The Role of Microorganisms in Vanilla Curing[a]

Part 2: Microbial transformation of phenols and other compounds

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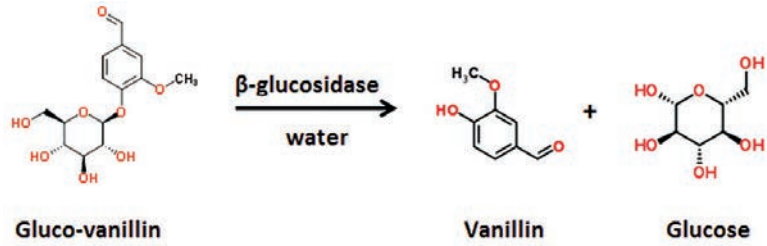
Vanilla bean curing is a complex and lengthy process that transforms an essentially flavorless green/yellow vanilla bean into the flavorful, chocolate brown, microbiologically stable commercial flavoring mainstay. From start to finish, the traditional curing process takes from four to six months to complete.[1]

Complex biochemical and chemical reactions occur during the different stages of curing. It's generally accepted that much of the sweating, or fermentation, stage involves the action of endogenous vanilla bean enzymes; among the more important of which are the β-glucosidases and oxidoreductases; the latter exemplified by peroxidase.[2]

All of the curing stages in the traditional process are protracted and variable due to weather dependence and may present opportunities for microbial participation in flavor generation and transformation.

Part one of this review discussed the stages during curing in which microorganisms could participate and the microorganisms identified in vanilla beans and contact materials during curing.[3] This review will address the potential role of microorganisms in the transformation of phenols and other vanilla flavor compounds.

β-Glucosidase hydrolysis of glucovanillin **F-1**



Evidence for Microbial Involvement in Flavor Formation and Transformation in Real or Simulated Curing Situations

The liberation of free phenols during the curing of vanilla beans involves the hydrolytic enzymic transformation of the corresponding glucosides (**F-1**).

A Japanese patent described the incubation of intact green vanilla pods or their pulverized equivalents with specific microorganisms comprising bacteria (the genus *Bacillus*), molds (the genus *Aspergillus*) or yeast (the genus *Saccharomyces*) for the conversion of vanilla bean precursors to the appropriate aroma material. This method was reported to reduce cost and time for the manufacture of vanillin.[4]

Two recent patents by Givaudan have provided additional information on the potential role of microorganisms during vanilla curing. An enzymatic process exposed ripe green vanilla beans to high drying temperatures (80–90°C), to reduce moisture content to <10% by weight. This was followed by extraction with a solvent and then treatment with β-glucosidase to generate vanillin from the glucovanillin precursor present in the bean as well as providing a well-balanced vanilla aroma without off notes.[5]

This patent indicated that the first drying stage contributed to reducing microorganisms that appear to be involved in vanillin degradation. Among the offending bacteria reported was *B. subtilis*.

A second patent from the same company used a fermentation process during which green, ripe, vanilla beans or previously dehydrated pods were incubated with *B. subtilis* to generate vanillin from glucovanillin in higher yield than traditional routes—as well as other related

[a] The first installment of this article, "The Role of Microorganisms in Vanilla Curing—Part 1: evidence for microbial involvement," appeared on page 24 of the May 2012 issue of *Perfumer & Flavorist* magazine; *www.perfumerflavorist.com/magazine/pastissues/*

phenols from their corresponding glucosides—while generating a fully developed vanilla aroma.[6] Off notes formation was reported to be limited by incubation with the microorganism.

The above findings offer routes to higher vanillin yield probably by microbial hydrolysis of glucovanillin and other related glucosylated phenols and apparently less off-flavor formation. However, these studied do not clarify the contribution of microorganisms to flavor generation during traditional vanilla curing despite providing a well-balanced vanilla aroma without off notes. Depending on conditions, the presence of *B. subtilis* appears to have both positive and negative effects on vanillin and aroma generation in fresh ripe or dehydrated vanilla beans.

