

Review

Vanilla flavour: production by conventional and biotechnological routes

S Ramachandra Rao and GA Ravishankar*

Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 013, India

Abstract: This review deals with the *Vanilla* plant: history; botanical description; chemistry of vanilla beans; curing of vanilla beans; commercial extraction of vanilla flavour; standard specifications and uses of vanilla flavour. The production of vanillin by both chemical and biotechnological methods is described. The biotechnological production of vanilla flavour metabolites by plant tissue/cell culture, microbial biotransformation and molecular approaches is also presented, together with a discussion on economic and safety considerations.

© 2000 Society of Chemical Industry

Keywords: vanilla flavour; biotechnology; biotransformation; precursor; biosynthesis; genetic engineering; safety

INTRODUCTION

Vanilla, which originated in Mexico, is a tropical orchid belonging to the family Orchidaceae.¹ About 110 species have been identified, but only three have been reported to be important in terms of commerce and cultivation: *Vanilla fragrans* (Salisbury) Ames; also known as *V planifolia* Andrews; *V pompona* Schiede; and *V tahitensis* JW Moore.^{2–5} Among these, *V planifolia* is the most valued for its flavour qualities and is therefore widely cultivated and used for the production of food additives.⁶ The fully-grown mature fruits of vanilla, also called beans or pods, develop characteristic aromatic properties by the process of curing. The cured beans are referred to as vanilla. Vanilla and its extracts are very important and popular natural flavourings used in food, beverages and confectionery. Vanilla is also used as a fragrance ingredient.^{2,7,8}

The conventional cultivation of vanilla is laborious, in that one has to manually pollinate the flowers. This labour-intensive process does not encourage farmers to cultivate vanilla plants on a large scale. To overcome this problem, there is a need to develop a viable and simple method for the production of vanilla flavour metabolites using cell culture systems.

In this context the recent emphasis has been on attempting to produce high-quality vanilla flavour products, using biotechnological methods, containing the range of metabolites produced by vanilla plants. The technology envisages bioconversion of cheap substrates using plant or microbial cells as biocatalysts

to produce flavours of high value. Consumer acceptability depends on the quality of the product and also on the safety of the organism used. The industrial interest lies in the cost-effective production of the flavour molecules, which are otherwise difficult to obtain in the proportions present in natural extracts.

This review focuses on vanilla plant cultivation, the chemistry of vanilla beans, curing of beans, extraction methods, uses of vanilla, biotechnological methods for production of vanilla flavour, molecular approaches, economic considerations and safety aspects.

HISTORY OF VANILLA

‘Tlilxochitl’, which means black pod, is the Aztec word for vanilla. Prior to 1500 AD, Aztecs, who were the natives of Mexico, cultivated vanilla and used it to flavour their drinks. In 1500 AD the Spanish invaded Mexico. They learnt how to use this spice to flavour a drink made from ground cocoa and sweetened with honey, called ‘xocolatl’ or ‘hot chocolate’. It was introduced into Europe around 1510 AD. Following this, Mexico remained the sole producer of vanilla for European consumption for the following 350 years. Efforts were made to cultivate vanilla in Europe prior to 1733, but owing to the absence of natural pollinators, the crop was a failure, as there was flowering but no fruiting. Professor Charles Morren of Liege developed an artificial method of pollination of vanilla flowers in 1841 and became the first to produce vanilla pods outside Mexico. Five years later,

* Correspondence to: GA Ravishankar, Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 013, India

Contract/grant sponsor: Council of Scientific and Industrial Research (CSIR), India

Contract/grant sponsor: Department of Biotechnology (DBT)

Contract/grant sponsor: Government of India

(Received 7 September 1999; accepted 20 October 1999)

Edmond Albins, who was employed on vanilla plantations on the island of Reunion in the Indian Ocean, devised a practical method of manual pollination using a bamboo stick. It was only then that the commercial production of vanilla became possible.

Using this process, the French soon started vanilla cultivation on many of their islands in the Indian Ocean, East and West Indies and French Oceania. The most important producing area is the northern part of the east coast of Madagascar. French vanilla beans are known as 'Bourbon' vanilla. Cultivation first started on the island of Reunion, east of Madagascar, earlier known as the island of Bourbon; on Nossi-Be, off the north-western tip of Madagascar; and on the nearby Comoro Islands. These were the three important French territories producing vanilla. The French contributed three-quarters of the world's vanilla beans. By 1890, vanilla was being successfully cultivated in Java, Tahiti, Madagascar, the Seychelles, the Comoro, Islands Mauritius, Reunion, Zanzibar, Jamaica and several other regions in the tropics.^{2,3,5}

BOTANICAL DESCRIPTION OF THE VANILLA PLANT

Vanilla is the only orchid family genus whose species produce a commercially important flavouring material. Vanilla is cultivated in a warm, moist, tropical climate where the annual, evenly distributed rainfall averages between 190 and 230 cm. The average minimum and maximum temperatures required are around 24 and 30°C respectively. It is cultivated by vegetative propagation.²

The vine has a fleshy, succulent stem; smooth, thick oblongate, acute bright green leaves 10–23 cm long and 4–6 cm wide; and numerous twining axial roots arising opposite each leaf, by which it clings for support. The yellow-coloured flowers occur in clusters and appear 2–3 years after planting. The flowers are about 10 cm across and borne on pedicels 4–5 cm long. The sepals and petals are linear, oblong, 5 cm in length and pale green (Fig 1). Owing to the closed structure of the flowers, self-pollination is almost impossible. It is observed that the vanilla flower stays in bloom for less than 24 h and pollination at just the right time (8–11 AM) is necessary for fertilisation and fruit development.⁹ Artificial pollination is carried out by hand using a bamboo stick to get a good yield. After fertilisation has taken place, it requires about 10–12 months for the beans to fully mature. At the fully-grown stage the pods are greenish yellow in colour, naturally cylindrical, three-sided, 10–25 cm long and 1–1.5 cm wide (Fig 2). The fresh beans have an unpleasant bitter odour and develop the characteristic pleasant aroma and chocolate brown coloration only upon curing. The harvesting time varies from one region to another, eg Madagascar: May–July; Comoro Islands: April–June; Reunion: June–August; Tahiti and other islands: April–July. Generally, a plantation is retained for 6–8 years with optimal yields.



Figure 1. Vanilla plant at flowering stage.

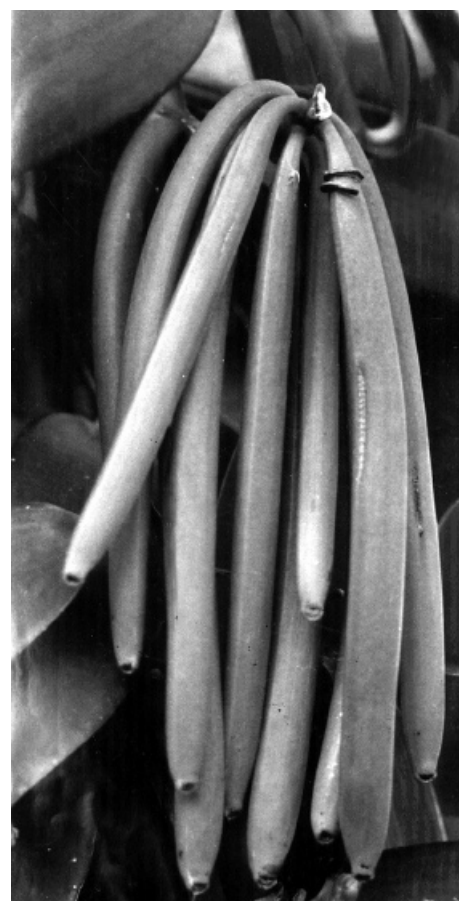


Figure 2. Green vanilla pods.

NATURAL VANILLA FLAVOUR

Natural vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most common flavour chemicals and is used in a broad range of flavours. It occurs in the vanilla bean at a level of 20 g kg^{-1} dry weight and is associated with many other compounds. Approximately 12 000 tons of vanillin is consumed annually, of which only 20 tons is extracted from vanilla beans;^{10–15} the rest is produced synthetically, mostly from petrochemicals such as guaiacol and lignin.¹⁶ During the past few years there has been considerable commercial interest in producing vanillin from natural raw materials using biocatalysis. Vanillin is a metabolic intermediate in the biodegradation of a variety of natural products, including stilbenes, eugenol, ferulic acid and lignin.^{17–20}

Natural vanilla is a complex mixture of flavour components extracted from the cured beans of the vanilla plant: *V planifolia* and *V tahitensis*.² More than 170 volatile aromatic components have been identified. The major components of vanilla flavour are vanillin, vanillic acid, vanillyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and *p*-hydroxybenzylalcohol, whose chemical structures are shown in Fig 3.^{5,21} The constituents of cured vanilla pod are given in Table 1. Pure natural vanillin flavour is priced at US\$4000 kg^{-1} while the synthetic equivalent costs about US\$12 kg^{-1} .^{11,13,22} A number of companies producing vanilla flavour are listed in Table 2. The limited supply and high price of the natural flavour compound have stimulated research on biotechnological substitution.^{23–29}

CHEMISTRY OF VANILLA BEANS

Various factors such as plant species, growing conditions, soil nutrition, harvesting maturity and curing method have to be taken into consideration when determining the relative concentrations of the constituents of vanilla. The cured beans contain proteins, sugars, lignocellulosic fibres, cellulose, organic acids, vanillin and other monohydroxy phenols, fixed oil, wax, resin, gum, pigments, minerals, volatile aromatics and essential oils. The major constituents of vanilla responsible for aroma and flavour are volatiles such as carbonyls, aromatic alcohols, aromatic acids,

Table 1. Major components of cured vanilla pod

Component	g kg^{-1} (dry weight basis)
Vanillin	20
Vanillic acid	1
<i>p</i> -Hydroxybenzaldehyde	2
<i>p</i> -Hydroxybenzyl methyl ether	0.2
Sugars	250
Fat	150
Cellulose	150–300
Minerals	60
Water	350

aromatic esters, phenols, aliphatic alcohols, lactones, aromatic and aliphatic hydrocarbons, terpenoids, heterocyclics, etc.³⁰ The non-volatile constituents which impart the characteristic vanilla flavour are tannins, polyphenols, resins and free amino acids. All these constituents together impart a delicate, rich and mellow aroma with sweet spicy, woody and balsamic notes. The fat content of vanilla beans ranges from 45 to 150 g kg^{-1} and the major fatty acids are oleic and palmitic acids. The sugar content in cured beans is reported to range from 70 to 200 g kg^{-1} and glucose and fructose are the main constituents, with traces of sucrose (Table 3).

