

## MICROBIAL PRODUCTION OF BIOVANILLIN

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### ABSTRACT

This review aims at providing an overview on the microbial production of vanillin, a new alternative method for the production of this important flavor of the food industry, which has the potential to become economically competitive in the next future. After a brief description of the applications of vanillin in different industrial sectors and of its physicochemical properties, we described the traditional ways of providing vanillin, specifically extraction and chemical synthesis (mainly oxidation) and compared them with the new biotechnological options, i.e., biotransformations of caffeic acid, veratraldehyde and mainly ferulic acid. In the second part of the review, emphasis has been addressed to the factors most influencing the bioproduction of vanillin, specifically the age of inoculum, pH, temperature, type of co-substrate, as well as the inhibitory effects exerted either by excess substrate or product. The final part of the work summarized the downstream processes and the related unit operations involved in the recovery of vanillin from the bioconversion medium.

**Key words:** vanillin, bioconversion, review, ferulic acid, *Escherichia coli*

### INTRODUCTION

Vanillin is an important flavoring and aromatic component that is mainly obtained from the bean or pod of the tropical orchid *Vanilla planifolia*, and in less extent of *Vanilla tahitiensis* and *Vanilla pompona*, which are commonly denominated as “vanilla”. Vanilla is produced mainly in Indonesia and Madagascar and China, as summarized in Table 1, published by the Food and Agriculture Organization of the United Nations (26).

**Table 1.** Amount of vanilla produced in 2008 (FAO-STAT, 2010).

Country or continent	Production (tons)
Indonesia	3700
Madagascar	2800
China	1400
Mexico	637
Tonga	150
Uganda	70
Asia	5270
Africa	2970
America	645
Oceania	180
European Union	15
World	9080

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However, less than 1% of the worldwide production of vanillin comes from natural vanilla, because its cost is very high (1.200-4.000 \$/kg) with respect to that (<15 \$/kg) of vanillin synthesized by chemical processes (46, 47). The high and variable cost of natural vanillin is due to various reasons, including limited availability of vanilla pods, fluctuations in crop yields associated with the climate, political and economic decisions, intensive cultivation, pollination, harvesting and ripening of pods. Since the use of a natural substrate or catalyst could allow the same product to be classified as natural (65), a biotechnological production of this compound is hoped for (52, 54, 58, 67, 85).

In the nineteenth century the chemical structure of vanillin was identified, and different processes of vanillin production were developed either by oxidation of coniferin (45), which is found in the tissues of various conifers, or from eugenol. This constituent of clove oil is currently the source of artificial vanillin, which is obtained from the sulfite spent liquor from paper mills (35, 73) and coal tar extract (79).

In 1997, the H&F Florasynth began the production of a vanillin identical to the natural one through a process that consists of two steps, one that leads to ferulic acid and another that turns it into vanillin. Recent research has identified the precursors and almost all enzymes involved in the metabolic pathway for the synthesis of vanillin in vanilla fruit, which could permit the production of synthetic vanilla just by the same way as plants do (53).

Currently, artificial vanilla production is performed at an industrial scale by the controlled oxidation of lignin from wood waste, which results in fatty product subsequently purified by distillation and crystallization.

### APPLICATIONS OF VANILLIN

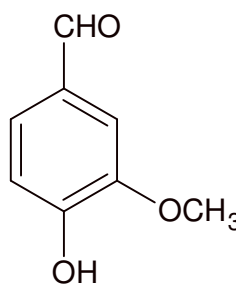
Along with aromas of citrus and mint, those of vanilla are the most important in the food industry, the world production of vanilla pods being at present more than 9,000 tons, and rising.

Vanillin is widely used as flavoring in sweet foods like cookies, muffins, cakes, ice creams and soft drinks. Because of its antimicrobial and antioxidant properties (14, 23), vanillin could be used as food preservative, but this potential use is hampered by its strong smell in the inhibitory concentrations required (28). It can also be used as a precursor in the manufacture of other flavorings. For example, it can be synthesized the zingiberon (main ginger flavoring agent) by condensation reactions, as well as the main component of jasmine oil (*trans*-jasmonate and (+)-*cis*-jasmonate) by reaction with heptaldehyde in basic medium and alcohol. It also has non-food uses, such as the implementation of fragrances or pharmaceutical preparations, where it is used as a flavoring agent or as a precursor of drugs among which L-Dopa, Aldomet and dopamine (81, 82).

