Vanilla: The Most Popular Flavour

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Abbreviations

°C	Celsius
cm	Centimetre
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
EU	European Union
g	Gram
h	Hour
kg	Kilogram
l	Liter
m	Meter
mM	Millimolar
sp.	Species
UDP	Uridine diphosphate glucose
US$	United States dollar

1.1 Introduction

Vanilla is a universally appreciated global delicacy and probably the most popular plant-derived flavour in the world. Vanilla flavour is obtained from the seedpods of the cultivated orchid, *Vanilla planifolia* and from several other vanilla species.

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W. Schwab et al. (eds.), *Biotechnology of Natural Products*,
https://doi.org/10.1007/978-3-319-67903-7_1

Vanilla pods, vanilla extracts (the isolated extract from vanilla pods) and vanillin (the main flavour of the vanilla extract) constitute a multimillion-dollar industry.

The history of human utilization of vanilla flavour began with the Aztecs, in current day Mexico, during the 1300s. The Aztecs utilized the seedpods from vanilla orchids for flavour and fragrance. The earliest documented consumption of vanilla pods dates back to 1520, when the Spanish arrived in Mexico. The colonists were exposed to the Aztecs' use of vanilla to flavour a drink, which is considered the ancestor of hot chocolate. The Spanish were impressed by the flavour of vanilla and transported the pods back to Spain. Subsequently, the demand for vanilla pods in Spain, France and the rest of Europe increased, and this demand led to domestication of the vanilla orchid, *V. planifolia* [1].

Despite the popularity of the flavour, there is still a great deal of uncertainty regarding the biology of vanilla. Natural pollinators of the vanilla orchid species are not well investigated [2, 3]. Symbiosis with fungi is required for seed germination and growth, but the interactions are poorly understood [4]. The question of why some *Vanilla* species produce aromatic fleshy pods while other *Vanilla* species do not is unclear, and it is unknown why some *Vanilla* species rarely flower in cultivation or even in their native habitat [5]. Moreover, little is known of pod and seed dispersal mechanisms, and the taxonomy and systematics of the *Vanilla* genus is in a state of ambiguity [6]. The biosynthesis pathway of vanillin (4-hydroxy-3-methoxybenzaldehyde), the main flavour component of vanilla extract [7], has remained elusive for decades in spite of dedicated efforts. In 2014, an enzyme designated *Vp*VAN was isolated from *V. planifolia* and shown to efficiently convert ferulic acid and ferulic acid glucoside into vanillin and vanillin glucoside, respectively [8] (**see also Chap. 9 of this book**).

In contrary to vanilla orchid biology, bioengineering approaches to biosynthesis of vanillin in microorganisms and its status as a 'natural' ingredient are in the spotlight. In this book chapter, we review our current understanding of the **vanilla plants**, **vanilla flavour**, and **vanillin biosynthesis in the vanilla orchid** and summarize **emerging bioengineering possibilities of vanillin biosynthesis in microorganisms**.

1.2 Vanilla Orchids, Vanilla Flowers and the Pod

Vanilla plants are climbing orchids [6, 9]. They belong to the *Orchidaceae* family, one of the largest families of flowering plants. However, more detailed classification of vanilla plants into tribes, subfamilies and genera has proven to be challenging. The increased use of DNA-based systematic sequence studies has governed the latest classification of the vanilla species. According to these studies, the vanilla orchid belongs to the genus *Vanilla*, tribe Vanilleae, subfamily Vanilloideae and family Orchidaceae [6, 10–12]. In the genus of *Vanilla* there are about 110 vanilla species, among which there are three species known to be important for commercial cultivation and for local low scale vanilla farmers: *V. planifolia*, *V. tahitensis* and *V. pompona*. *V. planifolia* originates in current day Mexico and is the most valued of these three species because

Fig. 1.1 *V. planifolia* climbing orchids that produce beautiful yellow flowers when the vine is about 4–5 m long. Pods in this picture are only a few weeks old (Photo was taken at CIRAD shade houses, at La Reunion. Photo credit: Nethaji J. Gallage)

of its vanilla flavour quality. *V. planifolia* is widely cultivated and provides 95% of the world vanilla production [9]. *V. planifolia* differs very little from its ancestors in the wild. The vanilla plants that are grown in La Reunion, Madagascar, Mauritius and Seychelles are derived from a single cutting of *V. planifolia*. Stem cutting propagation results in a lack of genetic variation in vanilla plants [9].

The orchid, *V. planifolia* is a climbing perennial vine with a large, green, fleshy and succulent stem that is photosynthetic. *V. planifolia* has oblong, smooth, bright green leaves and adventitious aerial roots that grow opposite of each leaf, aiding lateral support. The roots are associated with endotrophic mycorrhiza [13]. This symbiosis provides the fungus access to the sugars produced by *V. planifolia* through photosynthesis and in return, vanilla is able to get water and minerals through the fungal mycelium. This particular association involves 'endomycorrhizae'- fungi whose hyphae actually enter the plant's root's cell membrane [4].

When the *V. planifolia* vine is approximately 4–5 m long, the plant starts flowering. The vanilla flowers are yellow, bisexual and usually sprout towards the top of the plant (Fig. 1.1). Once opened, these flowers only survive for 24 h. Although flowers are bisexual, they are not able to self-pollinate. Pollination requires outside action either by transferring pollen from an anther to the stigma using a soft tiny paintbrush or by lifting a thin-membrane that prevents self-fertilization, and subsequently pressing the flower's anther towards the stigma [14]. The lack of specific flower pollinators was a key issue encountered when introducing *V. planifolia* to the rest of the world from Mexico. Vanilla's pollen is also largely inaccessible to most pollinating insects due to the shape of the flower [14]. In nature, flowers are pollinated by small bees and hummingbirds that are capable of penetrating the tough