CURING OF VANILLA BEANS

Green vanilla pods are almost odourless. They develop a faint phenolic odour, unlike that of cured beans, as the beans reach the stage of harvest maturity. During the curing process, vanilla pods develop flavour as a result of naturally induced enzymatic action. In green beans, flavour components are present as glycosides. The action of β -glycosidases on the glycosides releases the various vanilla flavour components.^{31,32}

Aroma and flavour precursors

The development of aroma and flavour during curing was found to be a type of fermentation process from quite an early date. Tieman (1885) reported the isolation of a heteroside, which he named glucovanillin, by the oxidation of a water extract of green vanilla beans.³¹ Subsequently, in 1900, Busse showed that vanillin could be obtained by the action of acid or emulsin on an unknown component of green beans. Lacompte (1913) postulated that the vanillin precursor present in green vanilla beans was coniferoside, which, under the action of an oxidase, first split to vanilloside, and this in turn yielded vanillin and glucose upon hydrolysis by acid or emulsin.³¹ The most abundant of three glucosides was glucovanillin, and the second was postulated as a glucoside of vanillyl alcohol. An alternative mechanism of vanillin formation was that the glucoside of vanillyl alcohol was oxidised to yield glucovanillin, which would then be hydrolysed to vanillin. Later, Goris³³ confirmed that green vanilla beans contained four components upon cleavage. The most abundant glucoside was gluco-

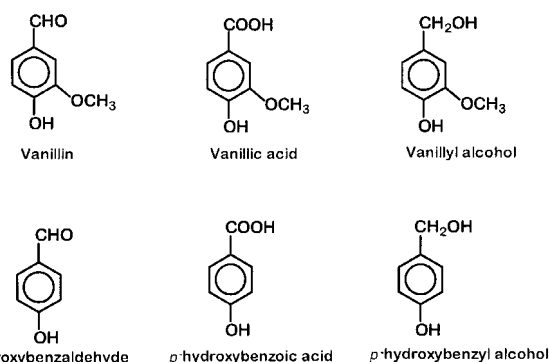


Figure 3. Chemical structures of major vanilla flavour compounds.

Table 2. Companies producing vanilla flavour

1.	ESC Agenetics Corporation, San Carlos, CA, USA
2.	David Michael Co Inc, Philadelphia, PA, USA
3.	Nielsen-Massey Vanillas Inc, Waukegan, IL, USA
4.	Premier Vanilla Inc, East Brunswick, NJ, USA
5.	Virginia Dare, Brooklyn, NY, USA
6.	Gernot Katzer, USA
7.	Aust and Hachmann Ltd, Montreal, Canada
8.	Food Research and Development Centre, Quebec, Canada
9.	Haarmann and Reimer (H&R), Holzminden, Germany

Table 3. Chemistry of vanilla extract^a

Constituent	Number of compounds
Hydrocarbons	40
Carbonyls, aldehydes	11
Acids	20
Lactones	2
Sulphur compounds	1
Ethers	5
Furans	10
Alcohols	25
Carbonyls, ketones	10
Esters	41
Acetals	1
Bases	3
Phenols	10
(Ep)oxides, pyrans, coumarins	2

^a Adapted from Ref 3.

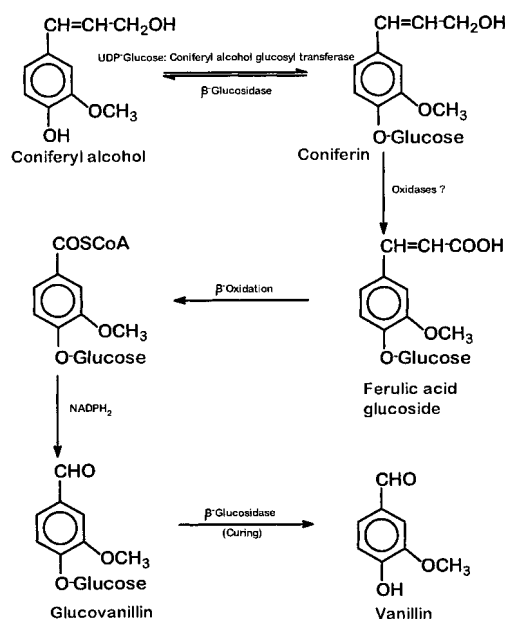
vanillin, while glucovanillyl alcohol was found in lesser quantities.⁶

Other enzymes acting on the vanilla bean during its growth period of 5 months are a protease, β -glucosidase, peroxidase, polyphenol oxidase, cellulase and hemicellulase. It was found that proteolytic activity decreased with pod age and peroxidase activity increased from the third month, while polyphenol oxidase and glucosidase activity increased less notably.³⁴ The presence of a glucosidase would act to hydrolyse coniferin (the glucoside of coniferyl alcohol) into coniferyl alcohol and glucose, and then an oxidase would oxidise coniferyl alcohol to vanillin. Alternatively, vanillin would be produced from its immediate precursor glucovanillin (the phenolic glucoside) by the action of a β -glucosidase. A hypothetical pathway for glucovanillin biosynthesis in green vanilla beans is presented in Fig 4.

A number of procedures have been developed for curing vanilla, but they are all characterised by four phases to allow transformation into a commercially viable product. These phases are killing, sweating, drying and conditioning.

Killing

Harvested vanilla beans are living tissues and continue to have most of the physiological functions intact. In order to cure the beans, it is necessary to stop

**Figure 4.** Hypothetical pathway for glucovanillin biosynthesis in green vanilla beans (adapted from Ref 2).

vegetative development and to disrupt the cell structure, so that various enzymes can come into contact with their substrates. The disruption of the cell structure can be carried out by hot water scalding, sun or oven wilting, scarification, treatment with ethylene gas or freezing. The process is called 'killing' because it disrupts the respiratory function by disrupting the cell membrane. Of the various methods, the most practical methods, namely sun, oven and hot water killing, are commonly used.²

Sweating

In this step, moisture is initially allowed to escape rapidly and attain a level that will reduce the risk of microbial spoilage during subsequent operations. This is also the most crucial step, since curing enzymes, indigenous to vanilla beans, are most active during this process. Under proper treatment conditions, indigenous enzyme activity is optimised, leading to high-quality cured beans. Vanillin and many related compounds are released from their glucosidic precursors in this step. Oxidation of polyphenolic compounds, which give cured beans their characteristic chocolate brown colour and some of their taste attributes, takes place during this step. The process lasts from 7 to 10 days.²

Drying

At the end of the sweating period the cured beans still have about 600–700 g kg⁻¹ moisture. The beans need further drying to reduce their moisture content to protect them from microbial spoilage. The lower moisture content in the beans after drying lowers undesirable enzyme activities and biochemical changes. At the end of drying, the beans contains about 250–300 g kg⁻¹ moisture.²

Conditioning

In this step the beans are stored in closed boxes for one to several months. Various chemical and biochemical reactions such as esterification, etherification, oxidative degradation, etc take place during this period to produce various volatile aroma constituents.²

There are two main methods of curing vanilla pods, namely the Mexican process (sun method) and the Madagascar process (Bourbon method).

Mexican process (sun method)

The Mexican process usually takes place in a series of buildings that cover huge patios. The beans are stored in sheds for a few days until they start to shrink. Next the beans are gathered rapidly and placed in large mahogany sweating boxes. A number of mats are placed over and around the sweating boxes so that the proper temperature can be maintained for the important flavour-developing enzymatic reactions, which are accompanied by slight fermentation. This important phase lasts for at least 24 h and the whole process may be repeated six to eight times during the early part of curing, which will extend over a period of 2–3 weeks. During this period the green beans acquire a dark chocolate brown colour.

Then moisture is reduced to the desired level of 250 g kg^{-1} by frequent exposure to sunlight and sweating. The final curing is done by placing the beans in aging boxes for a period of 2–3 months. Finally the beans are gathered and packed in special tins lined with waxed paper. The Mexican process of curing takes about 5–6 months.³⁵

Madagascar process (Bourbon method)

This method is of shorter duration than the Mexican method. The pods are dipped in hot water at 80°C for 7–15 min. Then, for the next 10 days, they are spread out on blankets and, when quite hot, are rolled up in the blankets to stand overnight for slight fermentation to take place. Next the beans are spread on large trays and allowed to cure and dehydrate naturally. Finally the beans are sorted and packed in small tins. The Madagascar process takes about 35–40 days.³⁶

COMMERCIAL EXTRACTION OF VANILLA FLAVOUR

Various methods of extraction have been used to elicit the flavour from the cured bean. The concentration of an extract is noted by its 'fold'. A single fold of vanilla extract contains the extractable material from 13.35 oz of vanilla beans per gallon of solvent or 100 g of extractable material per litre.

Vanilla extraction methods fall into two main categories, namely the percolation method and the oleoresin method.

The percolation method consists of circulating a solvent, which is an ethanol/water solution in the range 35–50:65–50 (v/v), over and through the beans under vacuum. This process may take from 48 to 72 h.³⁷ By

using this process, an approximately fourfold strength vanillin can be obtained.

The oleoresin method consists of pulverising whole beans and then circulating ethanol over the beans under vacuum at about 45°C . The excess alcohol is removed by evaporation. This process takes about 8–9 days.³⁷ However, by using the oleoresin process, an approximately 10-fold strength vanillin may be obtained. Commercially, natural vanillin is sold as a dilute ethanolic extract.²¹ Post-extraction processing involves clarification by centrifugation or filtration followed by aging of the extract for 1 year.

STANDARD SPECIFICATIONS

Standard specification is an important criterion for any commercially important product that determines its market value. Therefore specifications of quality of raw materials, processing methodologies and their influence on quality of the product are of vital importance. Vanilla bean qualities are hence graded in a systematic manner that determines the quality of vanilla flavour. Quality specifications of vanilla bean and vanilla flavour are given below.

Vanilla bean qualities

The qualities of vanilla (Bourbon variety) are the following.

- *Extra*. Whole, unsplit, fine, fatty, oily pods of a uniform chocolate brown colour, without defects or galls. Delicate aroma. Moisture content about $340\text{--}400 \text{ g kg}^{-1}$.
- *1st*. Same kind as the 'extra' quality, but the pods are not quite so fatty.
- *2nd*. Pods not so large, of chocolate brown colour, with a few small galls. Good aroma. Moisture content about $220\text{--}300 \text{ g kg}^{-1}$.
- *3rd*. Rather thin pods, hard, twisted, of a slightly reddish chocolate brown colour, with fairly numerous galls. Good aroma but nothing more. Moisture content about $120\text{--}160 \text{ g kg}^{-1}$.
- *4th (ordinary)*. Rather dry pods of a reddish colour. May have numerous galls. Rather ordinary aroma.
- *Inferior*. The lowest category. Very dry pods, very reddish colour. Rather 'uncultivated' aroma, which is, however, pleasant, especially in the kinds coming from Madagascar.

These six kinds are known as 'entire'. Six corresponding 'split' kinds exist; since they are of lower economic value, they come in between in the order of categories. 'Foxy splits' are reddish brown in colour, thin, hard-cured and woody, shrivelled, of short length and dry. These are high in vanillin and low in resin.

The residue, after preparing and packing pods which are too short, broken or twisted, is not put into bundles to form the lowest category known as 'in bulk'.³

British standards

According to Purseglove *et al.*,⁶ the British Standards Institution has published a specification for vanilla that is identical with the International Standards Organization specification 1969, 'Vanilla vocabulary'. This standard is specified for the following species of vanilla plant: *V fragrans*, *V tahitens* and certain forms obtained from seeds, possibly hybrids of *V fragrans*. It does not apply to *V pompona*. The standard defines such terms as green, prepared and cured vanilla, vanilla pods and split vanilla and provides a qualitative classification of vanilla types—supple, dry, frosted, mouldy, etc.

