water interface [32]. In contrast to esterases, which exhibit Michaelis-Menten kinetics, lipases show little activity in aqueous solutions with soluble substrates. The increase of activity at the lipid-water interface led to the suggestion that a conformational change takes place at the oil-water phase before substrate binding occurs. This change in conformation is supported by the X-ray structures of human [33, 34] and *Mucor miehei* [35, 36] lipases, and their complexes with inhibitors. In both the mentioned lipases the active centres contain structurally analogous Asp-His-Ser triads, whereas the *Geotrichum candidum* lipase has a catalytic triad consisting of Glu-His-Ser sequence [37], and the enzyme from *Humicola lanuginosa* requires Asp-His-Tyr for activity [38].

Lipases with very different specificity and stability have been reported. Regarding the broad range of properties, it seems to be impossible to compare lipases of various origins in a standardized activity assay. Nonetheless, the release of free fatty acids from olive oil at 37 °C and pH 7.8, and the release of butanoic acid from glycerol tributanoate at 30 °C and pH 6.8 have been agreed to serve as analytical parameters for lipase and esterase activity, respectively.

In organic synthesis, the pig pancreatic extract (PPE) and several microbial lipases, of which a number are outlined in Table 2, play an important role. They are used for the hydrolysis of esters, esterification (acid and alcohol), transesterification (alcohol and ester), interesterification (ester and acid), and transfer of acyl groups from esters to other nucleophils, such as amines, thiols and hydroperoxides. In all these reactions, several criteria of selectivity influence the catalysis, i.e.: (i) substrate selectivity [39]; (ii) regioselectivity [40]; (iii) stereoselectivity with endo-/exo- [41] and *Z*-/*E*- differentiation [42]; (iv) enantioselectivity [43]; (v) meso-differentiation [44]; and (vi) prochiral recognition [45].

Enantiomerically pure esters, acids and alcohols can be prepared by two major procedures as outlined in Fig. 1. The racemic ester can be activated ester [46], enol ester [47] or oxime ester [48]. Acids and acid anhydrides can also be used as acylating agents [46, 49]. It has to be pointed out that in many cases the stereoselectivity of a given enzyme in water and in an organic solvent is the same [46, 50]. Thus, reactions (1) and (2) (Fig. 1) yield complementary stereoisomers. For instance, if an enzyme prefers the (*R*)-enantiomer of a chiral ester over the (*S*)-ester, the hydrolytic reaction (1b) will produce (*R*)-alcohol and (*S*)-ester.

Table 2. Microbial lipases (EC 3.1.1.3) commonly used in organic synthesis [13]

Source	Trade name	Supplier
Alcaligens sp.	Amano lipase PL	Amano
Achromobacter sp.	–	Meito Sangyo
Aspergillus niger	Amano A	Amano
Bacillus subtilis	–	Towa Koso
Candida cylindracea[a]	Lipase OF Amano Lipase AY	Sigma; Boehringer Mannheim; Amano; Meito Sangyo
Candida lypolytica	–	Amano; Fluka
Candida antartica	SP-435[b]	Novo
Chromobacterium viscosum	Amano lipase LP	Sigma; Toyo Jozo
Geotrichum candidum	Amano lipase GC	Sigma; Amano
Humicola lanuginosa	Amano lipase R-10	Amano
Mucor miehei	Amano MAP-10 Lipozyme	Amano; Novo; Fluka
Pencillium camemberti	–	Rhône-Poulenc
Penicillium roqueforti	–	Fluka
Phycomyces nitens	–	Takeda Yakuhin
Porcine pancreas	–	Sigma; Amano; Fluka; Boehringer Mannheim
Pseudomonas cepacia[c]	Amano, P, PS PS30, LP80 SAM-I	Amano; Fluka
Pseudomonas sp.	Amano AK, K-10, SAM-II	Amano
Pseudomonas aeruginosa	LPL, PAL	Amano
Rhizopus arrhizus	–	Sigma; Boehringer Mannheim; Fluka
Rhizopus delemar	–	Sigma; Amano; Tanabe; Fluka
Rhizopus japonicus	Amano lipase FAP	Amano; Nagase Sangyo; Osaka Saiken; Fluka
Rhizopus niveus	Amano lipase N	Amano; Fluka
Rhizopus oryzae	–	Amano
Wheat germ	–	Sigma; Fluka

[a] Now called *Candida rugosa*
[b] The acrylic resin supported lipase which was produced by the host organism *Aspergillus oryzae*, after transfer of the gene coding for lipase B from *Candida antartica*
[c] Formely called *Pseudomonas fluorescens*

Conversely, since the stereochemical preference remains unchanged, transesterification in organic solvents (2b) will result in (*S*)-alcohol and (*R*)-ester. Ideally, if an enzyme has an absolute stereoselectivity, both reactions (1) and (2) (Fig. 1) will stop at 50% conversion and will give both enantiomers with 100% enantiomeric excess (ee = $[R - S]/[R + S] \times 100$; for $R > S$).

3.1.1 Lipolysis

One of the first flavours produced on a large scale by means of enzymes was a product known as lipolyzed milk fat. The original process involved subjecting

Hydrolysis of esters in water

Chiral acid

$$(\pm)\text{-}{*}R_1COOR_2 + H_2O \xrightarrow{\text{enzyme}} ({*}R_1COOH + {*}R_1COOR_2) + R_2OH \quad \text{(1a)}$$

racemic ester — optically active products

Chiral alcohol

$$(\pm)\text{-}R_1COO{*}R_2 + H2O \xrightarrow{\text{enzyme}} ({*}R_2OH + R_1COO{*}R_2) + R_1COOH \quad \text{(1b)}$$

racemic ester — optically active products

Acylation of alcohols in organic solvents

Chiral acid

$$(\pm)\text{-}{*}R_1COOR_2 + R_3OH \xrightarrow{\text{enzyme}} ({*}R_1COOR_3 + {*}R_1COOR_2) + R_2OH \quad \text{(2a)}$$

racemic ester — optically active products

where R_3OH is a primary alcohol.

Chiral alcohol

$$(\pm)\text{-}{*}R_3OH + R_1COOR_2 \xrightarrow{\text{enzyme}} (R_1COO{*}R_3 + {*}R_3OH) + R_1COOH \quad \text{(2b)}$$

racemic alcohol — optically active products

Fig. 1. Hydrolysis of esters in water and acylation of alcohols in organic solvents as two major procedures for the enzyme-catalyzed production of enantiomerically pure acids, alcohols, and esters [28]

cream to a controlled enzymatic hydrolysis using lipases [51]. Several lipases exhibit a high selectivity towards the flavour-active short-chain fatty acids, such as, for instance, the enzyme from *Mucor miehei* [18]. Other lipases preferably liberate long-chain fatty acids and others do not exhibit any particular preference. Lipases also show an additional type of selectivity, namely towards the position of the fatty acid residue in the triglyceride molecule. The fatty acids liberated in a lipase-catalyzed process can be isolated by steam distillation and the individual acids then further purified by fine distillation. In such a way, the short-chain fatty acids such as butanoic, hexanoic, octanoic and decanoic can be obtained in pure form. As recently reported, lipolyzed milk fat products find wide applications, e.g. for the enhancement of cream/butter-like flavours [52].

Another development of this type of enzyme technology led to a product known as enzyme-modified cheese (EMC). EMCs are products obtained by a controlled proteolytic and/or lipolytic enzyme treatment of a previously manufactured traditional cheese [53]. Such a product has significantly increased amounts of proteolytically and/or lipolytically derived flavour compounds characteristic of the cheese which was treated.

3.1.2 Kinetic Resolution of Racemates

Lipases are also used to perform enantioselective hydrolyses to yield pure optically active aliphatic and terpene alcohols. This type of reaction is particularly important in those cases in which the one isomer of a molecule has more desirable attributes than the other.

A typical example is (−)-menthol (*p*-menthan-3-ol), which occurs widely in nature and is the major constituent in peppermint oil. (−)-Menthol is the most important terpene alcohol and is used in high amounts in the flavour and fragrance industry. Only (−)-menthol has a characteristic peppermint odour and also shows a cooling effect. Due to the lack of this cooling effect, the other isomers are not considered to be "refreshing". Racemic menthol occupies an intermediate position; the cooling effect of (−)-menthol is distinctly perceptible. There are several chemical processes for the resolution of the two menthol isomers, but biochemical resolution methods have also been developed. As schematically outlined in Fig. 2, many microbial lipases preferentially hydrolyze (−)-menthyl esters, while (+)-menthyl esters remain untouched. Lipases from *Penicillium*, *Rhizopus*, *Trichoderma* and various bacteria, for instance, are able to perform this asymmetric hydrolysis on several esters of racemic menthol [54].

Candida rugosa lipase was used for the kinetic resolution of 2-methyl branched compounds via esterification and acidolysis [55]. Analogous reactions carried out with 2- and 3-hydroxyhexanoates yielded lower ee values.

Racemic alkanolides and alkenolides such as jasmolactone or tuberolactone have been separated using horse liver esterase [56]. Relactonization of the enantiomeric hydroxy acid yielded both optical forms as pure lactones.

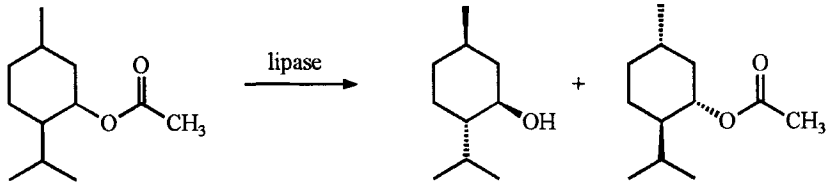


Fig. 2. Preferential hydrolysis of racemic menthyl acetate by microbial lipases [18]

3.1.3 Catalysis in Organic Media

Lipase catalyzed esterification and transesterification reactions have numerous food applications, e.g. in the synthesis of modified triacylglycerols, emulsifiers, peptides and oligosaccharides [57]. Enzyme catalyzed reactions in organic media are influenced by a number of parameters which determine yield and selectivity, i.e. water content of the medium – at present, it is generally agreed that enzymes only require a monomolecular water phase for their activity in organic solvents [58], pH value of the aqueous micro-phase [59], reaction temperature [60], type of solvent [61] and immobilization [62]. In addition, the selection of the appropriate enzyme is essential; in esterification reactions, for instance, *Candida rugosa* lipase has been reported to give high yields at low selectivity, while PPE and the lipases from *Aspergillus niger*, *Rhizopus arrhizus* and *Mucor miehei* have been found to exhibit high selectivity [43]. Recently, three enzymes were isolated from PPE, purified to electrophoretic homogeneity and their esterifying activity in organic medium studied in detail [63]. Surprisingly, purified porcine pancreatic lipase (PPL; EC 3.1.1.3) did not exhibit any esterifying activity. Purified cholesterol esterase (CE; EC 3.1.1.13) showed esterifying activity with low selectivity, but the third purified protein, identified as porcine pancreatic colipase, displayed esterifying activity with high ee-values ($> 95\%$).

In flavour biotechnology, attention is focused particularly on esters and lactones. Among the fungal lipases, the enzyme from *Mucor miehei* is the most widely studied [18, 64]. Esters of acids from acetic to hexanoic acid and alcohols from methanol to hexanol, citronellol and geraniol have been synthesized by lipases from *Mucor miehei*, *Aspergillus* sp., *Candida rugosa*, and *Rhizopus arrhizus* [65]. Methylbutanoates and methylbutyl esters, important components in many fruit flavours, are also amenable by *Candida* lipases. The lipases from *Penicillium simplicissimum* catalyzed the stereoselective esterification of menthol with fatty acids [66], a process which had been studied earlier in detail using *Candida lipolytica* lipase [19]. Recently, the potential of the lipases from *Staphylococcus warneri* [67] and *Pseudomonas cepacia* [68] in ester formation has been reported. Information about the selectivity of PPE catalyzed ester formation can be taken from the work of Lutz et al. [43].

The microbial synthesis of lactones, generally very pleasant, potent flavour materials, has attracted the attention of flavour researchers since the 1960s. In the meantime, this technology reached a high standard. Lactones can also be synthesized by lipase catalysis, e.g. via PPE catalyzed intramolecular transesterification of 4-hydroxycarboxylic esters (Fig. 3) [43]. In such a way, (*S*)-γ-lactones with chain lengths from C5–C11 have been obtained enantioselectively ($ee > 80\%$), while the preparation of optically enriched δ-lactones was hampered by the substrate selectivity of PPE. *Mucor miehei* lipase has been successfully applied to the synthesis of γ-butyrolactone and 15-pentadecanolide (Fig. 4) [18].

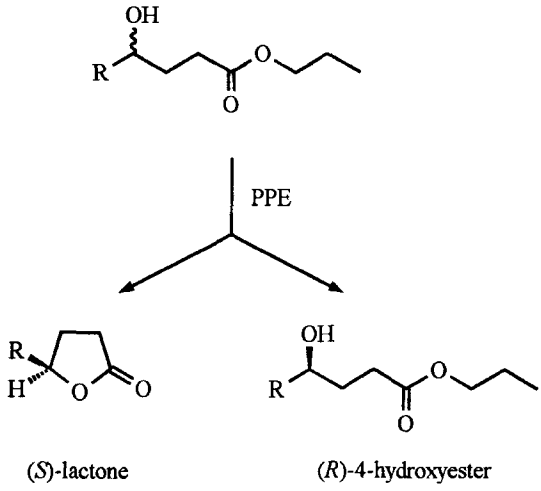


Fig. 3. Formation of (*S*)-γ-lactones via PPE-catalyzed intramolecular transesterification of 4-hydroxycarboxylic esters [43]. PPE, porcine pancreatic extract

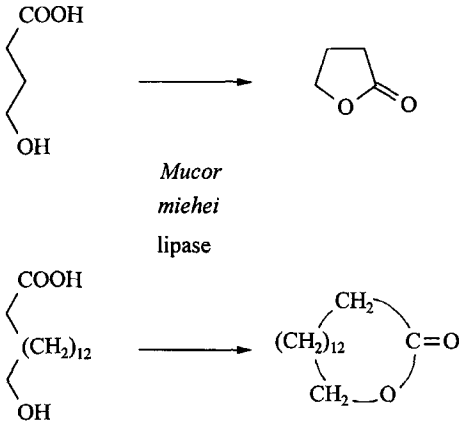


Fig. 4. Lactone synthesis from the corresponding hydroxycarboxylic acid using *Mucor miehei* lipase [18]

3.2 Glycosidases

In discussing the potential use of enzymes for the production of flavour compounds, the fact that in plant tissues a certain amount of the flavour constituents are bound as non-volatile sugar conjugates should be kept in mind. β-Glucosides appear to constitute the major part of the glycosidic pool of many plants, but other glycons such as pentoses, other hexoses and di- and trisaccharides have also been reported [69]. Recent information on conjugated flavour

compounds reveals the importance of acylated glycosides [70] and phosphate esters [71]. The state-of-the-art of the highly-sophisticated analysis of these labile polar natural products can be taken from the work of Herderich et al. [72].

Together with the structural elucidation of conjugates of flavour compounds, interest was also focused on the application of glycosidases to liberate aglycons from their bound forms. The development of the continuous process of enzymic treatment (*s*imultaneous *e*nzyme *c*atalysis *e*xtraction, SECE) [73] opened the way for potential technological applications in which industrially attractive plant volatiles will be accessible from their non-volatile conjugates. Major interest has been directed to wine, in which the grape β-glucosidase is rapidly inactivated during winemaking. Glucosidases from *Sacchoromyces cerevisiae* and *Candida molischiana* have been recommended to overcome this problem [74]. However, many fungal glycosidases are inhibited by glucose, fructose and ethanol, and exhibit low activity at the pH of wine. Some β-apiosidases, α-arabinosidases and α-rhamnosidases of *Aspergillus* sp. do not suffer from these drawbacks [75]. The formation of these enzymes was induced by the respective glycosides and their use has been patented for the liberation of flavour compounds from grape must [76].

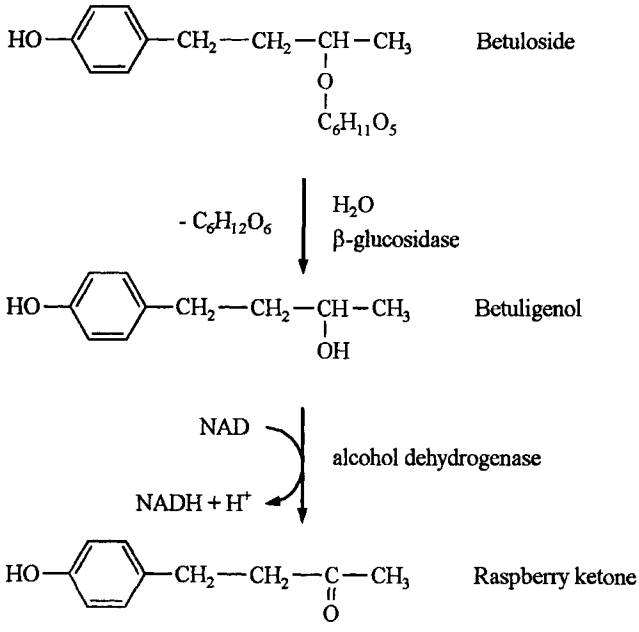


Fig. 5. Proposed biotechnological way to produce raspberry ketone [4-(4'-hydroxyphenyl)-butan-2-one] via β-glucosidase-catalyzed hydrolysis of the naturally occurring betuloside and subsequent enzymatic oxidation of the liberated betuligenol [79]

Microbial hydrolases have been reported to improve the sensory quality of food by the synergistic action of mono-, oligo- and polyglycanases. A patent claimed a process for the production of vanilla extracts involving the treatment of crushed green vanilla beans with enzymes capable of degrading both the plant cell walls and the glucosidic precursor [77]. Similarly, a cellulase exhibiting glucosidase side activity has been reported to liberate benzaldehyde during the processing of peach [78]. Recently, a biotechnological way for the production of raspberry ketone [4-(4′-hydroxyphenyl)-butan-2-one] was proposed involving the β-glucosidase-catalyzed hydrolysis of the naturally occurring betuloside to betuligenol which was finally transformed by microbial alcohol dehydrogenase into the raspberry ketone [79] (Fig. 5).

Even food of animal origin was found to contain metabolic conjugates of flavour compounds [80]. β-Glucuronidase, arylsulfatase and acid phosphatase liberated phenols in milk from their respective precursors.

Moreover, glycosidic forms of volatiles are becoming more and more recognized as technologically attractive flavour sources. The demand for stable precursors is illustrated, for instance, by synthetic acetal derivatives of flavour aldehydes such as benzaldehyde or cinnamaldehyde [81]. For example, phenyl β-D-glucoside as a model compound was added to corn meal prior to extrusion; the aglycone was formed during thermal processing [82]. As the chemical synthesis of glycosides is cumbersome, biotechnological transglycosidation using glycosidases attracts increasingly attention [83, 84].

4 Oxidoreductions

Most enzyme-catalyzed redox processes involve the transfer of the equivalent of two electrons by either two one-electron steps or one two-electron step. The one-electron process is a radical process which very often involves the use of cofactors such as flavin, quinoid-coenzymes and transition metals. The two-electron process can be a hydride transfer or a proton abstraction followed by two-electron transfer.

4.1 Nicotinamide Cofactor Dependent Oxidoreductions

Nicotinamide adenine dinucleotide (NAD) and the analogous 2′-phosphate (NADP) are involved in many two-electron oxidations catalyzed by dehydrogenases. The nicotinamide ring system is redox active, accepting a hydride or two electrons and a proton to form the 1,4-dihydronicotinamide derivatives NADH or NADHP. The reversible hydride transfer from a reduced substrate to NAD(P), and that from NAD(P)H to an oxidized substrate, is stereoselective and characteristic of individual enzymes. Each enzyme is able to transfer

stereoselectively one of the diastereotopic methylene hydrogens at C-4 of NAD(P)H to a substrate carbonyl group or an equivalent sp^2 centre (C=C or C=N) with high enantiofacial or diastereofacial selectivity.

The nicotinamide cofactors are usually not covalently bound to the enzymes and readily dissociate. They are too expensive to be used as stoichiometric reagents in large-scale synthesis. Thus, recycling of the cofactor is needed if enzymes requiring nicotinamide cofactors are to be used on the preparative scale [13].

4.1.1 Horse Liver Alcohol Dehydrogenase (EC 1.1.1.1)

Horse liver alcohol dehydrogenase (HLADH) has received more attention than any other alcohol dehydrogenase. HLADH can oxidize primary alcohols. In addition, the enzyme can reduce a significant number of aldehydes. HLADH is unable to oxidize methanol due to a lack of productive binding in the active site. For substrates insoluble in aqueous solution, biphasis systems, reverse micelles and organic solvents can be applied [13].

When using HLADH for the oxidation of alcohols to aldehydes, cofactor regeneration can be achieved using a second aldehyde as an oxidizing agent, and in biphasic solvent systems. For instance, cinnamic alcohol is oxidized in a solvent system consisting of 0.1% aqueous buffer/99.9% hexane, with octanal as a cosubstrate for cofactor regeneration [85]. HLADH has also been shown to give excellent enantioselectivity (ee > 95%) when racemic aldehydes or ketones were reduced in buffer saturated ethyl acetate containing ethanol as the NADH regenerating system [86]. As a representative example for the research activity in flavour biotechnology, the recently described conversion of (*Z*)-3-hexenol and 2-phenylethanol to the corresponding aldehydes using HLADH is mentioned (Fig. 6) [87]. A maximum yield of (*Z*)-3-hexenal of 50–60% could be obtained in a biphasic system with propanol and an alcohol oxidase as the cofactor regenerating system. In addition, immobilized alcohol oxidase, an unidirectional flavine enzyme, has been used for the production of acetaldehyde from ethanol in the gas phase [88].

In spite of the wide use of HLADH with its broad substrate acceptance, narrow stereoselectivity and bidirectional functionality, there is continuing research for novel reductases [13]. As a representative example, the extended study of Peters et al. [89] to search for suitable keto ester reductases from microbial origin can be mentioned.

4.2 Metalloenzyme Oxidoreductases

This group comprises the enolate reductase (EC 1.3.1.31) and related enzymes, galactose oxidase (EC 1.1.39), arene dioxygenase, the lipoxygenases (EC 1.13.11.12), the peroxidases and several monooxygenases [13]. In flavour biotechnology,

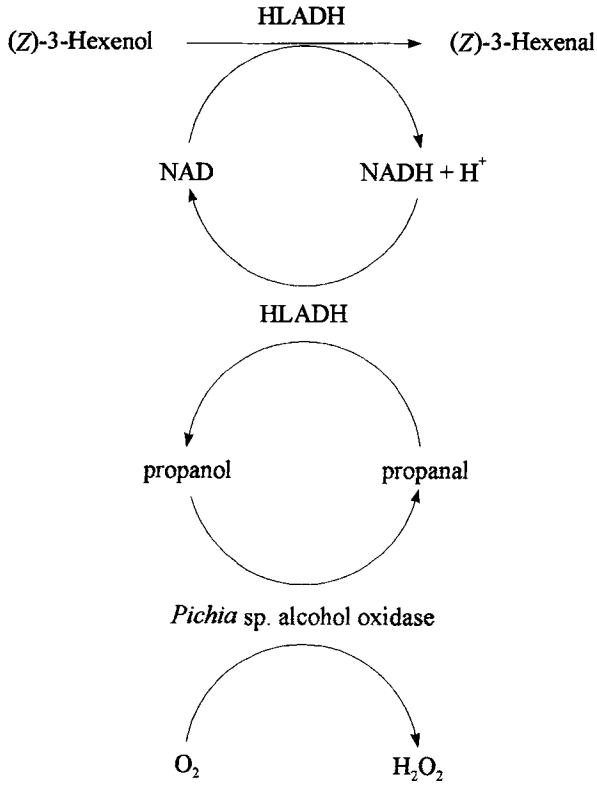
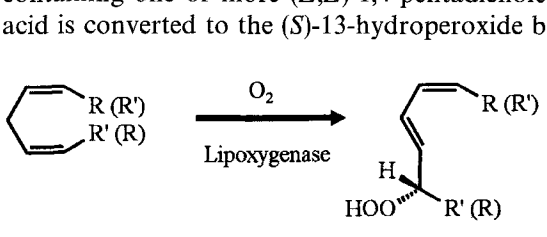


Fig. 6. Biotechnological process of (*Z*)-3-hexenal formation using HLADH in a biphasic system with propanol and an alcohol oxidase as cofactor regenerating system [87]

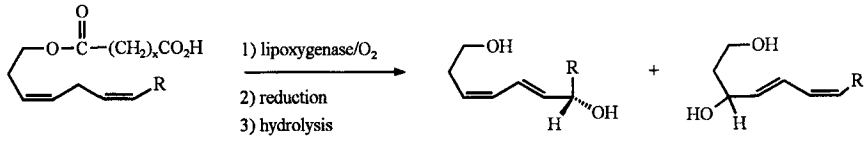
the lipoxygenases (LOX) play the most important role. LOX, together with horseradish peroxidase (HRP), will be treated in the following sections.

4.2.1 Lipoxygenase (LOX)

Lipoxygenase (LOX) is a non-heme, iron-containing dioxygenase that catalyzes the regioselective and enantioselective dioxygenation of unsaturated fatty acids containing one or more (*Z*,*Z*)-1,4-pentadienoic moieties; for instance, linoleic acid is converted to the (*S*)-13-hydroperoxide by LOX from soybean.



The catalytic mechanism was proposed to proceed through a free radical intermediate which reacts directly with oxygen or an organoiron intermediate [90]. The three-dimensional protein structure of the native form of LOX isoenzyme L-1 from soybean has been elucidated [91,92]. Recent investigation of soybean LOX regarding its application to the synthesis of chiral diols indicates that a decrease of the hydrophobicity for R and an increase of x lead to the increase of the product with the OH group next to R [93,94].



While with these studies a distinct insight into the structure-*regio*selectivity relationship has been evaluated, similar data on the observed *enantio*selectivity are still lacking.

In flavour biotechnology, LOX is an important factor in the industrial use of plant enzymes for the production of natural "green note" flavour compounds, i.e. the group of isomeric C6-aldehydes and alcohols [95]. In nature, the C6 compounds are synthesized in damaged plant tissues (leaves, fruits or vegetables) in a cascade of enzymic reactions, in which LOX, hydroperoxide lyase and an oxidoreductase are involved [96]. The recently proposed industrial process for the production of natural "green notes" follows the natural pathways (Fig. 7). The most effective hydroperoxide lyase has been detected in alfalfa. However, the proposed-also patented [97,98]–application of baker's yeast for in situ reduction of enzymatically produced aldehydes has been called into question concerning the effective production of (*Z*)-3-hexenol. According to Gatfield's report [99] the isomerization of (*Z*)-3- to (*E*)-2-hexenal is very rapid and the latter undergoes facile reduction to form 1-hexanol. In addition, baker's yeast is able to add activated acetaldehyde to (*E*)-2-hexenal, thereby forming 4-octen-2,3-diol.

Fungal LOXs exhibit different regioselectivity than LOX from plants; they catalyze the dioxygenation of linoleic and linolenic acid to 10-monohydroperoxides. The volatile impacts of mushroom aroma (Fig. 8) are generated by the activity of a hydroperoxide lyase and subsequent other enzymatic steps in damaged fungal cells. Industrial processes have been developed to produce (*R*)-1-octen-3-ol, based on the feeding of mycelia with linoleic acid [100]. More recently, agricultural treatments have been investigated to improve LOX activity [101].

The capacity of soybean LOX to co-oxidize plant pigments, such as carotenoids and chlorophyll in the presence of linoleic acid, has been known for a long time. Despite the abundant information on this process, the mechanism of the reaction mechanism remained undefined to date. Based on stereochemical studies of the unselective formation of epoxides during LOX-catalyzed co-oxidation, the hypothesis of a free peroxyl radical mechanism has recently been

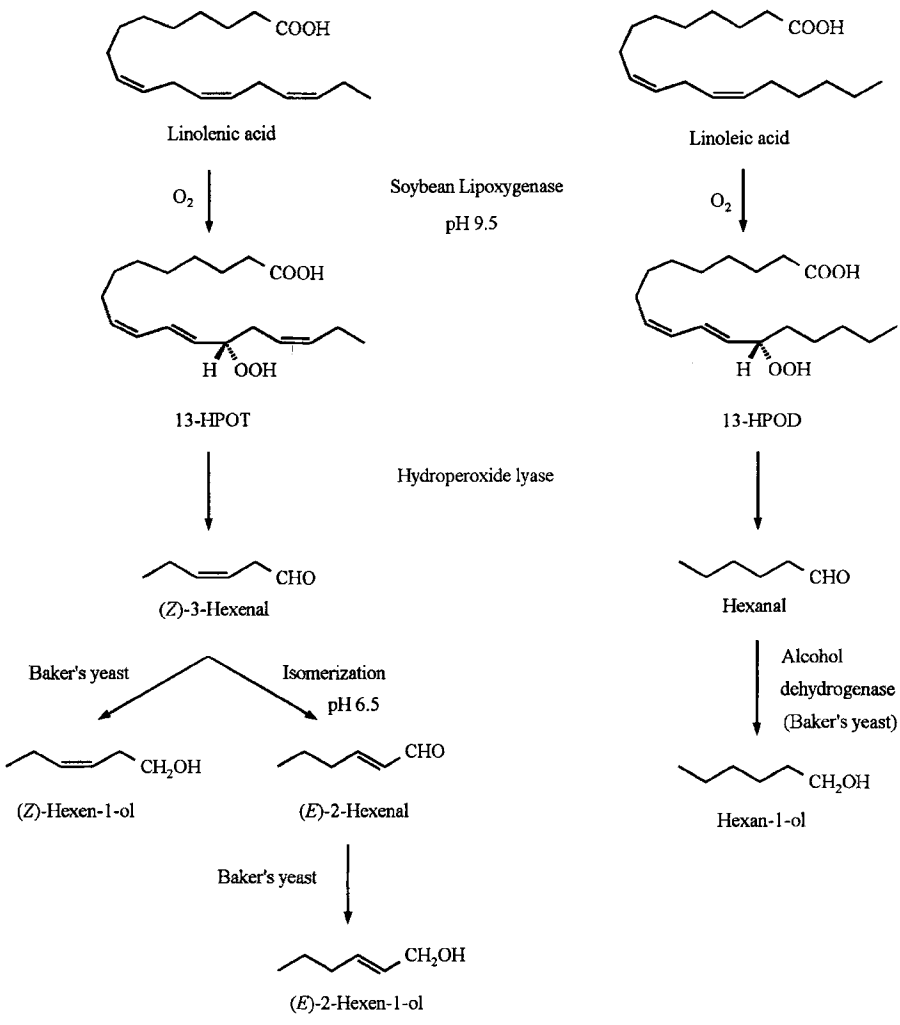
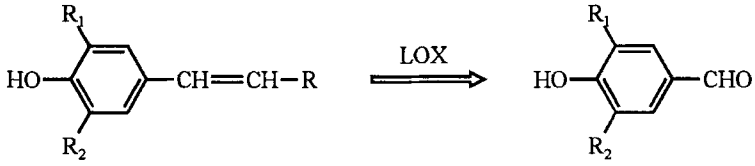


Fig. 7. Biotechnological process to produce C6-aldehydes and alcohols by the action of soybean lipoxygenase (LOX), hydroperoxide lyase and alcohol dehydrogenase (baker's yeast) [96]

supported (Fig. 9) [102]. A process for the production of α-and β-ionone by LOX-catalyzed co-oxidation of carotenes has been propagated [103]. According to another report, ionones are available via xanthine oxidase mediated degradation of carotenes [104]. The problem of limited availability of the raw material, however, has not been resolved to date.

Finally, the recent attention focused on LOX as biocatalysator for the vanillin production has to be stressed. A few years ago, a dioxygenase from *Pseudomonas* sp. was claimed to cleave *p*-coumaryl alcohol, coniferyl alcohol and some further substrates to yield vanillin. Interestingly, a similar broad spectrum of

phenylpropanoids was accepted by LOX [105], resulting in moderate (4–15%) yields of vanillin. The mechanism of this type of LOX catalysis is still unknown.



R_1 = H, OH, OCH_3 and C_{1-4}-alkyl

R_2 = H, OH, OCH_3 and C_{1-4}-alkyl

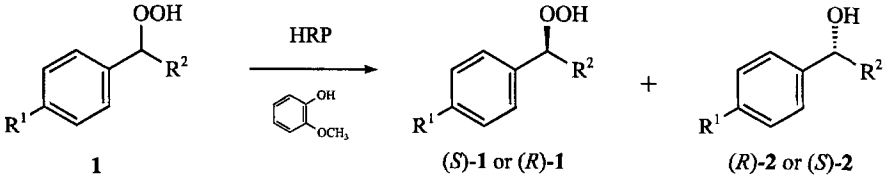
R_3 = H, C_{1-6}-alkyl, $-CH_2OH$, -COOH, $-OCH_3$, $-CO-CH_2CO-$ and $-CH_2O-R_4$

R_4 = benzoyl or C_{1-4}-alkanoyl

4.2.2 Horseradish Peroxidase (HRP)

The heme peroxidases are a superfamily of enzymes which oxidizes a variety of structurally diverse substrates by using hydroperoxides as oxidants. For example, chloroperoxidase catalyzes the regio- and stereoselective halogenation of glycals, the enantioselective epoxidation of distributed alkenes and the stereoselective sulfoxidation of prochiral thioethers by racemic arylethyl hydroperoxides [13]. The latter reaction results in (*R*)-sulfoxides, (*S*)-hydroperoxides and the corresponding (*R*)-alcohols, all in optically active form.

Recently, the horseradish peroxidase (HRP)-catalyzed kinetic resolution of racemic secondary hydroperoxides has been reported for the first time, yielding in one step (*R*)-hydroperoxides up to 99% ee and (*S*)-alcohols up to 97% ee [106]. The method allows the convenient preparation of optically active hydroperoxides as potential stereoselective oxidants.



1 (*S*)-**1** or (*R*)-**1** (*R*)-**2** or (*S*)-**2**

a: R^1 = H, R^2 = Me **b**: R^1 = Cl, R^2 = Me **c**: R^1 = H, R^2 = Et

d: R^1 = H, R^2 = nPr **e**: R^1 = H, R^2 = nBu **f**: R^1 = H, R^2 = nPen

g: R^1 = H, R^2 = nHex **h**:  **i**: 

j: R^1 = H, R^2 = iPr **k**: R^1 = H, R^2 = iBu **l**: R^1 = H, R^2 = CH_2OH

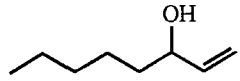
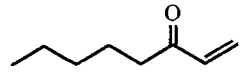
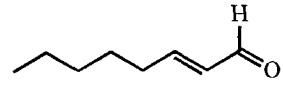
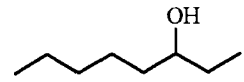
Compound	Structure	Threshold value (mg kg^{-1})
1-octen-3-ol		0.01
1-octen-3-one		0.004
(*E*)-2-octen-1-ol		0.04
(*E*)-2-octenal		0.003
3-octanol		0.018

Fig. 8. Threshold values and odour characteristics of main mushroom volatiles [100]

This type of reaction might be of interest in the transformation of natural hydroperoxides, as available, e.g. by LOX catalysis.

5 Carbon-Carbon Bond Forming Reactions

One of the most useful set of chemical methods for C–C bond formation is the catalytic aldol condensation. Complementary to these methods is the use of enzymes called aldolases, of which over 20 have been identified, catalyzing the stereoselective condensation of an aldehyde with a ketone donor [13].

5.1 Aldolase Reactions

Aldolases have been classified into two groups. Group 1 enzymes are obtained from animals, higher plants, and green algae and do not require metal ions for

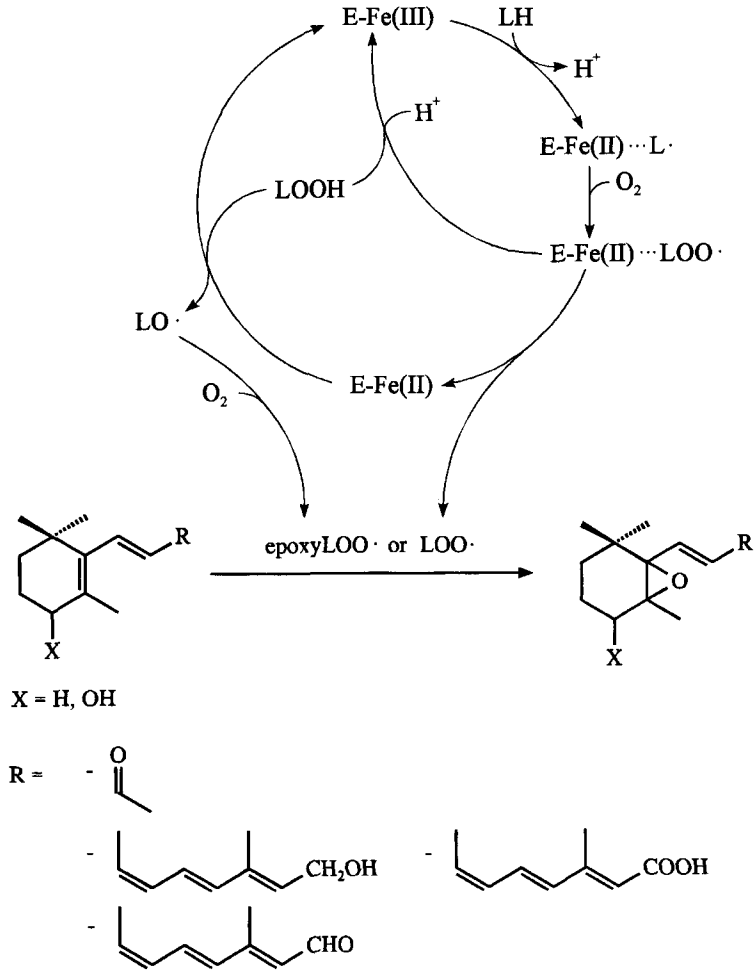


Fig. 9. Postulated mechanism of lipoxygenase-catalyzed aerobic co-oxidation. L, fatty acid; E, lipoxygenase [102]

their activity. Group 2 enzymes are isolated from bacteria, yeast and blue-green algae. The major differences from the Group 1 enzymes are lower pH optima, a requirement for potassium ions for activity and the presence of divalent metal ions in the active site [13].

In nature, there are four complementary aldolases involved in the carbohydrate metabolism which differ in their stereoselectivity (Fig. 10). Such a combination of enzymes provides a very powerful synthetic pool for the construction of new chiral centres with predictable absolute configuration.

In flavour biotechnology, first attempts have been made to use the high versatility of the aldolase reaction. As a representative example the enzymatic formation of 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (furaneol), an important

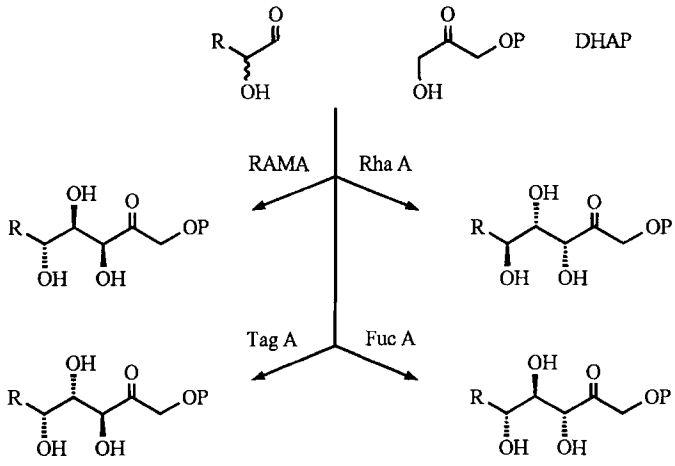


Fig. 10. Carbon–carbon bond formation by aldolases [12]. DHAP, dihydroxyacetone phosphate; RAMA, rabbit muscle aldolase (D-fructose-1,6-biphosphate aldolase); RhA, L-rhamnulose-1-phosphate aldolase; TagA, D-tagatose-1,6-biphosphate aldolase; FucA, L-fuculose-1-phosphate aldolase [11]

flavour compound, from fructose-1,6-bi-phosphate is mentioned. The proposed way comprises three enzymatic steps [107]: (i) the cleavage of the sugar biphosphate by rabbit muscle aldolase (RAMA) catalysis, yielding dihydroxyacetone phosphate and glyceraldehyde phosphate; (ii) the latter is isomerized by co-immobilized triose phosphate isomerase to give dihydroxyacetone phosphate which (iii) in turn is subjected to a RAMA-catalyzed aldol condensation with D-lactaldehyde, delivering 6-deoxyfructose phosphate. The monophosphate can easily be converted to furaneol.

6 Miscellaneous Enzymes

Other enzyme activities encountered in whole cell transformations have been used in flavour biotechnology. Lyase activity from *Pseudomonas putida* methionase has been described for the production of hydrogen sulfide and methanthiol, key odorants in cheese [108]. As to the formation of esters by sake yeast, the alcohol acetyl-transferase was characterized [109]. Cinnamoyl esterase and cinnamate decarboxylase may be prepared from commercial enzymes to produce 4-vinylphenols with impact attributes [110].

7 Conclusions

After intense research during the last 10–15 years, flavour biotechnology is an integrated part of industrial processes, in which enzyme technology is an

alternative to the use of microorgnisms. Lipase/esterase catalysis is state-of-the-art; in the near future, the current research in glucosidase activities will lead to new applications arising from both enzymatic and flavour precursor studies. As already achieved in bioorganic chemistry, in flavour biotechnology enzymes catalyzing both oxidoreductions and carbon–carbon forming reactions will provide selectively many valuable building blocks. For the production of "natural" flavours, the main drawback of enzyme technology still remains, however, the lack of appropriate "natural" substrates.

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Glycoconjugated Aroma Compounds: Occurrence, Role and Biotechnological Transformation

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The present paper reviews the occurrence of glycosidically bound aroma compounds in the plant kingdom and discusses different hypotheses concerning their role in plants. Emphasis is on biotechnological methods for flavor release and flavor enhancement through enzymatic hydrolysis of glycoconjugated aroma substances.

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List of Abbreviations

Ara	Arabinose
CCC	Countercurrent chromatography
CPC	Centrifugal partition chromatography
FAB	Fast atom bombardment
FTIR	Fourier transform infrared spectroscopy
Glc	Glucose
HPLC	High performance liquid chromatography
HRGC	High resolution gas chromatography (synonym: capillary gas chromatography)
HSCCC	High-speed countercurrent chromatography
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
ppb	part per billion
Rham	Rhamnose
RP	Reversed phase
SEC	Size-exclusion chromatography
XAD-2	Polystyrene resin for the adsorption of glycoconjugates
Xyl	Xylose

1 Introduction

The detection of glucoconjugated forms of monoterpene alcohols in rose petals more than 25 years ago by Francis and Allcock [1] has opened a new field in flavor research, i.e., the investigation of glycosidically bound aroma compounds. Nowadays, it is well established that flavorless glycosides represent one accumulation form of aroma substances in fruits and in many other plant tissues. Further nonvolatile constituents capable of generating volatile compounds are the terpene diphosphates. These phosphate esters, which are well-known intermediates in monoterpene biosynthesis [2], have been found to constitute a likely aroma reserve in, e.g., marjoram [3] as well as papaya fruit [4]. Since the vast majority of precursor compounds identified so far, however, have a glycosidic nature, this review will consider primarily glycoconjugated aroma precursors. Recent developments in the analysis of such polar plants constituents will be described and the occurrence of glycoconjugates in the plant kingdom will be summarized. The major emphasis, however, will be on their role in plants as well as on biotechnological methods for flavor release and flavor enhancement through enzymatic hydrolysis of the glycosidically bound volatiles.

2 Recent Reviews of the Field

The first period of research into glycosidic aroma precursors was centered on monoterpene glycosides, and this was independently reviewed in two papers in 1987 [5, 6]. In their overviews, Stahl-Biskup [5] and Mulkens [6] reported on the structures and the distribution of monoterpene glycosides identified until 1986. In the paper of Stahl-Biskup [5], 22 monoterpene glycosides, mainly glucosides, from 25 different plants are described. Whereas conjugates of geraniol, nerol, linalool, and α-terpineol are most often detected, glycosides with uncommon terpene structures are also described. Furthermore, the analysis of monoterpene glycosides and their functions in the plants are discussed. In the review of Mulkens [6], the glycosylesters of monoterpenoid aglycones are also included. From 28 plants, 25 different aglycones are listed. Special emphasis is placed on the methods of isolation and the role of monoterpene glycosides.

After this initial period, glycoside research has seen a rapid expansion, and an update on glycosidically bound volatiles was published in 1993 by Stahl-Biskup et al. [7], this time covering terpenoid and non-terpenoid glycosides. The distribution of approximately 200 aglycones (aliphatic alcohols, alkylphenols, monoterpenoids, sesquiterpenoids, and norisoprenoids) within 150 plant species is reported. In addition, possible roles of glycosides in essential oil plants, fruits,

and other plant tissues are discussed. A later review by Winterhalter and Schreier [8] was devoted to the class of carotenoid-derived aroma compounds, the so-called C_{13}-norisoprenoids. Recognition that the majority of C_{13}-norisoprenoid volatiles are similarly derived from glycosidic precursors prompted strong research activity in this field. In this review, data collected until 1993 on the occurrence and chemical composition of C_{13}-glycosides as well as their role as flavor precursor is presented.

An overall survey of the field of flavor precursors was provided in 1993 by Williams [9] and Williams and co-workers [10]. Emphasis was given to precursor occurrence, composition, reactivity, and analysis. Problems associated with the use of glycosidase enzymes and the need for sensory studies on precursors hydrolysates are discussed.

Günata et al. [11] reviewed the role of enzymes in the exploitation of the flavor potential from grape glycosides in winemaking. Nevertheless, the data presented is not restricted to winemaking, but equally important for all other applications of flavor enhancement through enzymatic release of glycosidically bound volatiles.

3 Methods of Precursor Analysis

Glycosidic aroma precursors are conveniently isolated from plant extracts, fruit juices, and wine by selective retention on C_{18}-reversed phase adsorbent [12] or Amberlite XAD-2 resin [13], respectively, followed by the desorption of the

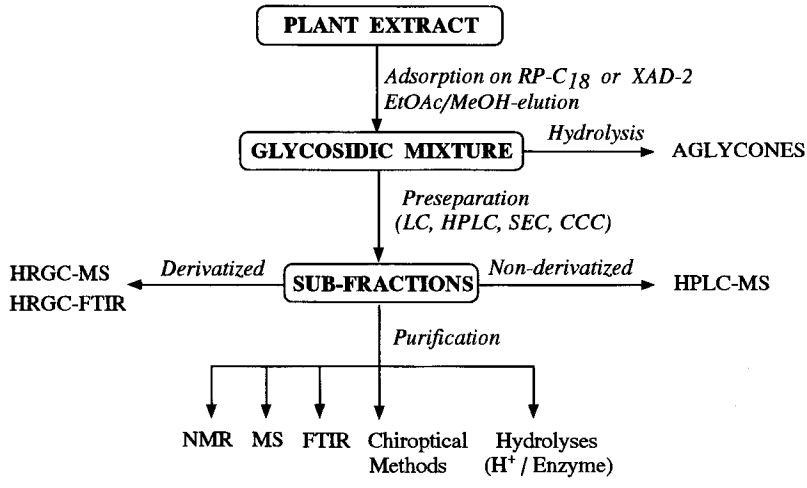


Fig. 1. Analysis of glycoconjugated aroma precursors (scheme)

retained glycosides using EtOAc or MeOH (cf. Fig. 1). Once a precursor concentrate has been obtained, two lines of investigations can be pursued. The first rapid approach consists of a HRGC-MS analysis of the aglycone fraction obtained after enzymatic and acid hydrolysis. If information on the structures of individual glycoconjugates is required, more thorough studies must be carried out. Due to the heterogenity and complexity of the glycosidic extracts, a whole set of sophisticated chromatographic separation steps is usually applied to yield finally the intact glucoconjugate in pure form. For preliminary separations, LC techniques are generally suited, such as, e.g., preparative HPLC, size-exclusion chromatography, or countercurrent chromatography (CCC). Especially the latter technique was found to have several great advantages with regard to the analysis of polar natural products, including glycosidic aroma precursors [14]. As an all-liquid chromatographic technique, CCC does not employ solid sorbents, i.e., adsorption losses and the formation of artefacts caused by active surfaces are eliminated or at least minimized. Instead of using solid packing materials, which in many cases – especially in preparative-scale workups – are very costly, CCC techniques rely exclusively on inexpensive solvent mixtures. Further advantages can be seen in the large sample load – several grams of crude extracts can be separated in a single run – and the total recovery of the sample material. Commercially available CCC-instruments which have been successfully used in flavor precursor studies are outlined in Fig. 2 ([14–17] and references therein).

Since, in most cases, the so-obtained glycosidic subfractions still contain a more or less complex mixture of glycosides, further purification steps involving, e.g., analytical HPLC or analytical CCC have to be applied. Alternatively, a derivatization (acetylation, methylation, silylation) of the glycosidic mixture offers a possibility to convert the nonvolatile glycoconjugates in more volatile forms, which are amenable to on-line coupled HRGC-MS or HRGC-FTIR analyses [18]. More recently, the application of FAB-MS/MS [19] as well as on-line coupled HPLC-MS/MS [20] has opened up a new dimension in precursor analysis, combining the high separation power of HPLC with the ability of

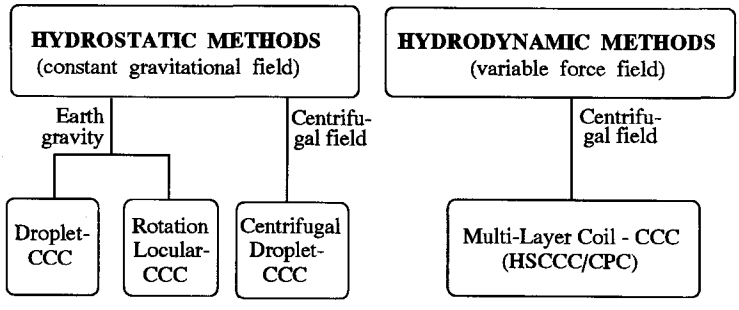


Fig. 2. Commercially available CCC-equipment used in flavor precursor studies. For details see [14–17]

MS/MS to provide significant information from the daughter and parent ion relationships. Especially in cases where reference glycosides are available, the on-line coupling of HPLC-MS/MS can be seen as method of choice for the analysis of trace amounts of glycosides in complex natural mixtures [20].