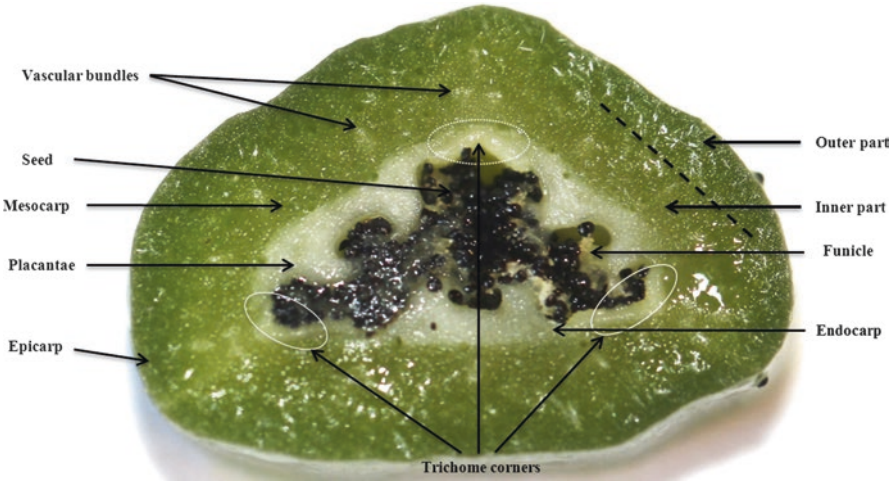


Fig. 1.2 Transverse picture of vanilla pod disc, photo is taken with Canon EF 200 mm f/2.8 L macro lens (Photo credit: Nethaji J Gallage)

membrane that separates the plant's pistil and stamen, although very little scientific information is available on this subject. In 1841, Edmond Albius had developed an artificial pollination technique of vanilla flowers by using bamboo sticks. Additionally, there are records by Charles Morren, in 1836, of the artificial pollination of vanilla flowers. Even today, hand pollination techniques are used for the flower pollination in vanilla production, and these techniques have not developed much further since 1841 [14, 15].

Botanically, the vanilla pod is a seed capsule, but is generally referred to as a vanilla bean or vanilla pod. The pod reaches its full size 10–15 weeks after pollination. Fully matured pods are about 15 cm long and are pale green to yellow in color. A transverse section of a mature green vanilla pod is triangular, containing a central cavity comprising numerous black seeds. From the outer part to the inner cavity, the pod consists of the following tissues: epicarp, mesocarp and endocarp. The mesocarp fills the majority of the fruit volume and consists of parenchyma cells. The cavity of the pod contains black seeds that are attached to a long narrow funicle [16] (Fig. 1.2).

Vanilla pods are harvested when they are 8–9 months old, before the pods begin to split from the end and become yellow in color. The immature green vanilla pods are almost odorless as the key flavour components are stored as glucosides. Freshly harvested pods are processed by curing to stop the natural vegetative process. This induces enzymes responsible for the formation of aromatic flavour constituents and prevents microbial growth, thus enabling long-term preservation of vanilla pods [9]. Curing methods can be different according to the country in which the plants are cultivated. As a result, the curing process has a major influence on the variety, quality and aromatic profile of the pods that are traded [17].

1.3 When the Green Becomes Black – Vanilla Pod Curing

In general, the curing process includes wilting, sweating, drying and conditioning of the pod. The main purpose of wilting is to stop the vegetative growth of the pod after harvest and to disrupt the cell structures. This process is also referred to as kilning, as it ceases the respiratory function of the plant tissues and promotes disruption of cell membranes thus creating better conditions for contact between certain metabolites and enzymes that release aroma compounds typically by catalyzing hydrolysis of a glycosidic linkage. Methods used to initiate kilning are sun drying, freezing, hot water, and ethylene gas treatments [18].

Sweating is the process by which the pod temperature is raised; moisture is initially allowed to escape to prevent harmful fermentation by microbial spoilage, but enough moisture is retained to promote enzymatic reactions. This is documented to be the most crucial step of the curing process, as most enzymes that are responsible for flavour and aroma development are active at this stage and are determinants of the quality of the cured pods. The sweating process is usually carried out for seven to ten days, and at the end of the process pods obtain a characteristic brown chocolate color due to the oxidation of polyphenolic compounds [13]. The pods are then dried at room temperature to reduce their moisture content so microbial spoilage can be avoided. The lower moisture content also reduces undesired enzymatic activities [9].

Conditioning/packaging is the last step of the curing process. Pods are placed in closed boxes for one to several months to initiate various biochemical reactions such as esterification, etherification and oxidative degradation etc., which produce the final high quality aromatic composition. After the curing process, the pod in general consists of sugars, proteins, free amino acids, fibers, cellulose, organic acids, oil, wax, resin, gum, pigments, minerals, volatile aromatics and essential oils. As mentioned, the most abundant aromatic compound in the vanilla extract is vanillin, followed by *p*-hydroxybenzadehyde, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzyl alcohol and vanillyl alcohol [18–20]. The ratio between these main flavour compounds in the pod is known to determine the quality and the final flavour of the vanilla pod and the vanilla extract. In addition to the curing process, the chemical constituents of the vanilla pod and its flavour are also determined by various other factors such as plant species, growing conditions, soil nutrition and maturity level at harvest [19].

Madagascar is the largest vanilla pod producer in the world. Madagascar, together with La Réunion islands, account for nearly 75% of the vanilla pod market. Vanilla pods from Madagascar and other islands east of Africa (La Réunion, Mauritius, the Comoros and the Seychelles) have the status ‘Bourbon’, and are considered the best vanilla pods in the current market. The vanilla growers in this region also produce the majority of the world’s natural vanilla extract, which is an alcohol extract from matured and cured vanilla pods. The vanilla extract isolated from the vanilla pods includes more than 200 aromatic compounds [21].

