The Impact of Recombinant DNA Technology on the Flavor and Fragrance Industry

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Biotechnology, of which recombinant DNA technology is an important sub-discipline, has a long tradition in the production of food and flavors. Around 3500 BC, humans first started to use microbes for the production of wine, beer, bread and many other foods that became an indispensable part of the daily diet.[1] In the beginning, such fermentations were carried out on a rather empirical level. Not until the discoveries of Louis Pasteur in the 19th century was the scientific basis laid. Isolation and controlled cultivation of microbes became possible, and about 20 years ago these techniques also found application in the production of various flavor chemicals.[2]

In the early 1970s, recombinant DNA technology emerged and soon started to become a significant part of today's biotechnology. Immediate impacts of this new technology were observed in pharmaceutical research. The first genetically engineered product, human insulin produced by bacteria, entered the market in 1982. Since then, more than 33 new drugs produced with recombinant DNA technology have been registered worldwide. In addition, 284 biotech drugs were in development in 1996, representing a three-fold increase since 1989.[3]

A similar change has been initiated in the food industry by the introduction of the FLAVRSAVR tomato in 1994 as the first genetically engineered whole food. Today, recombinant DNA technology has definitely found its way into the food industry, underscored by more than 3,600 transgenic field trials carried out by 1995. So far, 18 genetically engineered agricultural products have been approved for commercialization.[4] These include plants—such as corn, cotton, soybeans and potatoes—with improved pathogen/pest resistance, herbicide tolerance and food quality.[5]

The safety of genetically modified organisms has been assessed by the EC and the FDA.[6] Nevertheless, the public's acceptance of products from transgenic plants is still rather low. This is especially the case in Western Europe, where major concerns are expressed in Germany and Austria. On the other hand, little consumer reaction to genetically engineered food products has been observed in the USA. However, a recent European study showed that, as a potential food risk, genetic engineering was ranked similar to artificial food coloring but safer than food irradiation or pesticide residues.[7]

The application of recombinant DNA technology in the flavor and fragrance industries is less advanced than in the pharmaceutical and food industries. Nevertheless, the first products involving recombinant DNA technology in one way or another have been commercialized. Today, recombinant DNA technology has also become an important part of the research activities of flavor and fragrance companies. This article suggests several areas of the flavor and fragrance industry that will be increasingly influenced by the use of recombinant DNA technology, including the following:

- Production of aroma chemicals, such as cis-3-hexenol, via newly discovered pathways
- Heterologous production of tasty peptides, such as a beefy meaty peptide, using recombinant yeast strains
- Improved flavor and fragrance profiles through the genetic engineering of plants
- Removal of off-flavors, such as those sometimes associated with cheese flavors, via debittering proteases
- Enzymatic formation of flavor aldehydes
- Screening systems based on expressed olfactory and taste receptors

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Natural Aroma Chemicals

Nature is a rich source of aroma chemicals; several thousand have been identified and chemically synthesized in the past. With the on-going trend toward natural flavors, aroma chemicals were increasingly required to be of natural origin. Separation techniques such as extraction and distillation of natural materials are successfully used in our industry. When these can not be used economically, enzymatic or microbial conversions are used instead.

Today, fermentative processes are employed to produce many aroma chemicals, including various aliphatic and aromatic acids (such as 2-methyl butyric acid and phenylacetic acid), different esters (such as ethyl 2-methylbutyrate and methyl anthranilate) and lactones (such as γ- and δ-decalactones).

For the production of high impact chemicals (such as β-damascenone, methional or γ-nonalactone), none of the above-mentioned techniques has been reasonably applied so far. These chemicals are generally found in very small quantities in plant materials, making their recovery an expensive endeavor. No microbial or enzymatic conversions are evident, so other approaches are needed.

