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Detoxification of Corn Stover and Corn Starch Pyrolysis Liquors by Ligninolytic Enzymes of *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium (ATCC 24725) shake flask culture with 3 mM veratryl alcohol addition on day 3 was able to grow and detoxify different concentrations of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors [10, 25, and 50% (v/v)] in defined media. GC-MS analysis of reaction products showed a decrease and change in some compounds. In addition, the total phenolic assay with Dcs samples demonstrated a decrease in the phenolic compounds. A bioassay employing *Lactobacillus casei* growth and lactic acid production was developed to confirm the removal of toxic compounds from 10 and 25% (v/v) Dcs and Dst by the lignolytic enzymes, but not from 50% (v/v) Dcs and Dst. The removal did not occur when sodium azide or cycloheximide was added to *Ph. chrysosporium* culture media, confirming the participation of lignolytic enzymes in the detoxification process. A concentrated enzyme preparation decreased the phenolic compounds in 10% (v/v) corn stover and corn starch pyrolysis liquors to the same extent as the fungal cultures.

KEYWORDS: Corn stover; corn starch; detoxification; lignolytic enzymes

INTRODUCTION

In the biosphere, plant biomass is our largest supply of stored carbon. One physical process being evaluated to access this natural carbon reservoir is pyrolysis, which is a physical/chemical method for biomass saccharification (*1*). The pyrolysis process can be carried out at different temperatures (250−800 °C) in which the biomass is rapidly heated in the absence of oxygen (*2*). After cooling and condensation, a dark brown mobile effluent generated by the pyrolysis process contains anhydrosugars, such as levoglucosan (1,6-anhydro β-D-glucopyranose), and a wide range of oxygenated chemicals compounds (*2*). Levoglucosan is a major product of hexosan pyrolysis, which can range from 38 to 58% depending on initial cellulose content (*3, 4*). Levoglucosan can be utilized by prokaryotes and eukaryotes either directly as fermentable substrate or indirectly by mild acid hydrolysis to glucose (*5, 6*). Unfortunately, other compounds such as phenols, benzene, furan, and furfuryl derivatives (*2*) in the pyrolysis liquors inhibit microbial growth. Conventional methods of solvent extraction, chemical oxidation, and adsorbance on activated carbon have been employed to remove the inhibitor compounds from the pyrolysis liquor before its use as a fermentable substrate (*5, 7*). However, these methods are costly and generate environmental disposal concerns. The use of biological treatment to selectively degrade these toxic compounds while leaving the levoglucosan intact is a promising alternative (*8*).

Corn stover and corn starch pyrolyzed at 400−600 °C produce levoglucosan, which can be fermented to organic acid or alcohol; however, pyrolysis also produces a variety of toxic chemical compounds that inhibit microbial fermentation (*9*). Using GC-MS Khiyami (*8*) identified the inhibitory molecules as different phenolic and furfuryl derivative compounds that were present in diluted corn stover and diluted corn starch pyrolysis liquors.

White-rot fungi have been shown to degrade a variety of recalcitrant molecules including lignin, a polyphenylpropane molecule with β-aryl ether linkages (*10*). *Phanerochaete chrysosporium* has the lignin degradation system consisting of peroxidases, H_2O_2-producing enzymes, veratryl alcohol (3,4-dimethoxybenzyl alcohol), manganese, and oxalate (*11*). However, *Ph. chrysosporium* is also able to degrade chemicals via alternative pathways such as plasma membrane potential (*12*−*14*). *Ph. chrysosporium* also has other enzymes or associated activities that may be important in the remediation of organic pollutants including cellobiose dehydrogenase, a trans-membrane methyl transferase, trans-membrane redox potential, and laccase (*11, 15*). The lignin degradation system allows *Ph. chrysosporium* to biodegrade a wide range of compounds through oxidative−reduction mechanisms that use highly reactive, nonspecific redox reactions, which increases the number of molecules that can be effectively degraded via different alternative pathways (*11, 16, 17*). Because the ligninolytic enzymes and cofactors are secreted, it is ideal for use in the degradation

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of a wide range of chemicals compounds that are either soluble or nonsoluble in water. For example, *Ph. chrysosporium* can degrade lignin, munitions waste, pesticides, polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bleach plant effluent, synthetic dyes, synthetic polymers, and wood preservatives (*10*, *16*).

The purpose of the present study was to evaluate the ability of *Ph. chrysosporium* and culture filtrate to detoxify these pyrolysis liquors. *Ph. chrysosporium* was cultured on defined medium with different concentrations of diluted corn stover or diluted corn starch pyrolysis liquors. In addition, concentrated extracellular ligninase was added to these liquors to evaluate the extent of detoxification. Because *Ph. chrysosporium* degrades organic pollutants via ligninase-independent pathways (*11*), the involvement of the peroxidases in the detoxification process was confirmed via the addition of sodium azide, which inhibits the oxidation of veratryl alcohol in the presence of the lignin peroxidase and H_2O_2 (*18*). The importance of ligninases in pyrolysis liquor detoxification was determined via cycloheximide addition, which blocks protein synthesis (*19*). The detoxification process was monitored using different parameters including extracellular protein concentrations, ligninolytic activity, the decrease of total phenolics in pyrolysis liquors, bioassay tests, and GC-MS analysis.

