Component and Structure of Aspergillus flavipes sp.-Biodegraded Bayberry Tannins: A Potential Routine for Condensed Tannin Cleaner Degradation and Disposal

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ABSTRACT: Chemical degradation is widely used for producing lower-molecular-weight tannin compounds and tannin disposal, but it has negative effects on the environment, such as causing secondary pollution and consuming energy. For overcoming these disadvantages, a cleaner and sustainable degradation and disposal method for condensed tannins was developed through biodegradation. In this study, bayberry tannin solution, one kind of condensed tannin, was biodegraded by Aspergillus flavipes sp. at first; then, gel permeation chromatography and high-performance liquid chromatography were used for separating the biodegraded and original tannins to analyze the differences in components; finally, the changes in the tannin structure after biodegradation were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nuclear magnetic resonance. The results showed that the high-molecular-weight components decreased while the low-molecular-weight components increased when bayberry was subjected to A. flavipes sp. biodegradation; furthermore, the molecular weight of the biodegraded bayberry tannin decreased from 3371 to 2658 Da. Meanwhile, the structure of bayberry tannin polyflavonoids, especially A ring and C ring together with the galloyl group, was destroyed and some small fragments were generated during biodegradation. These structural changes resulted in the increase of low-molecular-weight phenols but the decrease of polyflavonoids after bayberry biodegradation. These would be the pieces of evidence showing that A. flavipes sp. consumed simple phenols as nourishment for growth and converted polyflavonoids into low-molecular-weight substances at the same time. To sum up, biodegradation can be used in every field where condensed tannins should be degraded or removed for a cleaner and ecofriendly routine.

INTRODUCTION

Tannins are naturally occurring polyphenols as a wide range of compounds considered to be secondary metabolites in different plant tissues: leaves, stems, flowers, fruits, seeds, and others. In general, tannins are divided into hydrolysable tannins and condensed tannins. Tannins could be used as vegetable tanning agents in leather making, adhesives for the wood industry, insulating foams, in the mineral industry, in the wine production industry, in animal nutrition, in the oil industry, in water treatment plants, and for protecting metals from corrosion. Many research studies have proven that different-molecular-weight tannins or their components have different properties and applications. For condensed tannins, polymeric compounds such as procyanidins, prodelphinidins, and propelargonidins, with subunits of catechin/epicatechin, gallocatechin/epigallocatechin, and afzelechin/epiafzelechin, are found to be potential antioxidant, antifungal, and antienzymatic agents. Hydrolysable tannin degradation products such as gallic acid, tannic acid, and ellagic acid are important chemical materials and have been reported to have many medically important properties as antioxidant, antimicrobial, antiviral, and antitumor properties. For obtaining low-molecular-weight tannin products, degradation is essential, in which chemical degradation is widely used, but it has many disadvantages such as high energy consumption, requirement of chemical reagents, and secondary pollution. For example, industrial ellagic acid is obtained by chemical degradation with defects of high production costs and environmental damage. Biodegradation is a gentle, eco-friendly, and clean process for tannin degradation, in which high-molecular-weight tannins being degraded into small...
molecules are more efficient and may have important biological activities and high-added value;\textsuperscript{17} in addition, some unique products might also be obtained in biodegradation.\textsuperscript{18}

The effluents from pulping, plant medicine, and forest chemicals contain tannins as they are widely distributed in plants.\textsuperscript{19} Tannin-containing wastewater has high chemical oxygen demand (COD) and biological oxygen demand and dark color, and it is difficult to treat the effluent with traditional dilution and adsorption.\textsuperscript{20,21} There are many methods to treat tannin-containing wastewaters in the lab scale, such as adsorption,\textsuperscript{22} membrane filtration,\textsuperscript{23} and photocatalytic\textsuperscript{24} and sonochemical degradation.\textsuperscript{25} However, these methods have high cost and complex operation, making them impossible to use for industrial application. Fortunately, some special microbes have high tolerance to tannins, so they could be a cleaner and effective method for tannin-containing effluent disposal.\textsuperscript{26,27}

Hydrolysable tannins consist of ester bonds and glycoside bonds, endowing the tannins with better biodegradation property. Therefore, the studies on hydrolysable tannin biodegradation are abundant and systematic. In 1969, research on the biodegradation of gallotannin and chestnut was carried out.\textsuperscript{28} An anaerobic bacterium which was able to degrade hydrolysable tannins was isolated from the ruminal fluid of goat.\textsuperscript{29} Some microorganisms were isolated to biodegrade hydrolysable valonea tannins, and the biodegradation processes were studied in detail.\textsuperscript{30,31} Furthermore, the dynamics of valonea biodegraded by the microorganisms and protease produced in the process were also illustrated fully.\textsuperscript{32,33} *Aspergillus* sp. and *Endomycopsis* sp. were also isolated and domesticated to biodegrade valonea tannins effectively. Condensed tannins, the so-called proanthocyanidins, are complex polymers of flavan-3-ol units that possess a typical C6–C3–C6 flavanoid skeleton.\textsuperscript{34} As the C–C bond is unaffected by microbe corrosion, condensed tannins are relatively difficult to biodegrade. Nevertheless, although condensed tannins could be biodegraded, the biodegradation rate and degree were usually limited compared with hydrolysable tannins.\textsuperscript{20,27}

