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**VANILLA FLAVOUR AND VANILLIN**

**Classical production of vanilla, chemical synthesis of vanillin, biocatalysis in vanillin and vanilla flavour production**

**1. ABSTRACT**

Vanillin is one of the most important aromatic compounds and is used as flavouring agent. It is employed in food industry, perfumes, cosmetics and pharmaceuticals. In this work vanilla plant, history of vanilla cultivation and production of vanilla beans are briefly reported and both chemical synthesis and bioproduction of vanillin are described.

Nowadays almost all the vanillin on the market is produced by chemical synthesis. The rising consumer request for natural flavours has generated great interest for new methods to obtain natural flavours. Biotechnology approaches are based on bioconversion of lignin, ferulic acid, eugenol and isoeugenol or aminoacids. Another possible procedure is the De novo biosynthesis, starting from glucose. Microorganisms used as biocatalysts are bacteria, fungi, yeasts, engineered organisms or plant cells and tissue cultures.

Bioproduction of vanillin is nowadays not economically suitable. Only few companies produce vanillin with one of these methods, but this natural vanillin has a very high cost. More studies on the optimization of vanillin bioproduction are necessary.

**2. INTRODUCTION**

VANILLIN
Vanillin is an organic compound, a phenolic aldehyde and it is the major component of vanilla beans extract. Vanillin is a colourless-white solid in crystal form, whereas the vanilla extract appears as a dark brown solution.

The vanilla flavour can be obtained by using vanilla beans directly, vanilla extract or synthetic vanillin. Nowadays, synthetic vanillin is more frequently used than natural vanilla extract as a [flavouring agent](https://en.wikipedia.org/wiki/Flavoring_agent), especially in industrial foods, beverages and pharmaceuticals.

Most of the vanillin is used in ice-cream and chocolate industry, while smaller amounts are employed in confections and bakery. Vanillin is also adopted in perfumes, cleaning products and pharmaceutical industry. Vanillin has a medicinal value too as it acts as an antimicrobial agent for the inhibition of moulds and yeast in fruit purees and fruit-based products.

VANILLIN STRUCTURE

**3. VANILLA PLANT [1]**

Vanilla is a tropical orchid belonging to the family Orchidaceae and represents the only orchid which produce a commercially important flavouring material. In specific, about 110 species of vanilla plant have been identified, but only three of them are important in commerce: *Vanilla fragrans*-*V. planifolia*, *V. pompona* *and V. tahitensis*. Among these, *V.* *planifolia* is the most valued for its flavour qualities and is largely cultivated and used to produce food additives.

The vanilla plant has green elongate leaves and clusters of yellow flowers. Flowers appear 2 years after planting and they have a very closed structure, that makes self-pollination almost impossible. Flowers bloom for less than 24h and a timely pollination is essential for fertilisation and subsequent fruit development.

Vanilla is cultivated in warm, moist, tropical climates and reproduced by vegetative propagation. Artificial pollination is performed using a bamboo stick.

The ripe fruits of vanilla are called beans or pods, they are green and cylindrical and fully ripened after 10 months. Fresh beans have an unpleasant bitter smell and a curing process is needed for developing the characteristic pleasant vanilla aroma and their typical brown colour.

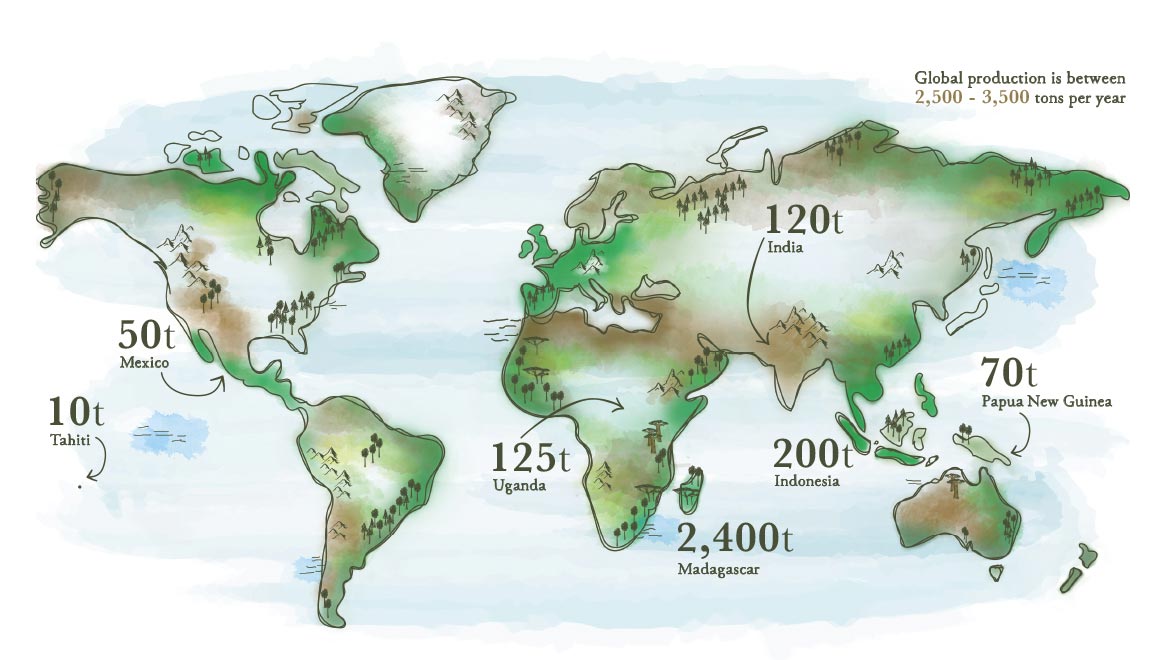
VANILLA FLOWER AND GREEN VANILLA PODS

**4. HISTORY OF VANILLA CULTIVATION [1]**

Aztecs in Mexico cultivated vanilla already around 1500 AD and they were using this spice for flavouring their drinks, such as chocolate. When the Spanish invaded Mexico, they learned from the native Aztecs how to use vanilla and successively they tried to export this plant in Europe. The crop was a failure because pollination was unfeasible and, consequently, plants were flowering but not bearing fruits.

Only in 1841 the Belgian professor Charles Morren developed a first artificial method for the hand-pollination of vanilla. However, a commercial production of vanilla out of its native land, the Mexico, was possible just five years later, when Edmond Albius devised a practical method with the use of a bamboo stick. Thanks to this technique, French soon started vanilla cultivation in many of their colonies. Nowadays, the most important production area is the north-eastern coast of Madagascar and French vanilla beans produced in this country are known as ”Bourbon” vanilla.