- *Green vanilla*. Fruit from the vanilla plant that has reached the correct stage of ripeness.
- *Cured vanilla*. Green vanilla that has been subjected to suitable treatment with a view to developing its flavour.
- *Vanilla pod*. Commercial name designating the fruit of vanilla plants.
- *Split vanilla*. Vanilla that is partially opened longitudinally from the stud owing to dehiscence. The stud is the end of the pod opposite the peduncular end, ie the hook.
- *Supple vanilla*. Fleshy and flexible vanilla.
- *Dry vanilla*. More-or-less dehydrated vanilla that has partially lost its suppleness.
- *Frosted vanilla*. Vanilla that bears crystals of naturally extruded vanillin.
- *Mouldy vanilla*. Vanilla that carries or has carried cryptogams, giving out a characteristic musty odour.

Code of Federal Regulations (CFR) USA specifications for vanilla-based products

Vanilla-based products such as vanilla extract, vanilla flavouring, concentrated vanilla flavouring, vanilla powder, vanilla–vanillin extract, vanilla–vanillin flavouring and vanilla–vanillin powder have been defined with standards of identity in the Code of Federal Regulations (21 CFR 169).³⁸ To reconcile these standards with the bulk flavour regulations found at 21 CFR 101.22, a natural vanilla flavour would consist solely of one or more of the following items: vanilla extract, concentrated vanilla extract, vanilla flavouring, concentrated vanilla flavouring and vanilla powder.

- *Vanilla extract*. This is an aqueous ethanolic extract containing soluble aromatic and flavour components of vanilla beans that is prepared using oleoresin. The ethanol content is 350ml⁻¹ or more. The extract contains glycerol, propylene glycol, sugar and corn syrup as thickeners or sweeteners—strength two fold. The anti-caking ingredients of finished products should not exceed 20kg⁻¹ by weight.
- *Concentrated vanilla extract*. Concentrated extracts of higher-fold strengths are prepared by removing part of the solvent under vacuum. The ethanol content should not be less than 350ml⁻¹. Concentrated

extracts are available in three fold to five fold strengths.

- *Vanilla–vanillin extract*. A combination of 1 gallon (3.8l) of two fold vanilla extract and 2oz (56.7g) of vanillin gives a four fold vanilla–vanillin extract. It is made by adding 1oz of synthetic vanillin for every unit of vanilla bean.
- *Vanilla flavouring*. This is similar to vanilla extract but contains less than 350ml⁻¹ ethanol by volume.
- *Concentrated vanilla flavouring*. The strength is three fold. The ethanol content is less than 350ml⁻¹.
- *Vanilla powder*. A vanilla powder is manufactured by using one unit per fold (13.35oz (378.5g)) in 8lb (3.63kg) of finished powder.

French regulations

In France, regulations were issued in 1966 which forbid the use of the word 'vanilla' or derivatives thereof except in reference to a product made from the properly dried and cured fruit of *V planifolia* or a related species. The regulations also stipulate that if the vanilla flavour used is a synthetic product or artificial, this must be stated on the label of the product. The International Standards Organization's 'Vanilla vocabulary' (ISO 3495)³⁹ was drawn up in collaboration with the Association Francais de Normalisation.

Analytical standards for vanilla extract

True vanilla extract should contain a minimum of 13.35oz (378.5g) (average) of vanilla beans per gallon, with no other ingredients except ethanol (350ml⁻¹) and/or glycerol, sugar and water.

The quality of vanilla extracts cannot be determined by chemical analysis alone. Analysis determines the amount of vanillin present, the lead number, the ash content and other parameters. It is used as a means of knowing whether the extract has been adulterated. However, tests for analysis do not indicate the quality of the beans used. Winton's analytical values are often used as a standard for detecting adulteration. These values are presented in Table 4.

USES OF VANILLA

Vanilla is the world's most popular flavouring for sweetened foods. Vanilla sugar is used in the manufacture of chocolates. Vanilla flavourings are used in countless commercial products, in liquors and in cheap brandy and whisky. In the USA, pure vanilla extracts are widely used as a flavouring par excellence for ice cream, soft drinks, chocolate, confectionery, candy, tobacco, baked foods, puddings, cakes, cookies and liquors and as a fragrance ingredient in perfumery.^{5,37,40,41}

It enhances the flavour of caramel, coffee and some dairy products. It is also used in spice oleoresin formulations, sausages, seasonings, etc. In all those

Parameter (g l ⁻¹ extract)	Minimum	Maximum	Average
Vanillin	1.1	3.5	1.9
Ash	2.2	4.32	3.19
Soluble ash	1.79	3.57	2.65
Lead number ^b	4.0	7.4	5.4
Alkalinity of total ash (0.1M acid ml l ⁻¹ extract)	300.0	540.0	—
Alkalinity of soluble ash (0.1M acid ml l ⁻¹ extract)	220.0	400.0	300.0
Total acidity (0.1M alkali ml l ⁻¹ extract)	300.0	520.0	420.0
Acidity other than vanillin (0.1M alkali ml l ⁻¹ extract)	140.0	420.0	300.0

^a Adapted from Ref 35.

^b This is a measure of organic acid content of vanilla extract determined by precipitation with lead acetate wherein insoluble lead salts are removed and excess lead is determined by chelometric titration with Na₂EDTA. From this titration and blank titration the Wichmann lead number is calculated (AOAC Official Methods of Analysis, 1995, Chap 36, pp 5–6).

Table 4. Composition of vanilla extract^a

formulations containing vanillin/ethyl vanillin, vanilla flavour gives a natural, 'rounded-off' flavour.^{6,9}

As well as its flavour qualities, vanilla also has medicinal value. Its acts as an antimicrobial agent for inhibiting moulds and yeast in fruit purees and fruit-based agar systems.^{42–44} Burri *et al*⁴⁵ have reported that vanillin acts as an antioxidant in complex foods containing polyunsaturated components. Vanillin and its isomer *o*-vanillin markedly inhibit mutagenesis in *Escherichia coli* caused by *N*-methyl-*N*-nitrosurea and UV irradiation.⁴⁶ Suppression effects of vanillin on chromosome aberrations in mice induced by X-rays⁴⁷ and mitomycin C⁴⁸ and in *Drosophila melanogaster*⁴⁹ have been reported. Glucosylvanillin has also been shown to have antimutagenic effects in bacteria.⁵⁰ A significant enhancement of repellence towards black fly species (*Silulium venustum* Say and *Prosimulium hirtipes* Fries) was observed when vanillin, along with toluamide, was used as a spray.⁵¹ The use of vanillin as an antidote for neutralising the effects of toxin from *Chinorex fleckeri* (box jellyfish) has recently been reported.⁵² This finding provides a novel application of vanillin as a pharmaceutically important compound.

PRODUCTION OF VANILLIN USING CHEMICAL METHODS

Vanillin can be obtained by several chemical methods using the starting materials coniferin, guaiacol, eugenol and lignin.

Preparation of vanillin from coniferin

This process was developed by Haarmann and Tiemann in 1874. The cambial sap of about 5000 freshly felled fir or pine trees was collected and coniferin, the glucoside of coniferyl alcohol, was obtained from it. In the second step the coniferin was oxidised in the presence of a mixture of potassium dichromate and sulphuric acid to obtain vanillin with a

yield of about 350 g kg⁻¹. This process is very expensive and is not economic.⁵³

Preparation of vanillin from eugenol

Tiemann (1876) developed a process for the production of vanillin from eugenol. Currently the following three methods are used to prepare vanillin from eugenol.^{53,54} In method 1, eugenol is isomerised to isoeugenol with potassium hydroxide in diethylene glycol. Isoeugenol is isolated and converted to the acetate, which is oxidised with osmium tetroxide/sodium metaperiodate. Vanillin is isolated upon acidification of the sodium sulphite extract. In method 2, isoeugenol is oxidised directly to vanillin with a vanadium pentoxide/H₂O₂ reagent in *tert*-butyl alcohol. In method 3, eugenol itself is oxidised with nitrobenzene in a basic solution of dimethyl sulphoxide (DMSO) to yield vanillin.

Preparation of vanillin from spent sulphite liquor

The starting material for vanillin production is the lignin present in sulphite liquor from the cellulose industry. The concentrated mother liquors are treated with alkali at elevated temperature and pressure in the presence of oxidants. The vanillin formed is separated from the by-products, particularly acetovanillone, by extraction, distillation and crystallisation.

Lignin is degraded with either sodium or calcium hydroxide solution and simultaneously oxidised in air in the presence of a catalyst (nitrobenzene or copper oxide). When the reaction is complete, the solid wastes are removed. Vanillin is extracted from the acidified solution with a solvent (eg butanol or benzene) and re-extracted with sodium hydrogen sulphite solution. Re-acidification with H₂SO₄ followed by vacuum distillation yields technical-grade vanillin, which must be recrystallised several times to obtain food-grade vanillin.^{55,56}

Preparation of vanillin from guaiacol and glyoxylic acid

Several methods have been used to introduce an aldehyde group into an aromatic ring. Condensation of guaiacol with glyoxylic acid followed by oxidation of the resulting mandelic acid to the corresponding phenylglyoxylic acid and, finally, decarboxylation continues to be a competitive industrial process for vanillin synthesis. Currently, guaiacol is synthesised from catechol, which is prepared by acid-catalysed hydroxylation of phenol with H_2O_2 . Glyoxylic acid is obtained as a by-product in the oxidation of glyoxal with nitric acid.

The condensation of guaiacol with glyoxylic acid takes place smoothly at room temperature in weak alkaline media. A slight excess of guaiacol is maintained to avoid the formation of disubstituted products; later, excess guaiacol is recovered. The alkaline solution containing 4-hydroxy-3-methoxymandelic acid is then oxidised in air in the presence of a catalyst (nitrobenzene or copper oxide) until the calculated amount of oxygen is consumed.⁵⁴ Crude vanillin is obtained by acidification and simultaneous decarboxylation of the (4-hydroxy-3-methoxyphenyl)glyoxylic acid solution. Commercial-grade vanillin is obtained by vacuum distillation and subsequent recrystallisation.

BIOTECHNOLOGICAL METHODS FOR PRODUCTION OF VANILLIN

The increasing trend towards natural food ingredients has helped to maintain buoyant demand for natural vanilla, but the worldwide demand for natural vanilla beans cannot be met by the available supply from the limited growing areas in countries such as Mexico, Madagascar, Tahiti and Indonesia. Therefore a need was felt to develop alternative natural biological sources of vanilla flavouring. The production of vanilla flavour is possible from *Vanilla* tissue cultures; another possibility is biotransformation-mediated production using either plant cells or microbes.^{8,57-59}

Plant tissue/cell culture-mediated production

Plant tissue cultures have been shown to produce a wide range of flavours and aroma compounds characteristic of their plant origin. The potential of plant tissue cultures to produce food flavours and aroma compounds has been reviewed.⁶⁰⁻⁶³ The rationale for the production of flavour compounds with cultured plant cells is based upon their unique biochemical and genetic capacity and on the totipotency of plant cells.^{64,65} Thus every cell of a vanilla plant contains the genetic information necessary to produce the numerous chemical components (or their precursors) that constitute natural vanilla flavour. Feeding intermediates of the biosynthetic pathway can enhance the production of vanilla flavour metabolites by precursor biotransformation.