4 Occurrence of Glycosidically Bound Volatiles

Since the first detection of monoterpene glucosides in rose petals in 1969, the knowledge about the distribution of glycosidically bound volatiles in the plant kingdom has dramatically increased. In both essential oil plants and non-essential oil-bearing plants, glycoconjugated aroma substances have been found [21–225, 232–250]. In many cases, the amount of glycosidically bound flavors was found to exceed the amount of the free aroma in a ratio range of 2 : 1 to 5 : 1. Table 1 summarizes our present knowledge about the occurrence of glycosidically bound volatiles. It is obvious that glycoside-bearing species belong to many different plant families; up to now they have been detected in almost 50 plant families. Moreover, the formation of glycoconjugates was found not to be

Table 1. Occurrence of glycosidically bound volatiles (adapted from [6–8])

Plant family	Species	Organ	References
Actinidiaceae	*Actinidia chinensis*	leaves	[169]
Alangiaceae	*Alangium platanifolium*	leaves	[21]
Anacardiaceae	*Mangifera indica*	fruits	[22, 239, 240]
	Spondias mombins	fruits	[23]
Apiaceae	*Apium graveolens*	rhizome	[24]
	Carum copticum	seeds	[25]
	Eryngium campestre	roots	[26]
	Ferula loscosii	aerial parts	[27]
Apocynaceae	*Vinca rosea*	leaves & stems	[28]
Aspidiaceae	*Polystichum tripteron*	aerial parts	[163]
Asteraceae	*Artemisia santolinifolia*	aerial parts	[176]
	Calendula arvensis	aerial parts	[29]
	Calendula persica	aerial parts	[30]
	Carthamus lanatus	aerial parts	[31–33]
	Carthamus mareoticus	aerial parts	[34]
	Carthamus oxyacantha	aerial parts	[35]
	Carthamus turkistanicus	aerial parts	[36]
	Conyza aegyptica	aerial parts	[37]
	Dendranthema shiwogiku	aerial parts	[180]
	Dicoria canescens	aerial parts	[191]
		whole plant	[38]
	Eupatorium tinifolium	whole plant	[39]
	Gaillardia coahuilensis	aerial plants	[40]
	Gibbaria ilicifolia	aerial parts	[41]
	Helenium donianum	aerial parts	[42]

Table 1. (continued)

Plant family	Species	Organ	References
	Hymenoxys ivesiana	aerial parts	[43]
	Hypochoeris radicata	whole plant	[44]
	Iphiona mucronata	aerial parts	[45]
	Iphiona scabra	aerial parts	[46,47]
	Ixeris repens	whole plant	[48]
	Melampodium divaricatum	roots & aerial parts	[49]
	Osteospermum auriculatum	aerial parts	[50]
	Osteospermum microcarpum ssp. *septentrionale*	aerial parts	[41]
	Osteospermum rigidum ssp. *elegans*	aerial parts	[41]
	Osteospermum scariosum var. *scariosum*	aerial parts	[50]
	Picris hieracioides var. *japonica*	whole plant	[51]
	Pluchea indica	aerial parts	[52]
		roots	[53]
	Psilostrophe villosa	aerial parts	[54]
	Sonchus asper	whole plant	[182]
	Syneilesis aconitifolia	roots	[55]
		aerial parts	[55]
	Tanacetum vulgare	leaves	[192]
		petals	[56]
Averrhoaceae	*Averrhoa carambola*	fruit	[178,213,237]
Berberidaceae	*Epimedium diphyllum*	aerial parts	[156]
	Epimedium grandiflorum var. *thunbergianum*	aerial parts	[57–59,156,177]
	Epimedium sagittatum	aerial parts	[155]
	Epimedium ssp.	aerial parts	[155–157]
Betulaceae	*Betula alba*	leaves	[60,103]
Bromeliaceae	*Ananas comosus*	fruits	[61,62]
Caesalpiniaceae	*Gymnocladus chinensis*	fruits	[63]
Capparidaceae	*Boscia salicifolia*	leaves	[179]
Caprifoliaceae	*Viburnum orientale*	leaves	[64]
	Viburnum phlebotrichum	leaves	[65]
	Viburnum urceolatum	leaves	[66]
Caricaceae	*Carica papaya*	fruits	[4,193,242,243]
Cistaceae	*Cistus laurifolius*	aerial parts	[171]
Cupressaceae	*Chamaecyparis lawsonia*	leaves	[68]
	Chamaecyparis nootkatensis	leaves	[68]
	Chamaecyparis obtusa	leaves	[69]
	Cryptomeria japonica	leaves	[68]
	Cupressocyparis leylandii	leaves	[68,70]
	Cupressus bakeri	needles	[68]
	Juniperus chinensis var.*pfitzeriana*	needles	[70]
	Juniperus communis	needles	[70]
	Juniperus sabina	needles	[68]
Ericaceae	*Arctostaphylos uva-ursi*	twigs	[71]
Gentianaceae	*Gentiana pneumonanthe*	leaves	[160,161]
Geraniaceae	*Pelargonium odoratissimum*	aerial parts	[72]
Lamiaceae	*Bystropogon plumosus*	leaves	[73]
	Hyssopus officinalis	whole plant	[74]
	Lavandula officinalis	leaves	[75]
	Majorana hortensis	leaves	[3,76]
	Melissa officinalis	leaves	[70,75,77–79]

Table 1. (continued)

Plant family	Species	Organ	References
	Mentha arvensis var.*piperascens*	leaves	[80,81,205]
	Mentha gentilis f.cardiaca	aerial parts	[82]
	Mentha longifolia	herb	[83]
	Mentha x piperita	leaves	[84,85]
	Mentha x piperita ssp.*citrata*	herb	[70]
	Mentha pulegium	aerial parts	[7]
	Mentha species		[194]
	Mentha spicata	herb	[83]
		leaves	[79]
	Monarda didyma	herb	[87]
	Monarda fistulosa	herb	[88]
	Ocimum basilicum	leaves	[89]
	Origanum vulgare	herb	[75,83]
	Rosmarinus officinalis	leaves	[75]
	Salvia officinalis	herb	[70,90]
		leaves	[75]
	Schizonepeta tenuifolia	aerial parts	[91]
	Thymus x citriodorus	aerial parts	[7]
	Thymus praecox ssp.*arcticus*	aerial parts	[7]
	Thymus vulgaris	herb	[70]
		leaves	[93]
Lauraceae	*Cinnamomum cassia*	stem bark	[94]
Liliaceae	*Liriope spicata* var.*prolifera*	roots	[95]
Martyniaceae	*Martynia lousiana*	leaves	[172]
		stems	[172]
Meliaceae	*Melia toosendan*	leaves	[181]
Olacaceae	*Scorodocarpus borneensis*	leaves	[190]
Oleaceae	*Jasminum sambac*	flower buds	[221–224]
	Ligustrum pedunculare	leaves	[212]
	Osmanthus asiaticus	bark	[249]
Orchidaceae	*Vanilla planfolia*	fruits	[96,97,246]
Orobanchaceae	*Aeginetia indica*	whole plant	[187]
Paeoniaceae	*Paeonia lactiflora*	roots	[153]
Passifloraceae	*Passiflora edulis*	fruits	[86,98]
	Passiflora edulis var. *flavicarpa*	fruits	[195]
Pinaceae	*Abies alba*	needles	[68]
	Cedrus atlantica	needles	[68]
	Cedrus deodora	needles	[68]
	Picea abies	needles	[68,99]
	Pecea omorika	needles	[68]
	Picea pungens	needles	[68]
	Pinus mugo	needles	[68]
	Pinus strobus	needles	[68]
	Pinus sylvestris	needles	[100]
Poaceae	*Melica uniflora*	leaves	[101]
Polypodiaceae	*Dryopteris filix-mas*	leaves	[101]
Rhamnaceae	*Hovenia dulsis* var. *tomentella*	leaves	[250]
	Ziziphus jujuba var. *inermis*	fruits	[102]
Rosaceae	*Chaenomeles japonica*	fruits	[60,103]
	Cydonia oblonga	fruits	[103–108,117,157]
	Eriobotrya japonica	leaves	[109,247]
	Fragaria ananassa	fruits	[110,244]
	Geum urbanum	roots	[111]
	Malus domestica	leaves	[168]
		fruits	[112–115,174,197]
	Prunus armeniaca	fruits	[22,116,154,232]
	Prunus cerasus	fruits	[118,119]

Table 1. (continued)

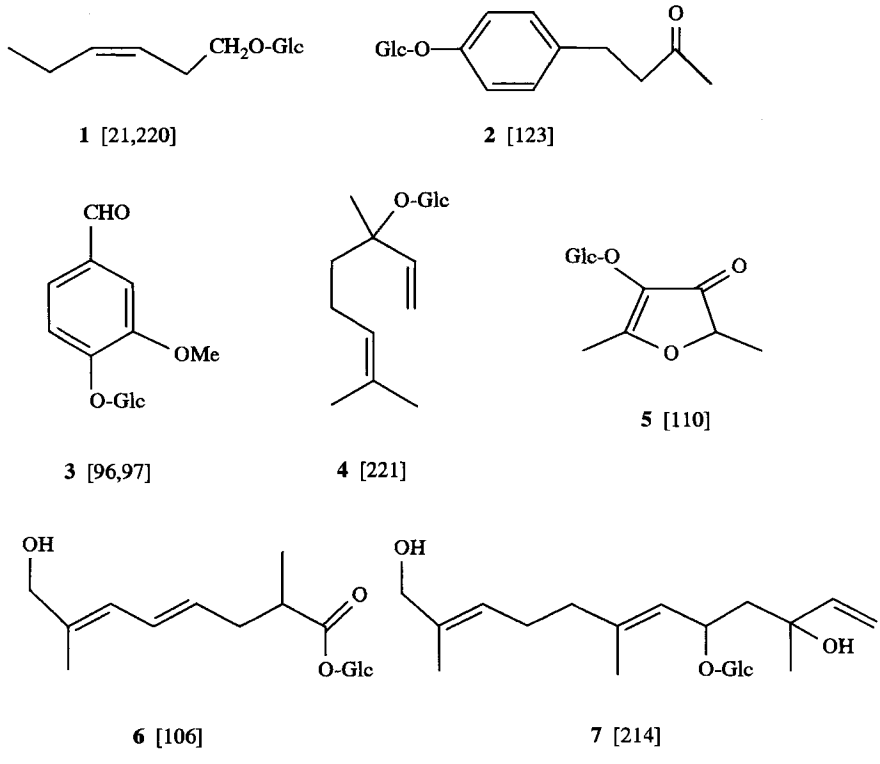
Plant family	Species	Organ	References
	Prunus domestica ssp. *syriaca*	fruits	[116]
	Prunus persica	fruits	[61, 116]
	Prunus spinosa	leaves	[122]
	Rosa alba	petals	[120]
	Rosa bourboniana	petals	[120]
	Rosa canina	petals	[120]
	Rosa centifolia	petals	[120]
	Rosa damascena cvs.	petals	[120]
	Rosa dilecta	petals	[121, 198–201]
	Rosa gallica cvs.	petals	[120, 121]
	Rosa hybrid Tearose cvs.	petals	[1, 120]
	Rosa moschata	petals	[120]
	Rosa rugosa	petals	[70]
	Rosa wichuratana	petals	[120]
	Rubus idaeus	fruits	[67, 92, 123, 124, 154, 158, 159]
	Rubus laciniata	fruits	[125]
		leaves	[125]
	Sorbus aria		[185]
	Spiraea cantoniensis	leaves	[126]
Rubiaceae	*Canthium subcordatum*	bark	[173]
Rutaceae	*Citrus unshiu*	leaves	[127, 162]
Salicaceae	*Salix fragilis*	leaves	[101]
Sapindaceae	*Sapindus delavayi*	pericarps	[128]
	Sapindus mukurossi	pericarps	[129]
	Sapindus trifoliatus	pericarps	[130]
Saxifragaceae	*Ribes rubrum*	leaves	[183]
	Ribes uva-crispa	leaves	[184]
Scrophulariaceae	*Penstemon digitalis*	leaves	[131]
	Picrorhiza kurrooa	roots	[132]
	Rehmannia glutinosa var. *purpurea*	roots	[133, 188, 189]
Solanaceae	*Atropa belladonna*	leaves	[134]
	Lycium halimifolium	leaves	[135]
	Lycopersicon esculentum	fruits	[17, 136, 245]
	Nicotiana accuminata	leaves	[137]
	Nicotiana alata	leaves	[137]
	Nicotiana repanda	leaves	[137]
	Nicotiana rustica	leaves	[137]
	Nicotiana sylvestris	leaves	[137, 248]
	Nicotiana tabacum	leaves	[137, 167]
		tobacco	[138–141]
	Nicotiana undulata	leaves	[137]
	Solanum tuberosum	leaves	[142, 214]
	Solanum vestissimum	fruits	[143, 241]
Taxaceae	*Taxus baccata*	needles	[101]
Theaceae	*Camellia sinensis*	aerial parts	[202]
		leaves	[144, 145, 215–220]
Urticaceae	*Urtica dioica*	leaves	[211]
		roots	[146]
Vitaceae	*Vitis vinifera*	fruits	[15, 17, 19, 22, 147–151, 166, 170, 175, 186, 203–205, 207–209, 233–236]
		leaves	[16, 164, 165, 186, 210]
Zingiberaceae	*Zingiber officinale*	juice	[152]

restricted to the aerial green parts of a species or the fruit, glycoconjugates having also been detected in the roots, rhizomes, petals, and seeds (cf. Table 1).

5 Chemical Composition of Glycoconjugated Flavors

5.1 Aglycone Part

The aglycone structures frequently reported are medium-chain alkanols and alkenols, shikimic acid metabolites as well as mevalonate-derived compounds with ten (monoterpenoids), thirteen (C_{13}-norisoprenoids), and fifteen carbon atoms (sesquiterpenoids). A selection of important glycosidic aroma precursors is presented in Fig. 3. Importantly, evidence is increasing that malonated glycosides may be more common in nature than previously thought. For a number of bound aroma volatiles, e.g., for structures **4** and **5**, substitution of the sugar part with a malonyl group was reported most recently [20, 221].



1 [21,220] **2** [123] **3** [96,97] **4** [221] **5** [110] **6** [106] **7** [214]

Fig. 3. Examples of important glycoconjugated aroma compounds, i.e., the β-D-glucopyranosides of Z-3-hexenol **1**, raspberry ketone **2**, vanillin **3**, linalool **4**, furaneol **5**, 2,7-dimethyl-8-hydroxy-4,6-octadienoic acid **6**, and 5,12-dihydroxy-β-nerolidol **7**. Reference numbers are given in brackets

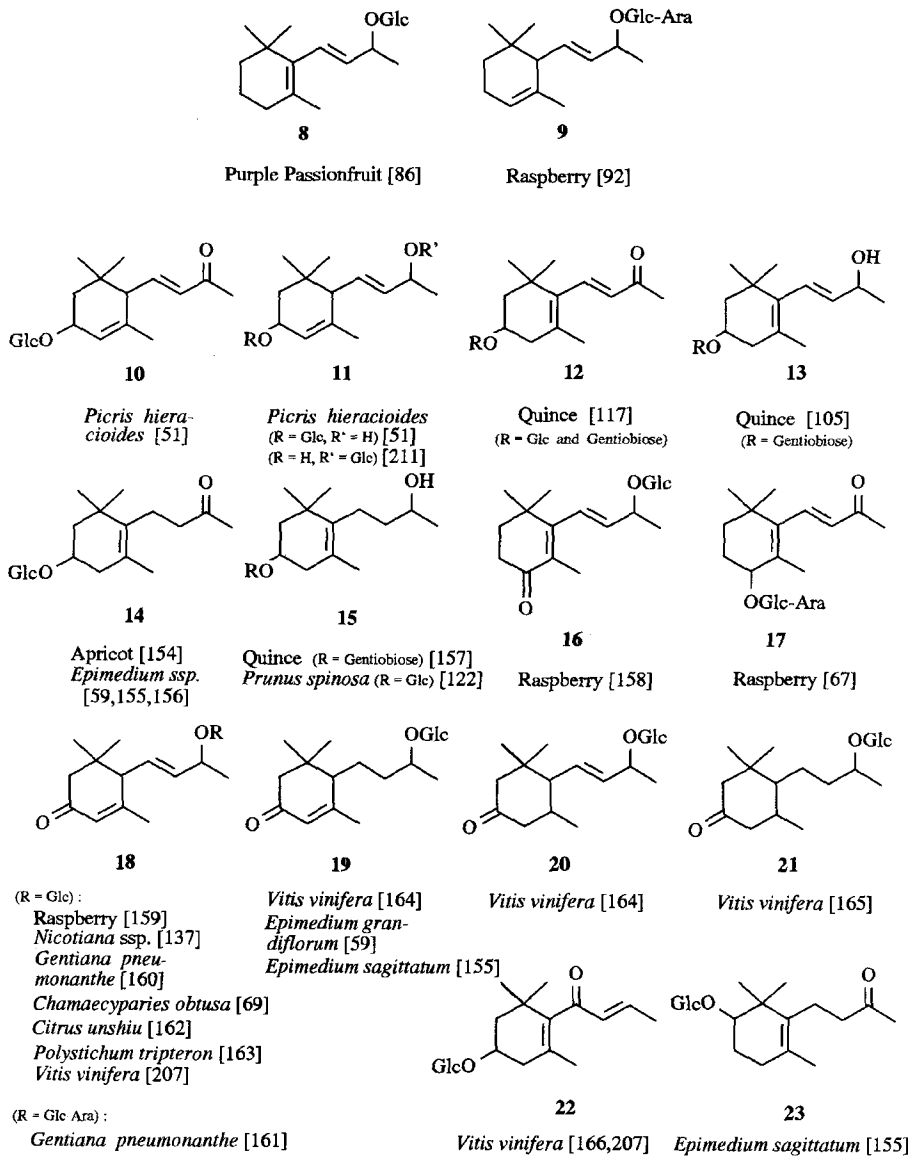
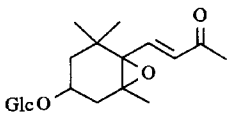


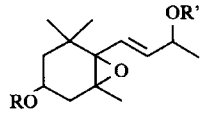
Fig. 4A, B. Structures and occurence of: **A** mono- and di-oxygenated C_{13}-glycosides; **B** higher-oxygenated C_{13}-glycosides. Reference numbers are given in brackets

Typically, the aglycones of plant glycosides are structurally complex and highly diverse. The group of carotenoid-derived C_{13}-norisoprenoids may serve as an example. The structures of C_{13}-norisoprenoid glycoconjugates that have been detected up to 1995 are summarized in Fig. 4. Also, for the shikimates, mono-, and sesquiterpenoids, a similar diversity in the aglycone structure is known. Details are given in [5–8].



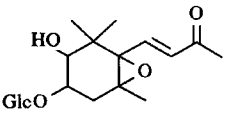
24

Epimedium ssp. [57,155,156]
Picris hieracioides [51]
Vitis vinifera [165]



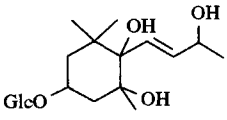
25

(R = H, R' = Glc):
Nicotiana ssp. [167]
Malus domestica [168]
(R = Glc, R' = H):
Prunus spinosa [122]



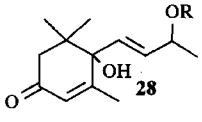
26

Epimedium diphyllum [156]



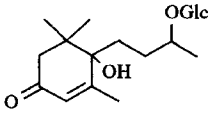
27

Kiwi [169]



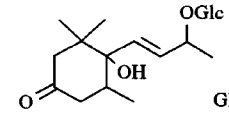
28

(R = Glc): [28,51,58,69,100, 103,154,162,165,170-173]
(R = Glc-Xyl): apple [113]
(R = Glc-Xyl-Glc): apple [174]
(R = Glc-Rham, Glc-Glc-Glc): [102]



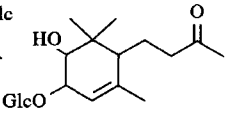
29

Pinus sylvestris [100]
Epimedium grandiflorum [59]



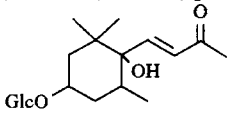
30

Vitis vinifera [175]
Artemisia santolinifolia [176]



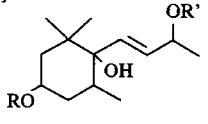
31

Epimedium grandiflorum [177]



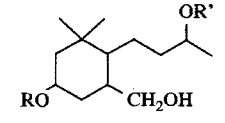
32

Starfruit [178]
Boscia salicifolia [179]
Denthranthema shiwogiku [180]



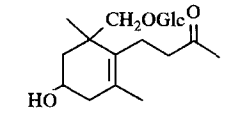
33

Starfruit (R = H, R'= Glc) [178]
Denthranthema shiwogiku (R = Glc, R'= H) [180]



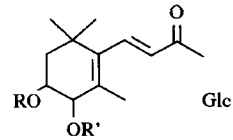
34

Melia toosendan [181]
(R = Glc, R'= H) and
(R = H, R'= Glc)



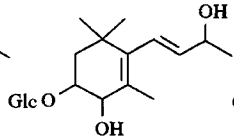
35

Epimedium grandiflorum [58]



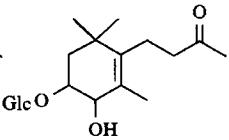
36

(R = Glc, R'= H) :
Vitis vinifera [164]
Picris hieracioides [51]
(R = H, R'=Glc / Glc-Api) :
Sonchus asper [182]



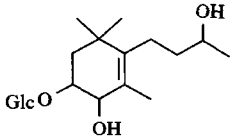
37

Pluchea indica [52]



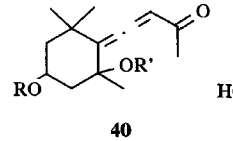
38

Epimedium diphyllum [156]
Ribes rubrum [183]
Vitis vinifera [164]



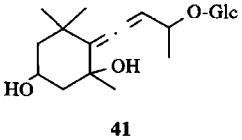
39

Ribes uva crispa [184]
Sorbus aria [184,185]
Vitis vinifera [186]



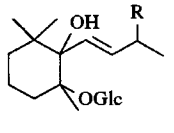
40

Vitis vinifera (R = Glc, R'= H) [165]
Sonchus asper (R = Glc, R'= H) [182]
Epimedium ssp. (R = Glc, R'= H) [57,156]
Scorodocarpus borneensis (R = Glc, R'= H) [190]
Citrus unshiu (R = H, R'= Glc) [127]
Cinnamomum cassia (R = Glc-Api, R'= H) [94]



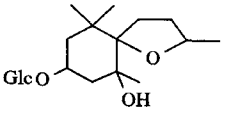
41

Lycium halimifolium [135]



42

(R: =O):
Aeginetia indica [187]
Rehmannia glutinosa [188]
(R: -OH):
Rehmannia glutinosa [189]



43

Scorodocarpus borneensis [190]

Fig. 4A, B. (continued)

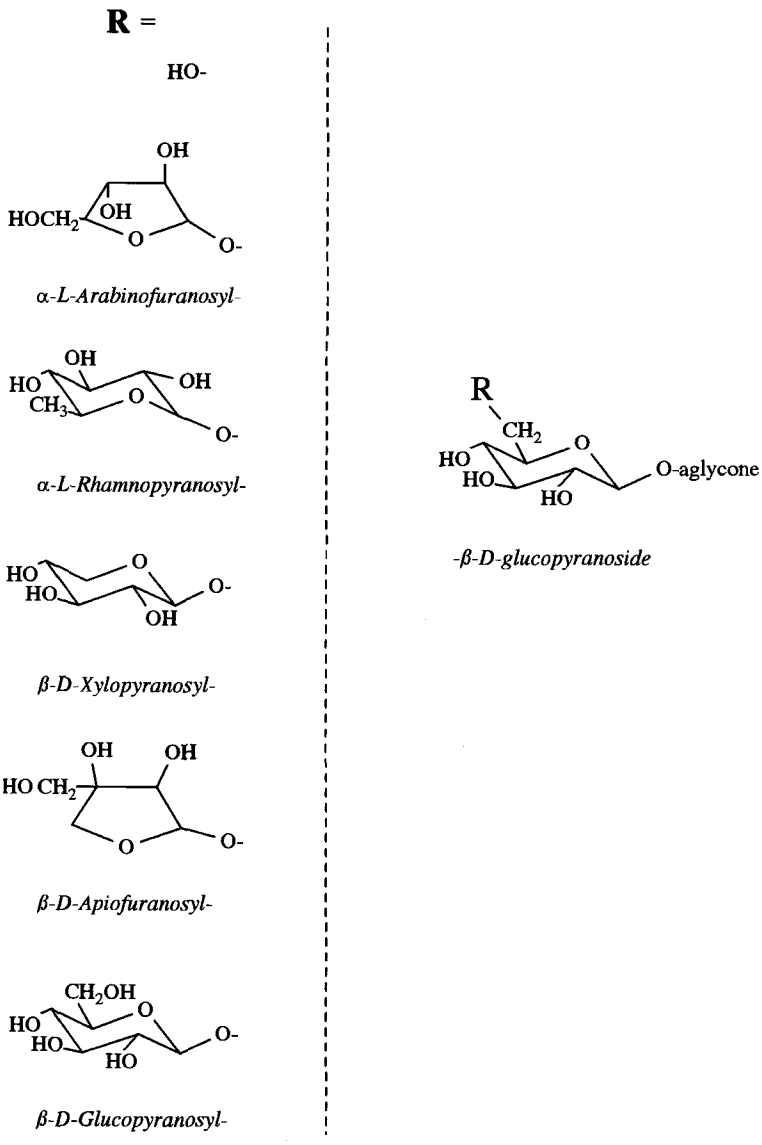


Fig. 5. Disaccharidic sugar moieties that have been identified in flavor precursors (adapted from [9])

5.2 Sugar Part

With regard to the glycone structure, a general feature of glycosidically bound volatiles is that the sugar directly bound to the aglycone is a β-D-glucose. This glucose moiety may or may not be further substituted with one or more

additional sugar units. So far, as a second sugar unit, the components outlined in Fig. 5 have been identified, i.e., α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, β-D-apiofuranose, and β-D-glucose [9 and references cited].

This finding has important consequences with regard to any envisaged biotechnological liberation of volatiles from glycosidic precursors. Since most of the applied hydrolases are *exo*-glycosidases, cleavage of such disaccharidic conjugates requires in the first step the action of an α-arabinosidase, α-rhamnosidase, β-xylosidase, or β-apiosidase for the enzymatic cleavage of the inter-sugar linkage, before in a second hydrolytic step a β-glucosidase activity is able to liberate the aglycone moiety [226]. Only in the case of gentiobiosides (β-D-glucopyranosyl-(1-6)-β-D-glucopyranosides), where the disaccharidic sugar moiety consists of two glucose units, is β-D-glucosidase activity able to liberate the aglycone in a two-step mechanism [227].

The fact that β-D-glucose is a general building block of glycoconjugates was used as basis for the development of a rapid method for the determination of the total glycoside content in wines, fruits, and other substrates (cf. Fig. 6). The procedure developed by Williams and co-workers [228] consists of (i) isolation of a glycosidic fraction by selective retention on C_{18}-reversed phase absorbent, (ii) acid hydrolysis of the so-obtained glycosidic fraction to liberate the glucose, and (iii) measurement of the released glucose in an enzyme assay (hexokinase/glucose-6-phosphate dehydrogenase). This method is already used by the wine industry to follow the development of glycoconjugates in ripening fruit and to monitor their decrease during the ageing of wines [229].

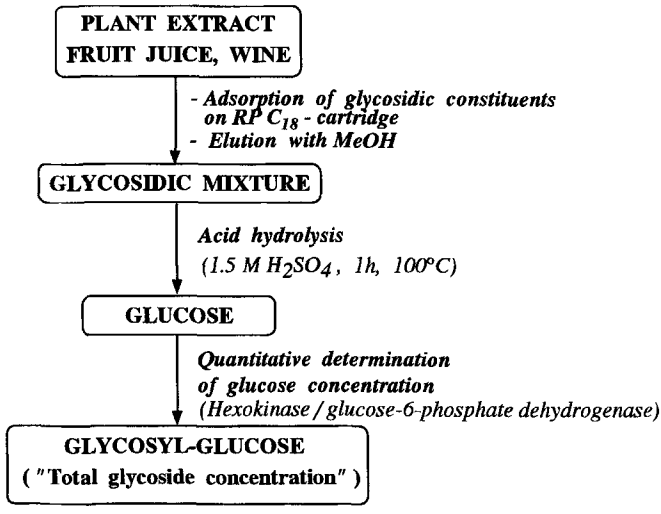


Fig. 6. Method for the rapid determination of the total glycoside content ("glucosyl-glucose assay") development by Williams et al. [228]

6 Role of Glycosidically Bound Volatiles in Plants

The discovery of a vast number of aroma glycoconjugates and their widespread distribution in the plant kingdom has led to speculations concerning their role in plants. This subject has recently been reviewed by Stahl-Biskup and co-workers [7], who have critically evaluated the different hypotheses published so far.

In summary, it can be concluded that plant glycosides are obviously involved in different processes. Glycoconjugates are considered to have importance as accumulation and storage forms as well as transport forms for hydrophobic substances. Furthermore, a general role as intermediates of secondary product metabolism is apparent.

6.1 Glycosides as Accumulation and Storage Forms of Aroma Substances

This hypothesis was initially proposed to explain, e.g., the accumulation of glycosidically bound volatiles during the maturation of fruits [204]. Indeed, in grapes and several other fruits as well as vegetables, the amount of "bound" volatiles was found to be considerably higher compared to the free volatile fraction. From a chemical point of view, glycosylated aroma compounds differ from the free aglycones in two main properties: they show enhanced water solubility and decreased reactivity. This may explain why glycosylated compounds, rather than the free aglycones, are accumulated in the plant kingdom [230]. Glycoconjugation allows a better storage within the plant vacuole and protects the plant from any form of toxicity exhibited by the free aglycone. Damage of labile cellular components, such as, e.g., membrane structures, is especially likely to be caused by high concentrations of lipophilic phenols or alcohols.

A protective mechanism through glycosylation is also apparent from experiments carried out by Berger and Drawert using plant cell cultures [231]. While rosemary and grape cells stopped growing, those cell lines that accumulated considerable amounts of glycosidically bound volatiles (conversion rate $> 40\%$), i.e., pear and peppermint cell cultures, continued growing. Obviously, their efficient glycosylation system was able to protect the cells from toxic effects exerted by an excess of added lipophilic substrates.

Besides this protective mechanism, studies of the accumulation profiles of bound monoterpenols in developing flowerheads of *Rosa* [1, 120] as well as *Jasminum* and *Gardenia* species [224] have thrown light on important additional functions of glycoconjugated forms of volatiles. Such glycoconjugates seem to play an important role in the mechanism of flower fragrance formation, and thus for the attraction of pollinating insects such as bee and wasp species.

In a recent study of Watanabe et al. [224], the following hypothetical mechanism of flower fragrance formation has been suggested (cf. Fig. 7). The

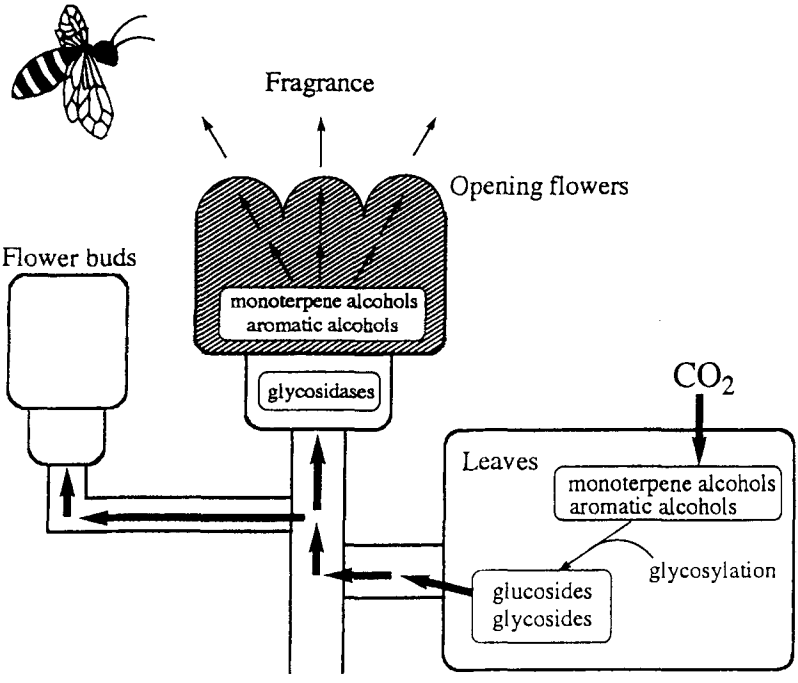


Fig. 7. Suggested pathway of flower fragrance formation according to Watanabe et al. [224] (reproduced with permission from [225])

aroma volatiles – biosynthesized in the leaves – are glycosylated and transported via the phloem to the flowers. Here, the glycosides accumulate as flavorless precursors in the flower buds. Upon flower opening, the precursors are enzymatically transformed into volatile compounds by the action of hydrolases. To substantiate this hypothesis, the Japanese research group designed an assay protocol as follows: (i) the accumulated fragrance precursors were extracted from flower buds, (ii) crude enzyme mixtures were obtained from the flowers at the opening stage, and (iii) the glycosidic precursors were then reacted with the crude enzyme mixtures. By monitoring the amount of enzymatically released volatiles, Watanabe et al. [224] could show that the aroma-producing enzyme activities of *Gardenia jasminoides* flowers rapidly increased to reach a maximum at the flower opening stage and decreased within 24 h after flower opening. The responsible enzymes were supposed to be newly induced and expressed or, alternatively, activated from inactive forms during flower opening.

6.2 Glycosides as Transport Form of Aroma Volatiles

Information about transport functions of glycoconjugates initially came from feeding experiments in which various substrates – being added exogenously – were

afterwards found in glycosylated form in a different part of the plant. An example is the study of Wills and Scriven on geraniol metabolism in apple fruit [197]. The authors observed that, after injection of geraniol into the core area of the fruit, the monoterpene alcohol was rapidly glycosylated before being transported to the flesh of the fruit, where it was further metabolized. In the case of menthone metabolism in peppermint leaves, Croteau and Martinkus found that (−)-menthone is partly converted into (+)-neomenthol, which is then transported into the rhizome as (+)-neomenthyl-β-D-glucoside [84].

Critical remarks concerning the transport role of glycosides in essential oil plants were recently published by Stahl-Biskup et al. [7]. Major weak points of the "transport theory" were seen in the facts that
(i) in some cases, glycosylation results in only a slight increase in the water solubility (example: thymol-glucoside), and
(ii) discrepancies are observed between the pattern of glycosidically bound volatiles and free volatiles, as, e.g., in the case of several *Coniferous* species [68]. For further details, cf. [7].

This still on-going discussion reflects that the specific role of glycoconjugated aroma compounds in plants has yet to be determined. A general role, however, appears to be that lipophilic aroma compounds – like many other secondary plant products – are accumulated and stored in form of their glycoconjugates, thus protecting the plant from any form of toxicity exhibited by the free aglycone. Questions regarding the extent of enzymatic release of the accumulated aroma glucoconjugates in vivo as well as possible physiological roles remain the subject for future research.

7 Applied Biotechnological Transformations of Glycoconjugated Aroma Compounds

The finding that many plant tissues accumulate aroma compounds in form of nonvolatile and flavorless glycosides prompted many research activities towards possible applications of hydrolases to liberate the "bound" aroma portion [9–11, 112, 251–253]. Due to the economical importance of winegrowing, grapes and wines were the substrates which research has focused on [9–11, 150, 226, 254–260].

Enzymatic cleavage of glycosidic aroma precursors, however, is not only considered as a possibility for aroma enrichment of wine or fruit juices. The enzymatic release of bound volatiles also offers a tool for the production of natural flavors from otherwise waste materials, such as, e.g., peelings, skins, and stems, from which – after enzymatic treatment – the liberated volatiles can be distilled off before the residues are discarded [261].

Table 2. Properties of endogenous glycosidases (adapted from [9])

Enzyme Source	Availability	Optimum pH	Inhibition by Glucose	Ref.
Plant-derived glycosidases				
Almond	commercial	5.0	10% at 50 mmol/l	[13,254]
Grape	isolated	5.0	40% at 50 mmol/l	[254,262]
Papaya	isolated	5.0	50% at 10 mmol/l	[263]
Yeast-derived glycosidases				
Candida molischiana	isolated	4.5	na	[258,264]
Hanseniaspora vineae	isolated	6.0	60% at 50 mmol/l	[265]
Candida wickerhamii	isolated	4.5	10% at 50 mmol/l	[11]
Hansenula sp.	isolated	na	50% at 50 mmol/l	[255]
Saccharomyces cerevisiae	isolated	5.0	0% at 280 mmol/l	[11,266]

(na: not available)

7.1 Properties of Endogenous Glycosidases

Both endogenous as well as exogenous enzymes have been tested with regard to practical applications in flavor release. In the study of Aryan et al. [254] the problems in using endogenous plant enzymes first became apparent. The authors investigated endogenous glycosidases of *Vitis vinifera* for their possible application during wine processing in order to enhance the aroma of wine. With pH optima at 5.0 and a strong inhibition in the presence of glucose, endogenous grape glycosidases were found to be virtually inactive in grape juice at its natural pH. Similar pH optima and competive inhibition by glucose have been observed for other endogenous plant or yeast derived glycosidases as summarized in Table 2 [9]. The only exception with regard to sugar tolerance was observed for a periplasmic glucosidase from *Saccharomyces cerevisiae*, which showed a strong activity even at a glucose concentration of 5% (280 mmol/l). Further restrictions in the use of endogenous glycosidases in fruit juice processing and winemaking are due to their known aglycone specificity and their low ethanol tolerance [9,11 and references therein].

For practical applications these results have important consequences. They indicate that most of the glycosides present in fruit or grape juices are not hydrolyzed by the endogenous glycosidases during processing. Also, in course of the fermentation process, glycosidase activities of yeast strains will barely affect the glycoside content in must. As a result, the majority of glyconjugated flavor compounds remains as a non-utilized source of potential aroma. Consequently, the use of exogenous glycosidases or, alternatively, the application of chemical processes (i.e., acid hydrolyses) have been suggested for aroma enrichment of wine and fruit juices [254].

7.2 *Application of Exogenous Glycosidases*

With regard to the envisaged exploitation of the glycosidic pool in plants through application of exogenous glycosidases, three important properties of the hydrolases have to be considered: (i) specificity, (ii) pH optimum, and (iii) glucose as well as ethanol tolerance.

7.2.1 Specificity of Exogenous Glycosidases

Although β-D-glucosides constitute the majority of the known glycoconjugated precursor compounds, disaccharide glycosides are frequently reported. In some fruits, e.g., grapes and quince fruit, they represent the preferred storage form of aroma substances [105, 117, 151, 203]. In rare cases, higher saccharidic conjugates have been reported [174, 267]. The disaccharidic aroma conjugates frequently found in natural substrates are: 6-O-β-D-xylopyranosyl-β-D-glucopyranosides (primverosides), 6-O-α-L-arabinofuranosyl-β-D-glucopyranosides (vicianosides), 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides (rutinosides), 6-O-β-D-glucopyranosyl-β-D-glucopyranosides (gentiobiosides), and 6-O-β-D-apiofuranosyl-β-D-glucopyranosides (cf. Fig. 5).

For the hydrolysis of these disaccharidic glycosides – except for the gentiobiosides – sequential activities are reportedly needed, i.e., in the first step a function (β-xylosidase, α-arabinosidase, α-rhamnosidase, or β-apiosidase) capable of hydrolyzing the inter-sugar link, and in the second step a β-glucosidase activity for the cleavage of the remaining β-glucosidic moiety [226]. Such multiple glycosidase activities are reported for fungal enzyme preparations [11, 254]. Günata et al. [11] have screened commercially available fungal enzyme preparations which have been applied in the studies of glycosidic aroma precursors. It was observed that enzyme preparations obtained from *Aspergillus niger* strains contain at various levels β-glucosidase, α-arabinosidase, and α-rhamnosidase activity, whereas β-apiosidase activity is rarely detectable as shown in Table 3 (Note: β-xylosidase activity has not been tested).

The production of β-apiosidase activity, however, was found to be inducible when apiin (a trihydroxyflavone-apiosylglucoside) was used as a carbon source in the culturing medium [260]. The above-mentioned application of sequential enzymatic hydrolysis in order to liberate bound aroma compounds is claimed in a recent patent [253].

Whereas most of the commercially available fungal enzyme preparations show *exo*-glycosidase activities, a fungus-derived *endo*-glycosidase has also been reported. Shoseyev et al. [268] isolated an *Aspergillus niger* strain that produces an extracellular *endo*-β-glucosidase when grown on a medium containing rutin as the sole carbon source. The enzyme was found to be able to cleave rutinosides in a one-step process into intact rutinose and the free aglycone.

Table 3. Glycosidase activities (nkat mg product) in commercial enzyme preparations according to Günata et al. [11]

Enzyme	β-apiosidase	β-glucosidase	α-arabinosidase	α-rhamnosidase
preparation[a]				
Cellulase A	–	6.1	0.6	0.07
Hemicellulase	–	7.1	7.0	0.9
Pectinol VR	–	0.2	0.1	–
Rohament GW	–	3.3	0.7	0.4
Pectinol D5S	–	0.5	0.7	–
Pektolase 3PA	0.3	1.5	3.8	0.04
Ultrazym 100	0.03	0.5	0.1	–
Pectinase 263	0.2	7.2	1.4	0.3

[a] Commercial sources: Ciba-Geigy (Ultrazym 100), Gist Brocades (Cellulase A, Hemicellulase, Pectinase 263), Gringsted (Pecktolase 3PA), Röhm (Pectinol VR, Rohament CW, Pectinol D5S)

More important, its pH optimum was found to be 3.4. The enzyme has already been applied in immobilized form to enhance the aroma of wine as well as passion fruit juice [259].

It is noteworthy, that according to Hösel [230], such *endo*-glycosidases have been known in plants for a long time. An *endo*-rutinoside was, e.g., purified from seeds of *Fagopyrum esculentum* [269]. A similar β-glycosidase acting on furcatin required the apiosyl (1-6) glucoside unit for activity, and neither the rutinose moiety nor the apisoyl (1-2) glucose moiety was accepted as substrates [270]. An *endo*-glycosidase acting on the latter substrate was purified to apparent homogeneity from leaves of *Cicer arietinum* [271]. For envisaged modifications of glycosidases using genetic engineering, these plant-derived *endo*-glycosidase may well constitute a valuable genetic pool.

7.2.2 pH Optimum of Exogenous Glycosidases

Contrary to the endogenous plant glycosidases with pH optima around 5 (cf. Table 2), several microbial enzymes were found to possess a pH optimum between 3 and 4. Thus, they are more suitable for practical applications under natural pH conditions in fruit juices or wine. These enzymes were either isolated from filamentous fungi [268] or from yeast strains [272, 273]. For the most commonly used glycosidase preparations from *Aspergillus niger* (e.g., Hemicellulose, Pectinol, Rohapect), however, β-glucosidase activity was found to be maximum in a pH range of 4.5–6.0 depending on the enzyme preparation used [11].

7.2.3 Glucose and Ethanol Tolerance of Exogenous Glycosidases

Aspergillus niger-derived β-glucosidases are strongly inhibited even at low glucose levels (inhibition constants 3–10 mmol/l). Quite resistant to inhibition

by glucose are the β-glucosidases from enological yeasts and the exocellular enzyme from *Candida wickerhamii*. *C. wickerhamii* β-glucosidase retained 44% of its initial activity in 500 mmol/l glucose medium, the usual level of glucose in grape juice. Under the same conditions the yeast enzyme lost only 30% of its initial activity [11].

With regard to ethanol tolerance, β-glucosidases from *Aspergillus niger* as well as from yeast and *C. wickerhamii* are almost not inhibited by 10% ethanol [11,254]. Even at a concentration of 15% ethanol, *Aspergillus niger* β-glucosidase activity was only reduced by 20% [254].

7.3 Sensory Properties of the Enzymatically Released Aglycones

Unlike glycosidically bound monoterpenols (e.g., conjugates of linalool) or phenolic constituents (e.g., conjugates of raspberry ketone) which, after enzymatic hydrolyses, liberate an "attractive" aglycone, a considerable number of glycoconjugates liberate a polyol structure, which in most cases is flavorless. For these structures, further modifications, e.g., acid-catalyzed conversions at elevated temperatures, are required to generate finally the odor-active form [135,157,241,274]. Two examples are shown in Fig. 8.

In this context, it should be mentioned that the contribution of glycosidically bound volatiles to the varietal wine aroma was recently determined by sensory descriptive analysis [229,275,276]. The experiments carried out by Francis et al. [275,276] showed that desirable flavor changes in the varieties under investigation could already be achieved by heat-treatment alone. Contrary to earlier results obtained for muscat varieties which liberated considerable amounts of aroma-active monoterpenols, this recent study revealed for Semillon and Chardonnay wines that the sensory contribution of acid-hydrolysates was much higher compared to the enzymic hydrolysates [275]. As an appropriate procedure for acid-catalyzed degradation of glycosidically bound volatiles in wine, storage of wines protected from air at 45 °C for several weeks has been suggested [276].

7.4 Acid vs Enzymatic Hydrolysis

For the understanding of the sensory differences observed for acid and enzymatic hydrolysates, respectively, differences in the reactivity of bound aroma compounds have to be considered. Apart from the aforementioned necessity to convert flavorless polyol structures into the odor-active form via acid and/or heat-treatment (cf. Fig. 8), recent results revealed a significant influence of the glycosidic linkage on the chemical reactivity of the bound aglycone.

It was not until 1991 that a comparative study was performed on the reactivity of aglycones and their corresponding β-D-glucopyranosides at a pH range typical of wines and fruits (pH 3–5) [277]. This study showed that the

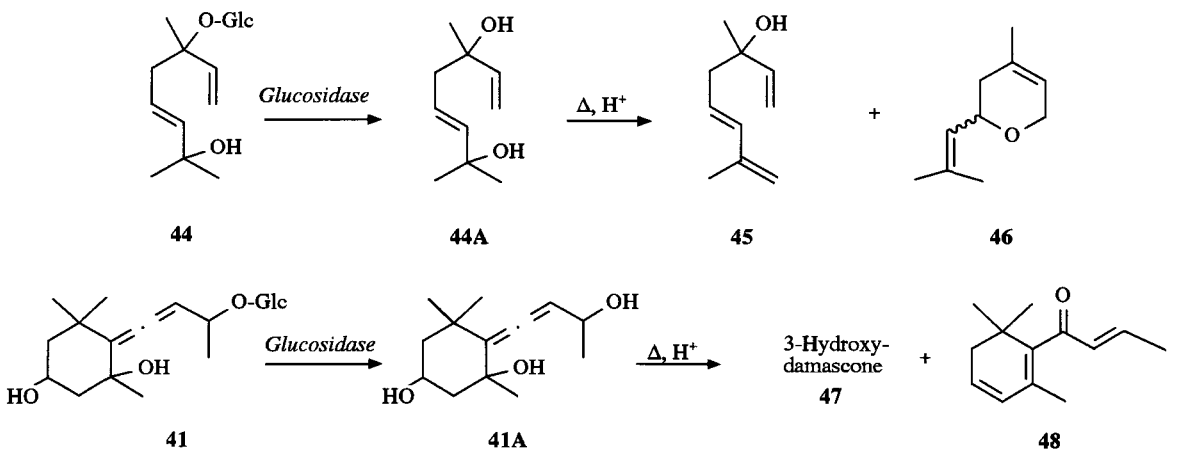
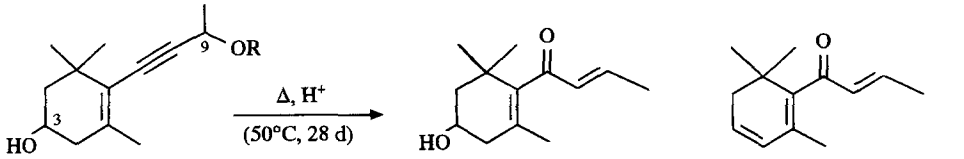


Fig. 8. Acid-catalyzed formation of the odoriferous compounds hotrienol **45** and neroloxide **46** as well as β-damascenone **48** from glycosidically released polyol structures **44A** and **41A**, respectively [135, 241]

allylic β-D-glucoside of geraniol hydrolyzed at pH 3.8 (100 °C), seven times more slowly to linalool than did geraniol itself.

This observed stabilization of the aglycone through glycosylation inter alia explains the finding that the glycoconjugated β-damascenone precursor **50** gave a greater proportion of β-damascenone **48** to 3-hydroxydamascone **47** than did the aglycone **49** (Fig. 9). It is assumed that through stabilization at the C-9 position, a dehydration at C-3 is favored, thus resulting in higher yields of the intensely odorous ketone **48**. Importantly, the flavorless by-product, 3-hydroxydamascone **47** was found to be stable under natural pH-conditions; neither the aglycone **47** nor its β-D-glucoside **22** will react further to give β-damascenone **48** [278]. Furthermore, in aglycones containing more than one hydroxyl function, glycoconjugation will not only affect the rate of formation of volatiles, but also the distribution of products formed from these precursors. This is demonstrated in the case of the known vitispirane-precursor, 3,4-dihydroxy-7,8-dihydro-β-ionol 3-O-β-D-glucoside **39** [184–186]. Whereas the free aglycone **51** was found to yield a whole pattern of volatile products, i.e., structures **52–57** (cf. Fig. 10), amongst which isomeric vitispiranes **52** were only present in minor quantities (15%) [196, 287, 288], the glucoconjugated triol **39** almost exclusively forms the isomeric spiroethers **52**. In the latter case, glycosylation stabilizes the hydroxyfunction at C-3 and, thus, cyclization to the furan ring is the preferred reaction (vitispirane yield > 90%) [199].

These examples demonstrate that different chemical pathways are followed depending on whether the free aglycone or the glycoconjugated form is present. In addition, due to the differences in chemical composition as well as relative proportions of volatiles formed, changes observed in the sensory profiles of acid and enzymatic hydrolysates can be rationalized.



	47 (*odorless*)		**48** (*odor-threshold: 0.009 ppb*)
49 (R = H)	17	:	1
50 (R = Glc)	11	:	1

Fig. 9. Influence of glycoconjugation on the rate of formation of volatiles according to Skouroumounis et al. [278]

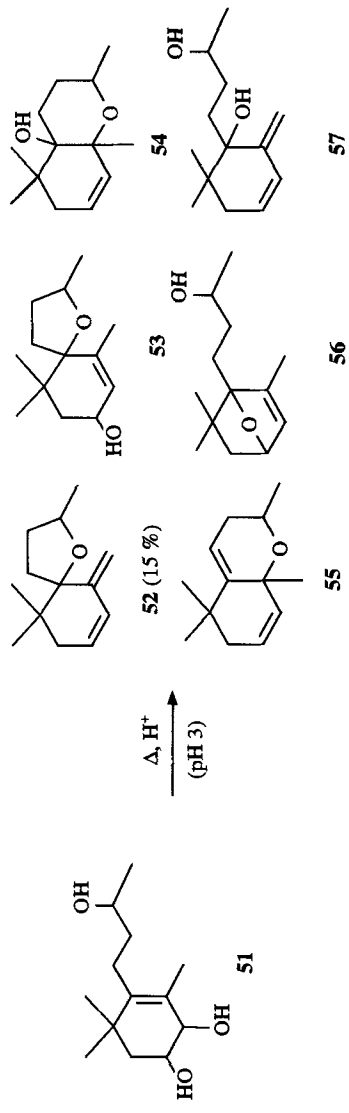


Fig. 10. Influence of glycoconjugation on the chemical composition of precursor-derived volatiles (example: vitispirane **52** formation; for details see text)

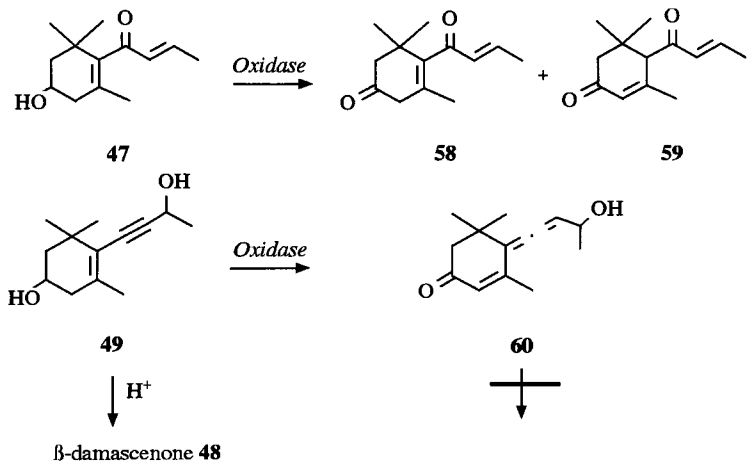


Fig. 11. Formation of oxidation products **58–60** from glycosidic forms of aglycones **47** and **49** after enzymatic hydrolysis using fungal enzyme preparations (adapted from [279])

7.5 Artefact of Off-Flavor Formation

In order to liberate the majority of glycosidically bound constituents, high concentrations of fungal glycosidases were routinely used in the past. The detection of aglycones that were clearly unable to form glycosidic linkages prompted more detailed studies into side-activities of the commercial enzyme preparations. Three different fungal enzyme preparations as well as a protease were reported to oxidize some of the reactive aglycones [279,280]. In particular, C_{13}-aglycones with a homoallylic glycosidic linkage were found to be susceptible to enzymic oxidation. This oxidation, however, alters the structure of the polyol aglycones in such a way, that they are unable to generate the desired aroma component, as depicted for β-damascenone formation in Fig. 11.

Due to the fact that the aroma precursors are present in highly complex mixtures, the likely formation of malodorous compounds after enzymic treatment has also to be considered. Although there is still little information available on the structure of enzymatically released, unpleasant aroma substances, an example may demonstrate the problem: after application of a commercial pectinase preparation during wine making, formation of malodorous vinylphenols was observed, which occurred following enzymatic release of cinnamic acid derivatives and subsequent transformation by decarboxylase activity from wine yeast [281]. This example shows that a careful sensory testing of the hydrolysates is recommended for each application and for each enzyme used.

8 Genetic Engineering

Genetic engineering is seen as a possibility to overcome the limitations in the practical application of glycosidases due to their – in general – low activity at natural pH values and strong inhibition by glucose. The enzymes that are needed should be able to hydrolyze glycosidically bound volatiles under native pH conditions (i.e., for fruits pH 2.5–4.5, for wine pH 3.0–3.8). They should furthermore tolerate high glucose concentrations, and, in the case of wine, also show a tolerance towards moderate (10%) ethanol concentrations.

Genes that code β-glucosidases have already been cloned from several species of bacteria and fungi, including *Saccharomyes cerevisiae* [282–284]. The construction of chimeric genes with improved hydrolytic properties and their overexpression in different hosts, such as, e.g., *Escherichia coli*, is the subject of actual research [285]. Thus, by using these genetically modified glycosidases, a more efficient release of glycosidically bound volatiles can be predicted in the near future.

Although genetic engineering may help to obtain enzymes with the desired hydrolytic activities, their practical application is still a question of legal regulations. In many states, current regulations do not permit the addition of such enzymes in fruit juice processing or in winemaking. In general, the legitimate use is restricted to pectolytic and maceration enzymes, which, of course, may show glycosidase side-activities.

For wine processing in particular, other legal possibilities of flavor release are searched for, e.g., by transferring the active gene into the enological yeast DNA as suggested almost ten years ago by Grossmann et al. [255]. It has already been shown that an *Aspergillus niger* β-glucosidase gene can be expressed in yeast. Penttila et al. [286] cloned a genomic DNA from *Aspergilus niger* in yeast and could show that the β-glucosidase gene was expressed. Moreover, it was found that the active enzyme was secreted into the medium. Thus, under fermentation conditions, activity of an appropriate glycosidase can be expected to hydrolyze the glycosidically bound volatiles.

9 Concluding Remarks

An overview has been given of the occurrence of glycosidically bound aroma compounds in the plant kingdom, and recent developments in the analysis of glycosidic aroma precursors have been described. Although questions concerning the specific functions of aroma glycoconjugates in plant tissues remain to be answered, their potential for aroma enrichment of foodstuffs, e.g., fruit juices and wine, has been recognized. In addition, the glycosidic pool in plant residues is seen as a valuable source for the production of natural flavor material. For an

efficient enzymatic release of glycosidically bound volatiles, however, genetically modified enzymes with improved hydrolytic properties are required. Construction of such enzymes, i.e., glucose insensitive, ethanol tolerant, and low pH active glycosidases, is the subject of actual research.

