Microbial Production of Natural Flavors

By degrading fatty acids from plants and other natural sources, microorganisms generate an array of aromas and flavors

Alex Häusler and Thomas Münch

For thousands of years, humans have used microorganisms, much of the time unwittingly, to produce more flavorful foods and beverages. Their use for producing wine and beer dates to very early times, with wine production in Egypt and Assyria traced at least as far back as 3500 B.C., and the brewing of beer in Babylon as long ago as 2800 B.C.

Despite such ancient uses for making familiar foods and beverages, however, the role of microorganisms as natural producers of flavor and aroma compounds was not well recognized and scientifically acknowledged until the early part of this century. Thus, for instance, one of the earliest reviews on this topic was written by the Russian microbiologist V. L. Omeliansky about 75 years ago.

Both before and since then, of course, microbiologists have often appreciated and shown keen interest in the smelly side of their subject matter. Indeed, even with the availability of sophisticated instruments, many modern microbiologists still use their noses as analytical tools to help in identifying the characteristic smells of various microorganisms on which they work—for instance, the earthy, musty odor of actinomycetes attributable to geosmin; the typical organic, sometimes fruity aromatics released during yeast fermentations that arise from alcohols and esters; and the unpleasant off-smelling scents of the indoles released by many enterobacterial species.

Flavor Industry’s Interest in Microbiology Intensifies

During the past 10 to 15 years, a more deliberate study of flavor- and scent-producing microorganisms has intensified, driven no longer strictly by the need to identify the subject matter but now also by an expanding flavor industry seeking new ways to meet the demands of its diverse clientele.

Most natural food flavoring ingredients are produced by means of traditional processes that depend on materials of plant origin, such as essential oils, fruit or vegetable juices, and plant concentrates or extracts. For example, more than 65% of all flavoring ingredients used commercially in the United States are labeled as natural and have a food market potential exceeding $9 billion. The variety of such natural flavor and aroma ingredients is somewhat limited.

However, the new molecular methods developed as part of the biotechnology industry now make it possible to supplement and enhance these basic plant-based flavor ingredients, converting relatively cheap starting materials into higher-value flavor and aroma additives useful in foods, beverages, cosmetics, and other consumer items. Intact microorganisms or microbially derived enzymes can transform natural precursors into valuable single-flavor molecules, called impact substances or top notes, or useful flavoring mixtures, known as flavor building blocks.

Microbiologically Modified Ingredients Qualify as Natural

In the U.S. and European regulatory systems, microbiologically modified natural ingredients are also considered “natural.” Thus, the term “natural flavor” is defined as follows in the United States (Code of Federal Regulation, 21 CFR 101.22.a3.1990):

“... the term “natural flavor” or “natural flavoring” means the essential oil, oleoresin,
essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating or enzymolysis, which contains the flavoring 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 flavoring rather than nutrition.”

Similarly, the European community (EC Flavour Directive 88/388/EEC) goes by the following definition:

“...flavouring substances or preparations which are obtained by appropriate physical processes ... or enzymatic or microbiological processes from material of vegetal or animal origin ...”

Both regulations explicitly include as natural those products that are produced through enzymatic and microbial processes, requiring only that the precursor material be natural and that both the precursor and the modified product can be found in nature or are part of traditional foods.

**How Microorganisms Produce Valued Flavors from Fatty Acids**

Among the wealth of microbial and enzymatic bioconversion processes, many act on fatty acids. Those fatty acids that derive from plant oils or animal fats are usually cheap and readily available on a large scale, and are the natural origin of many flavor and fragrance molecules. Such materials often are extracted in the form of triglyceride oils; commercially available lipases readily release commonly used fatty acids from such oils (see table). Established industrial processes are used to produce many fatty acid flavor and aroma derivatives, including compounds that provide so-called “green notes,” mushroom flavors, and specific aroma-conferring lactones and methylketones.

