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Chapter 2

Reduction of aryl acids by white-rot fungi for the biocatalytic production of aryl aldehydes and alcohols

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Abstract

Ligninolytic basidiomycetes were screened for their ability to reduce aryl acids to corresponding aldehydes and alcohols. Seven fungal strains converted *p*-anisic acid in high molar yields to the reduced products. The white-rot fungus *Bjerkandera* sp. strain *BOSS5* was one of the best reducing strains and was highly tolerant towards high concentrations of different aromatic acids. The reduction of *p*-anisic, veratric, 3-chloro-4-methoxybenzoic, 3,5-dichloro-4-methoxybenzoic, 3,4-dichlorobenzoic, 4-fluorobenzoic, and 3-nitrobenzoic acids by this fungus was tested. All of these compounds were reduced to their corresponding aldehydes and alcohols.

Introduction

The demand for organic synthons is rapidly expanding, requiring novel strategies for production. The conventional routes of chemical synthesis are still viable, but the transformation of organic compounds using biocatalysts has some advantages. Biotransformations can involve high degrees of regio- and stereospecificity and in most cases relatively mild reaction conditions are used. Rogers *et al.* (1997) described the biotransformation of benzaldehyde to 1-phenylacetylcarbinol, an intermediate in the production of 1-ephedrine, by various species of yeasts. Surprisingly, no reports could be found describing direct aryl acid reduction with a biocatalyst. An efficient route to synthesize aryl aldehydes and alcohols would be by starting from aryl acids, using the reductive enzyme system of white-rot basidiomycetes as biocatalyst. The reduction of aromatic acids and aldehydes released during lignin degradation has been reported (de Jong *et al.* 1994; Kirk and Farrell 1987; Shimada and Higuchi 1991). Aromatic acid and aldehyde reducing activities have been described in whole cultures of *Trametes versicolor* (Farmer *et al.* 1959), *Sporotrichum pulverulentum* (Ander *et al.* 1980) and *Phlebia radiata* (Lundell *et al.* 1990). An intracellular NADPH-dependent aryl-alcohol dehydrogenase (AAD) from the white-rot fungus *Phanerochaete chrysosporium* was purified and characterized (Muheim *et al.* 1991). This enzyme, produced during secondary metabolism of the fungus, presents a broad substrate specificity, reducing aromatic aldehydes such as veratraldehyde, *p*-anisaldehyde, vanillin and 3,5-dimethoxybenzaldehyde completely to the corresponding alcohols.

Several roles of the AAD or similar reductases in white-rot fungi are plausible. The reduction of aromatic acids, formed during lignin degradation, would convert them into more amenable substrates for ligninolytic enzymes, thereby enabling their continued degradation (Reddy *et al.* 1998; Schoemaker *et al.* 1989, 1990).

Aryl aldehydes and alcohols (e.g. veratryl, benzyl, anisyl and 3-chloro-4-methoxybenzyl) are produced as secondary metabolites of white-rot fungi and serve as substrates for extracellular aryl alcohol oxidase (AAO), generating H₂O₂ (de Jong *et al.* 1994; Field *et al.* 1995a). Intracellularly, the formed aryl aldehydes and acids are readily recycled by mycelium. Presumably reductive enzymes in the mycelium are necessary to maintain the redox cycle of the ligninolytic system.

This form of redox cycling was demonstrated in *P. eryngii* (Guillén *et al.* 1994) and *Bjerkandera* sp. strain BOS55 (de Jong *et al.* 1994).

The potential use of white-rot fungi as biocatalyst for aryl acid reductions was explored in this study. Basidiomycete strains were screened for their tolerance towards high concentrations of aryl acids. Tolerant strains were evaluated for their ability to reduce *p*-anisic acids. *Bjerkandera* sp. strain BOS55 was selected as a potent reductive strain and the reduction of several acids by this fungus was investigated. Time dependent reduction of the aryl acids to their corresponding aldehydes and alcohols was quantified.

Material and Methods

Organisms and culture conditions

Fifty-two fungal strains were obtained from different culture collections: Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; American Type Culture collection, Rockville, Maryland USSA Culture Collection of Industrial Microbiology Wageningen, Wageningen University and Research Center, The Netherlands; Centro de Inverigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain.

