African Journal of Biotechnology Vol. 9 (13), pp. 1955-1958, 29 March, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Bioconversion of ferulic acid and 4-vinylguaiacol by a white-rot fungus isolated from decaying wood

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Accepted 8 January, 2010

A white rot fungus isolated from decaying wood was investigated for its ability to convert ferulic acid to various valuable products. The fungus is able to convert ferulic acid to 4-vinylguaiacol, which is then metabolized further to acetovanillone. Both products have potential use in the chemical manufacturing and pharmaceutical industries and these results contribute to our knowledge of the biotransformation of ferulic acid.

Key words: White rot fungus, ferulic acid, biotransformation, 4-vinylguaiacol, acetovanillone.

INTRODUCTION

Ferulic acid and related compounds are members of a class of naturally-derived phenolic antioxidants and the health-related uses of this class of compounds are ascribed to this property (Rosazza et al., 1995). The use of naturally-derived ferulic acid from agro-industrial waste for microbial conversion to vanillin could provide a means of manufacturing vanillin of "natural origin (Li and Rosazza, 2000; Narbad and Gasson, 1998). Several microorganisms are able to convert ferulic acid to a wide range of aromatic compounds (Figure1). These include the following species: *Aspergillus*, *Bacillus*, *Candida*, *Corynespora*, *Fusarium*, *Pseudomonas* and many more (Dodd-o and Pearse, 2000; Engels et al., 1992; Lafeber et al., 1999; Pearse and Dodd-o, 1999; Peters et al., 2001; van den Worm et al., 2001; Walton et al., 2000). The major pathways of metabolism can be summarized as:

i) Nonoxidative decarboxylation to give 4-vinylguaiacol (4-hydroxy-3-methoxystyrene) (Rosazza et al., 1995; Lee et al., 1998; Donaghy et al., 1999).

ii) Reductive reactions forming saturated side chains, aldehydes and alcohols (Rosazza et al., 1995; Lee et al., 1998).

iii) Side chain β-oxidations including conversions of ferulic acid to vanillin and vanillic acid, a central metabolic product further yielding guaiacol, protocatechuic acid and methoxyhydroquinone (Rosazza et al., 1995; Lee et al., 1998; Li and Rosazza, 2000).

iv) O-Dealkylation reactions to give caffeic acid (Rosazza et al., 1995; Lee et al., 1998).

As an increasing demand for naturally-derived food flavours exists, there is considerable interest in ferulic acid and related phenolics as a cheap source for microbial conversion to vanillin and other flavours. In addition, the pharmaceutical use of steroidal drugs is becoming increasingly controversial, owing to multiple side-effects, creating a need for new and safe alternative anti-inflammatory agents (Lafeber et al., 1999; van den Worm et al., 2001).

This study involves the identification of conversion products of ferulic acid by an unidentified white-rot fungus isolated from decaying wood.

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Abbreviations: GC-MS, Gas chromatography and mass spectrometry; $\mathbf{R_f}$, retention fraction; **NSAIDs**, non-steroidal anti-inflammatory drugs.

MATERIALS AND METHODS

Chemicals

Ferulic acid, vanillin, Sabouraud dextrose agar and malt extract

were obtained from Merck Chemicals. Sabouraud dextrose agar was prepared according to the manufacturer's instructions and dispensed into Petri dishes (about 15 ml per dish) at least 24 h before use to ensure a dry surface. The plates were stored in plastic bags to prevent dehydration. 4-Vinylguaiacol was prepared by incubating a baker's yeast culture (30°C, 120 rpm, 48 h) with ferulic acid (Huang et al., 1993). Ferulic acid stock solution (1 mg/ml) was prepared in double-distilled water and filter-sterilized through a 0.22 µm membrane filter (Millipore Corp., USA) and stored in the dark at 4°C until use.