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Prospects for the Bioengineering of Isoprenoid Biosynthesis

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Over the last decade, our understanding of isoprenoid biosynthesis has progressed to the stage where specific strategies for the bioengineering of essential oil production can be considered. This review provides a current overview of the enzymology and regulation of essential oil isoprenoid biosynthesis. The reaction mechanisms of the synthases which produce many of the basic isoprenoid skeletons are described in detail. Coverage is also provided of the regulation of isoprenoid biosynthesis, including the roles played by tissue and subcellular compartmentation, and by partitioning of intermediates between different branches of isoprenoid metabolism. This provides necessary context for rationally targeting specific enzymes of metabolic pathways for bioengineering essential oil production. Wherever possible, emphasis is placed on research specific to essential oil isoprenoid biosynthesis, although relevant work related to other isoprenoids is also considered when it can provide useful insights. Finally, building upon this understanding of essential oil isoprenoid biosynthesis, several approaches to the bioengineering of isoprenoid metabolism are considered.

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List of Abbreviations

ACAT	acetyl-CoA acetyltransferase
BPP	bornyl diphosphate
DMAPP	dimethylallyl diphosphate
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
GPP	geranyl diphosphate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
IPP	isopentenyl diphosphate
LPP	linalyl diphosphate
NPP	nerolidyl diphosphate

1 Introduction

Throughout history aromatic plants and the essential oils derived from them have been highly valued not only for their fragrances but also for their medicinal and culinary uses. Traditionally, essential oil research has focused on the isolation, identification and cataloging of the characteristic aroma constituents present in the oil. Attempts to manipulate the composition and quality of the essential oil produced by the plant relied on conventional plant breeding and agronomic strategies. The composition of essential oils is usually complex and the overall olfactive character of the oil is often determined by a subtle interplay between numerous components. Despite the availability of low cost synthetic aroma chemicals, the use of natural aroma chemicals continues to attract interest for two main reasons [1]. The first derives from the public's understandable, but scientifically unfounded, desire for the use of natural (rather than synthetic) flavorings and fragrances. The second reason is that naturally occurring aroma chemicals often exist in an enantiomerically pure form which would be more expensive to obtain using synthetic routes. It is well known that different enantiomers of the same compound can often have distinctly different aroma characteristics [2, 3].

A family of natural products known collectively as isoprenoids are among the most common constituents found in essential oils. Isoprenoids are the largest single family of compounds found in nature with over 22000 known examples [4], the vast majority of which are produced by plants. The biosynthesis of isoprenoids involves the sequential assembly of branched five carbon isopentanoid units. Classification of the different families of isoprenoids is based on the number of five carbon units present in the skeleton of the compound [5]. The isoprenoids most commonly associated with flavor and/or aroma are the monoterpenes, although sesquiterpenes and to a lesser extent diterpenes also contribute to the overall aromatic character of natural oils. Monoterpenes are the major components of many essential oils of herbs, spices, citrus, and conifer species [6]. The characteristic volatiles of many flowers also contain monoterpenes [7]. Ionones and damascenones, which are common aroma components in tea, tobacco, and many fruits and vegetables, are derived from the degradative breakdown of the tetraterpenoid carotenoids. Turpentine-derived monoterpenes isolated from conifers are used in the manufacture of many low cost flavor and fragrance compounds [8]. In response to concerns about the long-term toxicity and environmental effects of synthetic chemicals, many naturally occurring isoprenoids (including monoterpenes) are also receiving attention in more non-traditional roles including use as agents for the biological control of insect pests and as potential herbicides (see, e.g., [9, 10]).

The following discussion provides an overview of isoprenoid biosynthesis at the biochemical level. Over the last decade, our understanding of the enzymology and regulation of isoprenoid biosynthesis has advanced to a point where specific strategies for the bioengineering of essential oil formation can now be

contemplated. Wherever possible, emphasis will be placed on studies carried out on essential oil biosynthesis. However, much of what is known about isoprenoid biosynthesis at the biochemical level comes from studies unrelated to the production of aromatic isoprenoids. These studies will be described where appropriate in order to provide a broader understanding of isoprenoid biosynthesis and regulation which can then be applied to the specific task of manipulating essential oil formation. Specific strategies for the rational manipulation of individual enzyme activities to influence product formation, the manipulation of pathway fluxes to influence essential oil composition, and approaches to increase yield in the plant without affecting the oil composition will be described. A brief discussion of the use of tissue cultures is also included.

2 Biosynthesis of Isoprenoids

The biosynthesis of isoprenoids from simple, primary metabolites can be conceptually divided into four overall steps: the synthesis of the fundamental precursor isopentenyl diphosphate (IPP) from acetyl-CoA; the formation of the allylic prenyl diphosphates from IPP which serve as the immediate precursors of the different families of isoprenoids; the elaboration of these allylic prenyl diphosphates into the manifold isoprenoid skeletons by specific isoprenoid synthases; and secondary chemical modifications of the parent isoprenoid products of these synthases. Although plants share much of the same enzymology of isoprenoid biosynthesis with animals and microorganisms, significant differences exist. In particular, plants produce a much greater variety of isoprenoids than either animals or microorganisms, and this is reflected in a more complex organization of isoprenoid biosynthesis at the tissue, subcellular and genetic levels. This organizational complexity has greatly hampered studies of isoprenoid biosynthesis in plants. As a result, several fundamental questions about the enzymology and regulation of isoprenoid biosynthesis in plants remain either unanswered or in dispute. A rational approach to the bioengineering of isoprenoid formation in plants requires an appreciation of the contribution of both the pathways and the organization of isoprenoid metabolism.

2.1 Formation of Isopentenyl Diphosphate

Isopentenyl diphosphate (IPP **1**) is the five-carbon building block for the biosynthesis of all isoprenoids. Although the formation of IPP in plants has been the subject of considerable study (reviewed in [11, 12]), many fundamental questions, particularly regarding the regulation of IPP formation, remain unresolved. The basic enzymology of IPP biosynthesis via the mevalonic acid pathway is widely accepted (Fig. 1). The initial steps involve the condensation of

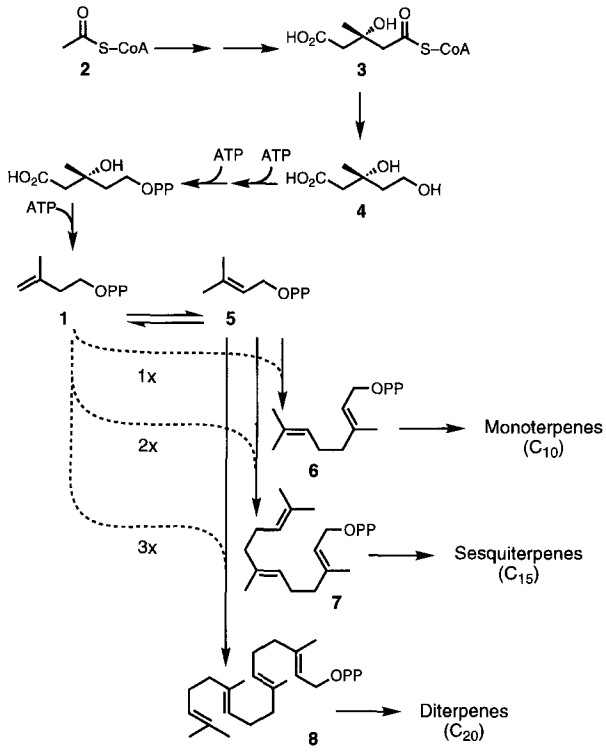

Fig. 1. Formation of the different families of aromatic isoprenoids by the mevalonic acid pathway. The diphosphate moiety is abbreviated as -OPP

three molecules of acetyl-CoA (**2**) to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA **3**), which is subsequently reduced to mevalonic acid (**4**) by HMG-CoA reductase (HMGR). Two sequential phosphorylations of mevalonic acid, followed by a phosphorylation-assisted decarboxylation yields IPP (**1**). In animals and yeast the formation of HMG-CoA from acetyl-CoA is catalyzed by two separate enzymes, acetyl-CoA acetyltransferase (ACAT) and HMG-CoA synthase. In plants, it has been reported that a single enzyme (ACAT/HMG-CoA synthase) may be responsible for both condensations [13], although more recent research is forcing a reevaluation of this earlier work (reviewed in [11]).

HMGR is considered to be one of the most highly regulated enzymes in animals [14] and evidence is also accumulating that HMGR is also highly regulated in plants (reviewed in [15]). In many of the plants examined so far, HMGR is encoded by small gene families which are distinguished from each other by sequence differences in the 3′ untranslated regions of the cDNAs (see, e.g., [16]), These gene families, each of which may contain multiple genes, are commonly designated as *hmg1*, *hmg2*, and *hmg3*. In the case of potato, 12 or more genes for HMGR have been found [17]. Expression of these different gene

families is complex with some members exhibiting tissue-specific expression, constitutive expression, or hormone-inducible expression. Induction of specific HMGR genes by wounding or pathogen infection also occurs. In the case of thale cress (*Arabidopsis thaliana*), the use of alternative promoters which select for different start codons has been shown to result in the tissue-specific expression of two distinct isoforms of HMGR from a single gene [18]. HMGR is also regulated by a protein kinase cascade in which phosphorylation inactivates the enzyme [19, 20]. Other controls involving calcium and calmodulin and proteolytic degradation may also regulate HMGR activity (reviewed in [15]). Despite this wealth of information available on the regulation of HMGR in plants, no unified picture has emerged for the role of the different species of HMGR in regulating the production of the different families of isoprenoids. (period) Indeed, even the subcellular compartmentation of HMGR is still controversial (see Sect. 3.2).

Recently, a new pathway for the biosynthesis of IPP in a wide variety of bacteria has been demonstrated [21]. Based on the labeling patterns observed with different ^{13}C-labeled substrates, it was proposed that IPP is formed in these bacteria by the addition of a two-carbon intermediate to a three-carbon phosphorylated intermediate of glycolysis, resulting in the direct formation of the branched carbon skeleton of IPP in a single step. A similar conclusion has been drawn from the ^{13}C-labeling patterns of the diterpenoid ginkgolides produced in embryos of *Ginkgo biloba* derived from different [^{13}C]glucose substrates, although the labeling patterns of the sterols produced in the same tissue were consistent with the operation of the classical mevalonic acid pathway (discussed in [11, 22]). Although the results of these in vivo studies appear convincing, none of the enzymatic activities involved in this novel pathway to IPP have been demonstrated. Anomalous ^{13}C-labeling patterns of the hemiterpene moiety of prenylated chalcones have also been observed in tissue cultures of mulberry (*Morus alba* L.) [23]. In this case, the labeling pattern was considered to be consistent with the involvement of the mevalonic acid pathway, although it was proposed that the first acetyl-CoA incorporated into IPP was derived from glycolysis, whereas the second and third acetyl-CoAs were derived from the pentose phosphate cycle. These in vivo feeding studies illustrate the pressing need for additional work on the enzymology, regulation and subcellular compartmentation of IPP biosynthesis.

2.2 Prenyltransferases

IPP is utilized in the formation of the allylic diphosphates which serve as the immediate precursors of the different families of isoprenoids. Isomerization of IPP (**1**) by an enzyme known as IPP isomerase produces the allylic isomer dimethylallyl diphosphate (DMAPP **5**). This enzyme has been characterized in a number of plants (reviewed in [5]) and extensively studied in a number of microorganisms (see, e.g., [24] and references therein). DMAPP is a reactive

primer which undergoes elongation by the sequential addition of one, two, or three additional IPP molecules to form either geranyl diphosphate (GPP **6**), farnesyl diphosphate (FPP **7**) or geranylgeranyl diphosphate (GGPP **8**) [25]. GPP and FPP are the ten and fifteen carbon precursors of monoterpenes and sesquiterpenes, respectively. A family of enzymes known collectively as prenyltransferases catalyze this electrophilic elongation sequence. Specific prenyl transferases exist for the formation of GPP, FPP, and GGPP [5] (Fig. 1). The reaction catalyzed by prenyltransferases involves the initial ionization of the allylic diphosphate to generate a delocalized allylic carbocation. This enzyme-bound cation then attacks the double bond of IPP, followed by deprotonation to generate the next allylic diphosphate homolog. In the case of GPP synthase, the first condensation product is released from the enzyme. In the case of FPP and GGPP synthases, the resulting enzyme-bound GPP undergoes further reaction with the addition of another IPP to generate FPP, which is either released in the case of FPP synthase or which undergoes reaction with a third IPP to generate GGPP in the case of GGPP synthase.

GPP synthase has been purified from *Vitis vinifera* [26, 27] and *Lithospermum erythrorhizon* [28] and characterized. However, the most extensively studied prenyltransferase is FPP synthase because of the pivotal role FPP plays in cholesterol biosynthesis in humans. Numerous studies with substrate analogs (see, e.g., [29, 30]) and using site directed mutagenesis [31–34] have been focused on dissecting the role of specific amino acid residues at the active site of the enzyme in substrate binding and catalysis. The crystal structure of recombinant avian FPP synthase has recently been reported at 2.6 Å resolution [35].

2.3 Isoprenoid Synthases

The family of enzymes responsible for the formation of isoprenoids from GPP, FPP, and GGPP are known as monoterpene, sesquiterpene, and diterpene synthases, respectively. These synthases use the acyclic, achiral allylic diphosphates as substrates to form the enormous chemical diversity of carbon skeletons characteristic of isoprenoids. Most isoprenoids are cyclic, and often contain multiple ring systems. The regio- and stereochemistry of the basic ring structure of the isoprenoids are in most cases determined by these highly specific synthases. Isoprenoid synthases that produce cyclic products are also referred to as "cyclases", although examples of synthases producing acyclic products are also known.

The greatest variety of monoterpene synthases that have been studied have been isolated from essential oil-producing plants. By contrast, relatively little is known about the synthases responsible for the production of aromatic sesquiterpenes and diterpenes found in plants. Much of what is known about sesquiterpene and diterpene synthases comes from research carried out on isoprenoids which are not important as aroma chemicals, such as fungal toxins, plant defensive compounds (phytoalexins), hormones, and resin acids. Because of

their central role in producing aromatic essential oils, most of the following discussion will focus on the monoterpene cyclases of plant origin. Consideration will also be given to the sesquiterpene and diterpene cyclases that have been studied, since many of these enzymes produce isoprenoids with the same cyclic carbon skeletons as important aroma compounds, presumably via the same basic reaction mechanisms.

2.3.1 Monoterpene Cyclases

Monoterpene cyclases catalyze the conversion of GPP (**6**) to cyclic monoterpenes with either one or two rings. A unified stereochemical scheme which describes the cyclization of GPP has been proposed (reviewed in [36]); all monoterpene cyclases examined to this point utilize this common strategy (see Fig. 2). Cyclization of GPP requires no cofactors other than a divalent metal ion, typically either Mg^{2+} or Mn^{2+}. The initial step of the cyclization reaction is the ionization of the diphosphate ester bond of enzyme-bound GPP to generate an allylic carbocation-diphosphate anion pair. This ionization is assisted by the divalent cation which helps neutralize the negative charge of the diphosphate, making it a better leaving group. Tight ion pairing of the diphosphate anion with enzyme-bound carbocationic intermediates greatly restricts motion of the diphosphate moiety [37–41] as demonstrated by studies with bornyl diphosphate synthase from sage (*Salvia officinalis*) and tansy (*Tanacetum vulgare*) which produce the phosphorylated products (+)-bornyl diphosphate ((+)-BPP **9**) and (−)-BPP (**10**), respectively [38]. During catalysis, the orientation of the two ends of the diphosphate anion remain fixed relative to the enzyme-bound carbocationic intermediates, and no rotation is permitted along the Pα-O bond axis. Thus, the diphosphate ester oxygen of the BPP product is derived exclusively from the same diphosphate ester oxygen of the GPP substrate.

The initially formed allylic carbocation-anion pair collapses to an enzyme-bound linalyl diphosphate (LPP) intermediate [42, 43] through a *syn*-isomerization process [44] to afford either the 3*R*-(**11**) or 3*S*-(**12**) enantiomer, depending on the helical conformation of the GPP substrate imposed by the binding site of the specific synthase; the chirality of the enzyme-bound LPP determines which antipode of the product is ultimately formed by the cyclase. The formation of enzyme-bound LPP is a prerequisite for cyclization since the *trans*-2,3-double bond of GPP prevents direct C1–C6 ring closure. Rotation about the newly formed C2–C3 single bond of LPP allows the intermediate to adopt a cisoid, anti-endo conformation from which ionization and subsequent cyclization of the resulting tertiary allylic cation yields the corresponding 4*R*- or 4*S*-α-terpinyl carbocation (**13**). The final product released by the synthase is determined by the subsequent steps which the α-terpinyl cation undergoes. These can include additional electrophilic cyclizations, hydride shifts and/or other rearrangements. The reaction is terminated when the carbocation is quenched, either by the addition of a nucleophile such as water (e.g., (−)-*endo*-fenchol (**14**) cyclase in

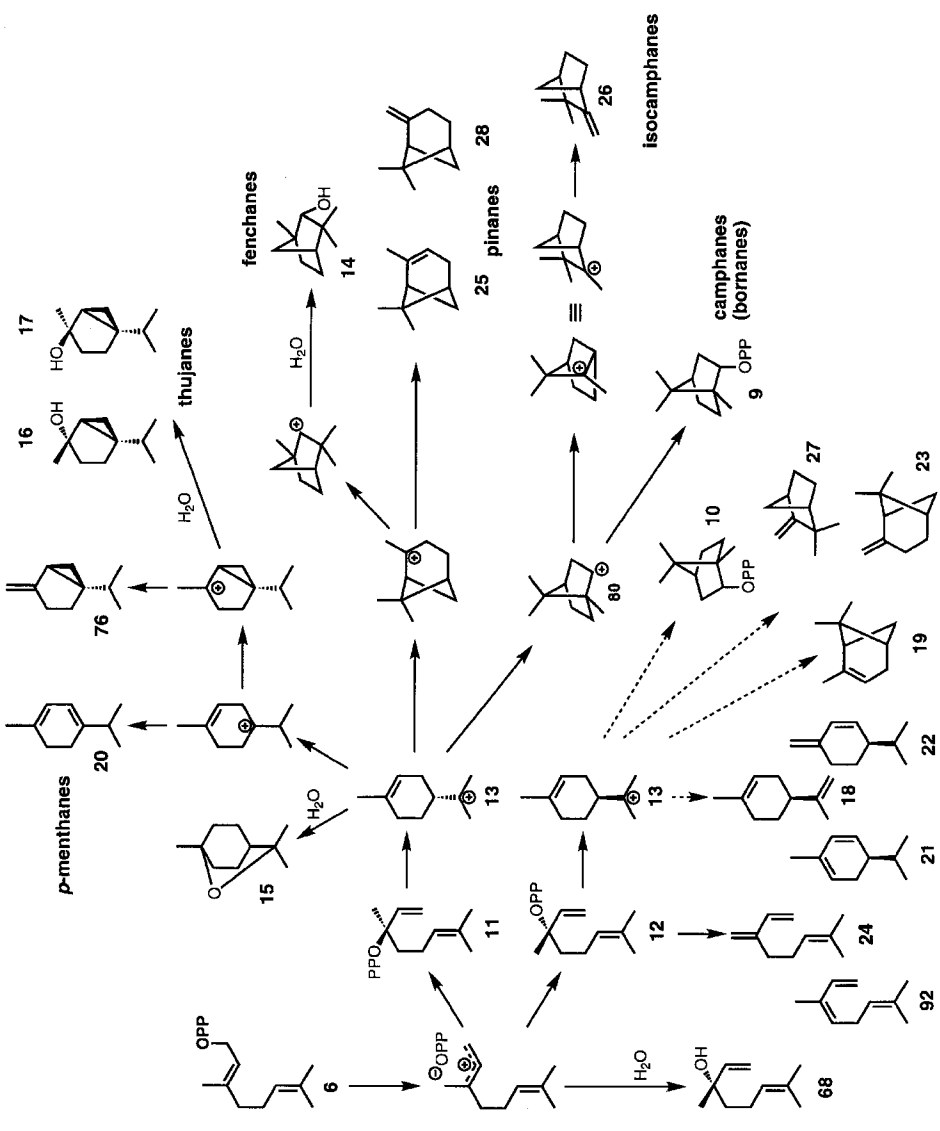

Fig. 2. Cyclization of GPP by monoterpene synthases through either 3*R*-LPP or 3*S*-LPP to produce different monoterpene skeletons

fennel (*Foeniculum vulgare*) [45], 1,8-cineole cyclase (**15**) in common sage (*Salvia officinalis*) [46] and (+)-*trans*-(**16**) and (+)-*cis*-sabinene hydrate (**17**) cyclase from sweet marjoram (*Majorana hortensis*) [47]), or by deprotonation to an olefin (e.g., limonene (**18**) cyclase in peppermint (*Mentha x piperita*) [48]). The terminating step of α-pinene (**19**) synthesis in sage may involve the original diphosphate anion as the base which deprotonates the enzyme-bound pinyl cation [49].

Although the monoterpene cyclases are highly stereospecific in employing only 3*R*- or 3*S*-LPP as the cyclizing intermediate to afford the corresponding 4*S*- or 4*R*-α-terpinyl intermediate and subsequent stereochemically related antipodes, an unusual feature of many of these enzymes is the production of multiple products at the same active site. This is a consequence of the reactive nature of the carbocationic intermediates, the many possible reaction routes, the variety of ways in which the reaction can be quenched, and the permissiveness of the enzymes in allowing multiple catalytic channels. Numerous studies have been carried out to distinguish between the formation of multiple products by enzyme preparations containing more than one synthase species, and the formation of multiple products by a single synthase. Initially, the production of multiple products by a single enzyme was suggested by studies demonstrating copurification of activities, and differential inactivation and inhibition by active site directed reagents [47, 50, 51]. Product analysis of γ-terpinene synthase from thyme (*Thymus vulgaris*) [50] and 4*S*-limonene synthase from peppermint [52] purified to apparent homogeneity demonstrated the formation of primarily γ-terpinene (**20**) and 4*S*-limonene (**18**), respectively, with minor amounts of other products. A more elegant approach has been to use isotopically sensitive branching to probe the partitioning of enzyme-bound carbocationic intermediates between different products [52–55]. This method relies on the kinetic isotope effect produced by deuterium atoms strategically placed in the substrate at positions which normally undergo deprotonation. An induced kinetic isotope effect [56] results in a decrease in the rate of formation of the product arising from deprotonation at the deuterium-bearing carbon with a concomitant increase in the rate of formation of other products, thus indicating that these products arise from a common enzyme-bound intermediate at a branch point rather than from the independent formation of multiple products by separate enzyme species. The formation of multiple products by different monoterpene synthases, as demonstrated by isotopically sensitive branching experiments, is summarized in Table 1. Although isotopically sensitive branching gives strong evidence for the formation of multiple products by a single cyclase species, the ultimate proof of this unusual phenomenon came with the cloning and expression in *Escherichia coli* of a catalytically active recombinant limonene cyclase from spearmint (*Mentha spicata*) which exhibited the same product profile as the native enzyme (see below).

Monoterpene and sesquiterpene cyclases exhibit rather low turnover numbers, typically in the range of 0.01–0.3 sec^{-1} [48, 50, 57–59]. In the case of monoterpene cyclases, the initial isomerization of GPP to LPP is considered to be the rate-limiting step of catalysis. This conclusion is based on the observation

Table 1. The formation of multiple products by monoterpene synthases demonstrated by isotopically sensitive branching

Plant species	Enzyme	Major Products	composition %	Reference
Grand fir (*Abies grandis*)	pinene cyclase	(−)-α-pinene (**19**) (−)-β-pinene (**23**)	35 65	[53]
Lodgepole pine (*Pinus contorta*)	phellandrene cyclase	(−)-α- phellandrene (**21**) (−)-β-phellandrene (**22**)	6 94	[53]
Peppermint (*Mentha* x *piperita*)	limonene cyclase	(−)-4*S*-limonene (**18**) α-pinene (**19**) β-pinene (**23**) mycrene (**24**)	94 2 2 2	[52]
Sage (*Salvia officinalis*)	cyclase I	(+)-α-pinene (**25**) (+)-camphene (**26**)	31 29	[54, 55]
	cyclase II	(−)-α-pinene (**19**) (−)-camphene (**27**) (−) -β-pinene (**23**)	25 31 24	[54, 55]
	cyclase III	(+)-α-pinene (**25**) (+)-β-pinene (**28**)	36 45	[54]

that most cyclases are capable of utilizing the correct antipode of LPP at considerably higher catalytic rates than GPP [38, 41, 44–46, 52, 60–66]. A surprising observation is that some cyclases are also capable of utilizing the incorrect antipode of LPP to form the opposite enantiomeric products from those produced with the natural substrate GPP. In some cases, the enzyme exhibits little catalytic discrimination between the enantiomers of LPP [41, 52, 62, 66], whereas others show a strong preference for the correct enantiomer generated in the natural reaction from GPP [46, 60, 61]. The ability of monoterpene cyclases to utilize either antipode of LPP indicates that formation of the enantiomerically pure end product from the native, achiral substrate GPP is determined by the helical conformation imposed on GPP by binding to the active site; i.e., the cyclase never encounters the incorrect enantiomer of LPP, and only one enantiomeric family of products is ever formed naturally. Utilization of the incorrect antipode of LPP, in addition to the formation of unnatural enantiomeric products, often also results in the formation of either aberrant product ratios or novel products not seen with either GPP or the correct antipode of LPP. This loss of catalytic fidelity in the reaction with the unnatural enantiomer of LPP has been observed with a range of cyclases from angiosperm and gymnosperm species [41, 46, 61, 62, 66].

In addition to sharing a common mechanism for the initial cyclization to the α-terpinyl cation (**13**, Fig. 2), the monoterpene cyclases examined to this point share many common structural features. In general, monoterpene cyclases are operationally soluble, albeit rather hydrophobic, proteins in the range of 40 to 100 kDa [46, 48, 50, 51, 67]. In the few cases where the subunit architecture is

known, the cyclases exist either as a monomer of 50–70 kDa [48, 67] or as an apparent homodimer with subunits of 40–50 kDa [50]. Although monoterpene cyclases from herbaceous angiosperm species have been studied extensively, only recently have the cyclases of conifers (gymnosperms) been examined. Significant differences in the kinetics and active site residues (based on substrate protection against chemical modification reagents) have been found for monoterpene cyclases of angiosperms and gymnosperms. These differences are summarized in Table 2. In general, monoterpene cyclases from gymnosperms exhibit a higher pH optimum and are incapable of utilizing Mg^{2+} as cofactor. Surprisingly, monoterpene cyclases from grand fir (*Abies grandis*) and lodgepole pine also exhibit an absolute requirement for a monovalent cation such as K^+. Angiosperm monoterpene cyclases appear to have one or more catalytically essential histidine and cysteine residues at the active site, whereas this has not been demonstrated for the gymnosperm cyclases examined. In contrast, an active site arginine residue has been implicated in gymnosperm cyclases but not in angiosperm cyclases. The use of polyclonal antibodies has also demonstrated significant structural differences between cyclases. Polyclonal antibodies generated against limonene cyclase from spearmint do not detectably cross-react with any of the cyclases present in either lodgepole pine or grand fir or other angiosperm genera [68]. Similarly, polyclonal antibodies generated against a wound-inducible pinene cyclase in grand fir fail to cross-react with cyclases present in either lodgepole pine, ponderosa pine (*P. ponderosa*), spearmint, peppermint, or sage, although they do cross-react with the other monoterpene cyclases of grand fir [69].

The limonene cyclases catalyze the formation of the corresponding limonene antipodes in a wide variety of plants, including (+)-4*R*-limonene in citrus [64] and (−)-4*S*-limonene (**18**) in *Mentha* species [48]. (−)-4*S*-Limonene synthase has been purified to homogeneity from peppermint and spearmint [48]; the corresponding cDNA has been isolated from spearmint, sequenced and the catalytically active enzyme has been expressed in *E. coli* [75]. The encoded protein contains a putative N-terminal plastidial transit peptide, consistent with

Table 2. Summary of monoterpene cyclase properties from angiosperms and gymnosperms

Property	Angiosperms	Reference	Gymnosperms	Reference
pH optimum	6.0–7.2	[36, 46, 50, 52]	7.5–8.0	[67, 71]
Divalent cation requirement	Mg^{2+}, Mn^{2+}	[36, 46, 50, 52]	Mn^{2+}, Fe^{2+} (Mg^{2+} ineffective)	[67, 71]
Monovalent cation requirement	no	[71]	$K^+ > NH_4^+ \gg Na^+$, Li^+	[71]
Active site residues[a]				
Cys	yes	[46, 47, 50–52, 72–74]	?	[66]
His	yes	[46, 50, 70]	no	[71]
Arg	no	[66]	yes	[66]

[a] Active site residues determined by substrate protection against chemical modification reagents

immunocytochemical localization of the mature enzyme in plastids (Gershenzon and Croteau, unpublished). The precise cleavage site of the transit peptide is not known because the amino terminus of the native limonene cyclase is blocked. Despite the presence of the transit peptide, the recombinant limonene cyclase preprotein is catalytically active, producing precisely the same spectrum of products as the processed native enzyme ($\sim$94% limonene (**18**) with small amounts ($<$ 2%) of α-pinene (**19**), β-pinene (**23**) and myrcene (**24**); see Table 1).

2.3.2 Sesquiterpene Cyclases

Sesquiterpene cyclases catalyze the conversion of FPP (**7**) to over 200 different cyclic skeletons in reactions very similar to those catalyzed by the monoterpene cyclases (reviewed in [76, 77]). However, with 15 carbons and 3 double bonds in the farnesyl substrate, there are considerably more possible reaction routes available compared to GPP as substrate. Few studies have examined the cyclases responsible for the formation of aromatic sesquiterpenes in plants. However, several plant sesquiterpene cyclases involved in the production of defensive compounds (phytoalexins) have been examined. The most extensively studied sesquiterpene cyclases are of fungal origin; these enzymes are involved in the biosynthesis of toxins or antibiotics. The production of these diverse classes of sesquiterpenes involves a relatively limited number of common mechanistic themes and, for this reason, it is instructive to consider the biosynthesis of phytoalexins and fungal toxins as it relates to the origin of aromatic sesquiterpenes.

As with the monoterpene cyclases, the enzymatic cyclization of FPP is initiated by ionization of the diphosphate ester to generate an allylic carbocation. Unlike the case of the geranyl cation with monoterpene cyclases, the farnesyl cation can attack either of two different double bonds. Only a limited number of binding conformations of FPP at the cyclase active site provide the proper orbital alignment necessary for interaction of the double bonds during cyclization (reviewed in [76, 78]). This limits the possible reactions that the farnesyl cation can undergo. The initial cyclization of FPP generates one of four possible carbocations [76, 79]. The formation of the macrocylic *trans*-germacradienyl cation (**29**) and the humulyl cation (**30**) can arise directly from cyclization of the allylic cation generated from FPP (Fig. 3a). However, as with the monoterpene cyclases, the *trans* geometry of the 2,3-double bond of FPP prevents direct cyclization to either the *cis*-germacradienyl cation (**31**) or the bisabolyl cation (**32**) (Fig. 3b). To form these intermediates, the cyclase must first isomerize FPP to the tertiary allylic isomer nerolidyl diphosphate (NPP **33**) by a process similar to the isomerization of GPP to LPP by monoterpene cyclases. The isomerization allows rotation about the newly formed C2–C3 bond of NPP to the cisoid orientation, and reionization of the diphosphate ester then permits cyclization of the resulting cisoid allylic carbocation. As with the monoterpene cyclases, further elaboration of the sesquiterpene cyclase-bound cationic intermediate(s) to the final products may involve additional electrophilic

cyclizations, rearrangements, and hydride shifts before ultimate termination of the reaction by either deprotonation or nucleophile capture.

The *trans*-germacradienyl cation gives rise directly to the widely distributed germacrane family of sesquiterpenes, such as germacrene D (**34**). The germacranes can readily undergo Cope rearrangement to form the elemanes (e.g., δ-elemene (**35**)). A cDNA encoding germacrene C synthase has recently been isolated from tomato (*Lycopersicon esculentum*) and sequenced (Colby et al., unpublished observations). Rearrangement of the *trans*-germacradienyl cation with a concomitant 1,3-hydride shift yields the patchoulane skeleton [80].

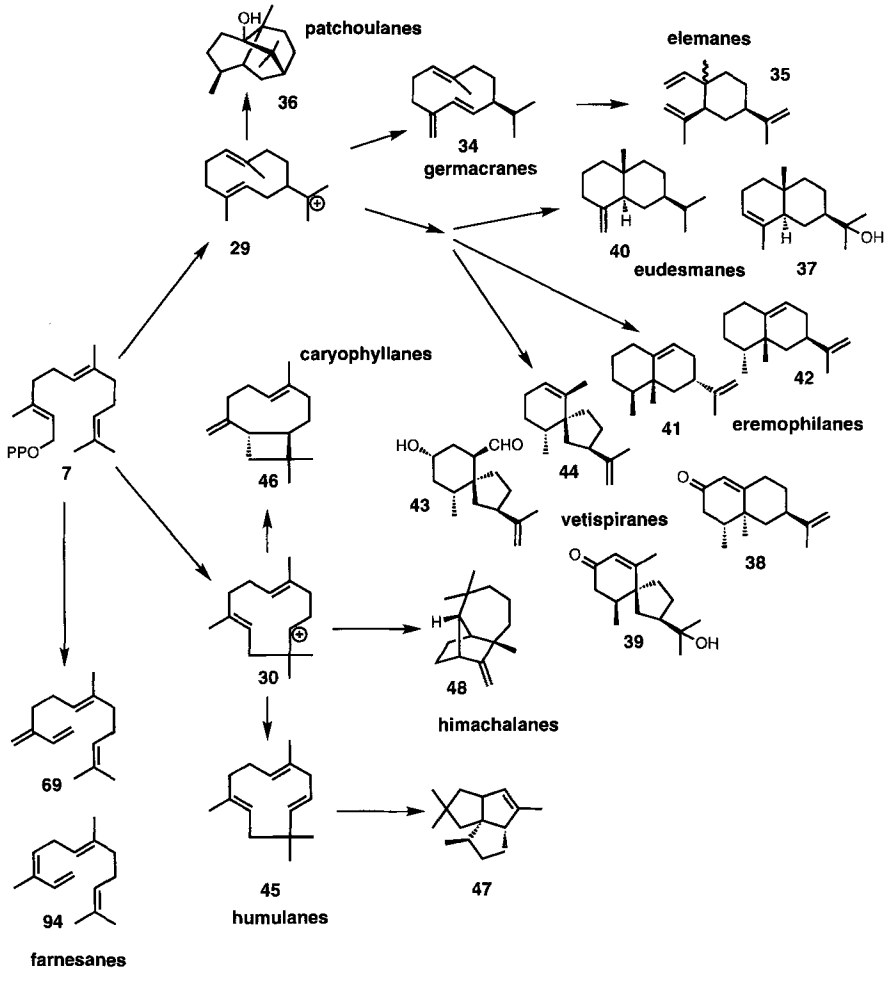

Fig. 3a. Cyclization of FPP by sesquiterpene synthases to produce different sesquiterpene skeletons: **a** direct cyclization of FPP

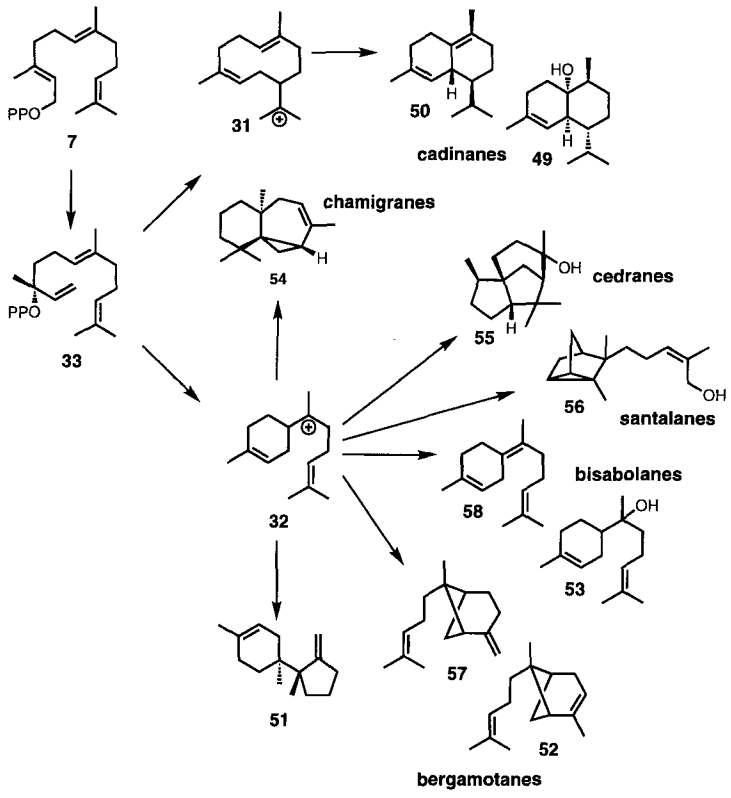

Fig. 3b. cyclization of FPP via an enzyme-bound NPP intermediate

Patchoulol (**36**) synthase has been purified to homogeneity from patchouli (*Pogostemon cablin*) and characterized [57]. Alternate rearrangement of the *trans*-germacradienyl cation leads to the eudesmane, eremophilane, and vetispirane skeletons. Examples of important sesquiterpenes belonging to these families include (+)-α-eudesmol (**37**), (+)-nootkatone (**38**), and β-vetivone (**39**) from eucalyptus, grapefruit, and vetiver, respectively [2]. β-Selinene (**40**) synthase has been partially purified from immature calamondin *Citrofortunella mitis*) fruit and characterized [81]. (+)-Aristolochene (**41**) is found in a variety of fungi while the (−)-enantiomer has been found in the leaf oil of several plants (reviewed in [76]). (+)-Aristolochene synthase from *Penicillium roqueforti* has been purified [82], and the corresponding cDNA overexpressed in *E. coli* [83]. Members of the *Solanaceae* accumulate a wide variety of phytoalexins in response to fungal infection. The formation of the phytoalexin intermediates *epi*-aristolochene (**42**) and lubimin (**43**) (derived from vetispiradiene (**44**)) is induced by fungal infection in tobacco (*Nicotiana tabacum*) [84] and potato (*Solanum tuberosum*) [85], respectively. *epi*-Aristolochene synthase has been purified from induced tobacco suspension cultures [84] and the cDNA has been

cloned and overexpressed in *E. coli* [86]. The formation of vetispirane-derived phytoalexins has been studied in cell-free extracts [85] although no enzyme activities have yet been purified or characterized. A cDNA coding for vetispiradiene synthase has been cloned from *Hyoscyamus muticus* by homology and expressed in *E. coli* [87].

The humulyl cation (**30**) can undergo either deprotonation to humulene (**45**), or an additional ring closure step followed by deprotonation to caryophyllene (**46**). Separate enzymes for the formation of humulene and caryophyllene have been isolated from sage [88] and characterized. Humulene is also believed to be an enzyme-bound intermediate in the formation of the fungal metabolite pentalenene (**47**) [76]. Pentalenene synthase is one of the most extensively studied sesquiterpene cyclases [25, 89–91]; it has been purified and the cDNA cloned and overexpressed at high levels in *E. coli* [58]. Recombinant pentalenene synthase has been crystallized and preliminary X-ray diffraction data have been reported [92]. Although extensive labeling studies have been carried out with the himachalane family of sesquiterpenes (e.g., (+)-longifolene **48**) (reviewed in [93]), none of the responsible enzymes has been characterized.

The cadinane family of sesquiterpenes is widely distributed in nature. Epicubenol synthase has been isolated from *Streptomyces* [79] and shown to catalyze the formation of (+)-epicubenol (**49**) through an initial isomerization of FPP to 3*R*-NPP (**33**), followed by formation of the *cis*-germacradienyl cation (**31**) and subsequent cyclization [94]. (±)-Epicubenol is found in *Juniperus rigida* oil [95] and the (−)-isomer in commercial cubeb oil [96]. δ-Cadinene (**50**) is a component of juniper leaf oil [97] and is also produced by cotton (*Gossypium hirsutum*) as a precursor of phytoalexins formed in response to bacterial pathogens [98]. The cDNA for (+)-δ-cadinene synthase from cotton has recently been cloned and overexpressed in *E. coli* [99].

The most extensively studied sesquiterpene cyclase is trichodiene synthase [100–102]. Trichodiene (**51**) is the parent olefin of the tricothecin family of mycotoxins which are produced by a variety of fungi. Trichodiene synthase has been purified from *Fusarium sprotrichioides* [59] and the gene isolated and overexpressed in *E. coli* [103]. The mechanism of trichodiene synthase is believed to involve the initial formation of enzyme-bound 3*R*-NPP (**33**) followed by cyclization to the bisabolyl cation (**32**) as an intermediate. However, trichodiene synthase exhibits a much lower V_{max} with 3*R*-NPP than with FPP; this has been suggested to result from a conformational change in the enzyme after binding of FPP [104]. Thus, binding of NPP to the enzyme may require a slow conformational change to the form capable of utilizing NPP.

The bisabolyl cation (**32**) also serves as the common intermediate for the formation of the bergamotanes, bisabolanes, santalanes, cedranes, and chamigranes. *trans*-α-Bergamotene (**52**) and α-bisabolol (**53**) are important aroma constituents of bergamot oil [2] and chamomile [105], respectively. Thujopsene (**54**) and (+)-cedrol (**55**), both major constituents of cedarwood oil [2], are typical examples of chamigrane and cedrane sesquiterpenes. α-Santalol (**56**) from sandalwood oil is a typical santalane sesquiterpene [2]. The formation of

trans-β-bergamotene (**57**) and γ-bisabolene (**58**) from FPP has been demonstrated in cell-free extracts of the fungus *Pseudeurotium ovalis* [106, 107] and of tissue cultures of *Andrographis paniculata* [108, 109], respectively. Other than the work with trichodiene synthase, little detailed enzymology has been carried out with sesquiterpene cyclases which utilize the bisabolyl cation intermediate.

2.3.3 Diterpene Cyclases

Because of their higher molecular weight and lower volatility, diterpenes are not commonly considered as aroma chemicals. A notable exception is sclareol (**59**) which is found in clary sage (*Salvia sclarea*) [110]. The aroma profile of clary sage is complex, with ambergris, tobacco-like characteristics. Sclareol isolated from clary sage is used as the starting material for the commercial preparation of the ambergris component Ambrox (**60**) [2]. The enzymatic formation of sclareol from GGPP (**8**) shares similarities with the formation of the labdanoid diterpenes of tobacco trichome exudates and of the abietane diterpene resin acids produced by conifers.

Unlike the monoterpene and sesquiterpene cyclases examined so far, conversion of GGPP to the labdanoid skeleton involves protonation-initiated cyclization from the olefinic terminus of GGPP without the direct involvement (ionization) of the diphosphate ester moiety (Fig. 4). The initial cyclic product formed is (+)-copalyl diphosphate (CPP **61**) which is subsequently converted by ionization-dependent cyclization to either the tobacco exudate components *cis*-abienol (**62**), labdenediol (**63**), or sclareol (**59**), or the resin acid precursor abietadiene (**64**). *cis*-Abienol [111], labdenediol [112], and sclareol [112] cyclase activities have all been localized to tobacco trichomes and partially characterized in cell-free extracts. Abietadiene cyclase has been partially purified from grand fir and lodgepole pine stem extracts and characterized [113], and the corresponding cDNA has recently been cloned and expressed in *E. coli* [114]. The enzymatic formation of these diterpenes shares the common bound intermediate (+)-CPP, and current evidence suggests that a single enzyme is responsible for both the formation of (+)-CPP and the subsequent cyclization of this intermediate to the final product(s). A unified scheme for the formation of several families of diterpenes from (+)-CPP has been proposed [113]. Diterpene cyclization via (+)-CPP contrasts with the formation of the gibberellin family of plant hormones. In this case, initial cyclization of GGPP yields (−)-CPP (**65**) which is subsequently converted to *ent*-kaurene (**66**) by the enzyme *ent*-kaurene synthase. The reaction is carried out by two distinct enzymes constituting *ent*-kaurene synthase A and B activities [115]. The A activity catalyzes the cyclization of GGPP to (−)-CPP; the *ent*-kaurene synthase A gene has recently been isolated from both Arabidopsis [116] and maize [117]. The B activity catalyzes the conversion of (−)-CPP to *ent*-kaurene. *ent*-Kaurene synthase B activity has recently been purified from pumpkin (*Cucurbita maxima*) and characterized [118].

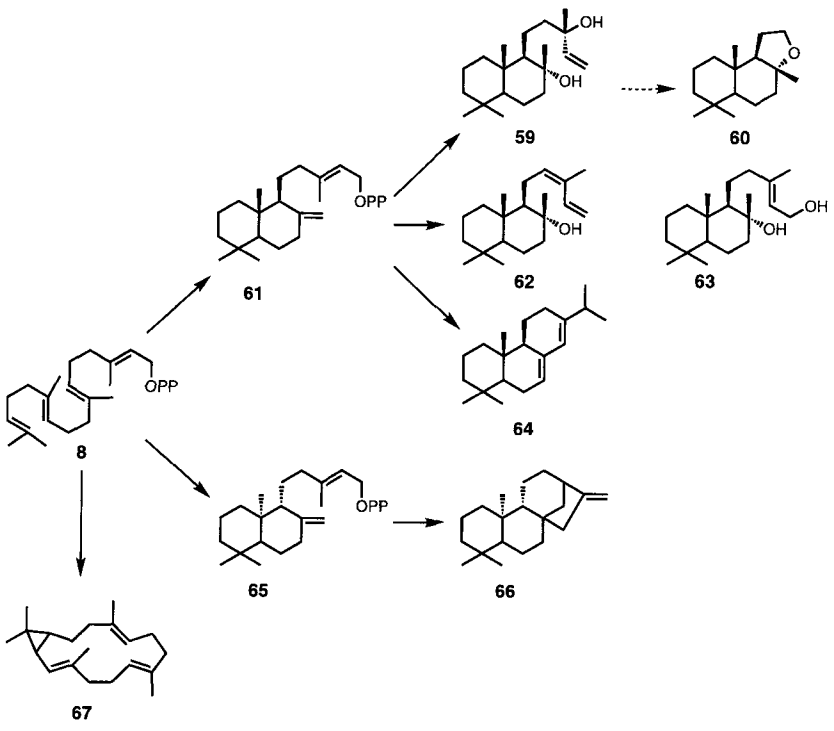

Fig. 4. Cyclization of GGPP by diterpene synthases

Although not involved in the formation of aromatic diterpenes, casbene synthase is another diterpene cyclase which has been extensively studied. Casbene (**67**) is a phytoalexin produced in castor bean (*Ricinus communis*) in response to fungal infection [119]. The cDNA for casbene synthase has been isolated [120] and transcriptional regulation of the gene in response to elicitation has been reported [121]. The reaction catalyzed by casbene synthase is similar to that catalyzed by monoterpene and sesquiterpene cyclases in that it involves the initial ionization of the prenyl diphosphate substrate to an allylic carbocation followed by cyclization and deprotonation to the olefin.

2.3.4 Acyclic Isoprenoid Synthases

Few examples of synthases that catalyze the formation of acyclic isoprenoids have been studied. *S*-Linalool synthase has been purified to homogeneity from the stigma of freshly opened flowers of *Clarkia breweri* [122]. This enzyme produces exclusively *S*-linalool (**68**) from GPP and is an apparent monomer of 76 kDa. The properties of the purified enzyme (pH optimum, divalent cation requirement, K_m for GPP) are similar to those of many of the monoterpene

cyclases. However, unlike most monoterpene cyclases, only a slow rate of conversion to product was observed with 3*S*-LPP as substrate, and the enzyme did not use the 3*R*-antipode. This suggests that formation of linalool involves addition of water directly to the initially generated allylic carbocation without the preliminary formation of enzyme-bound LPP (Fig. 2). This reaction is considerably simpler than that catalyzed by the monoterpene cyclases and may account for the observation that the turnover number of linalool synthase ($31.6\ sec^{-1}$) is much higher than those of the cyclases.

The sesquiterpene olefin *trans*-β-farnesene (**69**) is a common constituent of conifer resin (see references in [123]), is found in hop (*Humulus lupulus*) oil [124], and is also known as an aphid alarm pheromone [10]. *trans*-β-Farnesene synthase has been purified to apparent homogeneity from maritime pine (*Pinus pinaster* Ait.) needles [123]. This soluble enzyme is possibly a homodimer with 45-kDa subunits and its properties (pH optimum, divalent cation requirement, K_m for FPP, sensitivity to thiol-directed reagents) are similar to those of sesquiterpene and monoterpene cyclases.

2.3.5 Structural Similarities among Isoprenoid Synthases

With the availability of cDNA sequences for a number of monoterpene, sesquiterpene, and diterpene cyclases, as well as those for several prenyl transferases, a number of common protein structural features have become apparent. Common to most cyclases [58, 75, 83, 86, 87, 114, 117, 120, 125, 126] and prenyl transferases [127, 128] examined so far is the presence of at least one aspartic acid rich region with a consensus sequence of (I, L, V) XDDXX(D, E). This aspartate rich sequence is believed to be involved in binding of the Mg^{2+} (or Mn^{2+}) salt of the diphosphate substrate [129] and may assist in the initial ionization step of the reaction [130]. Consistent with this supposition is the observation that cyclases, which bind a single diphosphate ester substrate, contain only a single aspartic acid rich region, whereas prenyltransferases, which bind two co-substrates (IPP and an allylic diphosphate), contain two such aspartate rich regions referred to as domains I and II. Site directed mutagenesis of aspartic acid residues in either domain I or II of FPP synthase results in drastically reduced enzymatic activity [32, 34, 130]. The role of these aspartic acid residues in binding the divalent metal ion-complexed substrate is also supported by the X-ray crystal structure of avian FPP synthase [35]. As yet, no directed mutagenesis studies of these putative active site aspartic acid residues have been carried out with any isoprenoid synthase enzyme.

A significant level of homology exists among the plant monoterpene, sesquiterpene, and diterpene synthases. Typically, these enzymes exhibit 30–40% identity and 50–60% similarity at the amino acid level (reviewed in [114]). The organization of introns and exons of the genomic DNA for *epi*-aristolochene synthase [86], vetispiradiene synthase [87], and casbene synthase [120] is also very similar, suggesting that the exons encode functional domains of the enzyme

which have been highly conserved throughout evolution [120]. Other than the highly conserved aspartic acid rich consensus sequence, the plant isoprenoid synthases exhibit very little homology with the fungal sesquiterpene synthases.

Based on sensitivity to amino acid modification reagents, the presence of one or more histidine and cysteine residues at cyclase active sites has also been proposed (see Sect. 2.3.1 and 2.3.2). Supporting this proposal is the observation of three conserved histidine residues and a conserved cysteine residue in the deduced amino acid sequences for 4*S*-limonene synthase [75, 126], *epi*-aristolochene synthase [86], vetispiradiene synthase [87], casbene synthase [120], and abietadiene synthase [114]. When the sequences for the two *ent*-kaurene synthase A activities are taken into account [116, 117], only one of these residues, a histidine, is conserved in all synthases examined to this point. An active site cysteine residue in trichodiene synthase has also been demonstrated by both chemical modification and site directed mutagenesis [131]. The involvement of an active site arginine residue has also been suggested, based on the sensitivity of monoterpene synthases to the arginine-directed reagent phenylglyoxal [66]. This suggestion is also supported by the presence of two strictly conserved and five highly conserved arginine residues in the deduced amino acid sequences of limonene synthase [75, 126] *epi*-aristolochene synthase [86], *ent*-kaurene synthase [116, 117], and casbene synthase [120].

2.4 Secondary Transformations

The isoprenoid synthases previously discussed construct the basic carbon skeletons of the different families of isoprenoids. Once formed, these initial products are often further modified to generate the vast assortment of isoprenoids found in nature. These secondary enzymatic reactions include hydroxylations by P450 cytochromes, redox transformations, isomerizations, and conjugations. Although these secondary transformations are very common in both the sesquiterpene and diterpene series, emphasis will be placed on the secondary transformations of monoterpenes. Because of the great range of modifications possible, only selected, illustrative examples from the monoterpene literature will be presented here.