Synthetic vanillin is used as an intermediate in the production of herbicides, antifoaming agents (35), home products, deodorants, air fresheners and cleaning products such as floor polishes.

### PHYSICOCHEMICAL PROPERTIES

Vanillin is a white solid with a strong smell of vanilla, whose formula is C<sub>8</sub>H<sub>8</sub>O<sub>3</sub>. It is an aromatic aldehyde (3-methoxy-4-hydroxybenzaldehyde), belonging to the group of simple phenolic compounds (Figure 1). Structurally, its functional groups include aldehyde, ether, and phenol, and their physico-chemical properties are summarized in Table 2.



**Figure 1.** Chemical structure of vanillin.

**Table 2.** Physico-chemical properties of vanillin.

Property	Value
Solubility in water at 20°C (g/L)	10
Molecular weight (g/mol)	152.15
Melting point (°C)	80/83
Boiling point (°C)	~ 285

## PRODUCTION TECHNOLOGIES

Slightly less than 1% of world production of vanillin comes from natural vanilla; the rest is synthesized by chemical processes, making it cheaper.

### Extraction by Leaching

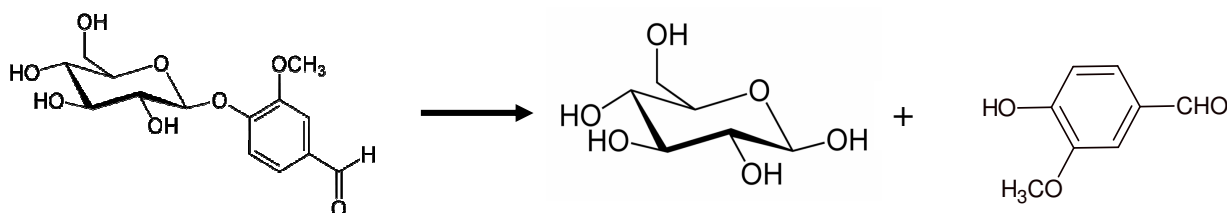
The processing of vanilla to produce vanillin begins with the curing of freshly harvested pods. The aim of the curing process is to stop the natural vegetative process and to accelerate changes responsible for the formation of aromatic flavor constituents. The curing method differs from one production area to the other, and this can have a major influence on variation in quality and aromatic profile of pods that are traded.

Although there are several ways of curing, all share four main phases:

- *Wilt:* It avoids post-harvest vegetative growth and promotes the enzymatic reactions responsible for the production of aroma and flavor. It is recognized by the appearance of brown spots on the pods.
- *Sweating:* In this phase the temperature is raised to promote the enzymatic reactions and rapid initial drying so as to prevent harmful fermentation.
- *Drying:* This process is performed at room temperature until the pods reach a third of their initial weight.
- *Packaging:* The pods are stored in closed boxes for a period of three months, which may stretch up to reaching the aroma and flavor desired.

Vanillin, which is linked to glycosidic molecules in the green pods, separates from them during the ripening process due to drying and heat; the vanillin  $\beta$ -D-glucoside is enzymatically hydrolyzed to give glucose and vanillin (Figure 2).

The production of vanillin is very laborious and expensive, so alternative routes are sought. Recently, Jadhav *et al.* (37), in their attempt to optimize vanillin extraction from cured vanilla pods, reported that the use of ultrasonic irradiation resulted in significant intensification of vanillin extraction in Soxhlet using ethanol as the solvent, being able to release no less than 140 ppm vanillin when the extraction time was reduced from 8 to 1 h and the temperature from 95 °C to room temperature at almost the same solvent-to-solute proportion (66.7 ml/g).

