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# The Use of Enzymes and Microorganisms for the Production of Aroma Compounds from Lipids

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**Summary**

Lipids are an important source of aroma compounds. In foods, lipids are degraded or modified by enzymatic activities that are originally present in the raw materials or that develop later due to the growth of microorganisms. Mimicking these natural pathways, some processes have been developed to produce aroma compounds in bioreactors. In this review we describe the production of aroma compounds from different families: lactones, green notes and ionones. We focus on points that are specific to these reactions in heterogeneous media: physicochemical forces involved in the interactions between the substrate, product and biocatalyst, transfers between the phases and, as the degradation of lipids often requires an oxidation step, on the problems of oxygenation of the reactors.

*Key words:* lipids, aroma compounds, biotransformation, microorganisms, enzymes, emulsions, surface properties, oxygen

## Introduction

The world of flavour is very attractive especially because it concerns the taste of what we eat. From a scientific and technological point of view, this field is also highly exciting since it brings together several different branches of science. Flavour is usually the result of the presence, within complex matrices, of many volatile components of various chemical and physicochemical properties. The processing of mixtures of raw food materials can have various sensorial impacts depending on the properties of each compound. Processing modifies the equilibrium between the different components and, as a result, the original flavour will be perceived as being weaker and as »artificial« or »chemical«. The work of the aroma formulator consists in »constructing« a flavour recalling a »true and original aroma« in a processed food product with a specific texture and composition. To be able to formulate this flavour, a technologist needs a wide spectrum of different aroma components.

These compounds can be extracted from fruits or vegetables but, as they are required in the product in concentrations comparable to those in the source material, this utilises high amounts of materials and is generally not economically realistic. Most of them can also be synthesised in a chemical way resulting in »chemical compounds« that are not well perceived by consumers whose demand, especially in Western Europe, is in favour of »natural products«.

As an alternative, biotechnology proposes to use enzymes or whole cells to produce aroma compounds

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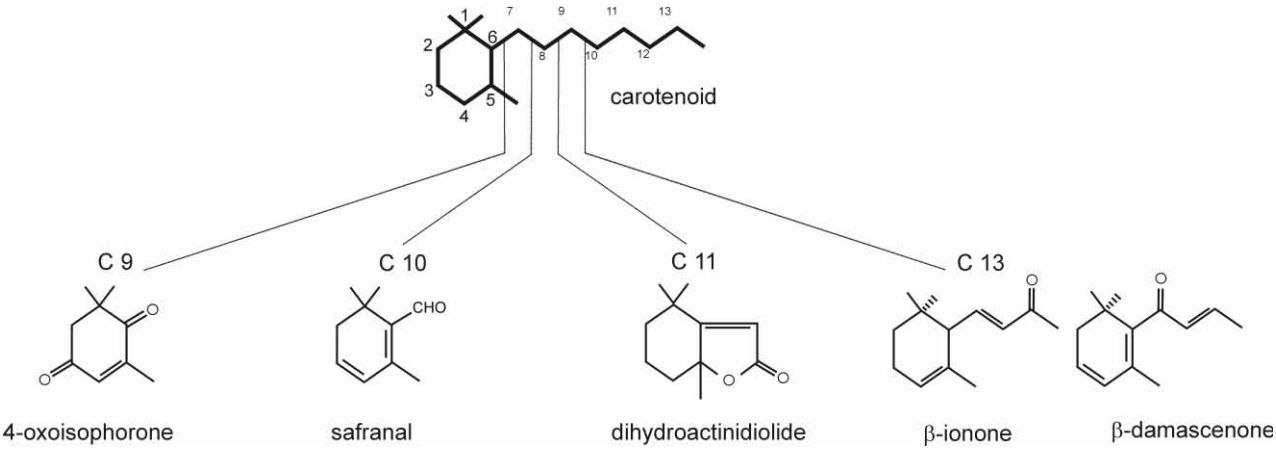


**Fig. 1.** Formation of some aroma compounds after cleavage of carotenoids

from natural substrates in a way often inspired by biochemical pathways encountered in nature. The challenge is to put a naturally rich source of substrate in contact with highly active enzymes. In adequate conditions, this can result in the production of flavour compounds in mass fractions of the order of several g/kg, instead of mg/kg encountered in raw materials. The resulting flavour compounds are called »natural« since they are produced from agro-products through natural biological activities. The ratio of isomers or isotopes is thus comparable to what can be found in extracted products and not to what results from chemical synthesis. However, although the productivity of some of these processes is good, the resulting products are usually more expensive than those from chemical synthesis.

There are various families of aroma compounds and the differences used to classify these families can be based not only on chemical structures, physicochemical properties or sensorial properties of the compounds but also, and in fact more commonly, on the chemical family of the substrate. On this latter basis, lipid-derived aroma compounds constitute one of the most important families, which include volatile fatty acids or esters, lactones, aldehydes, alcohols, ketones and some groups such as carotenoid-derived aroma compounds.