2.4.1 Mentha

The biosynthesis of the *p*-menthane monoterpenes in *Mentha* (mint) species is well characterized. The essential oils of peppermint and spearmint varieties are distinguished by the position of oxygenation of the *p*-menthane ring, and extensive classical breeding experiments and both in vivo and in vitro studies have firmly established the extended pathways for the formation of (−)-carvone (**70**), (−)-menthone (**71**), and (−)-menthol (**72**) (Fig. 5, reviewed in [132]). Hydroxylation at the C-3 position of the parent olefin limonene leads to the

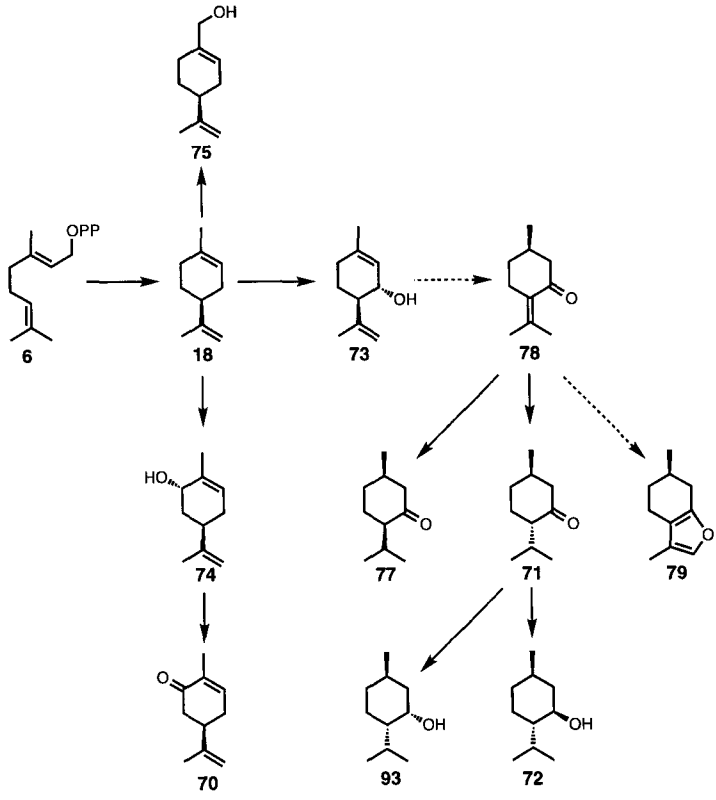

Fig. 5. Secondary transformations of *p*-methane monoterpenes in spearmint, peppermint and perilla. Dashed arrows indicate multiple enzymatic steps

characteristic family of monoterpenes found in peppermint (*M.* x *piperita*) and related species (*M. aquatica*, *M. arvensis*, *M. pulegium*), whereas the monoterpenes of spearmint species (*M. spicata*, *M.* x *gracilis*, *M. crispa*) are characterized by oxygenation at the C6 position of limonene. These reactions are catalyzed by stereospecific cytochrome P-450 oxygenases which hydroxylate (−)-4*S*-limonene at either the C3 position in peppermint varieties to produce (−)-*trans*-isopiperitenol (**73**) or at the C6 position in spearmint varieties to produce (−)-*trans*-carveol (**74**). The two cytochrome P-450s which catalyze these mutually exclusive positional hydroxylations can also be distinguished from each other by differential sensitivity to azole-type inhibitors [133]. Regiospecific, allylic hydroxylation of the parent olefin formed by monoterpene cyclases is a common theme in the biogenesis of many essential oils. Other examples include hydroxylation of (−)-α-pinene (**19**) and (−)-β-pinene (**23**) in hyssop (*Hyssopus officinalis*) [134], hydroxylation of (−)-limonene (**18**) to perillyl alcohol (**75**) in perilla (*Perilla frutescens*) [133], and hydroxylation of sabinene (**76**) to produce the oxygenated thujane monoterpenes (see Sect. 2.4.2).

(−)-*trans*-Carveol (**74**) is subsequently oxidized to (−)-carvone (**70**), the major monoterpene found in spearmint. A similar, but antipodal, pathway has been proposed for the formation of (+)-carvone in caraway seed (*Carum carvi*) [135]. In contrast, the metabolic transformations of (−)-*trans*-isopiperitenol (**73**) in peppermint are much more extensive. A series of redox reactions involving both double bonds and the oxygenated carbon, and a double bond migration, ultimately results in the formation of both (−)-menthone (**71**) and (+)-isomenthone (**77**), and all four stereoisomers of menthol (reviewed in [132]). The relative proportions of the two reductases producing menthone and isomenthone from (+)-pulegone (**78**) varies in commercial mint species from 3:1 to 10:1 [136, 137]. Pulegone is also located at the branch point for the formation of the commercially undesirable oil component (+)-menthofuran (**79**). The biosynthesis of menthofuran from pulegone is believed to involve a cytochrome P-450 catalyzed hydroxylation followed by cyclization and dehydration to provide the furan ring. Although conversion of pulegone to menthofuran by a cytochrome P-450 has been shown in mammalian liver microsomes [138, 139] this activity has not yet been demonstrated in peppermint or related species.

Further insight into the close relationship between the C3 and C6 oxygenated *p*-menthane monterpenes was obtained by examining the essential oil produced by a mutant of Scotch spearmint (referred to as mutant 643) [137]. Instead of producing carvone typical of the wild type spearmint, this mutant produced primarily C3 oxygenated *p*-menthanes, including significant amounts of menthone. Cell-free enzyme assays of both the wild type Scotch spearmint and the mutant 643 demonstrated that both plants contain the same complement of enzymes necessary to produce menthone from *trans*-isopiperitenol, with the only major difference being the specificity of the limonene hydroxylase activity. As expected, the wild type the wild type spearmint contained only the C6 hydroxylase whereas in mutant 643 this activity was entirely replaced by a C3 hydroxylase. The new C3 hydroxylase resembled that found in peppermint in differential sensitivity to azole-type inhibitors. Although spearmint clearly contains most of the same terpene-metabolic machinery as peppermint, these enzymes are normally catalytically silent because carvone is a poor substrate for these subsequent reaction steps.

2.4.2 Salvia

Common sage (*S. officinalis*) has proven to be a particularly rich source of monoterpene cyclases. At least six different major monoterpene cyclases have been isolated from sage oil glands and characterized: pinene synthase I produces mainly (+)-α-pinene (**25**) and (+)-camphene (**26**); pinene synthase II produces mainly (−)-α-pinene (**19**), (−)-β-pinene (**23**), and (−)-camphene (**27**); pinene synthase III produces (+)-α-pinene (**25**) and (+)-β-pinene (**28**) [54]; 1,8-cineole (**15**) synthase [46]; (+)-sabinene (**76**) synthase [140]; and (+)-BPP (**9**) synthase [63]. (−)-BPP (**10**) synthase has been isolated from tansy (*Tanacetum vulgare*)

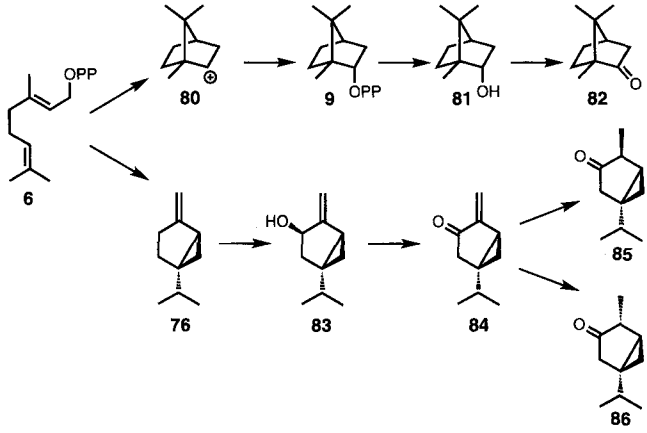

Fig. 6. Secondary transformations of camphane and thujane monoterpenes in sage and tansy

[63]. 1,8-Cineole synthase and BPP synthase illustrate two alternatives to cytochrome P-450 catalyzed hydroxylation for the formation of oxygenated monoterpenes. In the case of 1,8-cineole (**15**) synthase, the cyclic ether oxygen atom is derived by water quenching of the enzyme bound α-terpinyl cation (**13**) (Fig. 2) [46]. BPP synthase catalyzes a second cyclization of the α-terpinyl cation intermediate to produce the bicyclic bornyl (camphyl) cation (**80**), followed by capture of the diphosphate anion to yield the end product [38]. Specific phosphohydrolases cleave BPP (**9**) to borneol (**81**) [73] which is subsequently oxidized to camphor (**82**) [141] by a specific dehydrogenase (Fig. 6).

The thujanes are a widespread family of bicyclic monoterpenes containing an unusual cyclopropane ring (Fig. 6) [142]. (+)-Sabinene (**76**) synthase has been isolated from sage [140]. As with the *p*-menthanes described earlier, allylic hydroxylation of the parent olefin by a microsomal cytochrome P-450 follows to provide (+)-*trans*-sabinol (**83**). A series of redox reactions subsequently yield (+)-sabinone (**84**) and either (+)-3-thujone (**85**) in tansy or (−)-3-isothujone (**86**) in sage [142, 143].

2.4.3 Nepeta

Iridoids are a widely distributed family of monoterpenes that bear the characteristic methylcyclopentanoid-pyran ring structure. Interest in the biosynthesis of iridoids originated because of the role of these monoterpenes in the formation of pharmaceutically important indole alkaloids (reviewed in [144]). Although not known primarily for their aromatic properties, considerable interest in the biosynthesis of the nepetalactone family of iridoids has developed because of the role of these compounds as aphid sex attractants [145]. Nepetalactones are

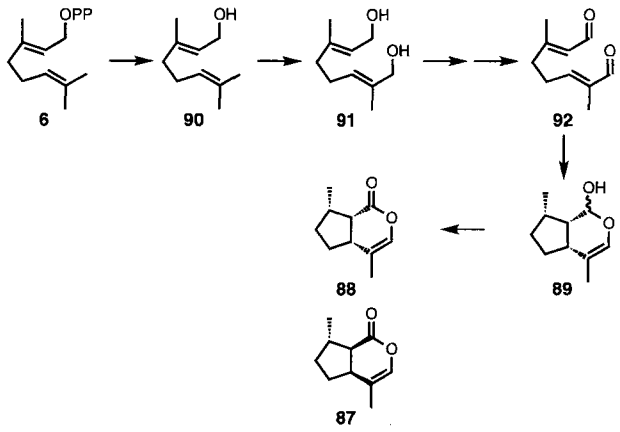

Fig. 7. Proposed biosynthesis of iridoid monoterpenes in catmints

found exclusively in the essential oils of catmints (*Nepeta* spp.) [146, 147], and (4a*S*, 7*S*, 7a*R*)-nepetalactone (**87**) from *Nepeta cataria* is well known as a feline attractant [148, 149]. Biosynthesis of the steroisomeric (4a*R*, 7*S*, 7a*S*)-nepetalactone (**88**) in *N. racemosa* has recently received attention because it possesses the same configuration as the nepetalactol isomer (**89**) employed as a sex pheromone by the damson hop aphid (*Phorodon humuli*) [150].

Unlike the biosynthesis of most cyclic monoterpenes, the formation of iridoids involves the oxidation of geraniol to a highly reactive dialdehyde which is subsequently cyclized to the iridoid skeletons (Fig. 7). After hydrolysis of GPP to geraniol (**90**), a cytochrome P-450 catalyzed hydroxylation yields 10-hydroxygeraniol (**91**). This enzyme has been demonstrated in *N. mussinii* [151] as well as in *Catharanthus roseus* which produces an iridoid glycoside used as an intermediate in indole alkaloid biosynthesis [152, 153]. Efforts to clone this cytochrome P-450 are currently under way [154, 155]. The resulting 10-hydroxygeraniol is then oxidized to the dialdehyde 10-oxogeranial (**92**). An oxidoreductase from *N. racemosa* capable of specifically oxidizing either of the alcohol groups of 10-hydroxygeraniol to the corresponding aldehyde has recently been purified to homogeneity [156]. Cyclization of this reactive dialdehyde affords the iridane skeleton. Although this novel cyclization has been demonstrated in cell-free extracts from tissue cultures of the monoterpene indole alkaloid producing plant *Rauwolfia serpentina* [157], no further work on this enzyme has been reported.

2.4.4 Catabolism

Catabolism normally refers to the degradative loss of an accumulated product through conversion to basic metabolic intermediates which can be recycled by

the plant. Common strategies utilized in catabolism include conjugations, hydroxylations, oxidations, and ring cleavage reactions designed to both increase the water-solubility of the compound and to allow the compound to be broken down into metabolites that can provide both energy and carbon. Non-volatile glycosides of aroma compounds, including many monoterpenes, are widespread in many plant tissues (reviewed in [158]) and are commonly considered as potential aroma precursors [159, 160]; in the plant they likely represent storage or transport derivatives. A consideration of the role of glycosides as precursors of aroma compounds is outside the scope of this paper and they will only be described here in the context of terpene catabolism.

Although the loss of accumulated essential oils during development and senescence has long been recognized (reviewed in [36, 161]) relatively few experiments have examined catabolism of monoterpenes at the biochemical level. Two systems which have been studied in some detail are the loss of essential oil observed in peppermint leaves after floral initiation [162] and a similar loss observed in sage leaves [163]. After floral initiation in peppermint, the composition of the oil changes, reflecting a conversion of menthone to menthol. A significant amount of the menthone is also believed to be converted to neomenthol (**93**), which is subsequently glycosylated to neomenthyl-β-D-glucoside. This glycoside is then transported to the rhizomes where it undergoes oxidative degradation, presumably to provide acetyl-CoA and reducing equivalents for rhizome metabolism (reviewed in [161, 164]). A similar process has recently been shown to occur in sage [165]. In this case flowering results in the loss of approximately 50% of the camphor in leaves [163]. Using both tissue cultures and leaf disks as experimental systems, camphor was shown to undergo hydroxylation to 6-hydroxycamphor followed by a series of oxidative ring cleavage reactions similar to those involved in the microbial degradation of camphor [166]. The cytochrome P-450 responsible for the initial hydroxylation of camphor has been isolated from tissue cultures of sage and characterized [167]. Glycosylation of 6-hydroxycamphor is also observed in both sage leaf disks and tissue cultures [165].

2.5 Regulation

The organization of isoprenoid biosynthesis in plants is complex, involving parallel pathways for the synthesis of IPP in different locales, multiple genes for the expression of different forms of HMGR, and multiple branch points for the partitioning of IPP by prenyltransferases into different families of isoprenoids. The partitioning of intermediates between the formation of isoprenoids essential for cell growth and development (such as sterols, carotenoids, and isoprenoid hormones) and the formation of secondary products such as aromatic oils and phytoalexins represents another crucial bifurcation in isoprenoid metabolism. The composition of the essential oils is determined by the efficiency of competition between multiple monoterpene or sesquiterpene synthases for their

respective precursors and by the relative rates of multiple routes for the secondary transformation of pathway intermediates.

Numerous analytical studies have documented the developmental regulation of essential oil formation at the whole plant level (reviewed in [5, 168]). For example, environmental factors such as photoperiod can affect the formation of specific monoterpenes and sesquiterpenes (see, e.g., [169, 170]). However, little is known about the regulation of essential oil biosynthesis at the biochemical level. HMGR has been suggested to be the primary enzyme that regulates the formation of IPP (reviewed in [11, 15, 171]), although no studies to date have addressed the role of HMGR in regulating essential oil biosynthesis. High levels of glandular trichome-specific expression of the histochemical marker β-glucuronidase fused to an HMGR promoter isolated from *Camptotheca acuminata* and expressed in tobacco has been observed [172] suggesting that specific forms of HMGR may be expressed in these tissues. IPP is a central branch point metabolite between the different families of isoprenoids (see Fig. 1). Several studies have begun to address the role that partitioning of IPP and other prenyl diphosphates plays in regulating isoprenoid biosynthesis. An increase in the levels of chloroplast IPP isomerase during light-stimulated carotenoid biosynthesis in maize has been observed [173]. Carotenoid biosynthesis in ripening bell pepper (*Capsicum annuum*) has also been shown to involve an increase in the levels of GGPP synthase [174]. Competition between monoterpene and sesquiterpene biosynthesis for a common pool of IPP has recently been demonstrated in peppermint oil glands [175] (see also Sect. 3.2). In a somewhat similar fashion, competition for a common pool of GGPP between diterpene and tetraterpene biosynthesis has been suggested by constitutive expression of a phytoene synthase gene in tomato [176]. Collectively, these studies indicate that the regulation of partitioning of either IPP or other prenyl diphosphates between different families of isoprenoids may play a central role in the regulation of isoprenoid biosynthesis.

Production of phytoalexins in tissue cultures in response to fungal elicitation can provide useful insight into the regulation of isoprenoid biosynthesis. Elicitation typically results in the inhibition of sterol biosynthesis and induction of either sesquiterpene [177, 178] or triterpene [179] phytoalexin biosynthesis. This readjustment of FPP partitioning from sterol to phytoalexin formation requires coordinated changes in several enzyme activities. Transient increases in HMGR activity, an increase in the levels of phytoalexin biosynthetic enzymes, and a decrease in sterol biosynthetic capacity all act in concert to redirect FPP metabolism [177–180].

3 Localization of Isoprenoid Biosynthesis

Several levels of organization exist for the compartmentation of isoprenoid biosynthesis and accumulation in plants. Although isoprenoids such as sterols

and carotenoids are found in all types of plant cells, this is not the case with essential oils which are normally synthesized and either stored in, or emitted from, specialized anatomical structures such as glandular trichomes. Complex organization of isoprenoid biosynthesis at the subcellular level also exists and represents a significant challenge to the understanding of how the isoprenoid biosynthetic machinery is integrated and coordinated to provide a diverse array of metabolites including the essential oils. The following sections provide brief coverage of the compartmentation of isoprenoid biosynthesis at both the tissue, cellular, and subcellular levels.

3.1 Tissue Localization

A wide variety of specialized anatomical structures are utilized by plants for the synthesis, and either storage or emission of volatile isoprenoids. These structures include the glandular epidermis of flowers [181, 182], resin blisters and ducts, glandular trichomes, secretory cavities, and secretory idioblasts [182, 183]. Variations in the distribution of structures such as glandular trichomes within a tissue, with attendant variations in the composition and amount of the stored essential oil, are common (reviewed in [183]). Flowers emit a wide variety of fragrant volatiles including fatty acid derivatives, phenylpropanoids, and isoprenoids [7, 184] which are believed to serve as both long- and short-distance attractants for animal pollinators [181]. Many isoprenoids accumulated by the plant in storage structures such as glandular trichomes and resin blisters are believed to serve defensive purposes against herbivores [183]. Storage in specialized structures which segregate the oils from the rest of the plant tissue may also serve to protect the plant from cytotoxic isoprenoids.

Glandular trichomes are perhaps the best studied tissue involved in the biosynthesis of aromatic essential oils. The localization of essential oil formation in such specialized tissues, which often constitute only a small fraction of the plant by mass, has hampered examination of the regulation and enzymology of isoprenoid biosynthesis. Thus, in whole plant extracts, it is often difficult to detect the activity of monoterpene biosynthetic enzymes due to the presence of large amounts of non-specific phosphohydrolases (which degrade substrates such as GPP) and other competing enzymes derived from non-glandular tissue. An early improvement over the use of whole-leaf extracts as a source of monoterpene synthases was the development of methods for preparing surface cell extracts from the leaves of essential oil-producing plants [185]. Subsequently, this method was improved to allow the isolation of intact secretory cells derived from the glandular trichomes of a number of plants [186]. The utilization of isolated secretory cells as a starting material has been instrumental in the purification of several monoterpene biosynthetic enzymes, including limonene synthase from peppermint [48], 1,8-cineole synthase from sage [46], limonene 6-hydroxylase from spearmint [187], and an NADPH-cytochrome P-450 reductase from spearmint [188]. Cell-free extracts prepared from isolated

glandular trichomes of tobacco have also been used as an experimental system to characterize the enzymatic synthesis of the labdane diterpenes *cis*-abienol [111], labdenediol, and sclareol [112].

Intact secretory cells from glandular trichomes of peppermint have also been used to examine the biosynthesis of monoterpenes from basic precursors [189]. This work established that the secretory cells of peppermint glandular trichomes, which are non-photosynthetic, contain all of the enzymatic machinery necessary to produce monoterpenes from an imported carbon source such as sucrose. Similar results have been obtained with photosynthetic glandular trichome cells isolated from tobacco leaves, which were shown to be capable of producing diterpenes from either acetate or carbonate [190, 191]. Several other methods have been reported for the isolation of intact secretory cells from glandular trichomes of sagebrush (*Artemisia tridentata*) [192], rose (*Rosa rugosa*) [193], geranium (*Pelargonium*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), squash (*Cucurbita pepo*), and velvetleaf (*Abutilon theophrasti*) [194], although the use of such cells for examining isoprenoid biosynthesis has been limited. All of these methods rely on abrading the leaf tissue under conditions in which the glandular trichomes can be selectively sheared, followed by separation of the isolated glandular heads from the rest of the leaf tissue. Insights gained from the biochemistry of essential oils in isolated secretory cells can be extended to isoprenoid formation in cells which are not so readily isolated, such as those of the secretory cavities of *Citrus* species and those of resin ducts and blisters in conifers.

Isoprenoids are among the most common volatiles emitted by flowers [184]. Emission of volatiles normally occurs during anthesis, with little aroma emission by flower buds. The most common isoprenoids found by floral head space analysis includes the cyclic monoterpenes limonene (**18**), 1,8-cineole (**15**), α-pinene (**19**), and β-pinene (**23**), and the acyclic monoterpenes myrcene (**24**), ocimene (**94**) and linalool (**68**), although sesquiterpenes such as caryophyllene (**46**) and α-farnesene (**95**) are also commonly encountered [184]. Different compositional patterns of isoprenoids are often emitted from different parts of the flower although few detailed studies of this phenomenon have been reported (reviewed in [181]). Diurnal rhythms in the emission of volatiles by flowers are also well known and are believed to play integral roles in the attraction of day or night pollinators [181]. The basis of this rhythmic emission of volatiles is not known, although the presence of glycosides of monoterpenes [195, 196] and phenylpropanoids [197] in flower tissues has led to the suggestion that release from non-volatile glycosidic pools is involved [196]. Emission of linalool (**68**) from *Clarkia* (Onagraceae) flowers has recently been shown to occur primarily from the petals and to a lesser extent from the pistil and stamens [198]. In contrast, two linalool oxides are emitted almost exclusively by the pistil. Linalool synthase was found in each part of the flower that actively emits either linalool or the linalool oxides. Recently linalool synthase from *Clarkia breweri* has been purified to homogeneity [122] (see Sect. 2.3.4).

3.2 Subcellular Localization

The wide variety of isoprenoids formed by plants, from primary metabolites such as sterols, carotenoids, and isoprenoid-derived hormones, to the natural products (secondary metabolites) such as monoterpenes and sesquiterpenes, has led to the evolution of parallel pathways for isoprenoid biosynthesis at several subcellular sites. This compartmentation can be conceptually divided into the pathways responsible for the formation of IPP and the pathways responsible for the transformation, and thus partitioning, of IPP into the different families of isoprenoids.

The subcellular compartmentation of the mevalonic acid pathway responsible for the formation of IPP in plants (Sect. 2.1) has been the subject of some disagreement [11, 199–201] especially regarding the subcellular localization of HMGR. As indicated earlier, HMGR is encoded by small gene families in plants, with some plants (e.g., *A. thaliana*) containing only 2 genes for HMGR and others (e.g., potato) containing 12 or more [15]. The N-terminal region of the different encoded HMGR proteins (approximately 200 amino acid residues) is highly divergent and does not resemble either a typical endoplasmic reticulum anchoring sequence or a plastidial or mitochondrial targeting peptide sequence [171]. However, the in vitro transcription and translation of both forms of *Arabidopsis* HMGR with subsequent insertion of the translated protein into mammalian microsomes [18, 202] has been used to argue for an exclusively microsomal (endoplasmic reticulum) localization for HMGR. Contradicting these results are studies showing that distinct forms of HMGR reside in plastids and mitochondria, in addition to microsomal fractions (reviewed in [203]). A series of experiments examining the formation of isoprenoids throughout chloroplast development in barley leaves has demonstrated that, early in development, chloroplast isoprenoids are formed from plastid-derived IPP, arguing for the presence of a plastidial mevalonic acid pathway [204, 205]. Purified, immature chloroplasts from barley leaves can synthesize isoprenoids from either pyruvate or acetate, and these organelles contain detectable levels of both mevalonate kinase and mevalonate phosphate kinase [206]. Only one study to date has specifically examined the compartmentation of IPP formation in an essential oil plant. Using secretory cells isolated from glandular trichomes of peppermint as an experimental system, this work demonstrated that the IPP utilized for both monoterpene and sesquiterpene biosynthesis is formed exclusively in the non-photosynthetic plastids (leucoplasts) [175]. The picture that emerges from these studies is that the compartmentation of IPP formation is complex, with parallel pathways existing in the cytoplasm, the plastid and possibly the mitochondria. The relative activities of these parallel pathways can change during development and must play an integral role in partitioning of IPP between the different families of isoprenoids.

In contrast to the situation with the compartmentation of IPP formation, the subcellular compartmentation of IPP utilization is quite well established (reviewed in [5]). FPP synthase, and the subsequent enzymatic steps involved in

sesquiterpene and triterpene biosynthesis from this precursor, are localized in the cytoplasm and endoplasmic reticulum. The formation of monoterpenes and diterpenes from IPP appears to be restricted to the plastid. GPP synthase [207] and GGPP synthase [208] have both been localized to this organelle. A cDNA for GGPP synthase has been isolated and encodes a typical N-terminal plastid targeting sequence [174, 209]. The presence of N-terminal plastid targeting sequences in the preprotein has also been reported for limonene synthase [75], as well as for the diterpene cyclases abietadiene synthase [114], *ent*-kaurene synthase A (involved in gibberellin biosynthesis) [116], and casbene synthase [120]. A cDNA clone for IPP isomerase isolated from the petals and stigma of *Clarkia breweri* flowers has recently been isolated and shown to encode a putative N-terminal plastid targeting sequence [210]. *epi*-Aristolochene synthase and vetispiradiene synthase, the only higher plant sesquiterpene cyclase genes cloned at this time, lack plastidial targeting sequences, consistent with the localization of the corresponding cyclases in the cytoplasm [86, 87].

The subcellular localization of IPP formation and utilization has important implications for the partitioning of IPP between the different families of isoprenoids. Recent studies with isolated secretory cells of peppermint have demonstrated the presence of high levels of the biosynthetic enzymes for both monoterpene and sesquiterpene formation from IPP [175]. However, this potentially high rate of sesquiterpene formation is not reflected in either essential oil composition or the rate of incorporation of basic precursors such as pyruvic acid. This discrepancy results from the formation of IPP exclusively in the leucoplasts of these isolated secretory cells. Thus, the cytoplasmic sesquiterpene pathway competes poorly for the pool of plastidic IPP relative to the plastidic monoterpene pathway. The presence of leucoplasts with well defined morphology has been closely correlated with the formation of monoterpenes in glandular trichomes, resin ducts, secretory cavities, and idioblasts [211]. Based on this observation, it has been suggested that the formation of IPP exclusively in the leucoplasts may be a common feature of these tissues and may play a critical role in the relative ratio of monoterpenes and sesquiterpenes produced [175].

4 Approaches to the Bioengineering of Isoprenoid Biosynthesis

The field of isoprenoid biosynthesis has advanced to the point where several approaches can now be considered for the rational manipulation of essential oil formation. These include the mutagenesis of specific isoprenoid synthases to alter product outcome, the manipulation of a biosynthetic pathway by either altering the activity of an existing enzyme or by introducing novel enzymes, and using genetic approaches to manipulate the formation of the specialized anatomical structures (such as glandular trichomes) involved in the synthesis and storage of

essential oils. An appreciation of the control mechanisms for regulating the flux of intermediates through the pathway, the partitioning of intermediates between branch points, and the accumulation of specific products of the pathway is essential for the rational targeting of specific enzymes for bioengineering. For this reason, metabolic control analysis is briefly described to introduce the concepts necessary for understanding the control of metabolic pathways. The application of tissue cultures for essential oil formation is also briefly considered.

4.1 Manipulation of Specific Enzymes to Alter Product Formation

An appreciation of the detailed kinetic mechanism of a wide variety of monoterpene and sesquiterpene synthases and related enzymes, and an understanding of the role of the active site residues in catalysis along with the availability of primary sequence information sets the stage for engineering product formation. Plant monoterpene and sesquiterpene synthases are particularly well suited for this approach because many of these enzymes already produce multiple products indicating a level of catalytic flexibility.

Two general approaches can be considered for the engineering of product composition by a specific enzyme, directed mutagenesis and domain swapping. Subtle changes in the amino acid residues at the active site of an enzyme can influence substrate binding conformation and the participation of side chain functional groups in catalysis. For example, in the case of monoterpene synthases, one could envision binding alterations to influence the isomerization of GPP to the opposite antipode of LPP, with consequent formation of alternate products. Similarly, modification of specific residues to affect the stability of carbocation intermediates could divert the reaction course along alternate routes to novel products. Although systematic studies of this type have not yet been carried out, two examples are known involving alteration of product outcome by mutation of a single amino acid residue. A yeast mutant which excretes large amounts of geraniol and linalool contains an altered FPP synthase [31] which has a single Lys197 to Glu mutation in a conserved position within a consensus sequence present in all known FPP synthases. The Lys at this position is believed to be involved in substrate binding. Mutation at this position aborts FPP production, although it was not reported if the product released by the mutant enzyme is GPP or geraniol. Recent studies on the site-directed mutagenesis of trichodiene synthase have demonstrated the ability of a fungal sesquiterpene cyclase to form aberrant products following minor modification of the active site [131]. Replacement of Arg304 with Lys results in a drastically decreased rate of trichodiene formation with concomitant formation of several novel sesquiterpene hydrocarbons. Replacement of Tyr305 with Thr results in the formation of approximately 50% of one of these novel products.

The second general approach to engineering product formation involves the construction of chimeric enzymes that combine functional domains from

different synthases. This approach is suggested by the high level of sequence homology observed for plant isoprenoid synthases, and by the nearly identical organization of introns and exons observed with *epi*-aristolochene synthase, vetispiradiene synthase, and casbene synthase (Sect. 2.3.5). The latter observation may indicate evolutionary conservation of functional domains in plant isoprenoid synthases [120], and suggests that chimeric enzymes with different functional domains can be created to synthesize novel products.

4.2 Bioengineering of Pathways

An understanding of the enzymology and regulation of a metabolic pathway (including the roles played by developmental changes, subcellular compartmentation, and the presence of multiple branch points) can permit the manipulation of the flux of intermediates through the pathway, with the goal of increasing the accumulation of a desirable product or decreasing the accumulation of undesirable ones. The bioengineering of the metabolic pathways for isoprenoid formation can involve either the modification of an existing pathway, directed to changing the composition and/or yield of the oil, or the introduction of new or altered enzymes to produce novel products not normally found in the plant.

It is now widely accepted that the control over the flux of intermediates through a metabolic pathway is distributed throughout the enzymes of the pathway, and it is rare that a single enzyme acts to control a truly "rate limiting" step (reviewed in [212]). The biosynthetic pathways used to provide energy and material for cell growth and development have evolved to maintain optimized flux distributions [213]. As a result of this distributed control over flux, metabolic pathways resist attempts to divert significant amounts of biosynthetic precursors from cell growth to the formation of secondary products (such as essential oils). In addition, the presence of branch points in metabolic pathways, and the nature of complex non-linear enzyme kinetics, often result in either minimal or unpredictable changes in pathway flux in response to changes imposed on the activity of a single enzyme (reviewed in [212]). Recent extensions to metabolic control analysis provide experimental strategies for manipulating the output of specific metabolites from a metabolic pathway by modifying two or more enzyme activities without affecting the steady state levels of other metabolites in the pathway (see, e.g., [214, 215]). An appreciation of this distributed flux control provides a sound framework for the successful bioengineering of the output of specific products from a metabolic pathway.

An illustration of the effect that partitioning of metabolites by branch points has on the quality of an essential oil is provided by the *p*-menthane monoterpenes produced in *Mentha* (Fig. 5) in which pulegone (**78**) acts as a branch point between the formation of menthone (**71**) and menthol (**72**), and the commercially undesirable component menthofuran (**79**). The major monoterpenes accumulated in pennyroyal (*M. pulegium*), peppermint (*M.* x *piperita*), and cornmint (*M. arvensis*) are pulegone, menthone and menthol, respectively. After flowering,

the menthone content of peppermint oil decreases with a corresponding increase in the menthol content of the oil. The relative amounts of menthone and menthofuran formed in peppermint oil are controlled by environmental factors such as photoperiod [170]. Thus, pulegone is a branch point between the formation of oils high in menthone and menthol, and those high in menthofuran. The accumulation of high levels of this branch point metabolite is characteristic of the pulegone-rich oil of pennyroyal. The formation of high levels of menthofuran by peppermint under conditions of short photoperiod or high temperature stress is largely beyond the control of the grower. Therefore, the branch point enzymes producing menthofuran and menthone represent promising targets for bioengineering efforts. The simplest approach would involve overexpressing the reductase which converts pulegone to menthone, thereby reducing the flux of pulegone into menthofuran. An alternate approach would involve decreasing conversion of pulegone to menthofuran by either repressing the expression of the putative cytochrome P-450 which converts pulegone to menthofuran, or removing the regulatory element(s) which result in increased expresssion of the P-450 under conditions of short photoperiod, resulting in a low, constitutive level of menthofuran formation.

An alternate approach to bioengineering of a pathway involves the introduction of new or modified enzymes not normally present in the plant. An illustration of the possibilities is provided by the production of C3 hydroxylated monoterpenes by a mutant of Scotch spearmint (see Sect.2.4.1). Replacement of the normal limonene-6-hydroxylase with a C3 hydroxylase activity results in the formation of numerous C3 hydroxylated *p*-menthane monoterpenes not naturally found in spearmint varieties [137]. Many monoterpenes, including limonene, exhibit insecticidal properties (reviewed in [10, 183]). The availability of cDNA clones for limonene synthase [75, 126] raises the possibility of engineering limonene production into crop plants to improve resistance to insect pests. The availability of cDNA clones for other cyclases and related enzymes of monoterpene metabolism provides the opportunity for manipulating composition in essential oil producing plants as well as introducing the formation of bioactive isoprenoids into new crops.

4.3 Trichome Formation

A third goal of bioengineering essential oil production is to increase the yield without affecting organoleptic quality. However, attempts to increase significantly the oil produced may be limited by the fact that these products are synthesized and stored in specialized anatomical structures, such as glandular trichomes, in which the primary metabolic pathways are already optimized to provide the energy and carbon sources necessary for isoprenoid synthesis. For this reason, attempting to increase the supply of IPP available for biosynthesis by increasing the activity of a single enzyme (e.g., HMGR) is unlikely to provide significant increases in total oil production.

Conceptually, the simplest approach to increasing oil production by the plant is to increase the tissue density of the specialized anatomical structures responsible for synthesis and storage. Recent work on the regulation of trichome development in *Arabidopsis* may lead to the development of approaches for increasing the density of glandular trichomes, which could result in a corresponding increase in total oil production. The identification of at least 21 different genetic loci involved in trichome development in *Arabidopsis* has been described [216]. Three of these loci (*GL3*, *TTG*, and *TRY*) appear to be involved in the initiation and surface distribution of trichomes [216–218]. The *GL2* gene has recently been cloned and shown to encode a regulatory transcription factor which is expressed in trichome progenitor cells [219]. Although the studies with *Arabidopsis* involve non-glandular trichomes, the development of glandular trichomes will undoubtedly share many of the same regulatory factors and control elements.

4.4 Tissue Cultures

Plant tissue cultures have long attracted interest as potential sources for the production of high value chemicals, including isoprenoids. However, significant economic and technical hurdles still exist in the utilization of these systems for commercial purposes (see, e.g., [220, 221]). For the most part, production yields of lower isoprenoids, such as monoterpenes and sesquiterpenes, by plant tissue cultures have been disappointing (reviewed in [222, 223]). Callus and suspension cultures established from essential oil-producing plants often produce only very low levels of the isoprenoids characteristic of the intact tissue. The low yield of essential oil isoprenoids is assumed to be due to the presence of undifferentiated cells in tissue culture; without specialized anatomical structures for production and storage, synthesis is suppressed and the activity of catabolic enzymes is enhanced. Studies with tissue cultures of sage have established that, although detectable levels of the enzymes for camphor biosynthesis are present, rapid catabolism of camphor prevents accumulation [224]. The capacity for the catabolism of camphor in tissue cultures of sage is so enhanced compared to intact tissue that cultured cells have been used for characterizing the catabolic pathway [165, 167]. The main value of tissue cultures to this point has been as convenient model systems for studying isoprenoid biosynthesis in response to different environmental stresses such as fungal infection [84, 177–180, 225].

5 Conclusions

This paper has attempted to provide a current overview of essential oil isoprenoid biosynthesis. An understanding of the mechanism of action of monoterpene and sesquiterpene cyclases, along with the availability of an increasing number

of cloned genes encoding isoprenoid synthases and related enzymes, has set the stage for the systematic bioengineering of product composition and yield. In many cases, the secondary transformations of isoprenoids are also understood well enough to rationally target specific enzymes for manipulation, with the goal of modifying essential oil composition. However, significant gaps remain in our understanding of the integration of essential oil biosynthesis with primary metabolism; in particular, the regulation of IPP biosynthesis and the partitioning of IPP remain poorly characterized. Too few studies have addressed the early steps of isoprenoid biosynthesis in essential oil-producing tissues and our understanding of the developmental regulation of essential oil biosynthesis is also fragmentary. These deficiencies need to be addressed before an integrated approach to the bioengineering of essential oil production can be attempted.

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6 References

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Opportunities in Microbial Biotransformation of Monoterpenes

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Monoterpenes are important flavour and fragrance compounds. The biotransformation of monoterpenes has been studied quite extensively during the past 30 years. Specific problems have been encountered during these studies which have prevented the commercialization of monoterpene biotransformation processes. The most important problems were the chemical instability of monoterpenes, the substrate and product toxicity and the presence of multiple transformation pathways in the microorganisms. Notwithstanding the encountered problems, the area of monoterpene biotransformation remains of great potential commercial interest to the food and perfume industry. The main advantages of the use of biotechnological methods for the production of flavours and fragrances are the fact that terpenoids produced in this way can be called natural and the fact that biocatalysts show, in general, a high regio- and stereoselectivity. Information regarding the enzyme systems involved in monoterpene biodegradation, except for the degradation of (+)- and (−)-camphor by *Pseudomonas putida*, is rather scarce. However, during the past decade new information has become available on the purification and description of several enzymes involved in other monoterpene degradation pathways. The genetic information encoding some of these enzymes has been cloned and sequenced. In the future, genetic engineering techniques may provide modified strains which can be used for the production of the desired product.

1 Introduction

Monoterpenes are naturally occurring branched chain C-10 hydrocarbons formed from two isoprene units. They are widely distributed in nature and over 400 different naturally occurring monoterpenes have been identified [1]. In particular, oxygenated derivatives of terpenes, commonly known as terpenoids, have strong and pleasant odours. Many of these terpenoids are considered as GRAS (Generally Recognized As Safe) and, when obtained from natural sources, they can be added to foods without being considered as additives [2]. On the other hand, various readily available monoterpenes, such as (−)-α-pinene, (−)-β-pinene, and (+)-limonene are used in large quantities in the chemical industry for conversion into more valuable terpenoids. The price of these bulk terpenes is relatively low; i.e. in the order of $2–4 per kg [3].

The microbial transformation of the monoterpene (+)-citronellal into (+)-citronellol by yeast was reported as early as 1915 [4]. More recent research has demonstrated that many terpenoids can be produced by microbial transformation of monoterpenes. The early work on terpene metabolism was highly influenced by the approach used in steroid biotransformations [5, 6]. As a consequence, the early literature is based mainly on terpenoids accumulated by fungal strains which do not mineralize the substrate but partly oxidize it by fortuitous co-metabolism. Later work has focused on the use of strains, mainly *Pseudomonas* spp., which are able to grow on the monoterpene substrates as sole carbon and energy source.

Several excellent reviews have appeared summarizing monoterpene biotransformation reactions [2, 7–13]. During the past decade, new information has become available on the purification and description of several enzymes involved in monoterpene degradation. The genes encoding some of these enzymes have also been cloned and sequenced, opening up the possibility of doing single step biotransformations in a neutral background. This review gives an overview of the characteristics of monoterpene biotransformations. It discusses the different problems encountered during biotransformation studies which have prohibited commercialization and discusses the niches in which biotransformation of monoterpenes can play an important role. The enzymes which have been purified and characterized so far are also discussed, and the genes cloned from monoterpene degradation pathways are documented. Finally, the potential of monoterpene biotransformation processes for the commercial production of flavour and fragrances is considered.

This paper will not discuss the biosynthesis of monoterpenes, and the interested reader is directed to the review by Croteau in this volume. Also the fermentative production of monoterpenes as secondary metabolites is not discussed and the reader is referred to the review of Welsh et al. [14] for information in this area.

2 Problems Encountered in Monoterpene Biotransformation Studies

Most of the monoterpene biotransformation studies described so far have been of more academic than practical value. The main accomplishment of these studies has been the description of monoterpene transformation pathways, sometimes revealing interesting and valuable catabolites. The microbial transformation of monoterpenes for the production of terpenoids is commonly recognized as being of great economic potential but is regarded as technically difficult. To our knowledge, no monoterpene biotransformation processes have been commercialized so far. In this section we summarize the different problems which have been encountered in the past, and identify the main reasons which have prevented the commercialization of the described biotransformations.

2.1 Chemical Instability of Monoterpenes

Monoterpenes are rather unstable compounds. They readily undergo spontaneous chemical autooxidation, *cis-trans* isomerization, racemization, hydration, cyclization, rearrangement and polymerisation reactions [15, 16]. Often, the chemically formed products are also formed by biotransformation, making it difficult to discriminate between chemical and biological reactions [6, 17]. Unfortunately, many papers do not state which products are formed in abiotic controls under the same reaction conditions.

Not only the monoterpene substrates, but also the products formed, are a target for chemical reactions [13, 18, 19]. This results in the formation of a mixture of products and in the reduction of the product yield, resulting in increased product recovery costs. The chemical stability of monoterpenes depends on several factors.

a. Intrinsic properties of the molecule; e.g. α-pinene undergoes acid catalyzed reactions 16 times faster than limonene [15].
b. Medium pH; pH values higher than 8 and lower than 6 result in increased rates of chemical reactions.
c. Metal-ion concentration; in particular, iron stimulates autooxidation.
d. Partial oxygen pressure.
e. Temperature.
f. Exposure to light (especially UV) results in an increased level of autooxidation [20].
g. Water content.

Most of these substrate instability problems can be minimized by growing the cells at moderate temperatures (i.e. below 30 °C), using a neutral pH, and using a mineral media containing a low salts concentrations.

Table 1. Water solubility and logP of selected monoterpenes; data from [21, 22]

Monoterpene	Solubility (mmol/l)	log P
α-Pinene	0.026	4.49
β-Pinene	0.049	4.42
Limonene	0.045	4.40
Myrcene	0.043	4.50
Citronellol	8.9	
Citronellal	18.0	
Linalool	12.3	
Linalylacetate	1.6	
Carvone	8.8	
Carveol	5.0	
Menthone	4.5	
Menthol	2.3	

2.2 Low Solubility of Monoterpenes

Monoterpenes, and especially the monoterpene hydrocarbons, are poorly soluble in water (Table 1). Depending on the affinity of the biological system (K_M) for the substrate, the reaction rate might, therefore, be limited by the bioavailability of the substrate. It is expected that at low cell densities this will not pose a problem, but at high cell densities the low solubility will affect the biotransformation rate.

2.3 Volatility of Monoterpenes

The main property of monoterpenes that makes them so interesting as flavour and fragrance compounds, their volatility, also causes serious problems during the large scale bio-production of these compounds. The commercially most interesting bioconversion reactions (see Sect. 4) are oxygen dependent. Vigorous aeration and agitation is, therefore, necessary to accommodate these reactions, resulting in substrate and product losses.

Fortunately, the terpenoids produced are, generally, less volatile than their terpene hydrocarbon counterparts [23, 24], and product losses are expected to be less of a problem than substrate losses. To date, only a few large scale bioconversion have been studied, and detailed studies to address these problems, e.g. by stripping of the outgoing air flow, have not yet been performed.

2.4 Toxicity of Monoterpenes

Another problem associated with terpene biotransformation studies is the toxicity of these compounds to whole cells [25–31]. Addition of the terpenes to

cell cultures has been observed to inhibit growth and the addition of high concentrations of monoterpenes can result in cell lysis [25]. Although most monoterpenes are barely soluble (see Sect. 2.1), the inhibitory effects were concentration dependent even at concentrations higher than the solubility limit [25, 30], indicating phase toxicity problems [32]. In general, at "concentrations" higher than approximately 0.05 vol.%, growth was no longer observed.

The main target for the toxicity of monoterpenes and, more generally, organic solvents is the cell membrane [33, 34]. Terpenes dissolve in cell membranes thus disturbing their integrity and effecting specific permeabilization [29, 35]. The hydrophobicity of the organic solvent, expressed as the partitioning of the solvent between octanol and water (log P value), is a good indicator for its toxicity. Substances with a log P value in the range 1–5 are, in general, toxic to whole cells [34]. Monoterpenes have log P values which generally fall in the range 2.5–4.5 (Table 1). The terpenoid products are, in general, even more toxic than the terpene hydrocarbon substrates [22], which might result in increased toxicity problems during the bioconversion. The toxicity of specific terpenes (e.g. citral) is enhanced by exposure to UV-A [36].

To avoid toxicity effects, in many instances the terpene substrates have been added to the culture after maximal growth was obtained. Also, by continuous or repeated addition of non-toxic quantities of the terpene, toxicity problems can be minimized. Another way to prevent monoterpene toxicity is by supplying the monoterpene substrate via the gas phase [19, 37] or, altertatively, to administer the monoterpene to the cells in a solvent which is non-toxic to the cells, resulting in the formation of an organic-liquid two-phase system.

In recent years, there have been several reports of bacteria exhibiting resistance to organic solvents [38–40]. These "solvent-resistant" strains can grow even in the presence of 50% monoterpene. Such strains should be very useful as hosts for bioconversion studies and they should eventually allow biotransformation reactions to be carried out in inhospitable monoterpene-liquid two-phase systems.

2.5 Absence of Product Accumulation and Product Degradation

The early studies on the biotransformation of monoterpenes used microorganisms which were able to transform terpenes in a co-metabolic fashion to prevent product degradation. More recently, however, research has focused on microorganisms which are able to mineralize the substrate, as these strains are expected to contain more efficient enzymes catalyzing the conversion of interest. Perhaps not surprisingly, most of these isolated microorganisms do not naturally accumulate products [8, 11, 19, 41], although in some instances the accumulation of metabolites could be detected when large volumes of culture fluid were extracted [47].

In cases where products did accumulate, often only a transient accumulation of the metabolite, generally in low yield, was observed during a specific growth

phase [6, 16, 42–45]. This requires the monitoring of product formation during the biotransformation to be able to isolate the metabolite.

Problems of the lack of product accumulation and product degradation could in some cases be solved by adding inhibitors to the growth media [46], by varying the growth conditions [17, 47] or by mutation of the strain [48]. However, as many strains have been found to contain multiple terpene metabolic pathways (see below), these approaches have, in general, not been very successful.

2.6 Multiple Metabolic Pathways Resulting in the Formation of a Mixture of Products

Microbial transformation of terpenes generally leads to mixtures of metabolites [e.g. 49–51] and the accumulation of a single reaction product is rare.

For instance a (+)-limonene degrading *Pseudomonas* sp. was found to accumulate 12 different products [47, 52] (Fig. 1). Using "paper biochemistry", these authors suggested that there are three different pathways for (+)-limonene metabolism present in this microorganism. However, only compounds of the perillic acid degradation route could support growth [47]. The other compounds were not further metabolised by (+)-limonene grown cells, suggesting that these were dead-end metabolites, formed as a result of the broad substrate specificity of some of the enzymes induced under these growth conditions. From the results presented it is difficult to judge if some of the dead-end metabolites formed resulted from biological transformation or from chemical reactions of the accumulated compounds.

In many cases different terpenoid metabolites have been observed during the first stages of transformation from those found towards the end, e.g. [16, 43]. Some of the terpenoids produced may give rise to off-flavours. As these complex mixtures often contain closely related products, it is difficult to purify the product of interest from the medium.

In this respect, some of the work performed by Kieslich and coworkers forms an exception. For instance, this group reported that the transformation of (+)-limonene by *Corynespora cassiicola* resulted in the formation of (1*S*, 2*S*, 4*R*)-*p*-menth-8-ene-1,2-diol as the only reaction product [53]. Unfortunately, this diol does not have a smell because it is not volatile enough [54].

2.7 Low Product Concentrations and Product Yields

Most monoterpenes produced during bioconversion studies were produced in trace amounts, sometimes even after extensive optimization studies [44]. Typically, concentrations in mg/l of culture media are reported. This will result in high down-stream processing costs. Also the yield of the bioconversion was generally very low; often the yield of specific compounds was less than 5% [16].

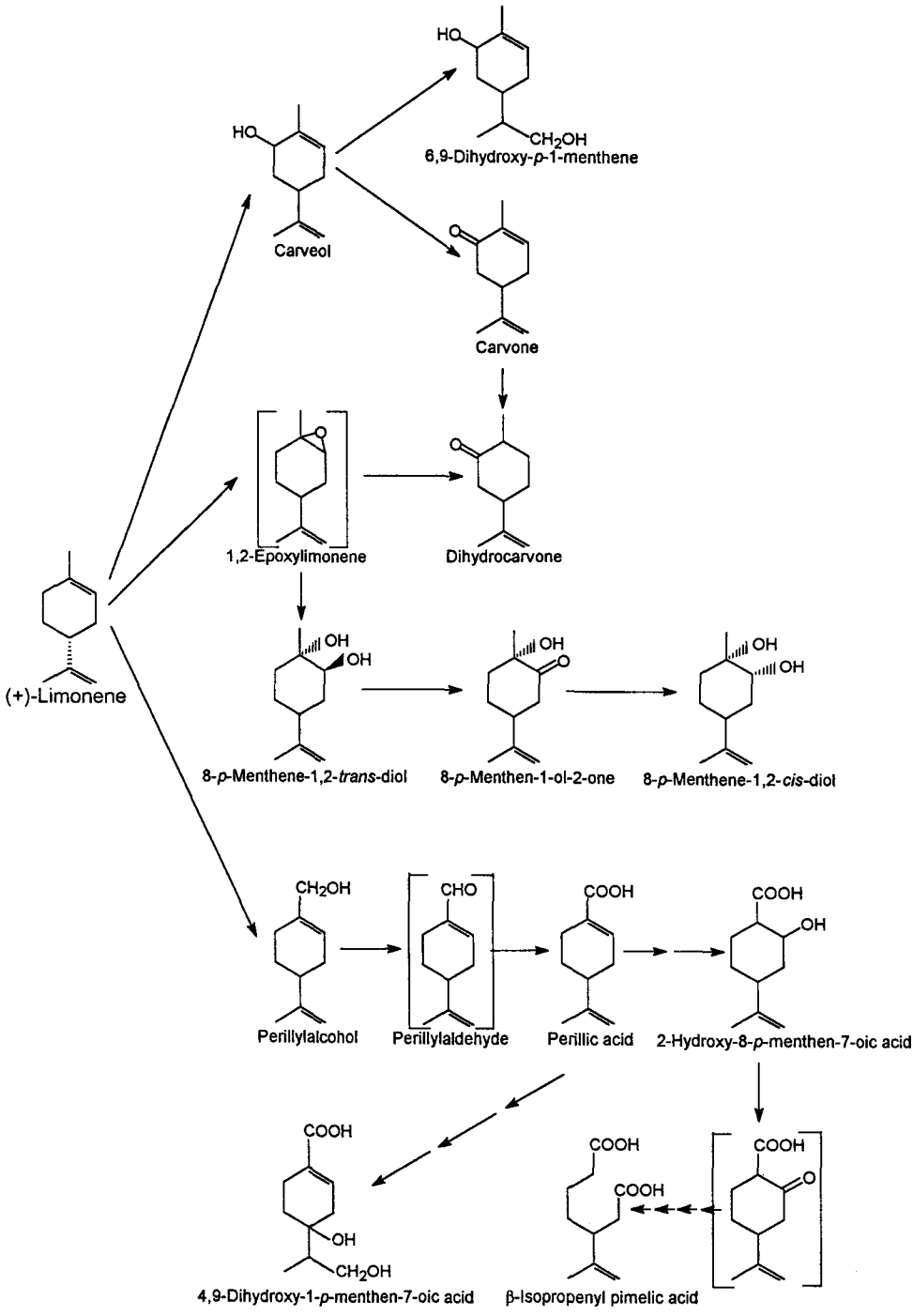

Fig. 1. Pathways for degradation of (+)-limonene in *Pseudomonas* sp. L [47, 52]

Again, the work performed in Kieslich's laboratory forms an exception. They reported the production of 900 g of (1*S*, 2*S*, 4*R*)-*p*-menth-8-ene-1,2-diol from 1300 g of (+)-*R*-limonene in a 70-l fermenter in 4 days [54]. His group also reported on the production of (+)-α-terpineol from *R*-(+)-limonene by *Penicillium digitatum* in concentrations of up to 0.46 g/l [55]. In general much higher product concentrations have been achieved when hydrolytic enzymes were used (see Sect. 4.2).

2.8 Enzyme Activity Not Detectable in Cell Extracts

The determination of monoterpene degradation pathways, and the use of isolated enzymes to transform monoterpenes, has been severely hindered by the fact that, after disrupting the cells, enzymatic activities could frequently not be measured at all [42, 45, 56], or were very unstable [42, 45].

2.9 Long Incubation Times

Many bioconversions, especially involving fungi, took many days to reach appreciable yields. The longer the fermentation time, the greater the cost and increased possibility of contamination by unwanted microorganisms. Chemical degradation of the substrate and products will also increase with length of incubation.

2.10 Short Biocatalyst Lifetimes

Often, repeated use of the microorganism resulted in a loss of the desired biocatalytic activity [8]. To be economically viable, biotransformation processes ideally require biocatalysts with long lifetimes and the potential for re-use.

3 Advantages of the Use of Biocatalysts in the Transformation of Monoterpenes

Notwithstanding the problems discussed above, the area of monoterpene biotransformation remains of great potential commercial interest to the food and perfume industry. The main advantages of the use of biotechnological methods for the production of flavours and fragrances are summarized below.

3.1 Production of Natural Flavours

Recent market surveys have demonstrated that consumers prefer footstuffs that can be labelled natural [57]. This preference is especially evident for flavouring compounds and by 1990, already 70% of the flavours used in the food industry in Germany were of natural origin [58]. This has resulted in a price premium for flavours isolated from natural sources.

Traditionally, natural monoterpenes have mainly been isolated from essential oils. Essential oils consist of the volatile materials derived by physical processes like extraction and distillation, from odorous plant material of a single botanical form and species [59]. This source of natural monoterpenes has several serious drawbacks, some of which are difficult to control.

a. Variability in the composition and yield of the final product from different geographical sources, but also from one source depending on the weather.
b. The risk of several plant diseases.
c. Seasonal variation in supply.
d. 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, leading to uncertainties of product supply.
e. Low concentrations of the desired monoterpene.
f. Expense of isolation.
g. Ecological drawbacks.