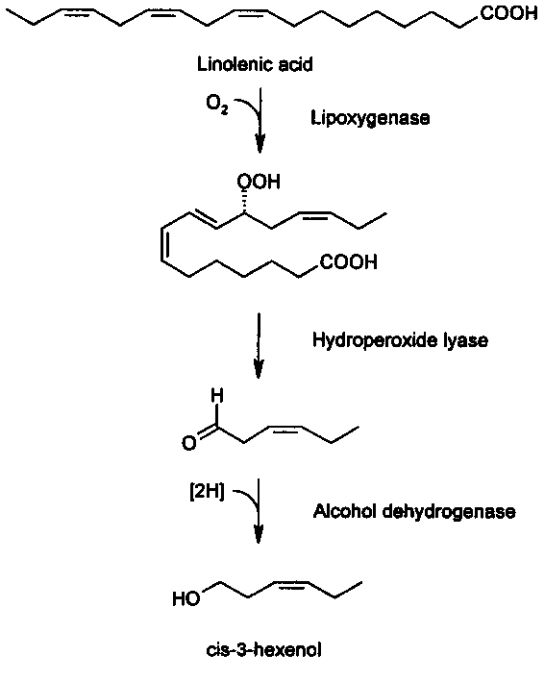

Figure 1. Biosynthetic pathway of cis-3-hexenol in plants. Three enzymes are involved in the production of cis-3-hexenol starting from linolenic acid. Various plant materials serve as sources for these enzymes allowing a conversion of the acid to cis-3-hexenol at industrial scale. Because the hydroperoxide lyase is the rate-limiting factor in such a reconstituted production system, the gene coding for this enzyme was heterologously expressed in yeast cells to yield a highly active lyase material.

In nature, these aroma chemicals are formed by specific yet sometimes unknown pathways. However, recently, some have been elucidated, as is the case for furaneol and γ-nonalactone.[8,9] With the help of recombinant DNA technology, the corresponding genetic information from the original source can be isolated and subsequently transferred into a suitable host strain. This allows efficient microbial production of natural aroma chemicals. We have applied this technique in the production of cis-3-hexenol, also referred to as leaf alcohol.[10]

Natural cis-3-hexenol and its esters are in high demand because they are widely used in various fruit flavors. Traditionally, cis-3-hexenol is isolated from mint terpene fractions. In the plant, cis-3-hexenol is formed from linolenic acid via hydroperoxide and cis-3-hexenal.

Peppermint oil fractions can not satisfy the global need for natural cis-3-hexenol, so an enzymatic route starting from linolenic acid was established (Figure 1).[11] The fatty acid is oxidized to the hydroperoxide, using, for example, soya flour containing lipoxygenase. The conversion of the hydroperoxide to cis-3-hexenal is achieved by using a fruit source, such as guava, that was found to contain high activities of the hydroperoxide lyase. Finally, reduction to the leaf alcohol is performed by yeast cells.

Although it is independent of peppermint oil fractions, this enzymatic transformation has a significant drawback: the rather large amount of fruit that has to be processed. The lyase was shown to be the rate-limiting factor in the enzymatic conversion of linolenic acid to cis-3-hexenol. We purified the linolenic-acid-hydroperoxide-lyase from banana plants, allowing the determination of four independent, internal amino acid sequences. Degenerate oligonucleotides and resulting polymerase chain reaction (PCR)-fragments helped to isolate the structural gene for the lyase from a banana complementary DNA (cDNA) library.[12] Interestingly, the DNA sequence shared 44% identity with the sequence of allene oxide synthase. The latter enzyme is important in the biosynthesis of methyljasmonate, another key flavor impact chemical.

The heterologous expression of the lyase gene helped to overcome the drawbacks of the enzymatic production route, supplying highly active lyase material. As can be seen in Figure 2, higher amounts of cis-3-hexenol have been produced in the presence of the recombinant yeast cells as compared to the route using homogenized bananas.

To unify all three enzymes involved in the formation of cis-3-hexenol in yeast, we also cloned and coexpressed the lipoxygenase gene. This generated an even more efficient system to produce cis-3-hexenol.

In addition, it should be pointed out that the literature includes reports that the degradation of fatty acids involves other lipoxygenases and lyases with different specificities.[13] Heterologous expression of such genes would allow the production of other important flavor chemicals, such as 1-octene-3-ol or 2,6-nonadienal.