Although there are many investigations into the natural generation of these compounds in food products, only a few aroma components are produced by biotechnological routes. In this review, we present the biochemical pathways leading to some of these compounds and the related processes that have been inspired by them. Far from aiming at being exhaustive, we present selected examples to illustrate the main characteristics of the field. Three main pathways will be presented using animal, plant or microbial enzymes: carotenoid degradation, lipoxygenase/hydroperoxide lyase route and β-oxidation for the production of β-ionone, hexenal/hexanal and γ-decalactone, respectively. Some aroma compounds are also produced by lipases but these enzymes constitute in themselves such important catalysts that their utilization is reviewed regularly and we recommend the reader to consult numerous recent works (*1–3*). The substrates involved in these systems are water-insoluble, while catalysts are usually used in aqueous phases. The problems inherent in this heterogeneous production medium will be discussed and finally, new trends and strategies will be presented.

## Generation of Carotenoid-Derived Aroma Compounds

Carotenoids are natural pigments that are widely distributed in the vegetal and animal kingdoms. They are usually C40 tetraterpenoids built from eight C5 isoprenoid units (Fig. 1). Due to their double bond system, carotenoids may be oxidised directly by activated species such as free radicals (*4*). Apart from crucial functions in photosynthesis, photoprotection and nutrition, carotenoids are important aroma precursors (*5*). The odours of the resulting constituents depend on the number of carbons and the position of the oxygenated groups. They are formed via enzymatic oxidation and photooxidation from the various carotenoids found in plants, flowers and fruits (Fig. 1). These volatile compounds are important in the flavour and perfume industries. More particularly, the ionones are found in many fruit flavours, such as blackberry, peach and apricot, and in the odour of flowers such as violets. Many works have investigated the production of ionones from carotenoids by enzymatic, thermal or microbial degradation.

### *Generation of ionones by physicochemical treatments*

Due to the instability of carotenoids at high temperature and in the presence of oxygen, much attention has been given to the formation of volatile compounds during physical treatments of carotenoids. Mordi *et al.* (*6*) analysed the products formed during the autoxidation of β-carotene in benzene: carotenyl and peroxycarotenyl were formed and reacted with β-carotene leading to β--ionone, epoxy-β-ionone and dihydroactinidiolide within the first few hours. These compounds were also found in the autoxidation of all–*trans*-β-carotene in a liposomal system where the radicals were generated by the thermolysis of AMVN (azo-bis(2,4-dimethyl valeronitrile) at 37 °C (*7*).

Crouzet *et al.* (*8*) showed that the structures of β-carotene are first epoxidised and then cleaved during heat

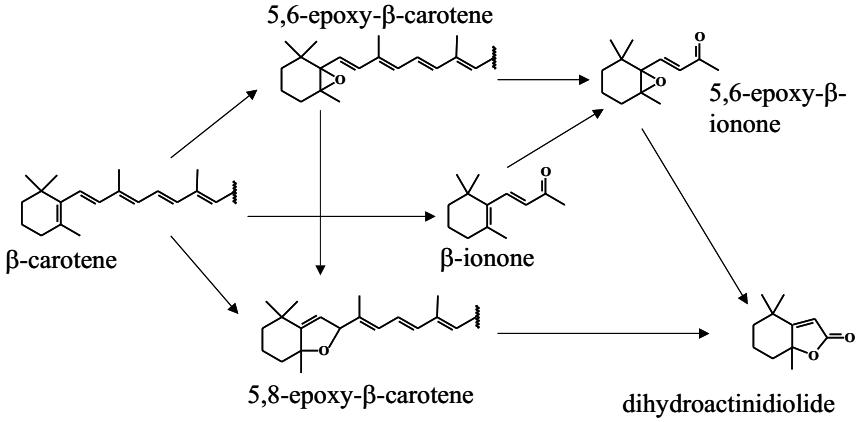


**Fig. 2.** Degradation of β-carotene to β-ionone and related compounds. Heat-induced degradation increases the epoxidation of β-carotene, resulting in epoxy-ionone and dihydroactinidiolide whereas oxidation-induced degradation favours first the cleavage of the C9-10 bond, leading to β-carotene

treatment, resulting in the formation of 5,6-epoxy-β--ionone, dihydroactinidiolide, β-ionone and β-cyclocitral (Fig. 2). These pathways have been reviewed recently (*9*).

In order to produce aromas from carotenoids that can be labelled as »natural«, other authors have studied the generation of volatile compounds by use of microorganisms or enzymes.

