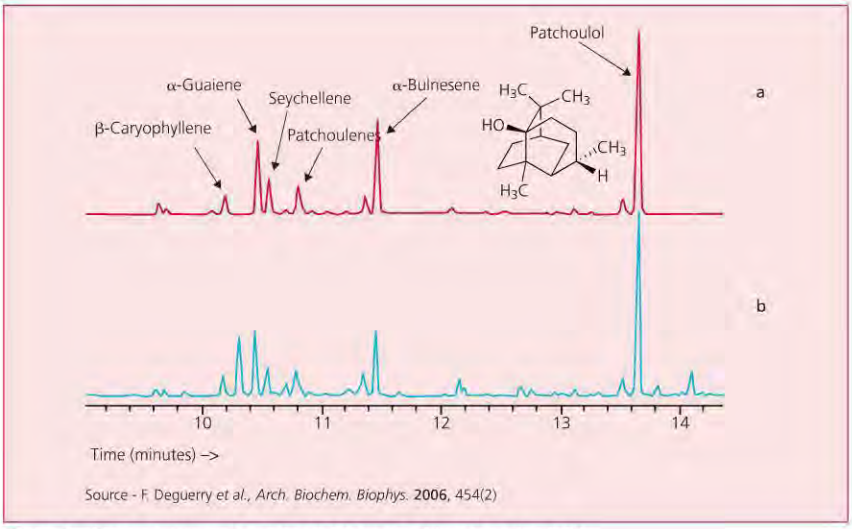
Flavours & fragrances: Recent advances in biotechnology

John C. Leffingwell and **Diane Leffingwell** look at how biotech routes have started to transform the making of flavours and fragrances

Recent years have seen progress in biotechnological manufacture of a number of important flavor and fragrance (F&F) ingredients. In this article, we will discuss this progress with respect to the following: amino acid-derived flavour enhancers (*umami* - monosodium glutamate (MSG) and *kokumi* – L-γ-glutamyl-valyl-glycine (γ-Glu-Val-Gly)), as well as patchouli oil (patchoulol), vanilla (natural vanillin) and sclareol (an ambergris scent intermediate).

2014 was a breakthrough year in the F&F industries' quest for sustainability and cost reduction for a number of important ingredients. It was a year where the value of biotechnical partnerships with companies like Amyris and Evolva began to payoff. In December 2014, Evolva acquired Allylix, a biotech producer of the important grapefruit ingredient nootkatone, which is used extensively in both industries.



Source - F. Deguerry *et al.*, *Arch. Biochem. Biophys.* **2006**, 454(2)

Figure 2 – Chromatograms of patchouli (a) & recombinant patchouli (b)

Amino acid-derived flavour enhancers

MSG is the largest production volume flavouring ingredient, at an estimated 2.96 million tonnes in 2013. It was originally discovered by Kikunae Ikeda at Tokyo Imperial University in 1907 as the unique flavour enhancing character (now called *umami*) in kelp (*kombu*). He patented a process for manufacturing MSG from hydrolysed protein in 1908.[1]

In 1909, production began by Saburosuke Suzuki at Suzuki Seiyakusho, which is now part of Ajinomoto. The product was introduced in Japan on 20 May 1909 with the trademark Aji-No-Moto, which means the 'essence of flavour'.* Ajinomoto is still a major producer, as are Fufeng, Vedan and CJ CheilJedang.

In 1956, separate groups at Tokyo University and Kyowa Hakko Kogyo (now Kyowa Kirin), found that the fermentation of carbohydrate and ammonia sources in a strain of a *Micrococcus glutamicus* (now known as *Corynebacterium glutamicum*) produced L-glutamic acid in good yields.[2] By the 1960s, glutamate production was rapidly moving to the fermentation method, with the *coryneform* bacteria (*Corynebacterium glutamicum* and *Brevibacterium* species) being preferred.[3]

Work on mutant strains began, with the first patents appearing in the early 1970s.[4] Since that time, over 4,000 patents and patent applications have appeared that use mutant microorganisms to produce amino acids, nucleosides and nucleotides. Publications have recently appeared on the metabolic engineering of glutamate in *C. glutamicum* and specifically for the genome sequence of a *C. glutamicum* mutant used in the commercial production of glutamic acid.[5]

L-Lysine is another approved flavourant to be manufactured using *C. glutamicum* strains. However, its main use is as an animal feed additive. Production of L-lysine in 2013 was estimated at 2.1 million tonnes.

