Journal of Industrial Microbiology (1996) 16, 286–294


# Biological potential of fungal inocula for bioaugmentation of contaminated soils

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**The suitability of the fluorescein diacetate hydrolyzing activity (FDA) assay for determining the biological potential (ie fungal biomass produced per unit of substrate) of solid pelleted fungal inoculum intended for use in the bioaugmentation of contaminated soils with white-rot fungi, was evaluated. FDA activity of the white-rot fungus *Phanerochaete chrysosporium* grown on pelleted substrates and on agar was found to be proportional to quantities of fungal ergesterol and fungal dry matter, respectively. Inoculum biological potential was found to be greatly influenced by substrate formulation and structure, and temperature. Biological potential and the type of carrier influenced the ability of *P. chrysosporium* to tolerate pentachlorophenol (PCP). *Phanerochaete chrysosporium* and *Trametes versicolor* introduced into PCP-contaminated soil on pellets with higher biological potential and higher nitrogen content (C:N ratio of 50:1), did not remove PCP more efficiently than when the fungi were introduced on pellets with a lower biological potential (C:N ratio of 309:1). However, under the latter conditions most of the PCP was transformed to pentachloroanisole (PCA). In soil inoculated with *T. versicolor* on pellets with high biological potential, higher manganese peroxidase activity was detected compared to soil inoculated with pellets with a lower biological potential.**

**Keywords:** fungal inoculum; bioaugmentation; biological potential; pentachlorophenol

## Introduction

Lignin-degrading basidiomycetes have the ability to degrade many persistent toxic organic chemicals, such as polychorinated biphenyls (PCBs), pentachlorophenol (PCP), and several polycyclic aromatic hydrocarbons [20]. Much attention has been directed toward the use of these fungi at various contaminated sites for soil remediation [11–14]. One of the barriers to successful implementation of fungal bioaugmentation is the development of inexpensive, high quality fungal inoculum with uniformly high biological potential. To address this need we are developing methods for the construction and production of pelleted fungal inocula for bioaugmentation of contaminated soils. These inocula are in the form of pelleted solid substrates that are coated with fungal mycelium. They are predominantly made from inexpensive agricultural and wood industry by-products, resist contamination by indigenous soil microbes, and can be produced with conventional machinery. One of the advantages of pelleted inocula over previously used fungal carriers with fixed nutrient compositions such as corn cobs, wood chips, or straw [7,17,19,26] is the ability to optimize the substrate composition of inocula to enhance fungal growth, pollutant-degrading abilities, and competitiveness against indigenous soil microbes. To optimize pellet composition to obtain maximum fungal growth and pollutant-degrading ability, methods that measure fungal biomass, or fungal biomass-associated metabolic activity per weight or volume of inoculum (ie biological potential of pelleted substrate) need to be developed.

Direct measurements of fungal growth on solid substrates is complicated because of the difficulty of separating fungal mycelia from solid substrates. Because of the complexities of making direct measurements, many indirect methods for estimation of microbial biomass and activity have been developed. They can be classified into three categories: (I) methods measuring cell constituents such as chitin, chitosan, nucleic acids, proteins; (II) methods measuring biological activity such as ATP, enzymatic activity, respiration rate, or immunological activity; and (III) methods measuring nutrient consumption [6].

A simple, inexpensive test for the rapid estimation of the biological potential of large numbers of samples is desirable for use in the development of high quality fungal inocula. The fluorescein diacetate hydrolyzing activity (FDA) assay, which is rapid and inexpensive, provides a measure of microbial biomass growth rate by measuring the activity of a number of enzymes (lipases, proteases, esterases), that are produced during fungal growth [25]. The product of this enzymatic activity is fluorescein, which can be quantified spectrophotometrically. The FDA method has been used successfully for differentiating metabolically active from inactive cells [9], to measure total microbial activity in soil and litter [24], and to measure the growth of filamentous fungi [6,9,22,24].

The purpose of the present work was to evaluate the suitability of the FDA assay for assessing the biological potential of pelleted substrates and for measuring the potential of fungi to proliferate from pelleted substrates to complex media contaminated with PCP. The influence of biological potential of the pelleted fungal inoculum on removal of

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Received 13 November 1995; accepted 6 March 1996

PCP from artificially contaminated soil, and on lignin peroxidase and manganese peroxidase activity in the soil was also assessed.

## Materials and methods

### *Organisms*

The white-rot fungi *Phanerochaete chrysosporium* (BKM F-1767, ATCC 42725) and *Trametes versicolor* (MD-277) were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, WI, USA. The fungi were grown and maintained on 2% potato-dextrose-agar (PDA) in slants or on master plates.

### *Chemicals*

Pentachlorophenol (PCP), pentachloroanisole (PCA), and fluorescein-diacetate were purchased from Aldrich Chemical Co (Milwaukee, WI, USA). Dimethyl-sulphoxide (DMS) was from Burdick and Jackson Laboratories Inc, Muskegon, MI, USA. All other chemicals were reagent grade. Aspen sawdust was obtained from Walter Brothers, Hokum, WI, USA. Rice hulls were from Riceland, Stuttgart, AR, USA. Nutrient-fortified grain-sawdust mixture was from the LF Lambert Spawn Co Inc, Coatesville, PA, USA. Lignosulphonate was from Lignotech, Greenwhich, CT, USA. Starch was from Staley, Decatur, IL, USA. Corn meal and corn steep liquor were from CPC, Summit-Argo, IL, USA.

### *Dry matter and FDA activity of fungi grown on agar plates*

Petri plates containing 20 ml of PDA were inoculated with 6-mm diameter agar plugs taken from *P. chrysosporium* master plates and incubated at 30°C. The fungal biomass was then removed together with agar medium after 3, 5, and 8 days. On each day, three of the plates were used to determine fungal biomass and four plates were used to assess FDA activity. Fungal biomass was determined by selectively dissolving the agar by microwaving it as described by Nout *et al* [18]. After microwaving, the melted agar and fungal hyphae from a plate were vacuum filtered and the fungal biomass was collected on pre-weighed Whatman No. 1 filter paper, rinsed with distilled water, dried for 24 h at 60°C, and weighed.

