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Full Length Research Paper

Transforming *p*-coumaric acid into *p*-hydroxybenzoic acid by the mycelial culture of a white rot fungus *Schizophyllum commune*

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The aim of this paper was to study the catabolic product(s) of *p*-coumaric acid by a white rot fungus, *Schizophyllum commune*. TLC and HPLC chromatogram showed that *p*-hydroxybenzoic acid was formed as a major degradation product of *p*-coumaric acid. The purity of *p*-hydroxybenzoic acid was further confirmed by mass spectrometry. A maximum amount 2.5 mg/l of *p*-hydroxybenzoic acid was detected in the culture medium on 12th day of incubation using 5.0 mM *p*-coumaric acid as sole source of carbon. It was assumed that during the process of *p*-coumaric acid degradation, *p*-hydroxybenzaldehyde was produced as an unstable intermediate, which was rapidly converted into *p*-hydroxybenzoic acid and accumulated in the media. An indication of a very slow reverse conversion, that is, *p*-hydroxybenzoic acid into *p*-hydroxybenzaldehyde was also traced.

Key words: *p*-coumaric acid, *Schizophyllum commune*, *p*-hydroxybenzoic acid.

INTRODUCTION

Hydroxycinnamates such as *p*-coumaric acid is one of the major components of plant cell wall particularly in monocotyledons (Harris and Hartley, 1980). For example, in maize bran, it constitutes around 4% of biomass dry weight (Faulds et al., 1997). A considerable proportion of *p*-coumaric acid is known to be esterified with lignin (MacAdam and Grabber, 2002). Could these large quantities of naturally available *p*-coumaric acid serve as a precursor for the biocatalytic production of value-added aromatic natural products? White rot fungi are well known for their capability to degrade lignin components upon attack on wooden logs (Kirk and Farrell, 1987) thus releasing hydroxycinnamates which otherwise would be locked away in lignin (Rosazza et al., 1995). *Schizophyllum commune* is one such widely distributed white rot fungus, recognized by its tiny fruiting bodies which lack stems and attaches itself on the dead wood of trees. *S. commune* cultures were earlier shown to induce ferulic acid esterase activity upon growth on agro-wastes such as wheat bran and liberate ferulic acid into the medium (Mackenzie and Bilous, 1988). Various white rot fungi have been reported for their ability to convert these hydroxycinnamates into hydroxybenzoates having commercial importance. *p*-Hydroxybenzoic acid and its derivatives find important applications as dietary antioxidant (Tomas-Barberan and Clifford, 2000), natural flavour (Walton et al., 2003), preservative, medicines and also as monomers for liquid crystal polymers currently used in various electronic devices (McQualter et al., 2005).

The present work reports for the first time, the capability of *S. commune* to degrade *p*-coumaric acid into *p*-hydroxybenzoic acid. The catabolic route of *p*-coumaric acid was also investigated on the basis of the identification of metabolic intermediate.

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MATERIALS AND METHODS

Microorganism

Aseptic mycelial cultures of *S. commune* were established from basidiocarps obtained from the surface of the wooden logs as described by Siva Lakshmi (2002). The cultures were grown in slants of Potato Dextrose Agar (PDA) and incubated at 37°C for 7 days. Stock cultures were maintained on PDA slant at 4°C.

Medium and culture conditions

Mycelial mats were grown in a minimal medium containing basal inorganic salts as essentially described by Ghosh et al. (2005). The initial pH of the medium was adjusted to 7.0 before autoclaving for 15 min at 121°C. All the carbon substrates were filter sterilized using disposable-syringe filter (0.2 µ pore size) before their addition to minimal media. Cultivation of *Schizophyllum* was carried out in conical flask of 100 ml capacity containing 25 ml of Potato dextrose broth (pH 6.0) at 37°C for 7 days. The 7-day old aseptic cultures of *Schizophyllum* (0.5 g fresh mass) were dried on sterilized filter paper and subsequently transferred to minimal media containing *p*-coumaric acid as sole carbon source. The cultures were incubated at 37°C and analyses were carried out in triplicate after 4, 8, 12 and 16 days of incubation.