Fungal strains were maintained at 4°C on agar slants. The agar medium contained (l^{-1}) 20 g glucose, 5 g mycological peptone (Oxoid Ltd., Basingstoke, Hampshire, UK), 2 g yeast extract (Gibco BRL, Life Technol. Ltd., Paisley, Scotland, UK), 1 g KH_2PO_4 , 0.5 g $MgSO_4 \cdot 7H_2O$, 15 g agar. Fungal strains were grown in a high-nitrogen peptone medium according to Kimura *et al.* (1990), containing (l^{-1}) 20 g glucose, 5 g mycological peptone, 2 g yeast extract, 1 g KH_2PO_4 , 0.5 g $MgSO_4 \cdot H_2O$, with the addition of 0.058 g NaCl.

Serum bottles (100 ml), containing 10 ml medium, were inoculated with a cylindrical agar plug (diameter 4mm), which was taken from the outer periphery of an agar medium plate covered with the mycelium of the fungal strain. Fungal cultures were incubated statically in the dark with loosely capped bottles. Control cultures containing no additon of aryl acids and were incubated and treated like acid-containing cultures to monitor for *de novo* production of metabolites. Sterile abiotic controls in presence of acids were also monitored to check for chemical reduction of acids. Unless indicated otherwise, the experiments were carried out in triplicate.

Plate screening

Two plate screening experiments were conducted. The first one was performed to screen for tolerance towards 1 mM *p*-anisic acid. The agar medium used for this screening contained (l⁻¹) 5 g glucose, 15 g agar and 3.5 g malt extract (Oxoid Ltd., Basingstoke, Hampshire, UK). 1 mM *p*-anisic acid was added and the pH of the medium was adjusted to 5.5. The second plate screening was performed to screen for tolerance towards 10 mM benzoic, *p*-anisic, veratric, 3-chloro-4-methoxybenzoic and 3,5-dichloro-4-methoxybenzoic acid. The agar medium contained (l⁻¹) 5 g glucose, 15 g agar and 3.5 g malt extract. 10 mM of one of the acids was added and the pH was adjusted to 5.5. Plates were inoculated with one cylindrical agar plug (diameter 4mm) and incubated in the dark at 25°C. Control plates without acid were inoculated and incubated the same way. After 6 days, tolerance was determined by comparing mycelial radial extension (diameter of mycelium) to the mycelial radial extension in the control plates. The experiment was carried out in duplicate.

Screening for p-anisic acid reduction.

Culture bottles of 100 ml, containing 10 ml high-nitrogen peptone medium were inoculated with a cylindrical agar plug (diameter 4 mm). Cultures were incubated in the dark at 25°C. To 4-day-old cultures, 1 mM *p*-anisic acid was added as a concentrated solution in acetone, to a concentration of 1 mM to the culture. The final concentration of acetone was 0.5% v/v which was not toxic when added to mycelium (Field *et al.* 1995b). After 3 days of incubation, 150 µl extracellular fluid was centrifuged and analysed by HPLC.

Aryl acid reduction by Bjerkandera sp. strain BOS55

Culture bottles of 100 ml, containing 10 ml high-nitrogen peptone medium were inoculated with a cylindrical agar plug (diameter 4mm) of *Bjerkandera sp.* strain BOS55. Cultures were incubated in the dark at 30°C. On the 4th day of growth, either *p*-anisic, veratric, 3-chloro-4-methoxybenzoic, 3,5-dichloro-4-methoxy benzoic, 3,4-dichlorobenzoic, 4-fluorobenzoic or 3-nitrobenzoic acid was added from an acetone stock solution to a final concentration of 1 mM. Reduction to the corresponding aldehydes and alcohols was followed during the first 16 hours of incubation. Samples

of 150 μ l extracellular fluid were taken every hour and were centrifuged and analysed by HPLC.

Determination of the dry weight of mycelium

Mycelial mats were separated from the culture fluids by filtration. Mycelia were rinsed with distilled water and filtered through dried and tared glass fiber filters (Schleicher & Schuell GF 50) (Dassel, Germany). Mycelial dry weights were determined after drying overnight at 105°C.

