Formation of vanillic acid from ferulic acid by *Paecilomyces variotii* MTCC 6581

Shashwati Ghosh, Ashish Sachan and Adinpunya Mitra*

Natural Product Biotechnology Group, Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur, Kharagpur 721 302, India

A filamentous fungus *Paecilomyces variotii* MTCC 6581 was analysed for its capability to transform ferulic acid into vanillate derivatives. When cultures of *P. variotii* were grown on minimal medium containing ferulic acid (10 mM) as sole carbon source, formation of vanillic acid was observed. In this case, a maximum amount of 115 mg/l of vanillic acid was accumulated after 16 days of incubation. Glucose supplementation (0.1% w/v) in the minimal medium resulted in an increased production of vanillic acid (226 mg/l), whereas with starch supplementation, a decrease in the accumulation of vanillic acid (85 mg/l) was observed. *In vitro* conversion of ferulic acid to vanillic acid was also demonstrated with cell-free extract.

Keywords: Ferulic acid, *Paecilomyces variotii*, vanillin, vanillic acid.

HYDROXYCINNAMIC acids, particularly ferulic acid and *p*-coumaric acid, occur widely in the cell walls of members of the family Poaceae. Ferulic acid is a ubiquitous phenolic acid found mainly conjugated with mono- and oligosaccharides, polyamines, lipids and polysaccharides and seldom occurs in free state in plants[1]. Biodegradation of hydroxycinnamic acids is important for the global carbon cycle from an environmental point of view, since these compounds are released from plants as breakdown products from lignin[2]. Over the past years, consumer demand for natural ingredients has widely increased. Thus, alternative natural sources of ingredients have been considered and production processes based on microbial biotransformation of natural precursors have become increasingly attractive[3]. A number of industrial and food applications were reported for ferulic acid. In principle, it seems feasible to produce vanillic acid from ferulic acid via phenylpropanoid side-chain degradation. Vanillic acid is one of the major components of biovanillin, the world's most highly prized natural flavour. Derivatives of vanillic acid are used in Europe as an analeptic medicine.

A green spore-forming fungus was isolated from dried mesocarp of tender coconut, which was identified as *Paecilomyces variotii* MTCC 6581 by the Microbial Type Culture Collection (MTCC), Chandigarh. Since *p*-hydroxybenzoic acid is one of the major phenolic constituents of mesocarp tissue of coconut[4], it was anticipated that this fungus might well be able to degrade a wide range of hydroxycinnamates and hydroxybenzoates. This communication reports the microbial transformation of ferulic acid into vanillic acid by *Paecilomyces variotii* MTCC 6581 (Figure 1). The biochemical route of ferulic acid degradation was partly investigated on the basis of identification of metabolic intermediate.

Pure cultures of *P. variotii* were initiated on Potato Dextrose Agar (PDA) slants from fungal spores at 37°C. After growth on PDA slant for 7 days, 1 ml spore suspension (10^{10-12} spores) was transferred into 100 ml flasks, each containing 25 ml of minimal medium[5]. Filter-sterilized ferulic acid was added to this as a sole carbon source. In order to study the effect of additional carbon source, filter-sterilized solution of either glucose or starch was added into minimal medium with a final concentration of 0.1% (w/v). Day basis analysis of culture media was performed to detect the degradation product of ferulic acid. Growth of the fungi was measured in terms of dry weight of mycelium after filtration on glass-fibre filters (GF/D, Whatman) and dry weight was taken at 60°C as essentially described by Estrada Alvarado and coworkers[3]. Each experiment was carried out in triplicate and repeated at least twice. Standard deviations of the analyses were less than 5%.

The culture filtrate was acidified (pH 1–2) and extracted with equal volume of ethyl acetate. Ethyl acetate was evaporated using the rotary vacuum evaporator and the residue was redissolved in 50% methanol as described by Ghosh and coworkers[6], and used for further analysis in thin layer chromatography plate (TLC) and high performance liquid chromatography (HPLC). TLC analysis was performed on Avicel® microcrystalline cellulose plate (E. Merck, Mumbai), as described earlier[4]. The supernatant free from any cellulose was collected and UV-spectral scan of TLC-elute was carried out in a SPECORD S 100 UV–VIS scanning diode-array spectrophotometer (Analytik Jena AG, Jena, Germany).