To determine FDA activity the agar in the four other plates was divided into sections that represented 25%, 12.5%, 6.25%, 3.13%, and 1.56% of the total area of the plate. The amounts of biomass on these sections were calculated from corresponding dry matter data from whole plates obtained as described above. The sections were removed and placed in glass tubes with 10 ml of 60 mM $NaH_2PO_4$ at pH 8 and a sterile pellet. The pellets were composed of 75% sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate, and cut to obtain similar length and weight. The whole suspension was then disrupted with a glass rod, shaken vigorously, and FDA activity was determined as described below. FDA determinations were performed on three replicate sections for each fractional area.

### *Substrate mixtures for determination of biological potential*

Substrate mixtures for determination of biological potential were based on either a nutrient-fortified grain-sawdust mixture with no further components except 2% lignosulphonate, sawdust or rice hulls as carriers enriched with starch, corn meal or corn steep liquor with 2% lignosulphonate as a lubricant. Substrate mixtures (Table 1) were sterilized by autoclaving them for 20 min. *Phanerochaete chrysosporium* was cultured on 2% PDA plates at 30°C for 7–10 days to produce conidia. The conidiospores were collected from agar plates with demineralized, sterile water and the spore suspension was filtered through glass wool. The spore concentration in the suspension was determined by measuring its optical density at 650 nm ($2.5 \times 10^6$ spores $ml^{-1}$ corresponds to $E_{650} = 0.5$ [10]). Ten milliliters of spore suspension were then mixed with 500 g of sterilized substrate mixture (moisture content of 27–30%), to achieve a final concentration of $4.5 \times 10^5$ conidia $g^{-1}$ of wet substrate. The spore-amended *P. chrysosporium* substrate mixtures were then pelleted aseptically in an Amandus Kahl (Hamburg, Germany) laboratory pellet press.

### *Determination of biological potential of the pelleted substrates*

Pellets made from different substrate mixtures (Table 1) and inoculated with $4.5 \times 10^5$ spores of *P. chrysosporium* $g^{-1}$ of substrate were prepared, as described above, and were used for measurements of biological potential. Under aseptic conditions, pellets of known moisture content were cut with a razor blade to obtain a similar length and then weighed. They were then incubated in Petri dishes (three pellets per plate) inserted in 20 ml of 1% noble agar containing 15 $\mu g\ g^{-1}$ PCP, at 24°C. The noble agar served as a source of moisture while nutrients necessary for fungal growth were obtained solely from the pellet. PCP was added to liquefied noble agar from a stock solution (0.075 g PCP 10 $ml^{-1}$ DMS). The PCP was included to slow the growth of the fungus and to serve as a stress factor to elicit possible differences in fungal growth among the various substrate mixtures. The pellets and the region of the agar that was overgrown with fungal hyphae were taken from the Petri plate immediately and after 2, 3, 4, 6, and 8 days of incubation for determination of ergesterol content or for measuring FDA activity, in triplicate.

To determine FDA activity, a pellet and the associated section of agar overgrown with fungus were removed from the plate and disrupted with a glass rod in a glass tube containing 10 ml of 60 mM $NaH_2PO_4$ [24], at pH 8, and shaken vigorously. Optimal FDA activity of *P. chrysosporium* in $NaH_2PO_4$ buffer was found to occur at pH 8 (data not shown). The hyphae and agar pieces were then separated from the buffer by filtration through a 45-$\mu$m pore size nylon, low protein retention filter (Cole-Parmer, Niles, IL, USA). The FDA activity of particles that remained on the filter was less than 5.5% of that in the filtrate solution. Then 10 $\mu$l of FDA from 10 mM FDA/DMS stock solution was added to the 5 ml of the filtrate solution to achieve a final concentration of 20 $\mu$M. This reaction mixture was incubated for 30 min at 24°C and then assayed spectrophotometrically at 490 nm. The remaining filtrate solution was

**Table 1** Biological potential of pelleted substrate mixtures and one non-pelleted substrate mixture at 24°C and 39°C

| Substrate mixtures | Biological potential (FDA activity) ($\mu$M fluoresc 30 min$^{-1}$ g$^{-1}$ ± s.d.) |
|---|---|
| [1,2]75% sawdust<br>15% starch<br>8% corn meal<br>2% lignosulphonate | 525.89 ± 84.11[a] |
| [3,4]98% nutrient-fortified grain-sawdust mixture<br>2% lignosulphonate | 72.28 ± 10.725[b] |
| [3] 98% nutrient-fortified grain-sawdust mixture<br>2% lignosulphonate | 41.32 ± 6.49[c,d] |
| [2,3]75% sawdust<br>15% starch<br>8% corn meal<br>2% lignosulphonate | 31.84 ± 3.82[d] |
| [3] 78% sawdust<br>15% starch<br>5% corn steep liquor<br>2% lignosulphonate | 24.46 ± 4.01[d,e] |
| [3] 53.25% rice hulls<br>17.75% sawdust<br>15% starch<br>12% corn meal<br>2% lignosulphonate | 17.9 ± 16.71[e] |
| [3] 80% sawdust<br>15% starch<br>3% corn steep liquor<br>2% lignosulphonate | 9.79 ± 3.55[e] |
| [3] 80% rice hulls<br>15% starch<br>3% corn steep liquor<br>2% lignosulphonate | 9.00 ± 8.01[e] |
| [3,5]83% sawdust<br>15% starch<br>2% lignosulphonate | 7.09 ± 0.90[e] |
| [3] 71% rice hulls<br>15% starch<br>12% corn meal<br>2% lignosulphonate | 5.85 ± 1.21[e] |
| [3] 81.5% sawdust<br>15% starch<br>1.5% corn steep liquor<br>2% lignosulphonate | 2.76 ± 1.29[e] |
| [3] 81.5% rice hulls<br>15% starch<br>1.5% corn steep liquor<br>2% lignosulphonate | 2.69 ± 1.91[e] |

