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Short communication

Degradation of ferulic acid by a white rot fungus *Schizophyllum commune*

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Summary

A ubiquitous white rot fungus *Schizophyllum commune* was used for the first time to study the degradation of ferulic acid. Vanillic acid was observed as one of the major products of ferulic acid catabolism, with vanillin formed as an intermediate. Almost 99.9% ferulic acid with a initial concentration of 5 mM was consumed by this fungus after 16 days of incubation at 37 °C.

Introduction

Many species of microorganisms are able to degrade plant aromatic compounds, thus releasing a vast amount of carbon which otherwise would be locked away in plant secondary metabolites such as lignin (Rosazza *et al*. 1995). Ferulic acid is the most abundant hydroxycinnamic acid in the plant kingdom and occurs mainly in the cell wall of cereal plants, where it is covalently linked to lignin with ether bonds (MacAdam & Grabber 2002).

A number of industrial and food applications have been reported for ferulic acid, especially based on its microbial degradation to vanillic acid. Vanillic acid is important for biotechnological applications as it is used as the starting material in the chemical synthesis of oxygenated aromatic chemicals such as vanillin, one of the most important flavour molecules (Walton *et al*. 2003). Microorganisms including basidiomycete fungi are known to be capable of degrading ferulic acid. For example, *Trametes* sp. was shown to reduce hydroxybenzoate derivatives (Nishida & Fukuzumi 1978). Vanillic acid was found to be formed during the degradation of ferulic acid by a white-rot fungus *Sporotrichum pulverulentum* (Gupta *et al*. 1981). The degradation of ferulic acid by *Pycnoporus cinnabarinus* has been well studied for biovanillin production (Bonnin *et al*. 2000). A yeast, *Rhodotorula rubra* was also shown to be capable of metabolizing ferulic acid to vanillic acid (Huang *et al*. 1993).

This work reports for the first time the capability of a white rot fungus S*chizophyllum commune* to degrade ferulic acid. *S. commune* grows on decaying logs and branches forming white fan-shaped basidiocarps. White rot fungi utilize non-cellulosic constituents of wood such as coniferyl alcohol and ferulic acid for their growth and development (Gupta *et al*. 1981). This is the rationale for choosing *Schizophyllum commune*. During the process of degradation via propenoic chain cleavage, vanillin was produced as an intermediate. This vanillin was further oxidized to vanillic acid which leached out into the medium.

Materials and methods

Microorganism

Schizophyllum commune basiodiocarps were obtained from the surface of wooden logs from a nearby forest and surface sterilization was done with 0.1% $HgCl_2$ solution. The fungus was identified at the Department of Botany, Visva-Bharati University, Santiniketan, India. Small mycelia from the fruiting bodies were transferred to potato-dextrose-agar slant and cultures were incubated at 37 °C. In order to obtain high-density cultures, the fungus was grown in potato dextrose broth (pH 6.0) for 7 days at 37 °C.

Medium and culture conditions

The fungus was grown in a minimal medium (Muheim & Lerch 1999) containing basal inorganic salts, ammonium nitrate (3.0 g/l) as a nitrogen source, hydrated magnesium sulphate (0.2 g/l), sodium chloride (0.2 g/l), potassium dihydrogen phosphate (1.0 g/l), disodium hydrogen phosphate (4.0 g/l), calcium chloride (0.05 g/l). The pH of the medium was adjusted to 7.0.

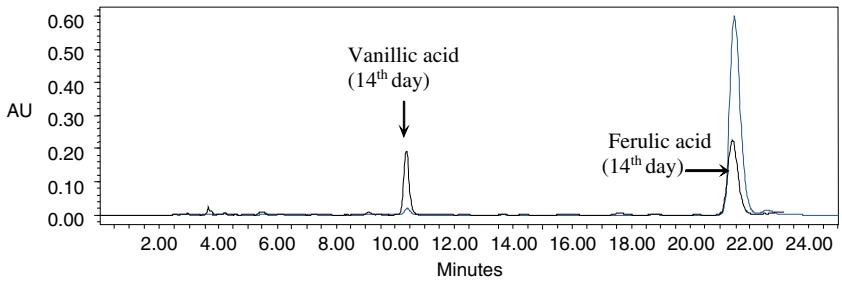

Figure 1. Overlay of HPLC chromatograms at 254 nm showing vanillic acid formation from ferulic acid on day 4 and day 14 of incubation.

