# Enzymatic Extraction and Transformation of Glucovanillin to Vanillin from Vanilla Green Pods

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Glucovanillin was extracted from green pods and simultaneously transformed to vanillin by a combination of enzyme activities involving cell wall degradation and glucovanillin hydrolysis. The reaction is best carried out with 47.5% v/v aqueous ethanol solution during 8 h at 70 °C, in a two-step enzymatic reaction using Viscozyme followed by Celluclast, two commertial enzymatic products containing mainly pectinase and cellulase activities, respectively. The extractive reaction proceeded with high efficiency with an amount of extracted vanillin 3.13 times higher than the one obtained with the Soxhlet method. The classical curing/extraction process results in 1.1−1.8 g of vanillin/100 g of dry pods. It is concluded that the enzymatic reaction may substitute the microbial process involved in tissue fermentation previous to vanillin extraction with the simultaneous hydrolysis of glucovanillin.

**Keywords:** *Glucovanillin; vanillin; β-glucosidase, cellulase, enzyme extraction*

## INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the principal flavor component of vanilla (*Vanilla fragrans*), the most widely used food flavoring, and, in its natural form, one of the most expensive spices. In many industrialized countries vanillin is chemically synthesized from guaiacol, but around 20 of 12000 tons of vanillin consumed annually is extracted from beans as a natural flavor (*1*). The natural process requires a curing process, and the characteristic flavor and aroma are developed as a result of different biochemical transformations. In Mexico, around 100 tons of vanilla pods is processed in one of the southeastern states, Veracruz, where a curing process developed in the 19th century is still practiced. The pods are cropped when ripe, placed in sacks, and treated in a highly humid oven for 36−48 h at 60 °C. Afterward, the pods are transferred to a wooden box for 24 h to allow water to drain out. The pods are then exposed to the sun during the day, and stored at night, in the wooden box. This drying process is repeated between 11 and 25 times until the pods dry, turn dark brown, and develop an elastic consistency. At the industrial level, vanilla is produced as extracts obtained from cured pods. Vanillin, the single most characteristic component of the flavor, is obtained by hydrolysis of its precursor glucovanillin by endogenous *β*-glycosidase during the curing process to release vanillin (*2*). Vanillin content varies with vanillin variety around the world. Madagascar produces the best quality vanilla, with a vanillin content of 2−3.4%, whereas the Mexican variety productivity is 1.1−1.8 g/100 g of pods (*3*). Despite the time required for the curing process, the enzymatic transformation of the glycosides is not very efficient. Ranavide also showed that cured beans from Tahiti, Tonga, Jamaic, and Madagascar increased their vanillin content up to 24% upon exogenous *β*-glycosidase treatment. Also, Mane and Zuccha (*4*) reported an increase of vanillin content of up to 14% after the treatment of cured beans with exogenous pectinase and *β*-glycosidase. More recently (*5*), Brunerie patented a process to increase the yield of natural vanilla flavor with the aid of enzymes. In this paper we propose a direct enzymatic extraction of glucovanillin, as well as other ethanol soluble compounds of the aroma, and their enzymatic transformation to vanillin from green pods, avoiding the curing step.

## MATERIALS AND METHODS

**Plant Material.** Vanilla pods were collected from the region of Papantla Ver. Mexico and were kindly provided by Mr. Vallejo, a local producer, before the curing process was begun. Pods were kept at 4 °C until the extraction was performed.

**Chemicals.** Viscozyme L. (Novo) is a mixture of arabinases, cellulases, hemicelullases, xylanases, and pectinases from *Aspergillus*, with a range of pH activity from 3.3 to 5.5 and temperature from 50 to 60 °C. Celluclast (Novo) cellulase was obtained from *Trichoderma reseii*, with an optimum pH 4.5−4.8 and temperature from 48 to 60 °C. Water, acetic acid, ethanol, and methanol of HPLC grade were from Mallinckrodt AR.

**Water Content Determination.** One hundred grams of green pods was weighed and cut in ∼0.5 cm pieces. A 1-g sample was frozen at −70 °C for 2 h and lyophilized (Labconco, Kansas City, MO). The water content is reported as the difference between the initial and final weights of 1 g of green pods.