[1]Biological potential at 39°C.
[2]High-N sawdust substrate.
[3]Biological potential at 24°C.
[4]Not-pelleted material.
[5]Low-N sawdust substrate.
Means followed by the same letters are not significantly different, according to the Scheffe's test ($P = 0.0001$).

used as a blank. FDA activity was calculated from a linear calibration curve of fluorescein concentration *vs* absorbance ($r^2 = 0.999$), corrected for additional absorbance by DMS. The spontaneous hydrolysis of fluorescein diacetate and hydrolysis caused by other factors in the substrate were taken into account by subtracting the value obtained with samples harvested at day 0 from values obtained after 2, 3, 4, and 6 days of incubation. The average biological potential of pelleted substrates was expressed as $\mu$M of fluorescein released in 30 min per g of dry pelleted substrate over 6 days ($\mu$M fluorescein 30 min$^{-1}$ g$^{-1}$). An analysis of variance (ANOVA) was performed to test for equality of mean biological potential response of each substrate formulation. If treatment means were shown to be significantly different by ANOVA, Scheffe's multiple comparison test was performed to determine which formulations were different from others.

Ergosterol content in the pellets and the associated region of the agar that was overgrown with fungal hyphae was determined by the method of Davis and Lamar [5]. Biological potential of pelleted substrates, based on ergesterol, was expressed as $\mu$g of ergosterol g$^{-1}$ of dried pelleted substrate.

### *Proliferation potential of the pelleted substrates*

The ability of *P. chrysosporium* to proliferate from several different carriers and the effect of PCP medium concentration and incubation temperature on proliferation potential of the various carriers were evaluated. Carriers included pelleted substrate mixtures of different composition, alginate capsules, and agar plugs. Substrate mixtures used were as follows: (A) 75% aspen sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate; (B) spawn mixture; and (C) 83% aspen sawdust, 15% starch, and 2% lignosulphonate. Substrate mixtures were inoculated with $4.5 \times 10^5$ *P. chrysosporium* spores g$^{-1}$ substrate (dry weight basis), pelletized as described below, and cut to equal length under aseptic conditions. The moisture content of one-fourth of the pellets prepared using substrate mixture A was adjusted to 45% with sterile $H_2O$ and then the pellets were incubated at 39°C for 4 days, at which time the pellets were overgrown with active mycelia. Alginate capsules were prepared by coating 3-mm wide by 8-mm long sterile plastic cylinders with 30–40% by weight of alginate hydrogel. The plastic cylinders were first dipped in 2% alginate which contained $40 \times 10^5$ *P. chrysosporium* conidia ml$^{-1}$, 2% malt extract (Sigma) and 2% glucose (Sigma). The pellets were then dipped in 5% $CaCl_2$ to form a stable hydrogel coating. Agar plugs, 6 mm in diameter were cut from PDA agar plates freshly overgrown with *P. chrysosporium.*

Pelleted substrates and alginate capsules were placed vertically into still-liquid malt extract agar (MEA) spiked with 10, 20, 30, or 50 mg L$^{-1}$ PCP. Agar plugs were placed on solidified MEA with the same PCP concentrations. The plates were incubated at either 24°C or 39°C as indicated in Table 2 and Figure 1. Mean extension rates (mm day$^{-1}$) of *P. chrysosporium* exposed to each treatment were determined by measuring the daily increase in colony diameter in two perpendicular directions on each of three replicate plates. If treatment means were significantly different by ANOVA, Scheffe's multiple comparison test was performed to determine which formulations were different from others.

**Table 2** Dependence of biological potential of pelleted substrate, measured as FDA activity

| | | |
|---|---|---|
| (A)[1] | Conidia concentration (spores g substrate$^{-1}$) | Biological potential (FDA activity) ($\mu$M fluoresc 30 min$^{-1}$ g$^{-1}$ ± s.d.) |
| | $9.0 \times 10^5$ | 34.0 ± 3.5 |
| | $4.5 \times 10^5$ | 31.3 ± 8.0 |
| | $1.8 \times 10^5$ | 27.9 ± 8.8 |
| | $9.0 \times 10^4$ | 24.9 ± 7.6 |
| (B) | Storage time (days) | |
| | 0 | 30.5 ± 13.4 |
| | 42 (–6°C) | 37.1 ± 8.0 |
| (C) | Dried pellets storage time (days) | |
| | 0 | 36.3 ± 14.8 |
| | 42 (–6°C) | 32.5 ± 5.0 |
| | 42 (24°C) | 25.6 ± 5.0 |
| (D) | No. of pelleting cycles | |
| | 1 | 31.3 ± 6.5 |
| | 4 | 31.9 ± 3.9 |

[1](A) *P. chrysosporium* conidia concentration used for inoculation of substrate, (B) time of storage of pellets in freezer and at room temperature, (C) drying of the pellets with heat gun in sterile conditions for 2 h at 45°C to final 6–8% moisture and storage of dried pellets, (D) pelleting regime (one or four cycles-passes of material through pellet press die).

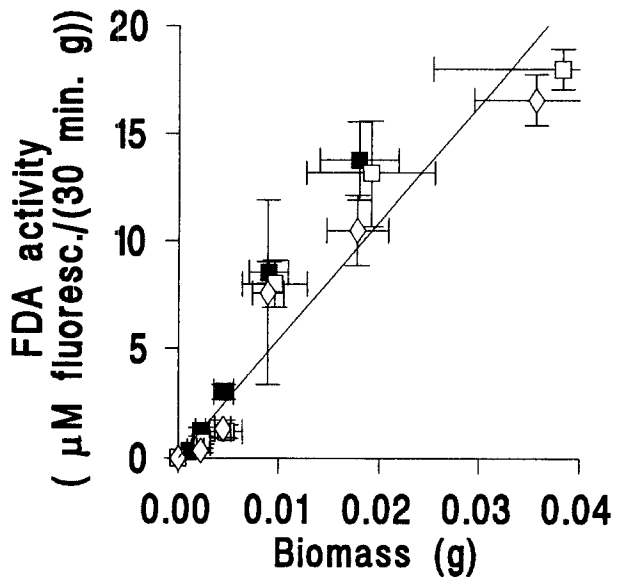


**Figure 1** FDA activity of different amounts of *P. chrysosporium* biomass grown on PDA and determined as fungal dry weight. Fungus from 3-day-old (■), 5-day-old (□), and 8-day-old (◇) cultures were tested. Error bars represent standard deviations of three determinations.

