Biological Systems and Flavors: Science, Technology and Applications

Natural, organic and clean aroma chemicals; unique complex bases; greener and ecologically friendly processes; taste and olfactory modifiers; and regulatory issues

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This article aims to capture the bond between science, technology and applications in the area of natural flavors. Demand for naturals has stimulated research to find effective and efficient solutions to scientific questions and practical problems. Imaginative implementation of new scientific understanding leads to original creations and applications. Recent developments are captured in a number of excellent reviews on natural flavor and fragrance molecules.[1] Here, the intention is to provide selected examples of how science, when transformed into technology, can successfully address the ever-changing market needs.

The biological systems considered here include plants, microorganisms and enzymes. For simplicity, the technologies that utilize such biological systems will be referred to as applied biotechnology. These processes employ whole or part of living cells, which can be natural or genetically engineered. One advantage of biotechnological processes is that they yield natural stereoselective and enantioselective products.

It is important to recognize that, at the present time, only a fraction of these processes involve genetically manipulated organisms and enzymes. This is a consequence of the market demanding clean, organic and all natural flavor and fragrance materials. At the same time the tools of molecular biology, metabolic and translational engineering are employed to build scientific understanding. These tools are also used as a guide for identifying important traits in production organisms and enzymes. These modern tools of DNA manipulation deliver more controlled and precise changes than the traditional hybridization techniques.

Sensory systems is another area where scientific progress has had critical impact, since olfaction and taste are the key instruments for evaluating flavors and aromas. The industry has greatly benefited from recent progress made in understanding receptors, signal transduction, neurobiology and sensory. In particular, molecular biology is an essential tool to elucidate the biochemistry of taste and olfaction. This new knowledge is translated into methods for the discovery of taste modifiers and masking compounds to offset undesirable taste and olfaction characters.

Additionally, biotechnology can be instrumental in manufacturing; for example in selecting raw plant materials, improving downstream processes and waste management.

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From these many topics, the focus will be on a few examples to illustrate how scientific progress translates into new technologies that advance the flavor and fragrance industry. Topics to be discussed include:

- Natural, organic and clean aroma chemicals
- Unique complex bases
- Greener and ecologically friendly processes
- Taste and olfactory modifiers

Natural, Organic and Clean Aroma Chemicals

Microbial and enzymatic biocatalysis has found application in manufacturing of natural aroma chemicals. However, the technology using plant cell cultures for flavor production is not yet cost competitive.

Biocatalysis is a natural alternative to chemical synthesis during which a structurally related precursor is converted into the final product using enzymes, plant, animal and microbial cells as catalysts. Advantages of these processes are: mild and more environmentally friendly manufacturing conditions, natural status, and correct stereochemistry. Almost any chemical process can be replaced by a biological system, even though some of them may not be economically practical. Numerous such processes have been patented and selected few were implemented.

In a search for novel compounds, the flavor industry may adopt new combinatorial biosynthesis technology that is now used for finding novel pharmaceutical biologics. This technology can prepare large libraries of modified natural compounds and combinations thereof.

