Enzymatic and Microbial Generation of Flavors

By Ian L. Gatfield, Haarmann & Reimer GmbH, Holzminden, Germany

During the last 10-15 years, biotechnological processes have established themselves in the flavor industry for the production of natural flavor materials.[1] The food laws of many countries recognize the fact that natural flavors and flavor materials can be obtained via biotechnology (Figure 1). However, certain conditions have to be met in order to guarantee the naturalness of the final product. These conditions stipulate that the raw materials used have to be natural, and that only physical processes are permitted for the isolation and purification of the materials so formed. Typical examples of such physical processes are extraction, distillation and crystallization.

Biotechnology has been used unwittingly by man to produce foods for thousands of years (Figure 2). The original benefit of this way of food preparation was the considerable increase in the shelf life of such products. The reason this traditional way of food preparation is still being performed is because of the positive flavor changes brought about by the enzymes and microorganisms used.

It is possible to obtain on a production scale both complex flavor mixtures and individual flavor components, via microbial fermentation and enzyme technology. Many different classes of flavor materials can be obtained this way including acids, esters, lactones, alcohols, aldehydes and ketones.

Acids

Probably the first individual natural flavor component made via biotechnology was acetic acid, produced when wine is oxidized to vinegar using *Acetobacter* species (Figure 3). This bioconversion was discovered by accident thousands of years ago, and the basic process has remained the same ever since. Production technology has become much more sophisticated, of course, and very large fermenters are now used to produce vinegar and acetic acid.

This type of bioconversion has been extended to other natural substrates in order to produce the corresponding natural acids that are of interest in the flavor industry. Thus, natural isobutanol, which is one of the components of fusel oil, can be converted by certain *Acetobacter* strains into natural isobutyric acid (Figure 4).[2,3] The other fusel alcohols such as 2-methyl butanol and 3-methyl butanol also can be converted efficiently to the

Extraction	Animal (beef, chicken, seafood ...) Vegetable (spices, mushroom ...)
Distillation	Citrus, spearmint, peppermint ...
Concentration	Extracts, fruit juices ...
Biotechnology	Fermentation (acids, alcohols ...) Enzyme modification (cheese, soy ...)

Figure 1. Processes for obtaining natural flavors

Cheese	Rennet, bacteria, mold	Camembert: *Pen. camemberti* Roquefort: *Pen. roqueforti* Emmentaler: *Propioni* bacteria
Yogurt	Bacteria	*Lactobacillus bulgaricus* *Streptococcus thermophilus*
Bread	Yeast	*Saccharomyces cerevisiae*
Meat	Enzymes	Proteases
Wine, beer	Yeast	*Saccharomyces cerevisiae*
Vegetables	Bacteria, yeasts	Sauerkraut, Olives, Cucumbers } *Lactobacillus*, *Saccharomyces*
Tea, cocoa	Enzymes, microorganisms	
Soya sauce	Mold	*Aspergillus oryzae*

Figure 2. Traditional food products from biotechnology

0272-2666/95/0005-0005$04.00/0

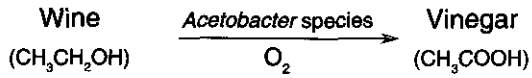


Figure 3. Bioconversion of wine to vinegar

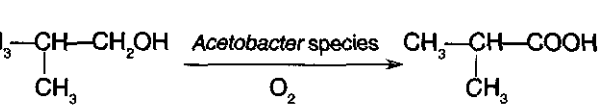
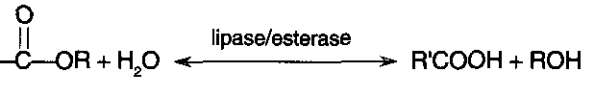


Figure 4. Bioconversion of isobutanol to isobutyric acid



Natural esters using: natural fusel alcohols + natural acids
natural terpene alcohols + natural acids