### *Preparation of fungal inocula*

Substrate mixtures used for preparation of soil inocula were composed of aspen sawdust, which served as the carrier, starch which served as a binder and as C source, corn meal, which contained 20% total nitrogen and served as the primary N source, and 2% Ca-lignosulphonate which was included as a lubricant for the pelleting process. Two substrate mixtures were prepared. The first, referred to as the high-N sawdust substrate, had a C:N ratio of 50:1 and was composed of 75% aspen sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate. The second, referred to as the low-N sawdust substrate, had a C:N ratio of 309:1 and was composed of 83% aspen sawdust, 15% starch, and 2% lignosulphonate. The moisture contents of both sawdust substrates was adjusted to 27–30% with deionized water and the substrates were sterilized by autoclaving them at 120°C and 1 atm. Sterile substrates were pelletized in a laboratory-scale pellet press aseptically. The resulting pellets were cylindrical and averaged 5 mm in diameter and 8 mm in length. They were further dried in a stream of hot air (*ca* 80°C) with a variable temperature heat gun (Master Appliance, Racine, IL, USA) for 1 h aseptically to 8–10% moisture content. Dried low-N sawdust or high-N sawdust pellets were then immersed in hot 2% PDA until pellets were coated with *ca* 30% of agar. Coated pellets were subsequently inoculated with agar plugs taken from *T. versicolor* or *P. chrysosporium* PDA master plates and incubated at 30°C or 39°C, respectively, for 4–6 days until thoroughly overgrown with fungal mycelia.

### *Preparation of soil cultures*

A Marshan sandy loam, collected from the A horizon, was air dried, mixed thoroughly, sifted through a 2.5-mm pore-size sieve, and stored in plastic bags at 4°C. The chemical characteristics and mineral trace elements of the soil were reported elsewhere [13]. Pentachlorophenol-amended soils were prepared by adding PCP in acetone to obtain final concentrations of either 100 $\mu$g g$^{-1}$ or 1500 $\mu$g g$^{-1}$. The moisture content of non-sterile Marshan soil was adjusted to 48% (dry weight basis) with deionized water. The soils were inoculated with approximately 3% (dry weight basis) of either *P. chrysosporium* or *T. versicolor* pelleted inoculum that was thoroughly overgrown with active fungal mycelium and prepared using both the high-N sawdust mixture and the low-N sawdust mixture, as described above. The inoculated soils were incubated for 28 days at 24°C or 39°C in the dark, in jam jars (30 g wet soil per jar) with modified covers to allow adequate air exchange. This was achieved by gluing a piece of microporous material over a 3.2-mm hole on the inside of the cover. Controls were prepared by amending soil with 3% of sterile pellets (dry weight basis). Two replicate cultures were prepared for each fungus and control by PCP concentration treatment.

### *PCP analyses*

Two 5-g soil sub-samples from two soil culture sample replicates were extracted at time 0 and after 7, 14, 21 and 28 days of incubation using the procedure described by Lamar *et al* [13]. To determine the efficiency of PCP extraction, the Marshan soil was spiked with *ca* 42 500 dpms $^{14}$C-PCP. The average recovery of $^{14}$C was 84.5 ± 3.8%. The extracts were analyzed by GC-ECD using the procedure described by Lamar *et al* [13]. PCP was analyzed as the trimethylsilyl derivate and quantified using the trimethylsilyl derivate of 2,4,6,-tribromophenol as internal standard.

### *Lignin and manganese peroxidase determination*

Lignin peroxidase (LiP) and manganese peroxidase (MnP) activities were measured in soils inoculated with *T. versicolor* and in control soils prepared as described above.

Moisture content was determined by weighing the soil before and after drying it at 60°C for 24 h. Two 2-g soil sub-samples from two soil culture sample replicates were extracted with 5 ml of 10 mM Na-tartrate buffer, pH 4.5, with 0.5% Tween 80 by shaking them for 5 min on a rotating tumbler shaker. Tween 80 was utilized to improve extraction efficiency and to protect LiP from mechanical deactivation [28]. Solid soil particles were separated by filtration through a 45-$\mu$m pore-size nylon, low protein retention filter (Cole-Parmer, Niles, IL, USA) and LiP and MnP activities determined by the method of Tien and Kirk [27] and of Paszczynski *et al* [21], respectively. Manganese peroxidase activity was expressed as $\mu$mol of oxidized vanyllil acetone $min^{-1}$ $g^{-1}$ of soil.

## Results

### *FDA activity*

FDA activity of *P. chrysosporium* grown on pellets was strongly and positively correlated with mycelial dry weight ($r^2 = 0.89$, $P = 0.0001$) (Figure 1) and ergosterol content of fungal mycelium ($r^2 = 0.89$, $P = 0.0001$) (Figure 2). After a lag time of 3 days, the FDA activity of pellets inoculated with *P. chrysosporium* conidia increased to a maximum after 6 days after which there was little or no further increase (Figure 3). FDA activity was not influenced greatly by pellet production and pellet storage conditions (Table 2). Increasing the concentration of *P. chrysosporium* conidia from $9 \times 10^4$ to $9 \times 10^5$ spores $g^{-1}$ substrate increased FDA activity only slightly. Pellets could be dried at 45°C and/or stored at –6°C for 6 weeks without detected loss of FDA activity. The number of cycles of substrate pelleting (substrate mixtures were passed several times through the pellet press die before mechanically stable pellets were formed) also did not influence measurements of biological potential based on FDA activity (Table 2).