Although there are opportunities for positive flavor formation during curing, contamination by bacteria and molds is also possible. Control of these processes are essential to avoid quality loss in the final product. As previously indicated, the drying phase of curing needs to be critically controlled to avoid bacterial fermentation which can lead to "creosoted vanilla" character. Such risks can be minimized by management of moisture content in combination with adequate aeration especially during the fermentation, sunning and drying phases of curing.[7]

Consideration of other plant fermentation processes may offer some insights into the microbial contribution to vanilla curing. Cocoa bean fermentation exhibits some parallels to vanilla curing.[b] Cocoa beans are embedded in a pulp which is sterile until the pods are broken. When damage is inflicted, they rapidly become contaminated with a variety of microorganisms from workers' hands, insects and vessels used for transport, which results in the onset of fermentation.

This process triggers a spectrum of chemical changes within the bean that are vital to the development of the final complex chocolate flavor. When the pods are broken open and the beans are scooped out, yeasts rapidly multiply in the sweet, fruity pulp by glycolytic transformation of glucose to pyruvate then to ethanol under anaerobic conditions. The yeast population peaks within 24 hours. Following this first phase, bacterial fermentation begins converting the formed ethanol into mainly acetic acid which then slowly penetrates the cocoa bean.

The latter bacterial phase of fermentation takes place under aerobic conditions. Beans are typically turned at least twice during this stage of the process in order to introduce oxygen and to ensure even aerobic fermentation. The bacteria population peaks roughly 72 hours after fermentation first begins and decreases rapidly over the following 72 hours; the entire fermentation process typically takes about six days.

Other than producing ethanol and acetic acid, the fermentation process also generates heat, which typically raises the temperature of the fermenting beans to about 45–50°C. The acid and heat generated by the

[b] *www.worldcocoafoundation.org/who-we-are/partnership-meetings/pdfs/MGilmour_Fermentation.pdf*

fermentation causes cell wall disruption, which facilitates enzyme contact with substrates. These enzymes bring about important chemical changes within the bean including acetic acid retention, transformation of flavonoids and formation of aroma flavor precursors. After the fermentation process, the cocoa beans are dried, to reduce the moisture content in the bean from around 55% to 7%, which promotes product stability.

The fermentation process in cocoa beans exhibits parallels to that of vanilla curing but clearly in the former, there are two stages in the process (an initial anaerobic and a second aerobic fermentation). In vanilla curing, on the other hand, the principal fermentation stage appears to be predominantly an aerobic process.

Microbial Transformation of Phenols

As indicated above, phenol glucosides in the ripe vanilla bean are important flavour precursors while the corresponding free phenols are key contributors to cured vanilla bean flavor.

A wide range of low molecular mass aromatic compounds are degraded by microorganisms. Aerobic metabolism is characterized by the extensive utilization of molecular oxygen. Monoxygenases and dioxygenases are essential for the hydroxylation and cleavage of aromatic ring structures. Typically, the central intermediates of the aerobic pathways (like catechol) are readily attacked oxidatively, which leads to ring opening. Anaerobic aromatic catabolism, on the other hand, requires a different strategy utilizing bacteria that degrade soluble aromatic substrates to carbon dioxide in the absence of molecular oxygen. Different central intermediates, such as benzoyl-CoA, are used in an anaerobic environment. Under these conditions, the aromatic ring is dearomatized by reduction rather than opened by oxidation.[8]