### *Generation of ionone by microorganisms*

Some fungi possess enzymes, such as polyphenol oxidase and lipoxygenase, responsible for cooxidation of β-carotene into volatile compounds. Lafosse (unpublished results) showed that fungi such as *Gyromitra esculenta*, *Clitocybe geotropa* and *Fibroporia vaillanti* are able to transform carotenoids to β-ionone and damascenone but the conversion yields are low. More recently, Zorn *et al*. (*10*) tested the degradation of β-carotene to flavour compounds with 10 fungal strains. Dihydroactinidiolide was formed as the sole conversion product of β-carotene in submerged cultures of *Ganoderma applanatum*, *Hypomyces odoratus*, *Kuehneromyces mutabilis*, and *Trametes suaveolens*. When mycelium-free culture supernatants from 5 species were used for the conversions, nearly complete degradation of β-carotene was observed after 12 h. Carotenoid-derived volatile products were detected in the media of *Ischnoderma benzoinum*, *Marasmius scorodonius*, and *Trametes versicolor*. β-Ionone was the main metabolite in each case, whereas β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone were formed in minor quantities. A photometric bleaching test was used to characterise partially the activities of β-carotene cleaving enzymes of *M. scorodonius*.

### *Generation of ionone by enzymatic action*

In nature, it is possible to obtain ionone from carotene by complex enzymatic degradation. Enzymatic oxidation of β-carotene through enzyme-generated free radicals has been investigated. The mechanisms are similar to those mentioned above in the physicochemical part. Different enzymes have been used to oxidise β-carotene in aqueous solution: phenoloxidase, lactoperoxidase, lipoxygenase and xanthine oxidase (*11*). The latter enzyme, which has a great potential for generating free radicals, was used to produce β-ionone by Bosser *et al.* (*12*).

#### Generation of ionone by lipoxygenase action

Our own group has investigated cooxidation of β-carotene in an aqueous medium in the presence of lipoxygenase. The process took place in two stages (*13*). Firstly, a lipase was used to liberate fatty acids from vegetable oil and then fatty acids reacted with a lipoxygenase to generate free radicals, which attack the carotenoid, leading to β-ionone and aldehydes. We have also worked with reverse-micellar systems but as the reactive oxygen species were only active in the aqueous phase, the amount of substrate was small and the production negligible (Waché *et al.*, unpublished results).

#### Generation of ionone by the action of xanthine oxidase

Xanthine oxidase (XO), an enzyme present in milk, was discovered a century ago and is still the subject of many studies dealing with kinetics (*14*) and with the identification of the radical species it generates (*15*). Bosser *et al.* (*16*) studied the production of β-ionone by the action of XO. The cooxidation reaction carried out by XO is complicated by the presence of two reactions: XO catalyses the oxidation of acetaldehyde-generating radical species that then cleave β-carotene to aroma compounds (Fig. 2).

The bleaching of β-carotene occurs quickly in the first hours of incubation resulting in β-ionone, 5,6-epoxy-β-ionone, and dihydroactinidiolide. However, β-carotene conversion yields to ionone are low because free radicals also react with ionone. In order to avoid the degradation of products, cooxidation of β-carotene in biphasic media with the action of XO was investigated (*17*). In an aqueous preparation, β-ionone is produced with a conversion of up to 15 % of the β-carotene after 6 h but the product is then degraded and only 9 % remains after 24 h. In biphasic medium containing 10 % hexane or 10 % benzene the amount of ionone increases about 11 times.

In biphasic systems, the preparation of β-carotene is important. If β-carotene is solubilised in the organic phase and if the enzyme and its substrate are in the aqueous solution, the bleaching of β-carotene is not observed. In

contrast, if β-carotene is dispersed in the aqueous solution within Tween droplets (*18*) then cooxidation of β--carotene occurs. The organic solvent is thus added only to extract reaction products (*17*). The conversion yields in the presence of organic solvents are higher than in aqueous media. The most important criterion for choosing a solvent is the ability to extract the reaction products without inhibiting the enzyme. Hexane and benzene enable a good enzymatic activity of XO, whereas for more polar solvents, such as dichloromethane, no cooxidation occurs, confirming earlier observations that enzymatic inhibition depends on the polarity of the solvent (*19,20*).

In order to obtain a better conversion yield, we continued to study β-carotene cooxidation in biphasic media. The problem was to extract β-ionone but not β-carotene and the intermediate compounds. With that aim we modified the ratio of the solvent and added the surfactant in excess (Ly *et al.*, unpublished results). By optimising these parameters, we obtained conversion ratios up to 34 % in a biphasic system containing 50 % solvent and an excess of Tween. These results highlight the importance of maintaining the substrate for sufficient time in the reactive aqueous phase before extracting the product.

Perspectives in enzymatic generation of ionones

Although conversion yields are becoming more realistic with optimised biphasic media, they can potentially still be improved by the use of cleavage enzymes specific for the C9-10 double bond. Such enzymes have recently been discovered (*21,22*) and, combined with the progresses in genetic engineering of carotenoids in microorganisms (*23*), the diversity of carotenoid-derived aroma compounds produced by biocatalysis could increase in the next years, including highly desired compounds such as β-damascenone or α-ionone.