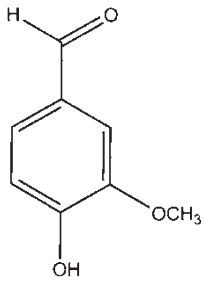
1.4 Vanillin Is the Key Flavour Compound of the Complex Vanilla Extract

Often the terms 'vanilla' and 'vanillin' are confused in non-scientific communications. The term 'vanilla' refers to the complete extract of the vanilla pod and, as stated above, is known to include more than 200 different flavour compounds. However, the term vanilla is commonly used also to describe vanilla plants, vanilla pods and vanilla flavour. Vanillin is the main flavour compound in vanilla extract. The chemical structure of vanillin is quite simple and known to give the characteristic flavour and aroma that we associate with vanilla extracts [7].

Vanillin (4-hydroxy-3-methoxybenzaldehyde) (Fig. 1.3) is the most abundant compound in the cured vanilla pod and corresponds to 2.5–4.5% of the dry weight [7]. The French chemist Theodore Nicolas Gobley was able to isolate vanillin as the main flavour constituent of vanilla pod extracts in 1858. He also elucidated the chemical structure of vanillin [22]. Recent studies have demonstrated that vanillin starts to accumulate in a free or glycosylated form from the 15th week and continues to accumulate until the 30th week after pollination [23]. Vanillin is an aldehyde. The aldehyde group is very reactive, forming Schiff bases with, for example, free amino groups present on the side of the protein-bound lysine residues. This is the reason it is toxic to living organisms in high concentrations [24]. Vanilla plants store vanillin almost entirely in the glucose-conjugated form, vanillin-β-D-glucoside (commonly referred to as vanillin glucoside or glucovanillin) (**see also Chap. 9 of this book**). Vanillin glucoside can account for up to 15% of the vanilla pod dry weight. The highest concentration of vanillin glucoside is reached in the inner part of the pod, including mesocarp and placenta, 6 months after pollination. Small amounts of vanillin glucoside can also be found in the papillae of the pod. There is no vanillin found in the black vanilla seeds and interestingly, black vanilla seeds are free of aroma [16] (Fig. 1.4).

Currently, the annual worldwide consumption of vanilla pods, vanilla extract and vanillin is over 18,000,000 kg [25]. However, due to the slow growth of the orchids and the low concentration of vanillin in the vanilla pods (about 2% of the dry weight of cured vanilla beans), only about 0.25% of consumed vanillin originates from vanilla pods [26]. The production of vanilla beans and the isolation of vanillin from vanilla pods is a laborious and costly process [7]. Production of 1 kg

Fig. 1.3 Vanillin (4-hydroxy-3-methoxybenzaldehyde)



vanillin

Fig. 1.4 The most popular flavour (Photo credit: Nethaji J. Gallage)

of vanillin requires approximately 500 kg of vanilla pods, corresponding to the pollination of approximately 40,000 vanilla orchid flowers. The market cost of natural vanillin derived from vanilla pods is therefore high and fluctuates because of the unpredictable availability of vanilla pods. Crop yield is tightly associated with weather conditions, the incidence of diseases, as well as local and international political and economic issues. Vanillin extracted from vanilla pods has a market price varying from around US$ 1200 kg^{-1} to more than US$ 4000 kg^{-1} [27]. Thus, the increasing global demand for natural vanilla flavour appeals other sources of vanillin [28].

Currently the main source of vanillin is chemical synthesis, while, as mentioned, less than 1% is derived from the vanilla pod industry. Less than 20 years after its initial isolation, synthetically produced vanillin was marketed. Nowadays, guaiacol and lignin are favoured starting materials for synthetic vanillin. Synthetic vanillin is able to meet the global market demands, and it is also rather cheap with a market price below US$ 15 kg^{-1} [1]. Chemical synthesis of vanillin suffers from serious drawbacks *e.g.* the use of hazardous chemicals. Chemical synthesis of vanillin via lignin has been calculated to require safe removal of 160 kg of waste per 1 kg of vanillin obtained. As a consequence, concerns are increasing regarding the negative environmental impact caused by chemical synthesis of vanillin [29]. Nevertheless, at present a substantial amount of synthetic vanillin is still derived from lignin [30]. Recent advances in biotechnology have allowed an alternative method to challenge the chemical synthesis of vanillin, namely bioengineering of natural vanillin.

1.5 How Does the Vanilla Plant Form Vanillin?

As the main constituent of the vanilla extract and the world's most popular flavour, vanillin is a compound of major interest to the flavour and fragrance industry. Although there are many research efforts and resources committed to engineering various microorganisms for vanillin biosynthesis, limited attention has been given to understanding the most efficient vanillin synthesizing machinery that is found in nature, namely the vanilla orchid. The vanilla orchid produces vanillin in the pods in such high concentrations that it cannot be compared to any other known biological system in nature.

This simple molecule, 4-hydroxy-3-methoxy benzaldehyde, was speculated to be formed through the operation of a multitude of pathways in the vanilla orchid. It was clear that vanillin biosynthesis in the vanilla pod proceeds from the amino acid phenylalanine and includes phenylpropanoid intermediates [31]. Vanillin glucoside and *p*-hydroxybenzaldehyde glucoside are the two most abundant components that produce aroma-active compounds upon hydrolysis in mature vanilla pods (**see also Chap. 9 of this book**). These compounds are structurally similar. Accordingly, a biosynthetic relationship between the formation of these two compounds had been hypothesized in the early literature, with *p*-hydroxybenzaldehyde as a putative precursor for vanillin glucoside biosynthesis [32–34]. Recently, the involvement of *p*-hydroxybenzaldehyde in vanillin biosynthesis has been ruled out by Gallage et al., who demonstrated that incubation with [^{14}C]-*p*-hydroxybenzaldehyde did not result in [^{14}C]-vanillin glucoside formation in the 6 month old vanilla pods after pollination [8]. This work also established a route to vanillin biosynthesis in the pod, namely via C3 side chain shorting of ferulic acid or its glucoside (Fig. 1.5). This argument was first brought forward by Zenk in 1965, who carried out radioactive precursor studies using [^{14}C]-ferulic acid and observed efficient conversion of [^{14}C]-ferulic acid to [^{14}C]-vanillin glucoside [31]. The conclusions of Zenk were confirmed by Negishi et al., who carried out a similar study employing radioactive precursors [35].