All the carbon substrates were filter sterilized through 0.2 μm nylon filters (Sigma, USA), before their addition to minimal medium. After growth on potato dextrose broth for 7 days, mycelial mass was collected under sterile conditions and cut into equal pieces of 0.5 g. These were individually added to the 100 ml flask each containing 25 ml of minimal medium with ferulic acid as a sole carbon source. The cultures were incubated at 37 °C and analysis was carried out on days 4, 8, 10, 12, 14 and 16 in triplicate.

Growth measurements

Growth was measured in terms of dry weight of mycelium after filtration on glass-fibre filters (GF/D, Whatman) and dry weight at 60 °C.

Analysis of degradation products

Culture supernatants prepared by centrifugation at $4000 \times g$ for 10 min, were acidified (pH 1–2) and extracted with an equal volume of ethyl acetate. Ethyl acetate fractions were evaporated to dryness under reduced pressure. These were resuspended in 1 ml of aqueous methanol (50% v/v), and applied to thin layer chromatograms. The TLC analysis was performed as described by Dey *et al.* (2003). The TLC plates were developed in 2% aqueous formic acid. The phenolics were viewed under a dual-wavelength (254/365 nm) UV-lamp (Uvitec, Cambridge, UK). The bands corresponding to authentic standards were detected on the plate (Figure 1). For further confirmation, 20 μl of crude extract was injected directly into HPLC.

HPLC analysis

Separation was performed on a Phenomenex™ (Torrence, CA, USA) C_{18} column (RP-HYDRO 4 μm, 250×4.6 mm) using a BREEZE™ HPLC (Waters, Milford, USA) equipped with a Waters 2487 Dual Absorbance Detector set at 254 and 310 nm. A guard column (Phenomenex Security Guard™ C_{18} ODS 4×3.0 mm) was positioned just before the analytical column. An isocratic linear solvent system of methanol (32%) and 1 mM aqueous trifluoroacetic acid (68%) with flow rate of 1.0 ml/min for 25 min at room temperature was used to elute the phenolic compounds. Data were analysed on Windows XP™ platform BREEZE™ software version 3.20 (Waters). The identity of each phenolic compound was confirmed by comparing retention times and u.v.-spectra with those of the authentic compounds. Quantification was also performed with BREEZE™ software using external standards.

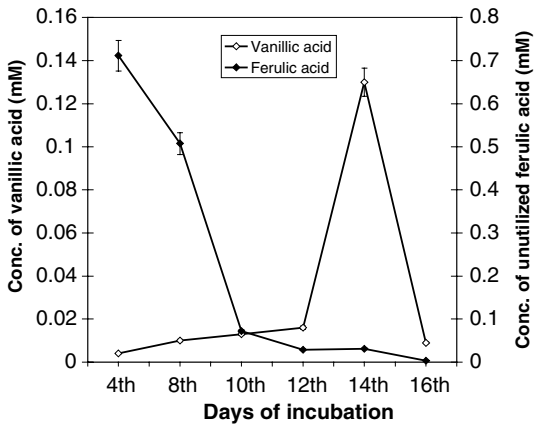

Figure 2. Time course of vanillic acid production and ferulic acid degradation by *Schizophyllum commune* grown on 5.0 mM ferulic acid.