γ-Glu-Val-Gly is a newly discovered *kokumi* flavour enhancer that is prepared from Val-Gly with a γ-glutamyl group donor in the presence of a mutant γ-glutamyltransferase. *Kokumi* is sometimes translated as 'heartiness' or 'mouth fullness' and can enhance the intensities of salty, sweet and *umami* tastes. In January 2015, it was reported that γ-Glu-Val-Gly can also enhance thick flavour, aftertaste and oiliness in reduced-fat peanut butter, suggesting that it can improve the flavour of low-fat foods.[6]

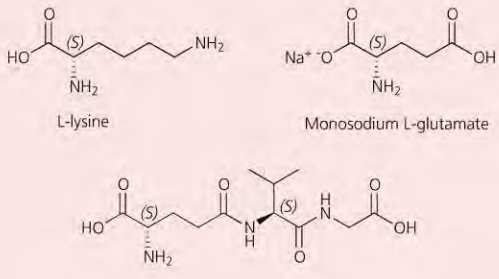


Figure 1 – Amino acid-derived flavour enhancers

Patchouli oil

In 2014, following on from its seminal work on patchoulol synthase, Firmenich introduced its Clearwood patchouli oil substitute, described as a "soft, clean version of patchouli without the earthy, leathery and rubbery notes found in the natural oil". This resulted from the scale-up and production of the Firmenich technology by Amyris. Figure 2 shows chromatogram comparisons of patchouli oil with that produced via a recombinant patchoulol synthase.[7]

In addition, as part of its overall ingredient sustainability programme, Firmenich is working with farmers in both Indonesia and Guatemala

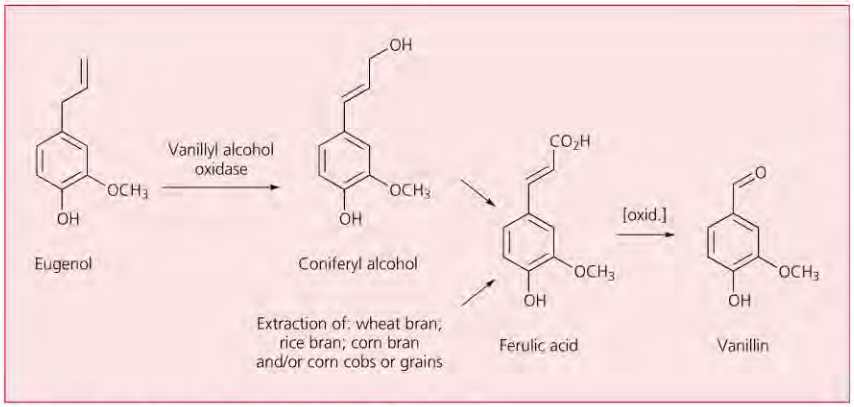


Figure 3 – Ferulic acid & eugenol processes for natural vanillin

on the production of patchouli oil to improve its own supply chain and revenues for farmers. Givaudan's patchouli oil sustainability programmes in Indonesia and Borneo also reflects this type of industry activity.

Vanilla

Vanillin was first isolated from vanilla extract in 1858.[8] In 1874, Tiemann and Haarmann were able to prepare vanillin by the oxidation of coniferin (coniferyl alcohol β-D-glucoside) present in softwood pine species. This led to the formation in 1875 of Haarmann & Reimer (now part of Symrise) for the commercial production of vanillin. By 1876, they had developed syntheses from both eugenol and guaiacol.[9]

Subsequently, other processes have been employed to produce synthetic vanillin from eugenol, lignin and guaiacol-glyoxalic acid. Because of environmental concerns, however, most of the vanillin producing facilities using lignin-containing waste liquor from paper plants have ceased operation. The guaiacol-glyoxalic acid route is now used extensively in China.[10]

Vanilla and vanillin are among the most important F&F products in the world, with a total market value of about $600 million and volume of about 18,000 tonnes. Only a small fraction of this consists of natural vanilla or natural vanillin. The ice cream and chocolate industries together comprise 75% of the market for vanillin as a flavouring, with smaller amounts being used in confectionery and baked goods.[11]

In the last two decades, consumers and marketers have increasingly expressed the desire for natural flavours in their products. For example, in February 2015, Nestlé USA announced that it will eliminate all artificial colours and flavours from its chocolates by the end of 2015 in response to consumer preference for natural ingredients. This follows similar moves by the company in other parts of the world.