<table>
<thead>
<tr>
<th>Sources of precursor fatty acids</th>
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<tbody>
<tr>
<td><strong>Precursor fatty acids</strong></td>
</tr>
<tr>
<td>(chain length: unsaturation)</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
</tr>
<tr>
<td>C4 to C12</td>
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<td>C8 to C12</td>
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<tr>
<td>C10 to C18</td>
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<tr>
<td>Unsaturated fatty acids</td>
</tr>
<tr>
<td>C18:1 oleic acid</td>
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<tr>
<td>C18:2 linoleic acid</td>
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<tr>
<td>C18:3 linolenic acid</td>
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<tr>
<td>C20:4 arachidonic acid</td>
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<tr>
<td>C20:5 eicosapentaenoic acid (EPA)</td>
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<tr>
<td>C22:6 docosahexaenoic acid (DHA)</td>
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<tr>
<td>Hydroxylated fatty acids</td>
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<tr>
<td>C18:1-OH ricinoleic acid</td>
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<tr>
<td>C16:0-OH 11-hydroxypalmatic acid</td>
</tr>
</tbody>
</table>

In general, when microorganisms degrade fatty acids, the first step involves detoxification by esterification with coenzyme A (CoA) as the fatty acid molecule is taken into the cell. Once inside the microbial cell, the CoA-fatty acid derivative is subject to oxidative breakdown by means of a number of metabolic pathways, with the β-oxidation cycle predominant. Microbial dioxygenases (lipoxygenases), monooxygenases (hydroxylating cytochrome P-450 enzymes), or hydratases acting on double bonds also can partly degrade fatty acids and can be important for producing sought-after flavors and aromas.

Lipoxygenases are dioxygenases that act on cis-cis pentadiene units of polyunsaturated fatty acids to form hydroperoxide derivatives of fatty acids. Although such enzymes are distributed ubiquitously in nature, only a few have been found in microorganisms. In mammals, for example, lipoxygenases are involved in the formation of prostaglandins and leukotrienes, which derive from polyenoic fatty acids.

Meanwhile, in disrupted plant tissues, lipoxygenases degrade unsaturated fatty acids such as linoleic or linolenic acid, forming hydroperoxides, which are subsequently cleaved by hydroperoxide lyases to form volatile aliphatic six- and nine-carbon compounds, all of which are important flavor and fragrance molecules (Fig. 1). The most important members of this family are cis-3-hexenol (leaf alcohol) and trans-2-hexenal (leaf aldehyde), which are responsible for
the “green” character of cut grass and the aromas of many fruits and vegetables. The enzymes used in this metabolic pathway, however, are not known to occur in bacteria or fungi.

Another plant fatty acid metabolite, jasmonic acid, an endogenous plant growth regulator with a variety of physiological functions, is produced by means of a similar metabolic pathway. After a lipoxygenase produces a hydroperoxide derivative of linolenic acid, this compound is converted to its allene oxide, which cyclizes. β-Oxidation and double-bond reduction yields jasmonic acid. The methyl ester of jasmonic acid is not only a volatile plant hormone, possibly involved in interplant communication, but is also an important flavor and fragrance molecule that imparts a sweet-floral, jasmine-like note (Fig. 2).

Otto Miersch and his collaborators at the Institute of Plant Biochemistry, Halle-Saale, Germany, who were studying fungal plant pathogens, including Botryodiplodia theobromae, discovered that such microorganisms produce jasmonic acid. The biosynthetic steps leading to jasmonic acid in this filamentous fungus are probably similar to those found in plants. Recently our laboratory, which is evaluating this strain’s capacity for producing jasmonic acid, found that B. theobromae yields only very low concentrations of jasmonic acid in liquid culture. Such findings suggest that the biosynthesis and excretion of jasmonic acid is strictly controlled during the growth cycle of this fungus on plants in its natural habitat.

Another lipoxygenase-dependent enzyme in certain fungi forms 1-octene-3-ol and additional eight-carbon alcohols and aldehydes. This group of molecules is responsible for the typical mushroom aroma reminiscent of the common mushroom, Agaricus bisporus. This fungal lipoxygenase, which oxidizes linoleic acid to form 10-hydroperoxy-linoleic acid, has unique specificity, according to Werner Grosch and his colleagues at Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany. This important flavor molecule is currently being produced on an industrial scale from otherwise wasted mushroom stems by adding linoleic acid to them as a precursor.

**Modified Lactones, Another Class of Microbially Modified Flavors**

Lactones derived from hydroxy fatty acids belong to the most important class of flavor components in many fruits, milk products, and fer-

![Figure 1](image1)

Degradation of unsaturated fatty acids by the lipoxygenase pathway. Products from the cleavage of linolenic acid hydroperoxide by the hydroperoxide lyase, the six-carbon aldehydes and alcohols (called green note compounds) are depicted on the left, and to the right a C12-oxoacid (12-oxo-Δ12-9-dodecenioic acid, not depicted), which is the direct precursor in plants for the “wound” hormones traumaat and traumatic acid, is produced. Epoxidation and cyclization of the hydroperoxide leads to another plant hormone, jasmonic acid, the precursor of methyljasmonate. Jasmonic acid but not the green note compounds can be found as a metabolite of microbial origin.