Culture medium and fungal isolation

Small pieces of unidentified very decayed wood collected from the indigenous forest near the edge of the Tyume river on Fort Hare farm were suspended in 0.9% saline water (10 min, room temperature) and then placed directly on Sabouraud dextrose agar plates. The plates were incubated at 28°C for 48 h, after which there was visible fungal growth. In order to obtain pure cultures, hyphal tips were cut off and transferred to sterile plain agar plates. The plates were incubated at 28°C for 48 h and a resulting pure isolate was transferred to Sabouraud dextrose agar slants and stored at 4°C for future work. These were kept as stock fungal cultures. Fresh cultures of the fungus were prepared by incubation at 30°C on freshly prepared Sabouraud dextrose agar plates.

Preparative culture conditions

The fungus was grown in Erlenmeyer flasks (250 ml) containing malt extract broth (100 ml) prepared according to the manufacturer's instructions. The broth was autoclaved (121°C for 15 min), allowed to cool and inoculated with fungus from the stock cultures (150 rpm, 30°C). Stock ferulic acid solution was added to 48 h old cultures to a final concentration of 0.1%w/v. Due to a limited availability of the prepared 4-vinylguaiacol, this was added to a final concentration of 0.01%w/v. The cultures were incubated with shaking for a further 24 h. Three blanks were run concurrently under the same fermentation conditions:

1. Malt extract broth + Ferulic acid (0.1%w/v)
2. Malt extract broth + 4-Vinylguaiacol (0.01%w/v)
3. Malt extract broth, inoculated with fungi.

Extraction of metabolites

After incubation, the cultures were centrifuged (Beckman J2-21, JA-10 head, 10 000 rpm, 10 min; RCF = 11 000 x g) at room temperature and the supernatant acidified to a pH < 2.0 with 1 N HCl. It was then extracted 3 times with diethyl ether (50 ml per 100 ml of supernatant). The organic phase was pooled, dried over anhydrous Na_2SO_4 and evaporated to dryness in a stream of air. The residue was dissolved in methanol for GC-MS analysis.

Combined gas chromatography and mass spectrometry (GC-MS)

Gas chromatography was carried out on a Hewlett Packard 6890 instrument, fitted with an HP-5 column (30 m x 0.25 mm i.d. x 0.25 μm phase thickness). The inlet temperature was set at 220°C and the column temperature was 70°C, programmed to rise at 5°C/min to 240°C. Carrier gas was helium at a flow rate of 0.7 ml/min. Mass spectrometry was performed with a Hewlett Packard 5973 mass-selective detector, with the MS source at 230°C and the MS quadrupole at 150°C. Identification of eluted compounds was based on comparison of retention times and mass spectra with authentic compounds (ferulic acid, 4-vinylguaiacol and vanillin) and by comparison of the mass spectral data with those in the Wiley 275 database.

RESULTS AND DISCUSSION

The fungal culture transformed ferulic acid into a wide range of products as shown in Tables 1 and 2 after removal of all background products present in test cultures and blanks. Table 1 indicates that most of the ferulic acid has been transformed after 24 h to various compounds, with 4-vinylguaiacol being the major product (~80% yield). Incubation for a further 24 h led to a decrease of 4-vinylguaiacol and a concomitant increase in acetovanillone.

With 4-vinylguaiacol as the substrate, the major product after 24 h of incubation with the fungus is acetovanillone (~39% yield, Table 2). Other minor products detected are listed in Tables 1 and 2.

This study aims to identify metabolic products of ferulic acid as a substrate of an unidentified white rot fungus. 4-Vinylguaiacol is recovered as the major degradation product after 24 h of incubation, with minor quantities of vanillin and vanillic acid. The fungus then converts 4-vinylguaiacol to acetovanillone, the major end product of extended incubation.

The bioconversion of ferulic acid to 4-vinylguaiacol has the value of providing quantities of an oxygenated styrene. This may be more difficult to achieve using organic chemistry. Phenolic styrenes are valuable starting materials for oxygenated biodegradable polymers, fragrances, flavours and intermediates for organic synthesis.