## Generation of Green Notes – Lipoxygenase/Hydroperoxide Lyase Pathway

Volatile aldehydes and alcohols are key compounds in the »fresh« and »green« sensorial notes of vegetables and fruits (*24*). They are produced by plants in response to various stresses and they play a major role in plant defence mechanisms (*25*). The pathway is presented in Fig. 3. C18-polyunsaturated fatty acids are oxidised into 9-, 10- or 13-hydroperoxides, depending on the specificity of the lipoxygenase catalyst. These compounds are then cleaved by hydroperoxide lyase into C6-, C9- or C10-aldehydes, which can be reduced into the corresponding alcohols by yeast alcohol dehydrogenase.

### *Biotechnological production of green notes*

The natural metabolic pathway generating green notes in plants has been utilised or mimicked in order to produce natural aldehydes and alcohols (*26–29*). The highest demand from the flavour industry is for *cis*-3-hexenol and *trans*-2-hexenal (*30*), but other compounds are also utilised, such as 2,4-decadienal. In the basic processes, sources of lipoxygenase and hydroperoxide lyase, such as apple pomace (*31,32*) or soybean flour (*26*), are added to sources of polyunsaturated fatty acids such as vegetable oil. However, there is a problem of scaling-up, resulting from the instability of hydroperoxide lyase. Whether this enzyme is sensitive to hydroperoxides or to the reaction products is not known, but its activity decreases rapidly within the medium, suggesting a suicidal behavior of the enzyme (*28*). Moreover, crude extracts are often associated with isomerase activities converting *cis*-3-hexenal into *trans*-2-hexenal (*24*).

### *Expression of plant genes in microorganisms*

The characterisation, cloning and expression of many hydroperoxide lyase-encoding genes in microorganisms has led to proposals of new processes involving lipoxygenase-produced hydroperoxides and microorganisms containing plant-hydroperoxide lyase and alcohol dehydrogenase. Hydroperoxide lyase specifically produces the highly demanded compound hexenal from the hydroperoxide of linolenic acid and the more common hexanal from the hydroperoxide of linoleic acid. However, expression in a microbial cell may modify the activity. In a mixture of hydroperoxides, recombinant hydroperoxide

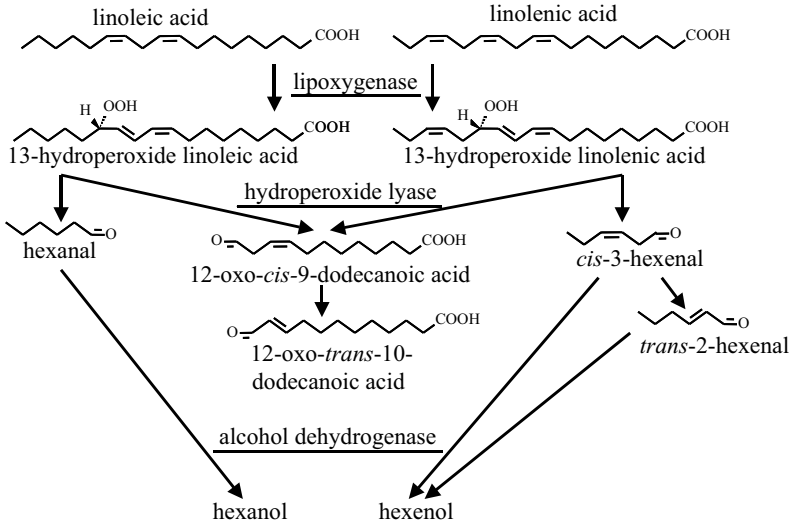


**Fig. 3.** Degradation of polyunsaturated C18-acids in the lipoxygenase/hydroperoxide lyase pathway and synthesis of green notes

lyase from alfalfa expressed in *E. coli* favours *cis*-3-hexenal production whereas the expression of the corresponding enzyme from green bell pepper in *Y. lipolytica* favours production of hexanal (*33*). Interestingly, the enzyme from green bell pepper favours the production of the unsaturated aldehyde (*cis*-3-hexenal) within the green bell pepper itself and when expressed in *E. coli* (*34*).

Other hydroperoxide lyases have been cloned and expressed in microorganisms: from tomato fruits (*35*), *Arabidopsis thaliana* (*36*), melon (*37*) and guava (*38*). This has resulted in several recently disclosed processes for the production of green notes (*39-42*). For expression of these heterologous genes, the wound-responding regulation system of the hydroperoxide lyase can be used (*43*) or, more conveniently, another expression system such as the lipid-inducible *POX*-promoter of *Y. lipolytica* (*33*).

## Generation of Aroma Compounds through β-Oxidation (γ-Decalactone)

β-Oxidation is the classical biochemical pathway involved in fatty acid degradation. It acts on an acyl-CoA and consists of a four-step reaction sequence, yielding an acyl-CoA which has two carbons less and an acetyl-CoA. This sequence is repeated several times until the complete breakdown of the compound (Fig. 4). Depending on many factors, the breakdown can be stopped before the theoretical end, liberating medium- or short-chain length volatile compounds. These metabolites can exit the pathway between two β-oxidation cycles or inside the sequence. This can lead to a variety of volatile compounds (Fig. 4).