MATERIALS AND METHODS

Chemicals. Cycloheximide was obtained from ICN Biomedicals Inc. (Aurora, OH). Veratryl alcohol was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide, syringic acid, poly(ethylene glycol), Lowry protein assay kit, 2-methoxy-5-methylphenol, 2,6-dimethoxyphenol, 1,2,4-trimethoxybenzene, 2(5*H*)-furanone, and 1,6-anhydro-β-D-glucopyranose (levoglucosan) were obtained from Sigma Chemical Co. (St. Louis, MO).

Microorganisms. *Ph. chrysosporium* (ATCC 24725) and *Lactobacillus casei* subsp. *rhamosus* (ATCC 11443) strain were obtained from American Type Culture Collection (Manassas, VA). The *Ph. chrysosporium* strain was maintained on potato dextrose agar slants at 4 °C. *L. casei* was maintained as freeze-dried culture and as working culture by monthly transfers in MRS broth (Difco Laboratories, Detroit, MI) and stored at 4 °C.

Pyrolysis Liquors Preparation. The pyrolysis process was carried out in a pilot-scale fluidized pyrolyzer at Iowa State University (*9*). The reactor consists of a 15-cm-diameter bubbling fluidized bed operated at 400−600 °C. It processes 2.5−5.0 kg/h of biomass to produce 1.3−2.6 L/h of pyrolysate oil. The heat required for pyrolysis was provided by burning pyrolytic gas or natural gas in an external burner and directing this hot gas through a heat exchange jacket surrounding the pyrolytic reactor. Recirculated pyrolytic gas serves as the fluidization agent with bubbling bed, thus avoiding addition of air to the reactor, which would reduce the yield of pyrolysate. The products of pyrolysis include gas (a flammable mixture consisting of hydrogen, carbon monoxide, carbon dioxide, and some noncondensable hydrocarbons), particulate matter (ash and char), and condensable vapors. Particulate matter was removed by insulated cyclones designed to keep the gas stream at high temperature. The gases and vapors then enter a series of four, water-cooled, shell-and-tube heat exchanges designed to condense water and organic vapors, which forms the pyrolysate used in the fermentation trials. In this research, the pyrolysates were prepared from corn starch and corn stover, which is the residual after corn is harvested. Corn stovers have 50% (w/w) cellulose with some hemicelluloses and lignin (*9*). Corn starch was purchased from a local grocery store, which could be subjected to a variety of chemical modifications aimed to improve functionality for food applications. The pyrolysates generated in this pyrolysis process were called corn stover pyrolysis liquor and corn starch pyrolysis liquor.

Preparation of Dilute Pyrolysis Liquors. Dilute pyrolysis liquors were prepared as described by Khiyami (*8*). Briefly, corn stover or corn starch pyrolysis liquors were vigorously mixed with deionized water at a 1:2 ratio and then stored overnight in a cold room at 4 °C to facilitate complete precipitation. The insoluble precipitates (IP) were removed by centrifugation at 10000*g* for 15 min at 10 °C. The supernatants were neutralized with $CaCO_3$ and then centrifuged at 15000*g* for 20 min at 10 °C. The supernatants of diluted corn stover (Dcs) and diluted corn starch (Dst) pyrolysis liquors were filter sterilized with a 0.45-μm PTFE Gelman Acrodisc filter (Pall Corp., East Hills, NY) to prevent any further changes in chemical composition that might result from heat sterilization.

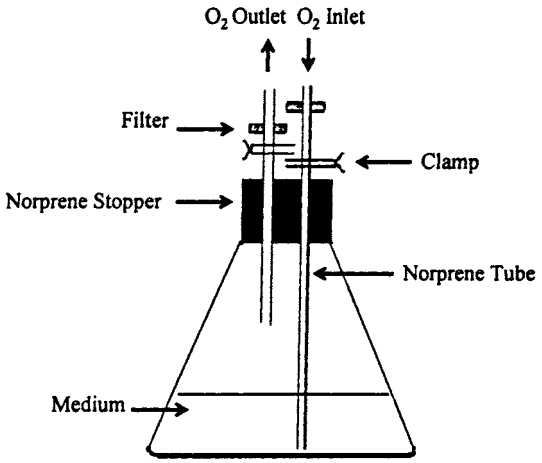

Figure 1. Setup for 2-L Erlenmeyer flask for agitated culture (120 rpm, 39 °C) containing 1 L of medium with Dcs or Dst and oxygen purging tubing and filters for *Ph. chrysosporium* ligninolytic enzyme production.