![Figure 2](image2)

Methyl-(+)-7-iso-jasmonate is the only stereochemistry which is active both as a flavor molecule and a phytohormone.
ment foods. The olfactory properties of such lactones depend on chain length, degree of saturation, size of the lactone ring, and chirality. Organoleptic descriptions for such lactones vary from fruity, peach, and coconut to fatty or even flowery. Such lactones are used extensively in the food industry. For instance, γ-decalactone, which is produced by fermentation on an industrial scale and is one of the few natural commodity flavor chemicals, is used in a wide range of products, enhancing both fruit and dairy flavors.

Many microbial species, particularly yeast strains such as *Sporidiobolus salmonicolor* (formerly *Sporobolomyces odoratus*), produce γ- or δ-lactones. They are formed by means of β-oxidation of saturated and unsaturated hydroxy acids followed by lipase-catalyzed or spontaneous ring closure—“lactonization”—under acidic conditions. The largest problem for industrial production of lactones is the limited natural abundance of hydroxy acids. The only precursor currently available in sufficient quantities and at reasonable prices is ricinoleic acid (12-hydroxy C18:1), the main constituent of castor oil.

A castor oil-based biotransformation process yielding high amounts of γ-decalactone was described and patented in 1983 by researchers at the flavor company Fritzche, Dodge and Olcott (now part of Givaudan Roure). This process involves use of the fungus *Yarrowia lipolytica* to hydrolyze and oxidize an ester of ricinoleic acid.

Similarly, δ-lactones can be generated from 1,1-hydroxy-palmitic acid in sweet potatoes (known as jalap resin). Only limited quantities of this hydroxy acid occur in sweet potatoes, making isolation for large-scale production too costly. Alternatively, *Mucor* and *Mortierella* species might be used to hydroxylate medium-chain fatty acids or their respective methyl or ethyl esters to form readily lactonized γ-hydroxy-octanoic and -decanoic acids. Very little is known about the mechanism of mid-chain hydroxylations. Such fatty acid hydroxylases may be related to cytochrome P-450 ω-hydroxylases found in bacteria and fungi, such as *Bacillus megaterium*, *Candida bombicola*, and *Fusarium oxysporum*. Similar end-chain hydroxylating enzymes activities are also found in *Mucor* species (Fig. 3), accounting for some of the side products found during lactone production.

**Flavorful Methylketones Derive from Fatty Acids**

Methylketones (2-alkanones), which also derive from medium-length fatty acids, confer strong cheese-associated flavors and are the basis for flavor development in cheeses such as Roquefort, Camembert, and Stilton. These methylketones form when the molds used to ripen such cheeses degrade medium-chain length fatty acids, using β-oxidation enzymes to generate methylketones that are one carbon shorter than their respective precursor fatty acids (Fig. 4). The most prominent methylketones include 2-pentanone, 2-heptanone, 2-nonanone, and 2-undecanone arising from fatty acids with 6- to 12-carbon chain lengths.

Many of the parameters that regulate this metabolic pathway in molds used for ripening cheeses are extensively documented in patents.
and other publications describing the production of methylketones. However, how this process is controlled at the cellular level is not known.

Microbial methylketone formation is strongly favored under conditions in which mold growth is constrained—for example, during sporulation. Perhaps methylketone synthesis is induced when cellular levels of coenzyme A become limited, thereby reducing 3-ketoacyl-CoA thiolase activity. Curiously, fungi do not produce methylketones from long-chain fatty acids even when growth is restricted. Instead, the β-oxidation cycle proceeds to completion. Presumably, such microbial cells contain more than one system for dealing with fatty acids of different chain lengths.

Methylketones are produced on a large scale by means of several different biotransformation processes. One of the simplest involves applying spores of Penicillium roqueforti onto medium-chain-length fatty acids or lipase-treated milk fats. Other more sophisticated technologies involve solid-state or two-phase fermentation systems. For example, according to a patent granted recently to the French food production company Sanofi, 2-heptanone is produced from octanoic acid by using an immobilized filamentous fungus from the genus Amastigomycota.

**Industrial Realization of Flavor Bioprocesses**

Developing new microbially based industrial processes for producing flavors represents a considerable challenge. For one thing, having economically operational fermentation equipment is almost a prerequisite before beginning bioengineering research and development efforts (see box, p. 559).

