

Informed strain improvement for lignin degradation by *Phanerochaete chrysosporium*

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The effect of breeding from the white rot fungus *Phanerochaete chrysosporium* ME446 on performance for lignin mineralization was examined. This model for informed strain improvement without mutagenesis is based on abundant restriction fragment length polymorphisms (RFLPs). Under optimized conditions for lignin mineralization, extracellular manganese peroxidase (MnP) but not lignin peroxidase (LiP) could be detected, so measurement of LiP activity is not a valid assay for lignin degradation. Mineralization of ¹⁴C-labelled synthetic lignin (¹⁴C-DHP) was used to compare the performance of the wild-type strain ME446 with those of sets of progeny strains. Meiotic progeny from strain ME446, heterokaryotic progeny of crosses between such strains, and meiotic progeny of one heterokaryotic strain were examined. In each case, a minority of strains performed more efficiently than the parental strain ME446. The greatest range of lignin-mineralization performance (70-fold) was found within the set of initial progeny of ME446 and the narrowest was within the set of secondary homokaryotic strains. This is consistent with the view that a moderate number of determinants contribute to lignin mineralization performance. However, performance did not correlate with the possession of any single allele of those for 38 previously defined RFLP markers. The results show that lignin mineralization performance can be improved by cycles of crosses and fruiting, without mutagenesis.

Keywords: lignin, *Phanerochaete chrysosporium*, RFLP, strain improvement, basidiomycete

INTRODUCTION

The paradigm of lignin degradation is the oxidative action of lignin and manganese peroxidases (LiP and MnP) by the white rot fungus *Phanerochaete chrysosporium* as a stress response, supported by an H₂O₂-generation system (Eriksson *et al.*, 1990; Gold & Alic, 1993). The sustained effort of an increasing number of research groups has yielded much physiological, biochemical and molecular genetical information on the system. If such laboratory knowledge is to be applied to this and other fungi to optimize biotechnological processes that include solid substrate fermentation (Agosin & Odier, 1985; Coughlan, 1989; Coughlan & Amaral Collaco, 1990; Kerem *et al.*, 1992) and bleaching of pulp (Addleman & Archibald, 1993; Kondo *et al.*, 1994; Michel *et al.*, 1991; Paice *et al.*, 1993), methods must be available to study which genes are expressed in the synergistic process of lignocellulose

degradation under scale-up conditions (Broda *et al.*, 1995). Strategies for genetic improvement of the organisms to be used are also desirable, and these are likely to be fundamentally different from those used, for example, in antibiotic production, which historically have involved cycles of mutagenesis and screening within one lineage, to produce strains that are high producers but otherwise crippled. In contrast, improved lignocellulose degraders must remain robust; in principle, the disadvantages inherent in random mutagenesis might be avoided by exploiting the variation that exists in natural populations (Raeder & Broda, 1986; Raeder *et al.*, 1989a, b).

Lignin degradation by white rot fungi presents a complex picture, several aspects of which are relevant to this study. The natural substrate is lignocellulose rather than lignin, and the other components of this complex material, cellulose and hemicellulose, are degraded concomitantly. *P. chrysosporium* cellulase genes have been identified and their expression studied (e.g. Uzcategui *et al.*, 1991; Sims *et al.*, 1994; Tempelaars *et al.*, 1994; Broda *et al.*, 1995). However, equivalent studies on the hemicellulases of *P. chrysosporium* are few (Copa-Patiño *et al.*, 1993; Copa-

Abbreviations: DHP, dehydrogenative polymerizate (model lignin); DMF, dimethylformamide; DMS, 2,2-dimethylsuccinate; LiP, lignin peroxidase; MnP, manganese peroxidase; NaOAc, sodium acetate.

Patiño & Broda, 1994), and the regulation of the overall synergistic process is not yet understood. The generation of the peroxide required for peroxidase activity is considered to depend on carbohydrate degradation, involving several possible pathways (Gold & Alic, 1993).

Low molecular mass compounds can be used as substrates (Eriksson *et al.*, 1990), but these are not convincing models for the high molecular mass insoluble three-dimensional polymer that is lignin. Chemically synthesized radioisotopically labelled high molecular mass model lignin (dehydrogenative polymerizate, DHP) (Chua *et al.*, 1983; Faix *et al.*, 1985; Haider & Trojanowski, 1975; Kirk *et al.*, 1975) is a better alternative, but this lacks the carbohydrate present in lignocellulose. In this study both ^{14}C -DHP and [*lignin*- ^{14}C]lignocellulose (Crawford, 1981; Mason *et al.*, 1990) were used to determine lignin degradation activity.

A study by Kirk *et al.* (1986) with different wild-type isolates of *P. chrysosporium* and also with a set of cellulase-deficient mutants isolated after irradiation (Johnsrud & Eriksson, 1985) showed differences in ^{14}C -DHP mineralization. There was little correlation between levels of supernatant LiP and ^{14}C -DHP mineralization. Pham *et al.* (1990) found with another white rot fungus, *Dichomitus squaleus*, that the ability of a set of meiotically-derived progeny to degrade lignin and cellulose varied greatly. They concluded that performance for these properties was determined multifactorially.

A genetic map has previously been derived for *P. chrysosporium* strain ME446 (which carries two genome equivalents) by the analysis of the distribution of RFLPs among a set of 53 basidiospore-derived homokaryotic derivatives (Raeder & Broda, 1986; Raeder *et al.*, 1989a). These strains varied widely in supernatant LiP activity, high levels of which were associated with one of the two alleles at locus 34 (Raeder *et al.*, 1989b). The demonstration of abundant RFLPs and this range of levels of LiP activity raised the possibility that there could also be extensive variation between strains in performance for the overall process of lignin degradation. The genetic composition of the 53 sibling strains was determined for RFLP alleles for 38 markers covering the whole genetic map (Raeder *et al.*, 1989a). Therefore this system provides, perhaps for the first time in a fungus, the opportunity to determine whether performance for a property of biotechnological importance (in this case lignin mineralization) can be related to the presence of specific alleles at particular loci. This would then provide the basis for an informed strain improvement programme.