HPLC analysis

Culture supernatants were centrifuged for 10 minutes (1,200 x g), and 50 μ l samples were analysed on a Hewlett Packard HPLC Chemstation (Pascal Series) (Waldbronn, Germany) equipped with a HP 1100 pumping station, series diode array, a HP1100 detector and HP1100 data processor. The column (200 mm x 3 mm) used for analysis of aryl aldehydes and aryl alcohols was filled with ChromSpher C18-PAH (5 μ m particles) and was from Chrompack (Middelburg, The Netherlands). Aryl aldehydes and alcohols were analysed with the following gradient: 90:10, 0:100 and 90:10 H₂O:acetonitrile at 0, 15 and 25 minutes respectively. The flow rate was 0.4 ml min⁻¹ and the column temperature 30°C. The UV absorbance was monitored at wavelengths of 230, 265 and 280 nm. The column (460mm x 150 mm) used for analysis of aryl acids was filled with Intersil ODS-3 (5 μ m particles) and was from Phase Sep (Deeside, UK).

All aryl acids were analysed under isocratic conditions with 0.01M H₃PO₄ (0.4 ml min⁻¹, 30°C). The UV absorbance was monitored at 255, 265 and 280 nm. Compound identifications were based on matching retention times and UV spectra with those of standards.

Chemicals

3,5-dichloro-4-methoxybenzoic acid and the corresponding standards (3-chloro-4-methoxybenzylalcohol, 3-chloro-4-methoxybenzaldehyde, 3,5-dichloro-4-methoxybenzaldehyde and 3,5-dichloro-4-methoxybenzaldehyde) were kindly provided by Henk Swarts, Dept. of organic chemistry, Wageningen Agricultural University. Protocols for their synthesis were published previously (de Jong *et al.* 1992, 1994; Swarts *et al.* 1996). All other chemicals were purchased from Aldrich

(Steinheim, Germany), Merck (Darmstadt, Germany) or Acros Chimica (Geel, Belgium), unless indicated otherwise.

Results

Of the 52 strains screened, 30 strains tolerated 1 mM *p*-anisic acid and grew in its presence. The tested strains were compared to strains grown on reference plates without added acids. Fourteen of the tested basidiomycetes showed a mycelial radial extension index > 0.75 and were subjected to another plate screening to screen for the tolerance towards high concentrations of selected aryl acids (table 2.1). The radial extension rate was measured on agar plates containing 10 mM (saturated concentrations) of either: *p*-anisic acid, benzoic acid, veratric acid or 3-chloro-4-methoxybenzoic acid and compared to radial extension on reference plates.

Table 2.1 Plate screening with mycelial radial extension as an indication of tolerance towards high concentrations (10 mM) of aryl acids^a.

Fungal strain	benzoic acid	<i>p</i> -anisic acid	Veratric acid	3 chloro-4-methoxy benzoic acid
<i>Phanerochaete chrysosporium</i> ATCC 24725	0.91	0.66	1.04	0.38
<i>Schizophyllum commune</i> PW 94.3	0.88	0.54	0.88	0.38
<i>Bjerkandera</i> sp. strain BOS55	0.89	0.33	0.97	0.33
<i>Phlebia brevispora</i> KBT 89	0.36	0.05	1.08	0.02
<i>Trametes hirsuta</i> CBS 282.73	0.58	0.07	0.28	0.38
<i>Trametes versicolor</i> 290	0.50	0.23	0.55	NG ^b
<i>Pleurotus eryngii</i> CBS 613.91	0.56	0.24	0.40	NG ^b
<i>Dichomitus squalenes</i> CBS432.34	0.26	0.07	0.58	0.11
<i>Stereum hirsutum</i> PW 93.4	0.59	NG ^b	0.36	NG ^b
<i>Ganoderma australe</i>	0.32	NG ^b	0.61	NG ^b
<i>Merulius tremellosus</i> ATCC 60027	0.23	0.05	0.63	NG ^b
<i>Lentinus tigrinus</i> PN 94.2	NG ^b	NG ^b	0.68	0.02
<i>Trametes gibbosa</i> RHEN 93.2	NG ^b	NG ^b	0.68	NG ^b
<i>Polyporus ciliatus</i> ONO 94.1	0.88	NG ^b	0.50	NG ^b

^a Mycelial radial extension was determined by diameter of mycelial colony incubated on 10 mM acid / diameter of mycelial colony on control plates after 7 days

^b No growth detected

D. squalenes, *Bjerkandera* sp. strain BOS55, *P. chrysosporium*, *T. hirsuta*, *P. brevispora* and *S. commune* proved to be the most tolerant and showed mycelial growth in presence of high concentrations of all the aromatic acids tested. Seven of the tested strains were totally inhibited by the presence of 10 mM 3-chloro-4-methoxybenzoic acid.