**Figure 2.** Separation of vanillin during ripening.

### Chemical Synthesis

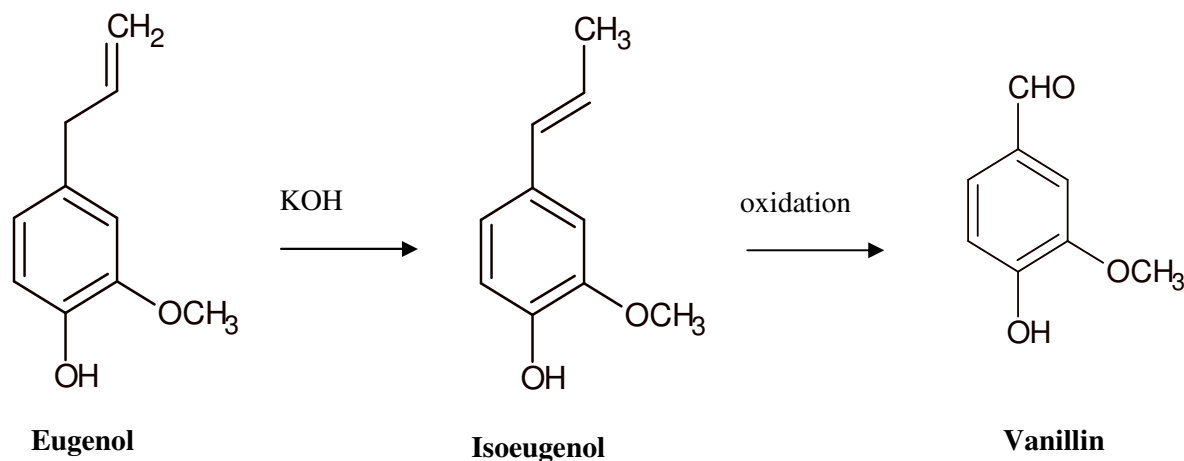
Between 1874-1875, it was developed a process of synthetic vanillin production from eugenol (Figure 3) that was used until the end of 1920.

The detection of a slight vanillin odor in a liquor made from sulfite pulping, which was subsequently confirmed by performing a pyrolysis of the dry residue of this liquor (29), suggested that vanillin could be produced from lignin-rich wastes. Later studies tried to establish its production from lignin-rich dry pulp (34, 36), but preliminary treatments were found to be too laborious.

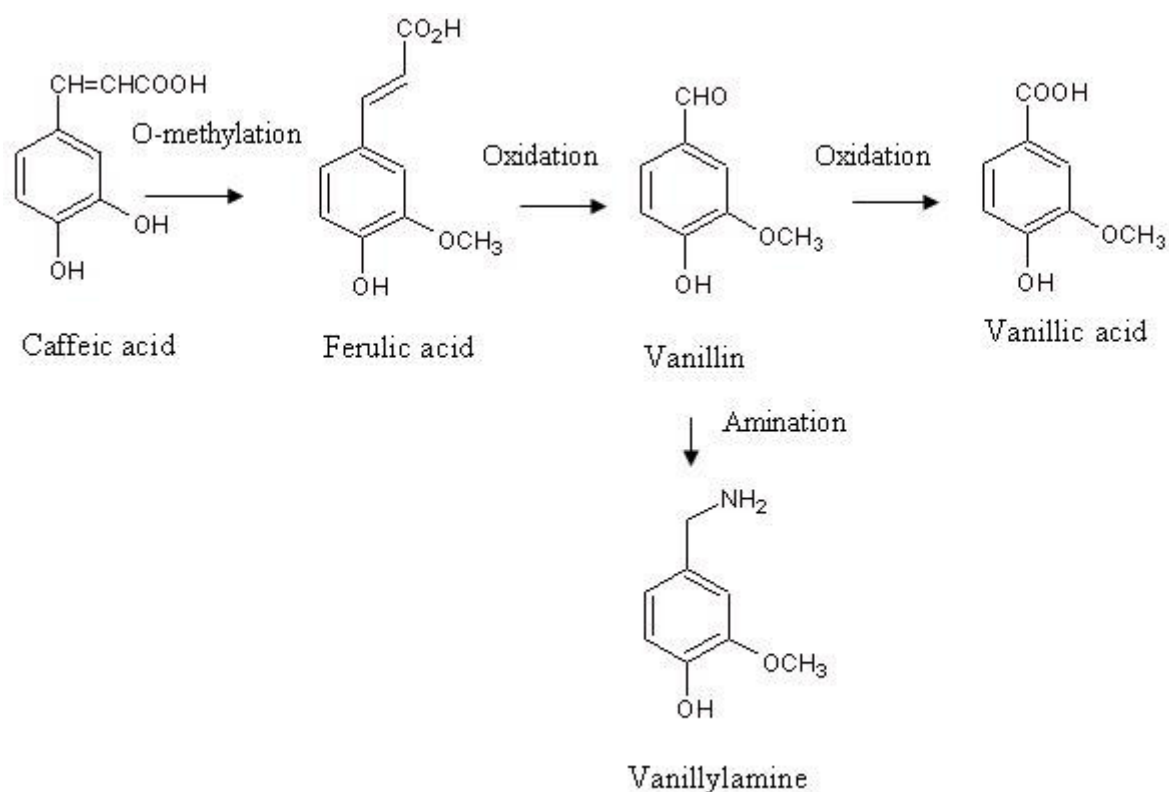
Finally, it was demonstrated that vanillin recovered from the sulfite spent liquor comes from guaiacyl units of lignin solubilized by alkaline oxidation during softwoods pulping. Its commercial production started from an aqueous solution of the liquor containing sodium vanillate, which was purified via carbonyl sulfate by addition of  $\text{H}_2\text{SO}_3$ .

