# Bioconversion of Ferulic Acid into High Value Metabolites by White Rot Fungi Isolated from Fruiting-Body of the Polypore Mushroom

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***Abstract*—The trend toward natural flavors has led to extensive research in this area. In the biotechnological process, ferulic acid was commonly used as a substrate and employs microorganisms as a biocatalyst. In this study, the potential of three white rot fungi- Ganoderma mastoporum, Ganoderma australe and Trametes pavonia isolated from the fruiting-body of the polypore mushroom and two strains of commercial mushrooms (Ganoderma lucidum and Schizophyllum commune) were investigated for their ability to convert ferulic acid into its metabolites using GC-MS. The results showed that G. lucidum, S. commune and T. pavonia were able to convert ferulic acid to 4-vinyl guaiacol as a major degradation product, while the extract of G. australe and G. mastoporum grown in the same medium were found to obtain vanillin as a major component. Moreover, various degradation products, including methoxy benzoquinone, 2-methoxyhydroquinone and methyl eugenol, were also found in this experiment.**

***Index Terms*—bioconversion, ferulic acid, 4-vinyl guaiacol, vanillin, white rot fungi**

## I. Introduction

The interest in using ferulic acid as a starting material in the bioconversion process has increased steadily since it is considered cheap, abundant and easily available [1]. Ferulic acid is the main phenolic compound that is found in the cell wall of plants in the form of an ester linkage. It could be converted to 4-vinyl guaiacol and vanillin through biocatalytic routes [2]. 4-Vinyl guaiacol (3-methoxy-4-hydroxystyrene), a styrene type molecule, is a valuable starting material for fragrances, flavors, oxygenated biodegradable polymers and is an intermediate for organic synthesis [3]. Moreover, ferulic acid can be used as a starting material to produce vanillin (4-hydroxy-3-methoxybenzaldehyde), an important flavor compound used in beverages and other food industries, such as in breads, cakes, ice cream, chocolate and confectionery products, as well as fragrances. In the previous study, several microbial strains were studied for the bioconversion of ferulic acid. However, white rot fungi seemed to have a great advantage in the bioconversion process of phenolic compounds as they are able to completely degrade lignin and to metabolize the resulting phenolic monomers into high value aromatic compounds [4], [5]. The bioconversion of ferulic acid by white rot fungi has been investigated intensively and it has been found that it can generate many high priced compounds. Thus, this study reports on the ability of both the isolated fungi (*Ganoderma mastoporum, G. australe* and *Trametes pavonia*) and the commercial mushrooms (*G. lucidum* and *Schizophyllum commune*) in converting ferulic acid into its metabolites.

Manuscript received May 2, 2013; revised July 3, 2013.

## II. Materials and Methods

### A. Chemicals

Ferulic acid (trans-, 99%) was purchased from Sigma-Aldrich. Potato dextrose agar (PDA) was obtained from Difco. All solvents used for High Performance Liquid Chromatography (HPLC) analysis were of the HPLC grade and all other chemicals were of the analytical grade.

### B. Selection, Isolation and Identification of Fungal Strains

#### 1) Selection of fungal strains

The mycelia of five fungal strains (three wild-type strains and two commercial strains) were used in this study to convert ferulic acid into aromatic biodegradation products. Wild-type strains (CMU-HM1, CMU-HM2 and CMU-HM3) were collected from two sites within the Medicinal Plant Garden and the Huay Kok Ma area of Doi Suthep-Pui National Park of Thailand. Commercial strains were purchased from the Biotechnology Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand (*G. lucidum* and *S. commune*). All fungal strains were grown and maintained on PDA slants and incubated at ambient temperature.

#### 2) Isolation and identification of wild type strains

Polypore fungi (CMU-HM1, CMU-HM2 and CMU-HM3) were collected from two sites within the Medicinal Plant Garden (18°48'20"N, 98°54'51"E and at altitude 1,055 m) and the Huay Kok Ma area (18°48'24"N, 98°54'36"E and at altitude 1,100 m) of Doi Suthep-Pui National Park of Thailand, during the period of time from mid-October to November 2009. Specimens were


doi: 10.12720/jomb.2.3.168-172

wrapped in aluminum foil during transport back to the laboratory. The mycelia were isolated from sporocarps by aseptically removing a small piece of mycelium from the inside and transferring them onto a PDA plate. The plates were incubated at 30°C in the dark. The mycelia emerging from tissue were selected and transferred to a new PDA plate. The pure cultures were kept on PDA slants for further use. The cultures were kept in both sterile distilled water at 4°C and 20% glycerol at -20°C for long-term preservation.