Quantitation of phenolic acids from processed culture filtrates was performed by HPLC, as described by Sachan

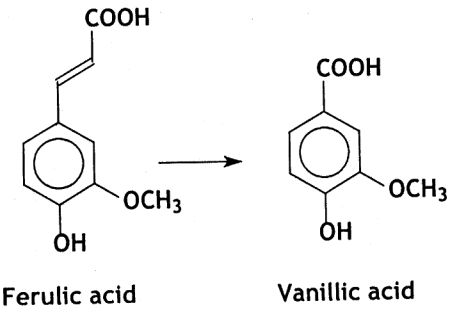

Figure 1. Structural representation of the substrate (ferulic acid) and degradation product (vanillic acid).

*For correspondence. (e-mail: adin@agfe.iitkgp.ernet.in)

and coworkers[7]. These phenolic compounds were identified by comparing the retention time and UV-spectra with authentic standards procured from Sigma-Aldrich (USA). Further confirmation of the chemical identity was carried out using Electron Spray Ionization-Mass Spectrometry (ESI-MS). HPLC-purified samples were injected using a syringe attached with Harvard Syringe Pump and analysed using Micromass® LCT™ Mass Spectrometer (Waters, Milford, USA), linked with MassLynx™ software version 3.5 (Waters) for data analysis. Phenolic acids were analysed in negative ion mode, while phenolic aldehydes were analysed in positive ion mode. Compounds in the sample were identified by comparison with authentic standards.

For *in vitro* bioconversion experiment, cultures were harvested during the exponential phase of growth by filtration. The mycelia were washed twice in 100 mM cold Tris HCl buffer (pH 7.8) and crushed in a chilled mortar containing acid-washed sand. This was finally extracted with 100 mM cold Tris HCl buffer (pH 7.8) containing 0.06% β-mercaptoethanol and 1 mM phenylmethylsulfonylfluoride (PMSF), a protease inhibitor[8]. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was desalted using ÄKTAprime™ Low Pressure Chromatography system (Amersham Pharmacia Biotech Ltd, Hong Kong) equipped with Sephadex G-25 column and monitored using Prime-View™ software. The elute was then collected and concentrated (20 times) using Amicon® Ultra-4 CFU (Millipore, USA) membrane. This concentrated elute was used as cell-free crude extract.

TLC and HPLC chromatograms of processed culture filtrate showed the formation of vanillic acid as a result of ferulic acid biotransformation (Figure 2). The purity of vanillic acid was further confirmed by UV-spectral analysis (Figure 3) and mass-spectrometry (Figure 4). In the control set without fungal culture, no ferulic acid degradation was observed. In preliminary experiments, the effects of temperature and various concentrations of ferulic acid on vanillic acid formation were examined by flask experiments. Cultures were grown aerobically in minimal media containing various concentrations of ferulic acid (1.0–10.0 mM) as the sole carbon source. Time-course studies of vanillic acid accumulation were carried out after 4, 8, 12 and 16 days of incubation. A maximum amount of 115 mg/l vanillic acid was accumulated in the minimal medium containing 10.0 mM ferulic acid, after 16 days of incubation. A decrease in the concentration of ferulic acid was observed with increase in production of vanillic acid (Figure 5). Growth of fungus was also monitored in terms of mycelial dry weight. An increase in mycelial biomass was observed with decrease in concentration of ferulic acid on a time-course basis (Figure 6). High-density culture of *P. variotii* was obtained by growing the fungus in minimal media containing 0.1% glucose or starch[9]. The aim of these experiments was to increase the production of vanillic acid by increasing the mycelial mass of the fungus. In order to reduce the production cost, starch (a cheaper carbon source) was also explored to replace glucose. However, no improvement in the yield of vanillic acid accumulation was observed in this case. When glucose was used, an increase in the production of vanillic acid (226 mg/l) was observed compared to the fungus grown in minimal media containing the same concentration of starch (Figure 7).