In this study we first established by three independent methods that, under the conditions to be used, lignin was degraded effectively. We then assessed whether strain improvement is possible without mutagenesis and also whether there are alleles of particular genes that make major contributions to the flux through the lignin-degradative system. We used *P. chrysosporium* strain ME446 (which carries abundant RFLPs) and progeny derived from it. These progeny were a set of meiotically-derived strains, heterokaryotic derivatives from crosses

between such strains, and meiotically-derived progeny obtained by fruiting one such heterokaryotic strain.

METHODS

Organism. *Phanerochaete chrysosporium* ME446 (ATCC 34541) and its derivatives were maintained on 2% (w/v) malt extract agar (MEA) slopes incubated at 37 °C and stored at 4 °C. The isolation of the basidiospore-derived strains referred to in the Introduction and their genetic composition with respect to RFLP alleles are described by Raeder & Broda (1986) and Raeder *et al.* (1989a) respectively.

^{14}C -DHP (lignin substrate). Three different batches of DHP were used. Substrates A and B were both derived from a single preparation of guaiacyl [*ring*- ^{14}C]DHP synthesized by the polymerization of [*ring*- ^{14}C]coniferyl alcohol (New England Nuclear) by Elke Heckman (INRA, Paris), according to the procedure described by Faix *et al.* (1985). The bimodal size distribution of this material (Eriksson *et al.*, 1990) was confirmed in this work. When the material was received in this laboratory in 1986, part was dissolved in dimethylformamide (DMF) (substrate A) and part was left as a powder (substrate B), both being maintained at -20 °C. Elution profiles for A were obtained in 1989 and 1991 and found to be similar; the greater part of the radioactivity was of higher molecular mass (> 10000 Da). This material was used in all of the experiments described except for those specified below. Substrate B was dissolved in DMF in 1991; its elution profile differed from that of substrate A, there being a larger proportion of radioactivity in the lower molecular mass fraction. This material was used for investigating the effect of molecular size of DHP on its mineralization by *P. chrysosporium*. Substrate C was a [β - ^{14}C]guaiacyl DHP kindly donated by Dr M. H. Gold (Portland, Oregon). This material was used to assess the performance of the homokaryotic progeny of strain 2/52.

In order to measure changes in the molecular mass distribution of ^{14}C -DHP after incubation with the fungus, samples of culture supernatants were applied to a 50 cm \times 1 cm Sephadex LH-60 gel column with DMF containing 0.1 M LiCl as eluant. Absorbance was measured at 278 nm. An untreated ^{14}C -DHP sample was included as a control. The void volume was calculated by measuring the elution volume of blue dextran (2 mg ml⁻¹; molecular mass 2 MDa). Polystyrene markers with molecular masses of 10200, 7000, 5050 and 2550 Da were used as standards to construct a calibration curve. ^{14}C -DHP was fractionated on an LH-60 Sephadex column with DMF as eluant. The high and low molecular mass peak fractions from two separate fractionations were collected, lyophilized to decrease the volume, and reappplied to the column.

Mature [*lignin*- ^{14}C]lignocellulose. Mason *et al.* (1990) produced [*lignin*- ^{14}C]lignocellulose substrates from barley crops of varying maturities that ranged from 30 d to 140 d growth. The substrate used here was from the 140 d material. The lignin component in the plants was labelled by injecting L-[^{14}C]phenylalanine into the plant stems at regular intervals during growth. The material was then homogenized and solvent-extracted; this was followed by repeated protease (Pronase E; Sigma) treatments until no further radioactivity was released. The lignocellulose was then treated sequentially in a similar way with two cellulase preparations (*Penicillium funiculosum*, *Trichoderma viride*; Sigma) and washed with distilled water. This yielded material in which not less than 90% of the radioactivity was located in the lignin fraction (Klason lignin) as determined by the methods described by Effland (1977).

Scanning electron microscopy. Samples of 140 d [*lignin*- ^{14}C]lignocellulose were examined, pre- and post-fungal in-

cubation. Each sample was frozen in nitrogen slush and transferred under vacuum onto the cold stage ($-160\text{ }^{\circ}\text{C}$) of a Cambridge S200 Cryo SEM. Surface water was sublimed under vacuum by raising the temperature in the cold stage to $-80\text{ }^{\circ}\text{C}$. The temperature was reduced again to $-160\text{ }^{\circ}\text{C}$ and the sample was moved into the ante-chamber, where it was sputter-coated with gold. The material was then replaced in the cold stage to be examined.

Culture medium. When ^{14}C -DHP was first used as a substrate, it was found that the majority of the radioactivity added to the culture fluid became attached to the flask. To alleviate this problem, the flasks were washed in dimethyldichlorosilane (DMDCS) solution, allowed to dry and then washed with distilled water to remove any HCl formed in the process.

Culture medium comprised 0.01 M 2,2-dimethylsuccinate (DMS) or sodium acetate (NaOAc) pH 4.5 containing (g l^{-1}): KH_2PO_4 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.013; thiamin, 0.001; $\text{NH}_4\text{H}_2\text{PO}_4$, 0.276; veratryl alcohol, 0.068; and trace elements solution, 7 ml l^{-1} . The trace elements solution contained (g l^{-1}): sodium nitrilotriacetate, 1.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5; NaCl, 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; CoSO_4 , 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.082; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.01; H_3BO_3 , 0.01; NaMoO_4 , 0.01; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.05. The carbon source was 2% (w/v) glucose unless otherwise stated, and the medium was dispensed in 25 ml amounts in 250 ml Erlenmeyer flasks.