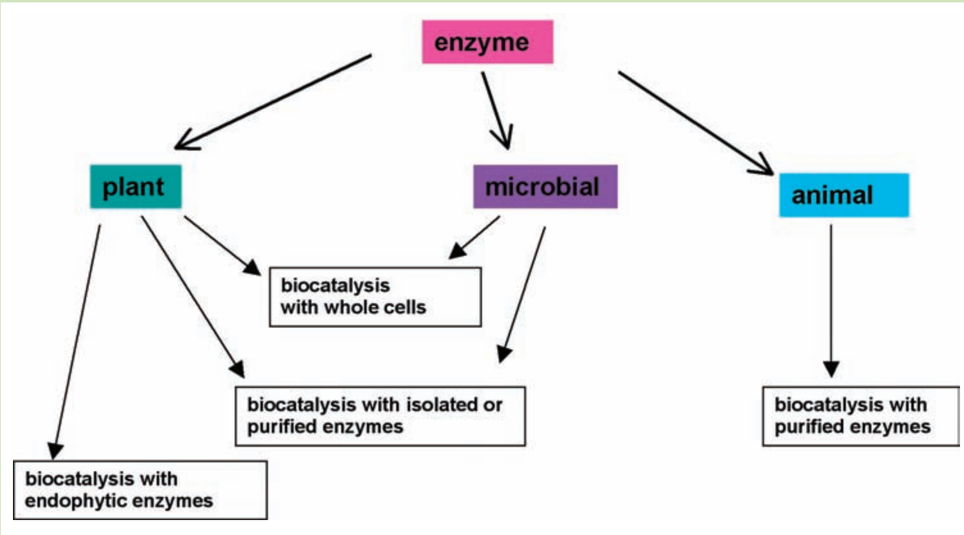
In a typical bioconversion, a single enzyme or a cocktail of enzymes is used. The source of the enzyme offers many trade-offs. Isolated or purified enzymes offer higher specificity. However, isolated or purified enzymes may be too costly, or not even available. Therefore, whole plant and microbial cells and their extracts are used as whole-cell systems containing the enzymes needed for the reaction. Whole-cell systems are advantageous when a multistep biocatalysis is needed. Such processes need to be designed carefully so that the cells accumulate the product and do not metabolize it. The downstream catabolism can be prevented by using bi-phasic systems, by continuous product removal or by disruption of genes responsible for the downstream metabolic pathways. Often the enzymes are immobilized so that they can be recycled and so lower the cost of manufacturing. **F-1** depicts the application of enzymes. (Enzyme manufacturers such as Novozyme, Biocatalysts, Valley Enzyme Research, etc. have created a portfolio of enzymes from a variety of sources, and with different specificities.)

Here, two examples of enzymatic processes are presented: single- and multi-enzyme biocatalysis. The first example is the oxidation of valencene to nootkatone using enzymatic biocatalysis (see **F-2**).[2] Valencene is first oxidized to a hydroperoxide by a number of fungal phenol oxidases. Valencene hydroperoxide is then converted to nootkatone. As prepared, nootkatone can be either isolated as a pure aroma chemical, or the whole citrus oil can be used as an enriched essential oil. Pioneering work on the production of laccase by plant pathogen *Botrytis cinnerea* by Professor James Nakas at SUNY laid the foundation for its usage. The biotechnology group at Givaudan invented a process using this enzyme for nootkatone production (US 6,200,786). Building on Givaudan's work, the gene for laccase was recently patented by an enzyme company Diversa (US patent application 20070105112).

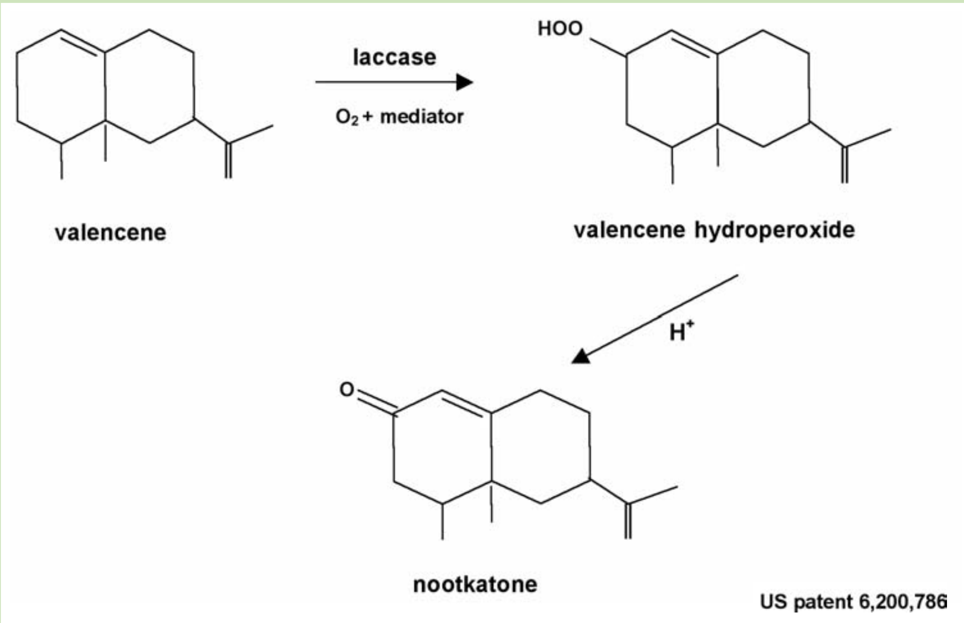
Multi-enzyme process is another example of biocatalysis. Formation of vanillin can serve as an example. Since vanillin is a high-volume aroma chemical, researchers have put great effort in identifying various biosynthetic and biotransformation processes (**F-3**). Vanillin can be formed via plant, microbial or enzymatic routes. Besides plants, microbial biotransformation of renewable ferulic acid has so far been the most promising for vanillin production. Biotransformation of ferulic acid is achieved in a series of enzymatic steps. Typically, for ferulic acid biotransformation, microbial cells are used in their stationary growth phase with an ample amount of alternative carbon source. The media pH determines the solubility of ferulic acid and thus the availability of the precursor to the biotransformation.