Figure 5. Schematic enzyme synthesis of esters

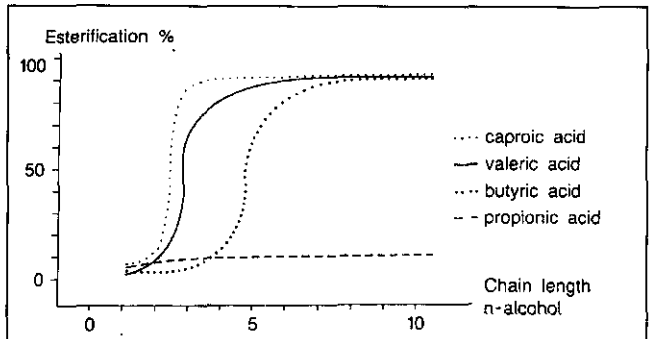


Figure 6. Substrate specificity of the lipase from *Mucor miehei*

Table I. Data from the enzymatic synthesis of isoamyl isovalerate using the lipase from *Mucor miehei*

Cycle	Degree of esterification (%)	Lipolytic activity (%)
0	–	100.0
1	85.2	9.8
2	88.0	9.5
3	85.0	10.0
4	86.4	9.0
5	82.1	8.5
6	53.7	8.0

corresponding acids. It can be shown that the conversion of 2-methyl butanol to 2-methyl butyric acid proceeds with very little racemization taking place.[3] This is surprising since the intermediate in the bioconversion step is the corresponding aldehyde which can undergo facile tautomerization and thereby give rise to a racemic product.

Esters

Such acids are valuable flavor materials and can be used as such in many types of flavors, including dairy, fruit and meat flavors. Carboxylic acids are also starting materials for the production of esters, which themselves are very important flavor materials. One way of making natural esters is by exploiting the synthetic capabilities of certain enzymes known as lipases (Figure 5).

This property of some lipases has been known for a few years and is well established.[4] Using this technique, a large number of different esters are accessible from the corresponding acids and alcohols under nonaqueous reaction conditions.[5] However, even in organic media, lipases can show a pronounced substrate specificity as, for example, can be shown for the lipase from *Mucor miehei*[6] (Figure 6).

It can further be shown that this enzyme apparently has both ester-synthesizing and ester-hydrolyzing capabilities which seem to be separate from each other.[7] Thus, when used in the synthesis mode, the lipolytic activity of the *Mucor miehei* lipase was lost very quickly. However, this enzyme can still synthesize esters efficiently (Table I). It was demonstrated that a conformational change in the protein structure takes place as a

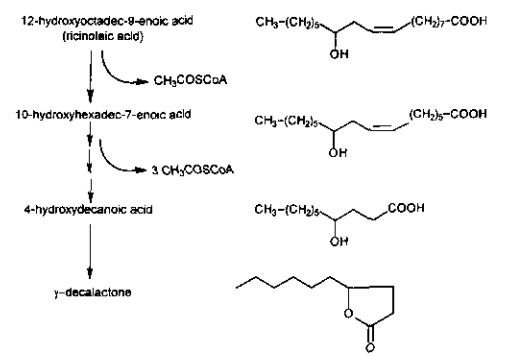


Figure 7. γ-Decalactone from fermentative β-oxidation of castor oil using *Candida lipolytica* (Note: Ricinoleic acid represents 90% of the triglyceride fatty acids in castor oil.)

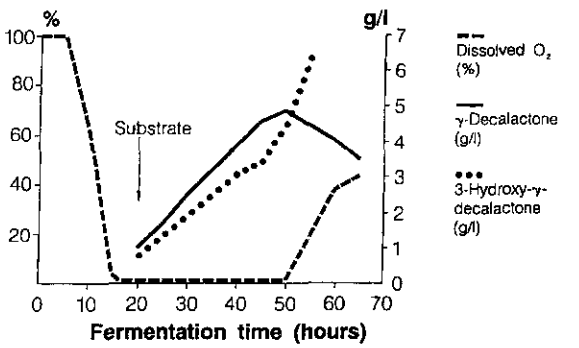


Figure 8. Fermentation profile for bioconversion of castor oil (ricinoleic acid) to γ-decalactone

result of the enzymatic esterification reaction.