The fungi were also screened for reduction of 1mM *p*-anisic acid to the corresponding aldehyde and alcohol in liquid medium (figure 2.1). Aldehyde and alcohol concentrations were found in high total molar yields, up to 97% for *D. squalenes*. The basidiomycete strains *Bjerkandera* sp. strain BOS55, *M. tremellosus*, *P. chrysosporium*, *T. hirsuta*, *L. tigrinus* and *P. cilatus* also showed a high molar recovery of aldehydes and alcohols (> 75%). A low recovery of the products (total molar yield < 30%) was found for *P. eryngii* and *S. commune*. Each fungal strain established an equilibrium between the produced aldehyde and alcohol.

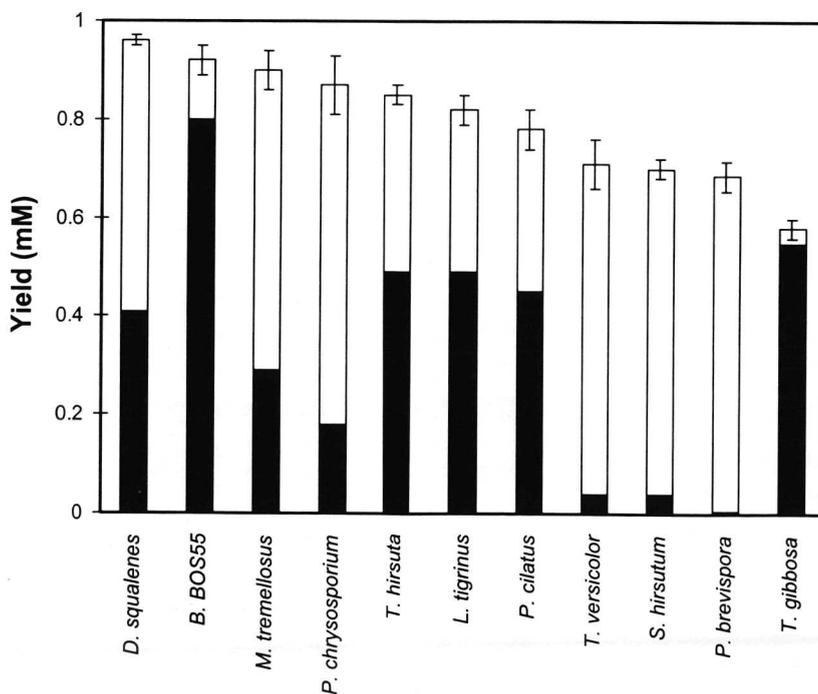


Figure 2.1 Reduction of 1 mM *p*-anisic acid to its respective aldehyde and alcohol by various basidiomycete strains after 3 days of incubation. ■: *p*-anisaldehyde (mM), □: *p*-anisyl alcohol (mM).

Most fungi had the tendency to reduce the acid to predominantly the alcohol, whereas the strains *Bjerkandera* sp. strain *BOS55* and *T. gibbosa* appeared to have the equilibrium on the side of the aldehyde. Since *Bjerkandera* sp. strain *BOS55* was one of the best reducers of *p*-anisic acid, was highly tolerant towards the different aromatic acids and has been intensively studied in our laboratory, this strain was used in further experiments.

The rates at which *Bjerkandera* sp. strain *BOS55* reduced 1mM *p*-anisic, veratric, 3-chloro-4-methoxybenzoic, 3,5-dichloro-4-methoxybenzoic, 3,4-dichloro benzoic, 4-fluorobenzoic, and 3-nitrobenzoic acids was studied (table 2.2). All tested compounds were reduced. The highest recovery of products was found for veratric acid (96%). Of all halogenated compounds, 3-chloro-4-methoxybenzoic acid was reduced with the highest yield (43% converted to alcohol and aldehyde). 3,4-dichlorobenzoic acid was reduced to the aldehyde, no alcohol was detected.

The recovery of unreduced acids was very low in the case of *p*-anisic and veratric acids, whereas for the other substrates from 40% to 71% was left unutilized. The total recovery of compounds was 94%-96% for *p*-anisic and veratric acids. In all other cases, the total recovery of acids, aldehyde and alcohol was between 74% and 84%. The equilibrium of the 3-nitrobenzoic acid reduction was on the side of the alcohol, whereas for all the other compounds tested, mostly aldehydes were formed.