### Biotransformation of Caffeic Acid and Veratraldehyde

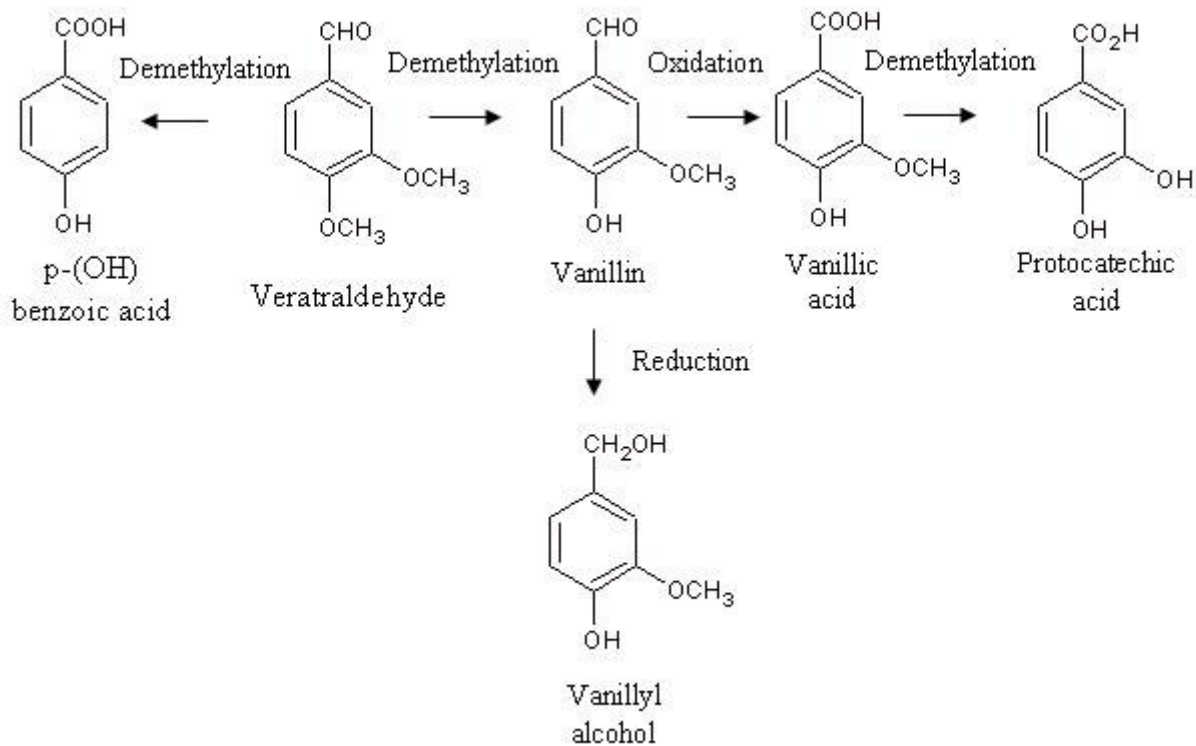
The biotransformation of caffeic acid and veratraldehyde is also reported in the literature as a possible alternative way to produce vanillin. To this purpose, the roots of *Capsicum frutescens* resulted to be an efficient source of caffeic acid (70). This biotransformation, which is illustrated in Figure 4, includes a first step of vanillin formation from caffeic acid and a subsequent oxidation of vanillin into vanillic acid. The biosynthetic pathway implying veratraldehyde as a starting carbon source is shown in Figure 5. In this case the substrate is demethylated enzymatically to form vanillin and *p*-hydroxybenzoic acid, the former being oxidated into vanillic acid successively (70). In both cases, the research efforts to get an effective accumulation of vanillin should be addressed to the way of hindering or suppressing the final vanillin-to-vanillic acid oxidation step.