As a result, the price of a given essential oil and/or related monoterpene is a highly fluctuating variable. Also, an increased demand for certain flavours might not be met by traditional production methods and a shortage in supplies of certain flavours, e.g. peppermint, have been encountered [58].

One method of alleviating this problem has been the development of biotechnological processes for the production of specific flavour and fragrance chemicals. In the USA, flavours produced by biotechnological means, both by whole cells and by enzymes, can be called natural if the precursor material is also of natural origin [2]. Some monoterpenes, such as (−)-α-pinene, (−)-β-pinene, (+)-limonene, citral and (±)-citronellal, can be isolated on a large scale from essential oils. These monoterpenes can be used as substrate for the bioproduction of more valuable monoterpenes.

3.2 Regio- and Stereoselectivity of Biocatalysts

The absolute configuration of the monoterpene, especially with respect to its stereochemical composition, strongly influences the sensory properties of a monoterpene (Table 2). The modification of monoterpenes requires, therefore, reactions with high regio- and stereospecificity. Biotechnological methods are

Table 2. Odour characteristics of separate enantiomers of selected monoterpenes [60]

Monoterpene	Enantiomer	Odour description
Camphor	R-(+)	camphoraceous
	S-(−)	camphoraceous
Carvone	R-(−)	fresh herbal, spearmint-like
	S-(+)	fresh herbal, caraway-like
Limonene	R-(+)	fresh, natural, citrusy, orange-like
	S-(−)	harsh, turpentine-like, lemon-note
Linalool	R-(−)	flowery-fresh, reminiscent of lily of the valley
	S-(+)	differs slightly in odour
Menthol	1R,3R,4S-(−)	refreshing, strongly minty, cooling
	1S,3S,4R-(+)	minty, musty, phenolic, medical
α-Pinene	1R,5R-(+)	harsh, terpene-like, slightly minty
	1S,5S-(−)	harsh, terpene-like, coniferous
α-Terpineol	R-(+)	heavy, floral, lilac-like
	S-(−)	tarry, coniferous, cold pipe-like

especially suited to achieve this type of transformation as enzymes generally show an absolute regio- and stereoselectivity.

Unfortunately, in many of the bioconversion studies reported so far, not only was the stereoisomeric composition of the end products not reported, but also it was frequently not stated which stereoisomer of the substrate was used or if it was a racemic mixture, e.g. [61, 62]. The use of racemic mixtures of the substrate makes it especially difficult to draw conclusions about the properties of the biocatalyst. The use of citral, a mixture of geranial and neral, as a substrate also has this drawback [63, 64]. In some reports the optical rotation of the product was stated, but no information was given about the optical purity of the product [53, 65].

In a few instances using whole cell bioconversions, it was reported that optical antipodes were produced when using the opposite enantiomer of substrate [53, 65–68] (Fig. 2). In many studies the enzymes involved were not identified, so it is not clear if one or two enzymes were involved in these reactions. On the other hand, the accumulation of both enantiomers of a product from a prochiral substrate, e.g. [69], might be the result either of an enzyme with a low stereospecificity, or from two enzymes catalyzing the same reaction with a high but opposite stereospecificity. For a racemic substrate the same argument may apply with respect to enantioselectivity. Some of these microorganisms were also found to catalyze the optical resolution of racemic mixtures by the preferential degradation of one of the antipodes [41] (Fig. 3).

The major problem in determining the stereo- and regiospecificity of many of the biocatalysts studied so far is the fact that they have been performed with whole cells, and that the enzymes involved have not been identified. This makes it difficult to judge how many enzymes were involved and if these enzymes are stereo- and regiospecific. However, in the few cases where enzymes have been purified, they were found to catalyze the reactions with a high stereo- and regiospecificity (see Sect. 4).

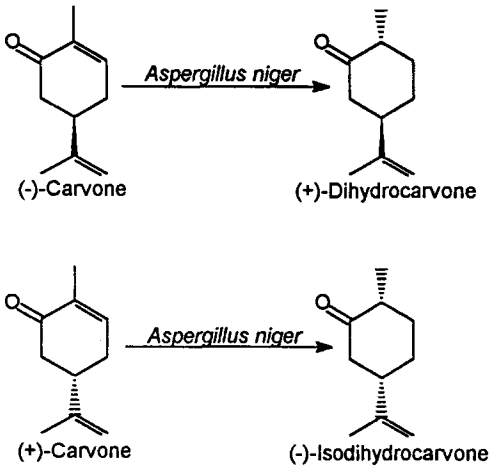

Fig. 2. Biotransformation of (−)-carvone and (+)-carvone by *Aspergillus niger* [66]

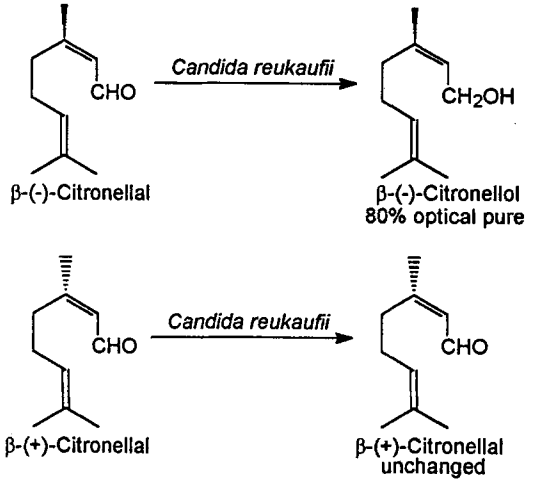

Fig. 3. Conversion of (±)-citronellal by *Candida reukaufii* [7,41]

3.3 Production of Novel (Natural) Flavours

The flavour and fragrance industry has an ongoing interest in the identification and description of new aroma chemicals that exhibit novel organoleptic characteristics [70]. Biotechnology has the potential to produce novel natural compounds using a combination of different enzymes. Biotechnology can also be

used to produce compounds which are not produced in appreciable amounts in nature.

3.4 Mild Reaction Conditions

The recent campaign for safer technology demands the use of milder reaction conditions. In this respect, the use of biocatalysis is especially suited. Further advantages of mild reaction conditions are the low energy costs incurred and the minimization of chemical side reactions.

3.5 Combination of Multiple Reactions in One Reaction Step

Microorganisms are biocatalysts which can catalyze the biosynthesis or degradation of compounds through multiple enzyme steps. The conversion of basic feedstocks to products of commercial interest may require more than one type of reaction. Whole cell biocatalysts, in contrast to conventional chemical synthesis, could potentially catalyze these transformations in a one step procedure. This phenomenon may be even more fully exploited by expressing recombinant enzymes in strains which are not able to degrade the product of interest (see Sect. 6), using recombinant DNA techniques.

3.6 Broad Substrate Specificity

Many enzymes of interest for the bioconversion of monoterpenes, especially oxido-reductases and hydrolases (see also Sect. 4), have broad substrate specificities. In this way, one biocatalyst can be used for the production of several related commercially interesting compounds, thus reducing research and production costs.

4 Microbial Transformation Reactions Involved in the Bioconversion of Monoterpenes

Information regarding the enzymes involved in microbial monoterpene biodegradation is rather scarce. The enzymes which have been studied most extensively are those involved in the (+)- and (−)-camphor degradation pathway of *Pseudomonas putida* ATCC 17453 [11, 71]. However, recently several other enzymes involved in monoterpene degradation, e.g. linalool monooxygenase, α-terpineol monooxygenase and α-terpineol dehydratase, have also been purified

and characterized [72–75]. This section gives an overview of the enzymes involved in monoterpene transformations described so far.

4.1 Oxido-Reductases

From a commercial viewpoint, the most interesting biotransformations are catalyzed by oxido-reductases. The major disadvantage of the application of this type of enzymes is their requirements for expensive cofactors and, in the case of monooxygenases, their instability. If instability is not a problem, the key for a commercial application of these enzymes will be to find ways for an efficient cofactor regeneration.

The reactions catalyzed by oxido-reductases affecting the transformation of monoterpenes can be divided into five groups of enzymes, discussed in Sects. 4.1.1–4.1.5.

4.1.1 Monooxygenases Catalyzing Hydroxylation

Terpenoids have, in general, more characteristic flavour properties than their terpene hydrocarbon counterparts. Therefore, the hydroxylation of cheap and readily available terpene hydrocarbons such as (−)-α-pinene and (+)-limonene is one of the target reactions for biotransformation studies. Monooxygenases catalyze the introduction of one atom of dioxygen into the monoterpene substrate [76]:

$$SH + O_2 + NAD(P)H + H^+ \rightarrow SOH + H_2O + NAD(P)^+. \quad (1)$$

Although sometimes used synonymously, the term 'hydroxylase' should strictly be limited to enzymes in which the inserted oxygen derives from water/hydroxyl. These enzymes may not be as complex as monooxygenases. In many of the bioconversion studies reported so far, the terpene substrate was hydroxylated, e.g. [5, 40, 49, 77, 78]. Unfortunately, however, monooxygenases are not very stable enzymes. In attempting to detect the monooxygenase activity involved in monoterpene oxidation, in many cases no [12, 19, 42, 56], or very low [79] activities were detected in cell extracts. Due to these low monooxygenase activities, a NAD(P)H generating system was added in many cases to prevent limitation of activity due to a high NAD(P)H oxidase activity present in the cell extracts [46, 47, 80]. In some of the cases where monooxygenase activity could be measured, this activity was reported to be present in the 100 000-g pellet fraction of cell extracts, indicating that the enzyme was membrane bound [47, 80, 81]. In contrast, several soluble terpene monooxygenases have also been reported [72, 73, 82, 83]. Some of these cell extracts were also described as having a distinct reddish brown colour, indicating the presence of cytochrome P-450 dependent enzyme system [73, 80]. The fact that cytochrome P-450 was

present in these extracts could also be concluded from the carbon monoxide difference spectrum [79, 80].

So far, three terpene monooxygenases catalyzing hydroxylation reactions have been purified and characterized; camphor monooxygenase from *Pseudomonas putida* catalyzing the conversion of (+)-camphor into (+)-5-*exo*-hydroxycamphor [84] and, to a lesser extent, linalool monooxygenase from *Pseudomonas putida* var. *incognita* catalyzing the conversion of linalool into 8-hydroxylinalool [72, 85, 86], and α-terpineol monooxygenase from a *Pseudomonas* sp. catalyzing the conversion of α-terpineol into 7-hydroxy-α-terpineol [62, 73]. In contrast to most P-450 enzymes reported so far, camphor monooxygenase, linalool monooxygenase and α-terpineol monooxygenase are soluble, making it easier to purify them [72, 73, 82]. All of these enzymes were found to consist of three components; an FAD-flavoprotein reductase catalyzing the electron release from NADH, an iron-sulphur protein acting as an electron carrier between the flavoprotein and the cytochrome P-450 component, and a cytochrome P-450 containing component which catalyzes the oxidation of the substrate. Also the P-450 component of cymene monooxygenase, $P\text{-}450_{cym}$, from *Pseudomonas aeruginosa* has been purified [85]. The crystal structure of both $P\text{-}450_{cam}$ and $P\text{-}450_{terp}$ has been determined to a resolution of 1.63 and 2.3 Å, respectively [87, 88].

The substrate specificity, stereospecificity/selectivity and regiospecificity have recently been studied extensively with $P\text{-}450_{cam}$, which is the best characterized P-450 enzyme so far. Besides the *exo*-hydroxylation of the natural substrate, (+)-camphor at the C_5-position (Fig. 4), this enzyme is also able to hydroxylate (−)-camphor at 70% of the rate of the "natural" isomer, resulting in hydroxy compounds of opposite stereochemical orientation [71]. This shows that, although the enzyme is stereospecific, it is not stereoselective. This poor stereoselectivity is probably related to the fact that the chiral carbon atom in the substrate is two carbon atoms removed from the site of hydroxylation. The hydroxylation of (−)-camphor also results in the formation of (−)-2,5-diketocamphane, probably due to the hydroxylation of the (−)-5-*exo*-hydroxycamphor formed, resulting in the formation of a carbon atom with two hydroxylgroups [93] (Fig. 4). This compound is chemically unstable and rearranges to the ketone with release of water. Besides the hydroxylation of camphor, $P\text{-}450_{cam}$ also catalyzes the hydroxylation of (in some instances both enantiomers of [84, 93]) several structural analogues of (+)-camphor [71, 89, 94–98], as well as the epoxidation of 5,6-dehydrocamphor [99] and 5-methylencamphor [100]. Also, several molecules which bear no resemblance to (+)-camphor were found to be substrates for $P\text{-}450_{cam}$ (Fig. 4); this enzyme also catalyzes the hydroxylation of several other compounds such as (*R*)- and (*S*)-nicotine [101] and ethylbenzene [102], but it also catalyzes other types of reactions such as the reduction of 2-methyl-1-tetralone trimethylsilyl enol ether [103], the sulfoxidation of e.g. thioanisole [91], both the oxidative and reductive dehalogenation of several low molecular weight chloroalkanes [92, 104], and the epoxidation of several styrenes [90, 105]. The transformation of some of

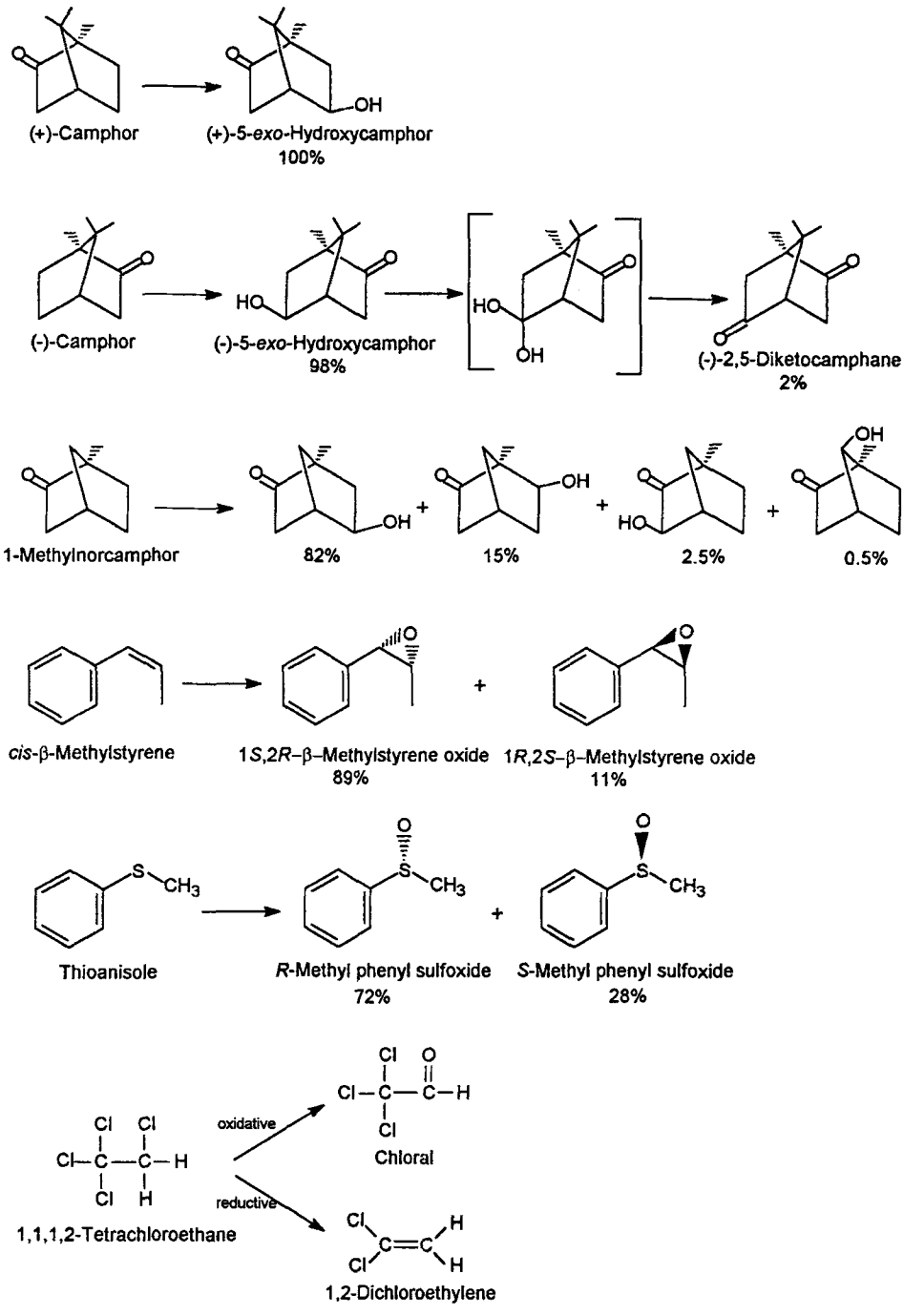

Fig. 4. Reactions catalyzed by purified camphor monooxygenase from *Pseudomonas putida* ATCC 17453 [68, 71, 89–93]

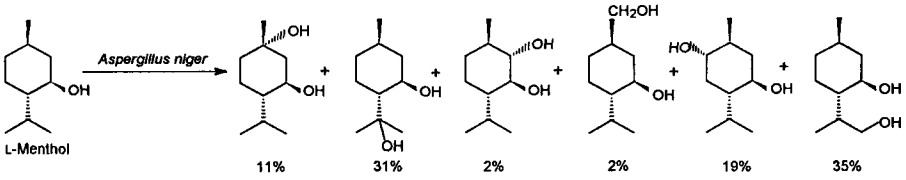

Fig. 5. Hydroxylation of L-menthol by *Aspergillus niger* [77]

these "unnatural" substrates also resulted in the uncoupling of NADH consumption from hydroxylation of the substrate, resulting in the production of hydrogen peroxide [106].

Camphor monooxygenase shows a strict regiospecificity when it catalyzes the oxidation of the natural substrate, (+)-camphor. However, when the enzyme catalyzes the hydroxylation of several of the camphor-resembling substrates, the regiospecificity was found to be much more relaxed. For instance, with 1-methyl-norcamphor, P-450$_{cam}$ catalyzes the hydroxylation of four different carbon atoms [89] (Fig. 4). The results suggest that the larger the discrepancy between the substrate and the "natural" substrate, the more hydroxylated products are formed [89, 93, 97]. This might also explain why, in some of the whole cell biotransformation studies and especially in the cases of co-metabolism, many different hydroxylated compounds are formed, e.g. [5, 77] (Fig. 5). When the transformation of "unnatural" substrates is catalyzed, the stereospecificity of the reaction is also much lower [90, 91, 105, 107]. For instance, the epoxidation of *trans*-β-methylstyrene resulted in the formation of a mixture of the stereoisomers of β-methylstyrene oxide [105] (Fig. 4).

Linalool monooxygenase and P-450$_{cym}$ are also able to catalyze the hydroxylation of a wide range of substrate analogues [85, 86]. Linalool monooxygenase was also able to produce 8-oxo-linalool from linalool by two sequential oxidations [72]. On the other hand, α-terpineol monooxygenase was also able to catalyze the sulfoxidation of several substituted thioanisoles and the epoxidation of several styrenes [62].

Because of the broad substrate specificity of the reported P-450 monooxygenases, these enzymes have been applied to the regio- and stereospecific production of chiral synthons [108], and a whole cells containing these enzymes have been applied in waste water [109] and waste gas [110] treatment.

4.1.2 Monooxygenases Catalyzing Epoxide Formation

Several monooxygenases catalyze the formation of epoxides from alkenes:

$$-CH{=}CH- + O_2 + NAD(P)H + H^+ \rightarrow \text{epoxide} + H_2O + NAD(P)^+. \quad (2)$$

The formation of monoterpene epoxides has so far been reported only in a few instances [46, 53, 111]. However, epoxides are thought to be intermediates in the production of monoterpene diols [53].

So far the purification of only one monooxygenase catalyzing the formation of a monoterpene epoxide has been described [112]. α-Pinene monooxygenase from *Pseudomonas fluorescens* NCIMB11671 catalyzes the NADH, FAD and Fe^{2+} dependent oxygenation of α-pinene to α-pinene oxide. The enzyme was comprised from two identical subunits and contains 2 FAD/mol enzyme. The enzyme was also able to catalyze the epoxidation of the 1,2-double bond of limonene [113].

4.1.3 Alcohol Dehydrogenases

Alcohol dehydrogenases catalyze the interconversion of alcohols to aldehydes/ketones:

$$\text{Alcohol} + X \rightleftharpoons \text{aldehyde/ketone} + XH_2. \quad (3)$$

This reaction is highly reversible and the direction of the reaction can be influenced by the pH of the reaction medium; at pH 9 the equilibrium of this reaction is towards aldehyde formation while at pH 7 the equilibrium lies in the direction of alcohol formation. This type of enzyme can be very effective for the conversion of the readily available aldehydes citronellal and citral for the production of e.g. (−)-citronellol and geraniol, compounds with a much higher flavour impact than the corresponding aldehydes. The stereospecific/stereoselective reduction of aldehydes or ketones by whole cells has been studied by several authors, e.g. [10, 41, 69, 114] (Fig. 3).

Three alcohol dehydrogenases involved in the metabolism of (+)- and (−)-camphor in *Rhodococcus rhodochrous* T1 and *Pseudomonas putida* C1 have been purified [71, 115, 116]. 6-*endo*-Hydroxycamphor alcohol dehydrogenase from *R. rhodochrous* T1 was purified to homogeneity and crystallized [71, 115, 116]. This secondary alcohol dehydrogenase requires a divalent metal ion for full enzymatic activity. The enzyme has a broad substrate specificity; it catalyzes the oxidation of six of the eight possible hydroxy camphors and it also catalyzes the oxidation of unsubstituted and 2- and 4-methyl substituted cyclohexanols [115, 116]. This enzyme catalyzes the oxidation of the enantiomeric alcohol intermediates produced in the degradation of both (+)-camphor and (−)-camphor. A 5-*exo*- and a 5-*endo*-hydroxy camphor dehydrogenase have been purified from *P. putida* C1 [71, 116]. 5-*exo*-Hydroxy camphor dehydrogenase is composed from two identical subunits of 38 kDa [117]. Both of these dehydrogenases were found to have an absolute stereoselectivity at the C_5-position; the 5-*exo*-dehydrogenase did not catalyze the oxidation of 5-*endo*-hydroxy camphor, while the 5-*endo*-dehydrogenase did not catalyze the oxidation of 5-*exo*-hydroxy camphor [116]. Several other compounds containing a hydroxy group on the bornane ring could also act as substrates for either dehydrogenase [71].

Two other terpene alcohol dehydrogenases have been partially purified; perillyl alcohol dehydrogenase from *P. putida* PL [118] and linalool-8-alcohol dehydrogenase from *P. putida* subsp. *incognita* [80]. Both of these enzymes were also found to have broad substrate specificities.

An example where an alcohol dehydrogenase catalyzes a reaction can be of great commercial potential, is in the reduction of menthone to L-menthol [119]. Natural L-menthol is isolated from the essential oil of the peppermint plant, *Mentha piperita.* Peppermint plants contain the highest level of volatile oils just before flower initiation [120]. At this time, 95% of the volatile oil consists of L-menthone. Unfortunately, upon further growth only 40% of the L-menthone is converted into L-menthol , while the remaining L-menthone is converted into D-neomethyl-β-D-glucoside [120]. Therefore, a separate process reducing L-menthone into L-menthol might be commercially viable. Although several microorganisms have been reported to catalyze the interconversion of L-menthol into L-menthone [121, 122], no L-menthol dehydrogenase activity could be detected in cell extracts [45, 122]. Recently, Kise and Hayashida [123] reported the continuous production of L-menthol from L-menthone by immobilized 3-α-hydroxysteroid dehydrogenase. This NADH dependent enzyme was purified from *Cellulomonas turubata* and found to be very stable. It catalyzes the reduction of L-menthone at 0.3% of the rate of the reduction of the natural substrate 5-α-androstan-17-β-ol-3-one [124]. During the continuous process, NADH was recycled by the oxidation of methyl isobutyl carbinol catalyzed by the same enzyme [123]. In this way, 65 g/l L-menthol could be produced. The half-life of this continuous system was found to be 25 days.

4.1.4 Reductases

The hydrogenation of unsaturated double bonds catalyzed by reductases is another potentially interesting reaction for the production of monoterpenes with important organoleptic properties:

$$-CH=CH- + XH_2 \leftrightharpoons -CH_2-CH_2- + X. \quad (4)$$

Up to now, the reduction of monoterpenes has not been studied very extensively [65, 66, 125–127]. *Aspergillus niger* and *Pseudomonas ovalis* were able to reduce stereospecifically two enantiomers of carvone and carvotanacetone, respectively [65, 66] (Fig. 2). However, in none of the cases where reduction has been reported has the enzyme involved been characterized.

4.1.5 Lactonases; Baeyer-Villiger Monooxygenases

In the degradation of monocyclic and some bicyclic monoterpenes, the ring opening is initiated by a monooxygenase which catalyzes a biological

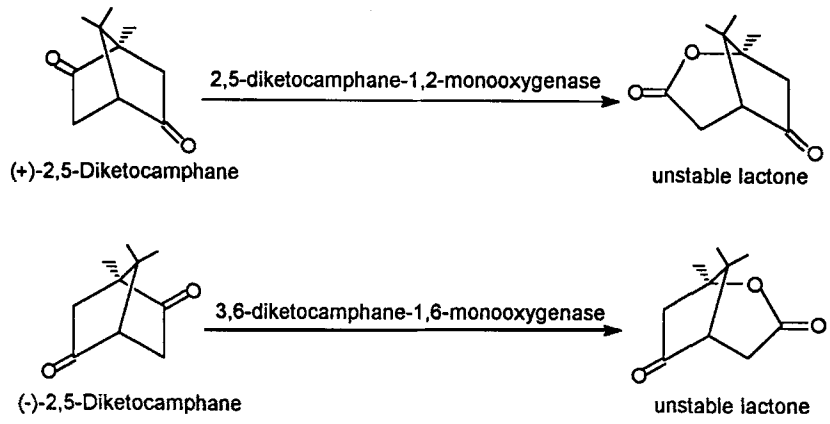

Fig. 6. Reactions catalyzed by 2,5-diketocamphane-1,2-monooxygenase and 3,6-diketocamphane-1,6-monooxygenase of *Pseudomonas putida* ATCC 17453 [128, 129]

Baeyer-Villiger reaction, inserting an oxygen atom in the ring next to a keto-group to form a lactone [11] (Fig. 6):

$$\text{-ketone} + O_2 + \text{NAD(P)H} + H^+ \rightarrow \text{lactone} + \text{NAD(P)}^+ + H_2O. \quad (5)$$

Lactones are potent, generally pleasant smelling compounds widely used by the flavouring industry [Review by Gatfield in this volume 2, 14, 130–132]. At present, in the few commercial processes in which lactones are formed bio-catalytically, they are formed by internal ester formation using a hydroxy fatty acid as the substrate [130]. This is the reverse of the reaction which occurs in cyclic monoterpene catabolism. Some of these lactones produced during the degradation of monoterpenes are chemically unstable and spontaneously ring open, e.g. [42, 71].

Baeyer-Villiger monooxygenases are flavoproteins and are generally very stable enzymes [133, 12]. Most of the Baeyer-Villiger monooxygenases reported so far require NADPH as the cofactor [133]. To date, three Baeyer-Villiger monooxygenases involved in monoterpene degradation have been purified; 2,5-diketocamphane-1,2-monooxygenase [128], 3,6-diketocamphane-1,6-mono-oxygenase [129], and 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase [134]. These three enzymes are involved in the degradation of (+)- and (−)-camphor in *Pseudomonas putida* C1. 2,5-Diketocamphane-1,2-mono-oxygenase and 3,6-diketocamphane-1,6-monooxygenase both consist of two enzyme components [128, 129]. One component is an NADH dehydrogenase, which transfers the electrons from NADH to FMN [129, 135]. The second component, the oxygenase component, is composed of two identical subunits which together firmly bind one FMN, which transfers the electrons from FMN to O_2, thus catalyzing the oxygenation of the substrate [128]. The common dehydrogenase component acts to transfer electrons to the oxygenase component

of 2,5-diketocamphane-1,2-monooxygenase and to that of 3,6-diketocamphane-1,6-monooxygenase [129]. These two Baeyer-Villiger monooxygenases showed an absolute stereoselectivity; 2,5-diketocamphane-1,2-monooxygenase catalyzes only the lactonization of (+)-2,5-diketocamphane, while 3,6-diketocamphane-1,6-monooxygenase only catalyzes the lactonization of (−)-2,5-diketocamphane [129, 136]. 2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase is composed of two identical subunits which together bind one FAD molecule [134]. The enzyme is NADPH dependent and catalyzes only the lactonization of a substrate containing a CoA ester.

L-Menthone-3,4-monooxygenase and 6-oxo-1,8-cineole oxygenase have been partially purified from a *Corynebacterium* sp. and *Rhodococcus* sp., respectively [42, 45]. Both enzymes required NADPH as the cofactor, and were not very stable.

The instability of some of these Baeyer-Villiger monooxygenase and the requirement for the use of CoA activated substrates will limit their use as biocatalysts. However, 2,5-diketocamphane-1,2-monooxygenase is a rather stable enzyme with a broad substrate specificity [11, 42, 137]. Recently, this enzyme has been applied in organic synthesis for the stereo- and regioselective lactonization of *endo*-bicyclo(3.2.0)hept-2-en-6-ol [138].

4.2 Hydrolases

Hydrolases (lipases, esterases) have found wide-ranging applications in biocatalysis [139]. These enzymes show different degrees of stereoselectivity, and as these enzymes catalyze equilibrium reactions, different approaches using these biocatalysts can be used for the production of (optically active) monoterpenes [140–141] Eqs. 6–10.

a. *Hydrolysis*:

(*R*, *S*)-monoterpene ester + water →

(*R*)-monoterpene + (*S*)-monoterpene ester + acid. (6)

b. *Interesterification*:

(*R*, *S*)-monoterpene ester + alcohol →

(*R*)-monoterpene + (*S*)-monoterpene ester + ester, (7)

(*R*, *S*)-monoterpene + ester →

(*R*)-monoterpene + (*S*)-monoterpene ester + alcohol. (8)

c. *Esterification*:

(*R*, *S*)-monoterpene + acid →

(*R*)-monoterpene + (*S*)-monoterpene ester + H_2O, (9)

(*R*, *S*)-monoterpene + anhydride →

(*R*)-monoterpene + (*S*)-monoterpene ester + acid. (10)

In water, the equilibrium of the reactions catalyzed by hydrolases lies in the direction of hydrolysis, thus reducing the yield of ester formed [61, 142, 143]. Fortunately, many of these enzymes have been found to be active in (water supplemented) organic solvents, thereby directing the equilibrium of the reaction towards ester formation. Another favourable aspect of the use of organic solvents as the reaction media is the fact that many monoterpenes are very poorly soluble in water (see Sect. 2.2).

Several factors affect the yield, the reaction rate, and the stereochemical configuration of the product obtained using hydrolases.

a. The enzymes used [144, 145].
b. The monoterpene substrate [144, 146].
b1. The ester group of the monoterpene substrate [140].
c. The co-substrate [142, 147].
d. The concentration of the substrate and the co-substrate [147].
d1. The substrate to co-substrate ratio [147].
e. The reaction catalyzed by the enzyme (see Eqs. 5–9). For example, while the conventional esterification reaction (Eq. 8), results in the production of water during the reaction, thus diminishing the yield, interesterification reactions [142] or the use of an anhydride as the co-substrate [140] does not suffer from this problem.
e1. The direction of the reaction, as the affinities/stereoselectivity of an enzyme for the substrate(s) and product(s) will differ.
f. The addition of organic solvents [148]. In some instances, the monoterpene substrate has been used both as substrate and as the solvent [149, 150].
f1. The type of co-solvent [147, 148, 151], especially optically active co-solvents [152].
f2. The water content of the organic solvent [61]. Water is necessary to maintain enzyme activity, but too much water negatively affects the equilibrium for esterification.
g. The temperature [147].

Several short-chain fatty acid esters of monoterpene alcohols, such as geranyl acetate and citronellyl acetate, are among the most widely used monoterpenes in the food, cosmetic and pharmaceutical industries as flavour and fragrance compounds [153]. The production of monoterpenyl esters by extraction from plant sources is often too expensive for commercial production [61]. Therefore, their biocatalytic production by hydrolases is an attractive alternative. The production of monoterpene esters has been investigated quite extensively, e.g. [142–144, 153, 154].

Hydrolases have been extensively studied for the production of L-menthol [140, 148], and several essential oil industries have filed patents covering biotransformation of menthyl esters by bacteria and fungi [155]. Racemic menthol

can be produced chemically by the reduction of thymol [156]. Hydrolases have been applied to separate L-menthol from the racemic mixture. Yamaguchi and coworkers were able to produce 44 g/l L-menthol in 24 h by asymmetric hydrolysis of a 30% (±)-menthylacetate mixture using a mutant of *Rhodotorula mucilaginosa* [157]. However, this process does not result in the production of natural L-menthol as the substrate is not natural, and at present "un"-natural L-menthol is produced commercially using an asymmetric synthesis approach [158].

Although in several instances the lipase/esterase catalyzed reactions were performed with commercially available purified enzyme preparations, e.g. [148, 159], so far only one hydrolase selected specifically for its potential to esterify monoterpenes has been purified [156]. Carboxylesterase from *Ochrobactrum anthropi* was found to consist of two identical subunits. The enzyme hydrolyzed the acetate and propanoate esters of a variety of organic compounds and only hydrolyzed L-menthyl acetate, but not its optical isomer [156].

Glycosidases have also been applied in the biotransformation of monoterpenes [160, 161]. Glycosidases can be used to improve essential oil yields, as many monoterpenes are present as odourless glycosides in plants.

4.3 Ring Closing and Ring Opening Reactions

Monoterpenes may be classed as acyclic, monocyclic and bicyclic. In several biotransformation studies ring closure of acyclic monoterpenes has been observed [162–164]. For instance, in a patent by Babička et al. [165] the cyclization of citronellal to pulegol and isopulegol by *Penicillium digitatum* was reported. Also the commercially interesting conversion of linalool into several linalool oxides by *Botrytis cinerea*, *Pseudomonas incognita* and *Diplodia gossypina* has been reported [41, 166–169]. On the other hand, the bicyclic α-pinene has been reported to convert into the monocyclic compounds limonene and sobrerol [17, 56, 170].

The only ring-opening enzyme purified so far is α-pinene oxide lyase. This cofactor independent enzyme catalyzes the conversion of α-pinene oxide into *cis*-2-methyl-5-isopropylhexa-2,5-dienal (isonovalal). This enzyme has been purified from a *Nocardia* sp. [171] and from *Pseudomonas putida* PX1 [13]. Both enzymes are composed of two subunits, but while the *Nocardia* enzyme is formed from two dissimilar subunits, the *P. putida* enzyme is formed from two electrophoretically identical subunits [13]. The enzyme of the *Nocardia* sp. is inactivated during catalysis and appears to have a narrow substrate specificity [171]. The commercial potential of the use of this enzyme in combination with α-pinene monooxygenase for the production of isonovalal has been exploited [172].

4.4 Hydration

Hydratases catalyze the addition of water to an unsaturated double bond:

$$-C{=}C- + H_2O \leftrightharpoons -CH-COH-. \quad (11)$$

The transformation of monoterpenes by hydratases could be of great commercial importance, as in this way the commercially more interesting terpenoids can be produced by a reaction which is independent of cofactors and oxygen. Hydratase catalyzed reactions occur with a high degree of stereospecificity and several hydratases have already been commercialized [173].

Only one biotransformation using a hydratase reaction has been studied in greater detail; the hydration of (+)-limonene to (+)-α-terpineol [41, 174, 175]. Conversely, the dehydration of α-terpineol to terpinolene has also been reported [176].

α-(+)-Terpineol dehydratase has been partially purified from *Pseudomonas gladioli* [74] and from *Pseudomonas alcaligenes* [75]. Unfortunately, this enzyme was in both instances membrane associated which has limited its further characterization.

In this respect the recently reported degradation of monoterpenes under denitrifying conditions is also of great importance [177]. Under anaerobic conditions, the only way to activate the substrate appears to be by hydration, and therefore interesting novel enzymatic activities might be detected in these microorganisms.

4.5 Allyl Rearrangement

The allyl rearrangement reaction can be described as follows:

$$R_1{-}C{=}C{-}CX{-}R_2 \leftrightharpoons R_1{-}CX{-}C{=}C{-}R_2. \quad (12)$$

Allyl rearrangement has been observed during several monoterpene biotransformation studies [5, 178, 179]. In all of these instances, the allyl rearrangement resulted in the formation of a tertiary alcohol (Fig. 7). In the case of biotransformation of piperitol by *Pseudomonas* NOF-5, both the primary alcohol and

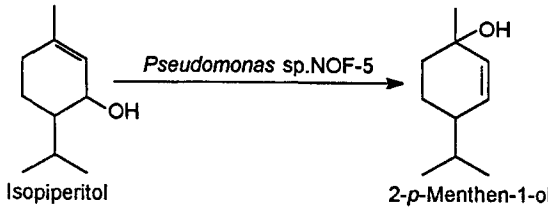

Fig. 7. Allyl rearrangement reaction catalyzed by *Pseudomonas* sp. NOF-5 [178]

the tertiary alcohol structure were isolated from the culture medium [178]. Unfortunately, the enzyme involved in this reaction has yet to be characterized.

4.6 Racemization

Racemases catalyze the racemisation of optically active compounds:

$$(+)\text{-monoterpene} \leftrightharpoons (-)\text{-monoterpene}. \quad (13)$$

In cases where the substrate contains more than one chiral centre, these enzymes are called epimerases.

Noma et al. [180] reported the epimerization of (−)-isodihydrocarvone to (−)-dihydrocarvone by *Pseudomonas fragi* IFO 3458. The enzyme was partially purified and was found to be very stable. The enzyme also catalyzed the epimerization of isomenthone to menthone.

5 Genetics of Monoterpene Degradation Pathways

Until now, only a limited number of genes coding for enzymes involved in the degradation of monoterpenes have been cloned and/or sequenced (Table 3). Some of the genes involved in monoterpene degradation have been found to be located on plasmids. The best known example is the CAM plasmid, encoding enzymes involved in camphor degradation. The CAM plasmid is a very large plasmid of $\approx$ 500 kb [185]. The plasmid contains two closely linked gene clusters, specifying the genes encoding enzymes of the early and middle pathway of camphor degradation [186]. The operon coding for the early camphor pathway, the monooxygenase operon, has been fully sequenced and characterized

Table 3. Genes coding for enzymes involved in monoterpene degradation which have been cloned and/or sequenced.

Enzyme	Genes cloned	Gene(s) sequenced	Reference
Camphor monooxygenase	*camA*, *camB*, *camC*	yes	181, 182
Linalool monooxygenase	*linC*	yes	183
α-Terpineol monooxygenase	*terpA*, *terpB*, *terpC*	yes	73
α-Pinene monooxygenase	*pinMO*	yes	112
5-*exo*-Hydroxycamphor dehydrogenase	*camD*	yes	184
(7-Hydroxy-α-terpineol) dehydrogenase	*terpD*	yes	73
(7-Oxo-α-terpineol) dehydrogenase	*terpE*	yes	73
Limonene degradative pathway	yes[a]	no	175

[a] It is not clear how many genes are present on the cloned DNA fragment, and which enzymes are encoded for (160)

[187, 188]. This operon (*camDCAB*) codes for the three components of camphor monooxygenase and for 5-*exo*-hydroxycamphor dehydrogenase. The operon is under the negative control of *camR* which is located immediately upstream of the *camD* gene, and shows maximal expression in the presence of (+)-camphor [188]. So far, no information has been reported on the second gene cluster on the CAM plasmid involved in (+)-camphor degradation.

The involvement of plasmid located genes coding for enzymes involved in the degradation of monoterpenes has been reported in two more instances; the degradation of citronellol and geraniol in *Pseudomonas putida* PPU2 [189] and the degradation of linalool in *Pseudomonas putida* var *incognita* [85]. In contrast, no plasmid could be detected in *Pseudomonas fluorescens* NCIMB 11671 which degrades α-pinene [190].

Also in a *Pseudomonas* sp. able to degrade α-terpineol, the genes were found to be clustered [73]. The operon (*terpDECAB*) coded for α-terpineol monooxygenase, and, on the basis of sequence homologies, an alcohol dehydrogenase and an aldehyde dehydrogenase were also identified in the operon. The enzymes involved in the limonene degradative pathway of *Bacillus stearothermophilus* appeared to be clustered on a 9.6 kb fragment [175]. However, neither the genes nor the enzymes coded for by this fragment have yet been characterized.

6 Future Outlook

The microbial transformation of terpenoid compounds has considerable potential for practical application in the flavour and fragrance industry as also illustrated by the patent literature, but the research area is generally perceived as difficult (see Sect. 2). Nevertheless, microbial transformation of various monoterpenes have challenging industrial possibilities. There are three areas in which the bio(techno) logical production of monoterpene can play an important role.

a. The formation of terpenoids from cheap and readily available monoterpenes such as (+)-limonene and (−)-α-pinene.
b. In the bioformation of terpenoids with a high flavour impact, which are not readily available from other biological or chemical sources.
c. The bioformation of novel (natural) flavours with unique organoleptic properties.

In the future, genetic engineering techniques may provide modified strains catalyzing a single pathway to the desired product resulting in the production of pure aroma chemicals. As many of the characterized enzymes involved in monoterpene degradation appear to have broad substrate specificities, these systems might also be used for the production of closely related terpenoids. Furthermore, by means of genetic engineering, the yields of the processes can be

improved by ad hoc control of the biosynthetic pathways and the exploitation of regulatory mechanisms.

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Special Transformation Processes Using Fungal Spores and Immobilized Cells

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Although many microbial processes have been described which are able to produce interesting aroma compounds, the number of industrial applications are limited. Reasons for this are in most cases low final product yield, low biotransformation rates, substrates and/or end-products inhibition, toxicity towards the microorganisms themselves and difficulties of recovery from the bioreaction mixture. This means that the development of specific catalysts and processes is an important challenge for researchers in this field. This review presents two special kinds of catalysts, fungal spores and immobilized cells, with emphasis on their production and on their use in the production of aroma compounds. The production of fungal spores by solid state fermentation is described in greater detail. In the second part, this review also offers examples of development of three production processes, the production of methyl ketones of spores of *Penicillium roquefortii*, the hydroxylation of β-ionone by immobilized *Aspergillus niger* cells, and the production of alkyl pyrazines by bacteria in liquid and solid media. For each of these processes, the analysis of limiting steps – biological and/or physico-chemical – is presented and the significant role of process conditions to increase aroma yield is discussed.

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List of Symbols and Abbreviations

a, b, c	constants	(dimensionless)
Bi	Biot number $= k_i L De_i^{-1}$	(dimensionless)
d_p	particle diameter	(m)
D_i	diffusion coefficient of compound i in the stagnant layer	$(m^2 s^{-1})$
De_i	effective diffusivity of i in the support liquid phase	$(m^2 s^{-1})$
De_{ic}	effective diffusivity of i in the support in the presence of cells	$(m^2 s^{-1})$
J_i	rate of transfer of compound i	$(mol\ m^{-2} s^{-1})$
k_i	mass transfer coefficient for the compound i	$(m s^{-1})$
L	characteristic length scale $\left(\frac{\text{volume of particle}}{\text{external area}}\right)$	(m)
M_T	Thiele modulus	(dimensionless)
M_w	Weisz modulus	(dimensionless)
r_{obs}	overall observed rate	$(mol\ m^{-3} s^{-1})$
Re	Reynolds number $= \rho u d_p \mu^{-1}$	(dimensionless)
S_{Li}	concentration of i in the bulk liquid	$(mol\ m^{-3})$
S_{Ri}	concentration of i at the surface of the gel	$(mol\ m^{-3})$
Sc	Schmidt number $= \mu \rho^{-1} D_i^{-1}$	(dimensionless)
Sh	Sherwood number $= k_i d_p D_i^{-1}$	(dimensionless)
u	relative fluid velocity	$(m s^{-1})$

Greek Letters

δ	thickness of diffusion boundary layer	(m)
η	effectiveness factor	(dimensionless)
μ	dynamic viscosity	$(kg\ m^{-1} s^{-1})$
ρ	density of the liquid	$(kg\ m^{-3})$
ϕ_c	cell volume fraction of support	(m^3 cells m^{-3} support)
ϕ_p	polymer volume fraction of support	(m^3 polymer m^{-3} support)

1 Introduction

Increase in the production of processed foods by industrial methods has resulted in an expanding market for food ingredients such as flavorants and flavor enhancers. In this area recent market surveys have shown that consumers prefer foodstuffs that can be labeled as natural. This preference for natural foods opens the door for bioprocessing because ingredients prepared using fermentation or enzymatic transformation are considered natural. Another factor encouraging development of biotechnological processes is the increased concern over depletion of natural raw materials. Such raw materials often contain only small amounts of the aromatic components useful in making flavorants or flavor enhancers; they are often in limited supply due to seasonal variations, climatic factors or political problems, their prices may become high and their availability and quality become variable.

However, food ingredients produced on an industrial scale by fermentation routes or microbial transformation are still very few. With the exception of glutamic acid and monosodium glutamate (annual global demand of approximately 700 000 metric tons), citric acid (400 000 metric tons), and gluconic acid (50 000 metric tons) as examples of non-volatile flavor production, many are produced in small quantities or even at the laboratory scale. In the flavor industry, as reported by Manley [1], of more than 1500 synthetic chemicals regularly used and considered "generally recognized as safe" for use in foods and beverages, only a small fraction is correctly produced commercially by biological routes.

There are many reasons for this situation; food-related industries use transformation operations characterized by low profit margin. Because of this low profit margin, there are limits to acceptable research and development costs for biological processes. However, there are opportunities for production of value-added materials such as flavors and aromas, provided methods are developed to enhance

- the production or transformation rates which are generally slow
- the yield from readily available substrates
- the final product concentration in microbial cultures, which are often low, in order to decrease recovery costs.

This requires one to identify the limiting steps in the process, whether these steps are of biological or physico-chemical nature. Among biological limiting steps are the cell physiology, nutrients concentration (carbon, nitrogen, phosphate and trace elements), metabolic precursors concentrations, energy source, inhibition by substrates and/or products, and remetabolization of products. Physico-chemical limitations can be as diverse as temperature, pH, water activity or osmotic pressure, diffusional transport, gas–liquid or liquid–gas transfer of substrates and metabolites (oxygen, carbon dioxide, synthesized

products etc.), interfacial interactions, gas–liquid and liquid–liquid equilibrium properties, mixing etc.

Notwithstanding these difficulties, a number of flavor compounds are already produced on an industrial scale. Every important flavor company claims to use microorganisms regularly for the production of aroma compounds, yet only a few of them mention the names of the products. This secrecy is typical for a sector with high competition where research and development of new products is extremely important [2].

This paper is divided into two parts. The first part offers a coverage of two "special" biocatalysts that can be used in bioprocesses, i.e., fungal spores and immobilized cells. The second part gives examples of integrated processes for aroma compounds production using these biocatalysts. Emphasis is laid on the analysis of limitating steps – biological or physical – and on the solutions adopted to circumvent them, leading to modifications of existing processes or implementation of new processes.

2 Special Biocatalysts

2.1 Fungal Spores

2.1.1 Generalities about Fungi

The fungi are eukaryotes, most probably derived from colorless representatives of unicellular algae [3]. They are heterotrophic since they are unable to grow in the absence of organic substances and do not possess photosynthetic pigments. They live as saprophytes and parasites and most of them can be grown on artificial culture media [4].

Fungi belong to a group of the most widely distributed living forms, and can be found nearly everywhere all over the world. All biotechnologically important fungi belong to the subdivision Eumycota of the division Mycota [4]. Eumycota may be divided into three classes, Phycomycetes (lower fungi), Ascomycetes and Basidiomycetes (higher fungi).

Numerous taxonomists also consider a special class, namely the Deuteromycetes or Fungi Imperfecti [5, 6]. It is an artificial division containing genera whose sexual reproduction has never been observed, and sometimes called "conidial fungi" [7]. Deuteromycetes are generally considered as anamorphs of Ascomycetes, and more rarely of Basidiomycetes, whose sexual states or teleomorphs have become permanently separate or temporarily disconnected [8]. Most fungi of industrial interest (*Penicillium*, *Aspergillus*, *Cephalosporium* etc.) belong to this class [9].

Fungal propagation may occur through asexual and sexual processes, which may take place together, except for the case of Deuteromycetes. Asexual

propagation is performed either by vegetative multiplication of asexual reproduction. The former involves no great cell modifications and leads to the formation of oidiospores (or arthrospores), chlamydospores (or gemmae), or sclerotia [4]. The latter leads to auxiliary fruiting forms supporting either planospores or aplanospores. Planospores are also called zoospores and correspond to mobile spores arising in planosporangia (zoosporangia) in aquatic fungi and some parasites. Aplanospores are produced by terrestrial fungi and may be endogenous (enclosed in sporangia) or exogenous. In the latter case, the spores are called conidiospores (conidia) and are usually formed on typical conidiophores [4]. These two processes involve no exchange of genetic material.

Sexual propagation occurs through the so-called main fruiting forms [4]. In the lower fungi a resting organ (e.g., a spore) arises from the zygote which usually germinates after meiosis to form a sporangium. In the higher fungi, formation of this resting organ is rare; the nucleus of the zygote divides meiotically, and typical meiosporangia arise. The ascus is the characteristic tubular or sac-like meiosporangium of Ascomycetes, which usually contain eight meiospores. The basidium is the club-shaped meiosporangium of Basidiomycetes, on which generally four spores are exogeneously generated [3].

In the following, only conidiospores are considered, and the terms "conidium" and "spore" correspond to these exogenous aplanospores obtained by asexual reproduction.

2.1.2 Characteristics of Fungal Sporulation

The most classical way for cultivation of filamentous fungi is, just like for bacteria or yeasts, the submerged technique. In these conditions, the microorganism grows as pellets or in a filamentous form [10]. When the vessel is stirred, hyphal elements are exposed to shear forces which may result in their break up. Moreover mycelial biomass exhibits a lack of uniformity, with young apical regions of hyphae and older, possibly less active, distal regions. These phenomena imply that the growth form of filamentous organisms in submerged cultures ranges continuously from dispersed mycelia to compact pellets [11]. This methodology has proven to be efficient for metabolite synthesis, such as penicillin [12], citric acid [13], or gluconic acid [14].

However, only a few fungi are able to exhibit asexual sporulation in these conditions [15]. Chemostat experiments indicate that the C/N ratio is a major determining factor for sporulation, and that this differentiation process occurs mainly during nitrogen limitation. Also, conidation is primarily observed at low specific growth rates [16, 17]. These features lead one to consider that submerged culture techniques are not well suited for fungal spore production.

It is therefore generally recognized that surface cultivation of fungi is the best methodology for spore production. This can be done by inoculating the surface of a liquid medium and allowing the growth to take place without stirring or agitating the flask or vessel. These conditions give a mycelial mat at the liquid

surface which may sporulate. A refinement of this system could be the use of a membrane separating the microogranism from the medium, as recently suggested for enzyme production [18, 19].

A second approach of surface cultivation is the use of solid substrates. It is now fully established that this represents the best way to achieve fungal sporulation [20]. The most common way involves the use the solidified media placed in Petri dishes or Roux bottles. In this case, mycelial growth leads to the formation of an organized colony with a peripheral growth zone of exponentially growing mycelium surrounding a central differentiated region [21–23]. This differentiated region supports reproductive structures.

Although numerous authors have paid attention to the modeling of fungal growth on solid media [24–29], few attempts have been made to describe growth and spore formation in the form of a mathematical model. One of the first attempts in this area deals with *Oidium begoniae* [30], but the authors stated that their model worked only with this fungus. The most complete approach in this area is the one presented by Georgiou and Shuler [31]. They consider the colony to consist of vegetative biomass, competent biomass (capable of differentiation [32]), conidiophore biomass, and conidial biomass. Competent biomass is able to differentiate usually if carbon and nitrogen sources concentrations meet some requirements. Hence, a high nitrogen concentration gives inhibition of conidiophore maturation while high glucose contents favor vegetative growth. On the other hand, if the two nutrients are limited, autolysis may occur even before conidiophore maturation. The model also considers that hyphae do not penetrate into the solified medium; diffusion of nutrients from the medium to the fungus is also taken into account, giving a spatial distribution of substrates within the medium. The results of their simulation studies suggest that mass transfer limitations are at least partially responsible for the proliferation of differentiated structures on solid substrates as compared to liquid cultures. They also show that the concentration profile depends on the depth of the substrate, and suggest that enhanced conidia production could be achieved by controlling the depth of the solid medium. This analysis shows that the main environmental factor affecting fungal conidiation in solid state cultivation is the medium heterogeneity. Young hyphae develop on rich substrate, giving vegetative proliferation. Older parts of hyphae are in contact with lower nutrient concentrations, which suppresses sporulation. This substrate heterogeneity is not encountered during submerged, stirred cultivations. These considerations explain why solid state cultivation is a method of choice for the production of fungal spores.

Surface cultivation of fungi is an excellent tool to investigate the behavior of a given micoorganism on a solid medium. Although it is well suited for conidation, it suffers from a lack of available surface for fungal extension, i.e., the ratio available area to volume is low. As a result, this methodology is not very efficient in view of spore production on a larger scale because it needs the use of huge numbers of containers (Petri dishes or Roux bottles, see above) which require tedious and time-consuming manipulations. However, it should be

noted that some industrial processes, mainly for cheesemaking, do involve gelosed media operated in Roux bottles. A refinement of this system, in which the medium is deposited on dishes stacked in a fermentor, has also been proposed [33].