Table 2.2 Molar yields of the conversion of 1mM aryl acids to the corresponding aldehyde and alcohol by *Bjerkandera BOS55* after 16 hours of incubation

substrate	produced aldehyde (mM)	produced alcohol (mM)	recovered acid (mM)	Total recovery (%)
<i>p</i> -anisic acid	0.80	0.12	0.02	94
veratric acid	0.74	0.21	0.01	96
3-chloro-4-methoxybenzoic acid	0.28	0.15	0.40	83
3,5-dichloro-4-methoxybenzoic acid	0.21	0.15	0.41	77
4-fluorobenzoic acid	0.17	0.08	0.49	74
3,4-dichlorobenzoic acid	0.13	0.00	0.71	84
3-nitrobenzoic acid	0.04	0.15	0.62	81

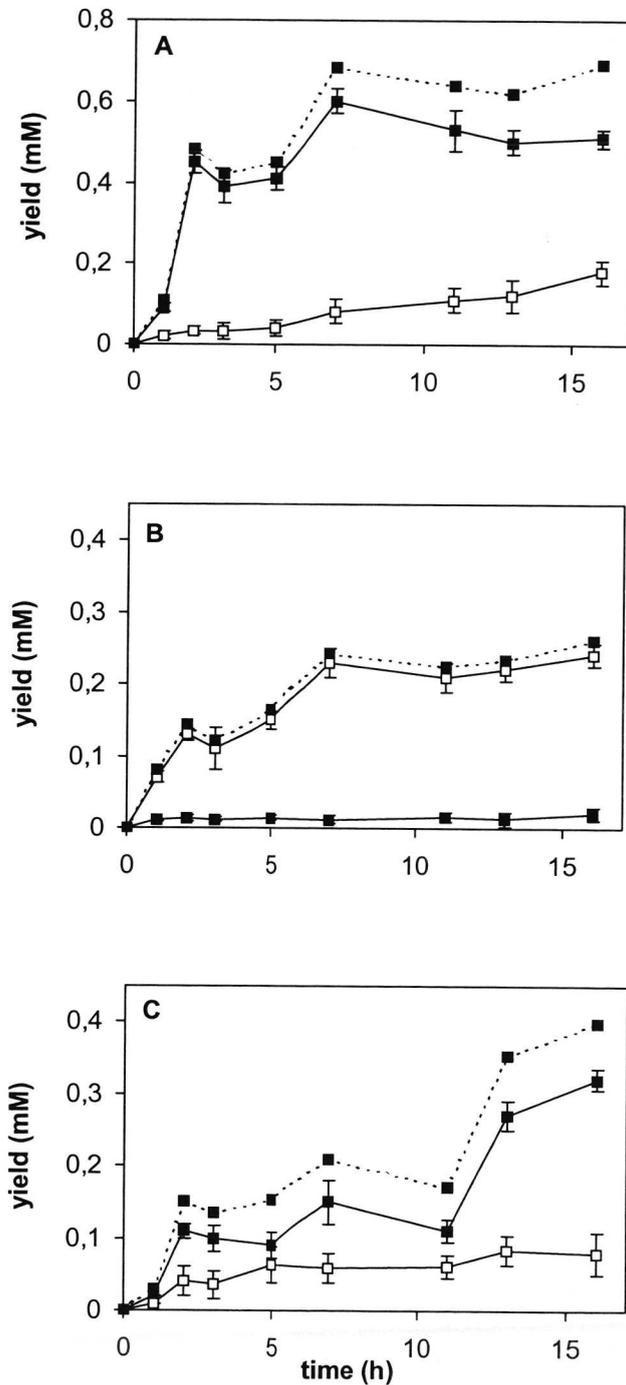


Figure 2.2 Reduction of 1 mM aryl acid by *Bjerkandera BOS55* during the first 16 hours of incubation. A: *p*-anisic acid reduction, B: 3-nitrobenzoic acid reduction, C: 3-chloro-4-methoxybenzoic acid reduction. ■: produced aldehyde (mM), □: produced alcohol (mM), dashed line: total molar yield (mM).

Furthermore, the time dependent reduction of the aryl acids supplied at 1 mM was monitored. During the first 16 hours of incubation, samples were taken every 2 hours and the conversion of the acids to the aryl aldehydes and aryl alcohols was measured as is shown for the examples of *p*-anisic, 3-chloro-4-methoxybenzoic and 3-nitrobenzoic acid in figure 2.2. The reduction rates for *p*-anisic, veratric, 3-chloro-4-methoxybenzoic, 3,5-dichloro-4-methoxybenzoic, 3,4-dichlorobenzoic, 4-fluorobenzoic and 3-nitrobenzoic acid were $1.0 \cdot 10^{-2}$, $1.0 \cdot 10^{-2}$, $4.7 \cdot 10^{-3}$, $3.9 \cdot 10^{-3}$, $2.7 \cdot 10^{-6}$, $1.4 \cdot 10^{-6}$ and $2.1 \cdot 10^{-6}$ mmol·h⁻¹·g⁻¹ biomass dry weight respectively.