**Figure 3.** Production of vanillin via eugenol.



**Figure 4.** Biosynthetic pathways of vanillin, vanillyl alcohol and vanillic acid production from caffeic acid.



**Figure 5.** Biosynthetic pathways of vanillin, vanillyl alcohol and vanillic acid production from veratraldehyde.

## Fermentation

The big difference between the prices of natural and synthetic vanillin, the increased demand for “natural” and “healthy” flavors have stimulated a great interest of the flavorings industry to produce natural vanillin by bioconversion from other natural sources. This common orientation in the production of flavors and fragrances was analyzed extensively (15, 32, 33, 42, 61).

To make the process economically viable, it is necessary to find a precursor chemically close to vanillin, cheap and easily available. Ferulic acid, a widely-known natural phenolic compound from lignin degradation by fungi (40) and bacteria (7), was deeply studied as vanillin precursor (61). As highlighted by Di Gioia *et al.* (25), the enzymatic hydrolysis is an especially interesting option to dissolve lignin by cleavage of the ester bonds present in lignin-polysaccharide complexes (27, 68, 83). Free ferulic acid can be obtained from common agricultural residues, grains and beet pulp through a combination of physical and enzymatic treatments (84).

Vaithanomsat and Apiwatanapiwat (78) obtained by steam explosion a *Jatropha curcas* stem hydrolysate containing 1.55 g/L of ferulic acid, which was successfully used as substrate for one-step vanillin production by *Aspergillus niger* and *Pycnoporus cinnabarinus*, while Torre *et al.* (74) optimized the release of ferulic acid (1.17 g/L) from corn cobs using NaOH hydrolysis.

Several bacteria belonging to different genera are able to metabolize ferulic acid as sole carbon source leading to the production of vanillin, vanillic acid and protocatechuic acid as catabolic intermediates (31, 71), the most interesting of which being *Pseudomonas fluorescens* (1, 4, 49).

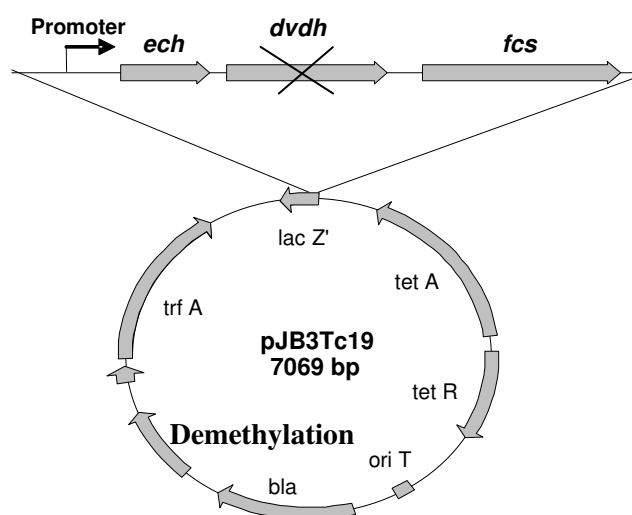
Interesting concentrations of vanillin were obtained from ferulic acid using the Gram-positive bacteria *Amycolatopsis* sp. (56) and *Streptomyces setonii* (30, 48). However, since the process optimization is very difficult, the development of new recombinant strains able to produce vanillin is quite attractive.