Ferulic acid and ferulic acid glucoside are ubiquitous phenylpropanoids that are derived from cinnamic acid. Ferulic acid is present as a constituent of the plant cell wall polymers. It is a component of lignocelluloses, where it confers rigidity to the cell wall by making the crosslink between polysaccharides and lignin. Ferulic acid is highly reactive and is often linked to a variety of metabolites including sugars as glycosidic conjugates, different esters and amides, thus forming a broad range of natural products [36]. Ferulic acid is formed by *O*-methylation of caffeic acid, and caffeic acid is formed from phenylalanine in approximately six enzyme catalysed steps. When produced from phenylalanine, the first intermediate is cinnamic acid, and the reaction is catalyzed by phenylalanine ammonia lyase (PAL) [37]. Subsequently, cinnamic acid 4-hydroxylase (C4H) [38] catalyzes the hydroxylation of cinnamic acid at the 4-position, resulting in the formation of *p*-coumaric acid. *p*-Coumaric acid-3-hydroxylase (C3H) [39] catalyzes hydroxylation of *p*-coumaric acid at the 3 position, resulting in the formation of caffeic acid. C3-hydroxylation step is shown to be proceeding via, *e.g*. quinate or shikimate

esters. 4-Hydroxycinnamoyl-CoA ligase (4CL) and hydroxycinnamoyltransferase (HCT) are involved in quinate and shikimate ester formation [39]. Caffeic acid could, in principle, be *O*-methylated by an *O*-methyltransferase (OMT) [40] to afford ferulic acid.

The key enzyme that is involved in catalyzing C3 side chain shorting of ferulic acid or its glucoside in the pods of *V. planifolia* was recently identified by Gallage et al., [8]. A single enzyme named vanillin synthase (*Vp*VAN) was characterized to catalyze the double carbon bond cleavage of ferulic acid and ferulic acid glucoside to vanillin and vanillin glucoside, respectively. *Vp*VAN was isolated from *V. planifolia* and functionally characterized *in vitro*, in yeast and *in planta*. A route to vanillin biosynthesis mediated by *Vp*VAN is illustrated in Fig. 1.5. *Vp*VAN belongs to the enzyme family of cysteine proteases [8]. In a recent paper including Hailian Yang, Daphna Havkin-Frenkel and Richard A. Dixon as authors, the role of *Vp*VAN in vanillin biosynthesis was questioned [41].

Cysteine proteases are known to possess versatile physiological functions and do not have well-defined substrate specificities. In general, cysteine proteases are expressed as a pre-protein, with a N-terminal ER-targeting signal peptide being part of a pro-peptide domain comprising 130–160 residues [42]. To form the mature cysteine-proteinases, the pro-peptide sequence is removed either by a processing enzyme or by auto-catalytical processing [43]. The *Vp*VAN protein has not shown any evidence of autocatalytic processing. This indicates that the removal of the pro-peptide requires the action of a separate processing enzyme [8].

As vanillin is almost entirely stored as vanillin glucoside, it is apparent that the glycosylation step in the vanilla pod is highly efficient (**see also Chap. 9 of this book**). The glycosylation step has not been explored in detail, and it is not known at which step glucose incorporation occurs in the course of vanillin glucoside biosynthesis. Gallage et al., demonstrated, that *Vp*UGT72E1 possesses vanillin-specific glycosyltransferase activity. However, the glycosylation step in the vanillin biosynthesis machinery needs further study [8]. The enzyme that catalyses the reverse reaction hydrolysing vanillin glucoside to vanillin, vanillin-β-glucosidase, has previously been characterized [44].

1.6 Flavour Synthesis by Brewing – Bioengineering of Vanillin Biosynthesis

The increasing global demand for natural vanilla flavour can no longer be met with pods of the vanilla orchid as the sole source. This is why the market for vanillin is increasing [1]. The major source of marketed vanillin originates from chemical synthesis. In recent years, demand from consumers for natural products has increased. Though the approved use of the attribute ‘natural’ is not well defined and not evident to most consumers [45], many consumers equate the term “natural” with food quality and food safety and maybe also with enhanced environmental friendliness [46] (see also Chap. 11 of this book). Accordingly, and guided by novel technologies, research on bioengineering of microorganisms for flavour production is rapidly growing.