Discussion

The results presented in this study demonstrate that several white-rot fungi appeared to have very good biocatalytic potential for the reduction of aryl acids. Selected strains reduced *p*-anisic acid in high molar yields and were tolerant to high concentrations of various aryl acids. The enzymes responsible for the reduction of aryl acids to their corresponding aldehydes are not yet known. An intracellular NADPH dependent aryl-alcohol dehydrogenase (AAD), purified from the white-rot fungus *Phanerochaete chrysosporium* has been described (Muheim *et al.* 1991), which reduces aromatic aldehydes to their corresponding alcohols. However it was not reported whether aromatic acids are also part of the substrate spectrum of AAD.

As many white-rot fungi also produce aryl alcohol oxidases (AAO) for the extracellular production of H₂O₂ (de Jong *et al.* 1994) an equilibrium between aryl aldehydes and alcohols is established. In the case of *p*-anisate, the strains tested here had the equilibrium on the side of the alcohol. *Bjerkandera* sp. strain BOS55 predominantly produced *p*-anisaldehyde. The oxidases and reductases in *Bjerkandera* sp. strain BOS55 appeared to maintain different stoichiometric ratios between the aldehydes and alcohols depending on the functional group of derivatives substituted on the aryl acid. The presence of the nitro group dramatically shifted the equilibrium towards the alcohol, probably because nitrated alcohols are more difficult substrates of AAO. Previously, alcohol was found to be the predominant product at equilibrium after incubation with homologous concentrations of benzaldehyde and benzyl alcohol in *P. eryngii* (Guillén *et al.* 1994) and in *Bjerkandera adusta* (Lauritsen and Lunding 1998; Spinnler *et al.* 1994). Furthermore it was found that incorporation of a functional group, e.g. methoxy, can shift the ratios between alcohol and aldehyde or even reverse them completely (Lauritsen and Lunding 1998).

From all the tested strains, the white-rot fungus *Bjerkandera* sp. strain *BOS55* was selected for further study due to its relatively high reduction rate of *p*-anisic acid and because it displayed high tolerance towards several aryl acids. Toxicity of aryl acids towards fungi is well known from the literature. An inhibiting effect of veratric acid at concentrations of 0.5 mM on AAO and AAD activities was described by Guillen *et al.* (1994). Consumption of glucose by the fungus *P. eryngii* was impeded in a medium containing 10 mM benzoic acid (Guillen *et al.* 1994).

Bjerkandera sp. strain *BOS55* showed to have a very broad substrate spectrum of aryl acids with several functional groups that are of interest to the pharmaceutical industry, such as nitro, chloro and fluoro substitutions. In support of our findings, Lauritsen and Lunding (1998) detected the occurrence of fluorinated aryl alcohols and aldehydes in cultures spiked with fluorobenzoates during their studies elucidating biosynthetic pathways in *Bjerkandera adusta*. The aryl acids were reduced with reduction rates varying from $1.4 \cdot 10^{-6}$ to $1 \cdot 10^{-2}$ mmol·h⁻¹·g⁻¹_{biomass dry weight}. The fastest rates were found for *p*-anisic, veratric, 3-chloro-4-methoxybenzoic and 3,5-dichloro-4-methoxybenzoic acids, which are natural metabolites of *Bjerkandera* sp. strain *BOS55* (Swarts *et al.* 1996) and are precursors to the corresponding aldehydes and alcohols (Mester *et al.* 1997).

Although oxidative enzymes of white-rot fungi have been extensively studied, relatively little is known about the reductive enzymes. Reductive enzymes from these fungi are capable of quinone reduction (Brock *et al.* 1995; Tuor *et al.* 1993), enantioselective reduction of ketones (Bernardi *et al.* 1991) and even reductive dechlorination of chlorophenolics (Reddy *et al.* 1998). This study demonstrates that white-rot fungi have outstanding reductive enzymes for the problematic conversion of aromatic acids to corresponding aldehydes and alcohols.