Lactones

Isolated lipases also can be used to synthesize lactones from the corresponding hydroxy acids.[5,8] However, a more efficient way to produce lactones would appear to be via the fermentation of certain fatty acids. Both γ- and δ-lactones are important flavor materials that enjoy widespread use. The most important lactone is probably γ-decalactone, which can be obtained via the fermentative β-oxidation of castor oil (ricinoleic acid) using the yeast *Candida lipolytica* (Figure 7).[1]

A typical fermentation profile for this bioconversion is shown in Figure 8, which indicates that product yields in the range of 5 g/L can be obtained after an incubation period of about 50 hours.[9] It is important to terminate the process at the correct point in time, otherwise a decline in product yield occurs as a result of further conversion of the 4-hydroxydecanoic acid (Figure 7) to 3,4-dihydroxydecanoic acid (Fig-

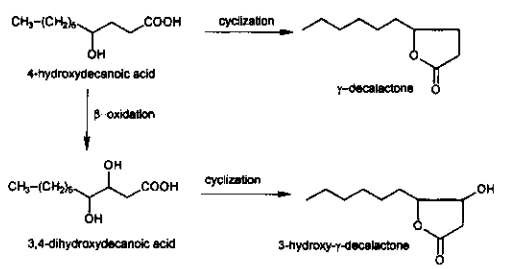


Figure 9. Conversion of 4-hydroxydecanoic acid to γ-decalactone and 3-hydroxy-γ-decalactone

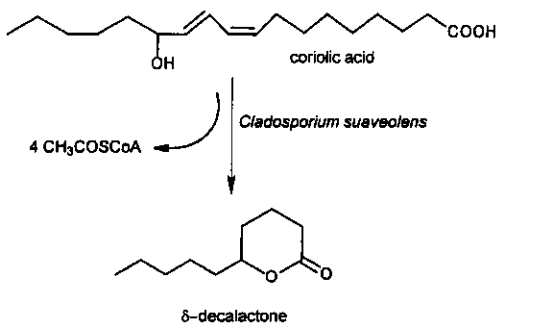


Figure 10. Bioconversion of coriolic acid to δ-decalactone

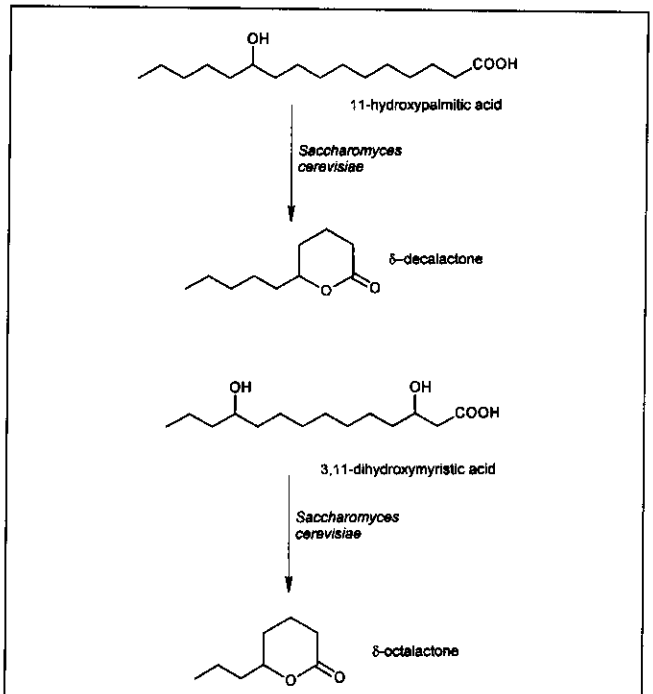


Figure 11. Bioconversion of hydroxylated fatty acids present in Jalap resin to δ-decalactone and δ-octalactone

ure 9). The latter cyclizes to form 3-hydroxy-γ-decalactone (Figure 9), which has no positive flavor properties.[9]

This concept of β-oxidation of hydroxylated fatty acids has been extended to coriolic acid for the production of δ-decalactone (Figure 10).[9,10] This lactone and its lower homologue δ-octalactone can be obtained via the fermentation of the hydroxylated fatty acids naturally present in Jalap resin (Figure 11).[11] Yet another method of obtaining lactones is via the site-specific hydroxylation of fatty acids using *Mucor* species. Thus, ethyl caprylate can be converted efficiently to γ-octalactone using *Mucor circinelloides*.[12] Soya lipoxygenase can be employed to oxidize unsaturated fatty acids into the corresponding hydroperoxides that can then be reduced to hydroxylated fatty acids and the latter converted into lactones via fermentation (Figure 12).[13]