Among the products presented in Fig. 4, γ-decalactone is the major compound produced by a biotechnological route although vanillin can also result from processes that use β-oxidation activities (*44,45*). Moreover, the production of this compound illustrates many of the problems encountered with this pathway and we will thus limit our presentation to it.

The possibility of producing a lactone using a biotechnological route was discovered in the 1960s by the group of Okui (*46,47*) during the studies of the catabolism of hydroxyacids in various organisms. *Candida tropicalis* degraded ricinoleic acid to C16, C14 and C12 acids and, interestingly, accumulated γ-decalactone, a lactone exhibiting fruity and oily notes important in the formulation of peach, apricot or strawberry aromas.

Since this observation, biotechnologists have devoted their efforts to the selection of yeast strains able to produce high amounts of this lactone. Many processes have been patented (*48-57*) that involve various microbial genera (including *Yarrowia*, *Sporobolomyces*, *Monilia*, *Pichia*, *Aspergillus* and *Cladosporium)* and reach values as high as 11 g/L (*56*). The production reached about 10 t in 1999 (*30*), which resulted in a sharp decrease in the prices from over 10 000 US$/kg in the early 1980s to approximately 300 US$/kg in 2004 (*58*). As a consequence, aroma producers are getting more and more interested in lowering the manufacturing costs. This has led to a change in the focus of the research in this field: previously it was largely devoted to the screening of strains or conditions, while now it addresses the biological

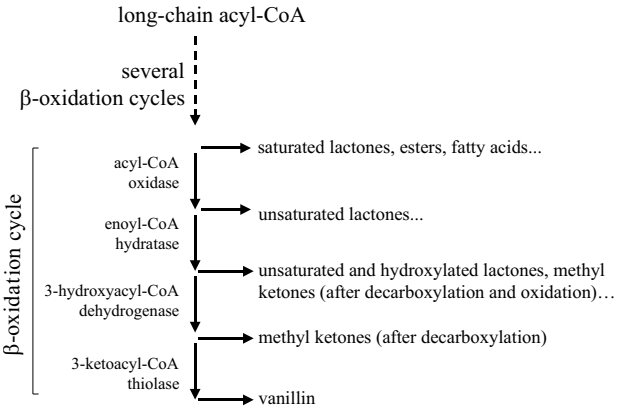


**Fig. 4**. β-Oxidation cycle and accumulation of aroma compounds

mechanisms of the catabolism of hydroxyacids. In fact, this has happened to such an extent that this widely applied field now generates a significant amount of basic results concerning yeast lipid metabolism.

### *Pathway of production*

The pathway from methyl ricinoleate to γ-decalactone and the involvement of β-oxidation have been deduced from the accumulation of intermediates. The first compounds detected were those accumulating between two β-oxidation loops (*47*), excluding the 4-hydroxydecanoic acid, which was probably lactonising spontaneously before detection, as has been reported for other 4-hydroxyacids (*59*). The detection of other intermediates improved with the use of biphasic media (*60*) or by processing the pathway *in vitro* (*61*).

The commonly accepted pathway from ricinoleyl-CoA to γ-decalactone is presented in Fig. 5: four β-oxidation cycles occur, yielding 4-hydroxy-decanoyl-CoA, which is then cyclised to γ-decalactone. Divergences occur within this general framework concerning how the double bond is dealt with. From the »β-oxidation auxiliary« enzymes present in *Saccharomyces cerevisiae*, Gurvitz *et al.* (*62*) proposed three possible pathways for C18-unsaturated fatty acids and intermediates detected during cultivation with methyl ricinoleate. One of these possibilities was confirmed in the case of *Y. lipolytica* (*63*) and added a new possibility in the case of *Pichia guilliermondii* (*64*). Due to our poor knowledge of the implication of auxiliary enzymes in β-oxidation fluxes, this point, up to now, has not been considered and investigations have focussed on the activities catalysing the four β-oxidation reactions.

*Y. lipolytica*, the species exhibiting the highest yields of production of γ-decalactone, is also the one possessing the highest number of genes coding for enzymes specialised in hydrophobic substrate degradation (*65*). It has thus become a model for the study of this pathway (*66*). This species possesses a family of six acyl-CoA oxidases (Aox1 to 6 encoded by *POX1* to *6*) comprising one short-chain-specific (Aox3) and one long-chain-specific (Aox2) enzyme. Interestingly, results obtained with *pox* mutants revealed differences between methyl oleate and methyl ricinoleate: most of the *pox* mutants exhibited