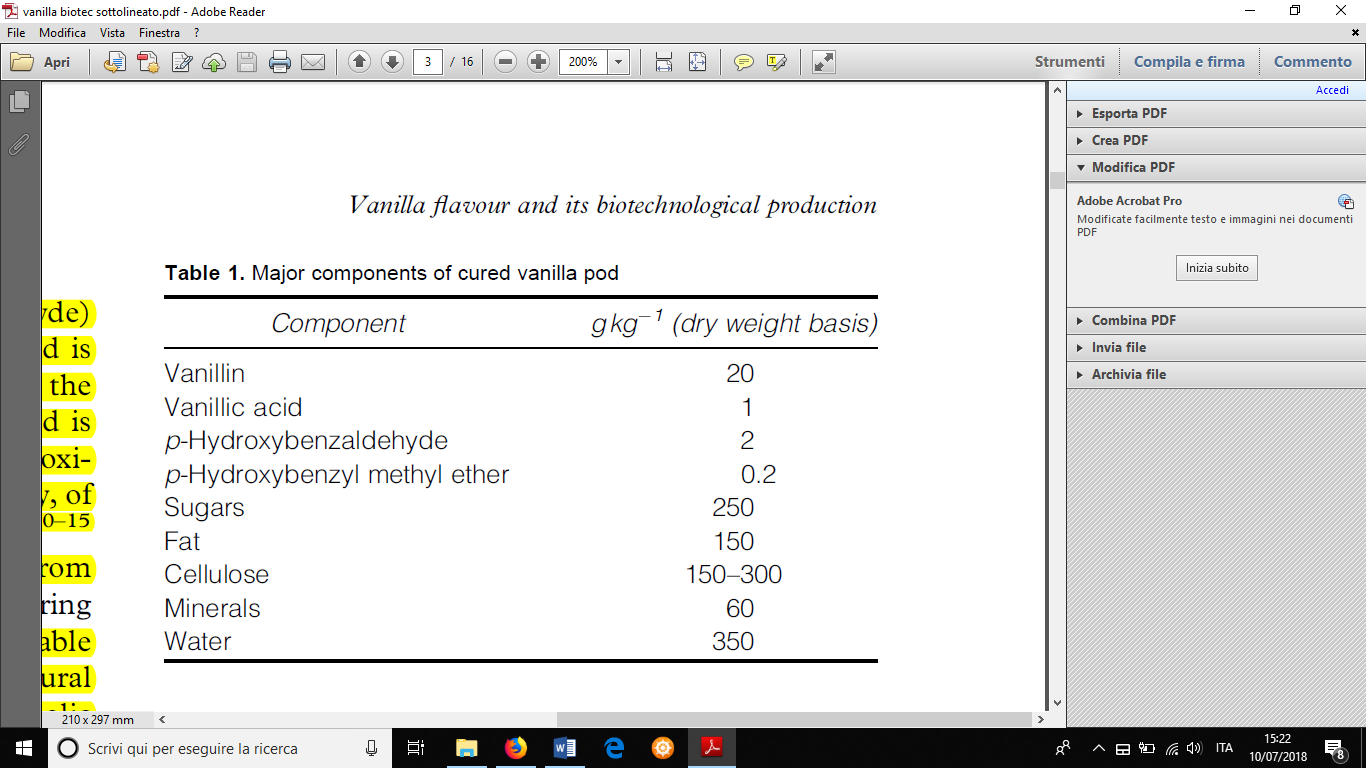
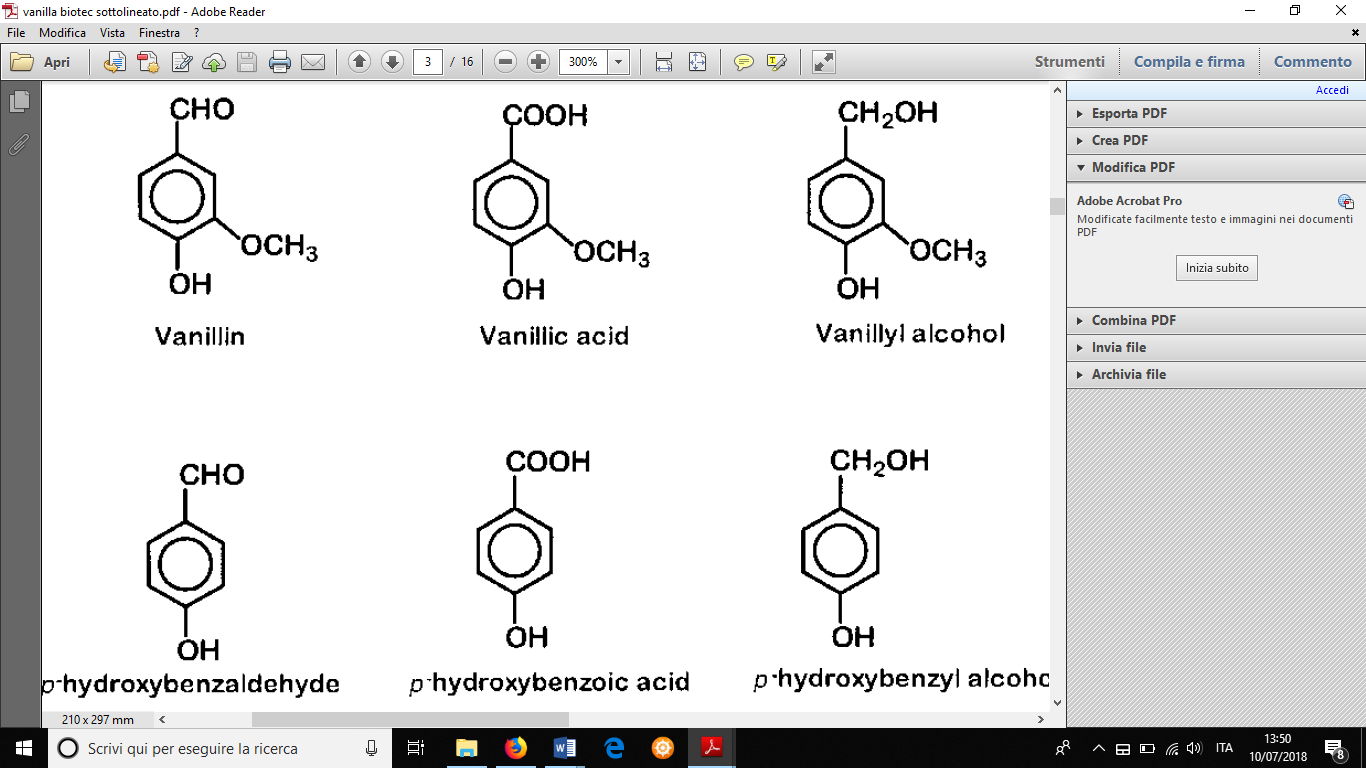
By 1890 vanilla was successfully cultivated in Java, Tahiti, Madagascar, the Seychelles, the Comoro islands, Mauritius, Reunion, Zanzibar, Jamaica and several other regions in the tropics.



MAP FOR VANILLA CULTIVATION

**5. COMPONENTS OF VANILLA FLAVOUR [1,2]**

Natural vanilla extract contains more than 170 volatile aromatic compounds, which are responsible for its aroma and flavour. The major components of the extract are vanillin (which its average content in vanilla pods is around 2%), vanillic acid, vanillyl alcohol, p-hydroxybenzaldehyde, p-hydroxybenzoic acid and p-hydroxybenzylalcohol. Also some non-aromatic components of the extract, such as tannins, poliphenols, resins and aminoacids, are important for the flavour too. In addition, cured beans contain also proteins, sugars, cellulose, organic acids, fixed oil, wax, resin, gum, pigments, minerals, volatile aromatics and essential oils. However, the composition of this mixture depends on various factors: plant species, growing conditions, soil nutrition, harvesting maturity and curing method.

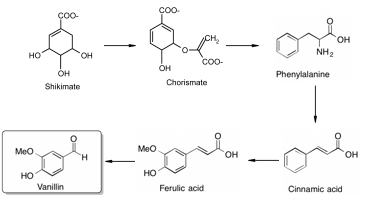


STRUCTURES OF VANILLA FLAVOUR COMPONENTS AND COMPOSITION OF VANILLA EXTRACT

**6. BIOSYNTHESIS [3,4]**

Vanillin is a secondary metabolite produced by some plants. Vanillin biosynthesis is part of Shikimate pathway. Shikimate is converted into Chorismate, a precursor of the amminoacids Phenylalanine and Thyrosine. This aromatic amminoacids can be transformed in various Cinnamic acids, such as Ferulic acid, which is the precursor of vanillin.

Vanillin can be also a precursor of other bioactive compounds, for example capsaicine (spicy peppers), which contains vanillin with a long chain substituent.