Extraction of the metabolites

Culture filtrate was acidified (pH 1-2) and extracted with equal volume of ethyl acetate. The ethyl acetate was dried out using the rotary vacuum evaporator and residue was redissolved in 50% methanol and spotted onto thin layer chromatography (TLC) plate.

Separation and identification of the phenolic acid derivatives by TLC/ UV-spectroscopy

TLC was carried out on Avicel® Microcrystalline cellulose plate (E. Merck, Mumbai, India). The TLC analysis was performed as described by Dey et al. (2003). The plates were developed in 2% aqueous formic acid. The phenolic acids were viewed under a dual-wavelength (254 nm / 312 nm) UV-lamp (UVItec, Cambridge, UK). The bands corresponding to authentic standards were detected on the plate. The individual band that corresponded to the R_f values of the authentic standard was eluted with methanol (50% v/v). The extract was centrifuged at 5000 g for 10 min. The supernatant free from any cellulose fraction were collected. UV scan of the supernatant containing the phenolic compound was performed in a SPECORD S 100 UV-VIS scanning diode-array spectrophotometer (Analytik Jena AG, Jena, Germany).

HPLC analysis

Separation and quantification of phenolic compounds from methanol extract was performed on a *Phenomenex*™ (Torrence, CA, USA) C_{18} column (HYDRO-RP 4 µm, 250 x 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 x 3.0 mm) was positioned just before the analytical column. An isocratic linear solvent system of methanol (32%) an 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 (Sachan et al., 2004). Samples were analyzed on a Windows XP™ platform with *BREEZE*™ software version 3.20 (Waters). Identification of each phenolic compound was confirmed by comparing retention time and UV-spectra with external standards. For further confirmation, mass spectrometry was carried out.

Mass spectrometric analysis

HPLC purified samples were subjected to electron spray ionization mass spectrometric (ESI-MS) analysis. Sample were analysed in Micromass® LCT™ Mass Spectrometer (Waters, Milford, USA), linked with MassLynx™ Software version 3.5 (Waters) for data processing. The samples were injected using a syringe attached with Harvard Syringe Pump. Phenolic acids were analysed in negative ion mode. Operational details are as follows: The electrospray capillary voltage and cone voltage in negative ion mode were 3.9 and 0.03 KV respectively. The source temperature was set to 100°C and the desolvation temperature was set to 120°C. Mass spectra were scanned at unit mass resolution from mass to charge ratio of 100 to 200 at a rate of 1 s per scan with an interscan time of 0.1 s. Compounds in sample were identified by comparison with their authentic standards.

RESULTS

Identification of biotransformed product of *p*-coumaric acid

In preliminary experiments, the time course study of *p*-coumaric acid degradation by *S. commune* was carried out at 3°C for 16 days. The cultures were analysed as described previously for the detection of the degradation products. The processed culture filtrate was subjected for TLC and HPLC. TLC chromatogram of processed culture filtrate showed two bands under UV light (Figure 1). The Rf value of lower band and upper band was compared with the authentic standards of *p*-coumaric acid and *p*-hydroxybenzoic acid respectively. The band corresponding to the authentic standard of *p*-hydroxybenzoic acid was scrapped from the TLC plate, processed and subjected to UV scan (Figure 2). Quantification of the substrate and its degradation products was performed in HPLC. The decrease in concentration of the substrate with an increase in product formation was observed in HPLC chromatogram (Figure 3). The chemical identity of *p*-hydroxybenzoic acid was further confirmed by mass-spectrometry. ESI-MS done in negative mode shows one mass less than the original mass of both the authentic standard and sample as shown here in case of *p*-hydroxybenzoic acid (Figure 4).