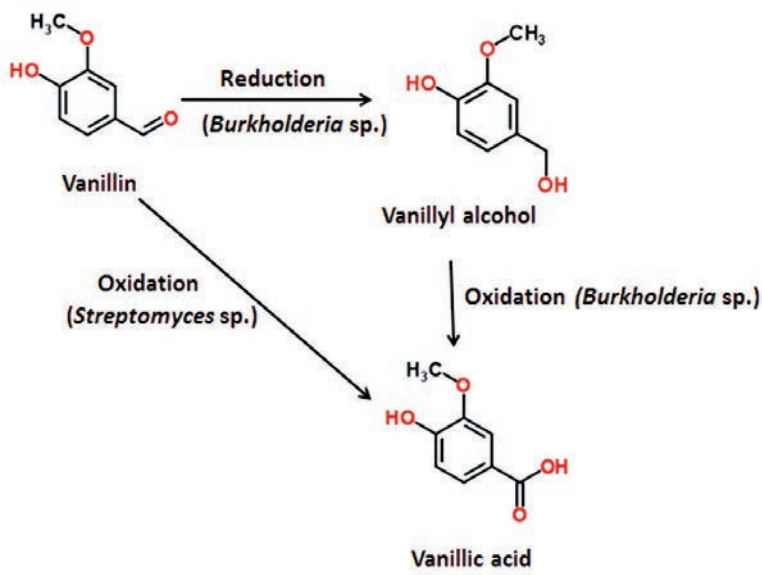
Bacteria, in addition to totally degrading phenols, are known to metabolize phenols to a number of different end products. Harazono et al. isolated aromatics degrading bacteria from the gut of a lower termite, *Coptotermes formosanus*.[9] Two species, *Burkholderia* sp. strain VE22 and *Citrobacter* sp. strain VA53 were isolated by aerobic enrichment culture with carbon sources veratraldehyde (3,4-dimethoxybenzaldehyde) or vanillin.

Strain VE22 was cultivated in a medium containing veratraldehyde as the carbon source and metabolized this compound to a mixture of veratryl alcohol and 3,4-dimethoxybenzoic acid.

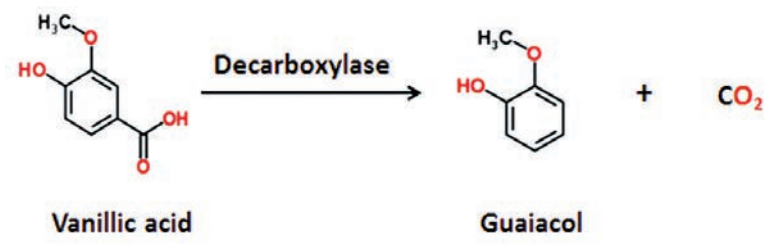
Strain VA53 was cultivated in a medium containing vanillin as the carbon source. Vanillin was metabolized during three days of incubation. Vanillyl alcohol was an intermediate detected in days one and two. The resting cell of the same strain incubated aerobically for three days at 30°C degraded all of the vanillin within three hours. Vanillyl alcohol maximized to ca. 50% mol/mol of the initial vanillin concentration after one hour, then declined. All of the vanillin was transformed to vanillic acid after five hours. Strain VA53 could also grow anaerobically transforming vanillin to vanillyl alcohol and vanillic acid, the mol/mol yield based on initial vanillin being 65% and 15% respectively. This anaerobic process took seven days at 30°C. These findings support a pathway from vanillin through vanillyl alcohol to vanillic acid in strains of VE22 and VA53, rather than direct oxidation of the aldehyde to the carboxylic acid.

Aromatic aldehyde oxidation directly to the corresponding carboxylic acid has been demonstrated for *Streptomyces* sp.[10] and *Pseudomonas* sp.[11] probably occurs directly via the aldehyde dehydrogenase (**F-2**).[10–11]

Microbial transformation of vanillin[9,10] **F-2**



Non-oxidative conversion of vanillic acid to guaiacol by *B. megaterium*[12] **F-3**



Guaiacol, as a product of microbial transformation of phenols, is well-established.

Several strains of *Bacillus megaterium* and a strain of *Streptomyces* were shown to convert vanillic acid to guaiacol (*o*-methoxyphenol) and carbon dioxide by a non-oxidative decarboxylation reaction (**F-3**).[12]

It revealed that numerous soils contain countable numbers (101 to 102 organisms per gram of dry soil) of aerobic spore formers, which are able to convert vanillic acid to guaiacol.