Nevertheless, the best way to improve the ratio area to volume is the use of particulate materials. This system is most commonly used for carrying out solid state cultivations.

2.1.3 Spore Production by Solid State Cultivation

2.1.3.1 Principle and Origins of Solid State Cultivation

Solid state fermentations, just like cultivation of immobilized cells in a liquid medium (Sect. 2.2) are systems which offer anchorage points for the microoganism. In all cases, cultivations involve heterogeneous media that may be classified with regard to the water distribution. Solid state cultivations (SSC or SSF) represent processes in which the water (or liquid) is fully retained by solid particles (Fig. 1).

Two kinds of systems exist; the first and most commonly used involves cultivation on an assimilable, polymeric matrix. The nature of the polymer may be starch, cellulose, or even lignin, depending on the enzymatic characteristics of the cultivated microogranism [34–39]. This approach derives from the well-known Koji process, which involves growth of a fungus, most often *Aspergillus oryzae*, on soya beans or rice for the production of a mixture of proteolytic and amylolytic enzymes that are used for the processing of other oriental fermented foods [40–42].

The second involves microbial development on an inert porous support impregnated with a liquid nutritive medium. The support consists of particles that offer anchorage points for the microorganism; materials proposed in this area are polyurethane foam, sugarcane bagasse, wheat bran, pozzolano or vermiculite (heat treated mica) [43–47].

It should be noted that some authors use the same acronym (SSC or SSF) with the meaning solid *substrate* cultivations (or fermentations). This terminology involves, for example, processes during which a microogranism is grown on straw particles suspended in water [20]. This case will not be treated in the following, and SSC or SSF will mean solid *state* cultivation or fermentation, respectively.

SSF is in fact a very old technology, since it has been used by man for many centuries, long before he even understood the underlying microbiological or biochemical processes involved [48]. One of the earliest records of SSF is the cultivation of paddy straw mushroom, *Volvariella volvacea*, in the Canton region of China's Kwangtung province during the Chow dynasty, about 3000 years ago [49]. Another very old application is breadmaking, and it is now considered that the Egyptians started making bread by the year 2600 BC, using methods essentially similar to those of the present time [38]. SSF has also been

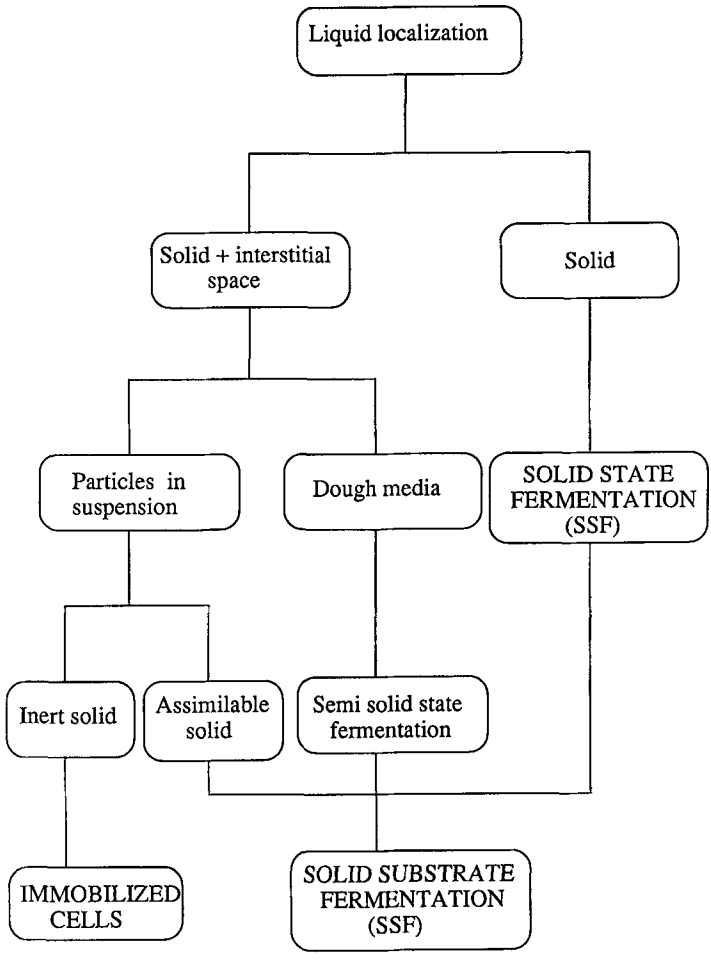

Fig. 1. Diagram of cultivation techniques involving heterogeneous media, from [273]

used extensively from ancient times in the Oriental, Asian and African countries; some of the first information on fermented oriental foods such as soy sauce Koji in China date back to 1000 BC [34, 35, 50]. Again, the records for the production of Roquefort cheese (a blue veined cheese) from sheep's milk in caves of southern France go back about a thousand years.

SSF has developed in the Western world after the Koji process was introduced in 1891 by Takamine. This process deals with the cultivation in trays of a fungus, generally *Aspergillus oryzae*, on rice or other cereal grains. It gives an enzymatic mixture which is also used, for example, for production of sake, the most traditional alcoholic drink in Japan. However, this methodology has been neglected from 1940 while efforts were directed towards the development of

submerged techniques for antibiotics production. Present knowledge about SSF allows one to consider that the arbitrary decisions taken at this time were not always appropriate [48, 51].

Nowadays, SSF is practised through three main traditional processes, i.e., fermented oriental foods, mould-ripened cheeses, and composting. Further developments include protein enrichment of agroindustrial residues, and metabolite and spore production.

Cultivations are carried out using two basic conceptions, i.e., static (Table 1) and agitated (Table 2) reactors. The critical parameters that are to be controlled are mainly substrate temperature and humidity [20, 38, 39, 41, 48, 49, 52–54]; fully controlled designs for SSF now exist.

2.1.3.2 Use of Solid State Cultivation for Metablites Synthesis

Exploitation of the SSF technique is mainly confined to processes involving fungi, because it is generally believed that they are not suitable for bacterial cultivation, mainly because of the material requirement for higher water activity. However, successful bacterial growth in these conditions is known in natural fermentations, for example composting or some food fermentations, and several

Table 1. Examples of solid state cultivation processes using static systems

Kind of fermentor	Process	References
Trays	Koji	20
	Single Cell Protein (SCP) production with *Sporotichum pulverulentum*	55
	Cellulase production by *Trichoderma reesei* and *Sporotrichum cellulophilum*	56
	Pectinolytic enzymes by *Aspergillus carbonarius*	57, 58
	Pigments by *Monascus purpureus*	59
	Protease by *Bacillus amyloliquefaciens*	60
	Rennet by *Mucor miehei*	61
	β-Glucosidase by *Aspergillus phoenicis*	62
	Composting	63, 64
Fixed bed columns	SCP with *Rhizopus oligosporus* and *Trichoderma viride*	65, 66
	Spores of *Penicillium roquefortii*	67
	Amyloglucosidase by *Aspergillus niger*	68
Other systems	Mason jars. Cellulolytic enzymes by *Trichoderma reesei* and *Aspergillus niger*	69
	Oven	70
	Rectangle vessel + heat exchange plates. Cellulase production by *Trichoderma harzianum*	71

Table 2. Examples of solid state cultivation processes using agitated systems

Kind of fermentor	Process	References
Rotating drum	Horizontal drum with 4 compartments, Ochratoxin A production	72
	Horizontal drum with blades, Koji production	38
	Cylindrical rotating fermentor (concrete-mixer type) many applications	36, 37, 73–78
Mechanical agitation	Kneading-trough, SCP production	79
	Vessel with agitation parts several applications	80–83
Pneumatic agitation	Fluidized bed	
	with agitation parts	84
	without agitation part	85–87
	Pulse aeration	88

Table 3. Examples of microorganisms cultivated in SSF conditions

Fungi	edible	*Agaricus bisporus* *Volvariella volvaceae.*
	entomopathogens	*Beauveria bassiana* *Metarhizium anisopliae*
	others	*Aspergillus* (*niger, oryzae, flavus, candidus*) *Penicillium* (*candidum, roquefortii*) *Rhizopus oligosporus* *Trichoderma* (*koningii, harzianum, viride*)
Yeasts	*Candida* (*lipolytica, pseudotropicalis*) *Saccharomyces cerevisiae*	
Bacteria	*Bacillus subtilis* *Clostridium acetobutylicum* *Pseudomonas putida*	

successful attempts at cultivation of bacteria in SSF conditions exist [89] (Table 3).

SSF is a powerful method for the production of enzymes, especially hydrolases. This is due to the fact that the microogranism generally develops on a polymeric substrate (starch, cellulose, etc.) which induces enzyme synthesis. Numerous works deal with this area (Table 4).

The suitability of SSF for other metabolite production has also been investigated and a wide variety, including aromas, may be obtained in this way (Table 5). These studies have been performed because it was sometimes claimed that SSF compared favorably with submerged fermentation in terms of energy requirements, productivity per unit volume of reactor, and water removal for product recovery [121]. However, it appears that SSF can rarely compete with submerged techniques for the production of high added-value compounds [48].

Table 4. Examples of enzymes produced by solid state cultivation

Enzyme	Microorganism	Substrate	Reference
Cellulolytic systems	*Trichoderma reesei*	Wheat straw	90
	Trichoderma harzianum	Wheat straw + bran	91
	Aspergillus phoenicis	Sugar beet pulp	62
	Neurospora crassa	Wheat straw	92
	Penicillium capsulatum	Sugar beet pulp	93
	Trichoderma harzianum	Sugar cane bagasse	94
Xylanases	*Aspergillus ochraceus*	Wheat bran	95
	Trichoderma reesei + *Aspergillus niger*	Extracted sweet sorghum silage	69
Pectinases	*Aspergillus carbonarius*	Wheat bran	57
Proteases	*Aspergillus oryzae*	Rice hulls + Rice bran	96
	Mucor miehei (Rennet)	Wheat bran	61
	Bacillus amyloliquefaciens	Wheat bran	60
Lipases	*Penicillium candidum* *Pencillium camembertii* *Mucor miehei*	Wheat bran	97
α-Amylase	*Bacillus megaterium*	Wheat bran	98
	Bacillus licheniformis	Wheat bran	99
	Aspergillus kawachii	Rice	100
Amyloglucosidase	*Aspergillus niger*	Wheat bran + corn flour	101
	Aspergillus niger	Wheat bran	102
Phytase	*Aspergillus carbonarius*	Canola meal + glucose	103

Nevertheless, it should be pointed out that SSF may be an appropriate process for in situ improvement of flavor content of solid foods [118]. Also, SSF should be considered as a valuable tool for testing the feasibility of an aroma production process to be used in parallel to liquid cultivations. Hence, the two approaches give complementary data, often allowing a faster process development.

2.1.3.3. Fungal Spore Production

Sporulation generally involves a rather restricted growth rate (Sect. 2.1.2), and so the temperature rise inside the medium is often relatively low and quite easy to control. One of the first attempts to control fungal spore production on a divided solid substrate was carried out at the Iowa Agricultural Experimental Station in 1935 [122]. It consisted of the cultivation of *Penicillium roquefortii* on cubes of wheat bread placed in salt-mouth bottles, the bottles being from about one-third to one-half full. The aim of this study was the production of a spore powder to be used as inoculum for blue cheese manufacture.

Since then, most work carried out in this area deals with the finding of versatile, non-bulky systems. On the laboratory scale, the most popular system

Table 5. Examples of metabolites synthesized by solid state cultivation

Compound	Microorganism	Substrate	Reference
Mycotoxins:			
Ochratoxin A	*Aspergillus ochraceus*	barley	104
Antibiotics:			
Surfactin	*Bacillus subtilis*	soybean curd residue	105
Penicillin	*Penicillium chrysogenum*	sugarcane bagasse	106
Tetracyclin	*Streptomyces viridifaciens*	sweet potato residue	107
Organic acids:			
Lactic acid	*Rhizopus oryzae*	corn, rice	108
Citric acid	*Aspergillus niger*	apple pomace	109
Others:			
Pigments	*Monascus purpureus*	tapioca starch	110
Gibberellic acid	*Gibberella fujikuroi*	wheat bran	111
Mycophenolic acid	*Penicillium brevicompactum*	Czapek-Dox agar	112
Substrate pretreatment for methane production	mixed fungal strains	orange processing waste	113
Ergot alkaloids	*Claviceps purpurea*	sugarcane pith bagasse	46
Ethanol	*Schwanniomyces castellii*	sugarcane bagasse	114
	Saccharomyces cerevisiae	fodder beets	115
		wooden pulp particles	116
	Zymomonas mobilis	sugar beets	117
Aromas:			
Cheese flavors	*Brevibacterium linens* *Propionibacterium freudenreichi sp shermanii* *Penicillium camembertii* *Penicillium roquefortii*	granular curds	118
Fruit-like flavors	*Ceratocystis fimbriata*	wheat bran sugarcane bagasse	119
Methyl ketones	*Aspergillus niger*	coconut	120

involves cultivation in plastic bags, a technique derived from in vitro plant cultivation methods. The bags are made either of polypropylene or polyethylene and may be porous [123–125] or fitted with pipes allowing gas exchange [123]. This very simple technique is also useful for enzyme production [126]. Static cultivations may also be carried out using thermostated fixed-bed reactors. This system allows a good aeration of the entire medium, ensuring homogeneous sporulation of the fungus cultivated [67].

Spores recovery may be carried out in two ways. The first involves their purification in a dry state. This can be done by pumping the spores under vacuum and collecting them on a membrane filter [123, 127]. This procedure may be employed if the conidia are dry enough, and located at the surface of the substrate, so that they may be easily released from the cultivation medium.

The second – and most commonly used – way involves soaking of the medium in a liquid solution, which generally contains a wetting agent, such as Tween 80, and sometimes a salt, such as sodium chloride. These solutes facilitate spore release from the mycelium. If necessary, the suspension obtained may be concentrated by centrifugation or diluted in order to get a defined conidia

content in the final solution. Also, the spores may be recovered as a pellet (or a filtration cake) and then dried or freeze dried [128].

2.1.4 Use of Spores as a Biocatalyst

Fungal spores represent both the end and the beginning of the life cycle of a fungus. These entities have for a long time been considered as dormant cells, unable to exhibit any metabolic activity before their outgrowth into mycelium. In fact, they own a complete enzymatic ability that may be activated when they are placed in appropriate conditions, and it is now generally considered that fungal spores are able to carry out the same reactions as the corresponding mycelium [129]. It should be noted that this feature is also claimed for bacterial and yeast spores [130].

From a practical point of view, the potential interest in the use of this kind of biocatalyst lies in the fact that it is generally easy to store, very often by a simple freezing at $-20\,^{\circ}C$ of either the full medium obtained after a solid state cultivation of the fungus or the spores obtained after extraction [131]. It is thus possible to anticipate the biocatalyst production, and so it can be considered that the methodology of use of conidia is close to the one involved with purified enzymes.

Another point to consider is that this material generally gives little mycelial proliferation during a biotransformation reaction. As a result, the reaction medium is not viscous and oxygen transfer is easier [132, 333]. Also, the lack of pellet formation ensures biocatalyst homogeneity [11]. Finally, product recovery may be considered to be easier [134].

The first observation that fungal spores could transform organic compounds may be attributed to Gehrig and Knight who reported in 1958 that octanoic acid could be converted to 2-heptanone by conidia of *Penicillium roquefortii* [135]. This pioneer work has led to the "spore process" which has been used to carry out transformation of several kinds of compounds (Table 6). Among these compounds, steroids represent the most extensively studied family [138]. For the most part, the transformations consist of hydroxylation of the nucleus or side chain, cleavage of carbon to carbon linkages, introduction of double bonds into rings A, B or D, oxidation of hydroxyl groups, and combinations of these reactions. It can thus be considered that the main reactions that are performed by microorganisms, i.e., oxidation, reduction, hydrolysis, condensation, isomerization, and bond formation [129] may be carried out by fungal spores.

In spite of these potentialities, fungal conidia have not been used so far in the area of aroma compounds synthesis, with the exception of methyl ketone production from fatty acids (Table 6). It is well established that vegetative fungal cells are able to produce a large variety of compounds [2, 152–159] and it might be expected that spores of these microorganisms could contribute to biotransformation processes in this domain.

Table 6. Reactions reported for fungal spores

Precursor family	Example of reaction	Microorganism	References
Steroids	– Great diversity of compounds obtained: Progesterone → 11α-hydroxyprogesterone *A. ochraceus*	*Aspergillus niger* *Aspergillus ochraceus* *Aspergillus terreus* *Cylindrocarpon radicicola* *Fusarium monoliforme* *Penicillium chrysogenum* *Sporotrichum epigaeum*	136–139
Triglycerides Fatty acids	– Methyl ketone synthesis	*Aspergillus niger* *Aspergillus oryzae*	
	– Octanoic acid → 2-heptanone *P. roquefortii*	*Penicillium caseicolum* *Penicillium roquefortii* *Trichoderma koningii* *Trichoderma viride*	135, 138, 140–143
Antibiotics	– Penicillin V → 6-aminopenicillanic acid (6-APA)	*Fusarium conglomerans* *Fusarium monoliforme*	144, 145
	– Antimycin A → mixture of 2 acids	*Aspergillus ochraceus*	
Flavonoids	– Naringin → naringenin + prurin	*Aspergillus flavus* *Helminthosporium sativum* *Cepholosporium rosam* *Penicillium charlesii* *Wojnowicia graminis*	146
Carbohydrates	Starch → glucose Sucrose inversion Glucose → D-mannitol Glucose → gluconic acid Tanin → gallic acid	*Aspergillus wentii* *Aspergillus oryzae* *Aspergillus candidus* *Aspergillus niger* *Penicillium chrysogenum*	147–151

2.2 *Immobilized Cells*

2.2.1 *Introduction*

The first successful experiments on the immobilization of active proteins (enzymes) were reported during the 1950s [160–162]. These attempts did not draw much attention to this principle, which was new in biotechnology, and biocatalyst immobilization was intensively studied only during the 1970s [163, 164].

Initial work in this area involved enzymes, but the feasibility of the immobilization of whole cells with retention of their biocatalytic activities has also been demonstrated, and this material is now accepted as biocatalyst [165]. So, immobilization of microorganisms, mammalian or plant cells is now considered as an efficient tool for biosynthesis, biotransformation and analysis [166]. Studies in this area were performed mainly because it is generally claimed that

living cells show a catalyzing ability to synthesize various useful and complicated chemicals using multi-enzyme steps and they have a regeneration activity which extends their catalytic life [167].

An immobilized cell system may be considered as a system in which cells are confined in a given space and which allows them to be easily reused [168]. So, in contrast to ordinary suspension culture systems, immobilized whole cells have the merits of: (1) avoiding wash-out of cells at a high dilution rate; (2) higher cell concentration in the reactor; and (3) easy separation of cells for reactors or the product-containing solution [167].

The methods used to immobilize cells should meet some criteria. They must be safe, and materials acceptable for use in foodstuffs should be preferred. The process must also be uncomplicated because the use of expensive supports and long procedures will add to the cost of the process. It must be gentle in order to maintain cell viability, membrane integrity or enzyme activity. The overall system (cells and support) must have a long life, and cell activity must be maintained as far as possible. This last criterion necessitates selection of materials reducing both cell leakage and diffusional resistance. Finally, the support should be as cheap as possible [169].

Many of the cell immobilization techniques are modifications of those developed for enzymes (Fig. 2). Two broad types of methods may be used, attachment to a support and physical entrapment, the latter being the most widely used technique [165, 169]. It may be achieved in two ways, entrapment and encapsulation.

2.2.2 Cell Entrapment in a Matrix

This involves enclosure of cells in a three-dimensional polymer network, the most commonly used being polyacrylamide, polyurethane, collagen, agarose, alginate, K-carrageenan, or chitosan (Table 7). Among these materials, alginate is one of the most studied, because it meets almost all the criteria listed in the preceding paragraph.

Alginates are copolymers of algal or microbial origin containing α-L-guluronic acid and β-D-mannuronic acid. Solutions of Na-alginate form gels when divalent or multivalent cations are introduced, through bridge formation between guluronate residues [191, 192–195]. Calcium ions are the most frequently used, giving Ca-alginate networks [191]. However, this material shows a high sensitivity towards chelating compounds such as phosphate, citrate, and lactate or anti-gelling cations such as Na^+, K^+, Mg^{2+} [191, 196]. Several methods may be used to overcome this limitation. First, alginate with a high content of guluronic acid blocks should be preferred because of its high mechanical stability, high porosity, and tolerance to salts [194]. Also, gel beads may be kept in a medium containing a few millimolar free calcium ions. Other approaches have also been proposed, among them replacement of Ca^{2+} ions by other divalent cations (Sr^{2+} or Ba^{2+} for living cells) having a higher affinity for

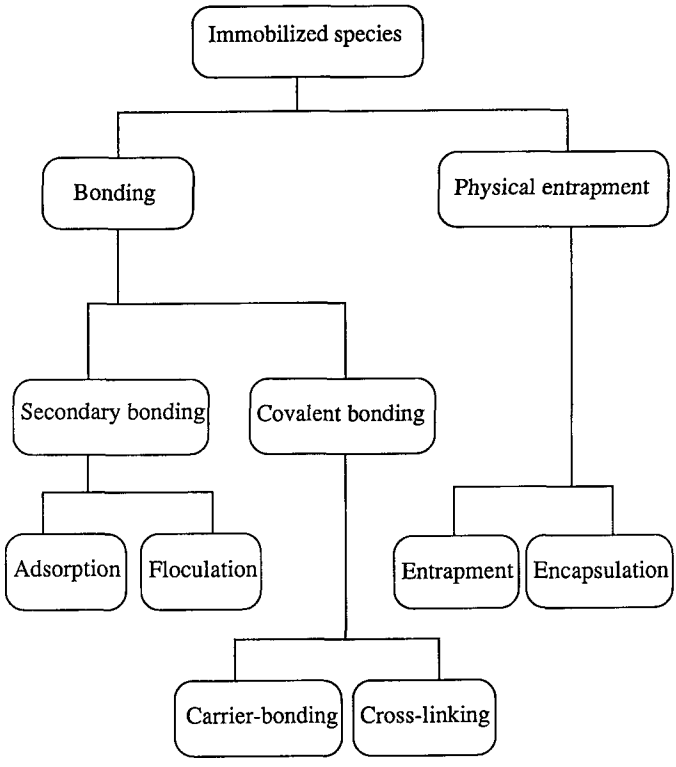

Fig. 2. Mechanisms and methods in cell immobilization, after [165]

alginate (Table 8). Also, it is possible to manufacture beads with an outer layer made of a Ca^{2+}-insensitive material, such as polyacrylamide or polyacrylate. Finally, Ca-alginate forms strong and stable complexes with polycations such as chitosan or synthetic polymers such as polyethylenimine (Table 8). However, one must consider that Ca-alginate remains the most popular matrix used with immobilized cells.

This network is generally used as homogenous beads. Some alternative shapes, including fiber formation or manufacture of complex particles, exist (Table 8). A gelation technique, involving in situ Ca^{2+} generation and giving one-piece matrixes, has also been suggested (Table 8).

The preparation of Ca-alginate gel beads is generally carried out by dripping a Na-alginate-cell sterile suspension into a solution containing Ca^{2+} ions, often in the form of $CaCl_2$. The sterile suspension is obtained by aseptic mixing of a cell suspension with alginate solution (2–4% w/v) sterilized by autoclaving or sterile filtration [191]; pasteurization at 60 °C for 3 h may also be used for this purpose. On the laboratory scale, dripping is performed by passing the alginate-cell suspension through a needle (inner diameter 0.22–1 mm) connected either to

Table 7. Main networks used for cell entrapment and methodology of use

Network	Methodology	References
Polyacrylamide	Free radial polymerisation of acrylamide with *N,N'*-methylenebis (acrylamide) as cross-linking reagent. Reaction initiated by a catalyst system made of – chemical system: persulfate ion and β-dimethylaminopropionitrile or *N,N,N',N'*-tetramethylethylenediamine (TEMED) – photochemical system: riboflavin, sodium hydrosulphite, TEMED Gives cubes or beads	170–174
Polyacrylamide hydrazine (PAAH)	PAAH crosslinked with a dialdehyde (glyoxal, glutardialdehyde or periodate-oxidized polyvinylalcohol) Gives cubes or beads if coated with alginate	175–177
Polyurethane	Isocyanate functional groups at both terminals of the urethane prepolymer (PU) molecule react with each other in the presence of water, forming a urea linkage and liberating carbon dioxide Prepolymers with different hydrophilic or hydrophobic character can be obtained Gives cubes	178–183
Agar, agarose	Thermal gelation Gives beads when dropped in a cold aqueous solution or an organic solvent	173, 184 185
Collagen	Cross-linking with glutardialdehyde Gives cubes	172, 186
K-carrageenan	Thermal gelation and ionotropic gelation (usually with potassium chloride) Gives beads	187
Chitosan	Ionotropic gelation, cross-linking with multivalent anions Gives beads	188, 189
Alginate	Ionotropic gelation usually with calcium ions Gives mainly beads	190, 191

a syringe or to a peristaltic pump [218]. This system may be modified by pressing the suspension through a syringe with a low flow rate [219], and droplets of a more uniform size may be obtained when an air flow is applied around the needle [191, 219, 220]. The rate limiting step of this method is the droplet formation at the tip of the needle, and two main procedures are proposed to scale up bead manufacture, i.e., an extrusion technique and dispersion of the gel suspension in liquid or air. In the extrusion technique or Hulst et al. [219], the aqueous alginate-cell suspension is pressed at such a high flow rate through a small orifice that a jet is formed. With a membrane, a sinusoidal vibration of a certain frequency is transferred to the liquid. The signal causes the

Table 8. Different uses of alginate gels for microorganism entrapment

Methodology	References
External gelation	
Bead formation	
Gelation with Sr^{2+} or Ba^{2+}	197–199
Ca-alginate stabilization	
polyacrylamide	200–203
polyacrylate	204
trivalent cations	205
polycations (polyethylenimine, polylysine...)	206–209
Fiber formation	210–212
Complex particles	
Hard porous core	213–214
Beads containing a polymeric substrate for cells	215
Beads with channels	216
Internal gelation	
One-piece matrix	192, 217

break-up of the jet in uniform droplets of 1–2 mm diameter. The vibration nozzle technique allows a production capacity two orders of magnitude higher than the conventional extrusion technique (the maximum capacity with a 1.1 mm nozzle diameter is 24 $l\,h^{-1}$). This system has recently been thoroughly investigated [220]. The second methodology is generally performed with a rotating-disk atomizer which disperses the Na-alginate solution in air, the droplets being collected in a calcium chloride solution for hardening. This system has been extensively studied, and beads with diameter less than 1 mm can be obtained [221–226].

Ca-alginate gels shrink during gel formation, leading to loss of water and an increase in the polymer concentration relative to the alginate solution, the greatest shrinkage being found with alginates with low guluronic acid residues contents [191]. The highly porous gel obtained (the pores range from 5 to 200 nm in diameter [227]) may be dissolved simply by immersing the beads in a solution containing phosphate or citrate. This process, which allows the recovery of entrapped cells, may take several hours if the alginate has a high guluronic acid content [191].

2.2.3 Engineering Aspects of Immobilized Cells

The most important problems encountered during the use of immobilized cells are the mechanical properties of the support and mass transfer phenomena. Mechanical properties of the support include gel strength and deformability, both parameters being influenced by the biomass loading [228]. Generally, high cell contents gives more brittle beads; it may be considered that microorganisms such as fungal spores can be added to final concentrations of up to 10^9 cells

ml^{-1} of gel, which corresponds to a volume fraction occupied by biomass close to 6.5% with these cells entrapped in Ca-alginate [228].

Mass transfers involve two main phenomena, i.e., external and internal resistances. When the reaction medium flows around the biocatalyst, a layer of liquid not disturbed by turbulence is formed near the surface of the carrier. Since the transport of substances in the laminar film is performed by molecular movements, it may be assumed that the resistance against the diffusion of the substrate is concentrated in the laminar film [229]. It is the concentration gradient between the two ends of this stagnant layer which represents the driving force for the substrate and product exchanges between the bulk medium and the support. This phenomenon is generally described with the aid of the film model which gives [162, 230]

$$J_i = k_i(S_{Li} - S_{Ri}) = \frac{D_i}{\delta}(S_{Li} - S_{Ri}). \tag{1}$$

The mass transfer coefficient k_i is strongly correlated with the hydrodynamics of the reactor system. It is commonly assessed using relationships among the Sherwood, Reynolds, and Schmidt numbers which take the form

$$Sh = cRe^aSc^b. \tag{2}$$

Values for a,b, and c may be found in the literature according to the type of reactor used [162, 230, 231].

External transfer resistance may be negligible towards internal transport phenomena if the Biot number (Eq. 3) is much larger than one [232]:

$$Bi = \frac{k_i L}{De_i}. \tag{3}$$

It is generally assumed that intraparticle transports occur through diffusion, i.e., the convection is neglected. Diffusional processes are found to obey the Fick's law, and numerous works report values for the effective diffusion coefficient in several diffusive component-support systems. Small molecular weight solutes in dilute gels of agarose, alginate, or K-carrageenan diffuse with rates similar to those in water [233, 234]. This result is in agreement with the equation of Mackie and Meares [235–237]:

$$\frac{De_i}{D_i} = \frac{(1 - \phi_p)^3}{(1 + \phi_p)^2} \tag{4}$$

where ϕ_p is the polymer volume fraction of the gel and De_i the effective diffusion coefficient.

This feature may change when biomass loading in the support becomes significant. In this case, the effective diffusivity of metabolites depends on the diffusivity in both the support material and the cells. It is generally found that the effective diffusivity decreases with cell fraction in the support regardless of cell type or diffusing species. As a result, De_i may vary between 0.01 D_i and 1.1

D_i [234, 237, 238]. Several expressions that may be used to predict De_i in the presence of cells are given by Westrin and Axelsson [237]. Among them is the exclusion model, which assumes that cells are impermeable to the solute and that only the volume fraction $1 - \phi_c$ remains available for the diffusional flux [232, 237] (Eq. 5):

$$\frac{De_{ic}}{De_i} = 1 - \phi_c \tag{5}$$

while other authors use the random-pore model [237] (Eq. 6):

$$\frac{De_{ic}}{De_i} = (1 - \phi_c)^2 \tag{6}$$

where De_{ic} is the effective diffusivity in the presence of cells and ϕ_c the cell volume fraction in the support.

The above results have been obtained with dead biomass. In fact, during a cultivation, living cells are used, and substrate and product migrations inside the particle occur in connection with biochemical reactions, i.e., substrate consumption and product synthesis; biomass formation may also take place. This gives the so-called diffusion-reaction coupling phenomenon. This leads to concentration gradients in the nutrient levels and can confine the cellular metabolic activity to the interface between the cultivation medium and the cell-containing support (Fig. 3).

This phenomenon gives a heterogenous growth of the biomass inside the support leading to high cell density in the outer layers near the support-solution interface. A steady state may be achieved, during which a cell concentration gradient in the network is established and additional biomass formation gives cell leakage into the external solution. For example, the maximal loading of the outer layers of agar was close to 5×10^{11} cells cm^{-3} for *Escherichia coli* [239]. In the case of filamentous microorganism, an outgrowth of filaments generally takes place; these filaments may be released into the medium after disruption, for example, due to mechanical stress arising from stirring [240]. Thus, immobilized cell cultivations rarely occur without any freely suspended microorganisms.

The overall effect of internal diffusion is generally quantified through the effectiveness factor η [229]:

$$\eta = \frac{\text{actual apparent rate}}{\text{reaction rate without diffusion effects}} \tag{7}$$

where η is a theoretical parameter which is not experimentally attainable. It may be calculated from the Thiele modulus M_T [238]. However, this calculation requires knowledge of the intrinsic rate parameters, and the use of the Weisz modulus M_W may be preferred [238, 241]:

$$M_W = \eta M_T^2 = \frac{r_{obs} L^2}{S_{Ri} De_{ic}}. \tag{8}$$

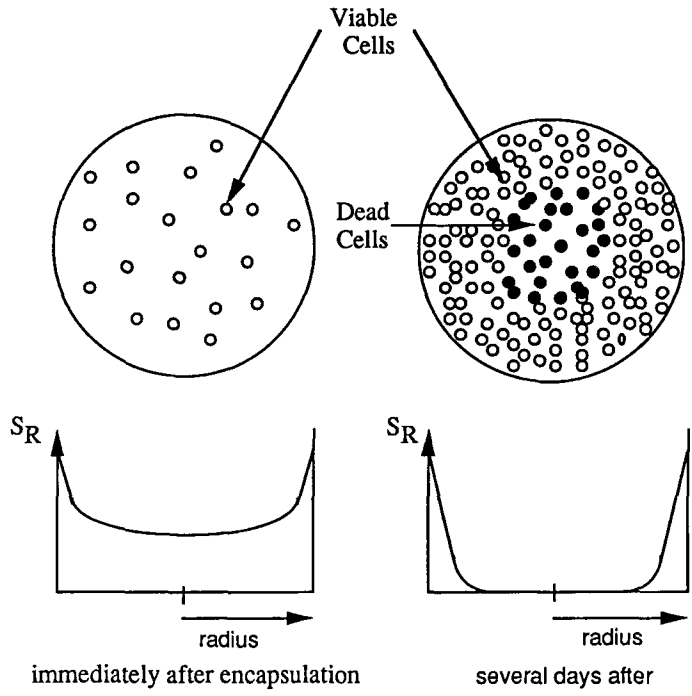

Fig. 3. Variations in cell concentration and nutrient profiles inside a gel bead during the course of a cultivation, according to [234]

Plots of η as a function of M_W for various reaction orders may be found in the literature [238, 241]. This approach, although very simple, does not constitute an infallible test for diffusional limitations because it does not take into account product inhibition [238].

Entrapped microrganisms may be operated in several kinds of bioreactors. Packed-bed reactors are well suited when substrate inhibition occurs during the reaction. It also gives no friction between the particles, and allows high substrate conversion. However, high pressure drops are often observed. Fluidized bed reactors allow good mixing and transport properties. The conventional stirred reactor remains largely used in spite of its major drawback, its high shear rate, which may damage the support particles [229, 242].

2.2.4 Aroma Production by Immobilized Cells

One application of immobilized cells is continuous metabolite production. In this case, a larger growing biomass may be retained in the bioreactor in comparison to values achieved with free material. Enhanced metabolic stability may also sometimes be observed. In this area, the cultivation of yeasts, mainly

Saccharomyces cerevisiae, for ethanol and volatiles production, is the most extensively studied system. The main support used is Ca-alginate, but other techniques, such as adhesion or passive immobilization in porous materials have also been studied [167]. Two points have been particularly actively investigated, long-term operational stability of the system and immobilization effects on metabolic pathways. Operational stability depends on feeding strategies [243], and the cell death-rate has to be taken into account [244]. Metabolic alterations may result from growth characteristics in the matrix. For example, increased specific hexokinase and phosphofructokinase activities have been found in Ca-alginate entrapped *S. cerevisiae* cells. This activation has been attributed to the formation of aggregates in the network during the growth [245]. The factors explaining this phenomenon are not clear, but it should be pointed out that the presence of free calcium ions in the medium may interact with the cellular metabolism, i.e., by modification of wall composition or membrane transports [246]. This point, important with the use of Ca-alginate support, should be more extensively studied.

Metabolite gradients in the network may also increase the effect of product inhibition. This point is illustrated by a study dealing with the contribution of immobilized cells and free cells released from the support to the production of ethabol and 4-ethylguaiacol to add the specific aroma to soy sauce [247]. Ethanol is found to be produced mainly by free cells and 4-ethylguaiacol by immobilized *C. versatilis* cells.

Attempts at the continuous production of soy sauce using immobilized cells show the potentialities of the use of this kind of biocatalyst for aroma production. The aim of these works was to reduce the time for soy sauce production, which takes six months for the conventional process. The first attempt was conducted by Osaki et al. [248], who continuously produced soy sauce for 80 days in 280-l column type reactors containing *Pediococcus halophilus*, *Saccharomyces rouxii* and *Torulopsis versatilis* cells entrapped in alginate gel beads. The total time for the process, including enzymatic hydrolysis of koji and refining, was only two weeks. However, the aroma pattern of the products was somewhat different from the commercial soy sauce. Further refinements of the method involve the use of two bioreactors in series, the first containing *Zygosaccharomyces rouxii* cells, and the second *Candida versatilis* cells, both immobilized on a cylindrical filamentous ceramic carrier or on ceramic beads. These supports have been used because the cultivation medium involves high salt contents, making Ca-alginate gel unsuitable for long cultivations [249–252]. The best results were obtained with ceramic beads as support. The process time in these conditions was only six days and the fermented product was claimed to be similar in composition to the commercial one [250].

Continuous production of cheese flavors using immobilized cells has also been investigated. The studies mainly deal with the use of lactic bacteria, with the aim of producing volatile fatty acids (acetic, propionic, lactic) and other aroma compounds (diacetyl, acetoin). Ca-alginate is the support most frequently used here [253]. Continuous production of emmenthal cheese flavors from

Propionibacterium sp entrapped in Ca-alginate gel beads has been reported; the authors claim the possibility of metabolite production in combination with cell recovery for use as propionic acid starter [218]. Continuous conversion of lactose into 2,3-butanediol was achieved over 25 days with coimmobilization of *Klebsiella oxytoca* and permeabilized *K. lactis* having β-galactosidase activity [254]. Also, 2-heptanone, a key compound of blue cheese flavor, may be continuously synthesized by Ca-alginate entrapped spores of *Penicillium roquefortii* in an aqueous synthetic medium [255].

Another use of immobilized cells is for repeated batch cultivations, i.e., biocatalyst reuse. For example, diacetyl may be obtained from *Lactococcus lactis subsp diacetylactis* coimmobilized in Ca-alginate gel fibers with homogenized bovine liver (source of catalase) [256]. Also, biotransformation of β-ionone may be carried out with *Aspergillus niger* mycelium entrapped in polyurethane foam [250]. An important area for cell reuse is biotransformations with plant cells, which represent an expensive biocatalyst [257]. Furthermore, close cell-to-cell contact in a high-density environment with gradients in levels of gases, substrates, and product intermediates may be critical in inducing or enhancing production of targeted metabolites [257]. Reviews on plant cell immobilization may be found in the literature [258–260]. Thus, immobilized cells of carrot enantioselectively transformed aromatic ketones, such as acetophenone or propiophenone, into (*S*) alcohols while the same substrates gave (*R*) alcohols with immobilized *Gardenia jasminoides* cells [261]. An example of enhanced reaction specificity is given by the selective hydrogenation of the C–C double bond of (−)-carvone and (+)-pulegone by *Nicotiana tabacum* [262].

Another example of easier immobilized cells separation from the medium is in sparkling wines manufacture. These effervescent wines (Champagne is the most prestigious of them) are produced by means of two successive alcoholic fermentations. The first is carried out at a temperature of 20 °C or below in large fermentors. The wine obtained is then drawn off, clarified, and supplemented with sucrose syrup. It is also inoculated with *Saccharomyces cerevisiae* or *S. bayanus*, then filled into the bottles in which it is sold. The secondary fermentation lasts for up to six weeks at 11–12 °C, and the pressure in the bottles increases by about $5–6 \times 10^5$ Pascal. An aging period follows this process, and is at least 12 months. The yeast lees are then settled on the stopper by means of riddling, which usually takes several weeks or months. The last operation is disgorging, during which the yeast sediment is removed; this has been automated [263]. It has been shown that the riddling process could be highly facilited using immobilized yeasts [264]. It should be noticed that immobilized cell technology may also be useful in beer production [265].

As already pointed out, immobilization of growing filamentous microorganisms may result in better cultivation conditions. The use of gel beads generally allows one to maintain growth and metabolic characteristics close to those observed with free pellets. Hydroxylation of β-ionone involves pellets of *Aspergillus niger*; this reaction has been reported to be efficiently carried out by Ca-alginate entrapped growing *A. niger* in a stirred reactor, and the conditions

allow reduction of mycelial proliferation on baffles, probes, and impeller in comparison to that observed with free cells [266].

An approach involving coimmobilization of substrate and microorganism in the support network has been proposed [267]. Several microorganisms, yeasts and filamentous fungi are entrapped in bilayered beads together with various incubation media. Among others, complex bioflavor generation is demonstrated by baker's yeast coimmobilized with apple juice, generating cider flavors. Also, brewing yeast (*Saccharomyces carlsbergensis*) gives a beer taste when incubated with malt. Beads processed in this way are claimed to be usable directly as a food additive, eliminating the need for product purification. Similar work, dealing with menthanethiol production from methionine by fat-coated microcapsule-encapsulated *Brevibacterium linens* cells has also been reported. These microcapsules could be added to cheddar cheese in order to improve its flavor [268].

It can be seen from the above considerations that relatively little work has been performed in the area of flavor production by immobilized cells. This is probably due to the fact that researchers on this subject mainly focus on microorganism screening and on medium formulation. Very often biotransformation processes for aroma synthesis are not advanced enough to think about cell immobilization for continuous or cell reuse purposes. An exception to this is perhaps for filamentous or plant cells. Thus traditional applications of immobilized cells involve alcohols, organic acids, antibiotics, and enzyme production. Biotransformation of steroids and waste water treatment are also areas of interest [167, 169, 179, 269, 270]. Some recent reviews also cover the applications of immobilized cells in the food industry, focusing on starter production, metabolite synthesis, and food bioconversion [271, 272].

3 Examples of Integrated Processes for Aroma Compounds Production

3.1 Production of Methyl Ketones by Spores of Penicillium Roquefortii

The manufacture of some salad dressings, soups, and savory items makes use of compounds conferring a blue cheese taste [142]. This flavor is mainly obtained by addition of methyl ketones, which are primarily recognized for their contribution to the flavor of blue veined cheeses. Although a large number of ketones have been isolated from various cheeses, the odd-numbered C_5 to C_{11} 2-alkanones predominate [274, 275]. They are generated during cheese ripening from lipid metabolism by the filamentous fungus *Penicillium roquefortii* [276].

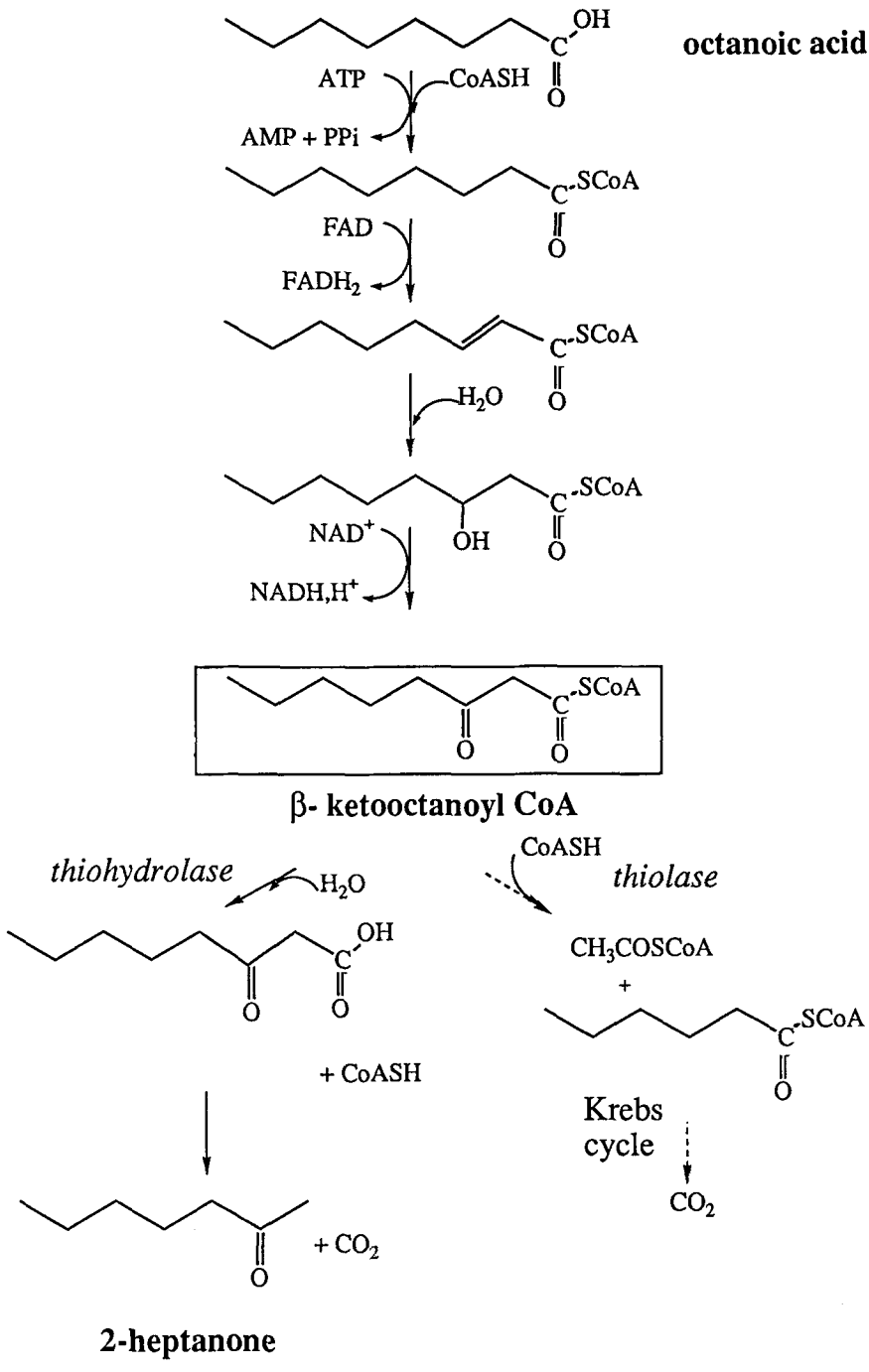

Fig. 4. Biosynthesis of 2-heptanone from octanoic acid by *P. roquefortii*, adapted from [143]

The need to produce methyl ketones that qualify for the natural label has prompted intensive studies, and several processes involving fatty acids breakdown have been proposed [140, 277–287].

The formation of methyl ketones from fatty acids is described by the following stoichiometric equation:

$$R\text{–}CH_2\text{–}CH_2\text{–}COOH + O_2 \rightarrow R\text{–}CO\text{–}CH_3 + CO_2 + H_2O.$$

This is an aerobic process. The biogenesis of methyl ketones is well known [142]; the fatty acid degradation pathway involves a key component, 3-ketoacylCoA, that could be further converted either into methyl ketone by hydrolysis through thiohydrolase action followed by decarboxylation, or into carbon dioxide by thiolase followed by the Krebs cycle (Fig. 4). The last possibility corresponds to the complete β-oxidation of fatty acids. A bioenergetic balance on all the reactions involved from fatty acids to methyl ketones shows that the reactions are energetically self-sufficient and thus capable of functioning with a biotransformation yield equal to unity, without the need for an energetic substrate such as glucose if the oxidative phosphorylation efficiency is greater or equal to one. The oxidative phosphorylation efficiency depends on the dissolved oxygen tension, and the preceding constraint may be achieved if the oxygen transfer from the gas to the liquid phase is sufficient to prevent the oxygen tension in the liquid phase from being the limiting step.

Several kinds of problems arise when producing the methyl ketones in submerged cultures. First, mycelial growth of *P. roquefortii* affects the apparent viscosity; the cultures become highly viscous, and suffer from low oxygen transfer rates. Second, a strong substrate inhibition of the reaction is observed. For example, *P. roquefortii* is unable to synthesize 2-heptanone at an initial concentration of octanoic acid higher than 10 mmol l^{-1} [140, 287]. Third, an important volatile loss, due to stripping by the air stream needed for the reaction, is observed [281]; this is due to the high activity coefficients of methyl ketones in water.

Fortunately, options exist to improve the process. For each of the three main limiting steps cited above, solutions were proposed. The first improvement consists of using spores of *P. roquefortii* instead of mycelium. Interestingly it was discovered [277] that if the vegetative cells were eliminated and only fungal spores were used in the inoculum, the reaction was faster. The utilization of spores, free or immobilized, in a submerged process avoids mycelium proliferation and oxygen limitation and the product recovery is easier. The second improvement is obtained by the mode of operation of the culture. A fed-batch technique enables one to avoid inhibition of *P. roquefortii* spores by the substrate. In aqueous media the process can be carried out at constant pH, the increase in pH resulting from the reaction being counteracted by direct substrate addition [297].

However, although the overall substrate metabolized can be increased using fed-batch procedures, this kind of cultivation does not lead to any improvement in the recovery of the ketones. Therefore the third improvement results from the

idea that, due to the hydrophobic nature of most aroma compounds, a water immiscible phase could act as a synthetic accumulation site. Experience gathered from the extractive production of ethanol, iso-butanol and iso-propanol etc. could be transfered to aroma biotechnology [288–290] provided the use of extraction solvents conforms to the European guidelines and regulations [291]. For methyl ketones recovery, the organic phase selected [292] consists of a technical isoparaffin solvent which could be regarded as tetradecane. The solvent volume ratio, taken as the ratio of the solvent layer volume to total liquid two-phase medium volume, was about 0.85–0.88. The aqueous phase was dispersed in the isoparaffin solvent when the spores were free and was fully entrapped in the beads when the spores were used immobilized.

The presence of an organic solvent in a medium may have adverse effects on the catalytic activity of microorganisms. Solvents with low polarity and high molecular weight exert the least toxic effects. The polarity may be quantitatively estimated by means of an easily obtainable parameter, logP [293, 294]. This term is the decimal logarithm of the partition coefficient of a given compound in a standard octanol-water two-phase system and should have a value higher than 4.0 [294]. Some authors [295] suggest that the three-dimensional solubility space of Hansen would be a more appropriate criterion, at least for enzymes.

The technical isoparaffin solvent used meets the requirements in terms of logP, its value being close to 7 [298] and *P. roquefortii* seems to follow the general rules stated by Laane et al. [294].

These considerations enabled the design of a process of C_5–C_9 methyl ketones production from the corresponding C_6–C_{10} fatty acids by spores of *P. roquefortii* in a water isoparaffin solvent, two-phase system, operated using a fed-batch procedure and carried out at constant substrate concentration. Spores are obtained by cultivation of the fungus by solid state fermentation on buckwheat seeds [67], and the simplest and best way to carry out the biotransformation is to use the whole sporulation medium without discarding the grains. These conditions allow the production of about 75 $g\,l^{-1}$ of 2-heptanone with an apparent yield close to 80% and the reaction rate observed is nearly twice as high as that achieved in an aqueous system. In the same way, about 20 $g\,l^{-1}$ of 2-pentanone and 60 $g\,l^{-1}$ of 2-nonanone can be isolated from the culture medium, making this process economically attractive for the synthesis of natural methyl ketones [292, 296].

When the spores are Ca-alginate entrapped, an improvement in catalyst stability was observed for C_6 and C_{10} acids with a slight increase in catalytic activity [296]. This suggests that diffusion into the beads is not limiting and that immobilization affords protection of the cell by reducing direct contact between the organic phase and the microorganisms. However, the drawback of the "immobilized spores" process is that it does not lend itself as well as the "buckwheat spores" process to large-scale production due to the more complex manipulations involved.

It can be pointed out that methyl ketones are produced on a commercial scale by the British company Stafford Speciality Ingredients, using a strain of

Aspergillus niger (see Table 5). In this process, coconut fat is converted into a mixture of methyl ketones by solid state fermentation. The fractional conversion is about 0.40 and 2-undecanone accounts for 60% of the end-product [120].

3.2 *Hydroxylation of* β-*Ionone by Immobilized Aspergillus Niger Cells*

A large number of compounds with a trimethylcyclohexane ring have been isolated from tobacco during the last few decades. The compounds of this group have characteristic and low threshold odors [299], and they are thought to be key flavor components in the essential oils of air-cured tobaccos [300, 301]. They probably originate in nature from carotenoids through complex enzymatic degradations [302].

Several investigators have attempted, therefore, to obtain tobacco flavorings by microbial transformation of carotenoids, or of ionones which are considered to be key intermediates of carotenoids [302–304] and are widely distributed in nature [305]. For examples, an essential oil characterized by burley tobacco flavor was produced by the oxidation of β-ionone by *Lasiodiplodia theobromae* [306]. The Swiss company Givaudan used resting cells of *L. theobromae* ATCC 28570 for the conversion of β-ionone and claimed in a patent that up to 10 g β-ionone per litre of medium were converted with a yield of 90%, giving β-cyclohomogeraniol as the main product [307]. *Aspergillus niger*, which is known to have an hydroxylating activity on several terpenes and terpenoids [2, 308], was used to synthesize hydroxylated derivatives of β-ionone [309].

In this latter case, the major products were 4-hydroxy-β-ionone, 2-hydroxy-β-ionone, 2-oxo-β-ionone, and 4-oxo-β-ionone in order of decreasing quantities. The neutral fraction of conversion product, when applied at parts-per-million concentration levels (1 to 5 ppm) caused low grade tobacco to assume desirable smoking qualities described as sweet and mild. The suggested transformation pathway of β-ionone was the enantioselective hydroxylation at C-2 and C-4 of β-ionone followed by further oxidation and dehydration, or both, or acetylation [309].

However, even if the products exhibit remarkable effects at very low level, it is absolutely necessary to maximize the final aroma content in the medium for industrial development.