There is a great deal of interest in producing vanilla

flavour compounds via *Vanilla planifolia* plant tissue cultures, and some commercial success has been demonstrated. Plant cell cultures of *V planifolia* and other *Vanilla* spp have been initiated from cells and tissue from various plant organs such as leaves and stems.⁶⁶

Influence of phytohormones

Knuth and Sahai⁵⁷ showed that a hormone mix containing 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl adenine was necessary and useful in the initiation of callus production and growth in *Vanilla fragrans* and other species. The phytohormones had a drastic influence on phenylpropanoid metabolism. 2,4-D suppressed secondary metabolism in this suspension culture. When naphthalene acetic acid (NAA) was substituted for 2,4-D, a significant increase in total extractable phenolics was observed, and combination with cytokinins resulted in the production of relatively large amounts of extractable phenolics. Under normal growth conditions the cultured cells did not produce any detectable benzoate derivatives (C_6-C_1 compounds).⁶⁷ Kinetin has been used as an elicitor to induce vanillic acid synthesis in cell suspension cultures of *V planifolia*.⁶⁸

Precursor biotransformation to vanilla flavour—*Vanilla* tissue culture studies

Feeding of cinnamic acid and ferulic acid to cell suspension cultures of *Vanilla planifolia* resulted in the formation of traces of *p*-hydroxybenzoic acid and vanillic acid.⁶⁹ Conditioned medium has been used for culturing *V planifolia* callus, which resulted in a twofold increase in vanillin production to give up to $15 \mu\text{g vanillin g}^{-1}$ fresh weight. Feeding of ferulic acid (1 mM) to *V planifolia* cells resulted in an increase in vanillin concentration by a factor of 1.7 compared with untreated callus, but precursor addition did not improve the ratio of the flavour components.⁷⁰

Fine suspension cultures of *V planifolia* have also been used to study the effect of radiolabelled precursors, cinnamic and ferulic acids, on vanillic acid production. The addition of 3,4-(methylenedioxy)cinnamic acid along with these precursors showed that 30 g kg^{-1} of fed ^{14}C cinnamic acid was incorporated into vanillic acid, but not ^{14}C ferulic acid. Based on this study, Funk and Brodelius⁷¹ showed that cinnamic acid, but not ferulic acid, was a precursor of vanillic acid.

The effect of light on *V planifolia* callus cultures in relation to the production of vanillin precursors was examined by Havkin-Frenkel *et al.*⁷² The influence of light on culture growth and on the accumulation of vanillin and relative vanillin precursors was observed over an incubation period of 3 weeks. Light quality had little effect on culture growth, and little difference could be detected in *p*-hydroxybenzoic acid, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde and *p*-coumaric acid concentrations.⁷²

Knuth and Sahai⁵⁷ found that the nature and

concentration of vanilla component precursors added to the medium were a factor influencing flavour production in *V fragrans* cultures. Phenylalanine and ferulic acid resulted in little enhancement of vanillin production, whereas addition of vanillyl alcohol resulted in a significant increase in vanillin content.

A process for producing natural vanillin from ferulic acid as precursor with aerial roots and charcoal as a product reservoir has been developed by Westcott *et al.*⁵⁹ Organised aerial roots of *V planifolia* plant detached and cultured in nutrient medium were used as biocatalysts in transforming ferulic acid to vanillin in the presence of charcoal. The charcoal was used as an adsorbent of vanillin, which enhanced the production to 400 mg kg⁻¹ tissue per day, amounting to 40% of the concentration found in vanilla bean. The aerial roots could be used several times, although their activity decreased gradually. The novelty of this method lay in the efficient bioconversion of precursor, wherein 5–10 times the amount of vanillin was obtained in comparison with vanilla bean or aerial roots not supplied with the precursor. The vanilla flavour produced using aerial root culture had a *p*-hydroxybenzaldehyde/vanillin ratio of 7.8:1 as compared with 12.8:1 in bean-derived vanilla. This was suggested to impart superior flavour note and to enhance acceptability.

Capsicum cell culture-mediated biotransformation

Biotransformation to vanillin has been reported in immobilised cell cultures of *Capsicum frutescens* treated with the phenylpropanoid pathway intermediates ferulic acid and vanillylamine.⁷³ The production of vanillin in *Capsicum* cell cultures is shown in Fig 5.

Production of vanilla flavour metabolites was observed when both freely suspended and immobilised cell cultures of *C frutescens* were treated externally with protocatechuic aldehyde and caffeic acid.⁷⁴ It was found that protocatechuic aldehyde-treated cultures showed higher vanillin production as compared with caffeic acid-treated *C frutescens* cultures. Other precursors such as isoeugenol, a clove principle,⁷⁵ coniferyl aldehyde and veratraldehyde were also biotransformed to a whole range of vanilla flavour metabolites in both freely suspended and immobilised cell cultures of *C frutescens*.⁷⁶ This range compared well with natural vanilla bean extract.⁷⁷

Release and recovery of vanilla flavour

V fragrans cultures were able to secrete flavour components into the culture medium. Vanillin production was improved by adsorption onto activated charcoal.⁷⁸ The vanillin yield obtained was 22 mg g⁻¹ on a dry weight basis, comparable with the 10–30 g kg⁻¹ vanillin content obtained in cultured vanilla beans. Subsequent optimisation improved the cell-doubling time from 106 to 50 h and the yield of vanillin from 100 to 1000 mg l⁻¹ (80 g kg⁻¹ on dry weight basis).⁶² The addition of Amberlite adsorbents XAD-4 and XAD-7 to the culture medium resulted in

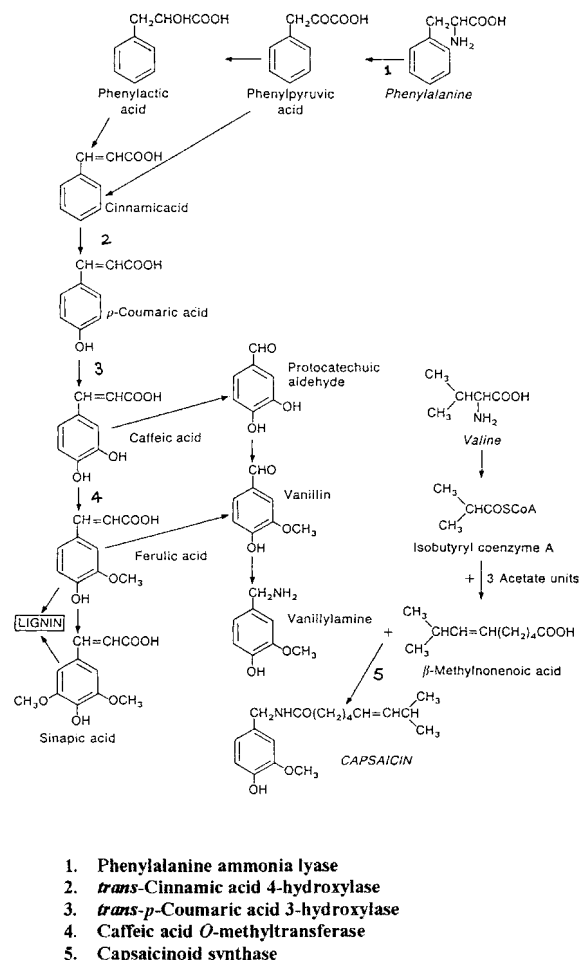


Figure 5. Biosynthetic pathway of vanillin production in *Capsicum* cell cultures through biotransformation.

excretion of metabolites from cells to medium. XAD-4 effectively adsorbed vanillin, leading to an enhancement in vanillin accumulation. On the other hand, XAD-7 showed more affinity towards *p*-hydroxybenzoic acid, which resulted in less formation of vanillin when compared with XAD-4 treatment of ferulic acid-fed *C frutescens* cell cultures.⁷⁷

Production of vanilla flavour from phenylpropanoid precursors using microbes

A large number of microbes such as bacteria, fungi and yeast have been used for the laboratory-scale production of vanilla flavour metabolites from various phenylpropanoid precursors—ferulic acid, eugenol, isoeugenol, vanillyl alcohol, vanillylamine, coniferyl alcohol, veratryl alcohol, etc. The organisms and precursors used for the production of vanillin are shown in Table 5.

Isoeugenol or eugenol as precursor

Abraham *et al.*¹⁰¹ reported that *Aspergillus niger* ATCC 9142 was capable of biotransforming externally fed isoeugenol to vanillin at 10% efficiency; vanillin was partially converted further to vanillyl alcohol and vanillic acid. Researchers from Haarmann and Reimer^{25,53} developed a process for the production of

Table 5. Biotransformation of phenylpropanoid compounds to vanilla flavour metabolites using micro-organisms^a

Micro-organism	Substrate	Product	Author	Ref
<i>Pycnoporus cinnabarinus</i>	Ferulic acid	Vanillin	Falconnier <i>et al</i> 1994	79
<i>Aspergillus niger</i>	Ferulic acid	Vanillin	Lesage-Meessen <i>et al</i> 1996	80
<i>Pycnoporus cinnabarinus</i>				
<i>Pseudomonas acidovorans</i>	Ferulic acid	Vanillin	Toms and Wood 1970	17
<i>Corynebacterium glutamicum</i>	Ferulic acid	Vanillin	Labuda <i>et al</i> 1993	58
<i>Paecilomyces variotii</i>	Ferulic acid	Vanillin	Rahouti <i>et al</i> 1989	81
<i>Pestalotia palmarum</i>				
<i>Spirulina platensis</i>	Ferulic acid	Vanillin	Ramachandra Rao <i>et al</i> 1996	82
<i>Haematococcus pluvialis</i>	Ferulic acid	Vanillin	Usha Tripathi <i>et al</i> 1999	83
<i>Pseudomonas fluorescens</i>	Ferulic acid	Vanillic acid	Andreoni <i>et al</i> 1995	84
<i>Escherichia coli</i>	Ferulic acid	Vanillin	Otuk 1985	85
<i>Alcaligenes paradoxus</i>	Ferulic acid	Vanillin	Krishnamohan and Khanna 1994	86
<i>Streptomyces setonii</i>	Ferulic acid	Vanillin	Sutherland <i>et al</i> 1983	87
<i>Fomes fomentarius</i>	Ferulic acid	Vanillin	Ishikawa <i>et al</i> 1963	88
<i>Polyporus versicolor</i>	Ferulic acid	Vanillin	Rosazza <i>et al</i> 1995	89
<i>Rhodotorula rubra</i>	Ferulic acid	Vanillic acid	Huang <i>et al</i> 1993	90
<i>Corynebacterium glutamicum</i>	Eugenol	Vanillin	Tadasa and Kayahara 1983	19
<i>Pseudomonas</i> spp	Eugenol	Vanillin	Rabenhorst 1996	91
<i>Serratia</i> spp	Eugenol &	Vanillin	Rabenhorst 1991	25
<i>Enterobacter</i> spp	Isoeugenol			
<i>Arthrobacter globiformis</i>	Eugenol	Vanillin	Cooper 1987	23
<i>Serratia marcescens</i>	Vanillin	Vanillin acid	Prestelo <i>et al</i> 1989	92
<i>Streptomyces viridosporus</i>	Vanillin	Vanillic acid	Pomanto and Crawford 1983	93
<i>Aspergillus niger</i>	Vanillylamine	Vanillin	Yoshida <i>et al</i> 1997	94
<i>Escherichia coli</i>	Vanillylamine	Vanillin		
<i>Pycnoporus cinnabarinus</i>	Vanillic acid	Vanillin	Lesage-Meessen <i>et al</i> 1997	95
<i>Proteus vulgaris</i>	<i>m</i> -Methoxytyrosine	Vanillin	Casey and Dobb 1992	96
Stillbene dioxygenase	Isorhaponin	Vanillin	Hagedorn and Kaphammer 1994	10
Lipoxygenase	Coniferyl aldehyde	Vanillin	Markus <i>et al</i> 1992	97
	Ferulic acid	Vanillin	Mane and Zucca 1992	98
<i>Brettanomyces anomalus</i>	Ferulic acid	Vanillin	Edlin <i>et al</i> 1995	99
	Caffeic acid			
	Coumaric acid			
<i>Penicillium simplicissimum</i>	Vanillyl alcohol	Vanillin	Fraaije <i>et al</i> 1997	100