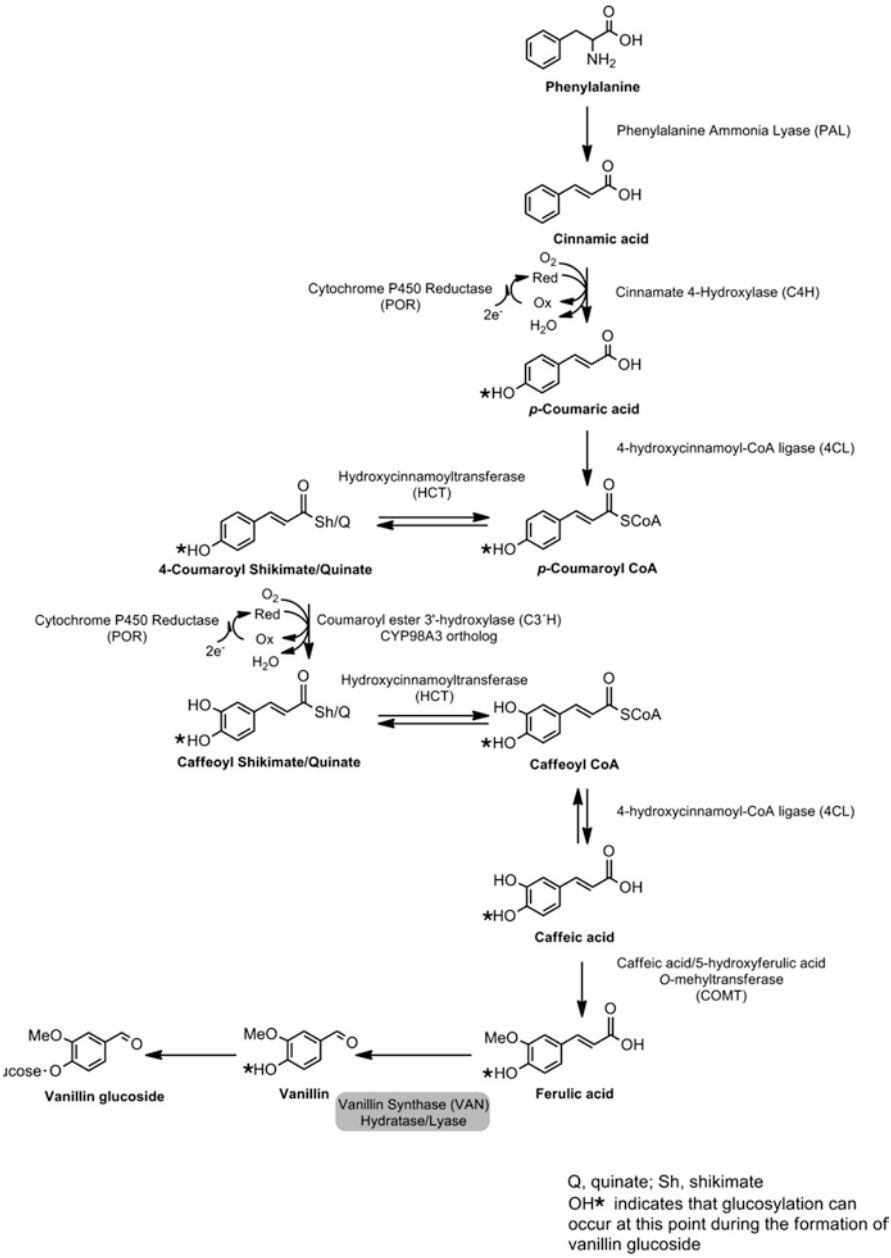


Fig. 1.5 A route to vanillin biosynthesis in pods of *V. planifolia* [8] (Figure reproduced with permission from Nature Communications)

Vanillin, obtained by bioengineered microorganisms by transforming a range of different substrates into vanillin, is entitled to the label 'natural vanillin' according to US and European legislation (EC Directive 88/388, OJ no. L 184 15/07/88) [47]. This affords significantly increased sales in the range of US$1000 kg^{-1} for the bioengineered vanillin and enables the vanillin produced by bioengineering to compete with the chemically synthesized vanillin that currently dominates the market [48]. Several of the bioengineering approaches have been successful, and biotechnologically derived vanillin products have been available on the market for more than a decade (Fig. 1.6). Rhovanil produced by Solvay (previously known as Rhodia) was the first commercially available fermentation-derived vanillin product and was obtained by bioconversion of ferulic acid [49]. Ex-tumeric vanillin is marketed by De Monchy Aromatics and produced from curcumin [50]. Sense Capture Vanillin is obtained by bioconversion of eugenol and marketed by Mane [51]. *De novo* synthesized bio-vanillin using glucose as a precursor was commercialized in 2014 by Evolva A/S and International Flavors and Fragrances (IFF) [52].

Fermentation and bioengineering have been used to produce beers, wine, cheese, food colorants and pharmaceuticals for centuries. Vanillin has now been added to that list. Most bioengineering approaches for the synthesis of vanillin are based on the bioconversion of certain natural substances such as lignin, ferulic acid, eugenol and iso-eugenol etc., using microorganisms such as yeast, fungi and bacteria as

Fig. 1.6 Different commercial routes to natural vanillin [1] (Figure reproduced with permission from Molecular Plant)

production hosts by fermentation [53–55]. Microorganisms that exhibit rapid growth rates and are amenable to molecular genetics are obviously preferred. Further bioengineering has focused on increasing tolerance to high concentrations of both product and substrate. Microorganisms and fermentation ingredients, which have been given GRAS status, are preferred. GRAS is an acronym for Generally Recognized As Safe under the regulations of the US Food and Drug Administration (FDA) [56].

Microorganisms that are able to metabolize a range of different precursors into vanillin have been subjected to further bioengineering to circumvent remaining pathway bottlenecks and other drawbacks. Bioengineering includes the use of tools such as genetic engineering, enzyme optimization and cost-efficient downstream processing. However, several major yet common issues have challenged the successful use of microorganisms for efficient bioconversion of various substrates into vanillin. Bottlenecks include: (1) cytotoxicity of the flavour products obtained and of their precursors; (2) inefficient metabolic flow; and (3) costly downstream processing methods due to the physicochemical properties of the substrate and the product. The increasing knowledge of enzymes that are involved in the bioconversion of ferulic acid and other substrates into vanillin, as well as identification and characterization of the corresponding genes, offers new opportunities for more targeted bioengineering of microorganisms for vanillin production. In the following sections, the bioconversion and bioengineering of vanillin by microorganisms (bacteria, fungi and yeast) are presented and commented upon, with emphasis on the major issues encountered and the solutions obtained.

1.7 Biotechnology-Based Production of Vanillin from Eugenol, Iso-Eugenol, Ferulic Acid and Glucose

Availability of the Substrate In general, bioengineering of vanillin in microorganisms is carried out using precursors that are structurally similar to vanillin *e.g.* eugenol (2-methoxy-4-(2-propenyl)-phenol), iso-eugenol (2-methoxy-4-(1-propenyl)-phenol) or ferulic acid (4-hydroxy-3-methoxy-cinnamic acid). These compounds are also relatively cheap and easily available. Ferulic acid is one of the most abundant hydroxycinnamic acid derived products, present as a constituent of the plant cell wall and as a lignin monomer precursor [57]. Ferulic acid is widely distributed throughout the plant kingdom and was recently shown to serve an additional function as the main precursor for vanillin production in the vanilla orchid [8]. The main source of the ferulic acid for bioengineered vanillin production is agricultural waste such as sugar beet, barley and wheat bran. Ferulic acid is ester-linked to pectic side chains in beet and ether-linked to lignin in cereals [57]. Eugenol and iso-eugenol are main components of natural essential oils of clove trees [58]. The pure eugenol and iso-eugenol substrates are inexpensive and cost no more than US$ 5 kg^{-1} [59].