The biotransformation of β-ionone in shake flasks by microorganisms shows the following patterns: first, β-ionone was found to have a high antimicrobial activity. Of a total of more than one thousand microorganisms tested by Mikami et al. [309, 310], bacteria and yeast strains were found incapable of accumulating significant quantities of aroma products and converted only a small portion of ionones; the ability to do so was prevalent among *Aspergillus* and *Rhizopus* cultures, and they finally retained a non-commercial *A. niger* strain, *Aspergillus niger* JTS 191. Larroche et al. [266] retained *A. niger* IFO 8541 which accumulated the same main compounds (Fig. 5). However the substrate

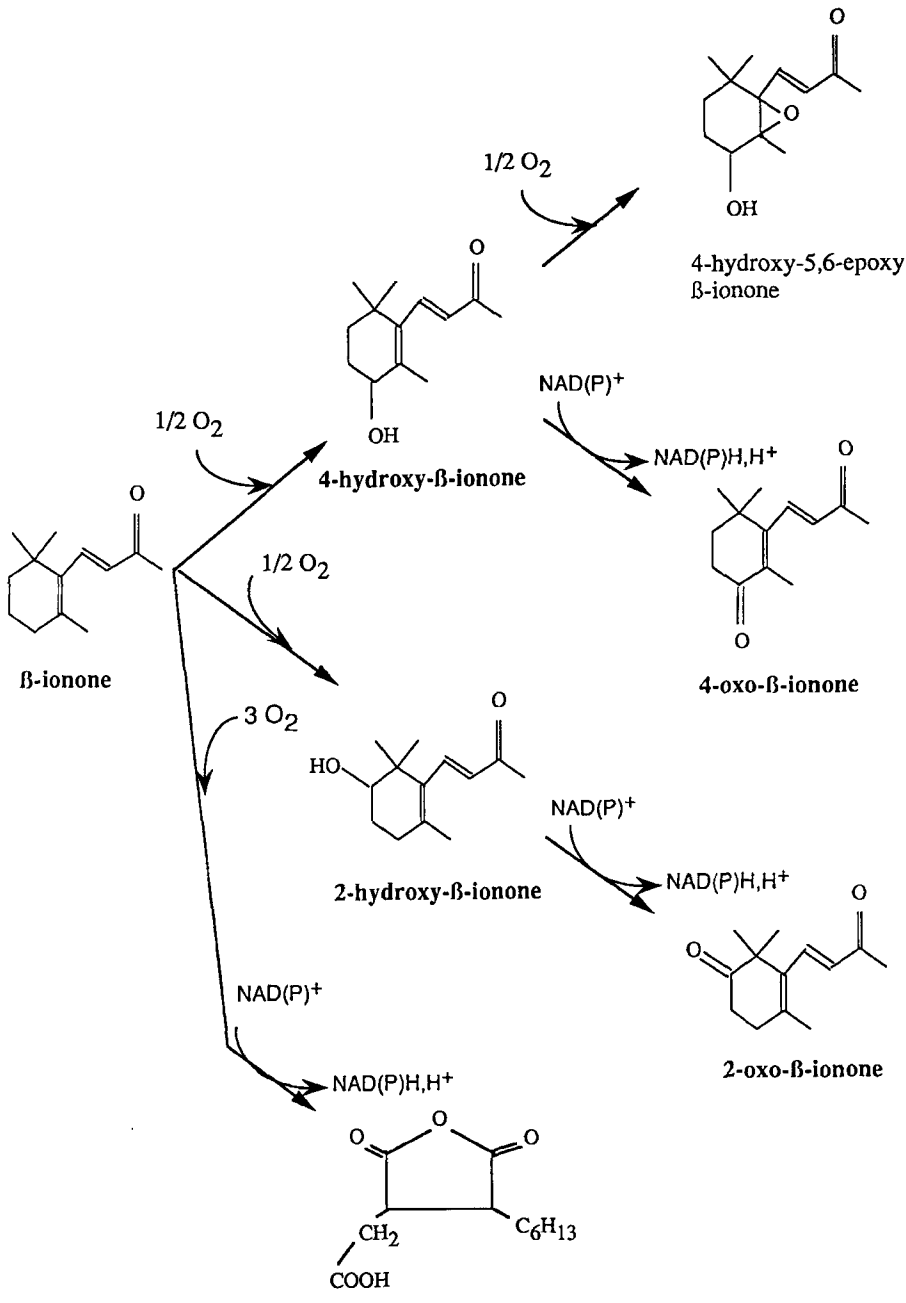

Fig. 5. Main pathways involved in the biotransformation of β-ionone by *Aspergillus niger* IFO 8541

concentration should be less than 1.5 $g\,l^{-1}$ to prevent inhibition. Second, the cultivation conditions for biotransformation carried out by *A. niger* involved formation of pellets during the fungal growth. Finally, the reaction is an oxidation and involves oxygen consumption, and an important feature is that the final aroma content remains constant after the synthesis is terminated. This means that metabolites are not degraded further.

These patterns suggest ways to improve the process and to define the conditions to maintain the environmental conditions for this microbial process on the reactor scale. The resistance of *A. niger* to the antifungal property of β-ionone could be increased by using previously grown mycelium entrapped in hydrophobic polyurethane cubes and by adding an organic solvent, isooctane, to the reaction medium [180]. Another approach, based on the use of a fed-batch technique, was used with *A. niger* IFO 8541 immobilized in Ca-alginate beads [266].

In these latter conditions, an integrated process of production in reactors was proposed. Spores of *A. niger* were obtained by a solid state fermentation technique and extracted with distilled water. Growth of *A. niger* after inoculation of the medium with free spores led to fungus gradually to attach itself to fermentor wall, baffles, impeller, and probes. This phenomenon, which was due to partial pellet disruption by the shear forces exerted by the mechanical stirring, made this mode of operation unsuitable. In order to maintain the desired morphology, i.e., pellets, the medium was inoculated with spores immobilized in Ca-alginate beads. The particles were directly generated in the stirred reactor in order to avoid contamination. After 48 h of preliminary fungal growth, the biotransformation was carried out using a fed-batch operation (sequential precursor addition). β-Ionone hardly reduced the fungal growth rate, but delayed the colonization of the fluid medium by the mycelium. The precursor was converted into metabolites provided the carbon source remained present in the medium. β-Ionone consumption by resting cells took place with a molecular yield close to unity. This allowed one to obtain about 4 $g\,l^{-1}$ aroma compounds from the broth. As already mentioned, these compounds are neither metabolized nor stripped by the air flow from the bioreactor, which facilitates further recovery [266].

Further optimisation of this process may be considered by finding conditions that completely stop the fungal growth and improve the time-life of active resting cells.

3.3 Alkyl Pyrazines Synthesis by Bacteria in Solid and Liquid Media

Pyrazines are heterocyclic nitrogen-containing compounds, which, since the mid-1960s, have been shown to contribute significantly to the unique taste and aroma of roasted and toasted foods [311, 312]. Pyrazines are widespread in nature. However, very little definitive information is available on their origin

[313]. Some are undoubtedly of biosynthetic origin, produced by enzymatic reactions which may be unique to the species in which the pyrazines are found. Others may be formed by non-enzymatic reactions between molecules which are present in plants or foods. The pyrazines formed in cooked, roasted, or toasted foods are probably produced by non-enzymatic reactions (Maillard reactions) between precursors found in the fresh foods, and are formed during the heating process.

The first evidence that microorganisms, among others *Bacillus subtilis*, were able to produce alkyl pyrazines was given by Kosuge and coworkers in 1962 [314, 315]. They showed that tetramethyl pyrazine is present in natto, a Japanese fermented soybean food. Later on they established that Japanese fermented foods such as miso, soy sauce, natto, beer, and sake all contain tetramethyl pyrazine [316]. Another study by Reineccius et al. [317] implicated microorganisms in the synthesis of the same pyrazine during the fermentation of cocoa beans.

The odor threshold values of alkyl pyrazines are very low [318], and they are generally produced at low levels in foods. The highest level (265 $\mu g\,kg^{-1}$) of tetramethyl pyrazines was found in miso stored for one year at room temperature [316]. Only 29 $\mu g\,kg^{-1}$ was found in 1-month old miso fermented by *A. oryzae*. Natto is made by fermenting soybeans with *Bacillus natto* and was found to contain 22 $\mu g\,kg^{-1}$ of this pyrazine compound, soy sauce only 4 $\mu g\,kg^{-1}$ (from *A. sojae*).

Pyrazines can be added to food for the generation of flavor notes such as nutty, coffee-, chocolate-, banana-like, etc. The need for roasty flavors, e.g., in microwave food where the non-enzymatic browning reaction does not take place, results in a demand for natural pyrazines [2]. The main problem hampering industrial applications is the low concentration in the fermentation broths, resulting in high cost for downstream processing.

The limiting step in that case is physiology, and attention has been focused on upgrading the strains through mutation, optimizing the medium formulation, and identifying precursors. A mutant of *Corynebacterium glutamicum*, deficient in a single enzyme of the isoleucine-valine pathway, was found [319] to accumulate 3 $g\,l^{-1}$ of tetramethyl pyrazine after 5 days. The accumulation was dependent on the addition of thiamine. This remarkable result obtained in 1967 seems not to have been repeated since that time. Concerning physiology, it was shown [320, 321] that, when *Bacillus natto* was cultivated in liquid medium, the addition of L-threonine was most stimulatory for pyrazine formation and L-serine ranked second. The main product in the L-threonine-containing culture was 2,5-dimethyl pyrazine and total pyrazine was more than 36 $mg\,l^{-1}$ of culture broth. The culture containing L-serine produced mainly tetramethyl and trimethyl pyrazine, nearly 5 $mg\,l^{-1}$.

It was thus postulated that, 2,5-dimethyl pyrazine was obtained from L-threonine according to the following global stoichiometric reaction:

$$2CH_3\text{–}CHOH\text{–}CHNH_2\text{–}COOH + \tfrac{3}{2}\,O_2 \rightarrow C_6H_8N_2 + 5H_2O + 2CO_2$$

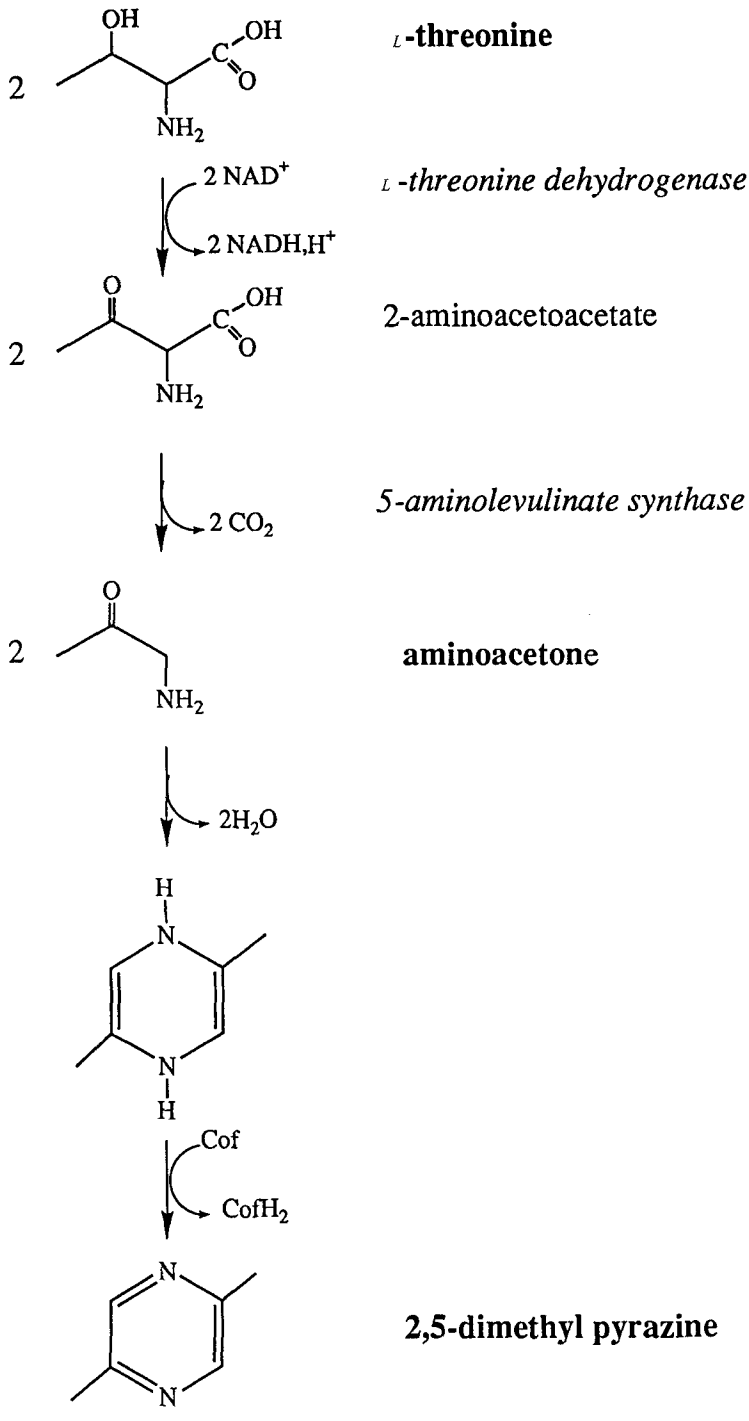

Fig. 6. Biosynthesis of 2,5-dimethyl pyrazine by *Bacillus subtilis*. Cof = unknown cofactor (NAD^+ or FAD)

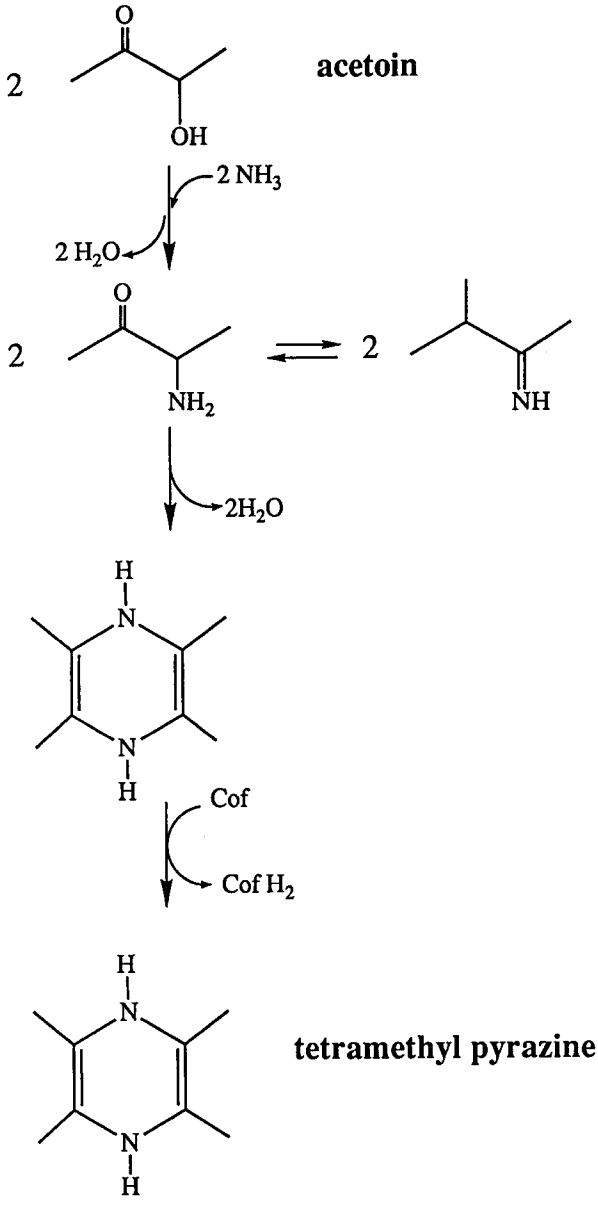

Fig. 7. Biosynthesis of tetramethyl pyrazine by *Bacillus subtilis*. Cof = unknown cofactor (NAD^+ or FAD)

and that tetramethyl pyrazine was synthesized from acetoin and ammonia according to

$$2CH_3\text{–}CO\text{–}CHOH\text{–}CH_3 + 2\,NH_3 + \tfrac{1}{2}O_2 \rightarrow C_8H_{12}N_2 + 5H_2O.$$

Figures 6 and 7 summarize the main intermediates from precursors to pyrazines. Aminoacetone is the main intermediate in 2,5-dimethyl pyrazine synthesis from L-threonine [320].

These two reactions are independent and correspond to two different physiological states of the cell. The precursors have inhibitory effects on each of the metabolic pathways. Acetoin is also inhibitory for 2,5-dimethyl pyrazine synthesis.

The French company Sanofi Bio Industries has recently patented a process [323] for the production on a commercial scale of di- and tetramethyl pyrazines. Two microorganisms are concerned, *Bacillus subtilis* and *Brevibacterium linens*. Preliminary work in this area involved solid state cultivations carried out under aerobic conditions with soy beans soaked and cooked in a manner similar to the one used in Japan for Itohiki-natto [324]. The cultures were performed in a fixed-bed column bioreactor. Results demonstrated the feasibility of pyrazine production with this system. However, liquid fermentation carried out with either ground soy beans or soy flour enriched with precursors appeared superior after extensive optimisation. The final medium therefore consists of 5 wt% soy flour dispersed in a water broth enriched with precursors (L-threonine, acetoin, ammonia) which are added separately and eventually by a fed-batch technique. An overall yield of $5\,g\,l^{-1}$ ($4\,g\,l^{-1}$ of 2,5-dimethyl pyrazine and $1\,g\,l^{-1}$ of tetramethyl pyrazine) can be reached in this way. The aroma compounds are separated by extraction-distillation processes.

4 Conclusion

The use of biotechnological routes for the synthesis and production of high value added compounds actually represents a challenge for researchers. The numerous works carried out in the area of flavor and aroma synthesis show that biotransformation may be considered as a valuable candidate for this purpose. However, only few processes have until now reached the industrial scale, mainly because the final metabolite contents in the medium remain too low.

It is more and more accepted that significant improvements in any biotransformation process can be obtained only with an integrated approach, considering both the biocatalyst and its environment. This means that the biocatalyst must have the highest intrinsic enzymatic activity possible and that its way of use must allow full expression of this activity. A biotransformation process may become efficient only if these two constraints are taken into account.

This review gives examples of results that can be achieved with such an integrated approach. It also shows that less conventional biocatalysts such as fungal spores or immobilized cells are poorly used in the area of flavor production. It also emphasizes the fact that unusual cultivation conditions like solid state fermentations may be of interest when elaborating a process.

Analysis of the physiology of the microorganism used together with the physico-chemical properties of the compounds involved in the reaction may lead to processes that may be considered as acceptable from an industrial point of view. It is possible to improve dramatically the results of a biotransformation process by using adequate, existing biochemical engineering tools without any modification of the biocatalyst at the genetic level. This demonstrates that previous works did not allow full expression of the microorganism potentialities. Examples given deal with methyl ketone synthesis from fatty acids, preparation of hydroxy derivatives of β-ionone, and alkyl pyrazines production.

Future prospects in this area should involve studies carried out with the aim of achieving final product concentrations in the range of those obtained with other classical compounds such as citric, gluconic, or glutamic acids. This aim will require a complete knowledge of the physiology and of the metabolism of the biocatalyst in order to elucidate whether the limiting step is of biological or of physical nature. This step appears to be essential for further work on the production of a given compound.

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Biotechnological Production of Flavour-Active Lactones

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Biotechnology lends itself to the production of natural flavour materials, which can either be obtained as complex mixtures or pure, individual flavour components. Examples of the latter category are acids, alcohols, esters, aldehydes, ketones and lactones. Biotechnological processes are reviewed which can be used to produce those γ- and δ-lactones which are important to the flavour industry. Emphasis is placed upon fermentative processes using microorganisms capable of performing β-oxidative degradation reactions. The preferred substrates in this type of biotransformation are hydroxy fatty acids which can themselves be obtained enzymatically or extracted from natural sources. Certain microorganisms are capable of hydroxylating fatty acids, thereby giving rise to the immediate precursors of γ- and δ-lactones. The intramolecular esterification of hydroxy fatty acids can be catalysed by certain lipases. The lipase from *Candida antarctica* is also capable of converting some cyclic ketones to the corresponding lactones via the Baeyer-Villiger reaction.

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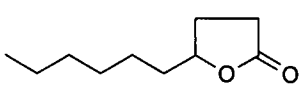
1 Introduction

Lactones are ubiquitous in nature and have been isolated from all major food systems [1]. The naturally occurring, organoleptically important lactones generally have γ- or δ-lactone structure, and are straight-chained, while a few are even macrocyclic (Fig. 1).

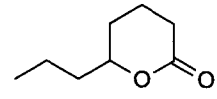
The importance of aliphatic lactones as aroma components of foods is based on their characteristic organoleptic properties. Among these are oily, peachy, creamy, fruity, nut-like, coconut, honey and so-on. Due to their mostly low odour thresholds averaging about 0.1 ppm [2], lactones often have a high flavour value. Whereas γ-lactones preferentially occur in plants, δ-lactones are mainly found in animal products.

Sensorily important lactones usually possess eight to twelve carbon atoms and some are very important flavour components in a number of foods such as strawberries, peaches, apricots, milk products and fermented foods. They are produced in plants in minute amounts when the fruit ripens, probably by catabolic processes involving the structurally related fatty acids [3]. However, the fact that both the optical purity and the absolute configuration can vary for identical lactones isolated from different sources supports the idea of the presence of different biosynthetic pathways, involving either anabolic or degradative mechanisms.



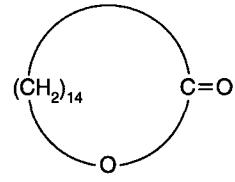
(1)

4-decanolide
(γ-decalactone)



(2)

5-octanolide
(δ-octalactone)



(3)

15-pentadecanolide

Fig. 1. Common lactone structures

The biosynthesis of lactones is complex and not that well understood. The following systems have been implicated:

a) from the corresponding keto acids via NAD-linked reductases;
b) from unsaturated fatty acids via hydration;
c) from fatty acid hydroperoxides via lipoxygenase action;
d) from esters of naturally occurring hydroxy fatty acids;
e) via the enzymatic cleavage in plants of long chain hydroxy fatty acids into fragments, including γ- and δ-derivatives capable of forming lactones.

It has not been elucidated whether lactones are formed intracellularly and excreted, or if the hydroxy acids are released from the cell and then undergo spontaneous lactonisation.

A variety of microorganisms can perform de novo lactone biosynthesis (Table 1) but, unfortunately, yields are invariably very low and amount to only a few milligrams per litre or less [13]. Such low yields usually preclude the use of such systems for production purposes. Nonetheless, quite a number of systems have been described in which lactones are produced by using enzymes and/or microorganisms. Those systems relevant to the formation of flavour-active lactones will now be covered in more detail. Emphasis will be placed on those individual lactones which are of importance in the flavour industry.

Table 1. Microbially produced lactones

Microbe	Lactones	Ref.
Candida globiformis	5-Decanolide	4
Candida pseudotropicalis	5-Decanolide	4
Ceratocystis moniliformis	4- and 5-Decanolide	5
Cladosporium butyri	5-Decanolide	4
Ischnoderma benzoinum	4-Butanolide, 4-pentanolide, 4-hexanolide, 2-hexen-4-olide, 4-heptanolide, 2-hepeten-4-olide, 4-octanolide, 2-octen-4-olide	6
Pityrosporum sp.	4-Hexanolide, 4-heptanolide, 4-octanolide 4-nonanolide, 4-decanolide, 4-undecanolide, 4-dodecanolide	7
Polyporus durus	4-Butanolide, 4-pentanolide, 3-penten-4-olide, 4-hexanolide, 2-hexen-4-olide, 5-hexen-4-olide, 5-hexanolide, 2-hepten-4-olide, 4-heptanolide, 2-, 5-, and 6-octen-4-olide, 4-octanolide, 2-nonen-4-olide, 2-decen-4-olide, 4-decanolide, 4-methoxy-6-methyl-2H-pyran-2-one	8
Saccharomyces cerevisiae	5-Decanolide	9
Saccharomyces fragilis	5-Decanolide	9
Sarcina lutea	5-Decanolide	9
Sporobolomyces odorus	4-Decanolide, 5-decanolide, *cis*-7-decen-5-olide, *cis*-6-dodecen-4-olide	10
Trichoderma viride	6-(pent-1-enyl)-α-pyrone, 6-pentyl-α-pyrone	11 12

2 Fermentative Production of Lactones

From the flavour point of view, the most important γ- and δ-lactones which are both accessible via biotechnology and are economically viable are those directly related to octanoic, decanoic and dodecanoic acids. Of these six lactones, γ-decalactone or 4-decanolide is by far the most important.

2.1 4-Decanolide

4-Decanolide (Fig. 1) is found in peaches and other fruits as well as in dairy products. It has creamy, fruity, fatty-oily flavour attributes. 4-Decanolide can be produced by both biosynthesis and biotransformation. Biotransformation routes are of major commercial interest because the yields are high compared with those from biosynthetic routes.

4-Decanolide is at present manufactured by the biodegradation of ricinoleic acid using *Yarrowia lipolytica* and other microorganisms [14–17]. The process originates from the early observation [18] that the former microorganism can perform β-oxidation of ricinoleic acid, thereby forming 4-hydroxydecanoic acid (Fig. 2), the open form of 4-decanolide. The 4-decanolide obtained in the above processes incorporates at position 4, the chiral centre originally present in natural (*R*)-ricinoleic acid which is the principle fatty acid in castor oil. The 4-decanolide so produced has a very high optical purity and usually contains more than 98% of the (+)-*R*-enantiomer.

The conversion of ricinoleic acid into 4-decanolide by *Yarrowia lipolytica* has been studied in some detail [14]. The experimental data indicate that ricinoleic acid first undergoes three separate β-oxidation cycles to form 6-hydroxy-3-dodecenoic acid before the carbon–carbon double bond is reduced by the yeast (Fig. 2). A fourth β-oxidation step then converts the so-formed 6-hydroxy-dodecanoic acid into 4-hydroxydecanoic acid. A further β-oxidation cycle results in the formation of 3,4-dihydroxydecanoic acid which cannot be utilised as a carbon source by the yeast and accumulates in the fermentation broth (Fig. 3) or cyclises to form 3-hydroxy-4-decanolide.

The normal distillative and/or extractive work-up of the fermentation broth gives a crude product consisting mainly of 4-decanolide together with minor amounts of both 2- and 3-decen-4-olide. The latter unsaturated lactones are formed as a result of the elimination of a molecule of water from either the 3-hydroxy-4-decanolide or the linear precursor 3,4-dihydroxydecanoic acid (Fig. 4). The concentrations of the two decenolides should be low, since they interfere with the overall flavour of the 4-decanolide [14].

The importance of natural 4-decanolide to the flavour industry is indicated by the number of patents already issued for its manufacture [15–17, 19, 20]. Most of the microorganisms claimed are yeasts, which exhibit suitable lipase

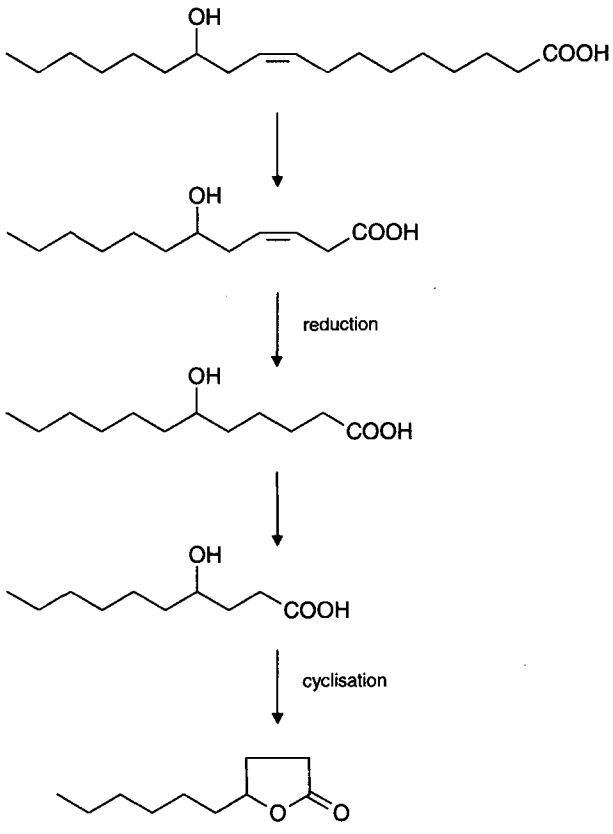


Fig. 2. Proposed modified degradation scheme of ricinoleic acid to 4-decanolide

activity necessary to hydrolyse the castor oil. Furthermore, the microorganisms have to be able to tolerate the fatty acids thus liberated and, most importantly, have to carry out the partial β-oxidation of the ricinoleic acid to form 4-hydroxydecanoic acid. This type of process can typically result in final product concentrations of about 5 g/l fermentation broth. A few reports suggest that concentrations in the region of 10 g/l are also possible [21].

In the case of *Yarrowia* fermentations, only partial cyclisation of the 4-hydroxydecanoic acid takes place during fermentation. Consequently, in order to obtain satisfactory yields, thorough lactonisation has to be achieved by acidification and/or heating.

A further development of this process entails the use of alkyl ricinoleates as substrate instead of castor oil [22]. The use of the simple esters instead of the oil apparently simplifies the production process since foam formation is reduced significantly and product isolation by solvent extraction is a lot easier.

Ricinoleic acid can also be produced by the fermentative hydration of oleic acid using various fungal, yeast or bacterial strains [23]. Considering the

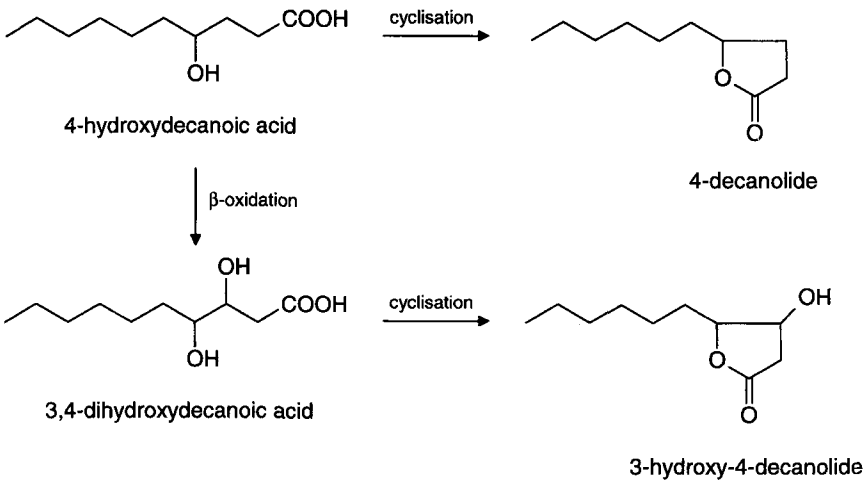


Fig. 3. Conversion of hydroxydecanoic acids to 4-decanolide and 3-hydroxy-4-decanolide

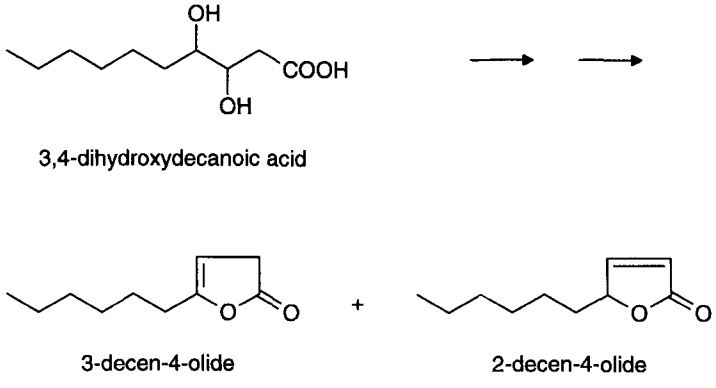


Fig. 4. Conversion of 3,4-dihydroxydecanoic acid to 2-decen-4-olide and 3-decen-4-olide

abundance of castor oil, this approach just has to be less economical. Nevertheless, it remains of interest for the production of other hydroxy fatty acids which are more difficult to obtain from natural sources.

Cardillo and co-workers [17] have claimed that *Aspergillus niger* and *Cladosporium suaveolens* are capable of producing both 4-decanolide and 4-octanolide from castor oil. Other microorganisms which are also claimed are *Phanerochaete cryosporium* and *Pichia etchellsii.*

Farbood et al. [19] have developed several patented processes for the production of 4-decanolide and other lactones from castor oil and ricinoleic acid. Thus, *Candida petrophilium* can convert castor oil to a mixture of various hydroxy fatty acids which can be recovered by acidification and extraction.

A variety of lactones was obtained after distillation of the hydroxy acids under acidic conditions, the major lactone being 4-decanolide.

Cheetham and co-workers [16] have applied for a similar patent using *Sporobolomyces odorus* and *Rhodotorula glutinis*. Both microorganisms are capable of the biotransformation of ricinoleic acid via β-oxidation, although both are apparently poorer than *Yarrowia* since a product concentration of only 0.6 g/l 4-decanolide was obtained.

The fungus *Monilia fructicola* is capable of producing 4-decanolide together with 4-octanolide [24] by the biotransformation of the corresponding fatty acids. Product yields of about 1 g/l fermentation broth were obtained.

A novel bioconversion process for the formation of 4-decanolide involves the baker's yeast reduction of 3-decenolide [25], a byproduct formed in the production of 4-decanolide from castor oil. It could be shown that this unsaturated lactone can be converted very smoothly by actively fermenting baker's yeast to the corresponding 4-decanolide. The overall reduction actually involves an enzyme-catalysed isomerisation step (Fig. 5) which is enantiospecific, thereby transforming the achiral substrate into (+)-*R*-4-decanolide with an enantiomeric excess of $\geqslant 95\%$. Reduction experiments in D_2O showed that a maximum of four deuterium atoms were incorporated into the molecule.

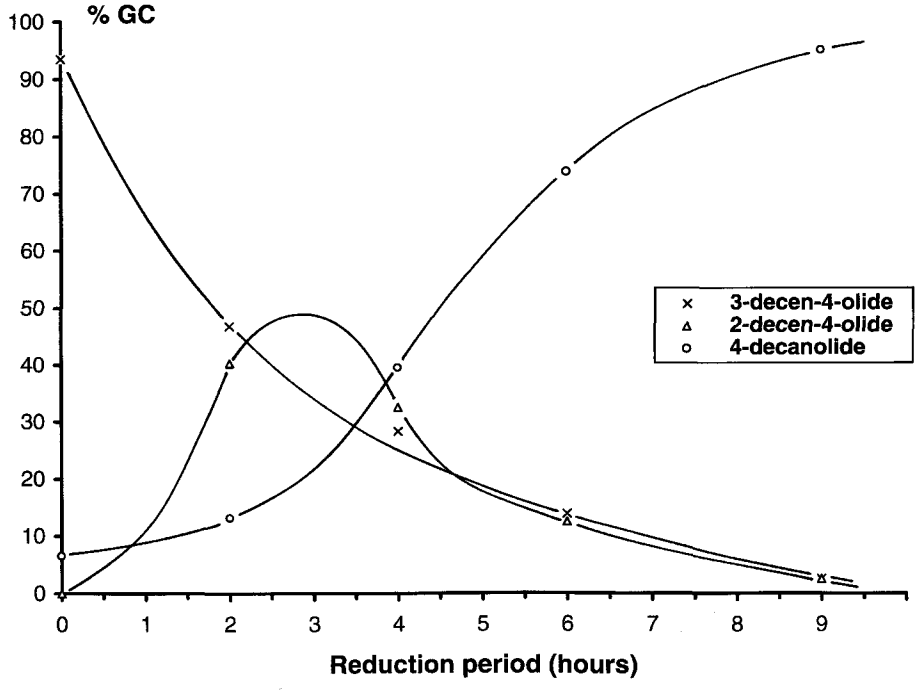


Fig. 5. Baker's yeast reduction of 3-decen-4-olide to 4-decanolide via 2-decen-4-olide as intermediate

2.2 5-Decanolide

5-Decanolide is naturally found in butter, coconut, white wine and in many fruits such as peaches, raspberries and strawberries. It exhibits creamy, sweet, coconut, milk-like flavour notes.

The successful conversion of ricinoleic acid into (+)-*R*-4-decanolide via microbial β-oxidation has resulted in the search for sources of other suitably oxo-substituted fatty acids which can be converted into natural lactones. Such fatty acids can be found in natural products. For example, sweet potatoes or Jalap resin contain 11-hydroxypalmitic acid and 3,11-dihydroxymyristic acid [26]. Baker's yeast is capable of converting both these acids into the corresponding δ-lactones, 5-decanolide and 5-octanolide (Fig. 6). In this process, the yeast has an absolute requirement for an odd number of carbon atoms between the carboxyl group of the fatty acid and its hydroxyl group.

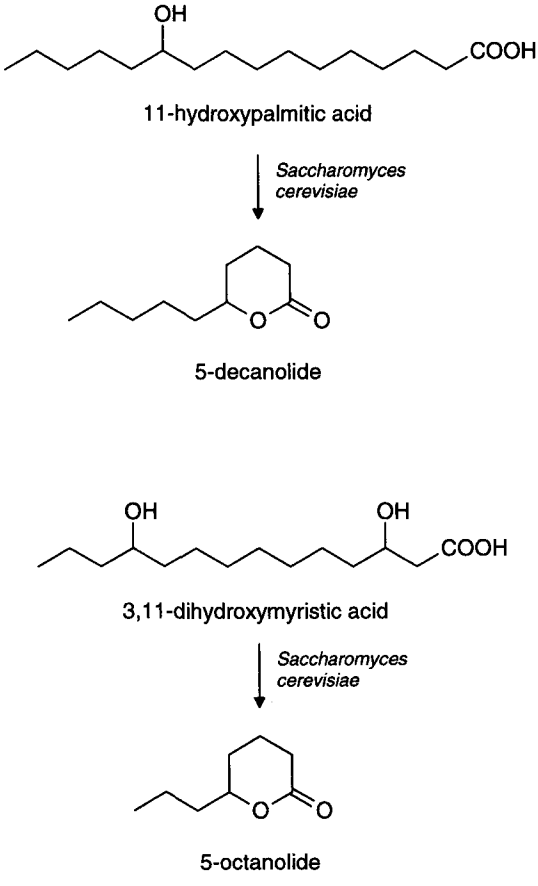


Fig. 6. Conversion of hydroxy fatty acids to δ-lactones by *Saccharomyces cerevisiae*

5-Decanolide can also be obtained by the β-oxidative degradation of coriolic acid (13-hydroxyoctadeca-9,11-dienoic acid) which is the principle fatty acid of the seed oil of *Coriana nepalensis* [27]. The microorganism originally described for this biotransformation is *Cladosporium suaveolens* (Fig. 7).

An alternative approach is to produce the hydroxy fatty acid intermediate by microbial hydroxylation of a fatty acid. Certain *Mucor* species can produce both γ- and δ-lactones from the corresponding fatty acids or their ethyl esters [28]. Such microbial transformations give rise to lactones having a high enantiomeric purity. However, the specificity of the enzymatic hydroxylation strongly favours the formation of γ-lactones.

Some hydroxy fatty acids can also be obtained via the reduction of the corresponding hydroperoxides which in turn can be synthesised enzymatically using lipoxygenase. Lipoxygenases are able to catalyse the incorporation of molecular oxygen into the 1,4-pentadienyl moiety of polyunsaturated fatty acids. Thus, linoleic acid can be converted using soybean lipoxygenase into (+)-coriolic acid. Under optimised conditions, a very high yield of hydroperoxide was obtained [29] which could be converted via chemical reduction into (+)-coriolic acid. Large scale reactions were able to generate approximately 25 g/l of the hydroperoxy fatty acid.

Probably the most promising method for producing larger quantities of natural 5-decanolide is via the microbial reduction of the corresponding α, β-unsaturated lactone present in Massoi bark oil. The oil consists mainly of 2-decen-5-olide (80%), 2-dodecen-5-olide (7%) and benzyl benzoate (6%). It was found that various microorganisms were able to hydrogenate effectively the ring double bond of both these unsaturated lactones [30]. The active microorganisms are fungi belonging to the *Basidiomycetes* and also baker's yeast. After optimisation, product concentrations of approximately 1.2 g/l 5-decanolide were obtained after a fermentation period of some 16 h. The minor product formed was 5-dodecanolide (Fig. 8).

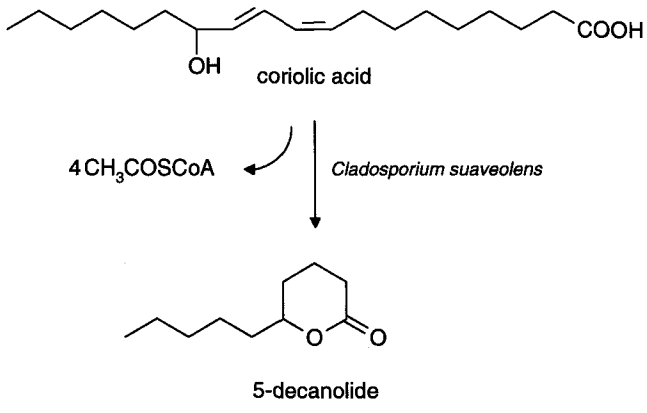


Fig. 7. Conversion of coriolic acid to 5-decanolide by *Cladosporium suaveolens*

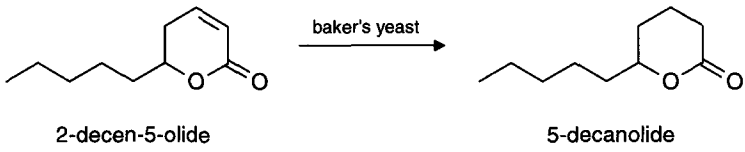


Fig. 8. Baker's yeast reduction of 2-decen-5-olide to 5-decanolide

The mechanism of this reduction reaction has been the subject of a number of investigations. Thus, NMR studies on 5-decanolide samples, obtained by reducing 2-decen-5-olide with baker's yeast in either D_2O or H_2O, showed that the double bond saturation involves *trans*formal addition of hydrogen atoms [31]. The hydrogen atoms themselves arise to a large extent from the water used as reduction medium.

Patent protection has been claimed for the original biotransformation using baker's yeast and various fungi including *Polyporus durus, Ischnoderma benzoinum, Bjerkandera adusta, Poria xantha* and *Pleurotus ostreatus* [32]. The use of other yeasts for the same biotransformation has also been claimed [33].

2.3 4-Octanolide

4-Octanolide or γ-octalactone, is found naturally in beer, pork, heated chicken, butter and in fruits such as raspberries, strawberries and peaches. It exhibits a sweet taste, very reminiscent of coconut.

The most efficient way of making 4-octanolide is via the site-specific hydroxylation of octanoic acid. This biotransformation was first reported by Drawert and co-workers [34] who reported the conversion of a triglyceride fraction rich in octanoic acid into the corresponding γ-lactone. The microorganism used was *Polyporus durus.*

Basically, the same biotransformation was patented for the production of γ- and δ-lactones from the corresponding carboxylic acids or their derivatives using *Mucor* species [28]. The bioconversion would appear to work best for the production of 4-octanolide. Thus, *Mucor circinelloides* converted ethyl octanoate into 4-octanolide very efficiently, resulting in a product concentration of some 11 g/l lactone in the broth after a fermentation period of 40 h.

The same biotransformation has been patented using microorganisms belonging to the *Mortierella* species [35]. Thus *Mortierella isabellina* is capable of converting either octanoic acid or ethyl octanoate into 4-hydroxyoctanoic acid which, after lactonisation, produces 4-octanolide with a high enantiomeric purity. High yields in the range of 10 g/l lactone in the fermentation broth were reported.

A similar bioconversion can be achieved by fermenting coconut oil, which is rich in octanoic acid, with *Aspergillus niger, Cladosporium suaveolens, Phanerochaete crysosporium* and *Pichia etchellsii* [17].

2.4 5-Octanolide

5-Octanolide or δ-octalactone has been found in nature in meat, cheese, fruits such as pineapple and strawberries and in fermented drinks such as wine and rum. It displays a fatty, creamy flavour impression with slight coumarin, coconut-like nuances.

This material is known to be a secondary metabolite of *Basidiomycetes* [36], together with very many other γ- and δ-lactones. 5-Octanolide can be obtained as a minor product when octanoic acid or its ethyl ester is fermented with either *Mucor* species [28] or *Mortierella isabellina* [35]. The most efficient way to produce this flavour compound, however, would seem to be via the β-oxidation of 3,11-dihydroxymyristic acid [26]. Baker's yeast is able to convert this dihydroxy fatty acid into 5-octanolide (Fig. 6). The substrate itself is present in sweet potatoes and is consequently readily available.

2.5 4-Hexanolide

4-Hexanolide or γ-hexalactone occurs widely in nature, for example in meat, cheese and various fruits such as apricots and strawberries. It displays a relatively weak, sweet, coumarin-like flavour which is somewhat reminiscent of hay. This is a relatively unimportant material for the flavour industry.

This lactone can be obtained together with 5-hexanolide upon the bioconversion of ethyl hexanoate using *Mucor subtillisimus* [28], although the yields are relatively low at < 0.5 g/l in the fermentation broth.

2.6 4-Dodecanolide

4-Dodecanolide exhibits a buttery, fruity, peach-like flavour and has been reported in fruits like strawberries and peaches and also in butter.

4-Dodecanolide has been known for many years to be a secondary metabolite of a number of microorganisms. Thus *Pityrosporum canis* was shown to be able to produce this material together with a complex homologous series of other γ-lactones [37]. In fact, many members of the genus *Pityrosporum* were able to convert lipid-rich substrates into the γ-lactone rich fermentation product. Likewise, the parasitic imperfect fungus *Fusarium poae* produces a complex mixture of volatiles, composed mostly of γ-lactones [38], the main component of which is 4-dodecanolide and its unsaturated derivative *cis*-6-dodecen-4-olide.

A number of publications describe the microbial oxidation of oleic acid to 10-hydroxyoctadecanoic acid [39]. Possibly the most efficient way of achieving this is by using resting cells of *Saccharomyces cerevisiae*. 4-Dodecanolide can be obtained by contacting the so-produced 10-hydroxyoctadecanoic acid with a microbe exhibiting β-oxidising activity. Thus, approximately 20% conversion

was achieved with baker's yeast and also with *Candida, Pichia* and *Hansenula* strains [40]. A similar process has been patented using *Pseudomonas* species and also with *Yarrowia lipolytica* which gives a yield of 3.5 g/l 4-dodecanolide after a fermentation period of 20 h [41].

Linoleic and linolenic acids can be converted by *Acetobacter* and *Rhodococcus* species in an analogous manner into their corresponding 10-hydroxy derivatives [42, 43] which themselves are the ideal substrates for the production of the appropriate unsaturated 4-dodecanolides.

2.7 6-Pentyl-2-pyrone

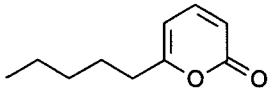
Another lactone having a strong coconut odour is 6-pentyl-2-pyrone (Fig. 9). This material was found to be the major volatile constituent of the fungus *Trichoderma viride* [44] and was produced at relatively high concentrations, namely approximately 200 mg/l.

It was claimed that the spores rather than the mycelia are responsible for the formation of this lactone. Other *Trichoderma* species are capable of producing 6-pentyl-2-pyrone and *Trichoderma koningii* also produces the homologue 6-heptyl-2-pyrone [45].

2.8 Macrocyclic Lactones

Certain macrocyclic lactones are of interest as musk fragrances. Such macrocyclic musk compounds are preferable to the traditional synthetic nitromusks, not only sensorially but also because of better tolerance by the skin and their superior degradation properties in the environment.

It has been known for many years that some microorganisms are capable of converting certain fatty acids into sophorolipids [46] (Fig. 10). Thus, starting from palmitic acid, the corresponding ester or even hexadecane, 16-hydroxyhexadecanoic acid is produced as a glycolipid by the yeast *Torulopsis bombicola* [47]. This must be one of the most efficient fermentation processes in the flavour and fragrance industry, since a yield of 40% is reached and a total production of 300 g/l glycolipid is achieved. The glycolipid is then subjected to acid hydrolysis to liberate the hydroxy fatty acid which is then cyclised to form the macrocyclic lactone, hexadecanolide.



6-pentyl-2-pyrone

Fig. 9. Major metabolic product of *Trichoderma viride*

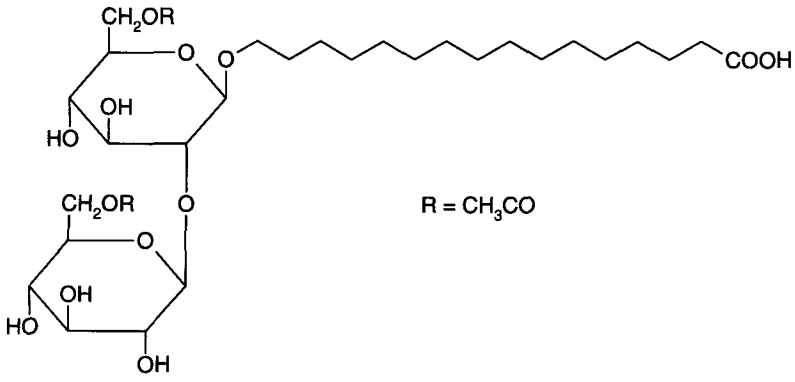


Fig. 10. Sophorolipid structure

3 Enzymatic Synthesis of Lactones

It is now well established that enzymes which hydrolyse esters in aqueous media can also catalyse esterification or transesterification reactions in monophasic organic solvents or in water-organic solvent biphasic media [48]. The ester-forming ability of lipases has been known since the beginning of this century [49].

3.1 Via Intramolecular Esterification

It was only first noted in 1982 [50] that, when certain hydroxy acids were exposed to the lipase of *Mucor miehei*, lactones were formed (Fig. 11). The macrocyclic pentadecanolide (3) was synthesised from 15-hydroxypentadecanoic acid and γ-butyrolactone from 4-hydroxybutyric acid [51]. This observation was subsequently confirmed by Yamada and co-workers [52], who reported the biocatalytic lactonisation of ω-hydroxy acid methyl esters in highly diluted solutions.

The factors affecting the enzymatic intramolecular lactonisation of 16-hydroxyhexadecanoic acid have been investigated in detail [53]. The ability of lipases to catalyse this reaction seems to vary considerably, some preferring to perform the intermolecular esterification reaction.

Gutman et al. [54] found that porcine pancreatic lipase in anhydrous organic solvents catalysed the lactonisation of a number of esters of ω-hydroxy acids with high degrees of enantiomeric specificity. The same enzyme, however, exhibits low enantioselectivity for alkyl-substituted 5-olides [55].

(*R*)-5-Hexadecanolide can be readily obtained by stirring the corresponding racemic hydroxyester with lipase P (from *Pseudomonas* species) in isooctane to approximately 40% conversion [56]. The enantiomeric excess was estimated to

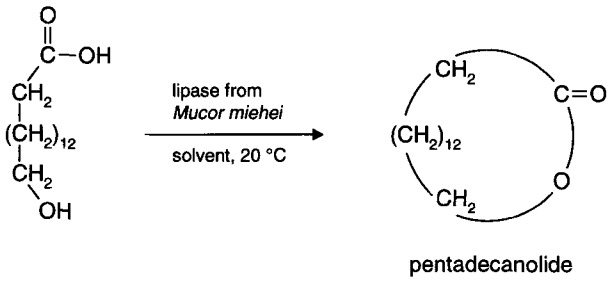


Fig. 11. Enzymatic synthesis of pentadecanolide from 15-hydroxypentadecanoic acid

be 70%. Immobilisation of the lipase led to an enhancement in the enantioselectivity. It was shown that an extensively denatured lipase prepared by heating in refluxing aqueous toluene for 24 h caused the formation of a racemic lactone. It was concluded that the lactonisation was catalysed not only by the active site of the enzyme, but was also promoted by the functional groups on the surface of the lipase.

These findings confirm those results reported on the normal ester synthesis using the lipase from *Mucor miehei* [57]. It could be shown that the ester-synthesising activity of this enzyme has little to do with its lipolytic activity. The latter is more or less completely lost early on during ester synthesis. Further synthetic cycles could be performed just as efficiently by using the enzyme which showed only very little residual lipolytic activity.

3.2. *Via Enantioselective Hydrolysis*

Optically active lactones can be obtained from the corresponding racemates by using a lipase or esterase capable of stereoselective ester hydrolysis. Thus, racemic 5-decanolide can be converted to (−)-(*S*)-5-decanolide with an enantiomeric excess of 64% when treated with horse liver esterase [58]. Other lipases capable of this stereospecific hydrolysis are porcine pancreas lipase and porcine liver esterase.

A similar conversion has been reported using a *Pseudomonas* lipase [59]. Thus, lipase P was allowed to react with racemic 5-decanolide. Asymmetric hydrolysis liberated the corresponding 5-hydroxydecanoic acid which, upon cyclisation, resulted in 5-decanolide with an enantiomeric excess of 83%

3.3. *Via Baeyer-Villiger Type Reaction*

The Baeyer-Villiger reaction is a reliable and useful reaction which allows the organic chemist to convert ketones into esters or lactones. Generally speaking, peracids are used to insert the oxygen atom into the substrate molecule.

It has been known for some time that certain microorganisms are capable of performing the Baeyer-Villiger reaction [60]. The enzymes responsible for this reaction are flavin-dependent monooxygenases.

Thus, the tridecanone-oxygenase from *Pseudomonas cepacia* is able to convert cyclopentanone into the corresponding δ-lactone, 5-pentanolide (Fig. 12). Similarly, whole cells of *Acinetobacter calcoaceticus* convert only one enantiomer of racemic 2-substituted cyclopentanones to give optically active δ-lactones [61] (Fig. 13).