A CTAB method [6], [7] was performed for the extraction of DNA from sporocarp or mycelium, amplifying for 18s rDNA. The sequences were compared with the Genbank database. A phylogenetic tree of DNA sequences were constructed using the neighbor-joining method [8]. Multiple alignments of sequences determined in this study and reference sequences obtained from databases were combined in the calculations of sequence similarity using CLUSTAL W 1.74 [9] with arithmetic averages for tree-making algorithms obtained from the MEGA package version 4.0 [10]. The topologies of the neighbor-joining phylogenetic trees were evaluated based on the bootstrap analyses of 1,000 replicates.

### C. Bioconversion of Ferulic Acid in Basal Medium

The analysis of the bioconversion products from ferulic acid by five fungal strains in the basal medium was analyzed. The basal medium for growth of all fungal strains was prepared as described previously by Zheng *et al.* [11]. Fungal strains were grown on PDA plates at ambient temperature for 7 days and three discs (6 mm diameter) of mycelia containing agar were separately inoculated in 125 mL flasks containing 25 mL basal medium. The media were incubated at 30°C and 200 rpm. After 48 hours of incubation, ferulic acid (1 mM final concentration) was added into the medium. The bioconversion was carried out at 30°C and 200 rpm. The samples were collected every 3 hours over a 36-hour period. The fermented broth was extracted with an equal volume of methanol (HPLC grade). After that, the samples were filtered quickly through a 0.2 μm membrane filter, and applied to HPLC for determination of the ferulic acid residue. The collection time of each fungal was different, *T. pavonia*, *G. mastoporum*, *S. commune*, *G. australe* and *G. lucidum* were collected at 9, 12, 12, 24 and 36 hours, respectively. The supernatants were filtered through Whatman No. 1 and then extracted twice with an equal volume of dichloromethane. Dichloromethane fractions were evaporated under reduced pressure and analyzed using gas chromatography coupled with mass-spectrometry (GC-MS).

### D. Analytical Methods

#### 1) HPLC

The HPLC analysis of ferulic acid was performed on an Agilent 1100 series equipped with a binary pump, a sample injector and variable wave length ultraviolet detector. The column used was a Zorbax SB-C18, 5 μm (4.6x150 mm) type from Agilent USA. The fingerprints were recorded at an optimized wave length of 280 nm. The flow rate was set to 0.8 mL/min. A linear gradient of two solvents was used: solvent A (0.5% acetic acid in water, v/v), and solvent B (acetonitrile). The linear gradient was run at 25°C over 15 min from 5% to 20% and over 15-40 min from 20–40% of B. The total running time was 45 min. The injection volumes for all samples were 20 μL. The solvent solutions were vacuum-degassed with ultrasonication prior to usage. The samples and standards were filtered quickly through a 0.2 μm membrane filter. Quantification of ferulic acid was calculated using calibration curves prepared from the HPLC peak areas of the ferulic acid standard.

#### 2) GC-MS

The analysis of ferulic acid conversion products were performed using a Gas Chromatography (GC) 6850 Agilent Technologies/MSD 5973 Hewlett Packard, equipped with a MS detector and HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane 30 m × 0.25 μm, film thickness 0.25 μm). The injector and detector temperature were set at 270 and 280°C, respectively. The oven temperature was held at 80°C for 2 min, 120°C for 4 min and 155°C for 4 min, then programmed to 270°C at a rate of 10°C /min. The total running time was 37 min. Helium was used as a carrier gas, at a flow rate of 1 mL/min. 1 μL of sample was injected in the splitless mode. GC-MS detection of an electron ionization system with ionization energy of 70 eV was used. Injector and MS transfer line temperature were set at 270 and 290°C, respectively. The components were identified based on the comparison of their relative retention times and mass spectra with those of the established standards (NIST05 library data of the GC-MS system and previous literature data).

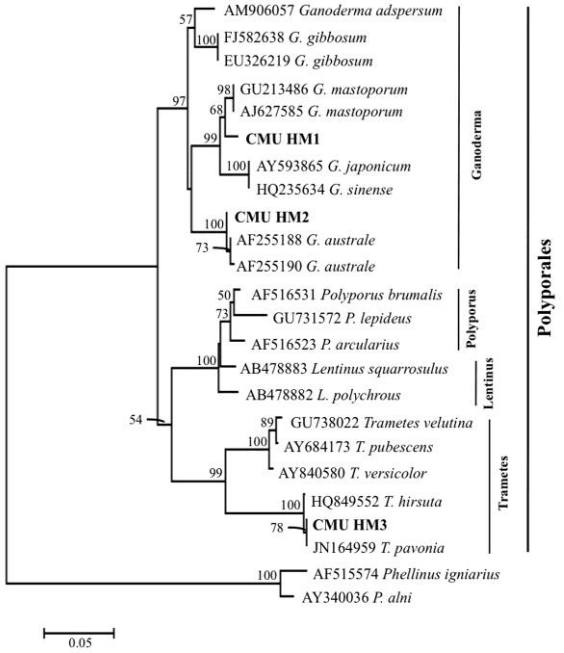



Figure. 1 The neighbour-joining tree based on the partial internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequence alignments of 24 isolates. *Phellinus igniarius* and *P. alni* were used to root the tree. Branches with bootstrap values ≥ 50% are shown at each branch and the bar represents 0.05 showing substitutions per nucleotide position.