However, in the case of vanilla, and vanillin, the very high cost of supplying 'natural' has been a major challenge for the flavour industry. In the case of vanilla extract, the price is dramatically affected by the cost of vanilla beans. As Madagascar exports about 80% of the world's supply, factors like cyclones, price to farmers, political turmoil and crop disease have historically caused some major price fluctuations.[12]

This was especially true in 2003, when vanilla bean prices rose to about $400/kg and then dropped to $32/kg in 2005. As cured vanilla beans contain about 2% vanillin, even at a more normal $25/kg the natural vanillin portion would cost at least $1,250/kg, compared to $16/kg for synthetic vanillin – hence the interest in bio-based natural vanillin production at a reasonable price.

In 1977, Tasada reported that a soil microorganism, tentatively identified as a *Corynebacterium* sp. and a *Pseudomonas* sp. converted eugenol to ferulic acid and vanillin.[13] The first patent on production of natural vanillin appeared in 1991 from Haarmann & Reimer, describing a process of converting isoeugenol or eugenol to vanillin using microorganism strains of *Serrati* sp., *Enterobacter* sp. or *Klebsiella* species.[14] Numerous publications and patents on bio-based vanillin from either ferulic acid or eugenol soon followed these discoveries.[15]

In 2000, Rhodia (now part of Solvay) introduced Rhovanil Natural®, a natural vanillin produced from ferulic acid based on technology from Givaudan.[16] Although it was priced at about $700/kg, the timing was perfect, considering the disastrous vanilla bean crisis of 2003.

In 2015, a Conagen/Givaudan patent application disclosed a new process for producing ferulic acid used for vanillin biosynthesis. In this case, ferulic acid is made from p-coumaric acid by a two-step enzymatic process, encompassing hydroxylation and O-methylation, with caffeic acid as an intermediate metabolite. This is potentially a significant advance, as p-coumaric acid accompanies ferulic acid at high levels in waste products like maize cobs, brewers' spent grain and rice husks.[11,17]

Also in 2015, BASF disclosed an improved biocatalytic process for producing vanillin from ferulic acid based on genetically engineered *Pseudomonas* strains, while in 2009 Shanghai Apple had developed an improved process from ferulic acid using a new a *Streptomyces* strain. In the same vein in 2014, Mane introduced its Sense Capture Vanillin obtained by an improved bioconversion of eugenol.[18] Figure 3 illustrates the eugenol and ferulic acid routes.

In 1998, John Frost's group at the University of Michigan published the *Synthesis of vanillin from glucose*. This laid the groundwork for the Evolva-IFF collaboration for the production of natural vanillin (Figure 4).[19] As vanillin is toxic to the micro-organisms employed, the process forms vanillin β-D-glucoside, which is later hydrolysed to vanillin. IFF began the use of commercial quantities of this material in vanilla-flavoured products in 2014.

In addition, companies such as Symrise, Firmenich, Takasago and Givaudan have active sustainability programmes with local vanilla bean farmers to improve both agricultural practices and wages in key producing areas because natural vanillin does not totally replicate the characteristic flavour profiles of high quality vanilla extracts.