In order to investigate the biochemical route of ferulic acid degradation, metabolites detected in the culture medium containing ferulate as the sole carbon source, were added to the minimal media as substrates[10]. The microorganism was allowed to grow in minimal medium containing anticipated degradation products such as vanillic acid (5.0 mM) or vanillin (5.0 mM), as reported earlier in *Schizophyllum* with ferulate as the sole carbon source[6]. Day-basis analysis was carried out for detection of degradation products after first, third and fifth days of incubation. The fungus transformed vanillin into vanillic acid (Figure

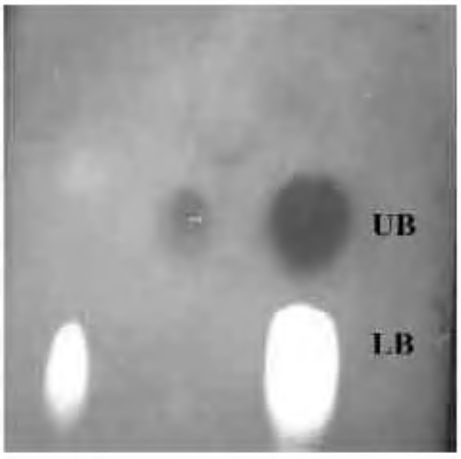

Figure 2. TLC chromatogram of processed culture filtrate of *Paecilomyces variotii* grown on minimal medium containing 10 mM ferulic acid. Lane 1, Vanillic acid standard; lane 2, Processed culture filtrate; LB, Lower band = ferulic acid; UB, Upper band = vanillic acid.

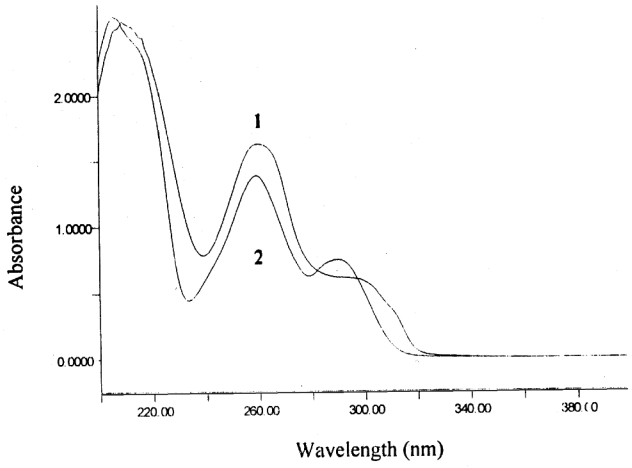

Figure 3. Overlay of UV-spectra of vanillic acid eluted from TLC-separated bands viewed at 254 nm (1) and that of standard vanillic acid (2).