Effect of various concentration of *p*-coumaric acid on the production of *p*-hydroxybenzoic acid

The major phenolic metabolite detected as a result of *p*-

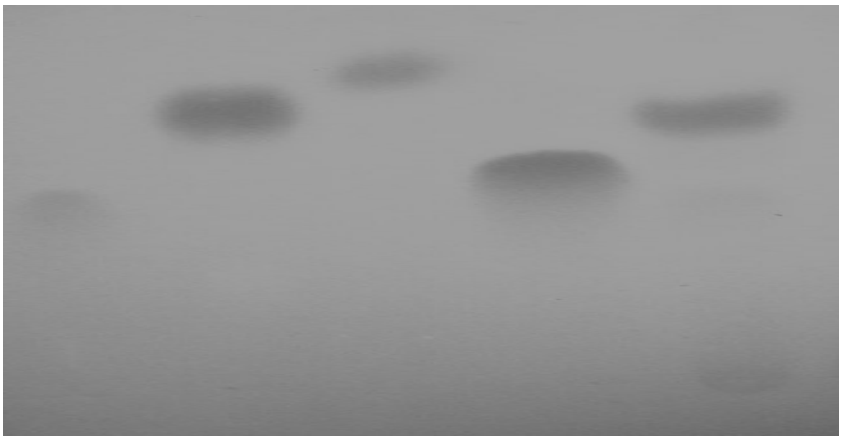

Figure 1. TLC chromatogram of *p*-coumaric acid and *p*-hydroxybenzoic acid detected from the processed culture filtrate of *Schizophyllum commune* grown on minimal media containing *p*-coumaric acid as sole carbon source.

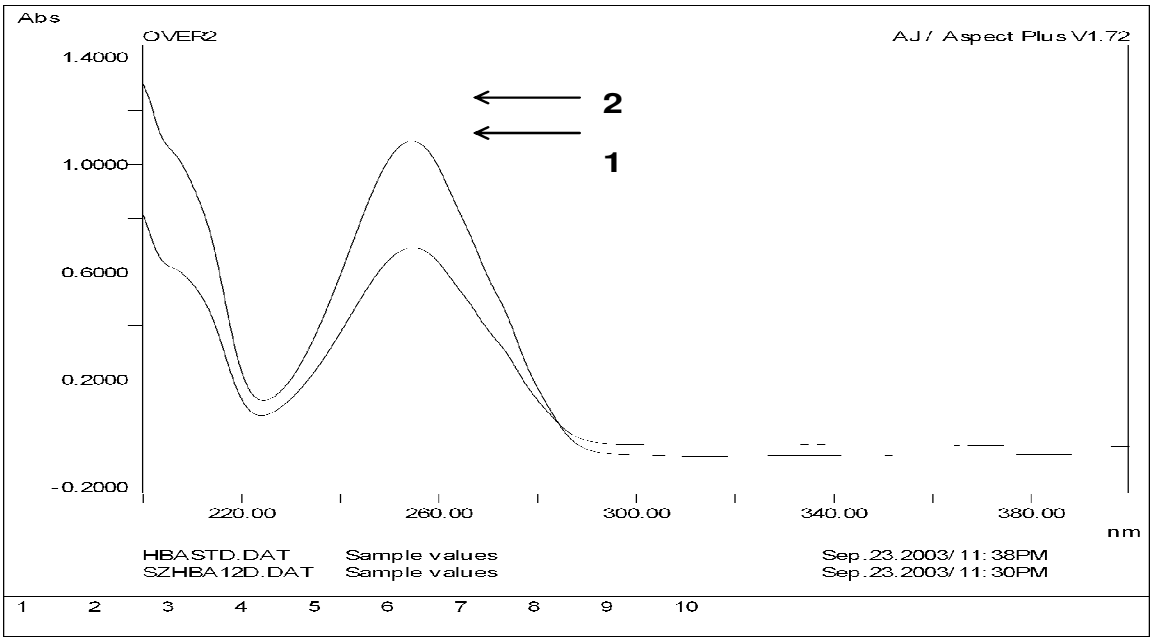

Figure 2. UV-Spectral overlay of standard *p*-hydroxybenzoic acid (1) and that produced from *p*-coumaric acid by *S. commune* (2).

coumaric acid degradation was *p*-hydroxybenzoic acid. This aromatic metabolite was only detected when *p*-coumaric acid was added into the minimal media, while control (without *p*-coumaric acid) cultures showed negative results. The effect of different concentrations of *p*-coumaric acid (1.0, 2.5, 5.0, 7.5, 10.0 mM) on *p*-