Of interest was the further degradation by fungal fermentation of 4-vinylguaiacol to give acetovanillone (4-hydroxy-3-methoxyacetophenone or apocynin), as established by GC-MS. Our initial conclusion was that the new metabolite was vanillin, as it had the same R_f as vanillin and gave the same colour after spraying with 2, 4-dinitrophenylhydrazine on a thin layer chromatoplate. Vanillin has been previously reported to be a ferulic acid and /or 4-vinylguaiacol metabolite (Karmakar et al., 2000; Lee et al., 1998).

Literature indicates that acetovanillone has a wide range of potential applications, mainly in the medical field. Most non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of rheumatoid arthritis (Lafeber et al., 1999). Undesirable side-effects can occur in the gastro intestinal tract (Lafeber et al., 1999), but acetovanillone treatment is reported to not show any side-effects (Dodd-o and Pearse, 2000; Engels et al., 1992; Lafeber et al., 1999; Pearse and Dodd-o, 1999; Peters et al., 2001). Acetovanillone has been found to be effective in the experimental treatment of several other inflammatory diseases, such as colitis and atherosclerosis (Dodd-o and Pearse, 2000; Engels et al., 1992; Lafeber et al., 1999; Muijsers et al., 2000; Pearse and Dodd-o, 1999; Peters et al., 2001; van den Worm et

Table 1. Products of ferulic acid degradation by a fungal isolate after 24 h of incubation at 30°C.

Peak	RT (min)	Major ions	% (Total)	R_I	Compound
1	7.51	122, 91, 65	1.24	1114.83	Phenylethyl alcohol
2	11.56	152, 137, 122	0.11	1281.92	4-Ethyl-2-methoxyphenol
3	12.87	150, 137, 107	80.40	1334.68	4-Vinylguaiacol
4	14.61	151, 109, 81	1.23	1404.96	Vanillin
5	15.96	181, 151, 93	9.80	1460.33	1-(2,3-dihydroxy-4-methoxy-6-methylphenyl)-ethanone
6	16.17	182, 137, 93	0.30	1469.42	Vanillic acid
7	16.32	166, 151, 123	0.27	1475.61	Acetovanillone
8	18.81	196, 168, 151	1.22	1581.90	Ethyl ester of vanillic acid
9	22.90	194, 179, 133	0.38	1768.72	Ferulic acid

(Identities based on comparison of retention times and mass spectra with authentic compounds and by interpretation of their mass spectra, and by comparison with mass spectra in the Wiley 275 database).
RT = Retention time, R_I = Retention index.

Table 2. Products of 4-vinylguaiacol degradation by a fungal isolate after 24 h of incubation at 30°C.

Peak	RT (min)	Major Ions	% (Total)	R_I	Compound
1	7.54	122, 91, 65	12.01	1116.10	Phenylethyl alcohol
2	12.42	150, 135, 107	15.20	1316.53	4-Vinylguaiacol
3	15.82	181, 151, 93	16.46	1454.96	1-(2,3-dihydroxy-4-methoxy-6-methylphenyl) Ethanone
4	16.23	168, 153, 93	6.47	1472.20	4,5-Dimethoxy-2-methylphenol
5	16.67	166, 151, 123	39.53	1490.08	Acetovanillone

(Identities based on comparison of retention times and mass spectra with authentic compounds, and by interpretation of their mass spectra, and by comparison with mass spectra in the Wiley 275 database).
RT = Retention time, R_I = Retention index.

al., 2001).

Acetovanillone affords the possibility of being one of a novel series of NSAIDs, creating a new therapeutic approach to the clinical problem of a wide range of inflammatory disorders (Muijsers et al., 2000; Pearse and Dodd-o, 1999; van den Worm et al., 2001). Acetovanillone, having anti-inflammatory and cartilage-protecting properties, is a drug deserving further *in vivo* study to evaluate it for long-term treatment of chronic inflammatory and degenerative joint diseases. Our findings contribute towards the understanding of the biosynthetic route, using fungi, for the production of acetovanillone from ferulic acid, a waste product generated by cereal industry.

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