Degradation of Ferulic Acid by the Endophytic Fungus

Colletotrichum gloeosporioides TMTM-13 Associated with Ostrya rehderiana Chun

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ABSTRACT: Biodegradation of ferulic acid, by an endophytic fungus Colletotrichum gloeosporioides TMTM-13 associated with Ostrya rehderiana Chun, was explored in this study. Ferulic acid was completely degraded by TMTM-13 as its initial concentration was lower than 400 mg L\(^{-1}\). Generally, the initial concentration of ferulic acid and fungal biomass of TMTM-13 kept synchronously growing up as the concentration was lower than 400 mg L\(^{-1}\). Fungal biomass reached a maximum of almost 1.77 g L\(^{-1}\) under concentrations of 400–450 mg L\(^{-1}\). HPLC-MS analysis indicated that ferulic acid ultimately degraded to vanillin, vanillic acid, acetovanillone, and dihydroconiferyl alcohol by TMTM-13. This study was the first report about an endophytic fungus associated with O. rehderiana Chun that has great potential for practical application in ferulic acid contaminated environments.

1. INTRODUCTION

Ferulic acid is one of the important allelochemicals inducing continuous cropping obstacles in crops.\(^1\) It can result in generalized cellular disruption, which inhibits mitochondrial respiration and photosynthesis, damages the membrane structure of cells, and produces autotoxicity in crops.\(^2,3\) Meanwhile, with the development of industry, ferulic acid is now presented in high concentrations in many industrial effluents and residues, including those produced in wine distilleries, olive oil processing industries, and pulp paper processing.\(^4\) Ferulic acid is considered an environmental pollutant because it is highly toxic to organisms and difficult to degrade in natural conditions.\(^5\) Now, research on the degradation of ferulic acid has become one of the key problems in the field of industrial and agricultural environments.

Several methods have been studied over the long term for promoting the degradation of ferulic acid, including photocatalytic degradation and chemical oxidation.\(^6,7\) The use of photocatalysis has proven to be a feasible method, while it has the main limitations of low stability and the difficulty in reusing.\(^8\) Similarly, chemical oxidation, such as advanced oxidation, has demonstrated its potential in degrading ferulic acid effectively. However, the high cost and almost no prospect in economy stop its practical application in ferulic acid contaminated soil environments.\(^9\) As an alternative, fungal bioremediation is an environmentally friendly approach to remove ferulic acid. As a large and novel microbial resource, endophytic fungi, which have a broad application prospect in degradation of organic acid pollutants, are now used in the field of bioremediation. For example, endophytic Phomopsis sp. could degrade ferulic acid and sinapic acid.\(^5,10\) Ostrya rehderiana Chun, an endangered species,\(^11\) has its uniqueness in both phylogeny and biogeography, which only lives in West Tianmu Mountain, Zhejiang. Upon our preliminary screening, an endophytic fungus TMTM-13 isolated from O. rehderiana Chun exhibited potent biodegradation activities to ferulic acid. The objectives of the present studies were to identify tolerance concentration of TMTM-13 for degradation and detect the metabolic mechanism, which provide a basis for the wide-spread application of endophytic fungi in the pollutions caused by ferulic acid.

2. METHODS

2.1. Experimental Materials. Leafy branches of O. rehderiana Chun were collected from West Tianmu Mountain, Zhejiang Province.

2.2. Isolation and Identification of Strain TMTM-13. The fresh leaves of O. rehderiana Chun were washed in running water to remove unrelated adsorbates. After being sterilized in 75% ethanol for 1 min, the leaves were immersed in 3%
NaClO for 1 min and washed with distilled water five times, each for 5 s. Then they were dried on the sterile filter paper. The sterilized tissues were cut into pieces and put in the PDA medium (potato 200 g L\(^{-1}\), glucose 20 g L\(^{-1}\), agar 15–20 g L\(^{-1}\), distilled water 1000 mL, pH 7.0). After incubating at 28 °C for 3–4 days, the fungus was further purified and transferred to the slope. The fungal strain was preliminarily differentiated by the morphological characteristics. The molecular identification of target strains was performed by the ITS rDNA sequence.