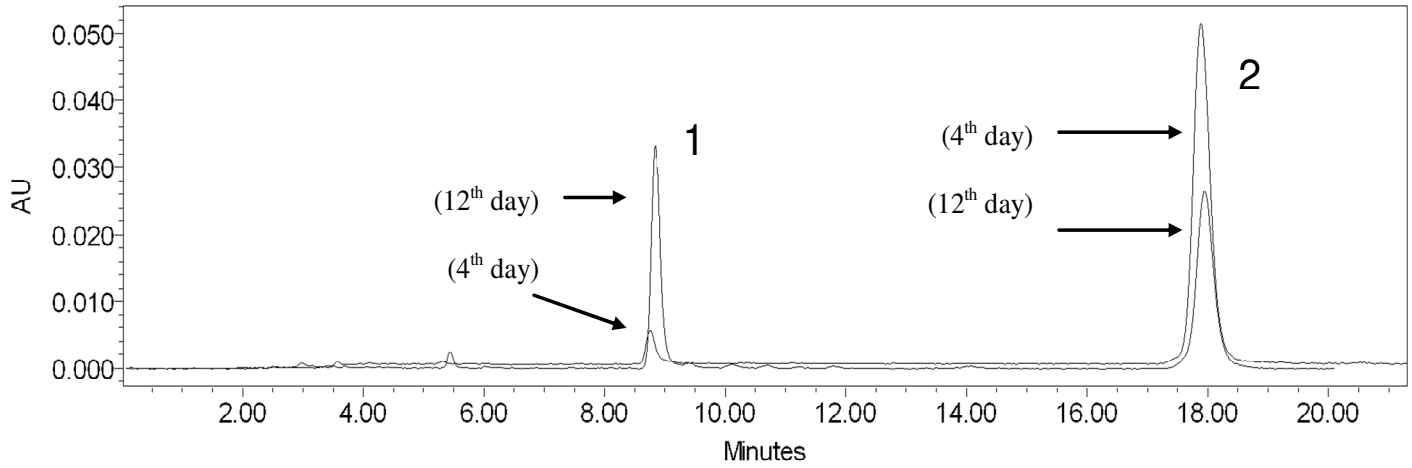

Figure 3. Overlay of HPLC chromatograms at 254 nm showing increase in the formation of *p*-hydroxybenzoic acid (1) and decrease in the concentration of *p*-coumaric acid (2) after 3rd and 11th day of incubation.