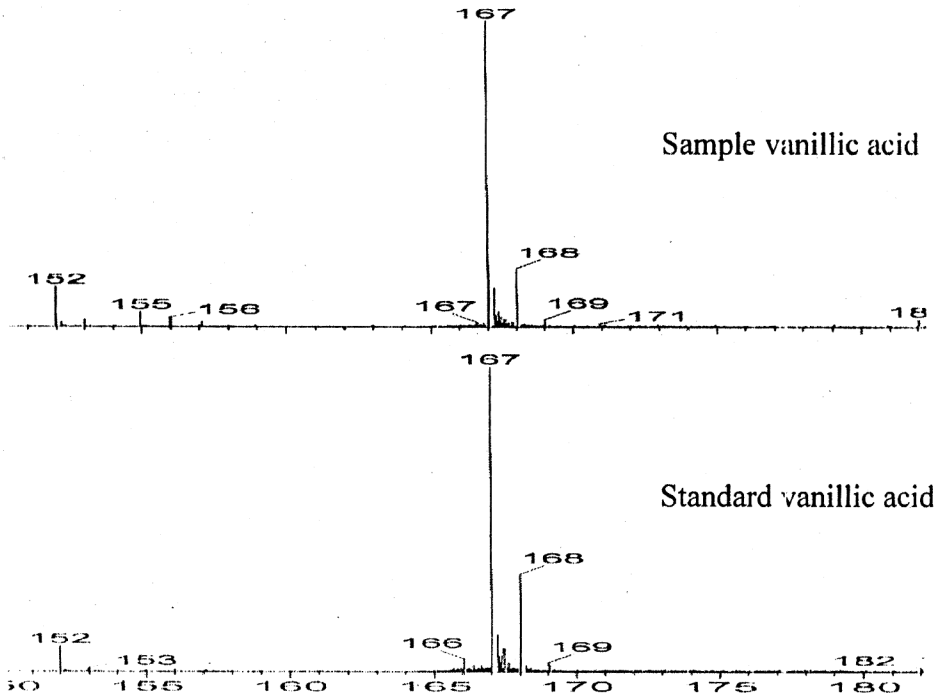

Figure 4. Mass spectral analysis of HPLC-purified standard vanillic acid and vanillic acid formed from ferulic acid by *P. variotii*. ESI-MS of vanillic acid was done in negative ion mode. *X*-axis shows mass-to-charge ratio, whereas *Y*-axis shows relative intensity.

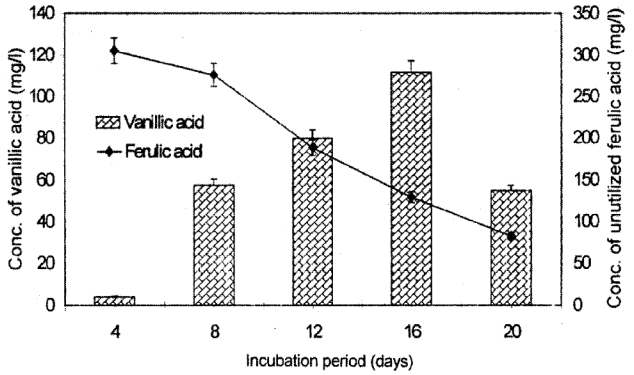

Figure 5. Time-course accumulation of vanillic acid and decrease in ferulic acid concentration in culture media of *P. variotii*.

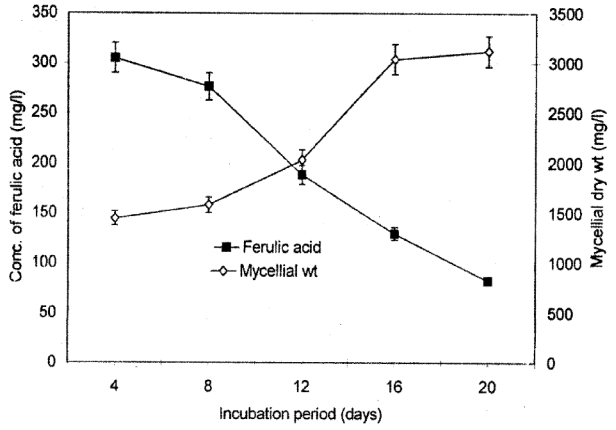

Figure 6. Effect of ferulic acid on the growth of mycelial biomass of *P. variotii*.

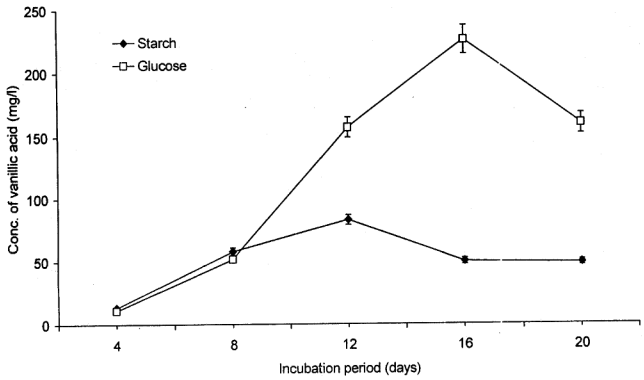

Figure 7. Time-course evaluation of vanillic acid production by supplementing additional carbon sources (glucose or starch) in medium containing 10 mM ferulic acid.

8 *a*, *b*), whereas reverse conversion was not observed (data not shown). In control experiments, where microorganism was absent, formation of vanillic acid from vanillin was not observed.