Bayberry tree is a kind of subtropical plant which belongs to the Myricaceae family and is considered as an important economic plant and widely distributed in South China.\textsuperscript{35} Bayberry tannins are extracted from the bark and classified as the condensed type.\textsuperscript{36,37} The high content of hydroxyl and galloyl groups in bayberry tannins is considered as its most obvious character and differentiates it from other condensed tannins.\textsuperscript{38} The bayberry tannin is an important vegetable extract and widely used in China.\textsuperscript{39} In order to study the biodegradation process of condensed tannins based on Chinese conditions, the bayberry tannin solution was naturally contaminated to get tannin-tolerant microbes. Then, *Aspergillus flavipes* sp. was isolated and identified.\textsuperscript{40} Previous study demonstrated that the COD, total phenol, and polyflavonoid removal efficiencies of biodegraded bayberry tannin were 18.6, 42.7, and 41.3%, respectively, after 15 days of fermentation, and in addition, there were changes in the pH, conductivity, and colloid properties of the tannin solution during biodegradation.\textsuperscript{41} However, the core and essential reason behind these changes has not been illustrated in detail.

In this work, gel permeation chromatography (GPC), high-performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and nuclear magnetic resonance (NMR) were used to determine the component and structural differences between the original bayberry tannins and bayberry tannins biodegraded by *A. flavipes* sp. This study reveals the bayberry tannin biodegradation mechanism and provides a cleaner degradation method for producing low-molecular-weight tannins and removing tannins in the effluent.

### Experimental Section

**Materials.** The bayberry tannin was purchased from the Wuming tannin extract factory (Guangxi, China). Other chemicals were all of research grade.

**Bayberry Tannin Biodegradation Process.** 300 mL of a 5 g L\textsuperscript{-1} bayberry tannin solution was prepared with the bayberry tannin sterilized by UV for 30 min in a SWCJ-1F bacteria-free operating floor (China), and then 10 mL of 2 × 10\textsuperscript{6} mL\textsuperscript{-1} *A. flavipes* sp. was introduced into the medium. The *A. flavipes* sp. used in the study was stored in the lab.\textsuperscript{42} Finally, the bayberry tannin solution was cultured in an oscillator at 28 °C for 15 days.

**Bayberry Tannin Component Determination.** The original and biodegraded bayberry tannin solutions were filtered using a 220 nm microporous membrane and lyophilized for GPC and HPLC analysis.

**GPC Analysis.** The tannins were dissolved in dimethyl formamide (DMF) and subjected to GPC analysis. A HLC-8320 GPC (TOSOH Corporation, Japan) system equipped with a differential refraction detector and a combination of a TSK gel and a Super AWM-H column was used to measure the molecular weight. DMF as a mobile phase was pumped into the column with a flow rate of 0.4 mL min\textsuperscript{-1} at 40 °C. A series of polymethylemethacrylate standards were used as the standard markers. The content of each peak was calculated using eq 1.

\[
\text{Content of fraction (\%) = peak area/total area } \times 100 \%
\]

**HPLC Analysis.** The tannins were dissolved in water and subjected to HPLC analysis. An Agilent 1100 HPLC (Agilent Corporation, America) system equipped with a diode array detector was used to measure the components with 50 µL of the sample. Methanol (A) and 1% acetic acid solution (B) as the mobile phase were pumped into the column with a flow rate of 1 mL min\textsuperscript{-1} at 40 °C. The process of the mobile phase is as follows: 0–20 min, A-10%, B-90%; 20–21 min, A-10 to 50%, B-90 to 50%; 21–40 min, A-50%, B-50%; 40–41 min, A-50 to 100%, B-50 to 0%; 41–60 min, A-100, B-0%. The content of each peak was calculated according to eq 1.

**Bayberry Tannin Structure Characterization.** The original and biodegraded bayberry tannin solutions were filtered using a 220 nm microporous membrane, and then, petroleum ether was added in equal volumes and stirred for 8 h at room temperature to remove esters. Then, the water phase was collected using a separating funnel and petroleum ether was eliminated by rotary evaporation at 50 °C. After the degrading process, glucose was removed by gel filtration on Sephadex LH-20 (GE, America) with 50% ethanol and 50% water, and then polyflavonoids were obtained with 50% acetone and 50% water. After the solvents were removed by evaporation and lyophilization successively, the original or biodegraded bayberry tannin samples were prepared for MALDI-TOF MS and \textsuperscript{13}C NMR tests.