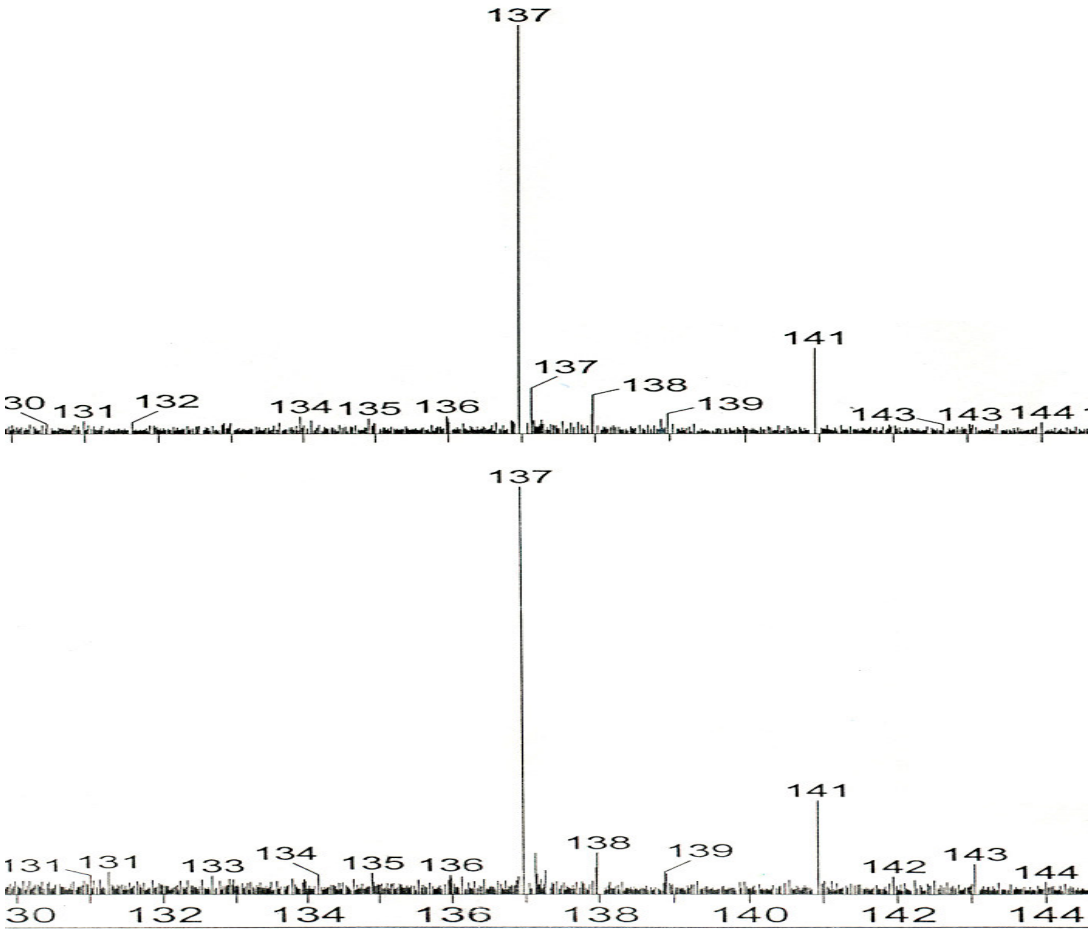

Figure 4. Mass Spectra of *p*-hydroxybenzoic acid produced from *p*-coumaric acid by *S .commune* (upper fragment) as compared to mass spectra of *p*-hydroxybenzoic acid standard (lower fragment). ESI-MS of HPLC purified samples was done in negative ion mode. X-axis shows mass to charge ratio, whereas Y-axis shows relative intensity.

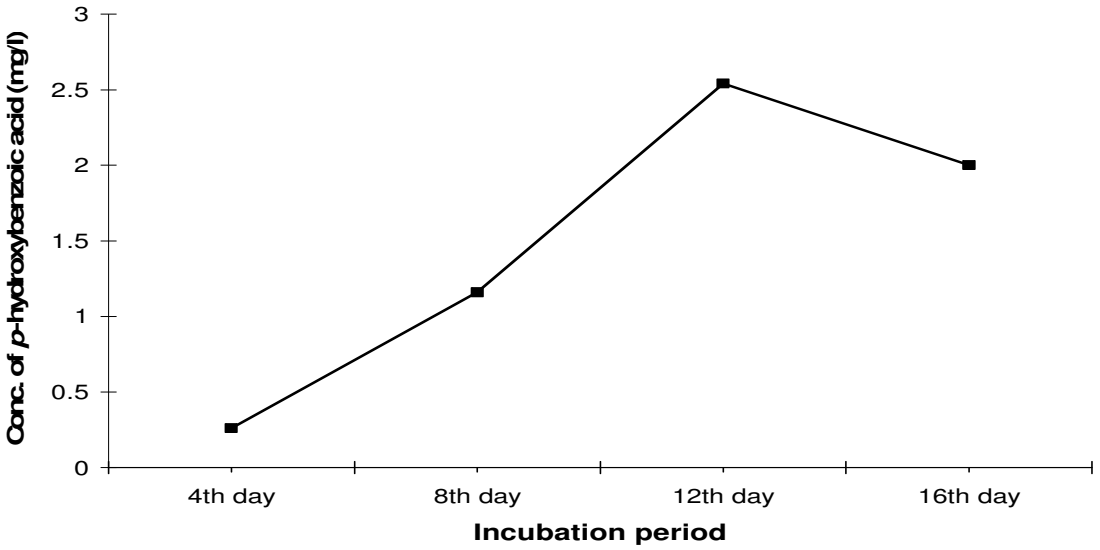

Figure 5. Time course analysis of *p*-hydroxybenzoic acid accumulation in culture media of *S. commune* grown on 5.0 mM of *p*-coumaric acid as sole carbon source.