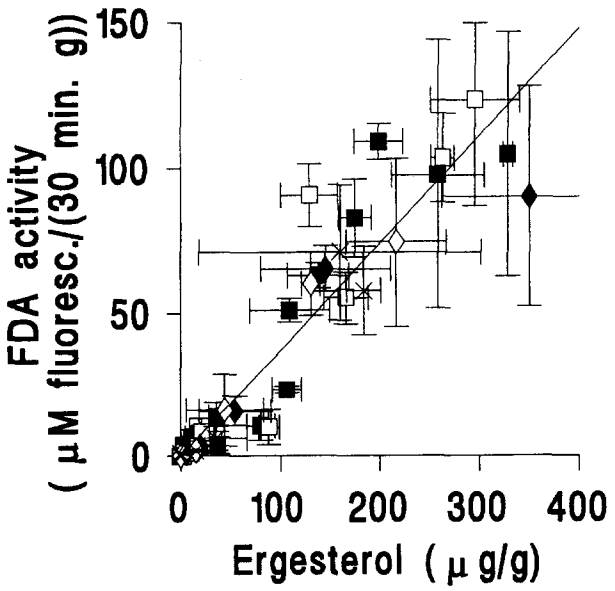


**Figure 2** Correlation between FDA activity and ergosterol content of *P. chrysosporium* grown from pellets inoculated with different amounts of conidia. Pellets were composed of 75% aspen sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate. (■) Pellets with $9 \times 10^5$ spores $g^{-1}$ substrate, (□) pellets with $8 \times 10^5$ spores $g^{-1}$ substrate, (◆) pellets with $4.5 \times 10^5$ spores $g^{-1}$ substrate, (◇) pellets with $1.8 \times 10^5$ spores $g^{-1}$ substrate, (X) pellets with $9 \times 10^4$ spores $g^{-1}$ substrate. Error bars represent standard deviations of three determinations.

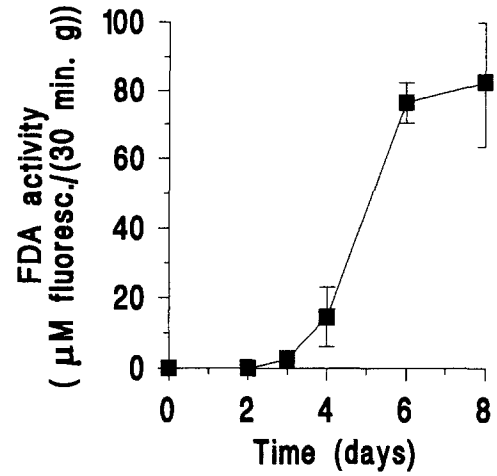


**Figure 3** FDA activity of *P. chrysosporium* grown from the pellets inoculated with $4.5 \times 10^5$ conidia $g^{-1}$ substrate, during the time of incubation. Pellets were composed of 75% aspen sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate. Inoculated pellets were dried and stored at –6°C for 6 weeks before they were used. Error bars represent standard deviations of three determinations.

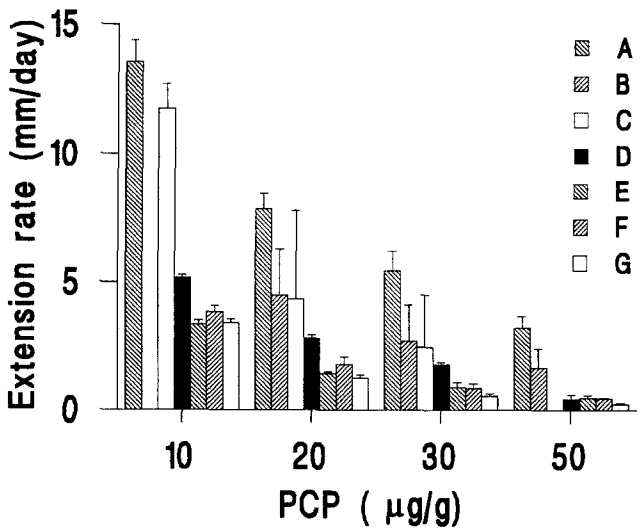


**Figure 4** Extension rates of *P. chrysosporium* from different carriers to MEA spiked with 10, 20, 30, and 50 $\mu$g PCP $g^{-1}$ of hydrogel. (A) pellets composed of 75% aspen sawdust, 15% starch, 8% corn meal, and 2% lignosulphonate and inoculated with $4.5 \times 10^5$ conidia $g^{-1}$ substrate and incubated at 39°C; (B) alginate capsules with $4 \times 10^5$ conidia $g^{-1}$ hydrogel and enriched with 2% of malt extract and glucose, at 39°C; (C) PDA agar plugs overgrown with active fungal mycelia, at 39°C; (D) spawn pellets inoculated with $4.5 \times 10^5$ conidia $g^{-1}$ substrate, at 24°C; (E) pellets composed as (A) pellets and overgrown with active fungal mycelium, at 24°C; (F) pellets composed as (A) pellets, inoculated with $4.5 \times$ conidia $g^{-1}$ substrate, at 24°C; (G) pellets composed of 83% aspen sawdust, 15% starch, and 2% lignosulphonate and inoculated with $4.5 \times 10^5$ conidia $g^{-1}$ substrate, at 24°C. Error bars represent standard deviations of three determinations.