The ability of cell-free extract of this fungus to convert ferulic acid into vanillic acid was examined. The complete reaction mixture (1 ml) contained 3 mM ATP, 3 mM $MgCl_2$, 1.3 mM NAD, 0.25 mM CoA, 100 mM Tris HCl buffer (pH 8.5), cell-free extract (200 µl) and 50 µM ferulic acid[11]. The mixture was incubated for 1 h at 37°C and the reaction was stopped by adding an equal volume of acetic acid : methanol (1 : 4). Vanillic acid formation was detected in the reaction mixture by HPLC (Figure 9). In

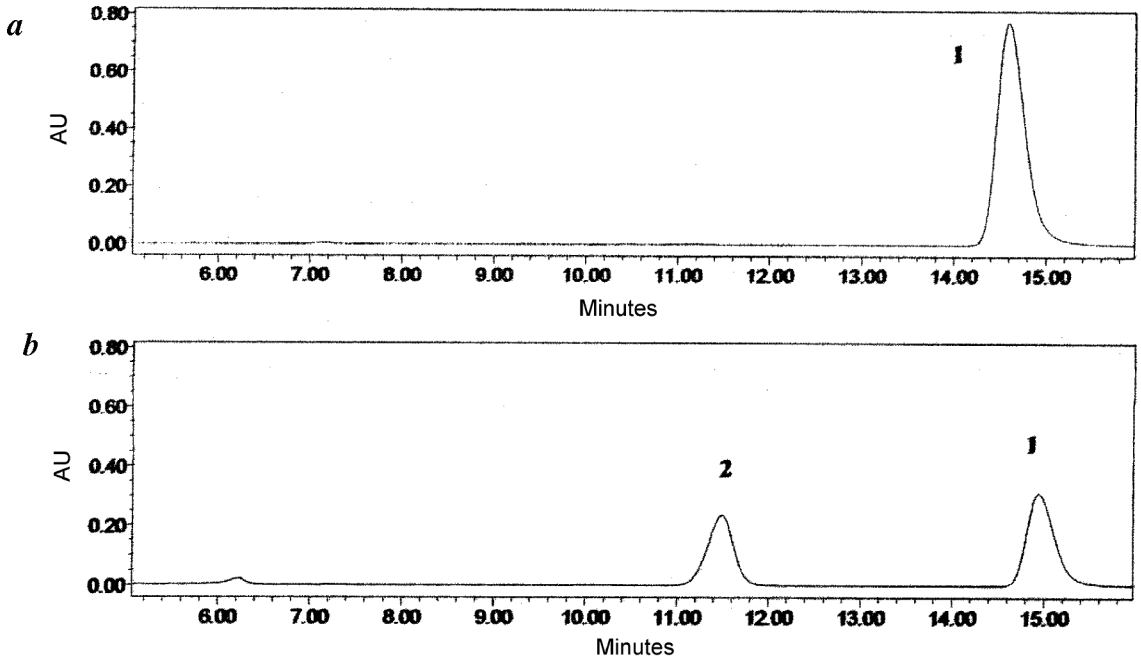

Figure 8. HPLC chromatogram at 254 nm showing (*a*), vanillin (1) as the only carbon source at the beginning of incubation (0 h); (*b*) Conversion of vanillin (1) to vanillic acid (2) by *P. variotii* after an incubation period of 72 h.

