The Role of Microorganisms in Vanilla Curing

Part 1: evidence for microbial involvement

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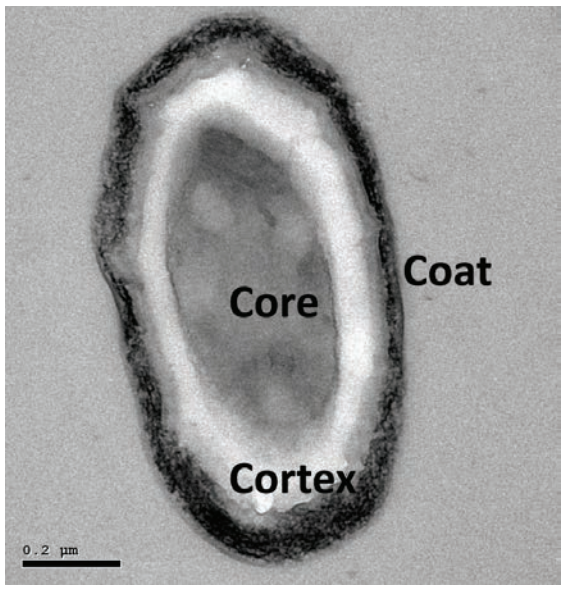
Vanilla is the number one tonality in the world because of its subtle, but complex, flavor. It is known that microorganisms are present during the different stages of vanilla curing, and it is conjectured that they may play a role in this process as well as in flavor generation and loss.[1] However, not much consistent information is available, probably due to the following:

- About 70% of the world supply of vanilla beans are grown and cured in Madagascar. There are more than 60,000 small, subsistent farmers and a significant number of them cure their own green vanilla beans in remote areas where for all practical purposes clean water and sanitation do not exist. The bacterial populations of these green vanilla beans and the curing equipment employed vary from one farmhouse to the next. As a result, it is not possible to anticipate or expect identical microbial profiles.
- Vanilla curing for the most part is based more on tradition than science, so there are no established curing procedures or sanitary standards except in a few locations where curing is conducted under more controlled operating regimens.[2]
- Only limited experimental information is available, testing the role of selected or mixed bacterial cultures on flavor formation and change during real or simulated curing operations.
- Collecting samples for microbial analysis is very challenging due to the previously mentioned 60,000 vanilla farmers in Madagascar. Most of them blanch, ferment, sun dry and sell their semicured vanilla beans to collectors or exporters. So there are thousands of vanilla farmers curing their vanilla beans in very remote villages. It is nearly impossible to achieve any standards of harvest and curing as farmers make their own schedules depending on their individual needs. Collective aseptic sampling and storage conditions are very difficult to achieve, and if not conducted correctly can result in microbial contamination.
- In-depth knowledge and techniques are essential to isolate and identify the microorganisms. To conduct such an operation effectively it is necessary to set up a university or a research laboratory where there is microbial expertise which not only has the required techniques but also the commitment to carry out relevant research activities rather than merely provide a microbiological service.



Bacillus subtilis *spore; by permission of Derek Atkins, electron microscopist Unilever*

The key questions to be considered in relationship to the microbial participation in curing are:

- What is the evidence for microbial participation in this process?
- Do microorganisms play a part in flavor generation and loss during the curing operation? If so, what function do they perform?

To try to answer this question a number of areas need to be explored. These include:

- The stages during curing in which microorganisms may participate
- What microorganisms have been identified in mature vanilla beans and contact materials during curing
- What evidence is there for microbial involvement in flavor formation and transformation in real or simulated curing situations
- Whether microorganisms participate in the transformation of phenols and other vanilla flavor compounds (This question will be addressed in part two of this review, which will follow in a later issue of this publication.)

Close inspection of the traditional curing process highlights a number of opportunities for microbial proliferation.

Stages at Which Microorganisms Could Participate in the Curing Process

Close inspection of the traditional curing process highlights a number of opportunities for microbial proliferation.