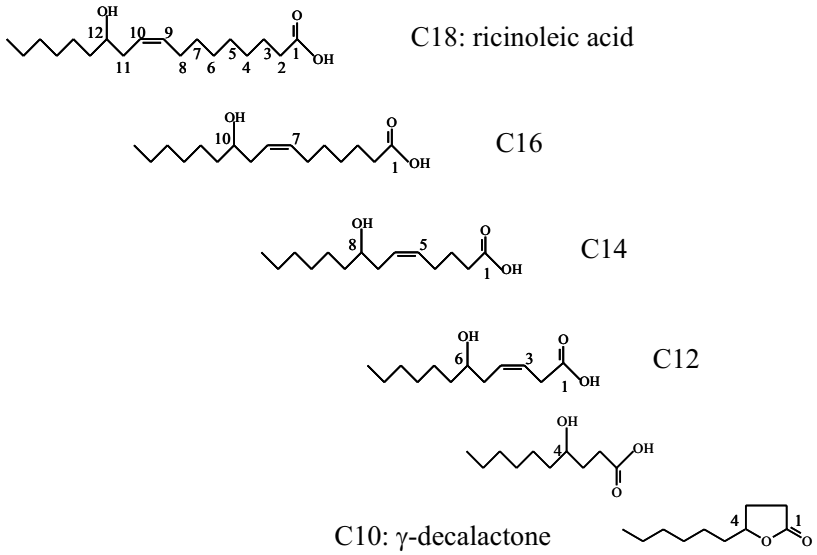


**Fig. 5.** Intermediates of γ-decalactone production from methyl ricinoleate

unaltered growth on methyl oleate while some of them grew more slowly on methyl ricinoleate. This highlights a very important point specific to ricinoleic acid: due to the hydroxy group, this acid is considerably more polar than straight acids. As a consequence, alterations of the genotype, which would not be detectable with methyl oleate, are significant with methyl ricinoleate. The fact that this hydroxylated substrate seems less fitted to β-oxidation enzymes probably explains why, at the C10 level, when the hydroxy group is in the γ-position, lactonisation occurs for a part of the substrate before oxidation, yielding γ-decalactone (Fig. 6). However, lactonisation can occur at the whole C10 stage resulting in other decalactones of variable interest (*63*): dec-3-en-4-olide has been described as exhibiting very powerful fruity notes but it appears at the same time as dec-2-en-4-olide, which is characterised by mushroom notes. These lactones are probably related to a deficient 3-hydroxyacyl-CoA dehydrogenase activity. This latter activity utilises $NAD^+$, giving rise to NADH. This cofactor is regenerated through a shuttle mechanism (*67*), which probably depends on mitochondrial respiration. The accumulation of these lactones occurs when the high cell density provokes oxygen limitation (*63*) and differences are observed between mutant strains with more or less altered growth (*68*).

Another point that was confirmed by *pox* mutations is that β-oxidation of C10 or smaller acyl-CoAs is responsible for a decrease in the yields and degradation of the γ-decalactone produced. Studies to construct mutant strains without acyl-CoA oxidase activity against short-

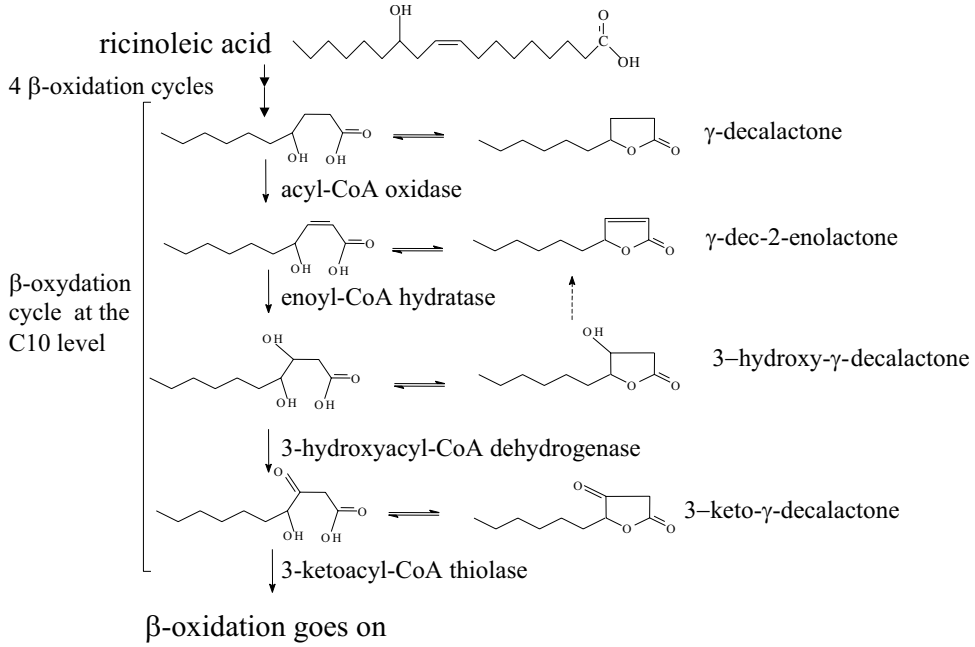


**Fig. 6.** Different lactones produced at the C10 stage of β-oxidation

-chain substrates were carried out, resulting in a strain unable to degrade this lactone (*69*,*70*). For some mutants, degradation was not reproducible, showing the difficulty of utilising genetically altered strains, which can sometimes utilise different pathways. It was also shown that acyl-CoA oxidase activities determined with straight chain acyl-CoAs might not be representative of the actual activity with hydroxylated substrates (unpublished results).