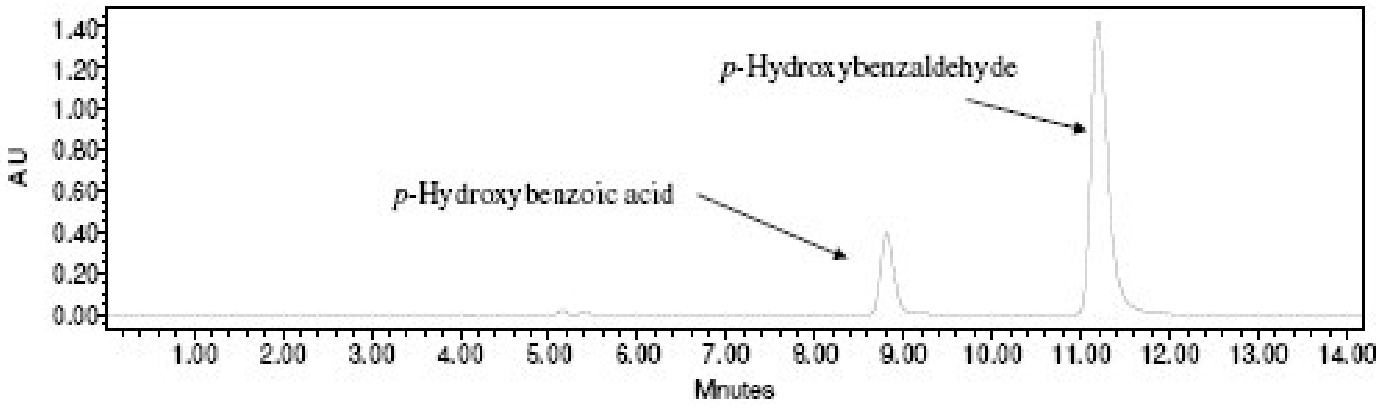

Figure 6a. HPLC Chromatogram showing the conversion of *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid at 254 nm by mycelial cultures of *Schizophyllum* after 5th day of incubation.

hydroxybenzoic acid production was examined by time-course experiments. The optimum temperature and pH were 37°C and 7.0 respectively. The time course of bioconversion was examined by sampling the culture at day 4, 8, 12 and 16. Maximum accumulation of *p*-hydroxybenzoic acid (2.5 mg/l) was obtained in the culture media containing 5.0 mM *p*-coumaric acid on 12th day of incubation (Figure 5). Consumption of *p*-coumaric acid by *S. commune* mycelial culture was very quick (data not shown), but only a part of utilized *p*-coumaric acid was biotransformed to *p*-hydroxybenzoic acid. It appears that the fungus for its growth consumed most of the *p*-coumaric acid, though visual changes in the mycelial growth remained unnoticed.

Catabolic pathway of *p*-coumaric acid

In order to investigate the route of *p*-coumaric acid degradation by *S. commune*, the metabolites previously detected in processed culture filtrate (Figure 3) or reported elsewhere (Alvarado et al., 2001) were used as sole source of carbon in minimal media. When *p*-hydroxybenzaldehyde was added in the medium as sole carbon source, it was oxidized by the fungus to *p*-hydroxybenzoic acid rapidly (Figure 6a). Interestingly,