The ^{14}C -DHP substrate in DMF was then added as a 15 μl aliquot. In addition, 1 mg unlabelled DHP in 5 μl DMF was added to each flask. The unlabelled DHP was produced by the polymerization of coniferyl alcohol using the *Zutropfverfahren* (drop by drop) method (Tanahashi & Higuchi, 1981). Alternatively, 3.35 mg 140 d [*lignin*- ^{14}C]lignocellulose was added per flask, equivalent to 8000 d.p.m. per flask. Flasks were then autoclaved at 15 p.s.i. (103.5 kPa) for 5 min.

Inoculation and processing of cultures. Spore suspensions of individual cultures were prepared by scraping conidiospores from agar plates into sterile distilled water. For each treatment, three or four replicate flasks were inoculated with approximately 10^5 spores per flask. A control uninoculated flask was included in each experiment. To determine the concentration of spores, the spore suspension was first filtered through sterile glass wool to remove hyphal fragments. The filtered spores were washed by centrifugation at 4000 r.p.m. for 10 min, followed by decanting the supernatant and resuspending the spores in sterile distilled water. The number of spores was then determined using a haemocytometer.

Each flask was stoppered with a bung with inlet and outlet tubes and the clips closed. Vacuum grease was smeared around the junctions of the tubes and the bungs, and Parafilm was wrapped around the bungs and tops of the flasks. The flasks were incubated at $37\text{ }^{\circ}\text{C}$ without shaking. After different periods of incubation the flasks were flushed with O_2 for 15 min, and the released $^{14}\text{CO}_2$ trapped in 2 ml Carbo-Sorb E (Packard). Two millilitres of Optiphase 'Hi-Safe' 3 (LKB) scintillation fluid were added after flushing, and radioactivity was counted to within 5% accuracy. About 97% of the released radioactivity was trapped within 15 min (unpublished data).

Enzyme assays. LiP activity was assayed as the oxidation of veratryl alcohol to veratryl aldehyde (Tien & Kirk, 1984). MnP activity was assayed by the oxidation of vanillylacetone in the presence of Mn(II) ions (Paszczynski *et al.*, 1985). The results were corrected for manganese-independent peroxidase by repeating the assay without MnSO_4 . Cellulase activity was measured using a cellulose-azure method (Rinderknecht *et al.*, 1967). Cellulose-azure (Sigma) was added to provide a 2%

(w/v) suspension in 50 mM sodium acetate buffer pH 9, containing 50 mM NaCl. Release of dye was measured at 595 nm, the assay being calibrated using commercial cellulase (Sigma C-0901).

Preparation of heterokaryons. The method used was essentially that described previously (Thompson & Broda, 1987). For pairings, the homokaryotic strains were subcultured onto 2% (w/v) MEA plates. After 2 d growth at $37\text{ }^{\circ}\text{C}$, 0.8 cm diameter plugs of mycelium and agar were taken sub-marginally. Plugs from the two strains to be mated were placed 1.5–2.0 cm apart in the centre of an MEA plate, which was then incubated at $30\text{ }^{\circ}\text{C}$ for 2 weeks. Pairings that produced no antagonistic reactions were considered to be putative heterokaryons. Unlike some basidiomycetes, heterokaryotic mycelia of *P. chrysosporium* are not macroscopically different from homokaryotic mycelia. Also the heterokaryon does not form clamp connections (Burdall & Eslyn, 1974), so that assessment of heterokaryon formation is subjective using such methods alone. Therefore the heterokaryotic status of 2/52 (a product of strains 2 and 52) was confirmed by demonstrating the presence of the expected pattern of RFLPs, using *EcoRI*, *PstI* and *SaI*. DNA samples from strains 2, 19, 35, 52 and ME446 were used as controls.

Fruiting medium for basidiospores. The medium was that of Gold & Cheng (1979) but with 5% (w/v) wet Whatman microgranular CC31 cellulose in 1/3-strength Vogel's N medium and with thiamin added at a final concentration of 1 mg l^{-1} instead of biotin; Bacto-agar (Difco) was added at 1.5% (w/v). Conidiospores were inoculated at 10^5 spores per plate, and the plates incubated for 1–2 weeks at $30\text{ }^{\circ}\text{C}$. Fruit-bodies appeared on the edges of the medium and basidiospores ejected from the fruit body were collected in sterile distilled water from the dish lid. There was no contamination with conidiospores. Monobasidiosporous cultures were obtained by plating approximately ten spores per MEA plate. The plates were incubated overnight and individual germinating spores were excised under a microscope and transferred to separate MEA plates. These were then cultured at $37\text{ }^{\circ}\text{C}$ for 7 d to allow profuse sporulation prior to storage at $4\text{ }^{\circ}\text{C}$.

RESULTS

Definition of standard conditions for ^{14}C -DHP mineralization

^{14}C -DHP mineralization by strain ME446 was examined over 63 d using two alternative buffers, NaOAc and DMS, both at pH 4.5 and 10 mM, in combination with three alternative carbon sources, glucose, xylose and glycerol. Glucose and DMS gave the highest initial and final levels of $^{14}\text{CO}_2$ release: up to 30% of the initial radioactivity was recovered as $^{14}\text{CO}_2$ (data not shown). The use of DMS was also attractive since prolonged incubation gave no significant change in the pH. In contrast, the use of NaOAc, particularly at low glucose concentrations, resulted in increases in pH, presumably due to utilization of acetate as a carbon source and a resulting loss of buffering capacity. Moreover DMS had previously been used by Kirk *et al.* (1986).