Considerable progress has been made in characterizing the biochemistry and molecular-genetics of the microbial catabolic pathways leading to vanillin. For example, scientific research has decoded the enzymes involved in formation of vanillin from ferulic acid in *Pseudomonas fluorescence*.[3] Identification and characterization of the genes coding for these enzymes will offer new opportunities for metabolic and translational engineering and for the construction of recombinant strains. Further work needs to be done to prevent oxidation of vanillin to vanillic acid by inhibition of vanillin dehydrogenase. Various other approaches,

Enzymes in flavor production **F-1**



Enzymatic biocatalysis of valencene to nootkatone **F-2**



microorganisms and conditions were reported. Some of the microbial processes are commercially viable and are used for vanillin production. Combination of microbial and enzymatic processes is another approach to form vanillin. For example, recombinant bacteria *Escherichia coli*, containing part of plant shikimate pathway, produces vanillic acid that is subsequently reduced to vanillin by purified fungal aryl aldehyde dehydrogenase.[4]

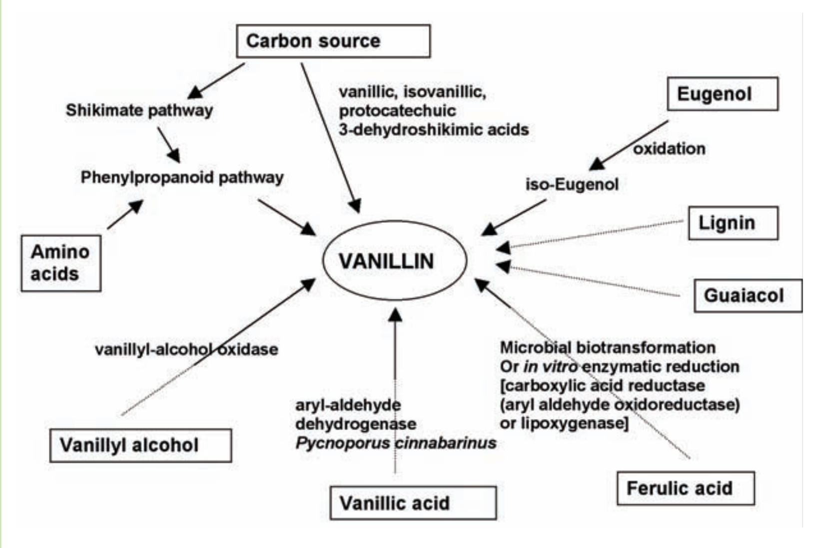
At the same time, vanilla plant biosynthetic routes need to be studied and better understood. Recent work by French scientists at Reunion Island discovered that the vanilla orchid has a very low genetic diversity, especially the cultivated types.[5] Since very few of the changes on the genetic level take place in the vanilla genome, the phenotypic variations that lead to variation in vanilla extracts may be due to epigenetics (different gene expression) or polyploidity (more than two sets of chromosomes). There remains a great need to uncover all the genes, their expression, silencing and translation, which are important for vanilla flavor formation. Metabolic engineering could help eliminate dramatic variations due to a number of factors, such as an impact of environment on genetic expression.

Also of interest was an attempt to reroute the tobacco plant phenylpropanoid pathway into vanillin. This was done by introducing a bacterial crotonase gene into a tobacco plant, which resulted in phenotypical abnormalities.[6] This work demonstrated that the accumulation of intermediate compounds can lead to a shuffling of different pathways leading to the variety of phenolic compounds.

This is in line with the complexity of vanilla extract, which boasts more than 80 compounds, resulting from numerous interconnected metabolic pathways that are interconnected. Identification of genes in vanilla orchids increases the understanding of the flavor-related biochemical pathways. This increases the impact of science in the art of vanilla extract manufacturing and can be translated into more predictable product.