Nonetheless, a wide variety of microorganisms capable of synthesizing potentially valuable flavor compounds is partly characterized, according to the scientific literature. Yields of these compounds, however, are often disappointingly low, rarely exceeding 100 mg/liter and almost never reaching a level that would make production by this means economically competitive.

Thus, the first challenge facing those interested in developing microbiologically based means of flavor production is to improve yields of the product dramatically, either by traditional means such as screening for better strains or by more recently developed genetic engineering methods.

Because most flavor compounds (and, often, their precursors) tend to be inhibitory or even toxic to microorganisms, this challenge is even greater than it first appears. For example, typical fatty acid flavor precursors, especially in the undissociated forms that are prevalent below pH 4.5–5, are significantly cytotoxic, and cellular uptake is limited. Different organism-specific mechanisms account for this limitation. In bacteria, for example, specific uptake proteins...
in the culture vessel, we obtained a fatty acid conversion rate of up to 0.8 g/liter per hour. Maintaining excess concentrations of precursor apparently detoxifies the microenvironment and drives selective product formation (Fig. 5). Indeed, if this microorganism is exposed for several hours to toxic fatty acid esters such as ethyl decanoate, the precursor for \( \gamma \)-hydroxydecanoic acid and the flavor compound \( \gamma \)-decalactone, morphology changes and the cells eventually lyse.

Separations, Purifications Affect Commercial Viability

In other cases, more complex measures may be needed to overcome the toxicity of the fatty acid precursor or flavor product. If the biocatalyst does not tolerate economically reasonable product concentrations in the fermentation broth, one countermeasure is to remove the product as it forms. The use of a two liquid-phase fermentation system, one of them being an organic phase that continuously extracts hydrophobic products, represents an attractive

restrict transmembrane transport of fatty acid precursors.

However, in yeast cells fatty acids added to the growth medium diffuse freely across cell membranes. The efficiency of this uptake system allows yeast species such as \textit{Yarrowia}, \textit{Rhodotorula}, and \textit{Sporidiobolus} to use a wide variety of fatty acids or derivatives as the sole source of nutrient carbon. In filamentous fungi, the uptake mechanisms are not yet well understood.

Serious precursor toxicity also occurs for many microorganisms when supplied with fatty acid esters that are hydrophobic at nonacidic pH values. This problem was described as the factor limiting production of \( \gamma \)-decalactone from the corresponding fatty acid ethyl and methyl esters in a patent granted to the German company BASE.

However, by using the filamentous fungus \textit{Mucor circinelloides} and carefully adjusting growth conditions

The coconut flavoring lectone 5-pentyl-\( \alpha \)-pyrone can be removed from \textit{Tichodermic} viride culture broth by pervaporation, a selective membrane separation technology.
Complicated Economics of Microbial Flavor Production

Classical fermentation technology dominates microbiologically based production of natural flavor compounds from fatty acids or other natural raw material sources. Thus, established fermentation and chemical engineering procedures used widely in the food industry provide an effective means for producing an array of flavorful metabolites. However, production costs are a major constraint on growth in this sector of the food industry.

Currently, about 100 flavor molecules of biological origin are available to the food industry. Among them, natural lactones represent one of the major large-volume flavor products. For example, the annual potential market for natural γ-decalactone is estimated to be 10 tons, which corresponds to a fermentation volume of about 2,500 m³. This volume represents a substantial potential market for such a high-value, fine chemical product.

Several years ago, a number of flavor companies indicated interest in this potential marketplace and thus began establishing programs for developing and producing flavors by microbial means. For example, during the last decade, at least 10 inventions relating to production of natural γ-decalactone by microbial fermentation received patents. Moreover, this natural flavor compound was then priced at $12,000/kg, justifying the estimated research investment of $1-2 million being made at the time.

Since then, however, production of this and other similar natural flavor molecules drastically increased, driving down the market value of γ-decalactone, which reached less than $700/kg in 1996 (above). This product thus became a commodity instead of a fine chemical and is now sold at a price close to its manufacturing costs. Economic production is only feasible for companies with a high market share, a well-performing manufacturing process, and access to a cheap, preferably fully paid-for fermentation capacity.

method with broad applicability for dealing with this challenge. However, use of high volumes of organic solvents raises safety concerns, and measures to protect against explosions are necessary.