Finally, applications based on our cloning of the lyase

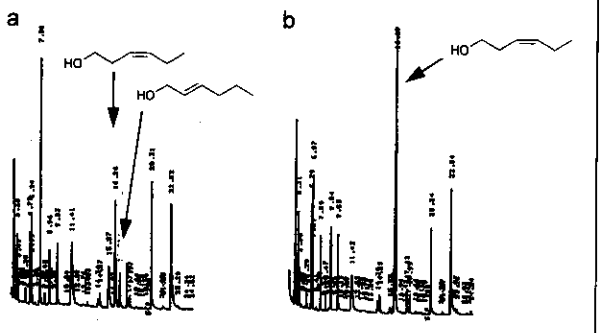

Figure 2. GC analysis of the reaction mixtures in which the hydroperoxide of linolenic acid was incubated with a) homogenized banana material and reducing yeast cells, and b) with recombinant yeast expressing the lyase gene and additional reducing yeast cells. The recombinant yeast process yielded higher amounts of cis-3-hexenol with no formation of trans-2-hexenal as a side product.

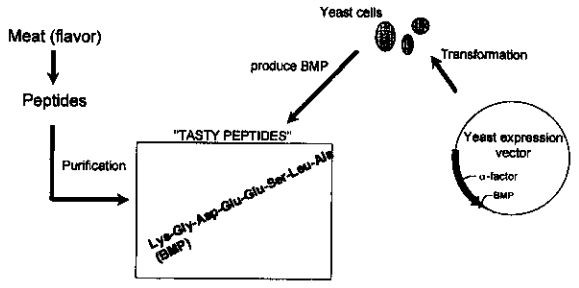

Figure 3. Recombinant DNA technology used to produce the beefy meaty peptide (BMP) in yeast cells. Yeast cells were transformed with an engineered yeast secretion vector. Upon induction, these cells started the synthesis of a fusion protein consisting of the desired peptide (in our case BMP) and the yeast α-factor. The desired peptide was then cleaved during the secretion process and could be easily recovered from the culture fluid in a semi-purified form.

gene are not limited to microbial systems. The gene also could be transferred into plant hosts, resulting in an increased formation of cis-3-hexenol/cis-3-hexenal upon maceration of the plant.

Ultimately, recombinant DNA technology could be used to enhance and to alter the flavor profile of fruits and plants by overexpressing key metabolic enzymes. As examples, one can imagine strawberries high in furaneol or especially green-smelling apples in which the lyase gene would be overexpressed.

Tasty Peptides

Tasty peptides, resulting from protease digestion of food proteins, have been found in various food products, such as meat, cheese, fish and yogurts.[14,15] Their organoleptic character and amino acid sequence can be determined after purification.

In order to improve or to boost flavors, specific tasty peptides have thus far been synthesized either chemically or enzymatically. However, neither strategy is feasible for a large-scale and commercial production of tasty peptides due to high production costs.[16] We have therefore investigated the heterologous production of peptides using recombinant yeast strains.

Several model peptides were chosen, among them an octapeptide known as beefy meaty peptide (BMP). This peptide was found in meat and was suggested to enhance the taste of beef gravy.[17] Figure 3 shows the recombinant DNA technology approach used to produce the BMP in yeast cells. Linking the genetic information of the octapeptide to the yeast mating pheromone α-factor in a suitable expression vector allowed the secretion of the desired peptide into the culture medium. From the culture filtrate, the peptide can be easily recovered and used in a semi-purified form.

Alternatively, intracellular accumulation of the peptide offers the possibility to generate specially flavored yeast extracts.[18] This approach can be seen as a further improvement of yeast strains that previously have been engineered to contain high content of 5'-nucleotides, inosine-monophosphate (IMP) and guanosine-menophosphate (GMP).

Improved Flavor and Fragrance Profiles

Plants are a major part of our daily diet. Due to their smell and taste, they are established sources for raw materials used in the flavor and fragrance industry. More than 3,000 different essential oils have been analyzed and many of them are used in the creation of fine fragrances or serve as starting materials for the isolation and modification of chemicals.[19]

As an illustration, 36,000 metric tons of d-limonene are extracted annually from citrus oils.[20] Other commercially used examples include l-carvone, geraniol and menthol, the latter with an annual sales volume of about US$2 billion.