^a Adapted from Ref 77.

vanillin by transformation of isoeugenol and eugenol, using *Serratia marcescens* DSM 30126. With isoeugenol as a substrate the initial yield was 5%; following optimisation, the yield increased to 20% (3.8 g l⁻¹). Eugenol was also transformed by this organism, but the yield of vanillin was much lower (0.018 g l⁻¹).

Tadasa and Kayahara¹⁹ reported that externally fed eugenol was biotransformed to vanillin via ferulic acid. It was further metabolised to vanillic acid and protocatechuic acid. Biotransformation of isoeugenol and eugenol to vanillin and vanillic acid was shown in cultures of the microalga *Spirulina platensis*. However, isoeugenol-fed cultures accumulated several-fold more vanillin than eugenol-treated ones.⁸² Recently, Rabenhorst⁹¹ demonstrated that *Pseudomonas* spp was capable of growing on eugenol as the sole carbon source and found vanillic acid, ferulic acid and coniferyl alcohol as metabolic intermediates. Washisu *et al*¹⁰² patented the production of vanillin from eugenol, using a strain of *Pseudomonas* spp TK2102, which accumulated vanillin up to 280 mg l⁻¹, and other metabolites found were coniferyl alcohol, coniferyl aldehyde, ferulic acid and vanillyl alcohol.

Researchers at Wageningen University have observed that *Penicillium simplicissimum* carries out two exceptional reactions. Firstly, it has an enzyme that converts eugenol into coniferyl aldehyde.¹⁰³ Secondly, it has an aromatic alcohol oxidase that converts vanillyl alcohol into vanillin.¹⁰⁴ BASF has obtained a patent on the biotransformation of eugenol to coniferyl aldehyde, using mutants of *Arthrobacter globiformis* DSM3597. Markus *et al*⁹⁷ developed a process for the production of vanillin using the enzyme lipoxygenase. The yield of isoeugenol conversion was 100–150 mg g⁻¹ and that of eugenol 3–5 mg g⁻¹.

Ferulic acid as precursor

Biotransformation of ferulic acid is effected by bacteria, fungi and yeast for the production of vanilla flavour metabolites. Cartwright and Smith¹⁰⁵ reported that vanillin, vanillic acid and protocatechuic acid were the metabolic intermediates of *Pseudomonas fluorescens* grown on ferulic acid. Toms and Wood¹⁷ reported that a strain of *P. acidovorans* oxidised vanillic acid and protocatechuic acid when grown on ferulic acid. Otuk⁸⁵ found that a strain of *E. coli* isolated from

decaying wood accumulated vanillin, vanillic acid and protocatechuic acid from ferulic acid. Ishikawa *et al.*⁸⁸ observed that the white-rot fungi *Polyporus versicolor* and *Fomes fomentarius* could degrade ferulic acid to vanillin, which could be reversibly reduced to vanillyl alcohol or oxidised to vanillic acid.

Biotransformation of ferulic acid in cell cultures of *S. platensis* showed that externally fed ferulic acid was biotransformed to vanilla flavour metabolites—vanillin, vanillic acid, vanillyl alcohol, vanillyl amine, *p*-hydroxybenzoic acid, protocatechuic acid and *p*-coumaric acid. The percentage conversion of ferulic acid to vanilla flavour metabolites was 45% in 1.25 mM ferulic acid-treated *S. platensis* cultures.^{77,82} The immobilised *Spirulina* cultures showed more vanillin accumulation than freely suspended cultures.

Falconnier *et al.*⁷⁹ reported the biotransformation of ferulic acid to vanillin, vanillic acid and vanillyl alcohol by the white-rot fungus *Pycnoporus cinnabarinus*, which also showed oxidative decarboxylation of vanillic acid to 2-methoxyhydroquinone. Cultures of *Streptomyces setonii* converted ferulic acid to vanillin, then to vanillic acid, which was subsequently demethylated to protocatechuic acid.⁸⁷ Biocatalytic transformation of ferulic acid to useful aromatic chemicals by various microorganisms was reviewed by Rosazza *et al.*⁸⁹ Biotransformation of ferulic acid to vanillic acid was reported to be effected by mutant strains of *Pseudomonas fluorescens* cultures.⁸⁴

Labuda *et al.*⁵⁸ reported that *Corynebacterium glutamicum* produced a mixture of vanillin and vanillic acid from ferulic acid. Cultures of *S. platensis* treated with ferulic acid showed accumulation of 116 mg l⁻¹ vanillin in the presence of the reducing agent DL-dithiothreitol.⁷⁷ The best bioconversion of ferulic acid to vanillin reported in *Corynebacterium* is that of accumulation of 76 mg l⁻¹ in the presence of DL-dithiothreitol.⁵⁸

Lesage-Meessen *et al.*⁸⁰ showed a two-step bioconversion process for the production of vanillin from ferulic acid. In the first step, *Aspergillus niger* transformed ferulic acid to vanillic acid. In the second step, vanillic acid was reduced to vanillin by *Pycnoporus cinnabarinus*. The channelling of vanillic acid transformation to vanillin in the presence of cellobiose was also reported in cell cultures of *P. cinnabarinus*.⁹⁵ Biotransformation of coniferyl aldehyde to vanilla flavour metabolites in *S. platensis* cultures was reported.⁸² Vanillin was formed via ferulic acid as a metabolic intermediate. Further reduction of vanillin to vanillyl alcohol or oxidation to vanillic acid and demethylation of vanillic acid to protocatechuic acid was demonstrated by using *Spirulina* cultures.⁷⁷ Biosynthetic pathways for the production of vanillin from various phenylpropanoids are given in Fig 6.

MOLECULAR BIOLOGICAL APPROACHES

Researchers at the Institute of Food Research (IFR), Norwich, UK applied both biochemical and molecular

methods with the aim of producing microbial biovanillin. In *Pseudomonas fluorescens* AN103, vanillin and vanillic acid are intermediates of the ferulic acid degradation pathway. A key feature of this pathway is the C2 side-chain cleavage activity of ferulic acid that requires the hydration and retro-aldol cleavage of feruloyl-CoA to produce vanillin and acetyl-CoA. Both of these reactions are catalysed by a newly discovered single enzyme, 4-hydroxycinnamoyl-CoA hydratase/lyase.^{106–108} The biosynthetic pathway of formation of vanillin and vanillic acid from ferulic acid using *Pseudomonas fluorescens* AN103 is shown in Fig 7.

Higher plants possess the phenylpropanoid pathway for the production of lignin and some secondary phenolic compounds. Coumaric acid and ferulic acids and their CoA esters are intermediates in these routes. Expression of the bacterial gene for the side-chain cleavage enzyme in plants may offer the potential to generate vanillin and related flavour compounds in a range of crops by diversion of phenylpropanoid metabolism. At IFR, attempts have been made to produce vanillin in hairy root cultures of *Datura stramonium* by expressing the *p*-hydroxycinnamoyl-CoA hydratase/lyase (HCHL) gene from *Pseudomonas fluorescens* AN103. The expression of the HCHL gene in *D. stramonium* hairy root cultures caused substantial changes in the content of *p*-hydroxybenzoic acid in the form of glucosides and glucose esters, along with traces of glucosides of *p*-hydroxybenzyl alcohol and vanillic acid. The potential of this line to biotransform added ferulic acid to vanillin is being studied.¹⁰⁹

4-Coumarate-CoA ligase (4CL) (EC 6.2.1.12) catalyses a branch-point reaction (conversion of hydroxycinnamic acids into the corresponding CoA esters) between the central phenylpropanoid pathway and pathways leading to various secondary metabolites such as flavonoids, lignans, lignin and related phenylpropanoid compounds.^{110,111} Brodelius and Xue¹¹² isolated and characterised 4CL from *V. planifolia* cell

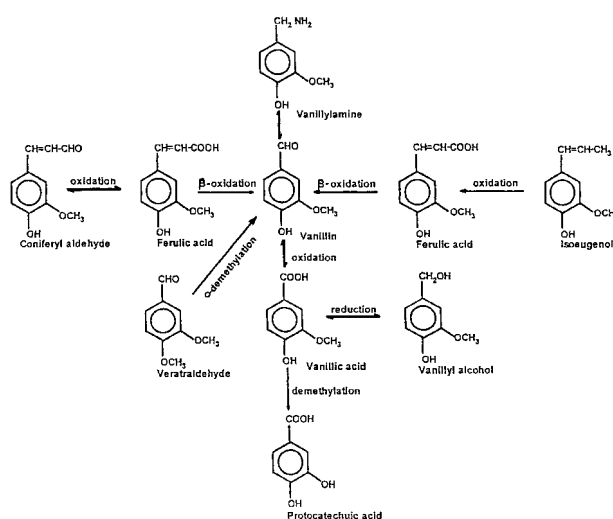


Figure 6. Vanilla flavour metabolite formation from various phenylpropanoid precursors in *Spirulina* cultures (adapted from Ref 77).