To reach a higher degree of sustainability, much effort has been focused on the use of agricultural waste as the main precursor for bioengineering. Several studies have attempted to remove ferulic acid from plant cell wall materials enzymatically

[60–62]. Feruloyl esterases are enzymes that are able to hydrolyze the ester bonds by which ferulic acid is attached to the cell wall polymers, and can be isolated from a wide range of fungi, yeast and bacteria [63]. Two feruloyl esterases, FaeA and FaeB, isolated from *Aspergillus niger,* are able to release ferulic acid from industrial by-products such as wheat straw, coffee pulp, apple core, maize bran, maize fiber *etc.*[64]. *A. niger* strain 1–1472 has been used to release ferulic acid from autoclaved maize bran [65].

Enzymatic hydrolysis of cell walls using a combination of commercial polysaccharide-degrading enzymes and feruloyl esterase has also been investigated [66]. Currently, these methods are not economically feasible as the commercially available polysaccharide-degrading enzymes are costly and would result in significantly increased production costs of vanillin. Ferulic acid can also be released from plant cell walls by alkaline treatment at high temperatures (85–100°C) [67, 68]. This kind of chemical release of ferulic acid would not be considered natural processing according to EU regulations, but would comply with registration as "natural" according to US legislation [47, 67].

Today, the ferulic acid used for commercial production of natural vanillin is mainly obtained as a by-product in the production of rice bran oil. The ferulic acid is liberated from the rice bran by enzymatic treatment to comply with the regulations for being classified as a natural product. The cost of naturally extracted ferulic acid is relatively high with a price around US$ 180 kg^{-1} [69].

The cost of glucose can be as low as US$ 0.30 kg^{-1}. It is the cheapest substrate used in vanillin production by bioengineering to date [53]. It is also valuable as a cheap primary energy source for the production strain. Moreover, glucose is a more attractive substrate in comparison to eugenol, ferulic acid and other phenolic compounds, because it is not toxic to the host microorganisms.

Host Microorganisms One of the key decisions in developing a vanillin bioengineering process is to choose a host strain that is highly tolerant to both the substrate and the product. Vanillin is rarely accumulated in high concentrations in living cells as it is toxic. In the vanilla plants, vanillin is glycosylated to vanillin glucoside while in many other living organisms it is expected to be oxidized to vanillic acid or reduced to vanillyl alcohol and thereby reduced in the toxicity [1, 8, 24, 53].

Studies of eugenol bioconversion and ferulic acid catabolism in *Rhodococcus sp.* I24 and *Rhodococcus sp.* PD630 have shown that *Rhodococcus sp.* I24, in contrast to *Rhodococcus sp.* PD630, can tolerate up to 2.4–3.0 mM eugenol, implying an effective eugenol catabolism naturally occurring in this strain [70].

Actinomyctetes, such as *Amycolatopsis sp.* [70] and *Streptomyces setonii* [71] are able to accumulate high concentrations of vanillin while at the same time exhibiting a high tolerance towards ferulic acid. *P. putida IE27* [72] and *Bacillus fusifomis* were reported to efficiently convert iso-eugenol into vanillin. *Bacillus fusifomis* is known to yield 32.5 g/l vanillin after 72 h incubation [73]. Similarly, the *P. putida* IE27 strain is able to produce 16.1 g/l of vanillin after 24 h incubation. The vanillin production was induced by continuously adding iso-eugenol to the cultures, which helps to prevent further oxidation of the vanillin formed into vanillic acid [72].

However, the filamentous growth of actinomycetes results in highly viscous broths, unfavourable pellet formation and a lot of fragmentation and lysis of the mycelium, thereby complicating downstream processing [74].

Compared to bacterial strains, yeast strains have not been as heavily exploited for bioengineered synthesis of vanillin. However, natural vanillin is produced via bioengineered *Saccharomyces cerevisiae* on a commercial scale, and more information on this strain is provided in the sections below. Vanillin biosynthesis in bioengineered algae and cyanobacteria is yet to be established.

Cytotoxicity The bioengineering of vanillin-producing microbial systems is challenged by the potential toxicity of precursors as well as products formed. It should be noted that compounds such as vanillin are produced in nature as part of the plants´ defense system against pathogens such as bacteria and fungi [48]. As pointed out previously, vanillin is toxic to living cells in high concentrations. Dealing with this issue is an important pre-requisite for building economically viable biotechnology-derived vanillin cell factories. In the case of *S. cerevisiae*, vanillin production beyond 0.5–1 g/l was toxic, as shown by hampered growth and low level of vanillin accumulation [53]. The natural vanillin biosynthesis pathway in the vanilla orchid *V. planifolia* has an elegant solution to cope with the toxicity issue, by glucosylation of vanillin to vanillin-β-D-glucoside (**see also Chap. 9 of this book**). The same strategy was implemented by Hansen et al. [53], in which the *A. thaliana* UDP-glucose glycosyltransferase UGT72E2 was employed to glucosylate vanillin, producing the less toxic vanillin-β-glucoside as the final product. Hansen and coworkers reported that extracellular concentration of vanillin β-D-glucoside even above 25 g/l had no effect on yeast growth [53]. Moreover, vanillin-β-glucoside has higher water solubility than vanillin and can potentially serve as a sink that can aid in directing the pathway towards vanillin synthesis.