**MALDI-TOF MS Analysis.** The analyses were performed on an Autoflex III MALDI-TOF MS (Bruker Daltonics, Switzer-
land) system, which was equipped with a N₂ laser (337 nm). The samples were analyzed in the positive-ion reflection mode with 3 nm of laser pulse. The acceleration and reflection voltage were set to 20.0. The samples were dissolved in acetone (80 mg mL⁻¹) and mixed with 2,5-dihydroxy benzoic acid at a ratio of 1:3 (v/v), and NaCl was added to enhance the ion formation process. The mixtures were applied onto a stainless-steel target and dried at room temperature; subsequently, MALDI-TOF MS analysis was carried out.

**RESULTS AND DISCUSSION**

Influence of Biodegradation on the Bayberry Tannin Component. Although previous study showed that the total phenols and polyflavonoids were removed during the biodegradation process, there was no research on the content change of each component. According to Figure 1, the bayberry tannins are divided into five components. Peaks 1, 2, and 3 are simple phenols, and peaks 4 and 5 are polyflavonoids. Every component of the tannins is calculated by the corresponding peak area, and the results are shown in Tables 1 and 2. During the biodegradation process, peak 1 and peak 2 increase by 5.32 and 4.89%, respectively, but peak 3, which represents the relatively high-molecular-weight phenols in simple phenols, reduces by 7.32%; peak 4 and peak 5 decrease by 2.46 and 0.43%, respectively. Consequently, the content of simple phenols increases by 2.89%, while the content of polyflavonoids reduces by 2.89%. These results demonstrate that phenols, especially the large-molecule phenols, are degraded by *A. flavipes* sp. Moreover, since the COD of the tannin solution decreased during biodegradation, we can infer that the polyflavonoids biodegraded to low-molecular-weight phenols, and the native low-molecular-weight phenols in tannins were used as nutrients for microbe growth.

As the GPC column elutes polyflavonoids by the size of the molecule, it is a useful method for calculating the average molecular weight and molecular weight distribution of polyflavonoids simultaneously. In the chromatogram, higher-molecular-weight (molecular size) components present a shorter retention time, and on the contrary, the longer retention time comes from smaller oligomers. There are obvious differences in bayberry tannin components in the GPC images. As shown in Figure 2, there are four fractions in the original tannins but only three in the biodegraded sample.

Table 1. Change of Simple Phenols in Bayberry Tannins during Biodegradation

| peak | before biodegradation | after biodegradation | difference between before and after |
|------|------------------------|---------------------|------------------------------------|
| 1    | 2.71                   | 8.03                | +5.32                              |
| 2    | 12.15                  | 17.04               | +4.89                              |
| 3    | 14.52                  | 7.20                | −7.32                              |
| total| 29.38                  | 32.27               | +2.89                              |

Table 2. Change of Polyflavonoids in Bayberry Tannins during Biodegradation

| peak | before biodegradation | after biodegradation | difference between before and after |
|------|------------------------|---------------------|------------------------------------|
| 4    | 64.47                  | 62.01               | −2.46                              |
| 5    | 6.15                   | 5.72                | −0.43                              |
| total| 70.62                  | 67.73               | −2.89                              |

Figure 1. HPLC images of bayberry tannins [(a) before degradation and (b) after degradation].

Figure 2. GPC images of bayberry tannins [(A) before degradation and (B) after degradation].
Also, both the retention time and content of each component are different (in Tables 3 and 4). As the contents of peak III and peak IV increase by 13.07 and 7.85%, respectively, it is clear that the component of bayberry tannins after biodegradation consists of more small-molecular-weight phenols. The average molecular weights before and after biodegradation are 3371 and 2658 Da, respectively, calculated based on the GPC results. The average molecular weight reducing by 713 Da, the retention time of peak I extending and peak II vanishing, and the peak III and IV contents ascending after biodegradation illustrate that the A. flavipes sp. biodegrade bayberry tannins through deteriorating polyflavonoids to low-molecular-weight products but depleting simple phenols for growth at the same time. During biodegradation, both polyflavonoids and simple phenols are consumed by A. flavipes sp., but their main tendency is to convert polyflavonoids into low-molecular-weight substances.

**Influence of Biodegradation on the Bayberry Tannin Structure.** MALDI-TOF MS has been used extensively to elucidate the complexity of polyflavonoids for providing the structural information of flavon-3-ol subunits. The MALDI-TOF MS spectra of polyflavonoids with the molecular weights ranging from 500 to 3000 Da are shown in Figure 3A,B. An obvious repetitive pattern of the peaks is observed in these spectra. The intervals of 152u, 304u, and 456u are discovered and peak II vanishing, and the peak III and IV contents ascending after biodegradation illustrate that the A. flavipes sp. biodegrade bayberry tannins through deteriorating polyflavonoids to low-molecular-weight products but depleting simple phenols for growth at the same time. During biodegradation, both polyflavonoids and simple phenols are consumed by A. flavipes sp., but their main tendency is to convert polyflavonoids into low-molecular-weight substances.