The molecular mass distribution of the ^{14}C -DHP that remained in suspension after 63 d was examined by gel filtration. In the control, the majority of the radioactivity resided in the $>10000\text{ Da}$ apparent molecular mass portion. After incubation with the fungus, under all conditions, the final molecular mass averaged 3000 Da

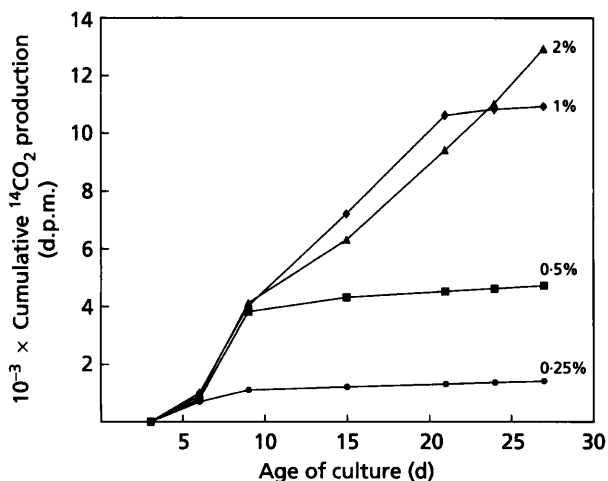


Fig. 1. Effect of glucose concentration on ^{14}C -DHP mineralization. ^{14}C -DHP was added at 46000 d.p.m. per flask. The cumulative values shown are the means of four replicate flasks for each glucose concentration. Flasks were flushed with oxygen on days 3, 6, 9, 15, 20, 24 and 28.

(data not shown). Thus, both depolymerization and mineralization were occurring.

The effect of glucose concentration on ^{14}C -DHP mineralization was examined in the concentration range from 0.25% to 2% (w/v). The duration of $^{14}\text{CO}_2$ release increased as a function of glucose concentration (Fig. 1). In this experiment, as in all others using ^{14}C -DHP, the d.p.m. released by replicate cultures varied. With the 0.5%, 1% and 2% glucose-containing cultures the means of the standard deviations for the different time points were 25%, 31% and 24%, whereas for the 0.25% glucose set the standard deviation was 85%. The reason for this difference was that, with the 0.25% set, one of the four cultures gave negligible mineralization; such aberrant behaviour of a minority of cultures was also observed in the experiments to be described below. The standard conditions that were then chosen were: 25 ml static cultures with 10 mM DMS pH 4.5 and 2% glucose, inoculation with 10^5 spores and incubation with sampling for $^{14}\text{CO}_2$ at 4 and 10 d. The cumulative values for d.p.m. were used in the subsequent calculations.

Use of ^{14}C -DHP as substrate

Published accounts of mineralization of ^{14}C -DHP to $^{14}\text{CO}_2$ show extensive variation between assays from replicate cultures (Kirk *et al.*, 1986). It is important to know whether such variation arises from the assay itself or from differences in the behaviour of replicate cultures. Since DHP is insoluble in water, and is added in a small volume of DMF, it is in suspension rather than in solution. This might be a source of variability in apparent performance between replicate cultures. Such variation was tested for by dispensing sets of replicate aliquots, with different amounts of carrier DHP. The variation between replicate samples was about 10%, and was unaffected by the amount of carrier.

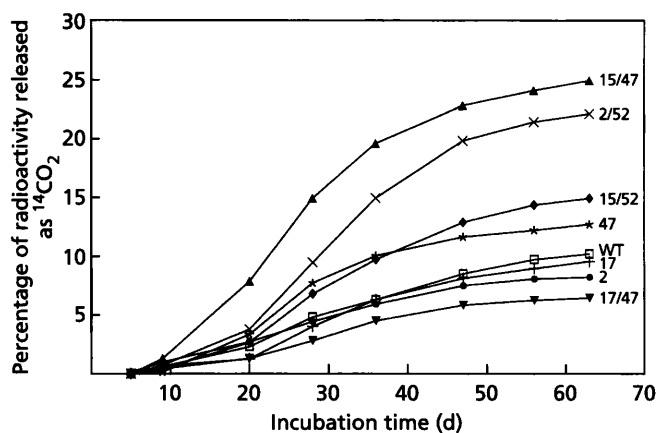


Fig. 2. Cumulative mineralization of 140 d [^{14}C]lignocellulose by strain ME446 (WT) and some homokaryotic and heterokaryotic derivatives. Substrate was added at 3.35 mg, equivalent to 8000 d.p.m., per flask. DMS pH 4.5 and 2% glucose were present. Four cultures were inoculated per strain. The cultures were regularly flushed with oxygen over a 63 d time course, $^{14}\text{CO}_2$ being trapped at each time point.

Gel filtration confirmed that the size distribution of the ^{14}C -DHP had two maxima. Such heterogeneity might lead to selective utilization of one size class. This hypothesis was tested with high and low molecular mass fractions. About 85% of the radioactivity released over a 10 d incubation arose from the lower molecular mass material.

Peroxidase activities in culture supernatants during DHP mineralization

Strain ME446 was cultured in DMS plus 2% glucose medium for 14 d. Fungal dry weight increased fourfold between days 3 and 10, and then started to decline. No LiP activity was detected in the supernatant at any time. In contrast, MnP activity appeared between days 5 and 6, with peak activity after 7 d; on day 14, supernatant MnP activity could still be detected. There was also MnP activity but not LiP activity during growth in DMS at lower glucose concentrations. In control experiments with NaOAc + 0.25% glucose, LiP appeared on day 5, peaked on day 6 and had disappeared by day 14. In this case biomass peaked earlier, at day 4, and then decreased (data not shown). However in NaOAc buffer with 2% glucose LiP was again not detected.