SCHEMATIC PATH FOR VANILLIN BIOSYNTHESIS, IMPORTANT INTERMEDIATES

**7. VANILLIN PRODUCTION**

7.1. LEGISLATION AND ECONOMY [2,3,5]

The annual consumption of vanillin is approximately 12000 tons, of which only 20 tons derive from the process of direct extraction from vanilla pods. The remainder of vanillin production arises from chemical industrial synthesis from petrolchemistry.

As seen before, the cultivation of vanilla plants presents various difficulties, such as climate restrictions, manual pollination and long curing methods, and these issues does not allow to cultivate vanilla plants on very large scale.

There are different processes for vanillin production in the lab. Vanillin was firstly synthetically produced in 1874 and it was one of the first flavour compounds available by synthesis.

In the last decades costumers prefer more naturally obtained products and, consequently, synthetic-chemical vanillin is nowadays less appreciated and it doesn’t have a high value.

The EC Flavour Directive (88/388/EEC) defines natural flavours as ‘… flavouring substances or preparations which are obtained by appropriate physical processes or enzymatic or microbiological processes from material of vegetal or animal origin’. This definition is used in US and EU legislations for labelling natural products.

It is noteworthy that with this definition the natural flavours include substances not only made by direct extraction from plants or animals, but also products of biosynthesis. So, natural flavours can be obtained also from natural precursors with biotransformations, using microorganisms or enzymes.

On the contrary, the same product, obtained by chemical synthesis, is called “nature identical” and, today, this type of product is no longer considered consumer friendly by the community. Anyway, from a chemical point of view, even if these molecules are obtained by different processes, they are structurally identical.

This new legislation and classification of natural and non-natural products, produced several changes in the market of flavours and fragrances. In fact, a typical consumer prefers to buy natural substances and consequently spends more for natural products.

The cost of vanillin extracted from vanilla pods lays between 1200 and 4000 US dollars/kg and only less than 1% of the global market is covered by extracted vanillin. Synthetic vanillin represents a cheaper product, costing less than 15 US dollars/kg.

Economics trends and costumer’s preferences have stimulated research for developing new biotechnological processes also in the obtaining of vanillin too and new methods to produce high value vanilla flavour were developed.

7.2. NATURAL PRODUCTION: EXTRACTION [1]

Physical processes to obtain natural flavours are, for example, extraction, distillation, concentration and crystallisation. Some of these processes are used also in the obtaining of the vanillin extract.

As seen before, some difficulties are faced during the process of natural production of this product. Firstly, the cultivation of vanilla plants is possible only in several climatic regions. Secondly, the short blooming period and the impossibility of spontaneous self-pollination implicates a manual pollination conducted in a very limited period (10 hours). Lastly, because green vanilla pods are odourless, vanilla beans should be cured in order to reach the final product.

The curing process is the most important step for developing the typical flavour and aroma of vanilla beans. Green pods contain aromatic components in form of glycosides and during the curing process enzymes such as glycosidases release from the pods the components of the final vanilla flavour. As an example, the glycoside glucovanillin was extracted from green pods for the first time in 1885 and the subsequent acid treatment of this green extract released vanillin. Also other enzymes work on green vanilla pods, like protease, glucosidase, peroxidase, polyphenol oxidase, cellulase and hemicellulase.

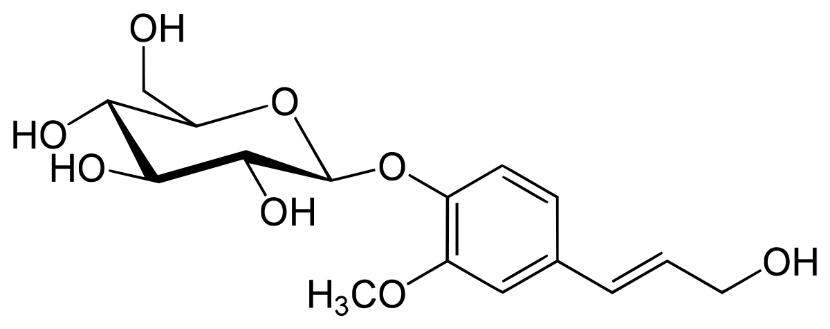
CURED VANILLA PODS

Two curing methods are known: the first is the Mexican process (sun method) and consists in storing the pods under the sun, at an appropriate temperature for 24 hours. This storage is repeated eight times in a period of three weeks and the final step imposes a 3-months aging. The second method is called the Madagascar-Burbon method: the pods are dipped in hot water, then they rest on blankets for 10 days, during which the lead fermentation takes place. This process lasts about 10-20 days.

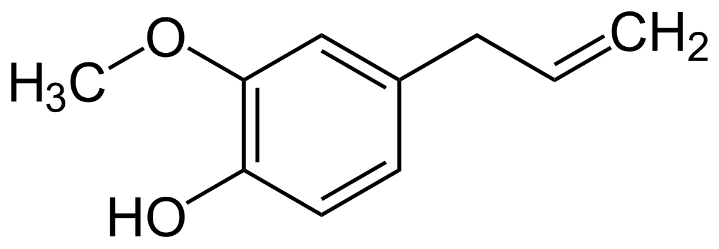
Normally, vanilla pods represent the final product of the natural extraction process and they are sold directly. Even if vanillin extraction from vanilla pods is not economically convenient, vanillin can be extracted using a classical Soxhlet method at 90-100°C with various solvents (ethanol, methanol, hexane).

7.3. CHEMICAL SYNTHESIS OF VANILLIN [1]

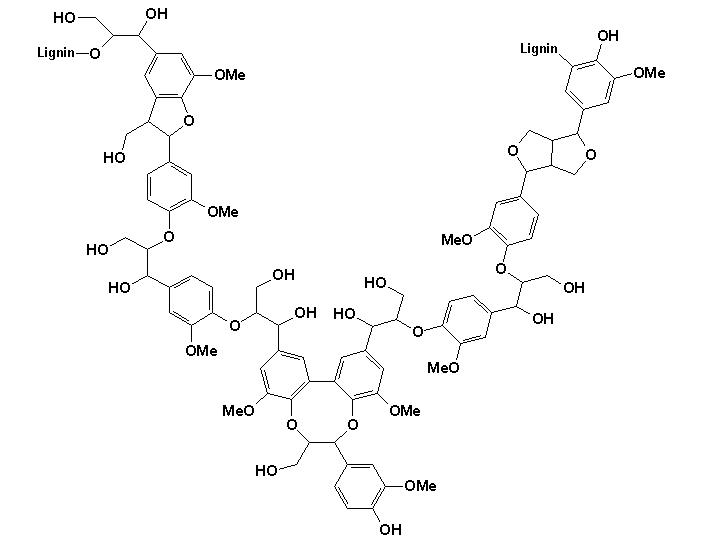
Vanillin can be obtained by several chemical methods using as starting materials coniferin, guaiacol, eugenol and lignin.

The first process was developed by Haarmann and Tiemann in 1874 and consisted in vanillin production from coniferin. Coniferin is the glucoside of coniferyl alcohol and was obtained from felled pine trees. This compound was oxidised in the presence of a mixture of potassium dichromate and sulphuric acid to obtain vanillin. The process is very expensive and is not economically suitable.

STRUCTURE OF CONIFERIN

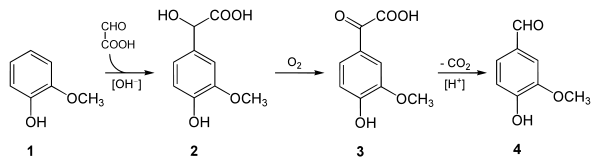
From 1876 Tiemann developed other processes for the production of vanillin, in which the starting molecule was eugenol. All these methods consist in oxidation reactions with different oxidants (osmium tetroxide, vanadium pentoxide, nitrobenzene).