Genes involved in metabolizing ferulic acid into vanillin have been heterologously expressed in engineered *E. coli* with high vanillin tolerance to bypass the problems related to product toxicity. This includes expression of the *Fcs* and *Ech* genes from *Amycolatopsis sp.* HR104 [75]. The vanillin-resistant mutant strain was obtained following NTG (N-methyl-N-nitro-N-nitrosoguanidine) mutagenesis and following a 48 h incubation period, as much as 1 g/l of vanillin was produced in a media containing 2 g/l of ferulic acid. To further circumvent the inhibitory effect of vanillin, XAD-2 ion-exchange resin was used to bind the vanillin formed in the medium. This increased vanillin yield to 5 g/l in 48 h when ferulic acid substrate was applied during incubation [75].

By-Products When various microorganisms metabolize eugenol, iso-eugenol and ferulic acid, vanillin is only produced as an intermediate, and is either readily reduced to vanillyl alcohol or oxidized to vanillyl acid by alcohol dehydrogenases and oxidases, respectively. General approaches used to circumvent undesired product formation are knock-outs and/or knock-downs of genes related to undesired catabolism of substrates and/or products. Several examples are listed below:

The actinomycete *Amycolatopsis sp.* ATCC 39116 is able to synthesize vanillin from ferulic acid but the vanillin formed is subjected to further undesired metabolism. Two to three times higher vanillin accumulation and a substantially reduced amount of vanillic acid was observed using the *Amycolatopsis sp.* ATCC 39116 Δvdh::Km(r) mutant when ferulic acid was provided as a substrate for biotransformation in a cultivation experiment using 2 l bioreactor scale. In the mutant strain, the *vdh* gene, which codes for the vanillin dehydrogenase activity, has been deleted [76].

Hansen et al. constructed glucose-based *de novo* vanillin biosynthesis in *S. cerevisiae*. Further metabolism of vanillin to vanillyl alcohol was circumvented by targeted deletions of alcohol dehydrogenase (ADH) encoding genes. From the tested enzymes, ADH6 was recognized as the most important enzyme catalyzing vanillin reduction in *S. cerevisiae.* The *adh6* mutants in *S. cerevisiae* grew normally under all growth conditions and showed a 50% decrease in converting vanillin to vanillyl alcohol [53].

Bacillus subtilis 3NA is a microorganism with enhanced capacity to metabolize lignin-derived compounds. The strain tolerates a high concentration of up to 20 mM of vanillin. However, *B. subtilis* 3NA further converts vanillin to vanillic acid and subsequently to guaiacol whereas ferulic acid is converted to 4-vinyl guaiacol. Gene deletion of phenolic acid decarboxylase *bsdD* resulted in an increased vanillic acid synthesis in *Bacillus subtilis* 3NA [77].

In yeast, ferulic acid is readily detoxified by the action of the decarboxylation enzymes PAD1 and FDC1, resulting in the formation of 4-vinylguaicol [78]. Mutation of *pad1* and *fdc1* is essential for improving vanillin production in yeast when ferulic acid is used as substrate [79, 80].

Inefficient Metabolic Flow One approach to bypass inefficiencies in the metabolic flux caused by inhibitory effects of substrates or accumulated intermediates is a continuous administration of the substrate to the cell culture. In the *P. putida IE27* strain, vanillin production was increased by continuous addition of iso-eugenol to the cultures, which reduced oxidation of the vanillin formed into vanillic acid. Using this method, *Bacillus fusifomis* was reported produce 32.5 g/l vanillin from isoeugenol over 72 h [72].

To reduce by-product formation, a two-step fermentation process can be carried-out using two different microbial organisms. This approach was employed by Lesage-Meessen and co-workers to optimize vanillin production by combining use of *Aspergillus niger* and *Pycnoporus cinnabarinus*. The micromycete *A. niger* metabolized ferulic acid to vanillic acid in high yield whereas the basidiomycete *P. cinnabarinus* reduced the amount of vanillic acid converted into vanillin. The vanillic acid titer from *A. niger* is reported to be 920 mg/l while vanillin titer from *P. cinnabarinus* strain was reported to be 237 mg/l [54].

The development of engineered production strains is an alternative route towards achieving efficient metabolic flow towards the desired product. Li and Frost [81] devised a route for microbial production of vanillin from glucose, in which *de novo* biosynthesis of vanillic acid in *E. coli* was combined with enzymatic *in vitro*

conversion of vanillic acid to vanillin. The recombinant *E. coli* KL7 strain was engineered to dehydrate 3-dehydroshikimic acid to protocatechuic acid (3,4-dihydrobenzoic acid) by the action of 3-dehydroshikimic dehydratase (3DSD), encoded by the gene *AroZ* from the dung mold fungus *Podospora anserina*. 3-Dehydroshikimic acid is an intermediate in the shikimate pathway resulting in biosynthesis of aromatic amino acids. Protocatechuic acid was then converted to vanillic acid by a human catechol-*O*-methyltransferase (COMT). Reduction of vanillic acid to vanillin was carried out *in vitro* using a cellular extract of *Neurospora crassa*, which contained the required aromatic carboxylic acid reductase (ACAR) activity [81].

Hansen et al., reported the first example of one-cell microbial vanillin biosynthesis from glucose in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* [53]. These strains encompass an ACAR from *Nocardia iowensis*, in combination with a phosphopantetheinyltransferase (PPtase), which is required for proper activation of the ACAR enzyme. ACAR catalyzes the ATP- and NADPH-driven reduction of protocatechuic acid to protocatechuic aldehyde and of vanillic acid into vanillin. The yeast strain utilizes the gene encoding 3DSD from *P. anserina* to mediate the formation of protocatechuic acid from 3-dehydroshikimate. From protocatechuic acid, the pathway may then proceed via vanillic acid formed by *O*-methylation catalyzed by human COMT, which is subsequently reduced to vanillin by ACAR [53]. Alternatively, protocatechuic aldehyde formed by the reduction of protocatechuic acid by the ACAR enzyme may subsequently be *O*-methylated into vanillin by COMT. To improve the metabolic flux through the *de novo* vanillin biosynthetic pathway in yeast, mutations were introduced into the production strains. These included a mutation in the AROM enzyme complex (ARO1) to increase the accumulation of 3-dehydroshikimate. This mutation resulted in an increased accumulation of protocatechuic acid, and thereby redirected the metabolic flux from aromatic amino acid production to vanillin precursor production. A more efficient and more specific ACAR enzyme, which was able to catalyse the conversion of the high concentrations of protocatechuic acid to protocatechuic aldehyde, was obtained from *Neurospora crassa.* Upon expression of the gene encoding *Nc*ACAR, the yeast efficiently catabolized the available high concentrations of protocatechuic acid. This was one of the key features for the successful generation of recombinant *S. pombe* and *S. cerevisiae* capable of *de novo* synthesizing vanillin [82].