Finally, yeasts also can be used to produce saturated lactones from their α– and β-unsaturated counterparts.[14] Thus, 2-decen-5-olide, present as the main component of Massoi bark oil, can be converted efficiently into δ-decalactone (Figure 13). It was recently shown that non-conjugated carbon-carbon double bonds present in lactone rings also can be reduced.[3] Thus, 3-decen-4-olide can be converted to (+)-R-γ-decalactone under very mild conditions and with a very high enantioselectivity (Figure 14).

Aldehydes and Alcohols

The lipoxygenase mentioned above is better known as an essential component in the enzymatic conversion of unsaturated fatty acids into low molecular weight aldehydes and

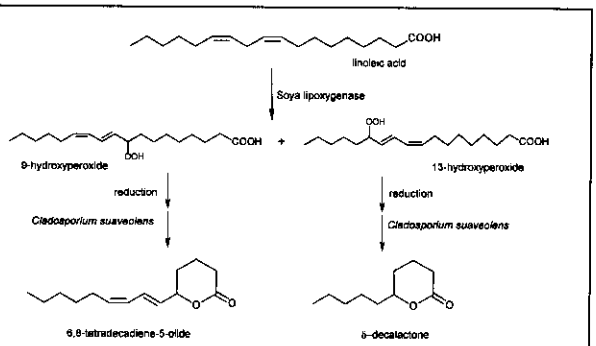


Figure 12. Bioconversion of unsaturated fatty acids to lactones via oxidation with soya lipoxygenase and fermentation with *Cladosporium suaveolens*

Figure 13. Bioconversion of 2-decen-5-olide to δ-decalactone using yeast

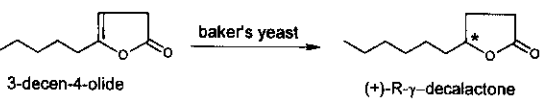


Figure 14. Bioconversion of 3-decen-4-olide to (+)-R-γ-decalactone using yeast

alcohols.[15] This enzymatic process is widespread in nature and is responsible for the production of cis-3-hexenol and trans-2-hexenal when green tissue is damaged. These two materials are very important in the flavor industry for imparting fresh, green notes to fruit and vegetable flavors. They can be obtained in limited quantities as by-products of the essential oil industry.

Processes have been described in which linolenic acid is incubated with enzymes such as soy lipoxygenase or with homogenized plant tissues such as lettuce leaves[16] or radish leaves.[17] In all cases, the major products of the enzymatic reaction are cis-3-hexenol and trans-2-hexenal, usually occurring as a mixture.

Some patents describe the use of baker's yeast to reduce the aldehydes as they are formed into the corresponding alcohols, thereby obtaining a purer product.[17,18] Our experience with such systems suggests that the isomerization of cis-3-hexenal to trans-2-hexenal is very rapid and the latter

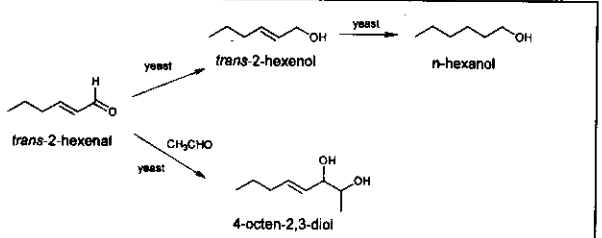
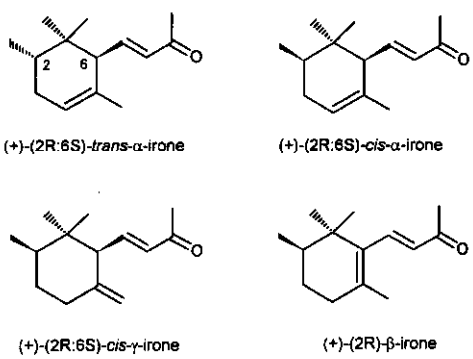


Figure 15. Bioconversion of trans-2-hexenal to n-hexanol and 4-octen-2,3-diol using yeast