Green vanilla beans are an agricultural crop that comes into contact with human hands, untreated water, harvesting tools, storage containers, wild and domestic birds and animals, and soil. Vanilla beans are harvested in the fields by hand with equipment that is not clean. Due to the small quantitites collected at any one time, vanilla farmers collect and store beans for a day or two before transporting them to a farmhouse or a curing station. It is common practice among farmers to leave the freshly picked green vanilla beans uncovered on the bare earth, grass, or a piece of dirty plastic or cloth.Transporting vanilla beans from remote areas to a curing station or a farmhouse is a difficult task and typically carried out with no hygenic consideration.These farming standards mean the green vanilla beans are prone to contamination by microorganisms (**F-1**).

Blanching is often carried out with untreated water and little control of the blanch temperature and time. Good hygiene is not always practiced subsequent to the blanching stage, especially with the extensive handling of the beans by workers (**F-2**).

During the sweating or fermentation stage, blankets are used and reused, without washing, to wrap the beans for maintainance of elevated temperatures during sweating (**F-3**).

Sunning proceeds in the same way, with unclean blankets contacting the beans (**F-4**). During air exposure at the sunning stage, the beans are spread on blankets, often in contact with the ground as opposed to a more hygienic practice of standing on elevated racks.

Drying is carried out under cover on open wooden or metal racks which are infrequently washed.

Conditioning is conducted in sealed wooden boxes, again without regular cleaning.

Microorganisms Identified in Vanilla Beans and Contact Materials During Curing

Roling et al. examined traditional Indonesian curing methods in relationship to microbial population before and during the curing process.[1] In this study, microorganisms isolated from green beans belonged to the genera *Pseudomonas, Chyseomonas, Flavimonas, Burkholderia, Enterobacter, Vibrio, Corynebacterium, Bacillus, Staphylococcus, Tsukamurella, Actinomycetes, Leuconostoc, Brevibacterium, Cellumonas* and *Rhodococcus*. Of these, only *Actinomycetes* and *Bacillus* strains were able to grow. After scalding, only *Bacillus* strains could be cultivated from the treated beans. The high temperature scalding step evidently had a large influence on the resident microbial communities.

Ripe vanilla beans **F-1**

Hot water blanching **F-2**

Fungi and yeasts disappeared rapidly during curing. Yeasts were not encountered after scalding. Fungi isolated during sunning and sweating were only able to grow at 30°C, but not at the high temperatures prevalent during these process stages. Curing conducted during unfavorable, cloudy, cooler weather conditions exhibited a temporary increase in fungal numbers, suggesting that fungal participation in vanilla curing is probably negligible and undesirable. Such relatively low temperature in conjunction with retained high moisture conditions

Fermentation in wooden boxes **F-3**

Sunning on blankets on the ground **F-4**

should be avoided. Beans visually infected with fungi were discarded during traditional Indonesian processing due to their poor appearance and off flavor.

Considerable numbers of thermotolerant and thermophilic *Bacilli* were detected on vanilla beans undergoing curing. The dominance of *Bacilli* in this study was the result of considerable growth capability of thermotolerant and thermophilic members of these genera during vanilla curing. Thermophilic *Bacilli* can sporulate and survive unfavorable conditions such as heat treatment or nutrient shortage on materials used during curing such as cotton blankets and wooden boxes.

The selection of the dominantly occurring strains is possibly favored by limitation of oxygen during autoclaving. Beans become brown in the sweat box as the result of enzymatic oxidation, which exhibits a dependence on molecular oxygen.[1] *Bacillus smithii, Bacillus licheniformis* and *Bacillus subtilis* are able to grow well under low-oxygen conditions.[3] These same strains possessed protease, hemicellulase, cellulase and β-glucosidase activity that may assist in the degradation of vanilla bean cellular components and specific compounds such as the phenol β-D-glucosides present in the ripe green beans.

In the Indonesian study, there were large differences in microbial abundance, communities and strain characteristics between different batches of beans, indicating that the effects of microbial activities on the development of vanilla flavor is probably both complex and variable. This finding must be due in part to the microbial status of the ripe green beans utilized as well as the many different curing options conducted by individual farmers and larger curing centers, each with their own operating procedures.