In general, biotransformation and fermentation processes tend to have lower yields than chemical reactions. Typical hurdles are the initial concentration of the precursor, its solubility, toxicity and the accumulation of the finished product. To make good yields economically feasible, the engineering of the process must be coupled with a detailed understanding of the pathways. Traditional recovery processes such as distillations and extraction may be sufficient to eliminate the product inhibition or toxicity of the product to the biocatalyst. More advanced technologies need to be incorporated to improve product yields, e.g. whole-cell biocatalysis in biphasic systems, cofactor regeneration for in vitro oxidation, immobilization, thermostable enzymes, membrane and immuno-based separation of the product, etc. These processes can also be adapted for the formation of fragrance materials when they present either economic advantage or distinctive end products.

Different routes to vanillin **F-3**



Unique Complex Bases

Fermentation processes are used to prepare complex bases and matrixes that have distinctive background notes. The flavor compounds are formed during the microbial growth on basic nutrients, typical of de novo synthesis. The type of microorganism determines which particular array of metabolic intermediates is created. This complexity gives a product a natural unique signature that is a competitive advantage. Frequently the entire flavor-intensive biomass is used to aromatize the foods, as, for example, in enzyme-modified cheese. In some instances, only a part of the flavor bouquet is created. For instance, a microbial fermentation can yield an intensive part of bread aroma and taste that is then used to enhance the taste of whole wheat products or chemically leavened dough. These bread biobases are generated by the bakers' yeasts, lactic acid bacteria fermentations, enzymatic reactions, etc. The nutrients, such as carbohydrates, proteins and fats, are converted into flavor chemicals such as lactic acid, acetoin, acetic acid, pyrazines, etc. Such a biobase carries strong bready and fermented notes. Their formation depends on the genetic makeup of the microorganism and on growth conditions. The beauty of these processes is the creation of a distinctive array of metabolic intermediates. As an example, impact of microorganisms and enzymes on dough flavor is summarized in **T-1**.

Meaty and savory flavor bases can be made from many protein-rich materials. Yeasts and vegetable proteins are both good sources of savory flavors. Savory flavors from these sources are generated through protein hydrolysis, peptide production and heat reaction. The nature of the protein and enzymes determine the flavor outcome.

Creation of flavor biobases requires knowledge of biochemistry, chemistry, microbiology and fermentations. However, the knowledge is mostly retained within the industry and rarely published. There are an infinite number of variations and permutations that can lead to a range of different products.

Flavorists and application chemists play an integral role in finding final applications for these complex biobases. Success in the marketplace for these complex flavor ingredients relies on the creative imagination of the flavorists and application chemists using them.

Improved Plant Extraction and Ecologically Friendly Processes

Many flavor and aroma materials are derived from plants. Plant materials are complex matrixes. Aroma chemicals are chemically intertwined into polysaccharides, proteins or lipids. These bonds can be efficiently hydrolyzed in a short time prior to the recovery processes such as extraction or distillation. Enzymes increase the yield by hydrolyzing the chemical bonds and the polysaccharides, protein and lipids. This enzymatic hydrolysis improves the yield and shortens the processing time. Depending on which enzymes are used, the addition of enzymes to the plant tissue can intensify and change the profile of the extracted flavors simply because of additional flavor release. Some of the processes use a cocktail of enzymes, whereas in some instances a sequential introduction of individual enzymes is more beneficial. As a result, more efficient manufacturing is coupled with higher yields and sometimes additional sensory attributes. A number of enzymes are naturally present in the plants and are involved in the hydrolysis during maturation, harvesting, pressing or distillation. De facto, in some instances, it is only an enhancement of the existing enzymes that is needed to increase the yields.

Downstream processing, such as filtration, is another application of enzyme technology. Enzymes are also helpful in eliminating the impact of annual crop variations by standardizing the extraction process.