**Soxhlet Extraction.** Two grams of pods was weighed in a cellulose thimble and placed in a Soxhlet apparatus (*6*). The vanilla compounds were extracted using a solvent containing 47.5% v/v aqueous ethanol solution during 8 h at ∼70 °C. Five milliliters of a 120 mg/mL ethylvanillin solution was added as internal standard to measure the extraction efficiency. Ethanol was evaporated and the extract adjusted to 100 mL

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in a volumetric flask. The extract was filtered in a 0.45 μm filter (Millipore). This extraction was used as a control.

**Enzymatic Extraction.** One hundred grams of green vanilla pods was chopped in pieces of ~0.5 cm; 50 g was placed in jacketed beakers to which 150 mL of distilled water was added. The extraction was performed using two procedures. First, 1% v/v of Viscozyme was added, with the temperature held at 50 °C for 8 h; afterward, 1% v/v of Celluclast was added, and the extraction/reaction was maintained for an additional 8 h at the same temperature. All of the reaction contents were kept in suspension by agitation using a magnetic stirrer. The amount of vanillin and glucovanillin was quantified using HPLC after the Viscozyme and Celluclast extraction. The analysis was carried out again after the addition of industrial ethanol to reach 47.5% v/v concentration in the enzyme reaction mixture, allowing 30 min to complete the extraction. It was observed that in order to enhance extraction and avoid enzyme denaturation a maximum of 30% v/v ethanol could be used as solvent for the extraction process, decreasing the reaction time (results not shown). The second procedure was carried out by inverting the treatments, adding first Celluclast and then Viscozyme, under the same reaction conditions. As a control, an extraction of 50 g of pods with 150 mL of distilled water, but without enzymes, was carried out under agitation at 50 °C for 8 h. Products in the control experiments were also analyzed after the treatment without enzymes as well as after ethanol addition (1:1 proportion). All of the samples were adjusted at 100 mL in volumetric flasks and filtered in a 0.45 μm filter. Experiments were carried out in triplicates.

***β*-Glycosidase Specific Activity Determination.** β-Glycosidase activity present in Celluclast and Viscozyme was determined using a solution of 0.5% of Cellobiose (Sigma). Ten milliliters of a 0.5% w/w of Cellobiose solution was used as substrate in test tube reactions containing 0.1, 0.15, and 25% v/v of each enzyme. The initial reducing sugars release was determined by the 3,5-dinitrosalicylic acid method on samples taken at 0, 10, and 20 min.

**HPLC Analysis.** Samples were filtered in a 0.45 μm filter (Millipore) prior to HPLC injection. The compounds were analyzed by HPLC on a Beckman System Gold, programmable UV−vis detector module 166 at 278 nm, 20 μL loop, column C-18, 4.66 mm × 25 cm (Beckman). Glucovanillin and vanillin and the internal standard ethylvanillin were determined using a gradient phase of methanol/water pH 4 (acidified with acetic acid) as follows: flow of 0.8 mL/min of 60−40% acidified water/methanol for 3 min; flow of 1 mL/min of 65−35% acidified water/methanol for 9 min, and flow of 0.8 mL/min of acidified 60−40%water/methanol. Standard curves were obtained using vanillin and ethylvanillin (Sigma).

## RESULTS AND DISCUSSION

As already shown (*2*), β-glucosidase is the most important activity required in the treatment of vanillin precursors in green or cured pod extracts; however, additional activities may enhance the extraction yield by hydrolysis of cell wall components as already demonstrated for other agricultural products such as olive oil, coconut oil, capsaicin from chili peppers, and rice (*7*−*9*). Actually, β-glucosidase may be part of the various activities present in commercial enzymes usually applied in extraction processes such as Viscozyme or Celluclast. We therefore evaluated this activity in the commercial products, following the release of glucose from Cellobiose at 45 °C and pH 4. It was found that both products contain similar levels of β-glucosidase, as Celluclast may release 5.0 ± 0.4 mg of glucose/mL of product·min and Viscozyme may release 5.8 ± 1 mg of glucose/mL of product·min. These measurements were carried out at three different enzyme concentrations.

Although a negligible amount of vanillin has been reported in green pods (*3*), the amount of vanillin found in this work was a function of green pod age or storage time. However, when fresh pods were available, the Soxhlet extraction showed no vanillin content in the 50 g samples used for extraction/reaction.