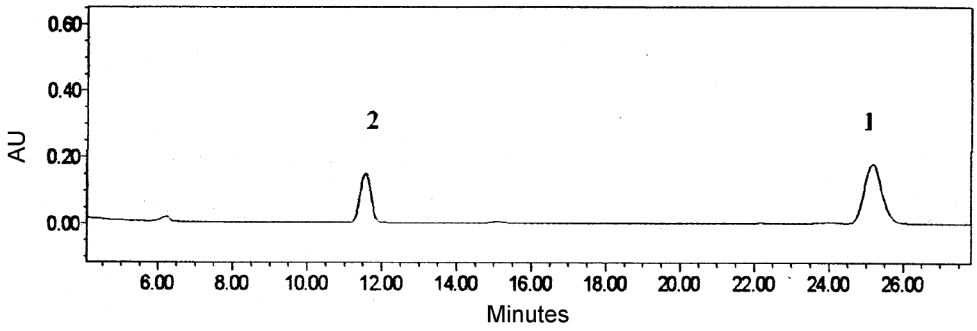

Figure 9. HPLC chromatogram showing vanillic acid formation from a cell-free extract of *P. variotii*. Separation was achieved using the Synergy™ 4 μ Hydro-RP C_{18} column (Phenomenex™, 250 × 4.60 mm) and the detection was monitored at 254 nm for tracing vanillic acid and ferulic acid[7]. Peaks 1 and 2 were identified as ferulic acid and vanillic acid respectively.

the absence of NAD in the reaction mixture, no such conversion was observed. When vanillin was incubated with the cell-free extract in presence of NAD alone, vanillic acid was observed.

Our results showed that *P. variotii* biotransformed ferulic acid into vanillic acid. Vanillin was not detected in the culture broth. Since vanillin is very reactive, it exhibits a toxic effect to most microorganisms[12]. It is conceivable that a rapid conversion of vanillin into a more stable vanillic acid occurred through oxidation. In fact, we have demonstrated that cultures of *P. variotii* were capable of transforming vanillin into vanillic acid but not vice versa (Figure 8 *b*). A number of fungi were shown to be capable of converting ferulic acid into vanillate derivatives[13]. 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[14]. Degradation of ferulic acid by another white rot fungus, *Pycnoporus cinnabarinus* was shown to catabolize ferulic acid via a different route, where vanillin was formed via vanillic acid[15]. Bioconversion of ferulic acid into vanillic acid was also demonstrated with a thermophilic fungus *Sporotrichum thermophile*[16], and more recently, with a white-rot fungus *Schizophyllum commune*[6]. Though in all the above cases, bioconversion or biodegradation was worked out in detail, surprisingly not much information is available on *in vitro* conversion of ferulic acid to vanillic acid in the fungal system. Our finding apparently suggests that a CoA-dependent phenylpropanoid side-chain degradation is in operation with *P. variotii* leading to vanillic

acid formation. A more or less similar situation was observed with a basidiomycetous yeast *Rhodotorula rubra*[17], where addition of CoA, ATP and NAD in cell-free extract was found essential for *in vitro* conversion of ferulic acid to vanillic acid. Work is being continued with *P. variotii* to characterize the enzymes involved in this biotransformation, the results of which will be presented elsewhere in the near future.

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Agrobacterium-mediated transformation of an elite indica rice for insect resistance

S. Ignacimuthu* and S. Arockiasamy

Entomology Research Institute, Loyola College, Chennai 600 034, India

In India, rice occupies the largest area among all crops and accounts for as much as 21% of the total cropped area. Rice plants are prone to attack from many pests and pathogens. Among them coleopteran pests are well known for causing extensive damage. *Sitophilus oryzae* causes severe damage to rice seeds during storage. Alpha amylase inhibitor gene isolated from *Phaseolus vulgaris* seeds was introduced into Basmati rice (PB1) through *Agrobacterium*-mediated transformation. A total of 174 hygromycin-resistant plants were regenerated. Most of these plants were GUS-positive. PCR analysis and Southern hybridization confirmed the presence of 4.9 kb alpha amylase inhibitor gene in transformed plants. Western blot confirmed presence of alpha amylase inhibitor protein. Results of the bioassay study revealed significant reduction in survival rate of rice weevil, *S. oryzae* reared on transgenic rice seeds.

Keywords: *Agrobacterium*, alpha amylase inhibitor, rice, *Sitophilus oryzae*.

THE Asian cultivated rice (*Oryza sativa* L.) is an economically important cereal crop in the world because more than half of the world's population depends on rice as a primary staple food[1]. In India, rice occupies the largest area among all crops and accounts for as much as 21% of the total cropped area. Rice plants are prone to attack from many pests and pathogens. Among them, coleopteran pests are well known for causing extensive damage[2]. Loss

*For correspondence. (e-mail: eri_lc@hotmail.com)