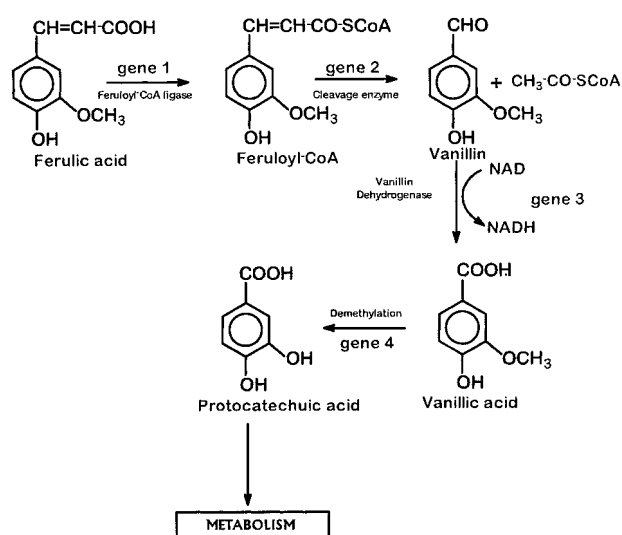


Figure 7. Vanillin pathway of *Pseudomonas fluorescens* AN103 (adapted from Ref 106).

suspension cultures. Based on studies using the inhibitors of 4CL,⁷¹ they concluded that expression of antisense mRNA for 4CL gene would redirect the flow of phenylpropanoid precursors from lignin biosynthesis into flavour compound biosynthesis in *V planifolia*.

Vanillin production from fermentative organisms

Isolation and characterisation of the metabolic genes should allow the introduction of the vanillin pathway into food-grade fermentative organisms such as *Lactococcus lactis* and *Lactobacillus* spp used in the dairy industry or the yeast *Saccharomyces cerevisiae* used in baking and brewing. These organisms have well-characterised genetics and well-developed vectorial systems which are available for the controlled expression of the novel genes. The production of flavours via fermentations based on these GRAS micro-organisms could be classified as 'natural'. The use of food-grade vectors for these organisms could avoid the introduction of resistance marker genes.¹⁰⁸

Recently, Li and Frost¹¹³ reported the synthesis of vanillin from glucose using a recombinant *E coli* as biocatalyst under fed-batch fermenter conditions using two different schemes. Scheme 1 comprised two stages. Firstly, glucose was converted to vanillic acid using *E coli* containing an engineered plasmid KL7/PKL5.26A having encoded catechol *O*-methyltransferase (P_{tac} COMT) or KL7/PKL5.97A with two loci of P_{tac} COMT. In the second stage, vanillic acid was reduced to vanillin using aryl aldehyde dehydrogenase isolated from *Neurospora crassa*.

In scheme 2 the *E coli* plasmid was engineered to produce 3-dehydroshikimic acid, incorporating genes for $aroF^{FBR}$ (3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase), $aroB$ (3-dehydroquinate synthase) and $aroD$ (3-dehydroquinate dehydrogenase). 3-Dehydroshikimic acid was converted by 3-dehydroshikimate dehydratase to protocatechuic acid.

Catechol *O*-methyltransferase converted protocatechuic acid using *in situ*-generated S-adenosyl-L-methionine (SAM) to produce vanillic acid and, in turn, vanillin. These systems have been projected to offer an ecofriendly technology in contrast to toxic substrates such as guaiacol.

ECONOMIC CONSIDERATIONS

Agroclimatic requirement, labour-intensive cultivation, pollination, harvesting and curing have all added to the high cost of vanilla flavour (US\$4000 kg⁻¹) obtained through conventional methods.¹⁵ In 1994 the market price of vanilla beans was in the range of US\$70–90 kg⁻¹.⁶² At 20 g kg⁻¹ (on dry weight basis) yield of vanillin, one will have to harvest 500 kg of vanilla pods to produce 1 kg of vanillin, which will involve pollination of nearly 40 000 flowers. Generally, the yield of green vanilla is around 1000 kg acre⁻¹. Twenty tons of vanillin could be extracted from vanilla pods, requiring the processing of nearly 10 000 tons of vanilla pods. The world market for natural vanilla flavour is US\$80 million. Since the cultivation area is not being increased commensurately with the demand, alternative technologies are gaining ground.

Plant tissue culture-based vanilla flavour

There are a number of reports on the economics of production of natural compounds by plant tissue culture.^{114,115} The economic aspect is governed by cell stability, growth rate, scale-up complexities, downstream processing and product recovery. So far, the maximum cell densities achievable are of the order of 50–70 g l⁻¹ in a bioreactor.¹¹⁶ Assuming a yield of 500 mg l⁻¹ as reported by Sahai,⁶² one may need to have a 2000 l reactor to get 1 kg of product. The medium cost is generally 10% of total cost of product and hence an input of US\$1500 is needed for medium alone and product cost is expected to be US\$15 000. Therefore improvement in yield is expected in the range of 3–4 g l⁻¹ by employing the cell culture process to bring down the cost of vanilla flavour and make the process a viable alternative.

Microbial biotransformation-based vanilla flavour

The method described for bio-vanilla or bio-vanillin production includes biotransformation of substrates such as ferulic acid, coniferyl aldehyde, isoeugenol and eugenol. The commercial costs of these compounds are in the range of US\$ 100–150 kg⁻¹. Since bioconversions are at the 50–60% level,¹¹⁷ the cost of vanilla flavour obtained by biotransformation will be of the order of US\$1000 kg⁻¹. Since it is regarded as a natural source of vanilla flavour, one will still be able to sell it at US\$2000 kg⁻¹.

SAFETY CONSIDERATIONS

There has been increasing emphasis on the production of naturally identical compounds through biotechno-

logical routes for food applications. This may be due to increased consumer preference on organoleptic attributes and also safety aspects. From the point of view of commercial production of novel foods through biotechnology, one would look for an alternative method to chemical synthesis, keeping in view environmental safety. In the case of vanilla flavour production through chemical means, guaiacol, a toxic material, is used as substrate, rendering the process environmentally unsafe. Vanilla flavour, being a highly preferred natural food additive with a high demand, has already attracted the attention of biotechnologists to produce a flavouring with comparable organoleptic properties to plant-derived vanilla. However, the risk, if any, of using genetically engineered organisms for vanilla flavour metabolite production still needs a detailed assessment. Generally, this involves biosafety experimentation for toxicological assessment using experimental animal models as per the OECD (Organization for Economic Cooperation Development) guidelines or FDA (Food and Drug Administration) regulations. OECD advocates a case-by-case approach for the analysis of novel foods, whilst FDA has a relatively liberal policy wherein a clear demonstration of single-gene expression and non-toxicity of the gene product should obtain clearance.¹¹⁸ Since several biotransformation-derived food additives and also those obtained from genetically engineered organisms have been cleared from the safety angle, it would not be difficult for these processes to be cleared. However, from a consumer preference point of view, labelling of the products as biotechnologically derived may be recommended.

FUTURE PROSPECTS

Biotechnological production of vanilla flavour components through plant cell culture systems or microbial cultures through biotransformation by selected cell lines or genetically modified cells is providing an interesting possibility as an alternative to natural or synthetic vanilla flavour as against synthetic vanillin.

Biotechnologically produced vanilla flavour is likely to meet the demands of the international market. It is envisaged that this biotechnological product may be priced at reasonably high levels, certainly not equivalent to vanilla flavour from plants. Moreover, the market for natural vanilla flavour should not be affected, since the biotechnologically derived product may not totally match the flavour from pods. However, the ever-increasing number of patents on biotechnologically based vanilla processes is a clear indication of the commercial importance of alternative methods for production of this flavouring.

The production of vanilla flavour through biotechnological processes certainly depends on the economics. As mentioned before, microbial bioconversions are at an interesting breakeven stage, but attempts are needed to bring down the costs by enhancing the biotransformation efficiencies and using cheaper substrates as well as easy systems for efficient downstream processing, employing immobilised cell cultures and spontaneous release of metabolites to the exterior, coupled with the use of adsorbents for recovery of the product.

Genetic engineering of the vanilla plant for over-expression of the genes for vanilla production has not yet been attempted and hence needs to be addressed immediately. The genes that will be cloned can be of plant or microbial origin. The recent work of Li and Frost¹¹³ has not only enhanced the prospects of vanilla production through micro-organisms, but has also given an insight into understanding which genes need to be cloned. Though Li and Frost have not mentioned the plant system, it is tempting to extrapolate their study to plants. Based on the present knowledge of biochemical pathways of vanillin production, a number of suggestions have been made for improvements through biotransformation and genetic engineering which are possibly realistic to achieve (Table 6). However, aspects of cloning of specific genes, their expression using suitable tissue-specific promoters, regeneration of plants from the transformed cell lines and expression of cloned genes in pod

Table 6. Approaches to improvements in yields of vanillin: suggested future trends based on research on metabolism of phenylpropanoids

Vanilla planifolia

1. *p*-Fluorophenylalanine-resistant cells for over-production of precursor phenylalanine¹¹⁹
2. Cloning and expression of 4-coumarate-CoA ligase gene¹¹² and caffeic acid *O*-methyltransferase¹²⁰ for over-production of vanillin/vanillic acid
3. Antisense gene for vanillin dehydrogenase to suppress conversion of vanillin to vanillic acid (not reported)
4. Cloning of 4-hydroxycinnamoyl-CoA hydratase/lyase for degradation of feruloyl-CoA to vanillin¹⁰⁹

Microbial systems

Ferulic acid

1. Cloning of 4-hydroxycinnamoyl-CoA hydratase/lyase for degradation of feruloyl-CoA to vanillin¹⁰⁷
2. Mutants/engineered cells for reduced vanillin dehydrogenase activity to obtain high yields of vanillin¹⁰²

Eugenol

Use of aldehyde dehydrogenase-deficient mutant or antisense gene for conversion of eugenol to coniferyl aldehyde and its subsequent conversion to vanillin²³

Vanillyl alcohol

Vanillyl alcohol oxidase, an inducible enzyme, bioconverts vanillyl alcohol to vanillin¹²¹

regions of the *Vanilla* plant need to be studied for the success of obtaining genetically modified plants for improved flavour characteristics.

After achieving success in the economical production of vanilla flavour through biotechnological inventions, it is also necessary to ensure safety. However, one can get clearance by using safer precursors, food-grade organisms, defined media for culture of cells, and downstream processing with permitted solvents. Ecological safety needs to be studied for genetically engineered plants and organisms. There is certainly a need to encourage conventional cultivation, since the demands should be met from various sources. Hence it is envisaged that biotechnological production will supplement and complement the existing methods.

ACKNOWLEDGEMENTS

One of the authors (SRR) thanks the Council of Scientific and Industrial Research (CSIR), India for providing financial support towards an extended Senior Research Fellowship. Financial support by the Department of Biotechnology (DBT), Government of India for biotransformation studies on *Spirulina* is gratefully acknowledged. The authors thank Mr VA Daniel for help in the preparation of the manuscript.