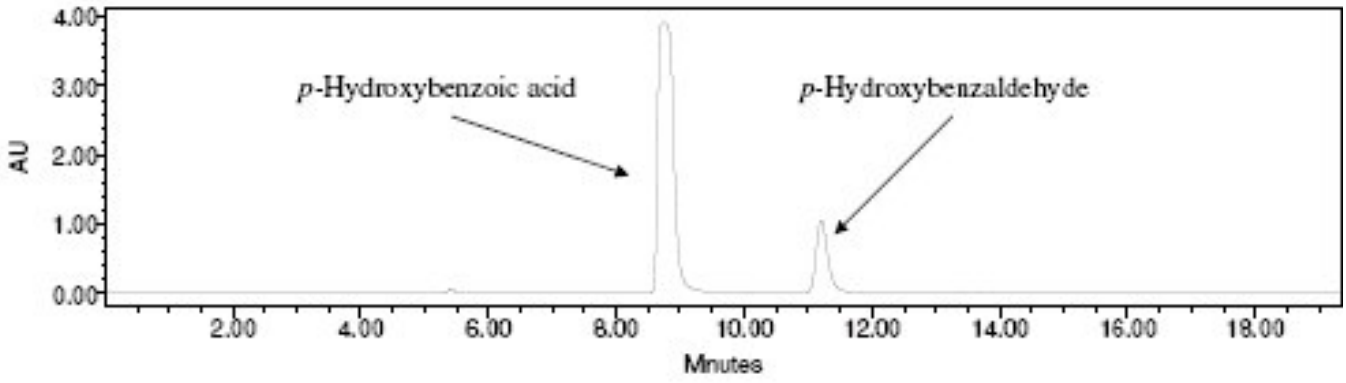

Figure 6b. HPLC Chromatogram representing the conversion of *p*-hydroxybenzoic acid to *p*-hydroxybenzaldehyde (reverse route) at 254 nm by mycelial cultures of *Schizophyllum* after 5th day of incubation.

with *p*-hydroxybenzoic acid as substrate, *p*-hydroxybenzaldehyde was also detected (Figure 6b). These findings indicate a both way conversion capability (that is, *p*-hydroxybenzoic acid to *p*-hydroxybenzaldehyde and vice-versa) by *S. commune*. In control experiments, where the microorganism was absent, no such conversion was observed in either of the substrates.

DISCUSSION

Much scientific interest has been focused on the ability of microorganisms to metabolize the hydroxycinnamic acids such as ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid into hydroxybenzoate derivatives which are of commercial importance. Of these, ferulic acid degradation by white rot fungi is well explored till date. For example, a white rot fungus *Trametes sp*. was shown to reduce ferulic acid into coniferyl alcohol, which was further degraded to vanillic acid, vanillyl alcohol and methoxyhydroquinone (Nishida and Fukuzumi, 1978). The degradation of ferulic acid by another white rot fungus, *Pycnoporus cinnabarinus* was shown to proceed via vanillic acid formation, which was subsequently reduced to vanillin, vanillyl alcohol or decarboxylated to methoxyhydroquinone (Falconier et al., 1994). Formation of vanillin, vanillic acid and methoxyhydroquinone were also detected as catabolic products of ferulic acid in *Sporotrichum pulverulentum* (Ander et al., 1980; Gupta et al., 1981). Recently, vanillic acid was demonstrated to be the only major product of ferulic acid catabolism in a white rot fungus *S. commune* (Ghosh et al., 2005).

P. cinnabarinus MUCL 39533 was the only white rot fungus where *p*-coumaric acid catabolism was investigated based on identification of metabolic intermediates. An oxidative side chain degradation pathway of *p*-coumaric acid conversion to *p*-hydroxybenzoic acid has been reported in *Pycnoporus*, where *p*-hydroxybenzoic acid was produced via β-oxidation pathway. This *p*-hydroxybenzoic acid was further reduced to *p*-hydroxybenzaldehyde and *p*-hydroxybenzylalcohol (Alvarado et al., 2001). A number of other fungi have also been reported to metabolize *p*-coumaric acid to their corresponding benzoic acid by various pathways. The conversion of *p*-coumaric acid to *p*-hydroxybenzoic acid was reported in cell-free extract of *Polyporus hispidus* (French et al., 1976). A related finding was reported recently in *Paecilomyces variotii* where this fungus was shown to be capable of degrading *p*-coumaric acid into *p*-hydroxybenzoic acid (Sachan et al., 2006). In our experiment with *Schizophyllum*, we indicate a different catabolic route for *p*-coumarate degradation as compared to *Pycnoporus* system, where *p*-hydroxybenzaldehyde was detected as an intermediate for *p*-hydroxybenzoic acid formation, though a relatively weak reverse route was also noticed.

Conclusion

This work demonstrates the catabolic capability of mycelial cultures of *S. commune* to convert *p*-coumaric acid into *p*-hydroxybenzoic acid by C_2 chain cleavage. It appears that during the process of *p*-coumaric acid degradation, *p*-hydroxybenzaldehyde was produced as an unstable intermediate. This *p*-hydroxybenzaldehyde was rapidly converted into *p*-hydroxybenzoic acid, which was accumulated in the media.

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