Recent studies have confirmed that the combination of genes of the feruloyl-CoA synthase and feruloyl-CoA hydratase from *Pseudomonas fluorescens* BF13 unable

different strains of *Escherichia coli* to produce vanillin from ferulic acid-rich substrates (3, 20, 50, 85). For this purpose, a recombinant plasmid (pBB1) was constructed which is made up of 5,000 bp (8). The donor fragment contains a mutation of the vanillin dehydrogenase (VDH) that prevents vanillin-to-vanillic acid oxidation, thus promoting vanillin accumulation during the process of ferulic acid bioconversion (Figure 6).

An earlier study, conducted with several strains of *E. coli* (*E. coli* DH5 $\alpha$ , *E. coli* JM109, *E. coli* JM Promega, *E. coli* Novablu, *E. coli* SureII, *E. coli* XL10 Gold), allowed selecting the second one as the best producer, being able to ensure the most interesting combination of vanillin yield and production kinetics as well as product stability (20). Nevertheless, this transformant showed in Luria-Bertani (LB) medium a duplication time more than twice (24) that of the wild type (0.5 h) under optimal growth conditions in glucose (13, 63).

The biotechnological process for vanillin production from various agro-products has been investigated using different microorganisms as biocatalysts. *Aspergillus niger* I-1472 and *Pycnoporus cinnabarinus* MUCL39533 were used in a two-step bioconversion using sugar beet pulp (11), maize bran (44), bran oil rice (88), and wheat bran (72) as raw materials.



**Figure 6.** Construction of plasmids pBB1 containing genes from *Pseudomonas fluorescens* BF13 to produce vanillin from ferulic acid using the vector pJB3Tc19. The plasmid was subsequently transformed into *E. coli* JM109.

## FACTORS INFLUENCING THE MICROBIAL PRODUCTION OF VANILLIN

### Age of Inoculum

To maximize the bioconversion yield, the microorganism has to be adapted to the substrate as much as possible (60); to achieve this result, the inoculum must be done once the microorganism has completed its exponential growth phase and before entering the stationary one [6 h in the case of *E. coli* JM109(pBB1)] (76). Previous studies have shown that biomass of this strain under non-proliferating conditions (*resting cells*) can be recycled up to 4 times without significant loss of bioconversion capacity, and used in a continuous process after immobilization in synthetic spongy material (76).

### Acidity

As is well known, the acidity level is a critical parameter in cell cultures since they can only grow in a narrow range of pH, outside of which the bioconversion yield decreases. The study of the effect of the acidity level on cultures carried out either in presence of H<sub>2</sub>O<sub>2</sub> at different concentrations or not revealed that decreasing values of pH below 7.4 accelerated the decay *E. coli* population (3, 57).

### Temperature

All microorganisms have an optimum temperature for growth. *E. coli* can be classified as a mesophilic microorganism, i.e., its optimum temperature range is between 20 and 40°C. Previous studies performed with *E. coli* JM109 (pBB1) cells demonstrated that the optimum growth temperature for this strain is 37°C. On the other hand, once the microorganism was grown and inoculated, the optimum temperature for carrying out the subsequent ferulic acid-to-vanillin bioconversion was 30°C (24, 77).

### Co-substrate

As detailed in the previous chapter, the production of vanillin occurs through a secondary pathway other than glycolysis, which does not imply any ATP formation. So, the

cell needs a carbon source for catabolism, i.e., an energy source that enables its long term-viability in view of possible application in a continuous system. A deep investigation on the components that make up the culture media is, therefore, very important. Rabenhorst (55) demonstrated the importance of yeast extract (YE), casein hydrolyzate and aeration to address the metabolism of eugenol in some isolates of *Pseudomonas* sp. toward the desired aromas (vanillic acid, ferulic acid and coniferyl alcohol).