REFERENCES

- Childers NF, Cibes HR and Hernandez-Medina E, Vanilla—the orchid of commerce. In *The Orchids, a Scientific Survey*, Ed by Wither CL, Ronald Press, New York, pp 477–508 (1959).
- Ranadive AS, Vanilla—cultivation, curing, chemistry, technology and commercial products. In *Spices, Herbs and Edible Fungi*, Ed by Charalambous G, Elsevier, Amsterdam, pp 517–577 (1994).
- Reineccius G, *Source Book of Flavours*, 2nd edn, Chapman and Hall, New York, pp 351–361 (1994).
- There is no such thing as plain vanilla. *Food Technol Eur* (September/October):40–42 (1995).
- Webster TM, New perspectives on vanilla. *Cereal Foods World* 40:198–200 (1995).
- Purseglove JW, Brown EG, Green CL and Robbins SRJ. In *Spices*, Vol 2, Longman, London, pp 644–735 (1981).
- Purseglove JW, Tropical crops: monocotyledons, vanilla. *Nature* 190:366 (1972).
- Funk C and Brodelius P, *Vanilla planifolia* Andrews: *in vitro* biosynthesis of vanillin and other phenylpropanoid derivatives. In *Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plants VI*, Vol 26, Ed by Bajaj YPS, Springer Berlin, pp 377–402 (1994).
- Vanilla—the queen of spices. *H&R Contact* 59:15–19 (1993).
- Hagedorn S and Kaphammer B, Microbial biocatalysis in the generation of flavour and fragrance chemicals. *Ann Rev Microbiol* 48:773–800 (1994).
- Berger RG, *Aroma Biotechnology*, Springer, Berlin (1995).
- Dornenburg H and Knorr D, Production of phenolic flavour compounds with cultured cells and tissues of *Vanilla* species. *Food Biotechnol* 10:75–92 (1996).
- Feron G, Bonnarne P and Durand A, Prospects for the microbial production of food flavours. *Trends Food Sci Technol* 7:285–293 (1996).
- Cleatham PSJ, Combining the technical push and the business pull for natural flavours. In *Advances in Biochemical Engineering Biotechnology*, Vol 55, Ed by Scheper P, Springer, Berlin, pp 1–50 (1997).
- Krings U and Berger RG, Biotechnological production of flavours and fragrances. *Appl Microbiol Biotechnol* 49:1–8 (1998).
- Clark GS, Vanillin. *Perfume Flavor* 15:45–54 (1990).
- Toms A and Wood J, The degradation of *trans*-ferulic acid by *Pseudomonas acidovarans*. *Biochemistry* 9:337–343 (1970).
- Chen CL, Chang HM and Kirk T, Aromatic acids produced during degradation of lignin in spruce wood by *Phanerochaete chrysosporium*. *Holzforschung* 36:3–9 (1982).
- Tadasa K and Kayahara H, Initial steps in eugenol degradation pathway of a microorganism. *Agric Biol Chem* 47:2639–2640 (1983).
- Shiotsu T, Samejima M, Habu N and Yoshimota T, Enzymatic conversion of stilbenes from the inner bark of *Picea glehnii* into aromatic aldehydes. *Mukuzai Gakkaishi* 35:826–831 (1989).
- Prince RC and Gunson DE, Just plain Vanilla? *Trends Biochem Sci* (December): 521 (1994).
- Dornenburg H and Knorr D, Generation of colours and flavours in plant cell and tissue cultures. *Crit Rev Plant Sci* 15:141–168 (1996).
- Cooper B, Method and microorganisms for the preparation of coniferyl aldehyde. *German Patent DE 3604874 A1* 870820 (1987).
- Groos B, Asther M, Corrieu G and Brunerie P, Production de vanilline par bioconversion de precursors benzeniques. *European Patent 0453368 A1* (1991).
- Rabenhorst J, Verfahren zur Herstellung von natuerlichem Vanillin. *European Patent 0405197 A1* (1991).
- Labuda IM, Goers SK and Keon KA, Bioconversion process for the production of vanillin. *US Patent 5128253* (1992).
- Labuda IM, Goers SK and Keon KA, Bioconversion process for the production of vanillin. *US Patent 5279950 A* 940118 (1994).
- Audras B and More J, Method for producing vanillin using the bioconversion of benzene precursors. *Patent WO 9634971 A1* (1996).
- Lesage-Meessen L, Delattre M, Haon M and Asther M, Obtaining vanillic acid and vanillin by bioconversion by an association of filamentous microorganisms. *Patent WO 9608576 A1* (1996).
- Cowley E, Vanilla and its uses. In *Proceedings of the Conference on Spices*, Tropical Products Institute, London, p 79 (1973).
- Arana F, Action of a β -glucosidase in the curing of vanilla. *Food Res* 8:343–351 (1943).
- Sorensen NA and Mehlum J, *US Patent 2752394* (1956).
- Goris MA, Formation du parfum de la vanille. *Ind Parfumerie* 2:4 (1947).
- Mane J and Zucca J, Process for the production of vanilla flavour by treatment of vanilla pods and vanilla flavour so produced. *French Patent FR 26918801 A1* (1993).
- Merory J, Natural flavourings from vanilla beans. In *Food Flavourings: Composition, Manufacture and Use*, 2nd edn, AVI Publishing, Westport, CT, pp 163–182 (1968).
- Rosegarten, *The Book of Spices*, Livingston Publishing, Pennsylvania, pp 453–466 (1969).
- Goodenough BR, Vanilla, vanillin, and vanilla derivatives. *Bakers Digest* (June):8–10 (1982).
- Code of Federal Regulations, *Foods and Drugs*, 21 CFR 100–169 (1996).
- International Standards Organization, *Vanilla Vocabulary*, ISO 3495 (1976).
- Harrison SG, Masfield GB and Wallis M, *The Oxford Book of Food Plants*, Oxford University Press, Oxford, pp 130–131 (1969).
- Manufacture of vanilla bean particle containing ice-cream. *Dairy Foods* 94:72 (1993).
- Lopez-Malo A, Alzamora SM and Agraiz A, Effect of natural

- vanillin on germination time and radial growth rate of moulds in fruit agar systems. *Food Microbiol* **12**:213–219 (1995).
- 43 Cerrutti P and Alzamora SM, Inhibitory effects of vanillin on some food spoilage yeasts in laboratory media and fruit purees. *Int J Food Microbiol* **29**:379–386 (1996).
 - 44 Cerrutti P, Alzamora SM and Vidales SL, Vanillin as an antimicrobial for producing shelf-stable strawberry puree. *J Food Sci* **62**:608–610 (1997).
 - 45 Burri J, Graf M, Lambelet P and Loiger J, Vanillin: more than a flavouring agent—a potent antioxidant. *J Sci Food Agric* **48**:49–56 (1989).
 - 46 Takahashi K, Sekiguchi M and Kawazoe Y, Effects of vanillin and *o*-vanillin on induction of DNA-repair networks: modulation of mutagenesis in *Escherichia coli*. *Mutat Res* **230**:127–134 (1990).
 - 47 Sasaki YF, Ohta T, Imanishi H, Watanabe M, Matsumoto K, Kato T and Shirasu Y, Suppressing effects of vanillin, cinnamaldehyde and anisaldehyde on chromosome aberrations induced by X-rays in mice. *Mutat Res* **243**:299–302 (1990).
 - 48 Inouye T, Sasaki YF, Imanishi H, Watanabe M, Ohta T and Shirasu Y, Suppression of mitomycin C-induced micronuclei in mouse marrow cells by post-treatment with vanillin. *Mutat Res* **202**:93–95 (1988).
 - 49 De Andrade HH, Santos JH, Gimmler-Luz MC, Correa MJ, Lehmann M and Reguly ML, Suppressing effects of vanillin on chromosome aberrations that occurs spontaneously or are induced by mitomycin C in the germ cell line of *Drosophila melanogaster*. *Mutat Res* **279**:281–287 (1992).
 - 50 Kometani T, Tanimoto H, Nishimura T and Okada S, Glucosylation of vanillin by cultured plant cells. *Biosci Biotechnol Biochem* **57**:1290–1293 (1993).
 - 51 Retnakaran A, Repellent for black fly. *US Patent* 4427700 (1984).
 - 52 Iekhsan O, Jaya V, Mustaffa MA and Rais MM, Antitoxin compounds from the plant extracts of *Ipomea pes-caprae* (L.) R.Br. *Abstr 8th FAOBMB Congr*, Kuala Lumpur, p 20 (1998).
 - 53 Hopp R, Some highlights of H & R research: a review of nearly 120 years of research at Haarmann & Reimer. In *Recent Developments in Flavour and Fragrance Chemistry*, Ed by Hopp R and Mori K, VCH, Weinheim, pp 14–28 (1993).
 - 54 Bauer K, Garbe D and Surburg H, *Common Fragrance and Flavour Materials*, VCH, Weinheim (1990).
 - 55 Mathias AL, Lopretti MI and Rodrigues AE, Chemical and biological oxidation of *Pinus pinaster* lignin for the production of vanillin. *J Chem Technol Biotechnol* **64**:225–234 (1995).
 - 56 Hocking MB, Vanillin: synthetic flavouring from spent sulfite liquor. *J Chem Educ* **74**:1055–1059 (1997).
 - 57 Knuth ME and Sahai OP, Flavour composition and method. *US Patent* 5057424 (1991).
 - 58 Labuda IM, Keon KA and Goers SK, Microbial bioconversion process for the production of vanillin. In *Progress in Flavour and Precursor Studies*, Ed by Schreier P and Winterhalter P, Allured Publishing, Carol Stream, FL, pp 477–482 (1993).
 - 59 Westcott RJ, Chetham PSJ and Barraclough AJ, Use of organized viable *Vanilla* plant aerial roots for the production of vanillin. *Phytochemistry* **35**:135–138 (1994).
 - 60 Westcott RJ, Progress and prospects in plant biotechnology. In *Biotechnology Challenges for the Flavour and Food Industry*, Ed by Lindsey RC and Willis BJ, Elsevier Applied Science, London, pp 45–53 (1989).
 - 61 Harlander S, Biotechnology for the production of flavouring materials. In *Source Book of Flavours*, Ed by Reineccius G, Chapman and Hall, New York, pp 151–175 (1994).
 - 62 Sahai OM, Plant tissue culture. In *Bioprocess Production of Flavour, Fragrances and Color Ingredients*, Ed by Gabelman A, Wiley, New York, pp 239–275 (1994).
 - 63 Scragg AH, The production of aromas by plant cell cultures. In *Advances in Biochemical Engineering Biotechnology*, Vol 55, Ed by Scheper P, Springer, Berlin, pp 239–263 (1997).
 - 64 Schleiden MJ, Beitrage zur Phytogeneseis. *Muller Arch Anat Physiol*, pp 137–176 (1838).
 - 65 Schwann Th, *Mikroskopische Untersuchungen uber die Uebereinstimmung in der Struktur in dem Wachstum der Thiere und Pflanzen*, Nr 176, Oswalds, Berlin (1839).
 - 66 Davidonis G and Knorr D, Callus formation and shoot regeneration in *Vanilla planifolia*. *Food Biotechnol* **5**:59–66 (1991).
 - 67 Funk C and Brodelius P, Influence of growth regulators and an elicitor on phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia*. *Phytochemistry* **29**:845–848 (1990).
 - 68 Funk C and Brodelius P, Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. IV. Induction of vanillic acid formation. *Plant Physiol* **99**:256–262 (1992).
 - 69 Funk C and Brodelius P, Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. II. Effects of precursor feeding and metabolic inhibitors. *Plant Physiol* **94**:95–101 (1990).
 - 70 Romagnoli LG and Knorr D, Effects of ferulic acid treatment on growth and flavour development of cultured *Vanilla planifolia* cells. *Food Biotechnol* **2**:93–104 (1988).
 - 71 Funk C and Brodelius P, Phenylpropanoid metabolism in suspension cultures of *Vanilla planifolia* Andr. III. Conversion of 4-hydroxycinnamic acid into 4-hydroxybenzoic acids. *Plant Physiol* **94**:102–108 (1990).
 - 72 Havkin-Frenkel D, Podstolski A and Knorr D, Effect of light on vanillin precursors formation by *in vitro* cultures of *Vanilla planifolia*. *Plant Cell Tiss Org Cult* **45**:133–136 (1996).
 - 73 Johnson TS, Ravishankar GA and Venkataraman LV, Bio-transformation of ferulic acid and vanillylamine to capsaicin and vanillin in immobilized cell cultures of *Capsicum frutescens*. *Plant Cell Tiss Org Cult* **44**:117–121 (1996).
 - 74 Ramachandra Rao S and Ravishankar GA, Production of vanillin and capsaicin in immobilized cell cultures of *Capsicum frutescens*. Poster presented at *Int Symp on Principles Regulating Biosynthesis and Storage of Secondary Products*, Halle, Abstr 38 (1996).
 - 75 Ramachandra Rao S and Ravishankar GA, Biotransformation of isoeugenol to vanilla flavour metabolites and capsaicin in freely suspended and immobilized cell cultures of *Capsicum frutescens*: study of the influence of fungal elicitor and β -cyclodextrin. *Process Biochem* **35**:341–348 (1999).
 - 76 Ramachandra Rao S and Ravishankar GA, Biotransformation of phenylpropanoid compounds to vanilla flavour metabolites in immobilized cell cultures of *Capsicum frutescens*. Paper presented at *4th Int Food Convention*, Mysore, Abstr D-30 (1998).
 - 77 Ramachandra Rao S, Studies on biotransformation to produce phytochemicals of importance using plant cell cultures. *PhD Thesis*, University of Mysore (1998).
 - 78 Knuth ME and Sahai OP, Flavour composition and method. *US Patent* 5068184 (1991).
 - 79 Falconnier B, Lapierre C, Lesage-Meessen L, Yonnet G, Brunerie P, Ceccaldi BC, Corrieu G and Asther M, Vanillin as a product of ferulic acid biotransformation by the white rot fungus *Pycnoporus cinnabarinus* I-37: identification of metabolic pathways. *J Biotechnol* **37**:123–132 (1994).
 - 80 Lesage-Meessen L, Delattre M, Haon M, Thibault J-F, Ceccaldi BC, Brunerie P and Asther M, A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *J Biotechnol* **50**:107–113 (1996).
 - 81 Rahouti M, Seigle-Murandi F, Steiman R and Eriksson KE, Metabolism of ferulic acid by *Paecilomyces variotii* and *Pestalotia palmarum*. *Appl Environ Microbiol* **55**:2391–2398 (1989).
 - 82 Ramachandra Rao S, Ravishankar GA and Venkataraman LV, An improved process for the preparation of vanillin. *Indian Patent* 1022/DEL/96 (1996).
 - 83 Usha Tripathi, Ramachandra Rao S and Ravishankar GA, A