Bacilli are also dominant species in other high-temperature processes, such as composting, and in the later stages of cacao fermentation.[4, 5] In these other processes microbial fermentation significantly contributed to heat generation. This appears not to be the case during vanilla curing, since in the latter elevated temperatures have to be maintained via heat input from scalding and sunning, and limitation of heat loss via insulated storage of the beans.

In addition to the data of Rollins et al., described above, the authors made some additional observations on the microbial status of traditional curing procedures.[1] Microbial analysis was conducted on beans at different stages of processing, and on the enrobing blankets used during the sweating and sunning operations. Preliminary analysis was conducted on six samples collected during the curing process from the July 2009 crop in Uganda. The samples employed were numbered 1–6 as listed below:

1. Whole beans ex the sweat box
2. Chopped beans ex the sweat box
3. The sweat blanket
4. Chopped beans after half of allocated time in the sun
5. Sweat blanket from contact with sample 4
6. Dry, chopped beans after curing

The results of the microbial analysis were:

Sample 1 (whole beans ex the sweat box): The aerobic bacterial plate count was 560,000/g, while yeasts and molds were in both cases <10/g. The bacterial isolate had a 99% match to *Acetobacter tropicalis.*

Sample 2 (chopped beans ex the sweat box): These beans had an aerobic plate count of 37,000/g with yeasts and molds at the same level as sample 1. The culture identity was 99.13% with *Bacillus pumilus.*

Sample 3 (the sweat blanket): This yielded yeast and mold counts of 960/sample and 450/sample, respectively. Aerobic plate count was 30,000/sample. Culture identity was 99.96% for *B. subtilis* subsp. *subtilis* ATCC =

6051, 99.72% for *Bacillus mojavensis*, *Bacillus subtilis* subsp. *spizizenii* ATCC = 6633, 99.5% for *B. subtilis* subsp. *spizizenii* DSM = 15029, 99.15% for *Bacillus atrophaeus*, 99.0% for *Bacillus amyloliquefaciens* DANI-QA-6CHG-1000115.

Sample 4 (chopped beans after half allocated time in the sun: ca. 10 days): Yeasts were <10/g, while molds were 180/g. The aerobic bacterial plate count was 320,000/g with 99.0% identity as *Acetobacter orleanensis.*

Sample 5 (sweat blanket from contact with sample 4): Aerobic plate count was 35,000/sample. Identities were 99.96% for *B. subtilis* subsp. *subtilis* ATCC = 6051, 99.73% for *B. mojavensis*, 99.56% for *B. subtilis* subsp. *spizizenii* ATCC-6633, 99.53% for *B. subtilis* subsp. *spizizenii* DSM = 15029, 99.14% for *B. atrophaeus* and 99.0% for *Bacillus amyloliquefaciens* DANI-QA6-CHG-1000115.

Sample 6 (dried chopped vanilla beans after curing): This sample had both yeasts and molds at <10 units/g. Aerobic plate count was 56,000/g with culture identity as 100.0% for *Bacillus thuringiensis* ATCC = 33679 and 100.0% for *B. thuringiensis* DSM = 6025.

All samples evaluated were low or very low in yeasts and molds. This is in keeping with the findings of Roling et al.[1]

As can be observed in the sample results, aerobic bacteria identified on the beans during various states of curing were from the genus *Bacillus* or *Acetobacter.* The blankets used in sweating and sunning carried organisms of the genus *Bacillus.* It was interesting to note that the predominant genus in whole beans from the sweat box was *Acetobacter*, while the corresponding chopped beans were dominated by *Bacilli.* The dried chopped vanilla beans (sample 6) also showed the *Bacilli* genus. Chopped beans (sample 4) after half the sunning treatment were dominated by *Acetobacter*. The two blanket samples (3 and 5) were dominated by *Bacilli.*

Microorganisms of the genus *Acetobacter* are obligate aerobic, nitrogen-fixing bacteria that produce mainly acetic acid as a result of metabolic processes. *Acetobacter* can be isolated from coffee plants and other vegetable materials. The genus is characterized by the ability to convert ethanol to acetic acid in the presence of oxygen. There are several species within this genus, and they all share this capability[a].