STRUCTURE OF EUGENOL

Another possible starting material is lignin, which is present in sulphite liquor that derives from cellulose industry. The concentrated liquor is treated with a base at high pressure and temperature in presence of oxidants.

STRUCTURE OF LIGNINE

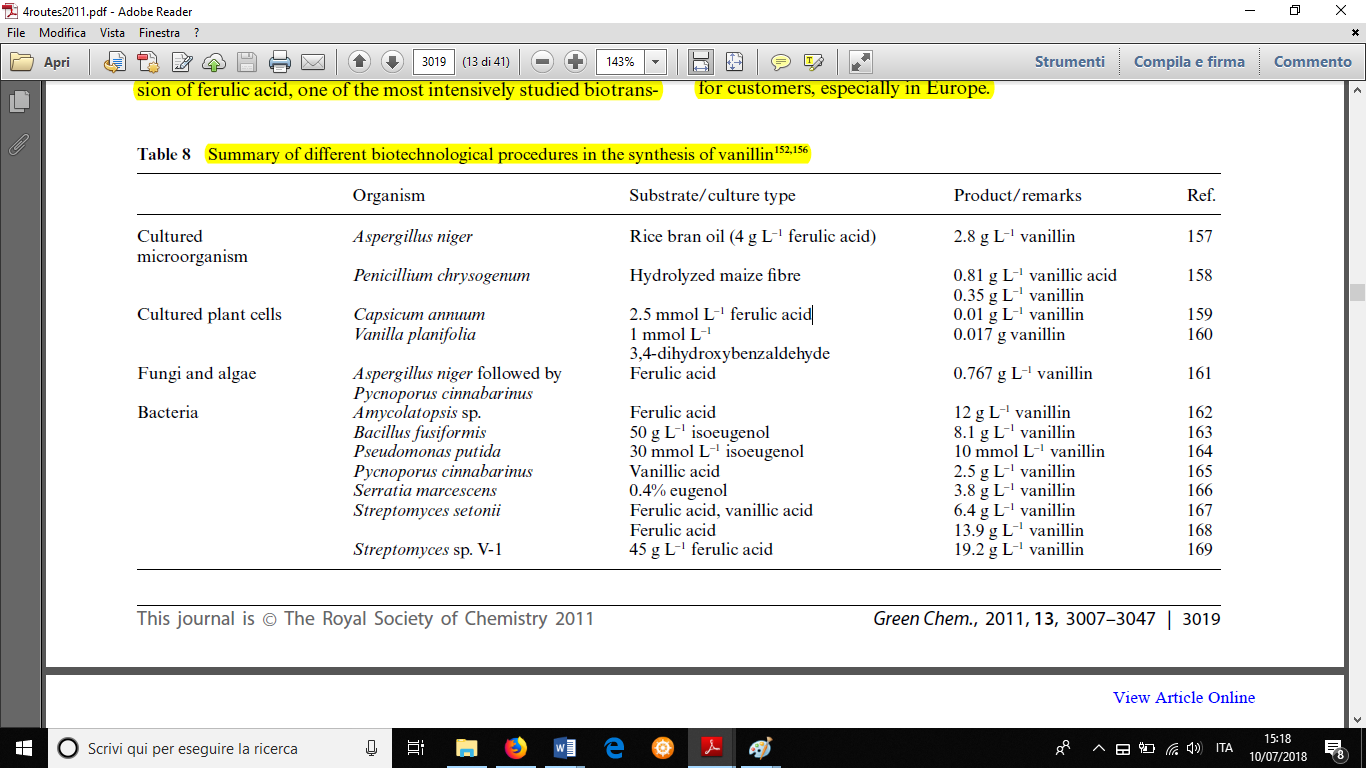
The last method is the introduction of the aldehyde group into the aromatic ring. This reaction is a condensation of guaiacol with glyoxylic acid followed by oxidation of the resulting mandelic acid to the corresponding phenylglyoxylic acid and, finally, decarboxylation. This is a competitive industrial process for vanillin synthesis.



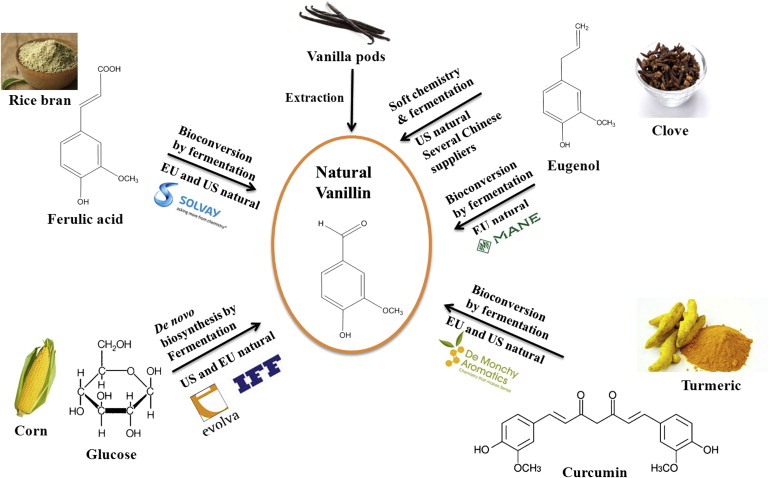
REACTION SCHEME FOR VANILLIN SYNTHESIS FROM GUAIACOL

7.4. NATURAL PRODUCTION: BIOCATALYSIS [3,5,6]

During the past years biocatalytic production of fine chemicals has been expanding rapidly and flavours and fragrances representnowadays a challenging target for both academic and industrial research. Therefore, also for the biocatalysis of vanillin some different techniques were developed.In the table below, some of the diverse types of biocatalysis approaches with the use of different organisms and substrates for the production of natural vanillin are reported.

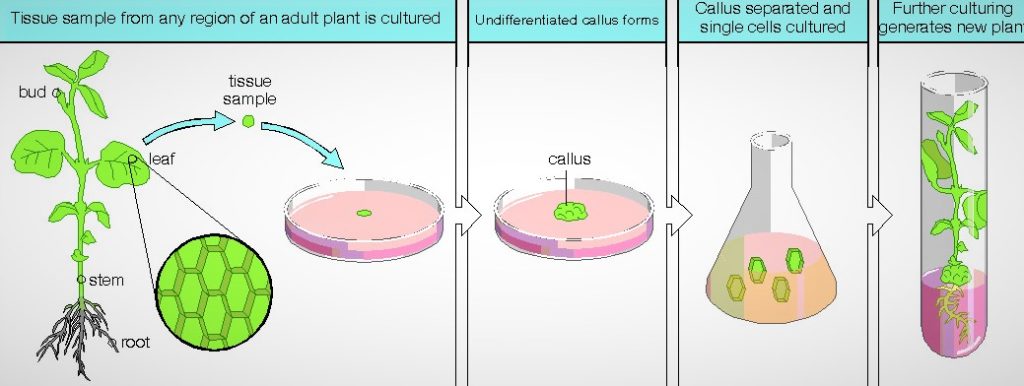


Actually, consumers can find on the market a very little amount of vanillin which is produced through different types of biotranformations. As an example, Rhovanil, which is now produced by Solvay company and represents the first commercially available vanillin, is obtained through fermentation using ferulic acid as precursor. De Monarchy Aromatic group produces vanillin for E-cigarettes with curcumin as precursor. The Sense capture vanillin is the product of bioconversion of eugenol, made by Mane company, whereas Evolva vanilla is made through a De novo synthesis from glucose. [7]



**7.4.1. PLANT TISSUE AND CELL CULTURES [1]**

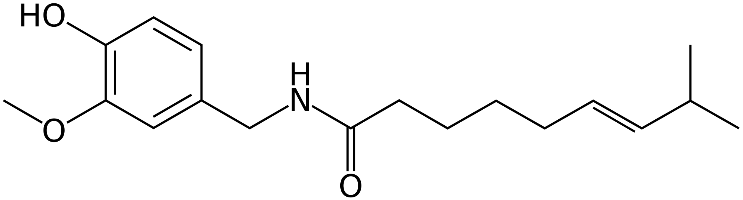
Plant tissue cultures produce flavour and aroma compounds of the plant from which they derive, because they possess the unique biochemical and genetic capacity to produce flavour compounds which are characteristic of that plant. Given that, every cell that composes the vanilla plant contains all the genetic information necessary to produce all the molecules responsible for the vanilla flavour. For this reason, vanilla plant tissue cultures can be used to product vanillin. The production of this molecule can be enhanced by feeding vanilla cell cultures with suitable precursors.