**Table 1. Average Vanillin Concentration Extracted from 2 g of Vanilla Pods Using Different Additives**

| extraction method | extracted vanillin (g/100 g of dry pods) | % of total |
|---|---|---|
| Soxhlet[a] | 1.14 ± 0.043 | 100 |
| water extraction (control) | 1.12 ± 0.05 | 97.36 |
| Viscozyme L and water[b] | 1.52 ± 0.08 | 132.17 |
| Viscozyme L and ethanol[c] | 1.96 ± 0.33 | 170.4 |

[a] Average of five extractions using cured pods. [b] One milliliter of Viscozyme was added to 9 mL of water, and the reaction was carried out during 8 h at 50 °C. [c] One milliliter of Viscozyme was added to 9 mL of water. The reaction was carried out during 8 h at 50 °C after which time 10 mL of ethanol was added for 30 min.

**Table 2. Average[a] Vanillin Concentration Extracted from 50 g of Vanilla Pods by Different Processes**

| treatment | extracted vanillin content (g/100 g of dry pods) | % of total |
|---|---|---|
| water[b] | 1.07 ± 0.08 | 93. |
| Viscozyme + water[c] | 1.17 ± 0.05 | 101.7 |
| Viscozyme + ethanol[d] | 2.45 ± 0.21 | 213 |
| Celluclast + water | 1.17 ± 0.06 | 101.7 |
| Celluclast + ethanol | 2.7 ± 0.17 | 234.8 |

[a] Average of three extractions. [b] Viscozyme (150 mL) was added to 50 g of green vanilla pods. [c] Viscozyme (15 mL) was added to 135 mL of water, and the reaction was carried out during 8 h at 50 °C. [d] Viscozyme (15 mL) was added to 135 mL of water, and the reaction was carried out during 8 h at 50 °C for 30 min. Afterward, a 5 mL sample was taken and 5 mL of ethanol added.

In Tables 1 and 2 the results of a series of extractions, including the control experiments, are presented. Two scales of experiments are reported corresponding to 2 and 50 g of vanilla of pods in the assays. On average, the maximum amount of vanillin that could be extracted was 1.14 ± 0.043 g/100 g of dry weight of pods as obtained using the Soxhlet method with cured vanilla pods.

Table 1 shows the beneficial effect of the enzymatic extraction using Viscozyme on vanillin extraction when compared to the control with water (1.52 vs 1.12 g of vainillin/100 g of dry weight pods). After ethanol extraction, this amount further increases to 1.96 g/100 g of pods. In both cases there are significant differences among samples using enzymes and the control at the 99% level.

To reduce the experimental error and to study the enzyme effect in more detail, the mass of pods was increased to 50 g and the same experiments were performed again, this time including a preparation with high overall cellulolytic activity. These results are shown in Table 2, where it may be observed that in the case of the enzyme treatment followed by ethanol extraction a high yield may be again observed. In these experiments, yields 2 and 2.3 times higher than the maximum value obtained by the Soxhlet method with cured vanilla pods were observed. However, no differences were found this time between the enzymatic aqueous extraction and the control. A synergistic effect was found between both enzymatic preparations as observed when sequential enzymatic reactions were studied, as shown in Table 3. It was previously observed that both preparations do not work efficiently when used together in the same reaction. Furthermore, as observed in Table 3, results using Viscozyme followed by Celluclast are similar to those observed when Viscozyme was used alone (Table 2), as if Celluclast had no effect. However, when Celluclast was added in a first reaction