## III. Results and Discussions

### A. Isolation and Identification of Wild Type Strains

Three isolates of polypore fungi were identified by both morphological and molecular technology. The partial internal transcribed 1, 5.8S ribosomal RNA gene and the internal transcribed spacer 2 sequences of all fungal isolates were obtained and compared with the GenBank database. In the neighbor-joining tree, two isolates, CMU-HM1 and CMU-HM2, were placed in the genus *Ganoderma* (Fig. 1).

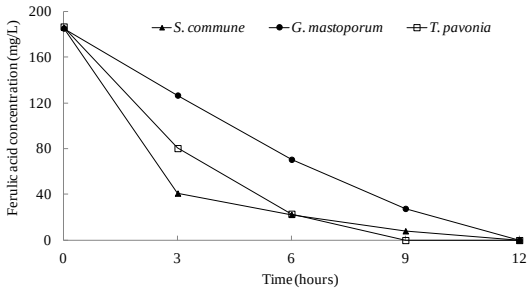
It was suggested that CMU-HM1 isolate was closely related to *G. mastoporum*, while CMU-HM2 was associated with *G. australe*, intimately with 100% bootstrap support. The remaining, CMU-HM3 was closely related to *T. pavonia*.

### B. Identification of Volatile Compounds in the Culture Medium

In this study, we found that all fungal mycelia used in this experiment could degrade ferulic acid. The three fungal strains, *G. mastoporum*, *S. commune* and *T. pavonia*, exhibited high degradation rates of ferulic acid, which was degraded completely within 12 hours (Fig. 2A). The results suggested that these 3 fungi possessed great degradation ability, which allowed them to metabolize ferulic acid dramatically.

In a previous published research study by Karmakar *et al*. [12], the use of a new strain of *Bacillus coagulans* BK07 isolated from decomposed wood bark was able to degrade entire ferulic acid within 7 hours. In addition, Li *et al*. [13] reported a rapid degradation of ferulic acid by *Enterobacter* sp. Px6-4 in the initial 12 hours. Whereas, the two fungal strains, *G. australe* and *G. lucidum* could completely degrade ferulic acid at 24 and 36 hours, respectively (Fig. 2B).

A.




B.

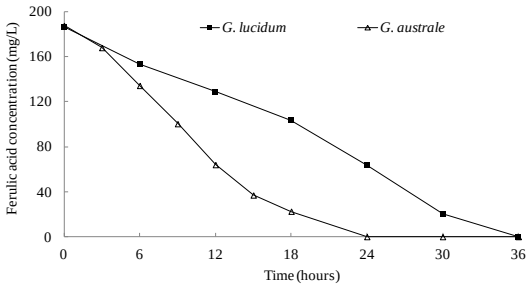



Figure 2. Ferulic acid concentration of (A) *G. mastoporum*, *S. commune* and *T. pavonia* (B) *G. australe* and *G. lucidum*.

TABLE I: PRODUCTS OF FERULIC ACID CONVERSION BY WHITE ROT FUNGI AT 30 ℃

| Compound[a] | RT[b] (min) | Major ions | Percent of total (%)[c] | | | | |
|---|---|---|---|---|---|---|---|
| | | | *G. mastoporum* | *G. australe* | *T. pavonia* | *G. lucidum* | *S. commune* |
| 1. Methoxy benzoquinone | 7.12 | 69,108,110, 138 | - | - | - | 4.32 | - |
| 2. 4-Vinyl guaiacol | 9.07 | 150,135,107,77 | 15.26 | 11.28 | 58.79 | 79.90 | 72.97 |
| 3. 2-Methoxyhydroquinone | 10.85 | 140,125,97 | - | - | - | 11.54 | - |
| 4. Vanillin | 10.95 | 151,152,181,109,123 | 84.74 | 88.72 | 41.21 | 4.24 | 18.64 |
| 5. Methyl eugenol | 11.01 | 178,151,147,152,163 | - | - | - | - | 8.39 |

[a]Compounds identified by comparing mass spectra data with NIST05 database.
[b]RT= retention time
[c]Relative percentages of the compounds were obtained electronically from FID area percent data.