Mineralization of [^{14}C]lignin from mature lignocellulose

Strain ME446 and seven of its derivatives were tested for their ability to release $^{14}\text{CO}_2$ from plant [^{14}C]lignocellulose substrate. This material had been subjected to repeated cellulase and protease treatments so that more than 90% of the ^{14}C was in the Klason lignin component (Mason *et al.*, 1990). Over 63 d, up to 26% of the ^{14}C label was released as $^{14}\text{CO}_2$, so that not less than 15% of the lignin in this mature material had been mineralized (Fig. 2). Although the preparation of the substrate had involved

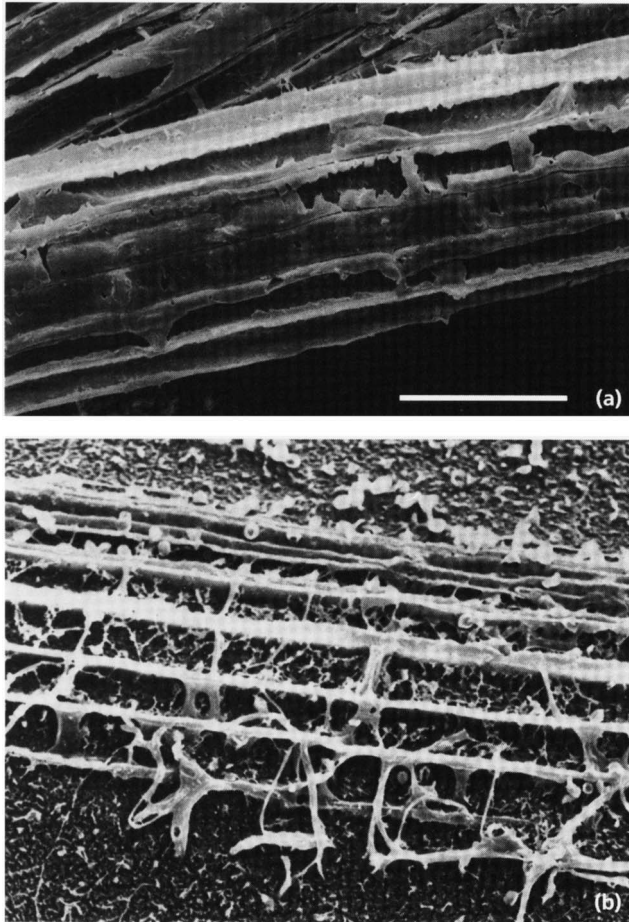


Fig. 3. Substrate prepared from 140 d [^{14}C]lignocellulose before inoculation (a) and after 63 d incubation with strain 15/47 (b). Bar, 50 μm (applies to both panels).

exhaustive treatment with cellulases, some of the $^{14}\text{CO}_2$ released in the experiment could still have originated from lignin that had first been solubilized by *P. chrysosporium* cellulases. However, no supernatant cellulase was detectable after 10 d incubation, using the cellulose azure assay; this was expected since cellulases are subject to catabolite repression in this organism (Eriksson & Hamp, 1978), and the medium contained 2% glucose. We infer that mineralization is due to lignin breakdown.

The variation in the cumulative d.p.m. released by replicate cultures of individual strains was greater than with the ^{14}C -DHP substrate using strain ME446 (see above). The mean standard deviation between replicates, across all strains and all time points, was 65%. It was hypothesized that this was due to the heterogeneous nature of the substrate and its relatively low specific activity with respect to ^{14}C . Therefore, although this material was valuable for the demonstration of lignin degradation by *P. chrysosporium* from mature lignocellulose, it was judged to be less useful than ^{14}C -DHP for

the studies to be described below on differences in performance between strains.

Untreated substrate and substrate incubated with one heterokaryotic strain (15/47) for 63 d in the above experiment were examined by scanning electron microscopy. Fig. 3(a) shows the material before incubation; although subjected to prior exhaustive protease and cellulase extractions the material appears reasonably intact. Fig. 3(b) shows extensive modification of the material after 63 d incubation, consistent with the conclusion that the fungus has achieved significant degradation of the lignin component during this period.

^{14}C -DHP mineralization by homokaryotic derivatives of ME446

Twenty-six homokaryotic derivatives of strain ME446 (Raeder & Broda, 1986) were tested for lignin mineralization performance in a series of experiments. Each experiment involved ME446 and five to nine other strains, with four replicate cultures per strain, and was repeated at least twice (Table 1). Determinations were based on the cumulative totals of radioactivity released as $^{14}\text{CO}_2$ by day 10; this was preferred to sampling at intervals for short-term determinations, since the latter method would be likely to reflect to a greater extent the preferential utilization of lower molecular mass ^{14}C -DHP demonstrated above.

As described above for strain ME446 there was substantial variation in d.p.m. released when the data from any set of four replicate cultures were compared. This variation included some cultures that gave negligible or very low levels of $^{14}\text{CO}_2$ release; these data were included in the calculations. Partly as a result of this there was considerable variation in performance of individual strains between experiments (Table 1) so the conclusions to be drawn are qualitative rather than quantitative. The calculated values for the highest- and lowest-performing strains differed by a factor of 70, and the values for the 26 strains fell on a line with no discontinuities or clustering. This suggests that there is substantial allelic variation in a number of determinants involved in ^{14}C -DHP mineralization.

Since the RFLP allele distributions for 38 genetic loci for these strains are known (Raeder *et al.*, 1989a), they were analysed for any correlation between the presence of particular alleles at specific loci on the one hand and high lignin mineralization on the other (Table 2). No such correlation was found, indicating strongly that alleles of a number of genes distributed throughout the genome contribute to the final ^{14}C -DHP mineralization rate, rather than there being a single major determinant of performance.