## Biotransformation from Lipids: Biological Catalysts within a Multiphase Medium

A biotransformation from lipids implies that a hydrophobic carbon source is present at a certain concentration in a medium that also contains an aqueous phase. Enzymes, organelles, cell extracts or cell populations can be used as catalysts. Improvements in process productivity require an understanding of the interactions and of the transfers within the system. We will focus in this second part on these aspects, taking as an example the production of γ-decalactone by *Y. lipolytica*, for which the biosynthesis pathway of the metabolite is now rather well known. Recent results highlight the impact of environmental conditions on the biotransformation and also on the regulation of β-oxidation fluxes.

### *Medium*

The heterogeneous medium containing an oil fraction turns into an emulsion with the aid of added surfactants and by absorbing part of the energy from the mechanical stirring applied. In most cases the oil forms a dispersed phase in a continuous aqueous phase containing the catalyst. The interfacial area between the organic and aqueous phases (and therefore the oil droplet size) becomes important in favouring the access of cells to the substrate and it is known that this characteristic influences the growth of microorganisms. The interfacial surface of emulsified methyl ricinoleate increases with agitation time and is not influenced by medium pH. The droplets present a wide range of sizes, from 0.05 to around 100 μm. The surfactant used and, to a lesser extent, its concentration in the medium also influences the interfacial surface, therefore these points have to be optimised (*71*,*72*).

During the production of aroma compounds, the organic phase also has the role of extracting the metabolites from the aqueous phase. This extraction may be important in minimising the contact between the metabolites and the cells, contact that can result in lactone or in yeast membrane degradation.

### *Interactions within the medium*

#### Cell-lipid interactions

The assimilation of lipids by microbial cells requires contact between the oil phase and the cells in order to have high rates of uptake of the substrate. Observations of *Y. lipolytica* during the biotransformation of methyl ricinoleate showed that the contact occurs mainly through the adhesion of small-sized droplets (with diameters less than 2.5 μm) on the surface of the yeast. Further investigations showed that these adsorptions are mediated in part by Lewis acid-base interactions rather than by hydrophobic interactions (*73*). Electrostatic forces, and thus the surface charges of the cells and of the droplets, are also implicated in the adhesion between both entities (unpublished results). Surprisingly, the cell wall of *Y. lipolytica* limits neither substrate assimilation nor γ--decalactone production, which can be deduced from the fact that protoplasts show the same behaviour as entire cells (*71*). This observation, together with the indication that polar interactions are required in the assimilation of the substrate (*73*), points to a mainly surfactant-mediated assimilation of methyl ricinoleate.

Surfactants, especially ionic ones, can have a toxic action on the cells, drastically reducing cell viability. On the other hand, neutral agents appear to be compatible with *Y. lipolytica*, however, the interfacial area obtained varies with the agent; the one giving the highest surface area is Tween 80 (*72*).

The surface properties may be intentionally modified in order to improve the cell-substrate adhesion. The surface charges may be altered, for example by modifying the medium pH or ionic force. Pre-culturing *Y. lipolytica* on a hydrophobic carbon source rather than on the classical glucose medium increases the subsequent adsorption of droplets on the cell surface (*73*).

Some other examples of surface modification for increasing adhesion are available in the literature. The addition of a chosen charged surfactant to the medium enables one to obtain lipid droplets with a controlled-charge and a consequent optimised adhesion (*74*); the cell surface charge can be modified by adding adequate salts such as $Al^{3+}$ to the medium (*75*).

The optimised conditions that we used for the production of lactone by *Y. lipolytica* enable a rather fast degradation of the substrate, with the maximum metabolite concentration reached after about 10 h. During the biotransformation, no intracellular accumulation of lipids occurs; such accumulation seems to happen mainly when the carbon/nitrogen ratio of the medium becomes very low (*71*). This point has to be checked since the intracellular accumulation of lipids can potentially lead to the entrapment of aroma compounds within the cells, since they take part in such hydrophobic environments.

#### Metabolite-cell interactions

High concentrations of aroma compounds reached during biotransformation may lead to toxic effects towards the producing microorganism. During lactone production by yeast, a progressive decrease in cell viability was correlated with the increase in metabolite concentration in the medium (*76*). Yeast cells are able to degrade γ-decalactone, however, the compound becomes toxic at a certain concentration threshold. Investigations of the mechanisms leading to toxicity showed that the hydrophobic lactone could take part in the cell membranes. This led *in vivo* to an increase in membrane fluidity, in a concentration-dependent manner. γ-Decalactone concentrations higher than 150 mg/L lead to a dissipation of cell membrane potential (*77*,*78*).

Retention of aroma compounds within cell membranes and a consequent toxic effect thus needs to be

considered for those compounds with hydrophobic properties. In order to reach high concentrations of the metabolites in the medium, existing strategies have to be applied (*76*), or new ones have to be imagined, notably by taking into account the »cell-membrane-fluidising« action of the compounds (*79*).