Irones	Maturation of rhizome: Traditional (%)	Maturation of rhizome: Microbiological (%)
trans-α-irone	4.0	6.5
cis-α-irone	44.0	34.0
cis-γ-irone	50.0	58.0
β-irone	2.0	1.5
yields	400 mg/kg (3 years)	1 g/kg (8 days)

Figure 16. Efficiency comparison of two processes to produce irones from orris rhizomes by incubation with *Serratia* and *Pseudomonas* species

undergoes facile reduction to form n-hexanol (Figure 15). Baker's yeast also is able to add activated acetaldehyde to trans-2-hexenal, thereby forming 4-octen-2,3-diol.[3]

Current Challenges

It would seem that many of the important flavor components are now available as natural materials as a result of the progress made in biotechnology during the last 10-15 years. This does not mean that all the problems have been solved in this very challenging area. The processes and problems now being worked on are undoubtedly much more complex, and consequently more difficult, than in the past. An example of such a process is the bacterial production of irones from their precursors in orris rhizomes through incubation with *Serratia* and *Pseudomonas* species (Figure 16).[19] The bacterial process is very much more efficient than the traditional process in regard to both the product yields and the speed of conversion.

A material that is closely related chemically and an important minor ingredient in certain fruit flavors is β-

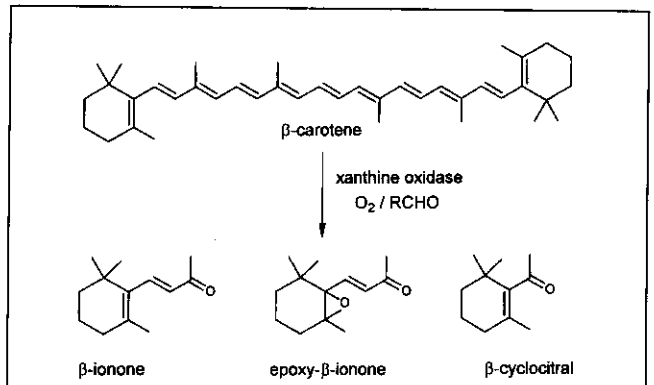


Figure 17. Xanthine oxidase mediated degradation of β-carotene

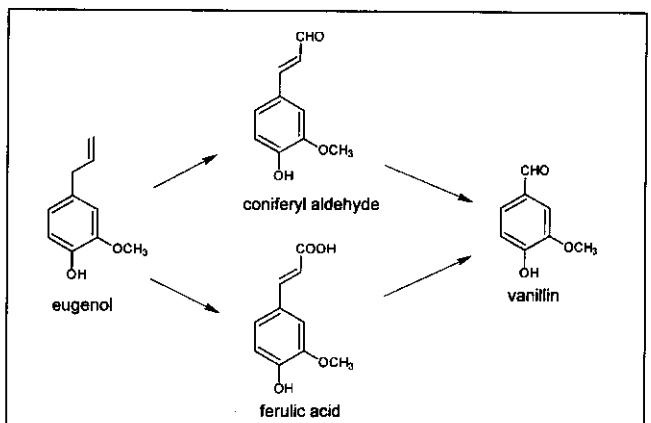


Figure 18. Bioconversion of eugenol to vanillin

ionone. This material is apparently one of the many products formed during the oxidation of β-carotene using the enzyme xanthine oxidase (Figure 17).[20]

One important—if not the most important—flavor molecule is vanillin, which in its nature identical form is used in food flavoring to the extent of approximately 10,000 tons annually. However, it would seem that an effective biotechnological process for its production has not yet been developed, despite the fact that many groups are working on the problem. In spite of its considerable bacteriocidal activity, eugenol would seem to be the most promising substrate. Eugenol can, for example, undergo bioconversion to coniferaldehyde[21] or ferulic acid,[22] both of which are useful intermediates and can be converted to vanillin (Figure 18).

Another approach involves the enzymatic conversion of coniferyl benzoate, present in Benzoe Siam, using an esterase and an alcohol dehydrogenase, to yield coniferaldehyde.[23] This procedure has been described as a one-pot process which gives rise to a product concentration of 1.5 g/L. According to other reports, both coniferaldehyde[24] and eugenol[25] can be converted directly to vanillin with the aid of the enzyme lipoxygenase.