### *Biological and proliferation potential*

The FDA activity of *P. chrysosporium* grown on several different pelleted and non-pelleted substrate mixtures based

**Table 3** Rates of hyphal extension of *Phanerochaete chrysosporium* from different carriers

| Carrier composition | Incubation temperature (°C) | Hyphal extension (mm day$^{-1}$ ± s.d.) |
|---|---|---|
| (A) 75% aspen sawdust<br>15% starch<br>8% corn meal<br>2% lignosulphonate | 39 | 6.95 ± 3.68[a] |
| (B) alginate capsules | 39 | 2.95 ± 1.76[b] |
| (C) spawn pellets | 24 | 2.46 ± 1.79[b,c] |
| (D) as in (A), pellets overgrown | 24 | 1.55 ± 1.2[c] |
| (E) as in (A) | 24 | 1.74 ± 1.36[c] |
| (F) 83% aspen sawdust<br>15% starch<br>2% lignosulphonate | 24 | 1.36 ± 1.29[c] |

Means followed by the same letters are not significantly different, according to the Scheffe's test ($P = 0.0001$).

on sawdust, rice hulls, and fungal spawn varied greatly (Table 1). In general: the FDA activity of pellets made from fungal spawn, a nutrient-fortified grain-oak sawdust mixture, was greater than that of pellets based on aspen sawdust or rice hulls; increasing the percentage of a nutrient source (ie corn steep liquor or cornmeal) resulted in greater FDA activity; incubation at 39°C, the optimum temperature for growth of *P. chrysosporium* [4], resulted in greater FDA activity than incubation at 24°C; and the FDA activity of non-pelleted spawn was higher than that of pelleted spawn (Table 1).

The proliferation potential and sensitivity of *P. chrysosporium* to PCP were greatly influenced by the type of carrier and the incubation temperature. At 24°C the fungus did not grow from nutrient-enriched alginate capsules or from agar plugs at PCP concentrations >10 mg L$^{-1}$. However, when growing from pellets based on sawdust or the nutrient-fortified grain-sawdust mixture, *P. chrysosporium* tolerated up to 50 mg L$^{-1}$ PCP. Overall the rate of hyphal extension was *ca* 2.8 times greater at 39°C (5.0 mm day$^{-1}$) than at 24°C (1.8 mm day$^{-1}$). The sensitivity of *P. chrysosporium* to PCP was decreased when the fungus was incubated at 39°C compared to 24°C. This decreased sensitivity can be illustrated by comparing the rates of hyphal extension of *P. chrysosporium* from high-N sawdust substrate incubated at the two temperatures (Figure 4). Rates of hyphal extension from this substrate at 39°C were from 72% at 10 mg PCP L$^{-1}$ to 86% at 50 mg PCP L$^{-1}$ greater than rates at 24°C. The greatest rate of hyphal extension was observed from the high-N sawdust pellets incubated at 39°C (Table 3). However, at 24°C, the greatest extension rate was from pellets made from the nutrient-fortified grain-sawdust mixture. The overall extension rate from this carrier was greater than rates from the high-N sawdust pellets and significantly greater than rates from the low-N sawdust pellets (Table 3). Finally, the rate of hyphal extension was slightly greater from high-N than from low-N sawdust pellets (Table 3).

### *PCP removal from soil*

In Marshan soil with 100 $\mu$g g$^{-1}$ PCP neither the availability of N source in pellets or temperature substantially influenced the extent of PCP depletion (Figure 5). However, biological potential of the pellets did influence the extent of transformation of PCP to PCA. In soil supplemented with low-N sawdust pellets with *P. chrysosporium*, a much higher transformation rate of PCP to PCA was observed than in soil supplemented with high-N sawdust pellets (Figure 5a and b). In soil where *T. versicolor* was introduced on low-N sawdust pellets, PCP was again mostly transformed to PCA (Figure 5e), while almost no PCA was detected in soil where *T. versicolor* was introduced on high-N sawdust pellets (Figure 5d). The growth of both fungi was well-distributed through the soil surface, with penetrations into the depth of the soil mass. In the 100 $\mu$g g$^{-1}$ PCP Marshan soil inoculated with sterile pellets, there was only a 10% decrease in the initial PCP concentration after 4 weeks (data not shown).

In 1500 $\mu$g g$^{-1}$ PCP Marshan soil, very little PCP depletion was observed both with *P. chrysosporium* and *T. versicolor* (Figure 6b,c,d,e) compared to control soil supplemented with sterile pellets (Figure 6a). The higher biological potential of the high-N sawdust pellets did not improve the ability of *P. chrysosporium* to decrease the PCP concentration when compared to the low-N sawdust. In soil inoculated with *P. chrysosporium* and incubated at 39°C the fungus was able to colonize the whole soil surface, while in other cultures, growth of *P. chrysosporium* and *T. versicolor* was limited to the pellet and the soil surface adjacent to the pellet.

Manganese peroxidase activity was detected only in Marshan soil cultures of *T. versicolor*. No LiP activity was detected in soils with either *P. chrysosporium* or *T. versicolor*. Higher MnP activity was found in soils inoculated with high-N sawdust pellets (Figure 7). Manganese peroxidase activity was higher in soil with 1500 $\mu$g g$^{-1}$ PCP than in soil with 100 $\mu$g g$^{-1}$ PCP.

## Discussion

The production of a high quality fungal inoculum with a uniformly high biological potential is necessary for successful implementation of fungal bioaugmentation. As more is learned about fungal pollutant-degrading mechanisms, biological potential may be more specifically defined in terms of fungal pollutant-degrading ability per unit weight or volume of fungal inoculum. However, the biological potential of fungal inoculum for use in soil remediation is currently defined as the amount of fungal biomass produced per unit weight or volume of fungal inoculum, on a dry weight basis.

In the present study, the usefulness of the FDA activity assay, which measures metabolic activity associated with microbial growth [2], was evaluated as an indicator of the biological potential of fungal mycelia grown on pelleted substrate. Strong positive correlations of FDA activity with both biomass (Figure 1) and ergosterol (Figure 2), a component of the fungal plasmalemma that is used as an indirect measure of fungal biomass [5], demonstrated the usefulness of this assay for estimating fungal biomass.