Range of performance among heterokaryotic strains

If alleles of a number of genes together determine performance for ^{14}C -DHP mineralization, the range of such performances of progeny of crosses between homo-

Table 1. Rank order for ^{14}C -DHP mineralization of 26 basidiospore-derived homokaryotic progeny of *P. chrysosporium* strain ME446

The medium contained DMS pH 4.5 and 2% glucose, and 8500 d.p.m. of ^{14}C -DHP substrate and 1 mg unlabelled carrier DHP were added to each culture. Determinations were based on the cumulative totals of radioactivity released by day 10. Results are expressed as percentages of the result for ME446 in each sub-experiment. Each value is itself the mean of the results from four independent cultures. Percentages of total counts are the aggregate percentages for all cultures in each sub-experiment.

Strain	Sub-expt:	1	2	3	4	5	6	7	8	9	10	11	12	13	Mean %	Rank order
1					3	25									14	24
2		38	75	129	3	82									65	11
3									16	4					10	25
4									35	58	12	31	28	18	30	19
8					230	131			105	58	84	112			120	4
9		7			60	73									47	15
13					71	44									58	12
14					163	136	66	74					48	51	90	7
15		6	35	4											15	23
17			55	128									59	45	72	9
18									81	201					141	1
24					192	112					72	150			131	2
26									119	198			107	89	128	3
27							56	76							66	10
28							20	26							23	20
29					85	36					103	82	105	103	86	8
30		5							38	54					32	18
31								21	18						19	21
32								4	1						2	27
34					74	27									50	14
38								22	85						53	13
42									55	38					46	16
43									34	178					106	5
47			15	1											8	26
50							13	19							16	22
52		26	35	61							32	34			38	17
ME446		100	100	100	100	100	100	100	100	100	100	100	100	100	100	6
% of total counts		1.8	7.2	3.3	8.7	6.7	2.0	3.4	4.3	1.4	5.2	2.5	1.7	3.0	3.9 ± 59%	

karyotic strains should be narrower than that of their parents. This is because all such progeny will be homozygous for about 50% of their genes. This prediction is also dependent on the validity of the values obtained using the ^{14}C -DHP mineralization assay. Strains were paired on MEA plates and 12 of the strains that appeared to have arisen by fusion were tested for ^{14}C -DHP mineralization, as above. In all cases strains ME446 (wild-type) and 2/52 (the product of the cross between strains 2 and 52) and an uninoculated flask were also included (Table 3). The range of levels of performance of the 12 strains was 5.3-fold, compared with 16-fold for that of their 11 parental homokaryotic strains. As with the homokaryotic progeny of ME446, a minority of strains performed better than ME446 itself.

^{14}C -DHP mineralization by siblings derived from strain 2/52

Comparison of DNA from strain 2/52 with DNA from strains ME446 and four homokaryotic derivatives showed that it was polymorphic with respect to the *SaI* and *PstI* sites on the sequence that hybridizes with clone 1 (Raeder & Broda, 1986). Basidiospores from this heterokaryotic strain were obtained by fruiting on Walseth cellulose medium, and cultures were derived from such basidiospores. Eighteen of these strains were assessed for mineralization of ^{14}C -DHP as compared with ME446 and 2/52 (Table 4). The range of mean degradation rates (3.2-fold) was much narrower than had been observed for the homokaryotic derivatives of ME446 and also narrower than that of the heterokaryotic strains described above.

Table 2. Test for correlation of RFLP allele distributions with high lignin degradation among homokaryotic derivatives of *P. chrysosporium* strain ME446

The loci are as in Raeder *et al.* (1989a) [col. (1)]. For each locus, strains with identified A or B alleles are taken and ranked; the rank-sum for the A-strains is obtained [col. (2)]. The numbers of A-strains [col. (3), n_A] and of A- or B-strains [col. (4), $n_A + n_B$] are given. The expected rank-sum [col.(5)] is calculated as $\frac{1}{2} n_A (n_A + n_B + 1)$. The Wilcoxon (Mann-Whitney) U -statistic [col. (6)] is obtained as $u = \text{rank sum} - \frac{1}{2} n_A (n_A + 1)$. The last column (7) is the probability $P (U \leq u)$ that the Wilcoxon u -value [col. (6)] will not be exceeded by chance; in single samples, values of P very close to 0 or 1 would be significant evidence of association between allele and performance. The significant level of P -values in an aggregate of cases is hard to evaluate because of their mutual dependence due to the linkage of loci. Nevertheless, a lower bound can be calculated that is based upon the number of linkage groups. It can be shown that with 10 linkage groups involved, the probability of at least one P value falling outside the range 0.02–0.98 – that is, outside the smallest centered interval covering all P values – is at least 0.33, which is not significant.

(1) Locus	(2) Rank- sum	(3) No. of A-strains	(4) No. of strains	(5) Expected rank-sum	(6) Wilcoxon u	(7) P ($U \leq u$)
65	100	9	26	121.5	55	0.13
9	193	15	26	202.5	73	0.32
52	167	14	21	154	62	0.84
1	233	16	26	216	97	0.82
47	233	16	26	216	97	0.82
54	213	16	26	216	77	0.45
76	199	14	25	182	94	0.83
39	218	16	26	216	82	0.55
34	180	14	26	189	75	0.33
5	222	14	26	189	117	0.96
44	180	13	26	175.5	89	0.60
56	178	15	24	187.5	58	0.30
41	223	16	26	216	87	0.65
79	170	12	25	156	92	0.78
18	233	17	26	229.5	80	0.58
7	215	17	26	229.5	62	0.23
19	176	14	26	189	71	0.26
58	229	17	25	221	76	0.69
62	232	18	26	243	61	0.28
45	174	15	24	187.5	54	0.22
11	206	16	26	216	70	0.31
cel	202	15	26	202.5	82	0.50
8	200	15	26	202.5	80	0.46
33	186	14	26	189	81	0.45
51	180	13	26	175.5	89	0.60
20	98	5	26	67.5	83	0.98
14	90	5	26	67.5	75	0.93
36	68	4	26	54	58	0.84
72	110	6	25	78	89	0.98
48	74	5	26	67.5	59	0.67
12	118	10	26	135	63	0.20
69	128	10	26	135	73	0.37
70	90	9	25	117	45	0.07
61	186	14	26	189	81	0.45
90	188	15	26	202.5	68	0.24
57	230	15	26	202.5	110	0.93
40	193	13	26	175.5	102	0.82
66	109	7	26	94.5	81	0.80