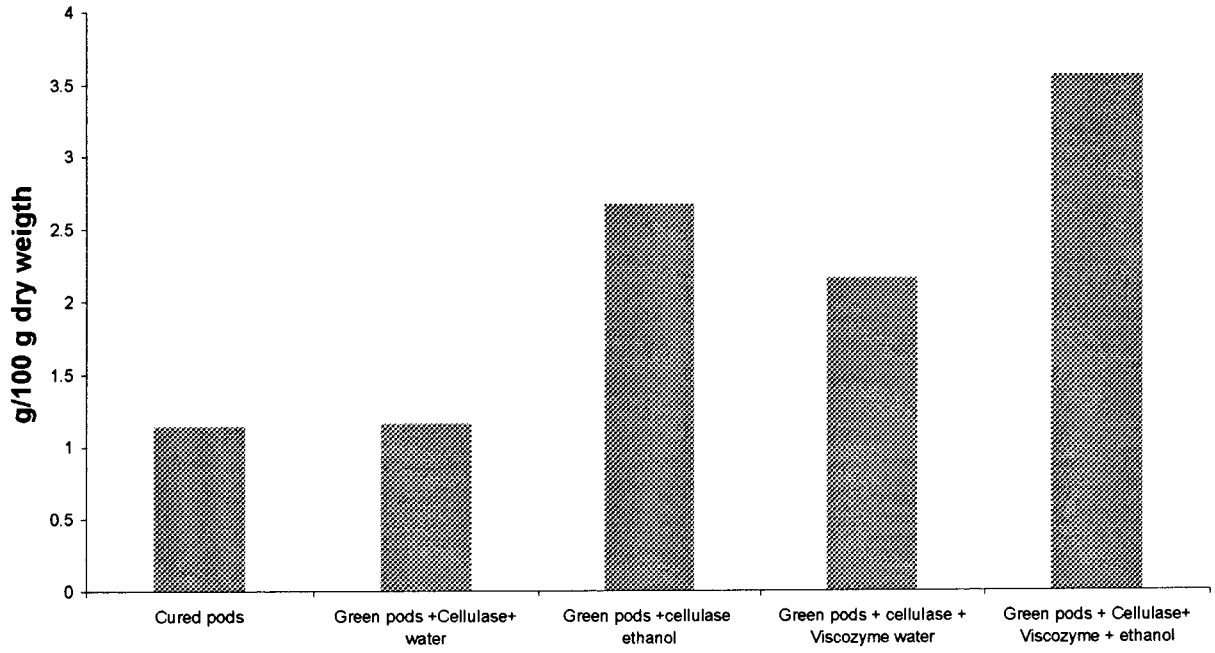


**Figure 1.** Extraction and glucovanillin hydrolysis to vanillin using commercial enzyme.

**Table 3. Average[a] Vanillin Concentration Extracted from 50 g of Vanilla Pods by Various Enzymes Mixtures and Processes[b]**

| treatment | extracted vanillin content (g/100 g of dry pods) | % of total |
|---|---|---|
| Viscozyme + Celluclast/water[c] | 1.17 ± 0.11 | 101.7 |
| Viscozyme + Celluclast/ethanol[c] | 2.66 ± 0.07 | 226 |
| Celluclast + Viscozyme/water[d] | 2.3 ± 0.1 | 200 |
| Celluclast + Viscozyme/ethanol[d] | 3.66 ± 0.04 | 313 |

[a] Average of three extractions. [b] One hundred and fifty milliliters was added to 50 g of green vanilla pods. [c] Viscozyme (15 mL) and Celluclast (15 mL) were added to 120 mL of water, and the reaction was carried out during 8 h at 50 °C for 30 min. [d] Viscozyme (15 mL) and Celluclast (15 mL) were added to 120 mL of water, and the reaction was carried out during 8 h at 50 °C. Ten milliliter samples were taken, and 10 mL of ethanol was added for 30 min.

step, previous to Viscozyme addition, the extractive reaction proceeded with high efficiency with an amount of extracted vanillin 3.13 times higher than the one obtained with the Soxhlet method. Finally, the amount of glucovanillin extracted and converted using the various options studied is presented in Figure 1, where it may be concluded that enzymes are useful not only in the conversion of the precursor glucovanillin to vanillin but also in the extraction from the pods, avoiding the requirements of a "fermentation−extraction process".

## CONCLUSIONS

The curing of vanilla pods using the traditional fermentation/extraction method is inefficient in terms of the amount of vanillin obtained; the Soxhlet extraction procedure results in lower yields when compared to the enzymatic extraction technique. The amount of vanillin transformed from green vanilla bean pods using Viscozyme and Celluclast enzymes was higher than the amount of vanillin extracted from cured vanilla beans in the classical process. It was found that when a two-step enzymatic reaction system with Viscozyme and Celluclast was used, glucovanillin extraction and conversion to vanillin may increase 3.13 times. The use of enzymes may be helpful to improve yield and productivity, as the enzymatic reaction may substitute the microbial process involved in the traditional tissue fermentation. Organoleptic evaluation of the enzymatic extracts is in progress.

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