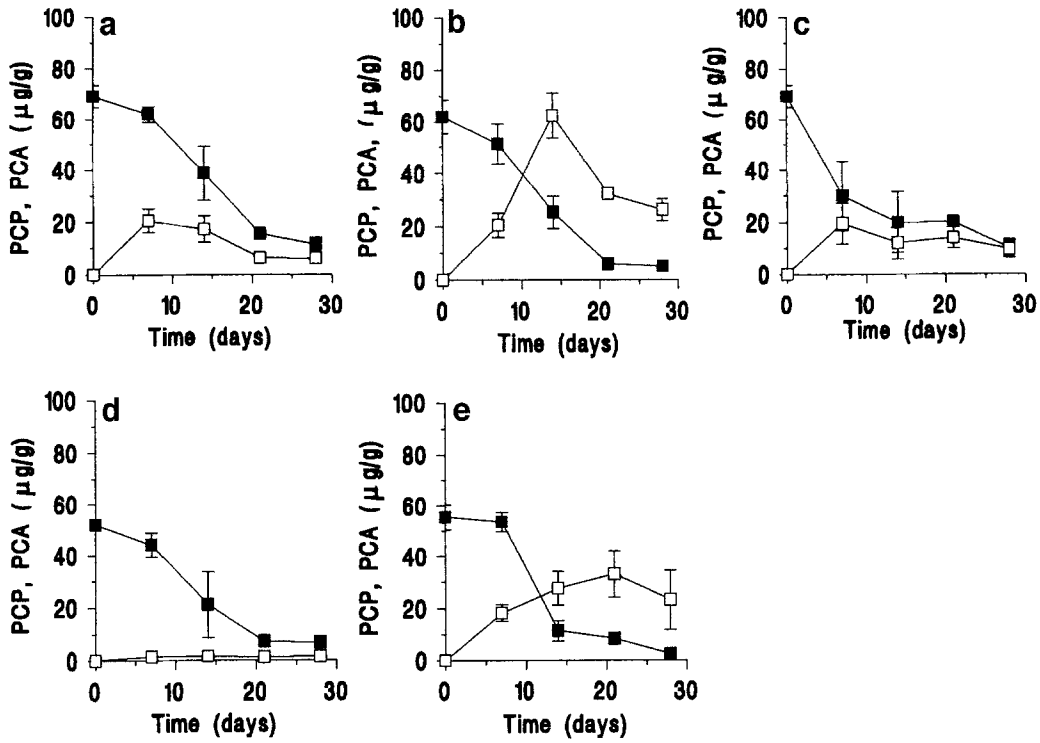


**Figure 5** Concentrations of PCP (■) and PCA (□) in Marshan soil supplemented with 100 $\mu$g g$^{-1}$ PCP and inoculated with 3% of pelleted fungal inoculum. (a) *P. chrysosporium* on pellets with C:N ratio 50:1, incubated at 24°C; (b) *P. chrysosporium* on pellets with C:N ratio 309:1, at 24°C; (c) *P. chrysosporium* on pellets with C:N ratio 50:1, at 39°C; (d) *T. versicolor* on pellets with C:N ratio 50:1, at 24°C; (e) *T. versicolor* on pellets with C:N ratio 309:1, at 24°C. Error bars represent standard deviations of four determinations.

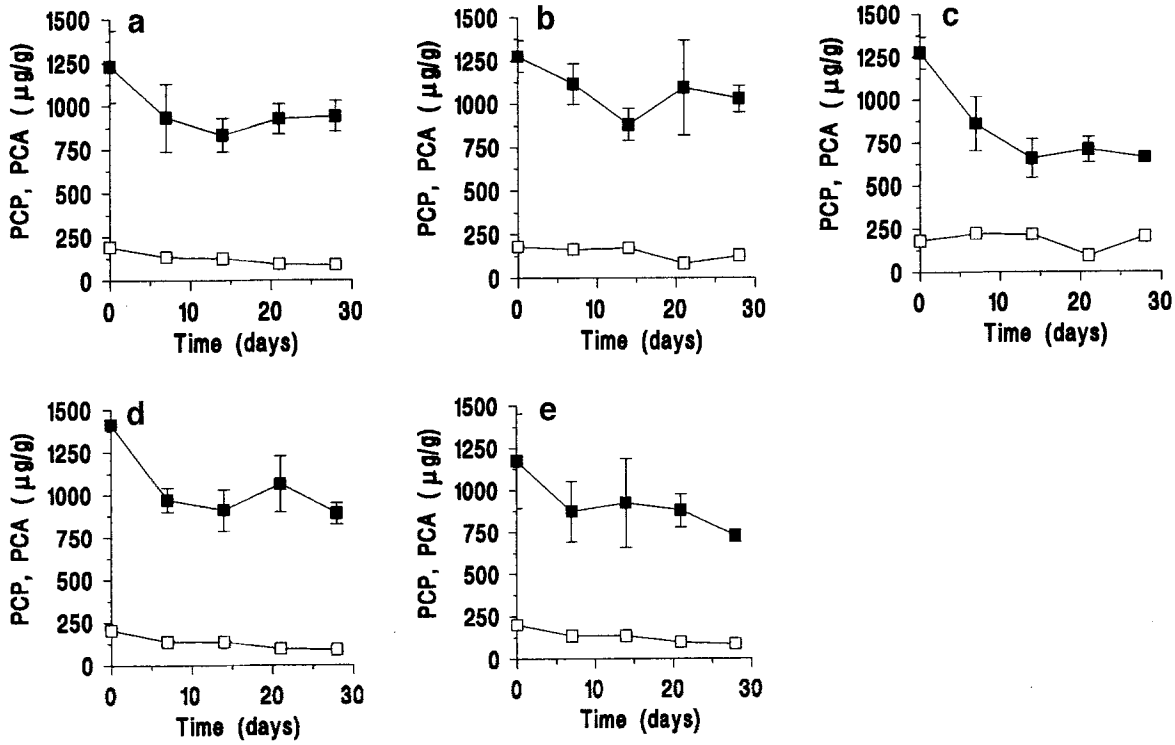


**Figure 6** Depletion of PCP (■) and increase of PCA (□) in Marshan soil supplemented with 1500 $\mu$g g$^{-1}$ PCP and inoculated with 3% of pelleted fungal inoculum. (a) Soil inoculated with sterile pellets and incubated at 24°C; (b) *P. chrysosporium* on pellets with C:N ratio 50:1, at 24°C; (c) *P. chrysosporium* on pellets with C:N ratio 50:1, at 39°C; (d) *T. versicolor* on pellets with C:N ratio 50:1, at 24°C; (e) *T. versicolor* on pellets with C:N ratio 309:1, at 24°C. Error bars represent standard deviations of four determinations.