Table 3. ¹⁴C-DHP mineralization by heterokaryotic strains as percentages of mean mineralization by strains ME446 and 2/52 in the same experiment

Experimental details as in Table 1. Each value is itself the mean of the results from four independent cultures.

Strain	Sub-expt:	2	3	10	11	12	13	Mean %	Parental means %*	% Heterozygous†
15/47		32	59					45	15, 8	66
17/47		45	80					63	72, 8	43
15/52		77	97					87	15, 38	46
2/52		110	114	71	100	86	84	94	65, 38	54
ME446		90	86	129	100	114	116	106	—	100
29/52				47	7			27	86, 38	43
8/52				43	56			50	120, 38	54
4/8				112	153			133	30, 120	58
8/29				152	100			126	120, 86	42
24/29				164	100			132	131, 86	42
17/26						46	4	25	72, 128	47
4/14						94	80	87	30, 90	53
4/29						124	84	104	30, 86	42
% of total counts		10.8	4.7	6.1	2.6	1.9	2.9	4.8 ± 3.3		

* The mean values for the parental strains were taken from Table 1.

† This value is derived from the RFLP analysis of Raeder *et al.* (1989a).

Table 4. Performance of homokaryotic derivatives of strain 2/52

Experimental details as in Table 1. The results are expressed as percentages of the means of the result for 2/52 and the result for ME446 in each experiment. Each value is itself the mean of the results from four independent cultures.

Strain	Sub-expt:	14	15	16	17	Mean
1		84	88			86
2		103	157			130
3		85	36			60
4		99	169			134
5		67	101			84
6		57	87			72
7		107	108			107
8		118	63			90
9		107	67			87
2/52		124	128	114	95	115
ME446		76	72	86	105	85
10				55	34	44
11				50	68	59
12				51	48	50
13				27	58	42
14				41	44	42
15				77	54	65
16				108	54	81
17				49	81	65
18				86	87	86
% of total counts		4.3	3.7	2.8	3.5	3.6 ± 0.6

DISCUSSION

There are major differences in efficiency of ¹⁴C-DHP mineralization among ME446 and its progeny strains, as predicted from the abundant RFLPs contained within ME446 (Raeder & Broda, 1986). Thus some alleles for genes encoding or regulating ¹⁴C-DHP mineralization functions differ quantitatively in their contributions to the flux through the system. We infer from the data that some strain improvement is feasible for lignin degradation by cycles of fruiting and crosses, without mutagenesis. To draw these conclusions with confidence we had first to examine the implications of two other features of the system of general interest: the variability in mineralization of ¹⁴C-DHP shown between replicate cultures, and the absence of supernatant LiP under the conditions used.

Initial experiments with ME446 established conditions that gave efficient release of ¹⁴CO₂ from ¹⁴C-DHP. The continuing release of label in the glucose dependence experiment indicates that ¹⁴C-DHP, diluted with unlabelled carrier, is not limiting. *P. chrysosporium* degraded the low molecular mass fraction of ¹⁴C-DHP preferentially. The considerable variation in yields between replicate cultures occurred both because some did not mineralize the substrate and because those that did so mineralized it to various extents. It was shown that very similar amounts of ¹⁴C-DHP entered each flask, and great care was taken to prevent leakage of gas from the system. We conclude that there is intrinsic variation in the behaviour of *P. chrysosporium* or that culture conditions are as yet uncontrolled in some crucial respect. Intrinsic variation

between replicate cultures has been described and discussed previously by Raeder *et al.* (1989b) for supernatant LiP using different conditions (0.1% glucose, NaOAc buffer). In that case, appearance of LiP activity was preceded by a decrease in fungal biomass; in about 5% of the cultures the decrease in biomass was less than in the other cultures, and these did not then produce supernatant LiP activity.

Lignin degradation in the absence of supernatant LiP

Under the chosen conditions, MnP activity was detectable in the culture supernatants but not LiP activity. Although ¹⁴C-DHP mineralization indicated lignin-degradative ability, we sought independent confirmation that such degradation was indeed occurring. Mineralization of material from mature barley straw provided a stringent test, since this substrate had been prepared by a long regime of labelling followed by exhaustive treatment with both polysaccharidases and proteases (Mason *et al.*, 1990; see Methods). Thus, as in some other white rot fungi (Péridé & Gold, 1991; Rüttimann-Johnson *et al.*, 1993), supernatant LiP activity is not necessary for lignin degradation by *P. chrysosporium* under these conditions. In contrast, MnP was always present in the supernatant. Moreover, whereas MnP activity appeared before mycelial biomass peaked (i.e. it was present in the primary growth phase), where LiP activity was present (in NaOAc medium), it appeared as mycelial biomass started to decline.