## Current Developments

Different aspects are being currently investigated, such as genetic engineering of the pathways (presented in the first part) and modifications of the redox environment in order to redirect fluxes. These investigations are resulting in significant improvements of the processes but they are not specific to lipid-derived aroma compounds and they have been discussed recently elsewhere (*80*,*81*). We will thus limit our discussion to a physical constraint that exists in every system but which attains higher complexity and importance in the heterogeneous media used in lipid degradation bioreactors, namely mass transfer.

### *Mass transfer within multiphase systems*

For bioprocesses in which oxygen is consumed, such as is typical for systems involving living cells, the mass transfer within the system is of crucial importance, especially if $O_2$ transfer can become a limiting step for the overall productivity. This question is gaining interest since the biotechnological applications of these systems have been increasing (*82*). It is of particular interest in the case of the production of γ-decalactone by *Y. lipolytica*, firstly because this yeast is an obligate aerobe, and secondly because not only the oxygen concentration but also the global redox environment (*83*) may influence the crucial step leading to the formation of other C10-lactones from 4-hydroxydecanoic acid, the direct precursor of γ-decalactone (*68*).

The addition of oil to an aqueous medium modifies its properties: the density, the viscosity and the solubility and diffusivity of $O_2$ are changed; consequently the access of the cells to $O_2$ may be altered. The presence of a dispersed oil phase in a continuous aqueous phase generally increases the oxygen mass transfer rates within the medium (*84*). However, for low concentrations of insoluble substrate, the presence of the organic phase can retard the gas to water mass transfer. Concerning the values for the overall $O_2$ mass transfer coefficient ($k_La$), the existing data are rather contradictory and general trends cannot be identified. Systematic measurements have to be done for a specific medium (*85*).

Within a two-phase system bioreactor, mass transfer most likely occurs in a series: from gas to water and from water to oil. Varying theories about the events at the gas--water interface also exist. The presence of a surfactant in the medium could complicate the transfer, but this point has still to be evaluated. Moreover, there is little data available on mass transfer from the gas phase to oil-in-water dispersions in biological systems in the presence of microbial cells, which can be considered as an additional solid phase.

Even if some means are available to increase the transfer of oxygen within the bioreactor, such as the use of oxygen carriers like perfluorocarbons (*86*) and silicone oils (*87*) or the use of hyperbaric air (*88*) or pure oxygen as sparging gas, it seems important to understand better the mass transfer in multiphase systems in order to predict and optimise the oxygen availability for the aerobic cells. This implies the measurement of oxygen transfer rates in order to determine $k_La$ and the use of models that involve these parameters to further optimise specific bioprocesses. Also, in the case of high oxygen concentrations, the possibility of cell stress must also be evaluated (*89*).

## Conclusions

The field of production of natural aroma compounds through biotechnological routes has evolved rapidly in recent years. The original process strategy of simply bringing together high concentrations of cheap substrates and enzymes seems to be over. There is now a need for better understanding of the mechanisms in order to improve significantly the processes. There are numerous developments involving genetic engineering although there are two difficulties. Firstly, the public perception of »natural aroma« produced through genetically modified organisms is not always good and, secondly, genetic engineering approaches are not always successful, due to the complexity of the potential biochemical pathways and of their regulation in the host microorganism.

However, it is probable that the data obtained with genetically engineered strains (for instance, regarding hydroperoxide lyase or β-oxidation) will help to improve classical processes and that the application of these strategies to the production of carotenoid-derived aroma compounds will revolutionise this field.

In complex pathways such as β-oxidation, the global redox equilibrium is responsible, through the regeneration of cofactors, for the accumulation of various products. This depends greatly on transfers inside the medium. These transfers are complicated by the heterogeneous nature of the medium and it is therefore probable that many improvements can be achieved through studies into reactor technology.

The field of production of natural aroma compounds is thus still in development and now requires highly innovative processes to increase the diversity of compounds that are produced in this way and to decrease the costs of production.

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# Korištenje enzima i mikroorganizama za proizvodnju aromatskih spojeva iz lipida

**Sažetak**

Lipidi su važan izvor aromatskih spojeva. U hrani su razgrađeni ili modificirani djelovanjem enzima izvorno prisutnih u sirovini ili naknadno razvijenih zbog rasta mikroorganizama. Oponašajući prirodni put, razvijeni su određeni procesi za proizvodnju aromatskih spojeva u bioreaktorima. U ovom je revijalnom prikazu opisana proizvodnja aromatskih spojeva u bioreaktoru iz laktona, jonona i spojeva neškodljivih za okoliš. Usredotočili smo se na uvjete specifične za ove reakcije u heterogenom mediju: na fizičko-kemijske sile u interakciji supstrata, produkta i biokatalizatora te transfera između faza. Budući da degradacija lipida često zahtijeva oksidacijsku reakciju, posebna je pozornost posvećena oksigenaciji u reaktoru.