Results and discussion

The white rot fungus, *S. commune* was found to be capable of transiently accumulating vanillic acid in the medium during ferulic acid degradation. In preliminary experiments, the study of the effect of various concentrations of ferulic acid on vanillic acid formation was examined by flask experiments. Cells were grown aerobically in minimal media containing various concentrations (1.0, 2.5, 5.0, 7.5, 10.0 mM) of ferulic acid as a sole carbon source. The time course study of ferulic acid depletion was carried out by sampling the culture at 4, 8, 10, 12, 14 and 16 day intervals. TLC and HPLC analyses showed that in all the cases, vanillic acid was detected as the major degradation product of ferulic acid (Figure 1). HPLC quantification indicated maxi-

mum amount of vanillic acid at 5.0 mM conc. on day 14 of incubation. The increase in the yield of vanillic acid is not linearly correlated to the increase in the concentration of ferulic acid. Ferulic acid was somewhat inhibitory to mycelial growth of the fungus at high concentration, though it promoted growth of *S. commune* at low concentration as reported for other white rot fungi (Nishida & Fukuzumi 1978).

On the basis of the above results, the time course study was carried out with 5.0 mM ferulic acid. Growth responses in the time course study using 5.0 mM ferulic acid were not visible. Probably at this stage, cells were keeping themselves metabolically active by utilizing ferulic acid as sole carbon source. It was found that *S. commune* cultures are capable of accumulating vanillic acid and small amounts of vanillin in the medium during the degradation of ferulic acid. These major degradation products of ferulic acid were quantified by HPLC. A maximum amount of vanillic acid, 0.13 mM was found to be accumulated in the culture media, on day 14 of incubation. After day 16 of incubation, 99.9% of ferulic acid had been consumed (Figure 2).

In order to investigate the degradation route of ferulic acid, the aromatic metabolites such as vanillin and vanillic were used as sole carbon sources respectively in separate experiments. Cultures were grown in minimal media containing either vanillin (5.0 mM) or vanillic acid (10.0 mM) as sole source of carbon. Culture media were analysed after 2-days and 6-days of incubation for detecting any degradation products formed as a result of those carbon sources used. When vanillin was added to the medium it was very slowly oxidized to vanillic acid, whereas the production of vanillin from vanillic acid was not observed. Traces of other unidentified compounds were also observed in culture filtrates (data not shown). These observations were consistent with the report of Ander *et al*. (1980), where aromatic aldehydes, such as vanillin and *p*-hydroxybenzaldehyde were shown to be toxic for white rot fungi and inhibit the growth of fungi, as compared to other phenolics. A similar inducible route for ferulic acid degradation was reported in *Pseudomonas fluorescens* (Narbad & Gasson 1998).

Our result suggests a novel pathway of ferulic acid catabolism. This white-rot fungus (*S. commune*) degraded ferulic acid into vanillic acid via vanillin as an intermediate (Figure 3). This route differs from the ferulate degradation pathways reported in another white-rot fungus, *Pycnoporus*, where vanillic acid was observed as an intermediate. Later, this vanillic acid was either converted into methoxyhydroquinone by oxidative decarboxylation, or reduced to vanillin and vanillyl alcohol (Krings *et al*. 2001). Earlier work of Nishida & Fukuzumi (1978) on *Trametes* sp. also demonstrated a different route of degradation, where ferulic acid was converted to coniferyl alcohol, which was further degraded to vanillic acid, vanillyl alcohol and methoxyhydroquinone.

Schizophyllum commune cultures were earlier shown to induce ferulic acid esterase activity upon growth on agro-wastes such as wheat bran, and liberates ferulic acid into the medium (Mackenzie & Bilous 1988). Our work complements this earlier finding by reporting a previously unexplored ferulate biotransformation route to vanillic acid, with vanillin as an intermediate. Work is in progress to characterize the enzyme responsible for this biotransformation.

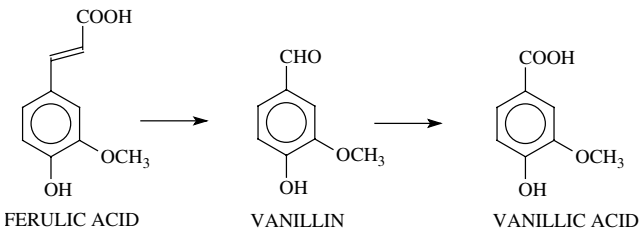

Figure 3. Proposed pathway of ferulic acid degradation in *Schizophyllum commune*.

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