SCHEME FOR PREPARATION OF A TISSUE CULTURE

The bioproduction of vanillin by cell culture makes use of a suspension of *V. planifolia* cells. Feeding the suspension with cinnamic or ferulic acid produces p-hydroxybenzoic acid and vanillic acid. Appropriate conditions of medium can increase vanillin production, but at high concentrations vanillin became toxic. This fact implicates that production of vanillin with cell cultures is still low. Some cultures can secrete flavour components into the culture medium. Possible approaches for increasing the production yield are feeding with precursors, use of hormones, inhibition of competitive pathways, cell immobilization, adjustment of conditions and the use of adsorbents to sequester the product. Nevertheless, none of the methods for producing vanillin from plant cell cultures is commercially suitable.

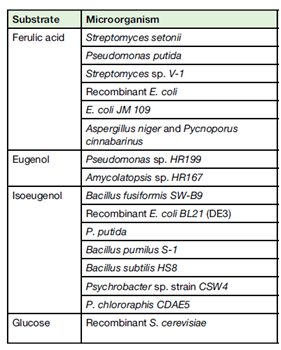
Even cell cultures of other plant species have the potential to produce some of the compounds that are present in cured vanilla pods. Cells of *Capsicum frutescens* (red hot pepper) have successfully been cultured and they accumulate vanillin and associated metabolites. This capacity is probably associated with the metabolism of peppers and its characteristic compound capsaicine, which is formed from vanillin. However, the quantity of produced vanillin by this technique is still too low. [4]



STRUCTURE OF CAPSAICINE

A new possible strategy to produce vanillin is metabolic engineering. It consists in the introduction of an enzyme or a pathway for the vanillin formation. The gene for one of the enzymes that forms bacterial-derived vanillin was isolated. This enzyme converts feruloyl-CoA directly into vanillin, which is thereafter rapidly converted into the glucoside. This gene was then expressed in plants of tobacco and stramonium and is characterized by high enzyme activity. These engineered plants produce the target molecules, but vanillin content in plant tissue is still low.

**7.4.2. BIOCONVERSIONS**

Biotransformations make use of microorganisms and isolated enzymes for producing various substances. One of these, the biocatalysis, represents a useful tool, because catalyses many stereo- and regio-selective chemical manipulations that are not easily achieved by classical synthetic procedures.

Biotechnological processes for natural vanillin production have recently been developed, including the bioconversion of lignin and some phenylpropanoids, such as ferulic acid, eugenol and isoeugenol.

Possible precursors of vanillin and organisms used as biocatalysts are listed in the table beside.

In addition to microorganisms, the use of immobilized enzymes is also studied [8]. Enzymes could be more unstable when used as biocatalysts, but we can reach a good operational stability using enzyme immobilization with proper carriers. Moreover, immobilized enzymes can be easily separated from reaction mixtures and then recycled. Use of immobilized enzymes in vanillin production is less reported than use of microorganisms, probably due to the complexity of enzyme systems, the need of factors and co-factors such as ATP and Coenzyme A which are expensive and non-recyclable.

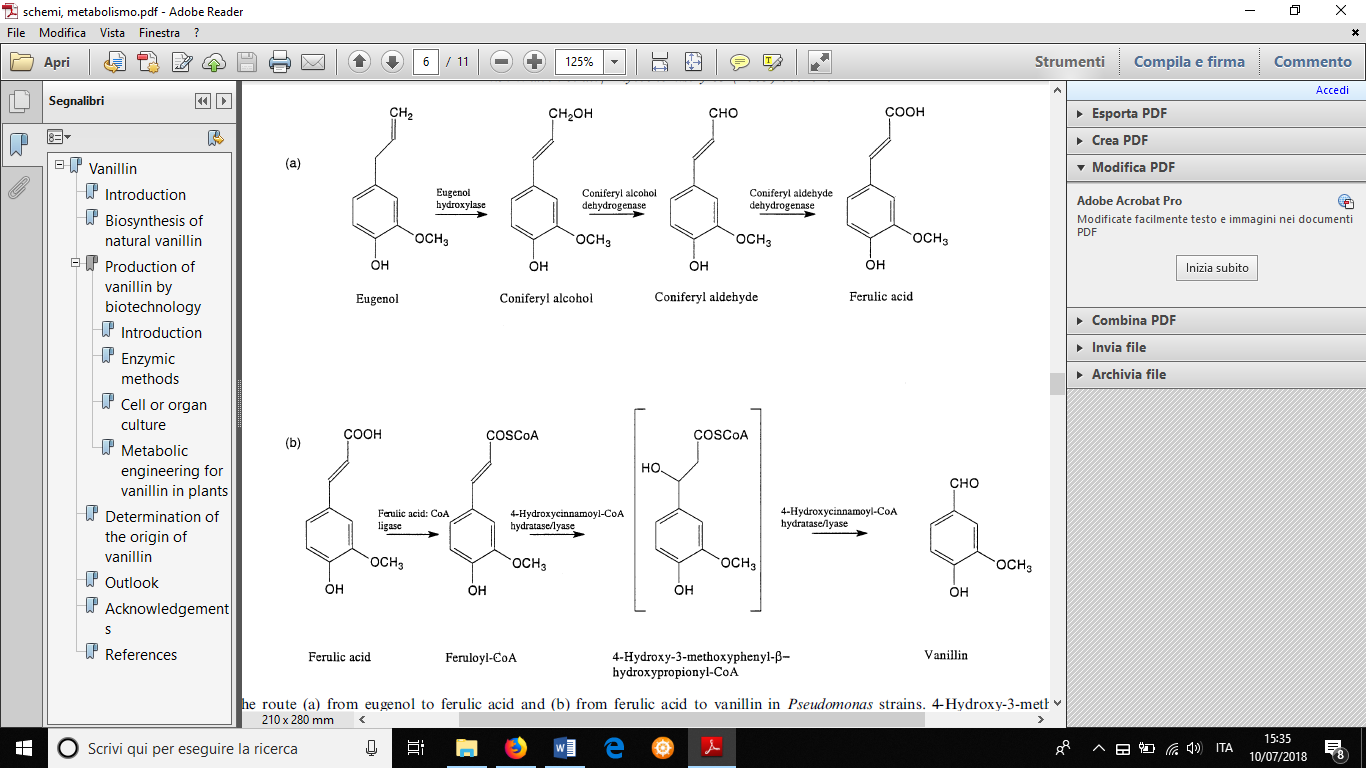
FERULIC ACID AS PRECURSOR [1,2,3,4]

One of the most intensively studied biotransformations to produce nature-identical vanillin is the conversion of ferulic acid. Ferulic acid is a cinnamic acid, present in many plant cells and in plant cell walls as a precursor of lignine. Even if ferulic acid can be easily obtained by alkaline hydrolysis from corn hulls, this does not represent a natural product. Thus, ferulic acid must be obtained via enzymatic conversion to be defined as a natural product, for example from lignine, using ferulic acid esterases and cinnamoyl ester hydrolases.