Downstream Processing Methods Based on the Physicochemical Properties of the Substrate and the Product Commercially viable vanillin production in microorganisms is dependent on efficient low-cost downstream processing and high product recovery. Few studies have reported on the potential advantages of using various product removal techniques to prevent further metabolism of the final product, *e.g.* by binding the product to absorbent resins that can be used in the fed batch fermentation.

Topakas et al., improved vanillic acid to vanillin transformation in *P. cinnabarinus* cultures grown at bioreactor scale by absorbing the toxic vanillin produced by the hydrophobic resin Amberlite XAD-2 [83]. The use of macroporous DM11

adsorbent resins has given promising results in the fed-batch biotransformation of ferulic acid to vanillin using *Amycolatopsis sp.* strain ATCC 39116. In this study, in the presence of a surplus of DM11, continuous addition of 45 g/l ferulic acid resulted in formation of 19.2 g/l vanillin within 55 h [84]. At 20°C, vanillin concentrations above 10 g/l resulted in vanillin crystallization and this provides a convenient way of isolation.

1.8 Future Perspectives and Final Remarks

Using synthetic biology, microbial organisms have been engineered for the production of various natural food components. Vanillin is one such successful example [56]. Major constraints like substrate or product inhibition have been overcome by bioengineering. The increased knowledge of enzymes involved in bioconversion of ferulic acid and other substrates to vanillin, as well as identification and characterization of the encoding genes, offers new opportunities for improved targeted bioengineering of microorganisms for vanillin production. The recent identification of *Vp*VAN as the enzyme converting ferulic acid into vanillin in *V. planifolia* provides the option to transfer the vanilla orchid pathway for vanillin synthesis into microorganisms. Yeast and cyanobacteria are apparently the best host organisms for this purpose, as the vanilla orchid enzymes may require post-translational modifications to be fully active.

The market launch of several synthetic biology derived vanillin products has generated some media attention. Bioengineering approaches may provide a more sustainable alternative to chemical synthesis [85]. In this context it is relevant to discuss whether or not it is justified and appropriate to label biotechnologically produced flavours such as vanillin as "natural" [45]. The general public cannot be expected to understand and adapt to definitions of flavour codes that are not self-evident and obvious. The main flavour codes such as, "natural", "nature identical flavour," and "artificial" (FDA), do not offer a proper and specific description of each category when the consumer is faced to choose between commercially available products from each category in a store. This situation obviously results in unsatisfied and insecure consumers. Multinational organizations like Friends of the Earth and Greenpeace exploit the situation to establish communication platforms voicing their general resistance to all products obtained using genetic engineering, even when no genetic material is present in the commercialized product [86]. It is also clear that unconscious or conscious lack of distinction between the meanings of the words vanilla and vanillin gives rise to misinterpretations and manipulation of the available facts [87].

It is important to highlight that from a commercial point of view, as well as from the point of view of the consumer, bioengineered natural vanillin competes with chemically synthesized vanillin, which currently dominates the market [88]. And not with the vanilla extract from *V. planifolia* which contains a wide range of flavour components in addition to vanillin.

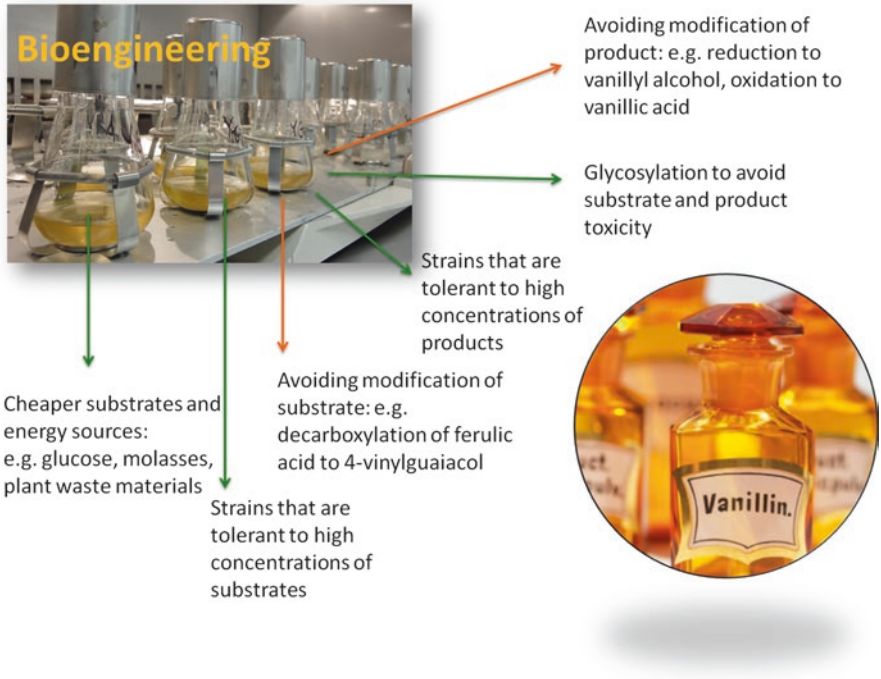


Fig. 1.7 Bioengineering approaches for vanillin production need key considerations as summarized here

In conclusion, the biotechnological production of vanillin from safe and cheap substrates by the use of food-grade production organisms and environmentally benign and economically feasible downstream processing is envisioned to result in a compatible and sustainable alternative to vanillin produced by chemical synthesis (Fig. 1.7).

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