- process for the preparation of vanilla flavour metabolites through biotransformation. *Indian Patent (pending)* NF269/99 (1999).
- 84 Andreoni A, Bernasconi S and Besetti G, Biotransformation of ferulic acid and related compounds by mutant strains of *Pseudomonas fluorescens*. *Appl Microbiol Biotechnol* **42**:830–835 (1995).
- 85 Otuk G, Degradation of ferulic acid by *Escherichia coli*. *J Ferment Technol* **63**:501–6 (1985).
- 86 Krishnamohan and Khanna S, Metabolism of ferulic acid by *Alcaligenes paradoxus*. *Indian J Microbiol* **34**:303–306 (1994).
- 87 Sutherland JB, Crawford DL and Pomento III AL, Metabolism of *p*-coumaric and ferulic acids by *Streptomyces setonii*. *Can J Microbiol* **29**:1253–1257 (1983).
- 88 Ishikawa H, Schubert WJ and Nord FF, Investigations on lignins and lignification. XXVIII. The degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally related to softwood lignin. *Arch Biochem Biophys* **100**:140–149 (1963).
- 89 Rosazza JPN, Huang Z, Volm T and Rousseau B, Review: biocatalytic transformations of ferulic acid: an abundant aromatic natural product. *J Ind Microbiol* **15**:457–471 (1995).
- 90 Huang Z, Dostal L and Rosazza JPN, Mechanism of ferulic acid conversion to vanillic acid and guaiacol by *Rhodotorula rubra*. *J Biol Chem* **268**:23 954–23 958 (1993).
- 91 Rabenhorst J, Production of methoxyphenol-type natural aroma chemicals by biotransformation of eugenol with a new *Pseudomonas* spp. *Appl Microbiol Biotechnol* **46**:470–474 (1996).
- 92 Prestelo F, Falcon MA and de la Fuente G, Production of vanillic acid from vanillin by resting cells of *Serratia marcescens*. *Appl Environ Microbiol* **55**:1660–1662 (1989).
- 93 Pomento III AL and Crawford DL, Whole-cell bioconversion of vanillin to vanillic acid by *Streptomyces viridosporus*. *Appl Environ Microbiol* **45**:1582–1585 (1983).
- 94 Yoshida A, Takenaka Y, Tamaki H, Frebort I, Adachi O and Kumagai H, Vanillin formation by microbial amine oxidases from vanillylamine. *J Ferment Bioengng* **84**:603–605 (1997).
- 95 Lesage-Meessen L, Haon M, Delattre M, Thibault J-F, Ceccaldi BC and Asther M, An attempt to channel the transformation of vanillic acid into vanillin by controlling methoxyhydroquinone formation in *Pycnoporus cinnabarinus* with cellobiose. *Appl Microbiol Biotechnol* **47**:393–397 (1997).
- 96 Casey J and Dobb R, Microbial routes to aromatic aldehydes. *Enzyme Microbial Technol* **14**:739–747 (1992).
- 97 Markus PH, Peters ALJ and Roos R, Process for the preparation of phenaldehydes. *European Patent 0542348 A2* (1992).
- 98 Mane J and Zucca J, *Patent WO 9402621* (1992).
- 99 Edlin DAN, Narbad A, Dickinson JR and Llyod D, The biotransformation of phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol Lett* **125**:311–316 (1995).
- 100 Fraaije MW, Pikkemaat M and Van Berkel WJH, Enigmatic grauituous induction of the covalent flavoprotein vanillyl-alcohol oxidase in *Penicillium simplicissimum*. *Appl Environ Microbiol* **63**:435–439 (1997).
- 101 Abraham WR, Arfmann HA, Stumpf S, Washausen P, Kieslich K, Microbial transformation of some terpenoids and natural compounds. In *Bioflavour '87, Analysis, Biochemistry, Biotechnology*, Ed by Schreier P, Walter de Gruyter, Berlin, pp 399–414 (1988).
- 102 Washisu Y, Tetsushi A, Hashimoto N and Kanisawa T, Manufacture of vanillin and related compounds with *Pseudomonas*. *Japan Patent 5227980* (1993).
- 103 Van Berkel JHW, Fraaije MW and de Jong E, *PCT Patent Application WO95/02 062* (1995).
- 104 Fraaije MW, Veeger C and Van Berkel WJH, Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*. *Eur J Biochem* **234**:271–277 (1995).
- 105 Cartwright NJ and Smith ARW, Bacterial attack on phenolic enters an enzyme system demethylating vanillic acid. *Biochem J* **102**:826–841 (1967).
- 106 Narbad A and Gasson MJ, Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly isolated strain of *Pseudomonas fluorescens*. *Microbiology* **144**:1397–1405 (1998).
- 107 Gasson MJ, Kitamura Y, McLauchlan WR, Narbad A, Parr AJ, Parsons ELH, Rhodes MJC and Walton NJ, Metabolism of ferulic acid to vanillin. *J Biol Chem* **273**:4163–4170 (1998).
- 108 Narbad A and Walton N, Vanilla flavour biotechnology. *Food Ingrid Anal* (May/June): 16–18 (1998).
- 109 Mitra A, Kitamura Y, Mayer MJ, Michael AJ, Narbad AJ, Parr AJ and Walton NJ, *p*-Hydroxycinnamoyl-CoA hydratase/lyase—a *Pseudomonas* enzyme expressed in hairy roots of *Datura stramonium*. *Biochem Soc Trans* **27**:A51 (1999).
- 110 Holton TA and Cornish EC, Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* **7**:1071–1083 (1995).
- 111 Douglas CJ, Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends Plant Sci* **1**:171–178 (1996).
- 112 Brodelius PE and Xue ZT, Isolation and characterization of a cDNA from cell suspension cultures of *Vanilla planifolia* encoding 4-coumarate:coenzyme A ligase. *Plant Physiol Biochem* **35**:497–506 (1997).
- 113 Li K and Frost JW, Synthesis of vanillin from glucose. *J Am Chem Soc* **120**:10 545–10 546 (1998).
- 114 Goldstein WE, Lasure LL and Ingle MB, Product cost analysis. In *Plant Tissue Culture as a Source of Biochemicals*, Ed by Staba EJ, CRC Press, Boca Raton, FL, pp 191–234 (1980).
- 115 Sahai O and Knuth M, Commercializing plant tissue culture processes: economics, problems and prospects. *Biotechnol Prog* **1**:1–10 (1985).
- 116 Fujita Y and Tabata M, Secondary metabolites from plant cell, pharmaceutical applications and progress in commercial production. In *Plant Tissue and Cell Culture*, Ed by Green CE, Somers DA, Hackett WP and Biesboer DD, Alan R Liss, New York, pp 169–185 (1984).
- 117 Muheim A and Lerch K, Towards a high-yield bioconversion of ferulic acid to vanillin. *Appl Microbiol Biotechnol* **51**:456–461 (1999).
- 118 Jonas DA, Antignac E, Antoine J-M, Classen HG, Huggett A, Knudsen I, Mahler J, Ockhuizen T, Smith M, Teuber M, Walker R and De Vogel P, The safety assessment of novel foods—guidelines prepared by ILSI Europe food task force. *Food Chem Toxicol* **34**:931–940 (1996).
- 119 Johnson TS, Sarada R and Ravishankar GA, Capsaicin formation in *p*-fluorophenylalanine resistant and normal cell cultures of *Capsicum frutescens* and activity of phenylalanine ammonia lyase. *J Biosci* **23**:209–212 (1998).
- 120 Xue ZT and Brodelius PE, Kinetin induced caffeic acid *O*-methyltransferase in cell suspension cultures of *Vanilla planifolia* Andr. and isolation of caffeic acid *O*-methyltransferase cDNAs. *Plant Physiol Biochem* **36**:779–788 (1998).
- 121 Benen JAE, Sanchez-Torres P, Wagemaker MJM, Fraaije MW, Van Berkel WJH and Visser J, Molecular cloning, sequencing and heterologous expression of the *vao A* gene from *Penicillium simplicissimum* CBS 170.90 encoding vanillyl-alcohol oxidase. *J Biol Chem* **273**:7865–7872 (1998).