A large literature exists on the conditions that correspond to maximum production of enzymes implicated in lignin degradation in supernatants. This literature describes different species, different strains of *P. chrysosporium*, different growth conditions (aeration, agitation, immobilization), pH and buffering, presence of veratryl alcohol, different concentrations of carbon and nitrogen sources and of Mn(II), and defined media versus wood decay and solid substrate fermentation conditions (Cancel *et al.*, 1993; Capdevila *et al.*, 1990; Datta *et al.*, 1991; Dosoretz *et al.*, 1993; Eriksson *et al.*, 1983; Faison & Kirk, 1985; Kerem *et al.*, 1992; Niku-Paavola *et al.*, 1990; Orth *et al.*, 1991; Perez & Jeffries, 1990; Tien & Myer, 1990; Tonon & Odier, 1988; Venkatrati & Irvine, 1990). Some authors appear to assume a relationship between supernatant enzyme levels and an efficient lignin degradation process. In contrast, others have shown by immunocytochemical methods that much of the LiP is mycelium-bound (Evans *et al.*, 1991; Garcia *et al.*, 1987; Lackner *et al.*, 1991; Moukha *et al.*, 1993; Ruel & Joseleau, 1991; Srebotnik *et al.*, 1988). The process of lignin depolymerization is likely to depend on structural organization that brings into proximity the peroxidases (and perhaps the peroxide-generation system) and the substrate; since such organization would depend upon the intact fungus, it seems to follow that supernatant enzyme is irrelevant for lignin degradation *in vivo*. Biochemical studies on the action of LiPs and MnPs *in vitro* show that polymerization occurs (Haemmerli *et al.*, 1986) as well as depolymerization (Hammel & Moen, 1991; Wariishi *et al.*, 1991; Hammel *et al.*, 1993). If peroxidases are responsible for lignin

depolymerization, the products must be rapidly partitioned to prevent their repolymerization. If mycelium-bound LiP is involved in lignin degradative ability and the appearance of supernatant LiP reflects senescence (Lackner *et al.*, 1991), LiP mRNA should be detectable in the absence of such supernatant activity. This has been reported elsewhere (Broda *et al.*, 1995). Such sequestration of LiP could explain the paradox that other fungi have LiP-related DNA sequences and/or proteins that cross-react with LiP-derived antibodies in Western blots but have no corresponding measurable supernatant LiP activity (Huoponen *et al.*, 1990; Orth *et al.*, 1993; Rüttimann *et al.*, 1992).

Performance and genotype

The range of levels of performance of 26 homokaryotic derivatives of ME446 was 70-fold; this demonstrates the consequences of the extent of genetic polymorphism present in ME446. Both this range and the absence of clustering in the values suggest that a number of determinants contribute to the overall efficiency of lignin mineralization. The markers included in the statistical analysis cover the whole genome of *P. chrysosporium* ME446. However, there was no evidence that for any loci the possession of any one allele has a disproportionate effect on the flux through the DHP mineralization system.

If performance for DHP mineralization is determined multifactorially, the range of values for the heterokaryotic strains should be narrower than that for the parental homokaryotic strains, and indeed this was the case. The range for the progeny of strain 2/52 was even narrower; this was also expected, since less polymorphism is contained in this strain alone than that present in the set of homokaryotic strains used to produce the heterokaryotic strains. The internal consistency of these results supports the validity of the DHP assay on which they are based.

The mean performance of the 26 progeny of ME446 was 58% of that of the parent, with five strains performing better than ME446 itself. The 12 heterozygous progeny from crosses between such strains had a mean performance of 83%. Their inferred degrees of heterozygosity were between 42 and 66%, on the basis of the RFLP study of Raeder *et al.* (1989a), but there was no correlation between the extent of this heterozygosity and the performance of individual strains. Of these 12 strains, eight performed better than either parent, two had performances intermediate between those of the parents, and only two performed less well than either parent. Strains 8/29 (126%) and 24/29 (132%) retained the higher levels of performance observed for the parental strains 8 (120%), 24 (131%) and 29 (86%). The 18 progeny of strain 2/52 (94%) had a mean performance of 79%, with 16 strains performing more poorly than the parental strain.

We conclude that within *P. chrysosporium* ME446 some strain improvement is possible for lignin mineralization by the use of breeding alone. This could be extended by inter-strain crosses. There is also scope for directed mutagenesis; potential targets of biotechnological rel-

evance include the elimination of specific cellulase activities by site-directed mutagenesis (thus sparing cellulose, as would be desired in biopulping) or the linking of peroxidase-specifying sequences to inducible promoters. A further possibility is the introduction of multiple copies of a number of genes simultaneously, which has been shown to increase flux in a *Saccharomyces cerevisiae* biosynthetic pathway (Niederberger *et al.*, 1992). Such approaches would depend on further development of transformation techniques for *P. chrysosporium* (Gold & Alic, 1993). However, it will be equally important to improve our understanding of the organism's physiology under scaled-up solid substrate fermentation conditions, and any genetical approaches must be firmly linked to such studies.

Conclusions

1. The ^{14}C -DHP mineralization assay allows genetically related strains of *P. chrysosporium* to be compared for performance for lignin degradation.
2. Under the conditions used, supernatant LiP activity is absent, so if LiP is necessary for ^{14}C -DHP mineralization, it must be as mycelium-bound enzyme. We infer that when supernatant LiP is present, as under other conditions, its levels cannot be used as an index of lignin mineralization.
3. Strain ME446 contains abundant RFLPs, and permutations of alleles in different meiotic progeny result in very different levels of performance for ^{14}C -DHP mineralization. However, no single allele is necessary for high performance.
4. Consistent with this interpretation, heterokaryotic strains obtained by crosses between such meiotically derived strains show a much narrower range of levels of performance. Moreover the meiotic progeny of one such heterokaryotic strain show a still narrower range.
5. With each set of strains, individuals were found with performances that were higher than that of the parental strain ME446. Moreover, most heterokaryotic progeny performed better than either of their parental homokaryotic strains.
6. Some strain improvement for ^{14}C -DHP mineralization is possible by breeding.

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