The unifying characteristics of the *Bacillus* genus are that they are Gram-positive, form endospores, and grow in the presence of molecular oxygen. Their collective capabilities include degradation of a wide range of substrates derived from plant and animal sources. These include cellulose, starch, pectin, protein and hydrocarbons. Endospore formation, universally found in the group, is considered to be a strategy for survival in the soil environment, where these bacteria predominate[b].

[a] *http://microbewiki.kenyon.edu/index.php/Acetobacter*
[b] *http://www.textbookofbacteriology.net/Bacillus.html*

The curing process converts an 80–82% moisture-containing product into a final processed bean with moisture content in the range 38–25%.[6] Water activity (a_w) of beans in the range of 38–25% moisture was determined as 0.89–0.84.[7] Most spoilage bacteria are inhibited at a_w below 0.90, while most molds require a value of ≤0.70. For most applications, however, final moisture levels in cured beans are around 20–25%, and as such would not indicate a significant microbial hazard. Despite this, care must be taken during the earlier, high-moisture stages of curing, particularly if they are prolonged, to prevent mold growth and bean spoilage.

Whether the growth and enzyme activities of *Bacilli* are indeed favorable for vanilla flavor formation has not yet been fully evaluated. This could be tested by comparing flavor profiles of inoculated beans subjected to curing to surface-sterilized controls.

The Microbial Status of Cured Vanilla Beans From Different Geographical Locations

Microbial analysis was conducted on cured vanilla beans from different geographical origins. The results are shown in **T-1**.

A number of interesting points arise from this data:

- All samples were free of the pathogenic bacteria *Salmonella* as well being low in *E. coli,* coliforms, *Staphylococci*, yeasts and molds.
- There was a wide range in values of aerobic plate count, from <100/g to 890,000/g.
- The water activity range was 0.503–0.895.
- The moisture content range was 16.35–21.35%.
- There was no clear correlation between water activity and moisture content of the cured beans.
- The vanillin content range was 1.12–3.29g/100 g on a dry weight basis.

General observation of vanilla curing conditions indicate blankets, wooden boxes, drying and conditioning surfaces, use of untreated water, and poor personal hygiene of operators are potential contamination and proliferation points for spoilage bacteria. Currently, cured vanilla beans do not seem to offer any health risk to humans. It appears that at the end of the curing process the only significant survivors are bacterial spores. This begs the question as to what in the curing process and the cured vanilla bean limits the growth of microorganisms.

Examination of the water activity values (a_w) in **T-1** shows a range of 0.503–0.985. This will limit microbial proliferation to some extent. According to ISO 5565-1:1999, the maximum moisture specification in cured beans is 38% for classes 1 and 2, 30% for class 3, and 25% for class 4.[6] These water contents correspond to water activities of 0.89, 0.86 and 0.84, respectively.[7] To prevent the growth of most molds, a_w needs to be ≤0.70. Most spoilage bacteria cannot grow at a_w ≤0.91. In fact, a combination of low water content with high phenolic content, of which the major representative is vanillin, seems to provide inhibitory conditions for microbial proliferation.[8]

Natural vanillin exhibits strong antimicrobial properties with activity demonstrated against *Aspergillus flavus, A. niger, A. ochraceus* and *A. parasiticus* in laboratory media and fruit-based agar systems. The most resistant mold was *A. niger.* The vanillin inhibitory concentrations were in general lower than 2,000 ppm, and its influence was related to the fruit base type.[9]

Nearly all antimicrobials can be classed into one or more of the following groups: (a) reaction with the cell membrane, (b) inactivation of essential enzymes, or (c) destruction or inactivation of genetic material.[10] Phenolic compounds are hydrophobic in nature and are likely therefore to be membrane-active. The mode of action of lipophilic molecules including hydrocarbons and related compounds accumulate in the lipid bilayer membranes of microorganism, affecting their structural and functional properties. As a result of the accumulated molecules, membranes lose their integrity and exhibit

Microbial analysis of cured vanilla beans from different geographical locations **T-1**