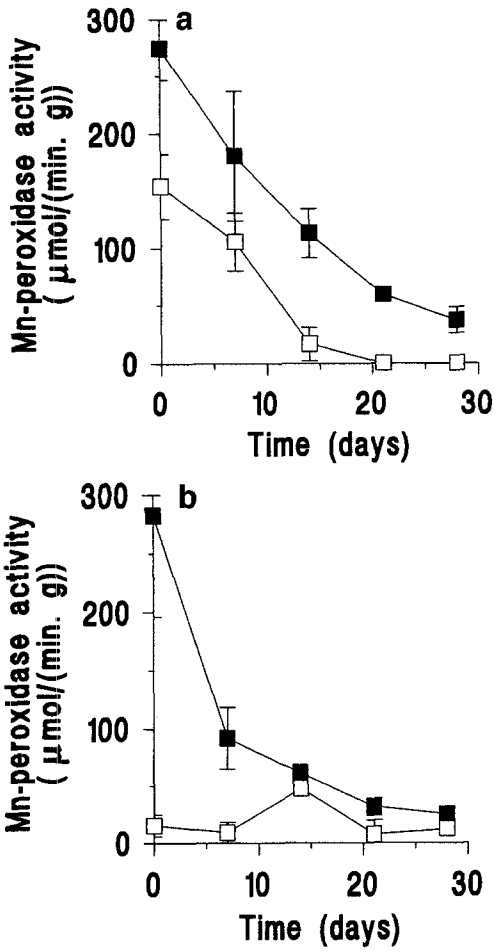


**Figure 7** Manganese peroxidase activity in Marshan soil spiked with 1500 $\mu$g g$^{-1}$ PCP (a) and 100 $\mu$g g$^{-1}$ PCP (b). Soil was inoculated with 3% of pellets with *T. versicolor* and incubated at 24°C. Pellets with C:N ratio 50:1 (■), pellets with C:N ratio 309:1 (□). Error bars represent standard deviations of three determinations.

The results of measurements of biological potential and proliferation potential of different substrate formulations indicated that in addition to optimal substrate composition, optimal temperature and pellet structure are important for high metabolic activity and for proliferation and PCP tolerance of *P. chrysosporium*. The influence of substrate composition of pellets on their biological potential and proliferation rate was cognate (Table 1, Figure 4). Pellets made from spawn had greater biological potential than high-N and low-N sawdust pellets and *P. chrysosporium* was more PCP-tolerant when grown from pellets with higher biological potential. This demonstrated the importance of substrate biological potential for growth and proliferation of fungi into contaminated environments. Biological potential of pellets at 39°C, the optimum temperature for growth of *P. chrysosporium*, was more than 10 times that at 24°C (Table 1). Tolerance of the fungus to PCP was also much higher when it was grown at 39°C (Figure 4).

The biological potential of the non-pelleted nutrient-fortified grain sawdust mixture was higher than that of the pelleted mixture, probably because the compacted structure of the pellets limited aeration in the interior of the pellet. Limited aeration in the structure of the alginate hydrogel [23] is also the probable reason for slower proliferation of *P. chrysosporium* from enriched alginate capsules than from sawdust pellets.

The depletion of PCP or transformation of PCP to PCA correlated with MnP activity detected in *T. versicolor* soil cultures. In *T. versicolor* soil cultures where no or little MnP activity was found (ie cultures inoculated with low-N sawdust pellets) (Figure 7b), PCP was mainly transformed to PCA (Figure 5e). This indicates a possible role of this enzyme in PCP removal from contaminated soils, as suggested earlier [3,8]. Significant transformation of PCP to PCA was also observed in *P. chrysosporium* cultures inoculated with low-N sawdust pellets (Figure 5b). This was consistent with previous results that examined the ability of *P. chrysosporium* to deplete PCP in the same soil using aspen wood chips, that also have a C:N ratio of approximately 300:1, as inoculum [15]. Less transformation of PCP to PCA in *P. chrysosporium* and *T. versicolor* cultures inoculated with high-N sawdust pellets suggests that this activity could be regulated (ie repressed) nutritionally.

Lignin peroxidase activity, as detected by the vertryl alcohol and two other assays, was shown to be strongly inhibited by the presence of components or conditions that resulted in loss of LiP activity in the presence of biobleaching liquor [1]. Similarly the absence of LiP activity in both *P. chrysosporium* and *T. versicolor* soil cultures may have been due to conditions or the presence of substances that resulted in the inhibition of LiP activity. Production of LiPs and MnPs by *P. chrysosporium* is nitrogen regulated [10]. However, no MnP activity was found even when *P. chrysosporium* was introduced in soil on low-N sawdust pellets with C:N ratio of 309:1. *Trametes versicolor* is an N-deregulated fungus [16] and higher MnP activity was detected in soils with high-N sawdust pellets (Figure 7a and b). Poor PCP removal from Marshan soil contaminated with 1500 $\mu$g g$^{-1}$ PCP can be attributed to poor fungal growth, with the exception of the *P. chrysosporium* soil cultures incubated at 39°C where *ca* 60% of the PCP was depleted after 4 weeks (Figure 6c). The transformation rate of PCP to PCA was comparable for all high PCP-contaminated soil cultures.

FDA activity was a useful tool for measurement and optimization of the biological potential of substrate formulations for production of fungal inoculum. Biological potential of pelleted substrates was an important factor in the ability of fungi to proliferate to the contaminated environment and for removal of PCP from soil, particularly to prevent the conversion of PCP to PCA.

## Acknowledgements

This research was supported by grant DW12931791-01-8 from US Environmental Protection Agency.

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