Similarly, Torre *et al.* (75) investigated the effect of different co-substrates (YE, tryptone or LB medium, which contains both ingredients) on the ferulic acid-to-vanillin bioconversion by *E. coli* JM109 (pBB1), trying to avoid or minimize the formation of other by-products. YE was shown to be a satisfactory stimulating agent, being able to provide, after only 3 h of bioconversion, a promising vanillin concentration (84 mg/L), which was 27% higher than that obtained with tryptone and only 3.6% lower than with the more expensive LB medium.

### Substrate Inhibition

The concentration of ferulic acid may influence the production of vanillin, both in terms of productivity and yield. A previous study showed that a progressive increase in ferulic acid concentration reduces the specific productivity of vanillin in a way similar to that observed for excess substrate inhibition of alcoholic fermentation (16, 21).

### Product inhibition

When the accumulation of the end product of a biochemical pathway stops the synthesis of the desired product, is said to occur product inhibition. The production of vanillin by microorganisms is associated to the formation of a number of compounds such as vanillic acid and vanillyl alcohol, which can act, together with vanillin, as potential inhibitors of the bioconversion, decreasing the amount of ferulic acid addressed to the production of the desired product (51). It is anyhow noteworthy the fact that, having both vanillyl alcohol and vanillic acid practically the same smell as

vanillin, a biotechnological production of a mixture of these three components could equally be of great interest for the flavor industry (19).

### Dissolved Oxygen Concentration and Agitation

The growth rate of biomass is influenced by agitation and by the coefficient of O<sub>2</sub> transfer. Torre *et al.* (75), to highlight the effect of the former variable on growth, inoculated *E. coli* JM109 (pBB1) in 250 mL Erlenmeyer flasks simultaneously varying the ratio of volumes of the medium and the flasks from 0.2 to 0.5 and the agitation in the range 100-200 rpm. The best conditions for the bioconversion resulted to be a ratio of 0.5 and an agitation of 100 rpm. Excess increase in aeration did in fact become deleterious for the bioconversion, probably because the activity of non-specific oxidases oxidized a significant portion of vanillin to vanillic acid, thereby reducing the product yield (75).

### Pre-cultivation of the Microorganism

The use of only one step of pre-cultivation can significantly reduce the bioconversion time, likely because of earlier adaptation to bioconversion conditions, thus reducing the overall costs of its potential industrial application. Rivas Torres *et al.* (60) investigated the possibility of omitting the second step of pre-cultivation on the bioconversion of ferulic acid to vanillin using *E. coli* JM109(pBB1). The results of their experiments highlighted that, when biomass from the first stage of growth in LB medium was directly employed in the bioconversion medium, vanillin volumetric and specific productivities increased from 1.7 to 3.6 mg/Lh and from 3.4 to 7.1 mg/g<sub>DM</sub>h, respectively using 0.5 g<sub>DM</sub>/L of biomass.

## VANILLIN RECOVERY

Vanillin, like several other aromatic aldehydes, is commonly considered toxic to microorganisms (51); therefore, it is difficult to get high volumetric yields of this compound. It is therefore worth not only selecting strains able to tolerate high vanillin concentrations, but also finding out bioconversion ways to remove the formed aldehyde from the reaction system. For this reason and because of the low

product concentrations in the bioconversion medium, also in the case of vanillin the major drawback that limits possible real-scale applications is, from both the technical and economic viewpoints, the operation at intermediate substrate concentration.

Since the international legislation about food products requires the exclusive use of physical separation operations for the recovery of natural flavors, vanillin isolation and purification can be performed by extraction, vacuum distillation and multi-stage re-crystallization from aqueous solutions and ultrafiltration (10, 12).