2.3. Identification of the Tolerance Concentration of TMTM-13 for Degradation. The endophytic fungus was inoculated in 50 mL of PDB medium, activated at 28 °C in a shaker rotating at 180 rpm for 48 h. Fungal mycelium was collected, washed twice with distilled water, and then diluted with 100 mL of MSM liquid medium (NaNO\(_3\) 2 g L\(^{-1}\), KH\(_2\)PO\(_4\) 1 g L\(^{-1}\), KCl 0.5 g L\(^{-1}\), MgSO\(_4\) 0.5 g L\(^{-1}\), FeSO\(_4\) 0.01 g L\(^{-1}\), agar 15–20 g L\(^{-1}\), distilled water 1000 mL, pH 7.0) consisting of glass beads. The cultures were incubated at 28 °C in a shaker rotating at 180 rpm for 2 h to eliminate the available nutrients adhered on the mycelium surface and make the mycelia as disperse as possible.

Fungal mycelia of 2 mL inoculums were added to the MSM medium with the sole carbon source, ferulic acid, from 50 to 600 mg L\(^{-1}\). The cultures were incubated at 28 °C with shading treatments in a shaker rotating at 180 rpm for 72 h. The dry weight of fungal mycelium contained in 2 mL of MSM medium was weighed three times, and the average was taken. The fermented broth and fungal mycelia were collected by filtering to detect the remaining ferulic acid concentration and dried fungal biomass, respectively.

The decrement of ferulic acid was gauged by HPLC (the HPLC conditions: Agilent LC chemstation and Diamonsil C18 chromatographic column; mobile phase:carbinol(A)-0.01 M KH\(_2\)PO\(_4\) solution (pH 3.7) (B); gradient elution: 0–15 min 30% → 85% A, 15–18 min 85% A; detection wavelength: 323 nm; flow rate: 0.8 mL/min; column temperature: 28 °C; sampling amount: 10 μL).

2.4. Identifying the Metabolic Products. The fungal mycelia were added to 2 L of MSM medium with the optimum concentration of ferulic acid as the sole carbon source. They were incubated at 28 °C with shading treatments in a shaker rotating at 180 rpm for 72 h. Then the fermentation broth was collected, and the metabolites were enriched with ethyl acetate using a rotary evaporator and gauged by the HPLC-MS technique.

2.5. Statistical Analysis. Basic statistics was processed by SPSS, and diagrams were drawn with GraphPad Prism 5.

3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of Strain TMTM-13. Morphological characteristics of TMTM-13 were observed in the MSM medium. The colony of strain TMTM-13 became circle-like with a diameter of 28 mm, and its hypha was similar to fluff in high density. The middle of the colony was protruding slightly with a gray color, while the edge was flat with a gray white color. The transparent hypha had no septum under the light microscope. Moreover, hyaline conidia were produced in cylindrical shape with rounded ends. The morphological characteristics were similar to those of Colletotrichum isolates.

The ITS rDNA region of strain TMTM-13 was sequenced, and the identification was performed by comparison with published sequences in GenBank using BLAST. 20 strains of fungi were used to construct a phylogenetic tree, which indicated that the TMTM-13 was closely related to C. gloeosporioides JF710562 (Figure 1). Therefore, TMTM-13 was identified as C. gloeosporioides according to morphological characteristics and ITS rDNA sequences analysis.

![Figure 1. Phylogenetic tree of strain TMTM-13 based on the 5.8S rDNA region sequence.](image)

3.2. Effect of Ferulic Acid Concentration on Degradation. The concentration of remaining ferulic acid and fungal biomass were examined after 48 h incubation (Figure 2).