In a similar manner, *Debaryomyces hansenii*, an isolated yeast strain, metabolized ferulic acid to 4-vinyl-guaiacol, also by the nonoxidative decarboxylation of the carboxyl group on the three-carbon side chain (**F-4**). *Debaryomyces* produced 1470 mg l^{-1} of 4-vinylguaiacol at the 10th hour of incubation, corresponding to a molar yield of 95% while the production of vanillin reached a maximum of 169 mg l^{-1} at the fifth hour.[13]

The formation of volatile compounds during *B. subtilis* fermentation (for 18 hours and 36 hours at 35°C) of cooked and roasted soya bean cotyledons was investigated.[14] Compounds formed in the largest amounts during fermentation were 3-hydroxy-2-butanone (acetoin); 2,5-dimethylpyrazine; and trimethylpyrazine. Compounds present at concentrations exceeding their odor threshold values included nonanal; decanal; 1-octen-3-ol; butandione; 3-octanone; 2,5-dimethylpyrazine; 3,6-dimethyl-2-ethylpyrazine; 2-pentylfuran; dimethyl sulfide; benzaldehyde; and 2-methoxyphenol (guaiacol).

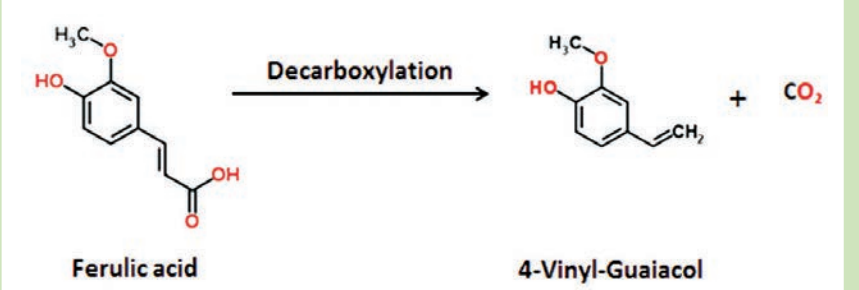
Bacillus pumilus PS213 isolated from bovine ruminal fluid was able to transform ferulic acid and *p*-coumaric acid to 4-vinylguaiacol and 4-vinylphenol, respectively, similarly by a nonoxidative decarboxylation route.[15] The enzyme responsible for this activity has been purified and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crude extract from a culture induced by ferulic acid or *p*-coumaric acid. The enzyme, molecular mass 45 kD, was absent from crude extracts of non-induced cultures. Enzyme activity was optimal at 37°C and pH 5.5 and was not enhanced by cations.

Resting cells of *Rhodotorula rubra* converted *trans*-ferulic acid to vanillic acid, then to guaiacol and protocatechuic acid, under aerobic conditions (**F-5**).[16]