In general, the flavor 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 flavor biotechnology has been introduced by most of the large flavor houses. In addition, the considerable interest devoted to flavor precursors by academic and industrial research groups indicates the importance of biotechnical processes for further development of natural flavors.

References

Address correspondence to Ian L. Gatfield, Haarmann & Reimer GmbH, Rumohrtalstrasse 1, 37603 Holzminden, Germany.

1. WD Murray and SJB Duff, *Food Enzymology*, v2, London and New York: Elsevier (1991) pp 105-141
2. PSJ Cheetham, The use of biotransformations for the production of flavours and fragrances, *Trends Biotechnol* **11** 478-488 (1993)
3. IL Gatfield, unpublished results
4. PJ Halling, Effects of water on equilibria catalyzed by hydrolytic enzymes in biphasic reaction systems, *Enzyme Microbial Technol* **6** 513-517 (1984)
5. German Pat DP 3,108,927, IL Gatfield and T Sand (1982)
6. IL Gatfield, The enzymatic synthesis of esters in nonaqueous systems, *Ann New York Acad Sci* **434** 569-572 (1984)
7. IL Gatfield, *Flavour Science and Technology*, Chichester, New York, Brisbane, Toronto and Singapore: John Wiley & Sons (1990) pp 167-170
8. FW Welsh, WD Murray and RE Williams, Microbiological and enzymatic production of flavor and fragrance chemicals, *Crit Rev Biotechnology* **9** 105-169 (1989)
9. IL Gatfield, M Güntert, H Sommer and P Werkhoff, Some aspects of the microbiological production of flavour-active lactones with particular reference to γ-decalactone, *Chem Mikrobiol Technol Lebensm* **15** 165-170 (1993)
10. R Cardillo, G Fronza, C Fuganti, P Grasseli, A Mele, D Pizzi, G Allegrone, M Barbeni and A Pisciotta, Stereochemistry of the microbial generation of γ-decanolide, γ-dodecanolide and γ-nonanolide from C_{18}13-hydroxy, C_{18}10-hydroxy and C_{19}14-hydroxy unsaturated fatty acids, *J Org Chem* **56** 5237-5239 (1991)
11. European Pat 409,321, ALGM Boog, ALJ Peters and R Roos (1990)
12. WO Pat 8912-104, GV Page and RG Eilerman (1989)
13. European Pat 412,880, R Cardillo, C Fuganti, M Barbeni, P Cabella, PA Guarda and A Gianna (1990)
14. PH van der Schaft, N ter Burg, S van den Bosch and AM Cohen, Microbial production of natural δ-decalactone and δ-dodecalactone from the corresponding α, β-unsaturated lactones in Massoi bark oil, *Appl Microbiol Biotechnol* **36** 712-716 (1992)
15. A Hatanaka, The biogeneration of green odour by green leaves, *Phytochemistry* **34** 1201-1218 (1993)
16. EM Götz-Schmidt, M Wenzel and P Schreier, C_6 Volatiles in homogenates from green leaves, *Lebensm Wiss u Technol* **19** 152-155 (1986)
17. French Pat 2,652,587, P Brunerie (1989)
18. WO Pat 9324-644, B Muller, A Gautier, C Dean and JC Kuhn (1993)
19. B Belcour, D Courtois, C Ehret and V Petiard, Rapid production of irones by maturation of orris rhizomes with two bacterial strains, *Phytochemistry* **34** 1313-1315 (1993)
20. A Bosser and JM Belin, Synthesis of β-ionone in an aldehyde/xanthine oxidase/β-carotene system involving free radical formation, *Biotechnol Prog* **10** 129-133 (1994)
21. German Pat DE 3,604,874, B Cooper (1987)
22. European Pat Appl 582,687, R Hopp and J Rabenhorst (1993)
23. C Leffemberg, A Gautier and E Ohleyer, Enzymatic preparation of coniferaldehyde from coniferyl benzoate ex Benzoe Siam, *Appl Biochem Biotechnology* **37** 43-52 (1992)
24. European Pat Appl, PH Markus, AL Peters and R Roos (1993)
25. WO Pat 9402-621, J Mane and J Zucca (1992)