The qualitative analysis of ferulic acid degradation products were analyzed by GC-MS. Each compound was identified based on mass spectral matching (≥ 90%) from National Institute of Standards and Technology (NIST) library. Only compounds that had a matching accuracy greater than or equal to 90% after removals of all background products present in blanks, are reported in Table I. The GC chromatograms showed that the main components were completely exhibited within 20 min.

By comparing the mass spectral data with the literature data, peaks at retention times of 7.12, 9.07, 10.85, 10.95 and 11.01 min were identified as methoxy benzoquinone, 4-vinyl guaiacol, 2-methoxyhydroquinone, vanillin and methyl eugenol, respectively. The results revealed that all fungal strains produce 4-vinyl guaiacol ranging from 11.28 to 79.90%. The highest 4-vinyl guaiacol was observed in *G. lucidum*. The GC chromatogram of *G. lucidum* containing 4-vinyl guaiacol and its mass

spectrum and structure were displayed in Fig. 3. Characteristic fragments were 150, 135, 107 and 77 m/z, which allowed for the confirmation of 4-vinyl guaiacol in all the extracts.

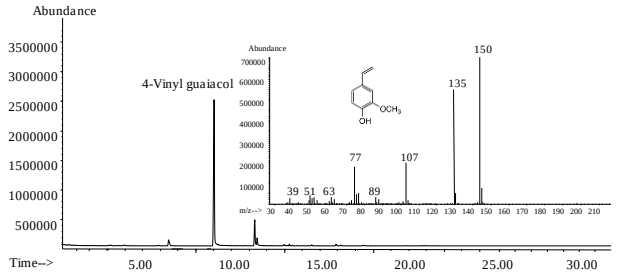



Figure 3. GC chromatogram of the extracts of *G. lucidum* and the mass spectrum and structure of 4-vinyl guaiacol.

Additionally, vanillin was found as a major component in the extract of *G. australe* (88.72%) and *G. mastoporum* (84.74%), and was also found in *T. pavonia* (41.21%), *S. commune* (18.64%) and *G. lucidum* (4.24%). The other trace compounds, such as 2-methoxyhydroquinone (11.54%) and methoxy benzoquinone (4.32%), were only discovered in *G. lucidum*, while methyl eugenol (8.39%) was found to barely exist in *S. commune*. Previous research studies have reported that ferulic acid could be converted to 4-vinyl guaiacol as a major degradation product. Donaghy *et al*. [14] suggested that ferulic acid is a precursor of 4-vinyl guaiacol, for which the bioconversion catalyzed by the enzyme ferulic acid decarboxylase, and 4-vinyl guaiacol could be further oxidized to vanillin and vanillic acid. Mabinya *et al*. [3] reported that a white rot fungus isolated from decaying wood converted ferulic acid to 4-vinyl guaiacol (80.4%), as the major compounds, while 1-(2,3-dihydroxy-4-methoxy-6-methylphenyl)-ethanone (9.80%), phenylethyl alcohol (1.24%), vanillin (1.23%), ethyl ester of vanillic acid (1.22%) and trace amounts (<0.5%) of vanillic acid, acetovanillone and 4-ethyl-2-methoxyphenol were also found. Adamu *et al*. [15] also reported that *Lactobacillus farciminis* was able to convert ferulic acid to 4-vinyl guaiacol as the major degradation product. In the study carried out by Li *et al*. [13], the rapid degradation of ferulic acid by *Enterobacter* sp. Px6-4 produced large amounts of 4-vinyl guaiacol (384.3 mg/L) and small amounts of vanillin (9.96 mg/L) in the initial 12 hours. However, 43.25% of 4-vinyl guaiacol was still detected in the medium, while the production of vanillin increased to 43.41% after 108 hours of incubation. Furthermore, Tsujiyama and Ueno [16] suggested that *S. commune* converted ferulic acid to 4-vinyl guaiacol and this product was then oxidized to vanillin and vanillic acid. However, based on our results, no accumulation of vanillic acid in the culture medium of *S. commune* was noticed, but methyl eugenol was detected instead.

## IV. Conclusion

This study demonstrated the bioconversion potential of ferulic acid by using the three white rot fungi isolated from fruiting-body of the polypore mushroom, *G. mastoporum*, *G. australe* and *T. pavonia*, and two strains of commercial mushrooms, *G. lucidum* and *S. commune* as biocatalysts. *G. lucidum*, *S. commune* and *T. pavonia* were able to convert ferulic acid to 4-vinyl guaiacol with high percentage yield, while *G. australe* and *G. mastoporum* could produce vanillin as a major component. Moreover, methyl eugenol, methoxy benzoquinone and 2-methoxyhydroquinone were also found in this experiment.

## Acknowledgements

We are grateful to Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education and Department of Chemistry, Faculty of Science and Graduate School, Chiang Mai University, Thailand for the financial support.

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