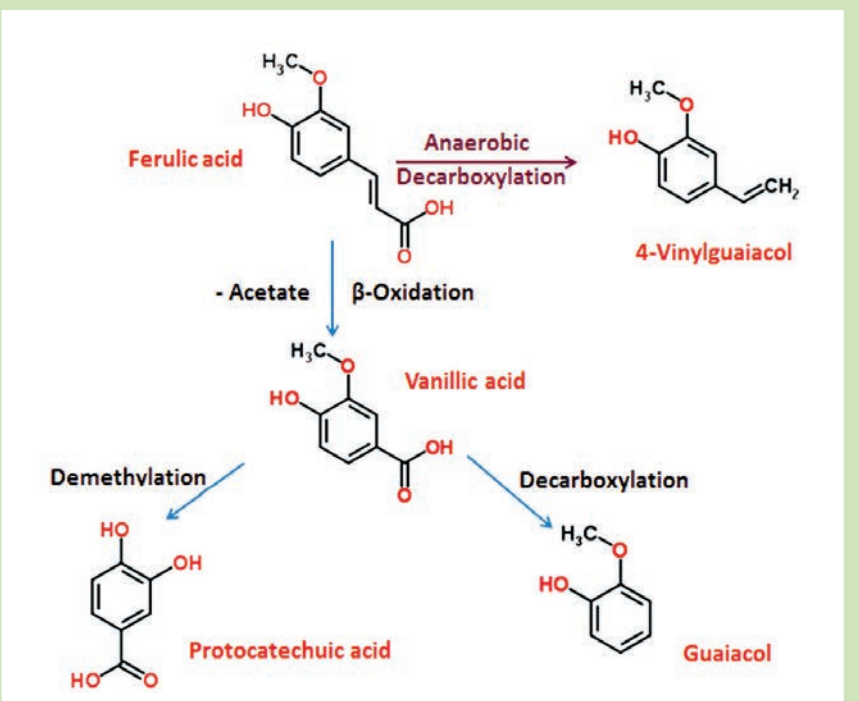
In an argon atmosphere, *R. rubra* transformed ferulic acid via β-oxidation to vanillic acid and also by direct decarboxylation to 4-hydroxy-3-methoxystyrene (4-vinylguaiacol). The biotransformation of ferulic acid to vanillic acid by *R. rubra* cell-free extracts required CoA, ATP, and NAD^+. Oxygen was incorporated from water during the conversion of ferulic to vanillic acid. These results suggest a parallel between this bioconversion and the β-oxidation pathway of fatty acids.[17] Additional deuterium studies implicated a quinoid vanillic acid tautomer as an intermediate in the decarboxylation reaction of vanillic acid to guaiacol.

The very high levels of vanillin in vanilla beans can provide a source of vanillic acid by oxidation of the aromatic aldehyde to a carboxyl group, which is a reaction catalyzed by plants, bacteria and yeasts. An additional bacterial transformation of phenols is the removal of a carboxyl group directly attached to the phenol ring, or as part of a propenoic acid residue also substituted on the phenolic ring structure as found in vanillic acid or ferulic and *p*-coumaric acids, respectively.

Nonoxidative decarboxylation of ferulic acid by *Debaryomyces hansenii*[13] **F-4**



Aerobic transformations of ferulic acid by *Rhodotorula rubra*[16] **F-5**



This could account for the elevated levels of guaiacol in traditionally cured vanilla beans. The prevalence of *Bacilli* in the traditional curing environment supports this proposition.

Other aromatic acids can also undergo microbial transformation. The degradation of tyrosine in pig feces and stored piggery wastes was determined using radioactively labeled compounds to characterize the intermediates in this anaerobic process.[18]

In feces, *p*-cresol and 3-phenylpropanoic acid were the end products of tyrosine metabolism. Meanwhile, phenol, *p*-cresol and minor quantities of phenyl propanoic acid were formed in anaerobically stored, mixed wastes.

These compounds were considered to contribute to the odor of pig feces. A scheme was proposed for the degradation of tyrosine in pig feces and in mixed wastes (**F-6**).

The pathway from tyrosine (I) to *p*-cresol (VIII) involves a classical transamination followed by two decarboxylation reactions. In addition, tyrosine was degraded to phenol (IX) by removal of the amino group and the three-carbon side chain. This latter reaction could occur from tyrosine via the transamination described above to form 4-hydroxy-3-phenylpyruvate (II). Further conversion to 4-hydroxy-3-phenyl-lactate (III) then 4-hydroxycinnamic acid (IV) is possible. The cinnamic acid derivative could undergo loss of acetate by β-oxidation to form p-hydroxybenzoic acid, which could form phenol by decarboxylation (this appears to be an important pathway in anaerobically stored pig wastes). The principal pathway in pig feces was via the same initial transamination step to 4-hydroxy-3-phenylpyruvate. This compound was then sequentially reduced to the corresponding secondary alcohol, dehydrated between carbons two and three of the side chain, followed by saturation of this 2-3 double bond. The final step leading to 3-phenylpropanoic acid (VI) required removal of the phenolic hydroxyl group.