In contrast to the classical breeding and selection programs, plants can nowadays be more efficiently improved by means of recombinant DNA technology. For example, huge efforts are presently undertaken to increase the content and quality of fatty acids in oil crop plants.[21] Genetically engineered grapeseed underwent the second most field trials after potato, and in 1995 an engineered canola crop with high laurate was commercialized.[22,23]

Commercial examples of genetically engineered plants used in the flavor and fragrance industry are not yet known, but the example of a transgenic *Pelargonium* plant, commonly referred to as lemon geranium, can illustrate the potential of recombinant DNA technology.[24] In this example, the titer of geraniol was increased four-fold and that of citronellol by 13-fold in the transgenic plant as compared to the wild type.

To optimally design such higher yielding plant species requires an improved understanding of metabolic pathways and the post-harvest biochemical reactions. DNA sequencing programs elucidating total plant genomes are expected to simplify the cloning of important gene sequences.

New Enzymes

The majority of industrial enzymes are used today in food preparations and in fabric care products. These markets each represent annual sales of roughly US$160 million.[25] Therefore, development of new enzymes is targeted mostly at these two segments.

These enzymes also find limited applications in the flavor and fragrance industry. Examples include proteases for the generation of food protein hydrolysates and lipases for the production of natural esters.[26,27] In the past, many enzymes involved in the generation of flavors or flavor precursors have been characterized. It is a well-known fact that during postmortem aging, various hydrolyzing enzymes are released within meat. This results in the formation of flavor precursors that are characteristic of the particular type of meat.[28] The use of such enzymes in the flavor industry is limited, since they are not available at a reasonable cost. For the time being it does not appear that enzyme manufacturers will produce them due to the rather small market. The advent of recombinant DNA technology, however, has now greatly facilitated their large-scale production, rendering it feasible for flavor companies as well.[29] With the availability of such enzymes, more authentic meat, cheese and other flavor mixtures could be generated.

Debittering proteases: An initial development in this direction can be seen in the area of enzyme-modified cheese. Treating milk proteins with commercially available proteases often results in bitter products. Though the occurrence of the bitter peptides has been extensively

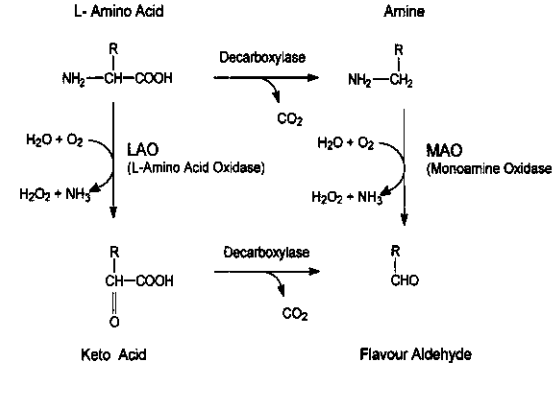

Figure 4. Enzymatic formation of flavor aldehydes such as methional or phenylacetaldehyde from the corresponding amino acid. The genes coding for LAO and MAO have been functionally expressed in microbial hosts allowing the formation of flavor aldehydes when fed with the required precursor.

studied, screening of various commercially available proteases and mixtures thereof was needed to prepare pleasant, non-bitter flavors. Because cheese flavors are the product of microbial activities, extracellular enzymes from various starter cultures have been characterized. Interestingly, some of them were shown to have debittering activities.[30] That such proteases remove off-flavors is of great interest to flavor industries that have started to clone and express the corresponding genes.[31] This opens new avenues to making, for example, cheese flavors that are more characteristic and intensive.

Flavor aldehydes from oxidases: In recent years, new classes of enzymes, such as oxidases (peroxidases and polyphenoloxidases), have been introduced for food and detergent applications. Oxidases are also important for the production of many different flavors. As an example, the oxidative degradation of amino acids yields various flavor aldehydes.

L-amino acid oxidase (LAO) deaminates various amino acids, resulting in the formation of the corresponding keto acids. After decarboxylation, these keto acids yield flavor aldehydes (Figure 4). We recently cloned the LAO gene from the filamentous fungus *Neurospora crassa* and overexpressed it homologously in the parent host.[32]

Alternately, flavor aldehydes can also be produced by decarboxylation of amino acids and deamination by a monoamine oxidase. We purified a novel monoamine oxidase (MAO) from *Aspergillus niger* and cloned the structural gene.[33] This flavin-adenine dinucleotide (FAD)-containing enzyme oxidizes various amines, such as phenethylamine and methylthiopropylamine, to the corresponding aldehydes. The gene coding for MAO was heterologously expressed in *Escherichia coli*. Incubating the above-mentioned amines with protein extracts of such induced *E. coli* cells resulted in the formation of methional and phenylacetaldehyde. The broad substrate specificity makes this enzyme attractive for the generation of various other flavor aldehydes.