Using cyclohexanone oxygenase, various lactones have been synthesised on a reasonably large scale [62]. Thus, monoterpenes such as L-fenchone, (+)-camphor and (+)-dihydrocarvone were converted in good yields to their corresponding lactones (Table 2) using the cyclohexanone oxygenase from *Leuconostoc mesenteroides.*

It is also possible to perform the Baeyer-Villiger reaction using isolated enzymes instead of microorganisms. Thus, the lipase of *Candida antarctica* converts some 2- and 3-substituted cyclopentanones and cyclohexanones into the corresponding δ- and ε-lactones [63]. This unusual reaction requires the presence of myristic acid and hydrogen peroxide. The lipase catalyses the formation of peracid through peroxide attack on the acylated enzyme. Thus, 2-hexylcyclopentanone and 2-methylcyclohexanone were oxidised to give the corresponding substituted lactones in 73% and 57% yield respectively. Not surprisingly, all the lactones thus synthesised were obtained in racemic form; the peracid obviously migrates away from the surface of the enzyme before interacting with the ketone.

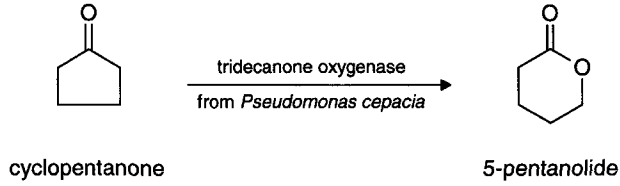


Fig. 12. Enzymatic conversion of cyclopentanone to 5-pentanolide

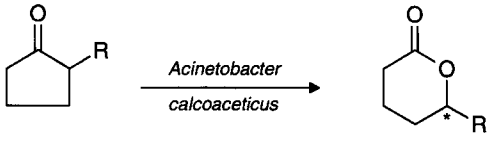
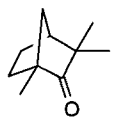
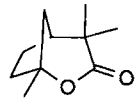
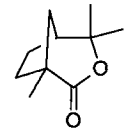
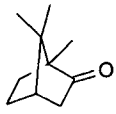
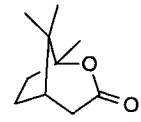
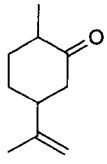
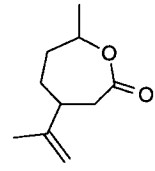


Fig. 13. Enantioselective conversion of 2-substituted cyclopentanone to the corresponding δ-lactone

Table 2. Synthesis of terpene lactones

Reactant (mmol)	Products	Reaction time (days)	Yield	
			mmol	%
	 and 	8	76	76
L-fenchone	1,2-fencholide 2,3-fencholide			
		10	45	89
(+)-camphor	2-oxabicyclo[3.2.1]1,7,7-trimethyloctan-3-one			
		10	30	75
(+)-dihydrocarvone	6-isopropenyl-3-methyl-2-oxacycloheptanone			

4 Conclusions

Generally, the flavour industry has been somewhat slow in understanding or accepting biotechnology as an integral part of industrial processes, but this attitude is changing. Microbial and enzymatic biotechnology have been introduced by many of the large flavour houses. In addition, the interest shown in flavour precursors by academic and industrial research groups indicates the importance of biotechnical processes for the further development of natural flavours.

A lot of progress has been made in the lactone field. Much of the success must be attributed to the low toxicity of the products towards the biocatalytical systems. Thus, amounts of lactones beyond the 1 g/l level can accumulate without significant inhibitory effect. Product yields in the range of 1 to 10 g/l are necessary if a fermentative production of lactones is to be viable. Such yields are only conceivable by exploiting the biotransformation of suitable natural precursors, which, in the case of lactones, are the corresponding hydroxy

carboxylic acids. Future processes will probably involve the enzymatic oxidation or the enzymatic stereospecific hydration of unsaturated fatty acids [64]. An alternative source of suitable fatty acids is modern agriculture which is striving to develop new crops. Thus, seed crops which contain hydroxylated or epoxy fatty acids are likely to be of importance in the future [65].

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The Production of Aromas by Plant Cell Cultures

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The adoption of a plant cell or tissue culture process for the production of various aroma compounds will depend on its ability to compete with current agricultural sources. To compete with the normal sources the plant culture process will, at least initially, involve low volume and high price compounds and high productivity, which requires a high yield of product. A wide range of aroma compounds have been detected in plant cultures, but the yields have been low (0.01–0.3% dry weight). Increases in yield have been obtained either by providing a site for the accumulation of the aroma compounds or by inducing some form of differentiation, such as root or shoots, into the culture. The scale-up of either suspension or organised cultures is possible as bioreactor cultivation of both types of culture has been successful, but there may be problems in obtaining high growth rates and biomass levels with organised cultures.

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1 Introduction

Higher plants are the source of many industrially important compounds. Plants serve as a source of industrial oils, flavours, fragrances, resins, gums, natural rubber, waxes, saponins and other surfactants, dyes, pharmaceuticals, pesticides, and other specialty products. Plants have been used for many years as a source of pharmaceuticals, starting perhaps in 1785 when William Withering reported the use of foxglove extracts to treat heart conditions [1]. However, due to advances in synthetic chemistry and the vast array of antibiotics produced by microorganisms the use of plant derived drugs has declined. Despite this trend some 25% of prescribed drugs are still extracted from plants [2], and with the recent interest in the ethnobotanical approach to new drug development, the targeting of plants used by native cultures as medicines [3, 4], plant derived products are still of great value. A recent example of an important plant derived drug is taxol, which is used to treat a number of cancers, and was isolated from *Taxus brevifolia*, the Pacific pine.

Plants as a source of flavours and aromas should not be forgotten as the world market for flavours is worth between two and three billion dollars per annum [5]. A great number of spices, condiments and beverages owe their characteristic properties of flavour and aroma to the secondary compounds that they contain. In many cases the flavour and aroma compounds were shown to be a very complex mixture which depends on the balance of the compounds to give the correct flavour or aroma. On the other hand, some flavours and aromas are due mainly to a single compound. The distinction between flavour and aroma is difficult, as when a flavour is volatile it becomes an aroma (Fig. 1).

The demand for aromas in the food and perfume industry, like that of the flavour industry, is increasing at a rate of 5–7% per annum. Some of the

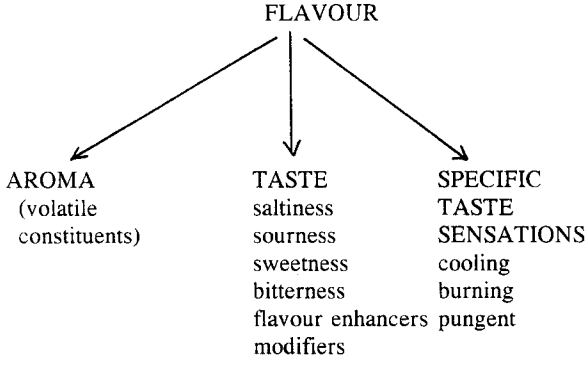


Fig. 1. Relationship between aroma and flavour

simple aromas and flavours can be produced chemically. Synthetic aromas are certainly important and play a major role in a number of industries due to their stability and strength. Despite the availability of synthetic aromas and flavours, many are still extracted from plants as the consumer has a preference for the natural product, and higher prices are paid for the product from natural sources [6]. In recent years the demand for natural products has increased greatly as a reaction against the increased use of chemicals. The meaning 'natural' may vary between countries. In the United States the term natural is defined as: the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavour constituents derived from a spice, fruit or fruit juice, vegetable, vegetable juice, edible yeast, herb, bark, bud, 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 nutritional (Code of Federal Regulations 21 CFR 101.22.a.3.) [5].

Therefore, although the aromas derived from natural sources are not as strong or as stable as many synthetic aromas, the industries that use them will be under pressure to increase their use. Most of the natural materials from which the aromas are extracted are not grown in their market areas but are found in less developed countries around the world. This means that the supply, quality and quantity can be variable for a wide range of reasons.

2 Plant Cell Culture

Plant tissue culture was originally developed as a research tool in order to study the biochemistry and physiology of plants. Interest in plant tissue culture began at the beginning of the century but it has been only in the last 30 years that the techniques have been sufficiently developed to encourage commercial interest. The first commercial interest in plant culture was the use of tissue culture for the micropropagation of plants, but more recently plant cell culture has been proposed as a alternative source of pharmaceuticals normally derived from plantation-grown plants. This has been encouraged by the detection of a large number of compounds over the years, some of which have been accumulated at quite high levels [7–10].

A factory system would be able to supply the product throughout the year in the right quantities and of the correct quality [11]. This could be especially useful for products derived from plants grown in remote districts, those subject to considerable variation in yield, limited supply and losses due to climate, disease, and pests. The same conditions apply to the use of plant cell culture for the production of those compounds responsible for aromas.

2.1 *Development of Plant Cell or Tissue Cultures*

The ability of an individual cell to grow and divide in a self-regulating manner was first expounded in the cell theory of Schleiden (1838) and Schwann (1839). Implicit in this theory is that the individual cells of an organism are totipotent, and it should be possible to regenerate a whole plant from a single cell, but despite considerable efforts this was not verified until 1965. Thus, plant tissue culture techniques enables plant cells to be separated and cultured on solid or in liquid media. The first plant tissues cultured were isolated root tips by Robbins in 1922, but since then much of the work has been carried out with callus. Callus is an amorphous mass of undifferentiated cells arising from the cells of the parent tissue.

A distinction needs to be made between organ culture and tissue culture. Root culture is an example of organ culture where the plant material maintains its morphological identity. Tissue culture is applied to any non-differentiated culture grown on solid medium or in liquid medium. The steps in the development of callus and other cultures are shown in Fig. 2. The first step is the removal of material from the whole plant, known as the explant. All multicellular plants are potential sources of explants, but the best material is that containing rapidly growing cells such as young leaves. Newly germinated seeds are especially good if the alternative is woody tissue. Most tissue culture is carried out under sterile conditions, as most contaminants will outgrow the cultured plant cells. Therefore, the explant is surface sterilised as even the cleanest plants have microorganisms associated with their surfaces. The nature of the explant will determine the form of sterilisation used, as tissues like roots will have a heavy infestation due to contact with the soil. Normal tissues are generally sterilised with sodium hypochlorite and/or ethanol. The disinfectants are non-specific, so the aim is to achieve sterilisation without killing the explant which will mean the optimisation of the time and concentration used. The explant once sterilised is normally placed on one of a number of media solidified with agar, although other gelling agents can be used. The medium contains all the major and minor salts required, and a carbon and energy source, usually sucrose. In addition to those components, the medium contains auxins and cytokinins. These growth regulators direct the growth elongation and differentiation of the explant cells, and, if the balance is correct, will cause them to grow rapidly in an unregulated state. The callus material which forms can be removed from the explant and maintained in vitro by routine subculture for long periods of time. Although useful for initial studies and storage, callus material grows slowly and is too heterogenous to be suitable for the development of a process. A process is best achieved by using plant cell suspensions which can be formed by placing friable callus material in a liquid medium of the same composition as the solid medium but without the gelling agent. Suspensions generally have a faster growth rate than the callus material and are more homogeneous. However, true homogeneity is not achieved since plant cells in suspension have a tendency to form aggregates. The main advantage is that the suspensions can be grown in

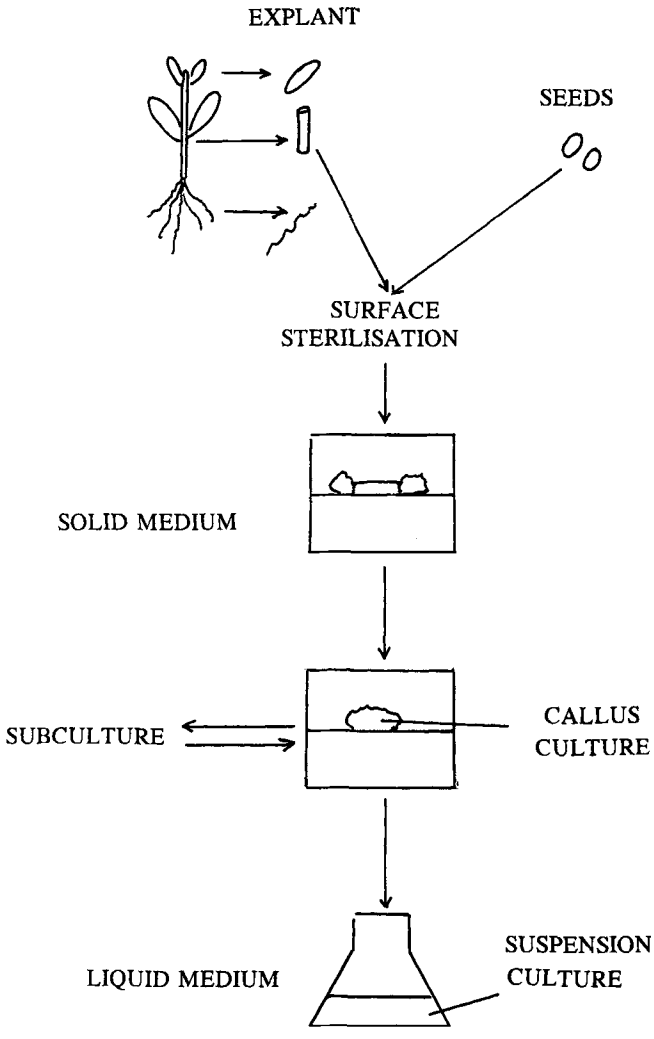


Fig. 2. The development of plant cell cultures

large volumes in bioreactors and thus have the potential for the development of a process. Callus and suspension cultures are not the only forms of culture that can be produced. By alteration of the growth regulator balance, excised roots and shoots can also be grown independently either directly or after excision from callus material (Fig. 3). In addition, infection of plant material with the bacteria *Agrobacterium rhizogenes* or *A. tumefaciens* leads to the formation of transformed root (hairy) or shoot cultures respectively.

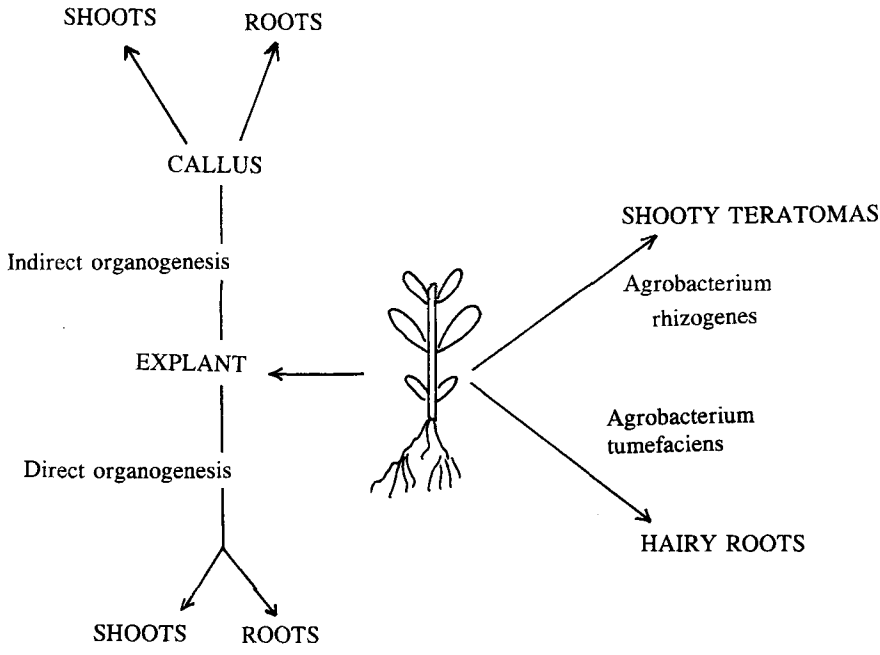


Fig. 3. The development of normal and transformed organ cultures

3 Potential Aroma Production by Plant Cell Culture

Commercial aromas, can be grouped into those used for perfumes, herbs, spices, fruits, and those miscellaneous aromas such as onion, peppermint and vanilla. The aroma components of any particular plant can be generally described as volatile and can be separated by distillation [12]. The distillate often is known as an 'essential oil', indicating that the extract is the essence of the plant odour. Early extraction was achieved at room temperature over a period of time by contact with odourless fats and was known as the 'enfleurage' method. Later the use of solvents or distillation replaced this method, as these were more rapid and cheaper. All these methods produce essential oils, but the compositions are somewhat different. For example, the enfluerage methods produces numerous oxygenated compounds not extracted by the other methods.

Essential oils are an extraordinary complex mixture of compounds, but are composed mainly of two types of compounds, terpenes and phenylpropanoids. The terpenes consist mainly of monoterpenes and sesquiterpenes and are far more abundant, but the phenylpropanoids are often the major aroma factor when they occur.

Table 1. Major components of thyme oil

Compound[a]	Content %
thymol	46.1
carvacrol	3.4
linalool	1.7
1-octen-3-ol	1.2
trans-sabinene hydrate	1.1
terpinen-4-ol	0.7
borneol	0.5
camphor	0.2
3-octanol	0.1

[a] Most significant volatiles of thyme oil, data from [13]

Aromas, like flavours, can be composed of specific mixtures of a large number of components. However, other aromas, although still mixtures, have a small number of components which are strongly associated with that aroma, for example jasmine with jasmone, geranium with geraniol and vanilla with vanillin. Table 1 shows the characteristic of the essential oil of thyme where the major component is thymol at 46.1% of the total oil.

4 Accumulation of Aroma Components in Plant Cell Cultures

A very wide range of volatile compounds has been detected in plant tissue cultures [12] and include terpenoids, phenylpropanoids, aliphatic compounds, polyines, glycosides and nitrogen-and sulphur-containing compounds. Table 2 lists some of those associated with the aromas from herbs, spices, fragrances, and other sources which have been detected in plant tissue culture. Many of the aroma compounds reported in Table 2 have been detected in callus material but only a limited number in suspension cultures (Table 3). From the 14 cultures that have been developed only 5 appear to produce the principle metabolite characteristic of the original plant. This confirms the view that, although there are some reports of monoterpene accumulation in plant tissue cultures, [39,44,45], more often the tissue, whether callus or suspension, fails to produce the correct monoterpenes, despite alterations in culture conditions and medium. In other cases there appears to be variation between species as *Mentha piperita* produces menthol [26], but *M. spicata* produces no monoterpenes [46], whereas *M. citrata* produces monoterpenes but no menthol [53]. Cultures of *Thuja occidentalis* produce monoterpenes but very different ones from the whole plant [45]. Thus the formation of aroma by plant cell cultures is very variable and will be difficult with complex mixtures.

Table 2. Aroma components detected in plant tissue cultures

Common Name	Botanical Name	Principal Metabolite	Ref[a]
angelica	*Angelica archangelica*	phellandrene/α-pinene	
anise	*Pimpinella anisum*	anethole/chavicol	[14–16]
balm (lemon)	*Melissa officinalis*	citral/geraniol	[17]
basil	*Ocimum basilicum*	methylchavicol/linalool	[18]
bay	*Pimenta officinalis*	eugenol	
bergamot	*Citrus aurantium*	L-linalyl acetate	
birch (sweet)	*Betula lenta*	methyl salicylate	
cade	*Juniperus oxycedrus*	cadinene	
calamus (flag)	*Acorus calamus*	β-asarone	
camomile	*Matricaria chamomilla*	α-bisabolene	[15, 19]
caraway	*Carum carvi*	D-carvone	
		D-limonene	
cardamon	*Elettaria cardamomum*	linalool/1,8-cineole	[20]
cedar wood	*Juniperus viginiana*	cedrene	
chocolate	*Theobroma cocao*	–	[21]
cinnamon	*Cinnamomum cassia*	cinnamaldehyde	
citronella	*Cymbopogon citratus*	geraniol	
(lemon grass)		citronellal	
		citral	
clove	*Syzygium aromaticus*	eugenol	
coriander	*Coriandrum sativum*	D-linalool	[22]
cumin	*Cuminium cyminum*	cuminaldehyde	
cypress	*Cupressus sempervirens*	furfural	
dill	*Anethum graveolens*	carvone	
fennel	*Foeniculum vulgare*	anethole/limonene	
fir	*Albies alba*	L-pinene	
gardenia	*Gardenia florida*	–	
geranium	*Pelargonium odoretissum*	geraniol	[23, 24]
grape	*Vitis vinifera*	–	[25, 26]
guava	*Psidium guajava*		[27]
hyacinth	*Hyacinthus orientalis*	esters	[28]
hyssop	*Hyssopus officinalis*	pinene	
jasmine	*Jasminum officinalis*	jasmone	[29, 30]
juniper	*Juniperus communis*	pinenes	
lavender	*Lavandula officinalis*	linalyl acetate	
	L. vera	pinenes	
linaloe	*Bursera delpechiana*	linalool	
marjoram	*Origanum majorana*	terpinene	
nutmeg	*Myristica fragrans*	myristicin	
onion	*Allium sativum*		[31]
orange flowers	*Citrus aurantium*	limonene	
		linalool	
oregano	*Origanum vulgare*	carvacrol/thymol	
patchouli	*Pogostemon cablin*	patchouli alcohols	[32]
chilli	*Capsicum frutescens*		[33]
pepper (Japan)	*Piper nigrum*		[34]
	(*Zanthoxylum piperitum*)		
peppermint	*Mentha piperita*	menthol	[35]
raspberry	*Rubus idaeus*	ketones	[36, 37]
rice	*Oryza sativa*	2-acetyl-pyrroline	[38]
rose	*Rosa damascena*	geraniol	[39]
rosemary	*Rosmarinus officinalis*	α-pinene/eucalyptol	
rue	*Ruta graveolens*	methyl nonyl ketone	
sage	*Salvia officinalis*	thujone/camphor	

Table 2. (continued)

Common Name	Botanical Name	Principal Metabolite	Ref[a]
sandalwood	*Santalum album*	santalols	
sassafras	*Sassafras albidium*	safrole	
spearmint	*Mentha spicata*	carvone/carvol	
spike	*Lavandula spica*	eucalyptol	
tarragon	*Artemisia absinthum*	methyl chavicol	[40]
thyme	*Thymus vulgaris*	thymol/carvacrol	[13,41]
vanilla	*Vanilla planifolia*	vanillin	
ylang-ylang	*Cananga odorata*	geraniol	

[a] Apart from where noted the data was obtained from [12, 42, 43]

Table 3. Possible sources of essential oils from suspension cultures

Common Name	Botanical Name	Principle Metabolite	Ref
anise	*Pimpinella anisum*	anethole	[14–16]
balm	*Melissa officinalis*	citral	[17]
basil	*Ocimum basilicum*	methylchavicol	[18, 52]
camomile	*Matricaria chamomilla*	α-bisbolene	[15, 19]
cocoa	*Theobroma cocoa*	–	[21, 53]
coriander	*Coriandrum sativum*	D-linalool	[22]
cypress	*Thuja occidentalis*	–	[45]
geranium	*Pelargonium odoretissum*	geraniol	[23]
grape	*Vitis vinifera*	–	[25, 26]
patchouli	*Pogostemon cablin*	alcohols	[32]
peppermint	*Mentha piperita*	menthol	[26]
rose	*Rosa damascena*	geraniol	[39]
tarragon	*Artemisia absinthum*	methyl chavicol	[51]
thyme	*Thymus vulgaris*	thymol	[13, 41]

Another important feature is the levels to which these compounds are accumulated, as a high yield is essential for the development of a process. Table 4 shows the concentration of essential oils accumulated in a number of suspension cultures. It is clear that even the best level of accumulation is low with values from 0.001–0.4% dry weight. Yields of 0.2–0.3% were only obtained when an accumulation site was added to the culture. These yields compare badly with the levels of other secondary products accumulated by suspension cultures (Table 5). It should be pointed out that many of the secondary products produced at very high levels, in excess of those values for some antibiotics, have no commercial value. In contrast, many of the high value pharmaceuticals are only produced at low levels, if at all, in suspension cultures.

The reasons for these low levels of aroma compound accumulating in plant cell culture are numerous and include the facts that aromas are complex mixtures, production of which is affected by the culture age and conditions, the essential oils have a high turnover, they are volatile and therefore can be lost

Table 4. Accumulation of essential oils by suspension cultures

Botanical Name	Concentration of Essential Oils	Refs
Matricaria chamomilla	0.06% F.Wt	[19]
	0.20% Dry Wt[a]	[15,16]
Mentha citrata	0.0035% F.Wt	[53]
Mentha piperita	0.0012% F.Wt	[53,54]
Ocimum basilicum	0.3×10^{-3}% Dry Wt	[18]
	0.026% F.Wt	[15]
Perilla frutescens	0.007–0.1% F.Wt	[44]
Pimpinella anisum	0.037% Dry Wt	[14]
Thuja occidentalis	0.3% Dry Wt[a]	[49]

[a]Yield achieved in the presence of Miglyol/activated charcoal.

Table 5. High yields of secondary products in plant cell suspensions[a]

Product	Plant Species	Yield (% dry wt)
Rosmarinic acid	*Coleus blumei*	21.4
Anthraquinones	*Morinda citrifolia*	18.0
Benzylisoquinolines	*Coptis japonica*	15.0
Acetoside	*Syringa vulgaris*	15.0
Shikonin	*Lithospermum erythrorhizon*	12.4
Berberine	*Berberis wilsonae*	10.0
Shikimic acid	*Galium mollugo*	10.0
Diosgeinin	*Dioscorea deltoidea*	7.8
Nicotine	*Nicotiana tabacum*	5.0
Serpentine	*Catharanthus roseus*	2.2

[a]Table adapted from [63]

from cultures, the essential oils are often cytotoxic, and accumulation normally occurs in specialised cells or structures such as trichomes or oil glands.

4.1 Complex Nature of Aromas

The essential oils are secondary products which are produced by pathways whose controls are not understood. Therefore, any technique for increasing the yield of essential oils can only be empirical, often using changes in the medium and cultural conditions to attempt to influence accumulation. This approach has been adopted for the accumulation of pharmaceuticals by tissue cultures with limited success [47] (Fig. 4).

Strain improvement via selection relies on the ability to arrange conditions so that those cells with a distinct characteristic survive, while all others are killed. In this case the characteristic required is the accumulation of aroma

1. Strain improvement (screening)	requires a non destructive method of detecting the product
2. Medium composition	
(a)carbon source	sucrose generally the best, an increase in concentration will often yield results
(b) nitrogen source	changes in form and concentration will enhance levels
(c) phosphate	high phosphate does not help accumulation
(d) growth regulators	changes can have considerable effect on accumulation, removal of 2,4-D particularly effective
(e) precursors	variable, but effective with some cell lines
3. Culture conditions	
(a) light	culture in the dark or light can have an effect in some cases
(b) temperature	lowering the temperature can increase accumulation
(c) osmotic stress	this can be caused by high sugar levels, and is effective
4. Specialized techniques	
(a) elicitation	treatment of cultures with fungal extracts or metal salts can give increased yields
(b) product removal	removal of product stops degradation and encourages production, effective for monoterpenes

Fig. 4. Approaches used to improve secondary product accumulation

compounds, and although there are some reports of selection schemes for some secondary products these are limited [8]. Far more success has been achieved with screening for high producing cell lines. Screening is favoured by the natural variation found in the heterogeneous plant cell population. Screening depends on the ability to detect the secondary product, preferably in a non-destructive manner, and therefore coloured or fluorescent products have been the easiest to work with. Those compounds which are colourless often require a sophisticated detection system such as the radioimmune (RIA) or enzyme linked immune (ELISA) system [75]. These systems are expensive, are not easy to use with mixtures and often require the destruction of the cell and extraction of the product. Despite this there have been some outstanding examples of screening of

Table 6. The effect of growth regulators and precursors on essential oil accumulation by thyme callus[a]

Growth regulators/Precursors	Total Oil[b]	Thymol	C15 aldehyde
IAA 1 mg/l: kinetin 1 mg/l	109.3	0.2%	50.4
IAA 10 mg/l: kinetin 1 mg/l	661.0	1.1%	47.7
2,4-D 1 mg/l: kinetin 1 mg/l	170.1	0.3	25.8
2,4-D 10 mg/l: kinetin 10 mg/l	554.3	0.4	27.9
mevalonic acid 100 mg/l	1242.5	0.3	9.6

[a] Data adapted from [13]
[b] Total oil μg/100 g fresh weight

both coloured and colourless products [76, 77]. Unfortunately the authors also noted that the line screens were unstable, and that any screen will also have to illustrate stability before the line can be incorporated into a process [78].

The accumulation of secondary products by cell lines is greatly influenced by the culture conditions and considerable work has been carried out on attempts to increase yields by alteration of the conditions. Figure 4 lists the possible changes and their effects. The best results have been obtained with changes such as reduction in 2,4-D or the use of other growth regulators, reduction in phosphate, increase in sucrose, and an alteration in the C/N ratio. The effect of growth regulators was best illustrated by the work on indole alkaloid accumulation by *Catharanthus roseus* [77]. Growth regulators have been shown to affect the accumulation of essential oils (Table 6), but the increases have only been small. The effect of changes in cultural conditions is often to impose stress on the cells which induces secondary product accumulation but reduces growth. This has given rise to the development of a two-stage process where the cells are grown in a growth medium, and once growth has finished the cells are switched to a production medium [11]. The relationship between growth and secondary product accumulation has been discussed in detail [79].

Stress can also be imposed on the cells by biotic (fungal extracts) and abiotic (metal salts) elicitors. In whole plants these types of elicitors initiate the formation of phytoalexins which are a plant's response to chemical and microbial attack [80]. In cell cultures this has stimulated secondary product accumulation but the results have been limited. The feeding of precursors has achieved variable success in increasing the yields of some products, but, as the rate-limiting step may be the enzyme activities, feeding may only work with cultures already generating the product.

4.2 Volatility

The volatile nature of essential oils means that they can be rapidly lost from a culture, which is particularly true if there are no sites of accumulation in the culture and the essential oils are exported to the medium.

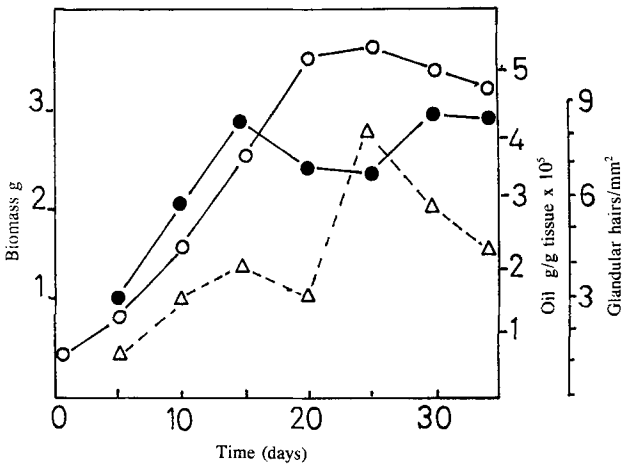


Fig. 5. Growth, essential oil accumulation, and glandular hair density during the growth of a shoot culture of *Pelargonium tomentosum*: (○) biomass g; (●) oil g/g tissue × 10^5; (△) glandular hairs/mm^2 (adapted from [63])

4.3 Cytotoxicity of Essential Oils

Many essential oils are cytotoxic [48] and because of this are often accumulated in the whole plant in specialised cells. Callus and suspension cultures do not have these specialised cells so that essential oils formed are often exported to the medium where they can inhibit growth, become degraded or lost due to their volatile nature. The effect of the presence of storage cells on accumulation of essential oil in a culture of *P. elegans* is shown in Fig. 5. It has been found that the provision of some form of external site of accumulation or continual removal of the essential oils increases the yield and in some case has allowed their detection for the first time [45]. The provision of an external site of accumulation may also avoid the feedback inhibition, intracellular degradation, removal or sequestering of the products. The external accumulation site can be in the form of a two-phase liquid culture incorporating organic solvents [81], a lipophilic organic phase like Miglyol [82, 83], perfluorochemicals [84], liquid paraffin [85] and polyethylene glycol [86]. In addition to a liquid phase, a solid phase can be used with adsorbants such as Amberlite resin XAD [87], RP-8 [16] and charcoal [58]. Table 7 gives examples of two-phase cultures and adsorbents used. Cells of *Pelargonium fragrans* in the presence of Miglyol gave increases of between 10- and 100-fold in monoterpene production and a shift of accumulation site from the cells to the medium [23]. An increase in growth rate was also observed, indicating that product toxicity may be operating. Addition of Miglyol to cultures of *Thuja occidentalis* increased the accumulation of

Table 7. Examples of artificial accumulation sites with plant cell cultures

Adsorbant	Culture	Reference
Miglyol	*Matricaria chamomilla*	[15]
	Pimpinella anisum	[15]
	Mentha canadensis	[56]
	Thuja occidentalis	[57]
Activated charcoal	*Matricaria chamomilla*	[58]
	Vanilla fragrans	[59]
RP-8	*Matricaria chamomilla*	[15]
	Pimpinella anisum	[15]
	Mentha piperita	[60]
XAD-2	*Thuja occidentalis*	[61]
XAD-4	*Vanilla fragrans*	[59]
	Thuja occidentalis	[62]
XAD-7	*Vanilla fragrans*	[59]

Table 8. Production of essential oils by various cultures of *Pimpinella anisum*[a]

	% Peak Area				
Essential Oil Component	Fruit	Shoot	Root	Callus	Suspension
anethol	92	50	4.9	60	–
methylchavicol	3.2	1.5	0.05	–	–
pseudoisoeugenol	2.7	16.2	4.8	0.4	0.5
epoxi-pseudoisoeugenol	10.5	25	63.5	17	0.2
myristicin	–	–	–	4	–
β-bisabolene	tr	6.2	15.5	8	1.5

[a] Data adapted from [14]

monoterpenes to 3 mg/g dry weight per day [49]. Anethole was only detected in cultures of *Pimpinella anisum* when RP-8 was present [16]. Addition of activated charcoal did not reduce the growth rate of *M. chamomilla* culture but increased the concentration of coniferyl alcohol 20-fold [58]. The disadvantage of the use of resin to accumulate essential oils can be their additional ability to remove growth regulators from the cultures.

4.4 Differentiation

One reason for the poor accumulation of aromas in callus and suspension cultures may be that they are under strict developmental control and that some form of differentiation is required for their production. It has been shown in some cases that as differentiation increases so does secondary product accumulation [70]. One example is shown in Table 8 where the accumulation of some of the aroma components of peppermint has been compared in the fruit, shoot, root, callus and suspension cultures of *P. anisum*. The main aroma component

Table 9. Shoot and root cultures of potential aroma plants

Botanical Name	Yield[a]	Reference
Shoots		
Lavandula augustifolia	n/a	[38]
Lavandula fragrans	50% plant	[63]
Mentha arvensis	10% plant	[64]
Pelargonium tomentosum	50% plant	[63]
Pelargonium graveolens	50% plant	[64]
Pimpinella anisum	0.056% plant	[66]
Rosmarinis officinalis	n/a	[38]
Zanthoxylum piperitum	n/a	[67]
Zingiber officinale	n/a	[68]
Transformed shoots		
Mentha citrata	25% plant	[102, 103]
Mentha piperita	n/a	[104]
Roots		
Pimpinella anisum	n/a	[69]
Zingiber officinale	n/a	[68]
Hairy roots		
Coriandrum sativum	n/a	[43]
Foeniculum vulgare	n/a	[43]
Pimpinella anisum	n/a	[43]
Cucumis sativus	n/a	[43]
Artemisia dracunculus	n/a	[43]
Ocimum basilicum	n/a	[43]

[a] n/a = not available

anethole is found in the shoot and callus cultures at levels above 50% of the fruit but the root and suspension cultures contained very little. Table 9 lists some of the root and shoot cultures, that have been developed, and, in a few cases, the yields of aroma compounds obtained. Shoot cultures have been shown to accumulate the correct balance of compounds for a culture of *Z. officinale*, but the overall level of essential oil was considerably lower than the whole plant [68].

4.5 Transformed Cultures

An alternative to root and shoot cultures are the genetically transformed roots and shoots which have been of considerable interest for the production of secondary metabolites. Most of the work has been carried out with "hairy" root cultures formed following the transformation by *Agrobacterium rhizogenes* [71, 72]. Transformation is generally induced on aseptic plants by inoculation with a culture of *A. rhizogenes*. After a few weeks roots emerge at the site of infection which is the result of transfer of plasmid DNA (Ri plasmid) to the plant genome. The transferred DNA expresses, among other functions, auxins which stimulate root formation. The roots formed can be excised, the bacteria removed by antibiotics, and the roots cultivated in growth regulator free medium. The

roots form a large number of lateral branches, grow rapidly, and are negatively geotropic [73]. The susceptibility of plant species to infection varies greatly, but at least 80 dicotyledonous plant species have been successfully infected [72, 74].

However, many secondary products are synthesised in the leaves and shoots of plants and therefore transformed shoot cultures have also been investigated for the accumulation of essential oils [81–83]. Shooty teratomas form after integration of the Ti plasmid from *A. tumefaciens* in a similar way to the formation of hairy roots. The exact nature of the response is not understood and there are only a limited number of reports of shooty teratomas (Table 9). Shoot cultures of *M. citrata* and *M. piperita* accumulated most of the major components found in the whole plant and oil glands were shown to be present on the shoots. However, the yield of essential oil was four times lower than in the whole plant [102]. After five years culture the pattern of accumulation in the *M. piperita* shoots changed with a reduction in menthol yields, but the *M. citrata* shoots remained stable.

5 Process Development

No matter what culture system is used, to be able to produce a secondary product able to compete with plantation grown material the productivity of the system has to be of a similar order as that for antibiotics, unless special conditions apply. Productivity is measured as g/l per day and is affected by the growth rate, biomass level, and the yield of secondary product [55]. Antibiotic yields of between 1 and 10% dry weight are often achieved, and it is this level that plant tissue cultures have been measured against. While it is true that high yield of secondary products have been obtained with cell cultures in the region of 18–21% dry weight [8, 9, 55], at present essential oils have only been detected at very low levels.

In contrast, the biomass levels and growth rates of large scale cultures of plant cells and tissues are encouraging. While most of the work on scale up has been carried out with suspension cultures, the production of aromas would appear to be best suited to differentiated cultures such as shoots and roots. This will require careful attention to the characteristics of the cultures used.

5.1 Suspension Cultures

The characteristics of suspension culture described in Table 10 affect the growth of plant cells in bioreactors. Many of the early cultures used stirred tank bioreactors and the largest reported to date is 75000 l [88]. The suspension cultures settle rapidly due to their aggregated nature and thus make sampling

Table 10. The differences between plant cell suspensions, organised cultures, and microbial cells

Characteristic	Microbial	Plant Cell Suspension	Organised Cultures
Size	2–10 μm	10–200 μm	200 μm <
Individual cells	often	not often, aggregates	cells in organs
Inoculation density	low	high, 5–10%	high, 5–10%, except hairy roots
Growth rates	rapid	slow	slow
(doubling times)	1–2h	2–5 days	2–8 days
Aeration	high	low	low
Shear tolerance	high	partial	low

difficult. The cultures have a slow growth rate and low aeration requirements. Much as been made of their shear sensitivity due to their large size, rigid cell wall, and large vacuole. However, research has shown that plant cell suspensions are not as sensitive as expected and that some cultures can be regarded as shear tolerant [89, 90]. Thus plant cell suspensions require good mixing and low aeration, both of which can be accomplished at a moderate level of shear. The level of mixing will, however, need to be increased greatly if high biomass concentration are required (20–40 g/l) to intensify the process.

In order to maintain good mixing at low shear rates the standard microbial stirred tank bioreactor was operated either at reduced impeller speeds or with a modified impeller. Many types of modified impellers have been used, and these include inclined blade turbine [91], anchor, helical [92], Intermig [88], sail [93], gate [94] and cell lift [95] (Fig. 6). The airlift bioreactor was used to cultivate plant cell cultures as it gave good mixing with low shear. The design proved to be successful but has problem with foaming and meringue formation [96], and it may not be so effective at high cell densities.

Other bioreactor designs have been used for the cultivation of plant cell suspensions with the same criteria of aeration and mixing at low shear. Examples are shown in Fig. 7 and include the rotating drum [97], silicone tubing [98], Taylor-Couette [99], and bubble column. The rotating drum mixes and aerates as it rotates slowly. It was developed for the growth of *Lithospermum erythrorhizon* for the production of shikonin, and was in some cases shown to be better than a stirred tank bioreactor [100]. The silicone tubing bioreactor was developed from animal cell culture where bubble-free aeration is required. The tubing is permeable to oxygen and carbon dioxide and therefore a coil inside the bioreactor will supply oxygen without the introduction of shear-forming air bubbles. In this case mixing was carried out by rotating the silicone tubing coil. The Taylor-Couette based bioreactor relies on the vortex created between a stationary drum and one rotating to mix the culture, and the aeration is supplied by a permeable inner drum [99]. All these designs were successful in cultivating plant cell suspensions, although only in a few cases were the designs

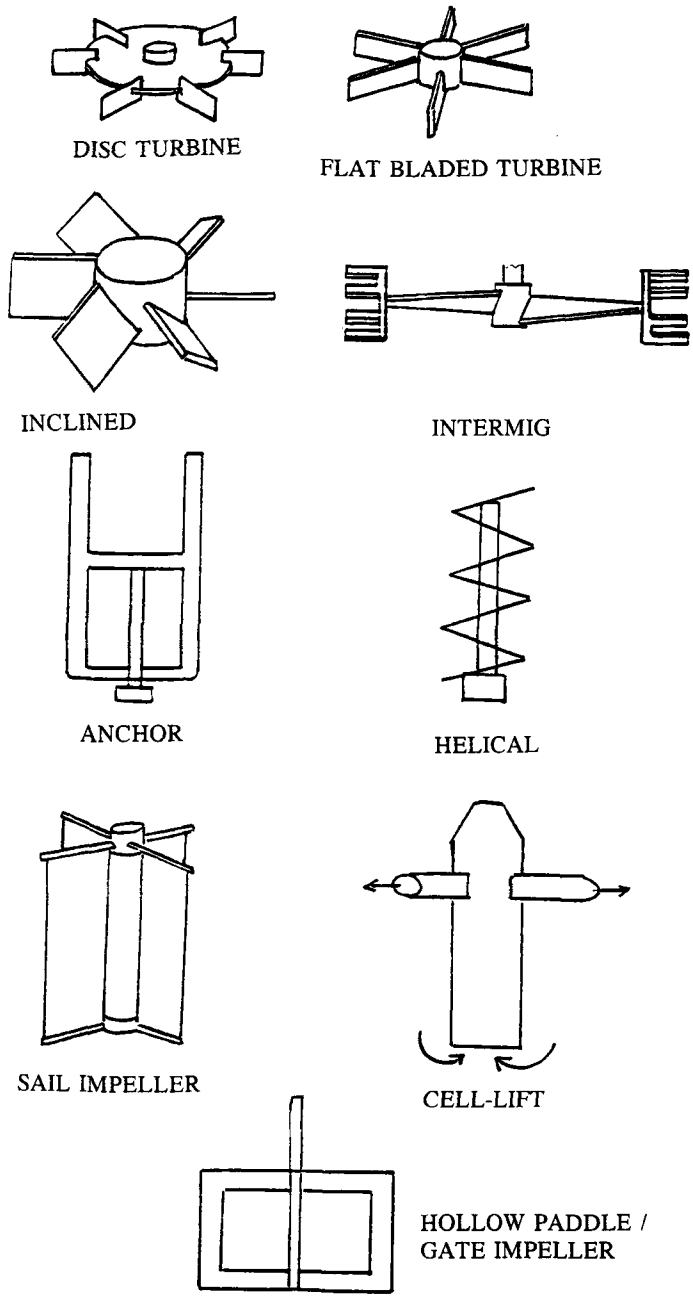


Fig. 6. Impeller designs for stirred tank bioreactors culturing plant cell suspensions (adapted from [10])

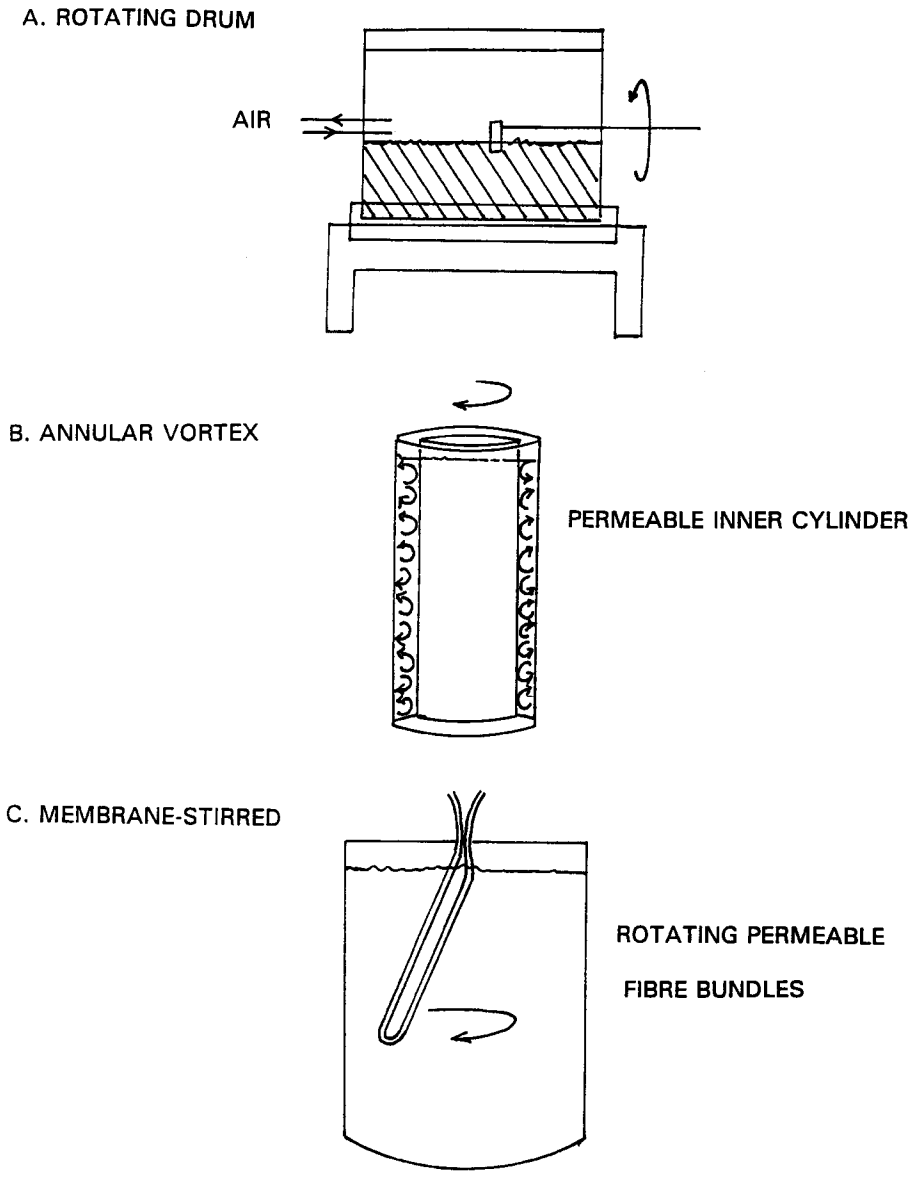


Fig. 7A–C. Three bioreactor designs for the cultivation of plant cell suspensions (adapted from [11])

directly compared with the same cultures [101]. However, with the tolerance of a number of plant cell suspensions to shear, these designs and modification may not be required. Where they may be required is in the cultivation of organized cultures where the large size of the roots and shoots will make them susceptible to shear.

Table 11. Bioreactor designs used for organised plant cultures[a]

Bioreactor		Culture	Form
Stirred-tank	0.3-l	*Armoracia rusticana*	hairy roots
Stirred-tank	(10-l)	*Atropa belladonna*	roots
Airlift	9.0-l	*T.foenum-graceum*	hairy roots
Airlift	0.3-l	*Armoracia rusticana*	hairy roots
Airlift	(square 2-l)	*Artemisia annua*	plantlets
Bubble column	(1-l)	*Nephrolepis exaltata*	shoots
Bubble free	(2-l)	*Euphorbia pulcherrima*	embryo
Mist		*Musa, Cordyline, Nephrolepis*	shoots

[a] Adapted from [105]

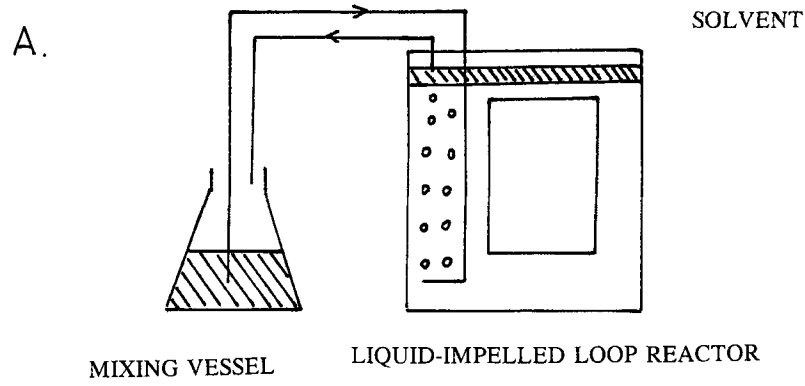
5.2 *Organised Cultures*

It has been shown that organised cultures are more likely to accumulate essential oils and that these organised cultures may need to be cultivated at high densities and volumes. Structures like roots, shoots and the rapidly growing and highly branched hair roots are sensitive to intense mixing [72]. These characteristics have encouraged the use of a number of different designs for the cultivation of organised cultures (Table 11) including many of those developed for suspension cultures. The largest bioreactor used to date for hairy roots is a 500-l modified stirred tank used to culture *Datura stramonium* hairy roots [106], and a review of other bioreactors is given by Toivonen [72].

The large scale cultivation of shoots has been mainly restricted to the production of whole plants and a number of bioreactor designs have been described [107]. Growth rates of 0.14 per day have been obtained for shoot cultures of *M. citrata* in shake flasks and biomass levels of 129 g/l (wet weight) in 500-l bioreactors [108]. Transformed shoots of *M. citrata* and *M. piperita* have been grown in 14-l modified stirred tank bioreactors. The yields of biomass were 2.5 kg for *M. citrata* and 3.5 kg for *M. piperita* after 60 days culture with essential oil yields of 0.007% and 0.033% respectively [104].

5.3 *Provision of an External Accumulation Site*

From the data presented in Sect. 4.3, the provision of an external accumulation site may also be needed to be incorporated into the bioreactor system. This can be the use of a two-phase system, such as the incorporation of Miglylol or a liquid- solid two-phase system incorporating resins such as XAD-7. There are reports on the use of XAD-2 to collect terpenoids released by a culture of *T. occidentalis* [62], and the release of scopolamine by a culture of *Duboisia leichhardtii* [109]. In the liquid-impelled loop reactor [81] (Fig. 8), the solvent added is lighter than water, and, when introduced into the base of the reactor, will rise and cause a circulation in addition to collecting metabolites.



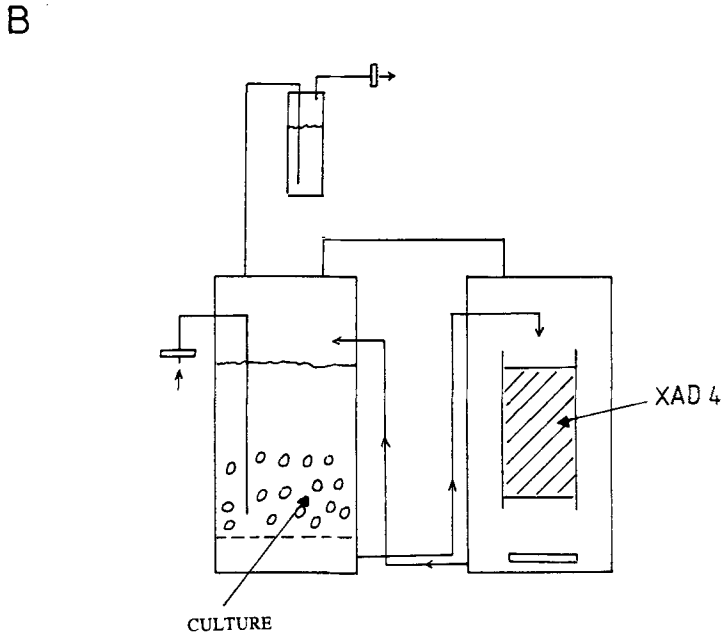


Fig. 8A. Liquid-impelled loop reactor. The lighter than water solvent is introduced at the bottom of the reactor and rising causes a circulation as well as accumulating essential oils (adapted from [81]). **B** A bioreactor in which the medium is circulated through XAD-2 resin (adapted from [62])

6 Conclusions

It is clear that if aroma production is to be achieved using plant tissue culture, then the productivity of the systems will need to be improved. Productivity is a combination of yield, biomass and growth rate. The yield of essential oils is

low when compared with other secondary products accumulated by plant cell cultures. The essential oils are more difficult than other secondary products because they are volatile and can be lost from the culture, often cytoxic so that accumulation slow or stops growth, and, due to toxicity, they are mainly stored in specialised cells or organs which are not found in normal plant cell cultures. These problems can be reduced by the provision of an external site of essential oil accumulation or the development of organised cultures which also contain these specialised cells. The addition of Miglyol or the use of resins like XAD-2 has been shown to increase the essential oil levels considerably but only to 0.3% dry weight.

Conventional techniques of media variation and screening have been successful in increasing the level of spontaneously accumulating compounds, but not where little or no product accumulates. This also appears to be true for essential oil accumulation.

The other problem of essential oil accumulation is that, in many cases, a complex mixture is required, and, in the case, production will be very difficult. The production will best be suited to an aroma where there is a single main component.

The problems of scale up, biomass, and growth rate should also be included in any discussion of the development of a process. Suspension and organised cultures have been grown in bioreactors to biomass levels of up to 10 g/l; however only suspension cultures have been cultivated at much higher biomass values. The growth rates of suspensions in bioreactors are as good as those in shake flasks, and shoot cultures have shown growth rates of 0.14 per day (doubling times five days). Due to the probable shear sensitivity of the organised cultures, modified or alternative bioreactor designs may be needed, but there are sufficient data on the growth of organised cultures to suggest that there should be few problems.

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