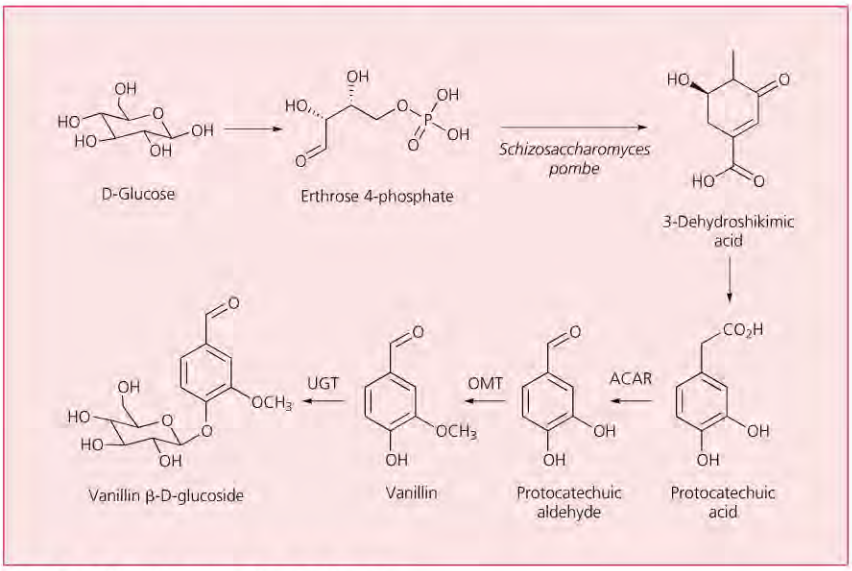


Figure 4 – IFF-Evolva process for natural vanillin

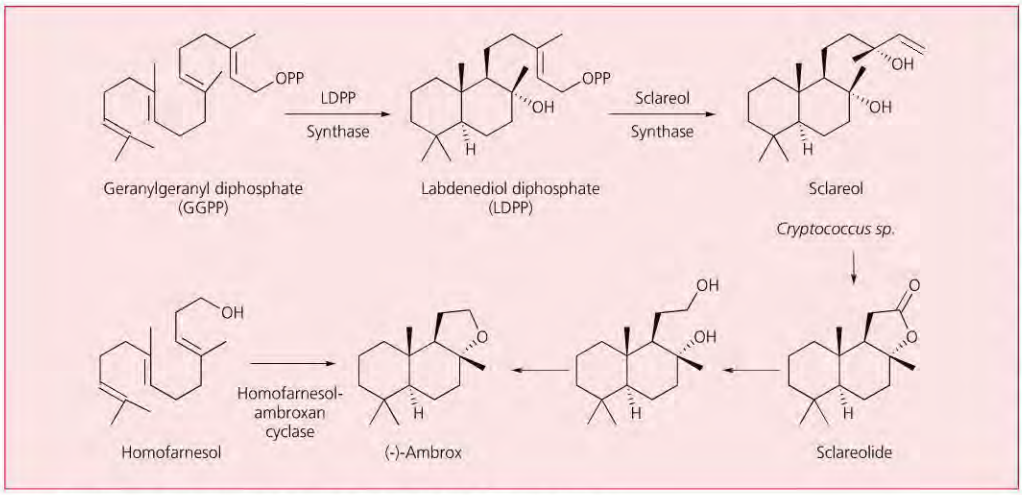


Figure 5 – Biotech processes for (-)-Ambrox from sclareol & homofarnesol

Sclareol & homofarnesol

(-)-Ambrox* was first disclosed as one of the most important odour constituents in ambergris by Max Stoll's group at Firmenich in 1950, which determined both its structure and a process for producing it from sclareol. Normally, clary sage oil, also a valuable perfume ingredient, is steam-distilled from the Clary sage plant (*Salvia sclarea L.*), after which sclareol is extracted with a hydrocarbon solvent, then separated by methanol extraction with subsequently conversion to sclareolide, the key intermediate for the classic Ambrox.[20]

In 1988, a shortage of sclareol and sclareolide occurred and work began on the development of alternative synthetic routes.[21] Avoca, the world's largest producer of sclareol and sclareolide, originally used a permanganate oxidation of sclareol to produce sclareolide but today uses a biochemical process.[22] As both sclareol and sclareolide are relatively expensive and are subject to potential volatility in availability, investigations into biosynthesis have been under way for over a decade.

Both Firmenich and Allylix appear to have developed viable biosynthetic routes for production of sclareol from geranylgeranyl diphosphate via a labdendiol intermediate which can be converted to Ambrox by classical procedures (Figure 5).[23] Another approach, recently developed by both Kao and BASF is partial biosynthesis using homofarnesol. The Kao process uses a squalene-hopene cyclase while the BASF process employs a polypeptide with the activity of a homofarnesol-ambroxan cyclase (Figure 5).[24] These type processes may eventually be scaled up for commercialisation.

Outlook

In September 2014, at the International Federation of Essential Oils & Aroma Trades (IFEAT) convention in Rome, the role of biotechnology and its importance to the F&F industry was discussed extensively.

Panchapagesa Murali of Evolva pointed out that the biotech sector is currently addressing the 'low hanging fruit', but complex offerings may logically follow. Toine Janssen of Isobionics, which produces the important natural citrus ingredients valencene and nootkatone via biotechnological routes, indicated that fermentation technologies could produce many of the terpenes and sesquiterpenoids used in the industry, including the carvones, menthol and the pinenes.

Pascal Longchamp, also of Evolva mentioned that new projects to produce agarwood (*Oud*) and saffron were now under way. Jason Kelly of Ginkgo BioWorks indicated that many of the new biotech companies are in partnerships not only with F&F companies but also with those in fuels and biofuels and that the number of entries is expanding. Relative to the types of products that can be produced, Longchamp remarked "The world is the limit".[25]

*AJI-NO-MOTO is a registered trademark of Ajinomoto Corporation; Rhovanil is a registered trademark of Solvay SA; Ambrox is a registered trademark of Firmenich SA

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