**Figure 3.** MALDI-TOF MS spectrum of bayberry tannins ((A) before degradation and (B) after degradation).
between the peaks with the strongest signal in the original bayberry tannins, indicating the presence of galloyl, (epi)-gallocatechin, and (epi)gallocatechin gallate subunits, respectively (Figure 4). However, the 16u differences are not observed between these peaks, indicating bayberry tannins consisting of prodelphinidin.45 After biodegradation, the 152u peak is eliminated and replaced by 75u, and at the same time, slight 88u and 60u patterns are generated. These results demonstrate that bayberry tannin is fragmented in A. flavipes sp. biodegradation. This is additional evidence for proving the GPC and HPLC results that small-molecular-weight substances are produced during the biodegradation process. Unfortunately, MALDI-TOF MS could not provide sufficient proof to clarify the exact structure of the biodegraded products, but it is enough to explicate that bayberry tannins are broken during biodegradation and convert into low-molecular-weight products partly.

NMR analysis is considered to be the most powerful tool for characterizing polyflavonoids’ detailed structural information, including the composition of the subunits, average polymerization degree, and stereochemistry. It can be obtained through resonances of carbons from the A ring, B ring, and pyranoid C-
phenols. As observed in $^{13}$C NMR, if the concentration of one weight substances and consumption of large-molecular-weight the main interaction for the generation of low-molecular-might occur in the galloyl group shows that the ring opening and decarboxylation process. The disappearance of C-1 indicates that the B ring is not damaged obviously in the biodegradation are shown in Table 5.

The signal of C-3 vanishing at about 64 is evidence that the A ring and C ring generate the ring-opening reaction. However, the existence of C-1′, C2′, C3′, C4′, C5′, and C6′ indicates that the B ring is not damaged obviously in the process. The disappearance of C-1″ and C-3″ related to the galloyl group shows that the ring opening and decarboxylation might occur in the A. flavipes sp. degradation process; this is the main interaction for the generation of low-molecular-weight substances and consumption of large-molecular-weight phenols. As observed in $^{13}$C NMR, if the concentration of one substance was too low, the resonance signal would vanish during the measurement. Although GPC, HPLC, and MALDI-TOF MS spectra indicate that polyflavonoids still exist in the degraded tannin, the content of polyflavonoids reduces because of microbial growth; therefore, the C-3, C-4a, C-6, C-7, C-8, C-1″, and C-3″ resonance lines disappeared.

**CONCLUSIONS**

Through comparing the differences of the component and structure between the original and A. flavipes sp.-biodegraded bayberry tannins, it could be concluded that the biodegradation mechanism is A. flavipes sp. growth and metabolization damage of the A ring and C ring of bayberry tannins and hydrolysis of the ester bond to release the galloyl group, which are responsible for the increase of low-molecular-weight phenols but the decrease of polyflavonoids; A. flavipes sp. consumes simple phenols as nourishment for growth and biodegrades polyflavonoids into low-molecular-weight substances, which is responsible for the increase of simple phenols but the decrease of large-molecular-weight phenols. This study proves that bayberry tannins, one kind of condensed tannins, could be biodegraded and illustrates the biodegradation mechanism. As a cleaner and ecofriendly degradation method, it might be used in every field where condensed tannins should be removed or degraded and where tannin products are needed.

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**Notes**

The authors declare no competing financial interest.

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**Table 5. $^{13}$C NMR Chemical Shift Data of Bayberry Tannin before and after Biodegradation**

|        | C-2 trans | C-2 cis | C-3 terminal | C-3 extending | C-4 | C-4a | C-5 |
|--------|-----------|---------|--------------|---------------|-----|------|-----|
| before | 78.61     | 76.80   | 64.11        | 70.52         | 45.93 | 99.69 | 160.18 |
| after  | 78.14     | 76.78   |              | 71.00         | 45.65 | 99.78 | 160.18 |
| C-6    | C-7       | C-8     | C-8a         | C-1′          | C-2′ | C-3′ |
| before | 97.20     | 161.49  | 95.91        | 156.23        | 133.56 | 107.53 | 146.14 |
| after  | 156.55    | 134.13  | C-1″         | C-2″          | C-3″ | C-4″ |
| C-4′   | C-5′      | C-6′    | C-1″         | 121.50        | 110.96 | 152.60 | 139.98 |
| before | 139.98    | 146.14  | 108.51       |               |      |      | 139.84 |
| after  | 139.84    | 146.20  | 109.16       |               |      |      |      |
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