Also other organisms can metabolise ferulic acid into vanillic acid and vanillin, as for example rot fungi and bacteria.

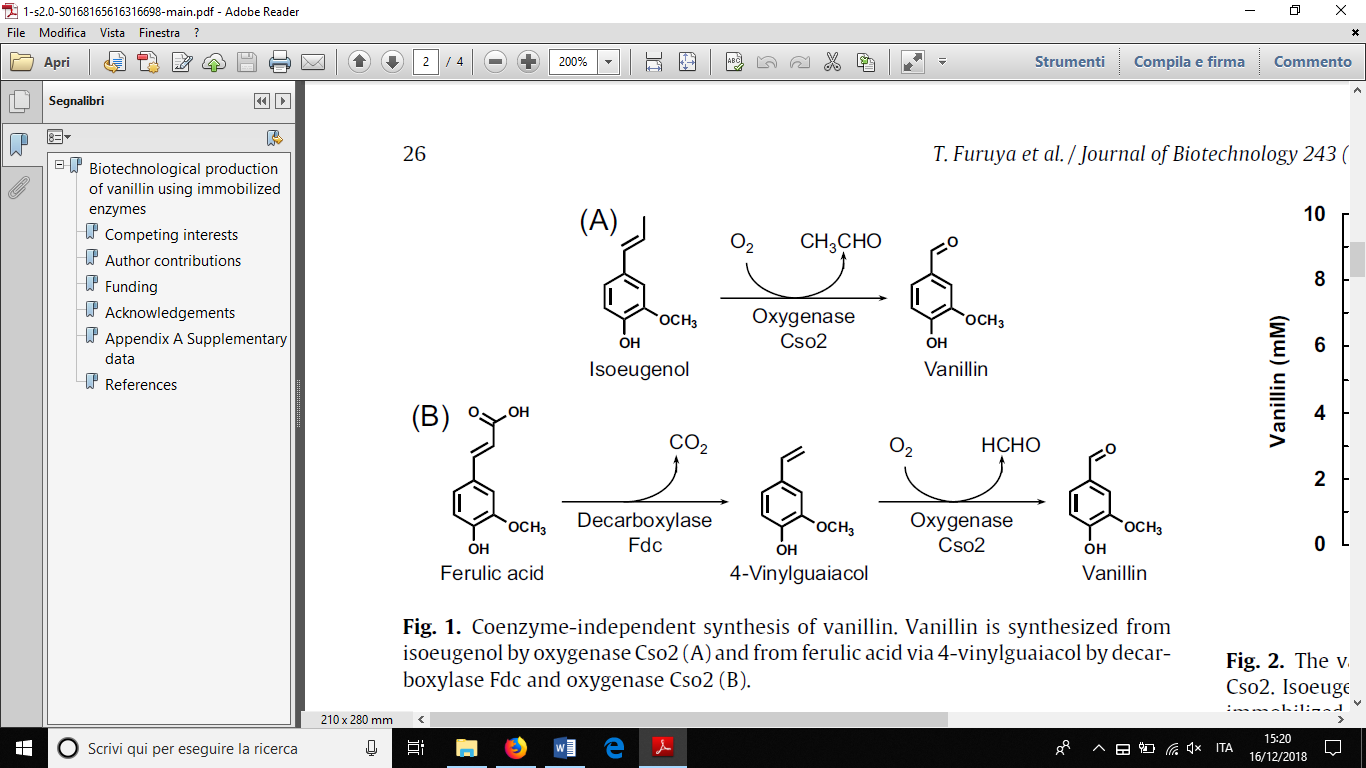
*Aspergillus niger* transforms ferulic acid into vanillic acid and this product can be successively converted into vanillin by some basidiomycetes. This fermentation process was optimized, leading to a yield of 1g/L. As seen before, vanillin becomes toxic at high concentrations, but adsorption on Amberlite resins can reduce the toxicity by entrapping the produced vanillin, leading to a yield increase to 1.5g/L.

Almost the same reaction, by some different steps, can be carried out with *Pseudomonas putida* and *Streptomyces setonii*, bringing the final level of vanillin around 6.5g/L. High yields of vanillin can be achieved also by using bacteria *Amycolatopsis sp.* (up to 12 g/L).



REACTION SCHEME FOR PRODUCTION OF VANILLIN FROM FERULIC ACID WITH MICROORGANISMS AND INVOLVED ENZYMES

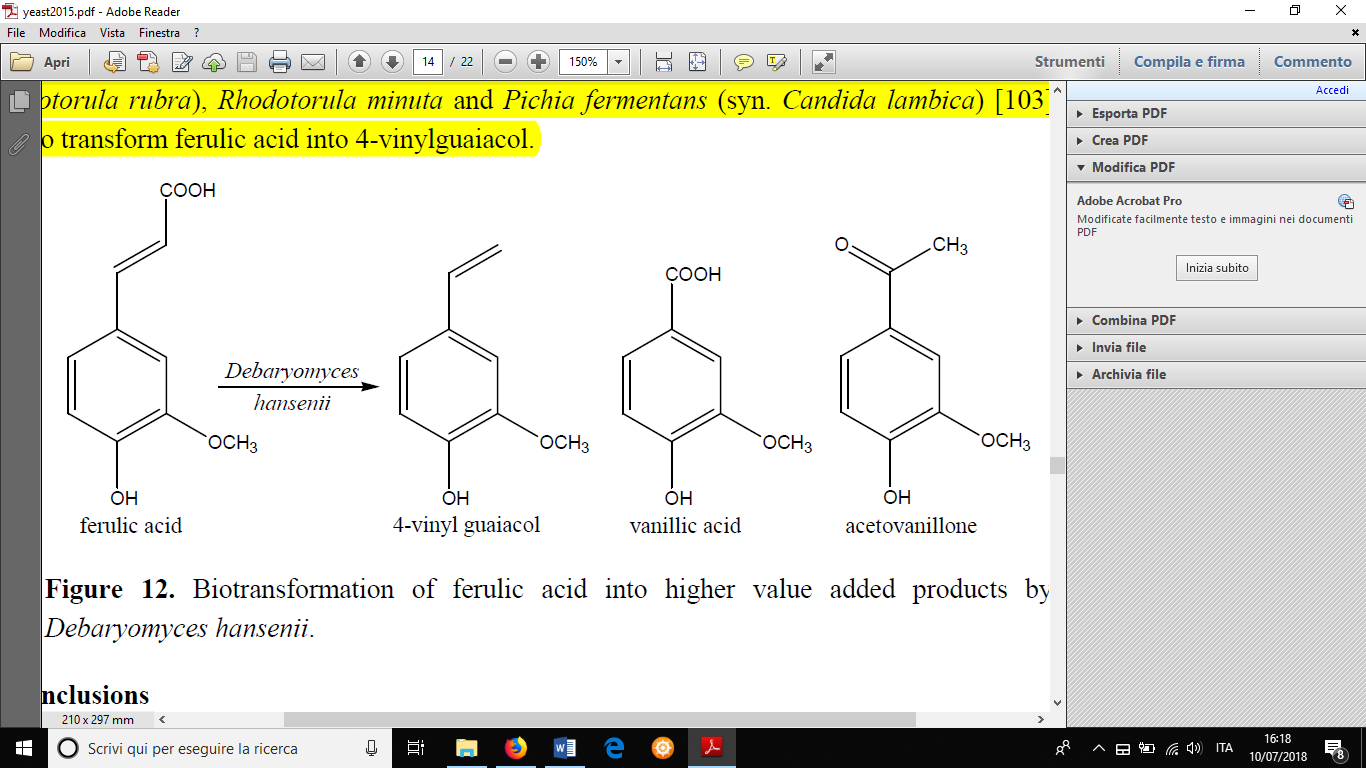
Recently, a new route for vanillin production with immobilized enzymes [8], were developed. This pathway makes use of a coenzyme-independent decarboxylase and a coenzyme-independent oxygenase. Ferulic acid is converted into 4-vinylguaicol and subsequently to vanillin. In the same way, isoeugenol can be converted into vanillin. Enzymes can be immobilized with different types of adsorbent resins-Sepabeads: polymethacrylate, styrene-divinylbenzene polyamines or quaternary alkylamines.