Molecular Olfaction and Taste

Olfaction: Recombinant DNA technology can be expected to have a rather large impact in the field of molecular olfaction and taste. The first putative olfactory receptors were cloned in 1991.[34] Since then, the understanding of olfactory receptors and their signal transduction mechanisms has been drastically increased. It became widely accepted that olfactory receptors belong to the G-protein coupled seven transmembrane receptor family that represents 60% of the targets for all drugs sold today.[35] A possible interaction of odorants with a heterologously expressed mammalian receptor has been suggested in the case of Lilial[a] and Lyral.[b, 36]

Such ligand-receptor models form a broad and scientific basis for the pharmaceutical industry to find new drugs targeting diseases such as AIDS, cancer or arthritis. Research in the field of molecular olfaction and taste has

[a] Lilial is a trade name of Givaudan-Roure, Geneva, Switzerland
[b] Lyral is a trade name of IFF, New York, New York

benefited from the techniques and know-how developed for the discovery and screening of pharmaceutical drugs. Structure-odor relationship studies, for example, have been widely applied in the search for novel fragrance molecules, but the present lack of a three-dimensional structure of an olfactory receptor has hampered the efforts to model and study the odorant-receptor interactions.[37,38] Thus, many recent efforts have been directed toward functional expression of olfactory receptors.

A specific receptor can be expressed at the surface of a cell (Figure 5). These receptors are linked via ordinary signal transduction mechanisms to a reporter gene that signals receptor binding of a potential odorant. Such engineered screening systems are widely used in the pharmaceutical industry to test low-molecular-weight drugs.

Taste: In contrast to olfaction, much less is known about taste receptors. Nevertheless, a simplified screening system for bitter and sweet compounds has been established.

Ruiz-Avila et al. isolated receptors from the bovine tongue papillae, added recombinant G-protein gustducin or transducin and incubated this reconstituted tongue receptor system in the presence of guanosine-5'-*O*-thiotriphosphate (GTP-γ-S) and potential tastants.[39] Trypsin digestion followed by Western blot analysis indicated if interaction occurred between tastant and receptor. So far, this system is applicable to tissues isolated from bovine but not human tongues. However, it allows for the molecular screening of potential tastants. More interestingly, it also allows for the molecular screening of taste enhancing or blocking agents.

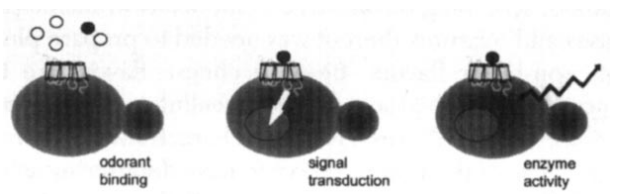

Figure 5. Recombinant yeast cells expressing olfactory receptors at the cell surface. These yeast cells can be used to screen a mixture of odorants. Upon specific odorant binding the intracellular signal transduction cascade is activated, stimulating a reporter gene. The resulting enzyme activity correlates to the binding strength of the odorant and can be easily monitored.

In summary, screening systems based on gustatory and olfactory receptors are feasible and will certainly become part of future investigations in flavor and fragrance industries.

Outlook

The flavor and fragrance industry is now at the point where the pharmaceutical industry was 20 years ago with respect to recombinant DNA technology. At that time, recombinant DNA technology entered pharmaceutical research without much notice. However, the benefit of this technology was clearly seen with the rather sudden emergence of the first products, and it has since developed to become an essential part of the research and production of new drugs.

It can be foreseen that the flavor and fragrance industry will go through a similar phase until the commercial benefit of recombinant DNA technology is clearly recognized. It already is evident that recombinant DNA technology will become an important tool for the discovery and production of cheaper and novel flavor and fragrance chemicals. Furthermore, the technology is essential to ultimately advance our understanding of olfaction and taste. This will lead to the discovery of novel odor- and taste-modifying compounds, changing the way flavor and fragrance compositions will be formulated in the future.

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