![Figure 2. Determination of optimum concentration for degradation and fungal biomass.](image)

When the initial concentration of ferulic acid was 350 mg L\(^{-1}\) or lower, there was almost no ferulic acid left in solution. If the initial concentration went up to 400–450 mg L\(^{-1}\), the remaining ferulic acid was 0.05–0.15 times the concentration of the ferulic acid at the beginning. However, the degradation ability of TMTM-13 showed an apparent decline as the initial concentrations were more than 450 mg L\(^{-1}\). Generally, the initial concentration of ferulic acid and fungal biomass of TMTM-13 kept synchronously growing up as the initial concentration was lower than 400 mg L\(^{-1}\). Furthermore, fungal biomass reached a maximum of almost 1.177 g L\(^{-1}\) under concentrations of 400–450 mg L\(^{-1}\). Nevertheless, fungal biomass presented a decreasing trend as the concentrations were more than 450 mg L\(^{-1}\), opposite to lower than 400 mg L\(^{-1}\). To our knowledge, this study was the first report about an...
endophytic fungus associated with *O. rehderiana* Chun that has great potential in the biodegradation of ferulic acid.

TMTM-13 performed well in the degradation of ferulic acid in comparison with *Phomopsis liquidambaris*, an endophytic fungus isolated from *Bischaja polycarpa*. TMTM-13 showed stronger capability of tolerance with its optimum concentrations of 400–450 mg L\(^{-1}\), while the latter reached a maximum of degradation effect when the concentration was 200 mg L\(^{-1}\). Nevertheless, the decrement of ferulic acid and the increment of fungal biomass in TMTM-13’s experiment
were a little bit lower than those in *P. liquidambaris* when both were put in the same conditions, which indicated that TMTM-13 was slightly inferior to *P. liquidambaris* in degradation efficiency.

Based on the results above, when the initial concentration of ferulic acid was under 400 mg L\(^{-1}\), almost no negative impact was put on TMTM-13, while higher ferulic acid concentrations could be poisonous. The finding was highly consistent with that of Mendonça et al.\(^9\) whose study suggested that high concentrations of xenobiotic substances were related with abnormal microbial growth. As a kind of microorganism coevolving with plants for a long time, endophytic bacteria associated with plants may be capable of eliminating environmental pollutants due to the unique living environments and metabolic pathways.\(^14\) Tian et al. found an endophytic strain B4 from *Artemisia annua* L., which presented its superior performances in absorbing, degrading, and eliminating toxicity in triclosan (TCS) bioremediation.\(^15\) *Ceratobasidium stevensii* B6 isolated from *B. polycarpa* showed high efficiency in degrading phenanthrene.\(^16\) In addition to organic compounds mentioned above, Cycon et al. demonstrated that diazinon-degrading *Serratia marcescens* had bioremediation potential to remove the organophosphorus pesticides from soils.\(^17\) Moreover, various microorganisms have been found and studied, which were useful in degrading environmental pollutants.\(^18,19\)

### 3.3. Identification of Metabolic Products

All compounds in the solution were analyzed by the HPLC-MS technique to detect relative molecular masses. According to Figure 3, four major biodegradation products were observed separately, and HPLC-MS analysis further showed that the metabolite products were vanillin, acetovanillione, vanillic acid, and dihydroconiferyl alcohol with \([M+H]^+\) \(m/z\) 151, 165, 167, and 181, respectively. Also, ferulic acid was found with a relatively high concentration. Based on the possible pathway of ferulic acid degradation in microorganisms,\(^20,21\) the proposed metabolic pathway in TMTM-13 degradation is displayed in Figure 4. However, the intermediate products 4-vinylguaiacol and coniferyl alcohol could not be examined by HPLC-MS. This might be attributed to their low concentrations, which were hard to be detected by equipment.

### 4. CONCLUSIONS

The endophytic fungus *C. gloeosporioides* TMTM-13 could grow and multiply using ferulic acid as the sole carbon source. The fungus degraded ferulic acid effectively in the MSM liquid medium. The biodegradation pathway of ferulic acid by TMTM-13 was also preliminarily proposed. This was the first to find that an endophytic fungus associated with *O. rehderiana* Chun could be used for ferulic acid degradation, which has great potential for practical applications in solving ferulic acid contaminated environments.

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