REACTION SCHEME FOR PRODUCTION OF VANILLIN FROM ISOEUGENOL AND FERULIC ACID WITH IMMOBILIZED COENZYME-INDIPENDENT ENZYMES [8]

Non-conventional yeasts (NCYs) are domesticated microorganisms with a biotechnological potential. *Saccharomyces cerevisiae* which is the first and most studied yeast is a conventional yeast. In the last decades other uncommon organisms, called non-conventional yeasts, such as ascomycetous and basidiomycetus were described.

Production of vanillin with NCY *Debaryomyces hansenii* whole cells requires ferulic acid as precursor. The final mixture contains other minor metabolites, including guaiacol, vanillic acid, acetovanillone, vanillyl alcohol, dihydroferulic acid, coniferyl alcohol, dihydroconiferyl alcohol and homovanillic acid. This process produces in 10 hours a product made for 95% of vinyl guaiacol, but containing only 170mg/L of vanillin. For this reason, the described process is not very inexpensive. [10,11]



REACTION SCHEME FOR PRODUCTION OF VANILLA FLAVOUR WITH NCY

*EXAMPLE: procedures for production of vanilla flavour with fungi [9]*

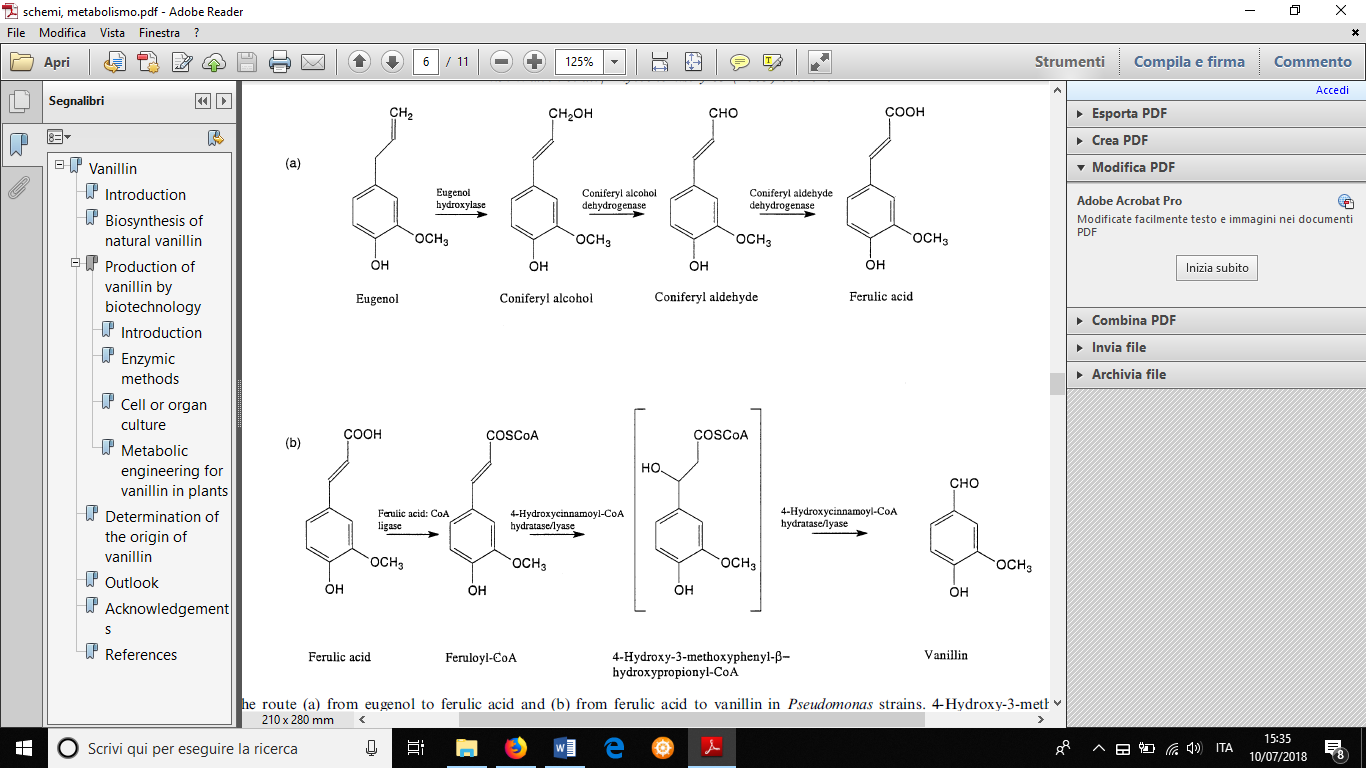
Filamentous phytopatogenic fungi have as principal metabolic pathway the decarboxylation of ferulic acid to produce guaiacol, vanillin and other compounds. All these compounds are economically important for fragrance industry.

In the past, during the procedure to obtain vanilla flavour, mycelia of filamentous phytopatogenic fungi *Colletotrichum acutatum* and *Lasiodiplodia theobromae* were used to be incubated in culture medium and ferulic acid as substrate. The process was carried out at room temperature, under agitation, for 300h. After this period, medium and mycelia were separated by filtration and metabolic products were isolated.

The same process was previously reported to be driven also by other fungi, as *Sclerotum rolfsii, Aspergillus niger, Rhizopus oryzae*.

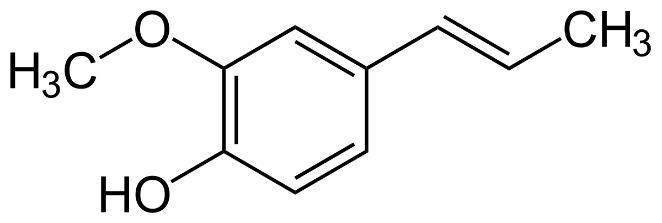
However, this procedure is nowadays unsuitable for industrial scaling.

EUGENOL AS PRECURSOR [1,2,3,4]



REACTION SCHEME FOR PRODUCTION OF FERULIC ACID FROM EUGENOL AND INVOLVED ENZYMES

Eugenol is the main constituent of clove oil and it is a commercially aviable and cheap vanillin precursor.

Bioconversion of eugenol via ferulic acid, ferulaldehyde or coniferylaldehyde by *Arthrobacter*, *Corynebacterium* or *Pseudomonas* bacterial strains produce vanillin and vanillic acid. The bacteria *Serratia marcescens* produces vanillin too, with a 20% yield in the optimized process with eugenol and isoeugenol as substrate. Some bacteria of *Pseudomonas spp.* can grow on eugenol as the sole carbon source and produce vanillic acid, ferulic acid and coniferyl alcohol as metabolic intermediates.