The crystallization is, perhaps, the most widespread technique utilized for the recovery of bioproducts from fermentation or bioconversion media; however, in the case of vanillin, it will become competitive only when it will be possible to obtain the product with much higher yields with respect to those reported in the literature (54), the only exceptions being the high concentrations of vanillin (10 g/l) obtained with *Amycolatopsis* sp. (56) and *Streptomyces setonii* (48). The minimum concentration of vanillin to allow for its effective crystallization is in fact considered to be just 10 g/l at 20°C (17), which strongly influences the recovery process.

The recovery of biomolecules by two-phase systems utilizing an aqueous phase and an organic phase is promising (18, 59, 62, 64), even if the selection of new microorganisms with greater resistance to organic solvents (2) seems to be necessary when this technique is employed for the continuous removal of the product. Interesting two-phase extraction processes, employing either sulphhydryl compounds, such as dithiothreitol, dithioerythritol, glutathione, or L-cysteine (43), or alkaline KOH solutions (5), are reported in the literature for the recovery of vanillin as well as for its separation from other aromatic substances coming from the hydrolysis of lignocellulosics, among which ferulic acid.

The pervaporation, as a technique for the recovery and/or purification of volatile solutes on hydrophobic membranes and desorption into a vapor phase, has been described for numerous bioproducts (6, 80). In particular, its use for vanillin recovery from aqueous solutions is suggested by the excellent



permeability of some types of membranes toward aromatic hydrocarbons (9). Promising results have been obtained in this field by vanillin adsorption and pervaporation from bioconverted media using polyether-polyamide copolymer membranes (10, 89).

The selective adsorption of a given product can enhance the production by avoiding further undesired reactions (83). For example, the use of polystyrene resins allowed minimizing the reduction of vanillin to vanillyl alcohol in *Phanerochaete chrysosporium* cultures (69). Recently, Zhang *et al.* (87) reported the use of macroporous adsorption resins with crosslinked-polystyrene framework to recover dissolved vanillin from aqueous solutions. The highest adsorption capacity was 416 mg/g (vanillin/resin), and the recovery efficiency was more than 95% when using 3–5 bed volumes of absolute ethanol that is an acceptable solvent in food grade application. The resin was used repeatedly by simple regeneration, and its adsorption capacity kept almost unchanged. Alternatively, Knuth and Sahai (41) described a process for the continuous removal of vanillin using activated charcoal as an adsorbent, but the cost of this material is notoriously too high for industrial purposes.

Membrane processes have all the requirements needed to act as key separation units in biorefineries due to their excellent fractionation capability, low chemical consumption and low energy requisites (38). The ultrafiltration technique has been evaluated to recover vanillin from Kraft lignin oxidation (86). Membrane technologies allow separating molecules that normally do not permeate the membrane and are collected in the “retentate” fraction from those that permeate the membrane and are collected in the “permeate” fraction. Retention of the concentrated molecules strongly depends on the size of membrane pores, though shape and chemical nature of molecules (physico-chemical interactions) often play an important role as well, especially in those processes, like nanofiltration and reverse osmosis, involving molecules of compounds with very low molecular weight. Recently, Sciubba *et al.* (66) evaluated the potential of using membrane contactor (MC) technology for the recovery of vanillin from

bioconversion broths in view of an integrated bioconversion separation process.

A filtration device usually consists of a system with plate or modular membrane; in both cases, the permeate flux is radial with respect to the flux so as to reduce the membrane fouling, with consequent reduction of the filtration effectiveness (18). The most typical parameters involved in a membrane process are the permeate flowrate, which defines the filtering performance of the membrane, the working pressure, which is strictly correlated with the filtration rate, and the recirculating flowrate of the waste (39).

Cost analysis of this kind of processes is basically depending on the working pressure and on the average membrane life. Lastly, the concentration yield indicates the degree of concentration of the treated waste. In fact, among all the technologies that can be applied to this purpose, the use of membrane separation systems comes out to be very interesting and attractive thanks to the effectiveness and low costs involved (22). This would represent a simple way both to recover vanillin and to recirculate the residual substrate. Coupling the extraction to the biocatalytic system is expected to allow operating continuously so as to achieve higher bioconversion productivity.

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