T-1 Microbial and enzymatic contribution to bread flavors

Enzyme / organism	Product	Flavorant
Yeasts Lactic acid bacteria	High and low molecular components Small molecular weight volatile compounds	Taste modifiers such as peptides, nucleotides, oligosaccharides Carbonyls, furanones, pyrazines, alcohols
Protease	Amino acids as intermediates for flavors, peptides, taste enhancers, sweeteners, bitter agents	Carbonyls, furanones, pyrazines
Lipases	Short chain fatty acids	Off-notes and off-aromas
Invertase	Glucose, fructose	Carbonyls, pyrazines,
Lipoxygenase	Oxidation products of fatty acids, vitamins	Carbonyls
Oxidases	Strengthen gluten	
Carbohydrases	Oligosaccharides from xylan, hemicellulose, pentosans	Furanones, pyrazines, pyrroles

Taste Modifiers

Throughout the last 15 years, scientists have uncovered the basis of taste and olfaction little by little. This knowledge is invaluable to progress in the food industry. Prolonging the taste experience, enhancing the positive attributes and suppressing the negative ones are the major areas of interest. In all cases, science can be an engine behind progress in the art of extending, enhancing, masking, changing and reducing tastes.

Extension of the pleasant and desirable tastes can be achieved through sustained release of flavorants from its matrix. On the other hand, suppression of the negative attributes can be achieved using different approaches: (I) interaction with taste receptors, (II) coating of the undesirable compounds, (III) larger particle size, (IV) modification of the active compound to bypass the receptors, etc. For example, microencapsulation, used widely in the flavor industry, is one of the methods used to bypass taste receptors and improve taste and eliminate off-taste. It also helps with extending of product shelf life.

Knowledge of the basic logic behind taste coding and interaction between taste receptors and tastants is critical for future developments. It will allow for more targeted modifications, such as competition for receptor sites, or changing the receptor binding site. For example, early taste receptor work by Robert Margolskee, MD, PhD et al. at Mount Sinai Hospital in New York, New York resulted in patent identifying adenosine monophosphate analogues as bitterness suppressors.[7] The unpleasant, medicinal, or harsh taste of thymol can be masked by effective amounts of a sugar alcohol, or a mixture of sugar alcohols, and anethole. Elucidating different aspects of the taste mechanism is an area of active research. Recent patents by Senomyx and National Institutes of Health teams demonstrated that an expression of heterologuous receptors is needed for tasting a variety of sweet compounds. This knowledge is being used in rapid throughput screening.

Scientific work on starch derivatives, natural polymers and nanotechnology succeeded in bringing novel materials to the market. Materials can be selected based on their functionalities for either sustained release of desired flavors or masking of undesirable ones. Flavorists have additional options to target the taste perception by creating flavors that complement the off taste. For example, bitter taste is more acceptable in chocolate or grapefruit flavors where they are expected.

Identification of many of the masking and enhancements has been done through trial-and-error types of experiments relying on sensory panels to identify compounds with certain attributes.

Regulatory Aspects

Regulatory status is a key factor for any flavor product. Flavors can be natural, all-natural, organic or artificial. The process through which they are prepared determines the regulatory status. However, the interpretation of the processing conditions depends on the country of origin and its regulatory system. It also depends on the intended product; naturalness is preferred in flavorants and relatively unimportant for fragrances. Compounds derived from microbial, plant and enzymatic reactions are considered natural. From a regulatory perspective, products derived from biological processes can be classified as natural, organic-compliant and organic. Typically, a flavor is considered natural when manufactured with genetically modified microorganisms (GMO), plants or enzymes. Such flavor does not require a special labeling in the United States; however, GMO cannot be applied to manufacture organic, organic-compliant and natural flavors. It should be stressed that genetic engineering using molecular biology tools is the modern version of a traditional mutation protocol employed by farmers and breeders. Classical breeding of plants and microbes has been with us for many centuries, and it can be expected that, in time, molecular biology tools will be fully accepted as more efficient and safe. Only new technologies leading to more precise changes in DNA are considered GMO. Transgenic organisms and their usage in the food chain are an entirely different category, since they cross DNA between species. However, tools such as molecular genetics, metabolic and translational engineering are indispensable in gathering knowledge about the genes and their functionalities of microorganisms, plants and their enzymes. Overall, this scientific backbone is essential for future advances.

Conclusion

Recent progress in biological sciences provides exceptional opportunity to create innovative products. Translation of these innovations into successful processes and products heavily depends on investment into research, on being able to predict the shifting markets and regulatory environment, in addition to factors such as cost, rapid delivery, etc. Successful processes are only those that offer difference from competition, cost-effectiveness and return on investment. A captivating product is a result of a synchronized and progressive management that brings together basic and applied sciences, applications, manufacturing and marketing.

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