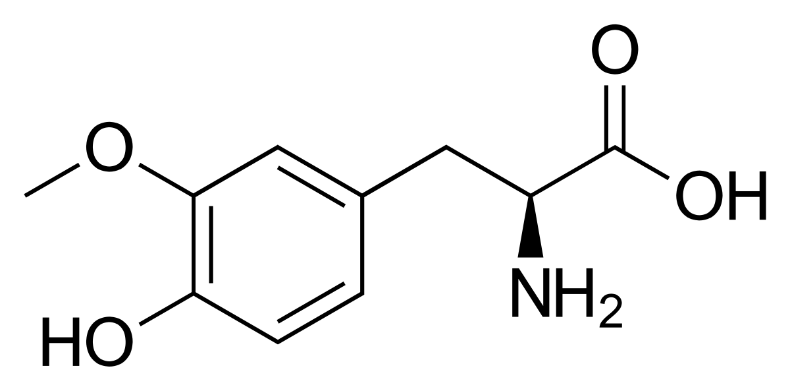
STRUCTURE OF ISOEUGENOL

Also feeding of *Aspergillus niger* yeast cells with isoeugenol leads to a production of vanillin. Part of vanillin is converted to vanillyl alcohol and vanillic acid.

In addition, biotransformation of isoeugenol and eugenol to vanillin and vanillic acid was shown in cultures of the microalga *Spirulina platensis*.

Yields for this type of bioconversion are very low and this approach is still not economically attractive.

AMMINOACIDS AS PRECURSORS [1]

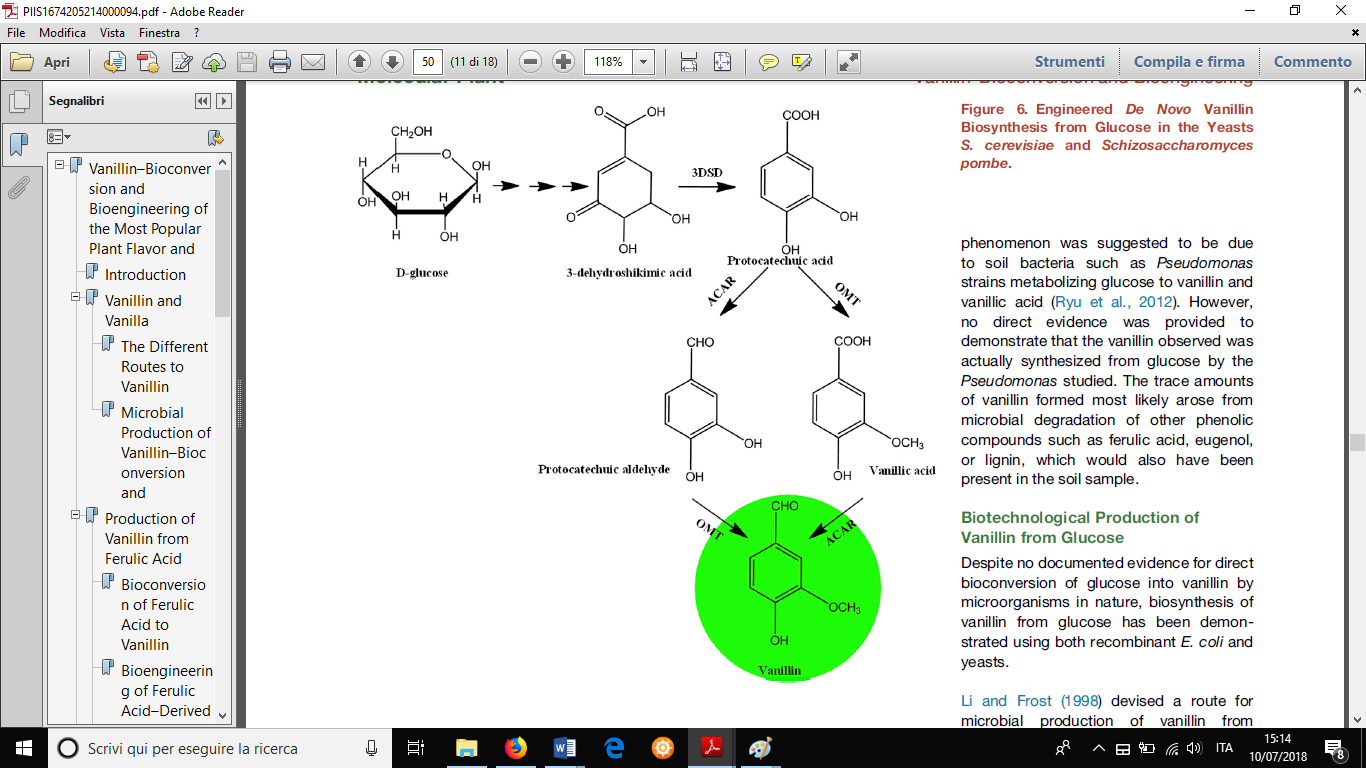
Phenylalanine and methoxytyrosine are possible vanillin precursors.

Methoxytyrosine is deaminated with the enzyme ammonia liase, producing phenylpyruvic acid, which is converted to vanillin with a mild caustic treatment. The yield is low and this method is not applicable in the industrial production of nature-identical vanillin. Metabolic engineering could increase yields, but might be problematic for costumers.

STRUCTURE OF METHOXYTYROSINE

**7.4.3. DE NOVO SYNTHESIS [1,7]**

Recombinant *Escherichia coli* is a biocatalyst for the conversion of glucose to vanillin via fermentation (patent applications). Glucose is converted into vanillic acid with appropriate enzymes. Then, vanillic acid is reduced to vanillin with the isolated enzyme dehydrogenase from *Neurospora crassa*.



REACTION SCHEME FOR DE NOVO SYNTHESIS OF VANILLIN FROM GLUCOSE WITH POSSIBLE INTERMEDIATES

**8. CONCLUSIONS**

Vanillin is a very important research target. Nowadays, production of vanillin is not sufficient to satisfy costumers request. Natural products have higher commercial value and there is a great demand of them. Natural products can be obtained by extraction or biocatalysis with natural precursors as substrate. Biotechnological production of vanillin and vanilla flavour components through biotransformation is an interesting possibility as an alternative to natural vanilla extract or synthetic vanillin.

In the past years different procedures for producing natural vanillin by biocatalysis were reported. Possible precursors are represented by ferulic acid, eugenol and lignine. These processes make use of different microorganisms, such as bacteria, fungi, algae and yeasts and plant tissues or cell cultures. Engineered organisms could be good catalysts too, but they are considered costumer not friendly.

Nowadays only a few companies produce natural vanillin with biocatalysis and this type of vanillin has a very high cost. Optimization of reaction parameters could allow the production of natural vanillin at lower cost. Strengths of biotransformations are mild reaction conditions and consequently lower energy consumption, decreased or eliminated use of solvents and other chemicals.

The selection of appropriate precursors is an important factor. Lignine is a good substrate for vanillin biosynthesis and a waste product of other industrial processes, such as paper production. According to principles of green chemistry and bioeconomy, in which wasting is not allowed, this type of reactions should be the appropriate approach.

Another problem in vanilla production are concentrations of added substrate and released product. Many compounds are inhibitory or even toxic. Fed batch fermentation is a good improvement and this method consists in slow continuous feeding of low precursor levels. Furthermore, released product can be adsorbed and eliminated from reaction medium.

Also, detection, identification and characterization of flavour compounds must be optimized. In addition, the development of new methods for analysing food sophistications is needed. Nowadays is almost impossible to discriminate between synthetic vanillin or natural vanillin, because the molecules are identical; the only difference is the production path.

Different microorganisms are capable of synthesis of flavourings, however yields are low and these processes are economically unattractive. One of the problems that must be solved is that natural biosynthesis is not yet completely understood and not all the involved enzymes and intermediates are known.

The number of patents on biotechnologically based vanilla processes is an indication of the importance of studying alternative production pathways.

It is necessary to decrease the costs by enhancing efficiency, using inexpensive substrates, making downstream processing, product release and recovery easier.

Biocatalysis in production of flavours and fragrances is a powerful tool and there is a considerable variability in the different methods used. New strategies for natural flavour will take advantage and the preference of costumers will economically support their production.

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