Microbial Transformation of Non-phenols

Bacillus polymyxa produces (R,R)-2,3-butanediol from a variety of carbohydrates.[19]

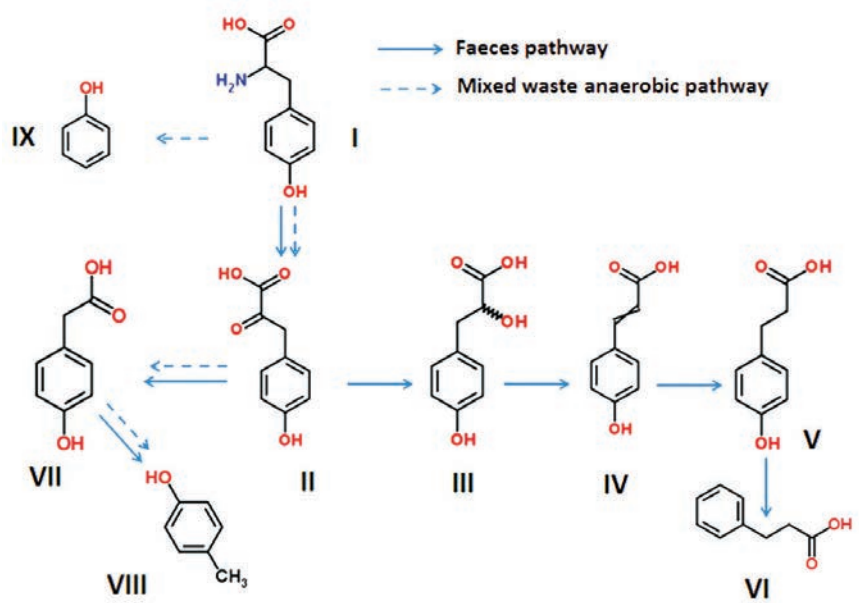
Other metabolites are also produced including acetoin, acetate, lactate and ethanol. The excretion of each metabolite was found to depend on the relative availability of oxygen in the culture. When the relative oxygen uptake rate was high, enhanced yields of acetate and acetoin were noted. At intermediate oxygen availability, the butanediol yield was maximal. When the availability of oxygen was more restricted, higher yields of lactate and ethanol occurred. The cells appeared to regulate themselves so that energy generation was optimal subject to the constraint that the cells didn't produce more reducing equivalents than could be oxidized by the bacterial electron transport chain.

The significant levels of acetic acid in vanilla beans could reflect the presence of the *Acetobacter* genus. Acetic acid bacteria are applied on a large scale for the industrial production of this organic acid as exemplified by the classical vinegar process, in which acetic acid is generated via the incomplete oxidation of ethanol by *Acetobacter* species.[20, 21]

Ethanol oxidation occurs via two membrane-associated dehydrogenases—namely alcohol dehydrogenase and aldehyde dehydrogenase. The typical acetic acid producers can use very few compounds as carbon sources for growth. Most of these strains only grow on ethanol, acetate, and in some cases, lactate as a carbon source.

Acetic and its derivatives play a central role in both carbohydrate and fatty acid metabolism in both plants and microorganisms and may therefore arise from both sources.

Proposed pathways for the degradation of tyrosine in pig feces and anaerobically stored, mixed wastes **F-6**



I. Tyrosine; II. 4-hydroxy-3-phenyl-pyruvate; III. 4-hydroxy-3-phenyl-lactate; IV. 4-hydroxycinnamic acid; V. 4-hydroxy-3-phenylpropanoic acid; VI. 3-phenylpropanoic acid; VII. 4-hydroxy-2-phenylacetic acid; VIII. *p*-cresol; IX. phenol [18]

Summary

The case for microbial participation in vanilla curing remains an open question. However, a microbial role is supported in several ways:

- The presence of a number of key aroma compounds in cured vanilla beans that could arise by microbial as well as plant metabolic pathways
- Many of the microbial transformations of phenols

described in the literature give tacit support for this position

Further experimentation needs to be conducted under simulated vanilla curing conditions in sterile environments, under normal curing conditions and in the foregoing situations with the addition of selected bacteria as well as vanilla bean-associated microorganisms.

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