Origin of cured beans	Salmonella	*E. coli*	Coliforms	Yeasts and molds	Aerobic plate count	*Staphylococcus*	Water activity	Vanillin (g/100 g dry weight)	% Moisture
Indian	Negative/375g	<3/g	<3/g	<10/g	<100/g	<10/g	0.786	1.95	21.35
Bourbon better	Negative/375g	<3/g	<3/g	<10/g	730000/g	<10/g	0.586	2.03	16.31
PNG *tahitensis*	Negative/375g	<3/g	<3/g	<10/g	2000/g	<10/g	0.715	1.12	17.20
Ugandan	Negative/375g	<3/g	<3/g	<10/g	6700/g	<10/g	0.761	3.29	20.61
Bourbon best	Negative/375g	<3/g	<3/g	<10/g	200000/g	<10/g	0.787	2.38	20.02
PNG *planifolia*	Negative/375g	<3/g	<3/g	<10/g	890000/g	<10/g	0.503	2.26	16.44
Mexican	Negative/375g	<3/g	<3/g	<10/g	100/g	<10/g	0.895	1.73	19.26
Ugandan organic	Negative/375g	<3/g	<3/g	<10/g	380000/g	<10/g	0.701	1.70	18.19
Madagascan organic	Negative/375g	<3/g	<3/g	<10/g	1800/g	<10/g	0.826	1.67	18.17
Indian organic/FT	Negative/375g	<3/g	<3/g	<10/g	46000/g	<10/g	0.615	1.95	19.01

an increase in permeability to protons and ions in many instances. This results in the impairment of intracellular pH homeostasis. In addition, proteins embedded in the membrane are affected, probably as the result of changes in the lipid environment, as well as direct effects on membrane proteins.[11]

Mode of action studies using plant essential oils (oregano, thyme and tea tree) or some of their phenolic constituents (carvacrol, eugenol and thymol) against several pathogenic bacteria and yeasts have shown that their activity resides in their ability to perturb the cell membrane, resulting in the loss of chemiosmotic control leading to cell death. Oregano and thyme essential oils possess significant in vitro colicidal and colistatic properties, which are exhibited over a broad temperature range and substantially improved by the addition of agar as a stabilizer. Extracts of oregano and thyme may be effective in reducing the number, or preventing the growth of *E. coli* O157:H7 in foods.[12]

Fitzgerald et al. investigated the mode of action of vanillin with regard to its antimicrobial activity of several food-related bacteria, namely *E. coli*, *Lactobacillus plantarum* and *Listeria innocua*.[13]

The inhibitory action of vanillin at its minimum inhibitory concentration (MIC) was found to be bacteriostatic in contrast to the more potent phenolic antimicrobials such as carvacrol and thymol, which are bactericidal in their function.[14]

Vanillin activity resided primarily in its ability to damage the integrity of the cytoplasmic membrane, with the resultant loss of ion gradients, pH homeostasis and inhibition of respiratory activity. Energy generation remains largely unaffected or can indeed be stimulated for at least a short period of time (1 h). The extent of the membrane damage appears to be sublethal in the majority of cells within an inhibited microbial population, exhibited as a bacteriostatic action of inhibition at the minimum inhibitory concentration.

Vanillin is not the only phenol present in cured vanilla beans and it may be a combination of the total phenolic complex that could contribute to any antimicrobial activity. Whether vanilla extracts, at the levels applied to foods, are effective in modifying microbial activity remains an open question.

Summary

The case for microbial participation in vanilla curing remains unclear, though such a role is supported by:

- The presence and survival of particularly the genus *Bacillus* and *Acetobacter* during curing
- The presence of these organisms on contact materials or the beans themselves are evidence for the existence of conditions for their growth and proliferation
- Phenolics naturally present during the curing process and in cured vanilla beans may contribute to the limitation of the final microbial status of the beans

Further consideration needs to be given to the potential for the participation of microorganisms in the transformation of phenolic and other flavor compounds during the curing process (this will be the subject of part two of this paper). Additional experimentation needs to be conducted under simulated vanilla curing conditions in sterile, controlled environments, under normal and simulated curing conditions and in the foregoing situations with addition of vanilla bean-associated bacteria.

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