Alternatively, available membrane separation technologies for extracting potentially toxic products may be compatible with certain single aqueous-phase culture conditions. For instance, Karl W. Bödeker and his collaborators at the GKSS Research Centre in Geesthacht, Germany, coupled a membrane pervaporation system to a bioreactor to recover the lactone 6-pentyl-α-pyronone (6-pp) from Trieboderma viride cultures (Fig. 6). Continuous removal of this coconut flavor component, which inhibits its own synthesis, markedly improves yields.

Only the desired product passes across the highly selective membrane, providing as much as a 20-fold enrichment and yielding 85% 6-pp in this compartment after two weeks of productive fermentation. However, overall productivity is still too low to justify the investment that would be required to establish this process on an industrial scale. Another complicating factor inhibiting wider use of this technology is that almost all flavor fermentation facilities produce a wide variety of products, meaning that their equipment needs to remain adaptable for multipurpose operation.

Reducing raw material costs is another critical factor affecting the commercial viability of biotechnology-based flavor production. Unspe-
cific side reactions also affect yield and cost, particularly if they result in by-products that are difficult to separate from the desired product. Even traces of less than 1% impurities may adversely affect the taste and smell of the product, effectively reducing its price.

Sometimes manufacturers have had to invent methods for dealing directly with troublesome by-products. For instance, to improve the efficiency of a microbiologically based process for producing γ-decalactone, the flavor company Haarman & Reimer GmbH in Holzminden, Germany, developed and patented a process for recovering the side-product 3-hydroxy-γ-decalactone, which forms from ricinoleic acid during the fermentation and is coextracted with γ-decalactone. During a subsequent distillation, the by-product is converted to a 3,4-unsaturated γ-decalactone. In the patented process, this dehydrated derivative is stereoselectively reduced by Saccharomyces cerevisiae as part of a second biotransformation, improving the yield of the desired γ-decalactone.

Progress Depends on Better Understanding Microbial Metabolism

To make better and broader use of microbiologically based procedures for producing natural flavors, we need to obtain a better understanding of how microorganisms synthesize flavor components and the underlying metabolic pathways that make these reactions possible. Doing so involves identifying novel enzymatic activities and characterizing entire metabolic pathways, which includes learning how they are regulated and how metabolite yields may be maximized. Some studies may entail identifying and cloning the genes coding for key enzymes or regulatory elements.

Another novel approach to this challenge involves identifying microbial compounds with signature aromas. We have been screening microbial isolates grown on a variety of potential precursor fatty acids for their abilities to generate characteristic odors. Such organoleptic compounds typically have very low odor thresholds in the range of parts per billion to parts per million, meaning that they are easily detected by the human nose.

However, to establish and automate a systematic screening procedure, we have adapted sensitive detection systems—in this case, gas chromatography coupled to mass spectrometry—for continuously analyzing volatile substances released into the head space from microbial cultures. Using our large in-house mass spectral library of flavor molecules, we are identifying metabolites produced during the microbial breakdown of fatty acids added to the culture media. Already, we have identified promising microbial isolates that produce lactones or methylketones from fatty acids and esters.

Another approach is to genetically engineer microorganisms with genes encoding enzymes used by plants to degrade fatty acids, making recombinant organisms that can generate unusual flavor molecules by fermentation. For example, we isolated the fatty acid hydroperoxide lyase from banana plants and subsequently cloned the corresponding gene, which can be expressed in Saccharomyces cerevisiae. In engineered yeast cells, the plant-derived lyase cleaves the hydroperoxide of a fatty acid supplied in the culture medium, and yeast enzymes, including alcohol dehydrogenase, convert the lyase-modified product into the "green note" flavor compound cis-3-hexenol. A further refinement will involve adding a plant gene encoding lipoxigenase, which will enable production of the green note compound through direct degradation of linolenic acid.

Identifying and cloning genes encoding other crucial enzymes will help to elucidate further the biochemical basis of flavor molecule formation. The hydroperoxide lyase amino acid sequence, for example, closely resembles that of the allene oxide synthase involved in the metabolic pathway leading to jasmonic acid. According to Kenji Matsui and his colleagues in the Department of Biological Chemistry at Yamaguchi University in Japan, the hydroperoxide lyase of bell pepper, which is specialized for the metabolism of lipid hydroperoxides, apparently is a novel member of the cytochrome P-450 enzyme family.

To characterize flavor molecule metabolism, researchers will need to study many more examples of such genes and enzymes as well as the metabolic pathways to which they belong. Closer interactions between academic and industrial research institutions will help to form the synergistic ties needed to continue these studies. The flavor industry welcomes that heightened cooperation.
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