Inoculum Preparation. *Ph. chrysosporium* was cultured on a medium containing 10.0 g/L of glucose, 10.0 g/L of malt extract, 2.0 g/L of peptone (Difco Lab) 2.0 g/L of yeast extract (Ardamine Z; Sensient Flavor, Juneau, WI), 1.0 g/L of asparagine, 2.0 g/L of KH_2PO_4, 1.0 g/L of $MgSO_4 \cdot 7H_2O$, 0.001 g/L of thiamin, and 20 g/L of agar (Sigma Chemical Co.) (*20*). The slants were incubated for 5 days at 39 °C. Spore suspensions were prepared in sterile water followed by passage through sterile glass wool to remove mycelia. Spore concentration was determined by measuring absorbance at 650 nm (*20*). The average dry mycelium dry weight was determined gravimetrically via filtration using preweighed Whatman no. 52 filter paper (Whatman Inc., Clifton, NJ) and then dried for 48 h at 70 °C.

Detoxification of Pyrolysis Liquors. The Dcs and Dst incubations were carried out in 2-L Erlenmeyer flasks. The flasks were capped with solid norprene stoppers with two holes containing norprene tubing, which were attached to a glass microfiber filter (0.2-μm pore size and 50-mm diameter; Whatman Inc.) to provide filter sterile air inlet and outlet (**Figure 1**). Each flask contained 1-L total volume of *Ph. chrysosporium* ligninase production media with 10, 25, or 50% (v/v) filter sterilized Dcs or Dst. The ligninase production media consist of 20.0 g/L of glucose, 0.22 g/L of ammonium tartrate, 2.0 g/L of KH_2PO_4, 5.0 g/L of $MgSO_4 \cdot 7H_2O$, 0.1 g/L of $CaCl_2 \cdot H_2O$, 1.2 g/L of acetic acid, 0.4 g/L of NaOH, 11.7 mL/L of trace elements (*20*), 1.0 g/L of Tween 80 (*21*), and 25 mL/L of spore suspension (A_{650} of 0.83). The flasks were purged with oxygen immediately after inoculation, the tubing was clamped, and after day 3, the flasks were purged daily with oxygen. The flasks were incubated with shaking at 120 rpm for 14 days at 39 °C. On day 3 of the incubation, 3 mM veratryl alcohol was added to the culture flasks. On day 4 the flask cultures were amended with 10, 25, or 50% (v/v) (final concentrations, respectively) of filter-sterilized Dcs or Dst, which also resulted in a disproportionate dilution of the original culture medium. To determine the total decrease of phenolic compounds in Dcs samples, the above experiment was performed under the same conditions with 10 mL of medium in 1250-mL Erlenmeyer flasks. The total phenolic compounds were determined in triplicate flasks ($n = 3$) every 2 days for 14 days.

Effect of Ligninase Inhibitors on Dcs and Dst Detoxification. To determine whether detoxification of the Dcs and Dst was due to *Ph. chrysosporium* lignolytic enzymes, sodium azide and cycloheximide,

which inhibited the enzyme activity and production, respectively, were added to *Ph. chrysosporium* culture medium. The experiment was carried out in replicates of three ($n = 3$) with 10% (v/v) Dcs or Dst concentration. Sodium azide was added in 0 (control), 0.5, and 2.5 μM to 10 mL of medium in a 125-mL Erlenmeyer flask. Cycloheximide was added at 0 (control) and 70 μg to 10 mL of medium in a 125-mL Erlenmeyer flask. These concentrations were added to culture on day 2 before ligninase production and on day 4 after mycelium growing and ligninase production (*22*, *23*). All experimental flasks were inoculated with 150 μL of spore suspension (A_{650} of 0.83) and incubated for 6 days under the same conditions as described previously for ligninase production and detoxification experiments.

Detoxification of Pyrolysis Liquors with Concentrated *Ph. chrysosporium* Enzymes. The ligninolytic enzymes were produced as described above in 2-L Erlenmeyer flask cultures, but without the addition of Dcs or Dst. Peroxidase activity in the culture supernatant reached a maximum on day 6 [19 and 31 units/L for lignin peroxidase (LiP) and manganese peroxidase (MnP), respectively]. The culture broth was then centrifuged at 10000*g* for 5 min at 4 °C. The yellow supernatant, which contains ligninase activity, was added to dialysis tubing with a molecular weight cutoff 6000−8000 Da (Fisher Scientific, Spectrum Lab. Inc., Rancho Dominguez, CA), placed in a tray covered with poly(ethylene glycol) 15000−20000 MW flakes (Sigma Chemical Co.), and stored at 4 °C overnight. The enzyme activity of concentrated supernatant was 28 and 43 units/L for LiP and MnP, respectively. Enzyme concentrate was evaluated in detoxification experiments in replicates of three ($n = 3$) of 10 mL each at 39 °C without shaking. The reaction mixture contained 10% (v/v) Dcs, 3.0 mL of concentrated enzyme, 0.1 M sodium tartrate (pH 4.5), 0.1 mM veratryl alcohol, and 0.1 mM $MnSO_4$. The reaction was initiated by the addition of 0.1 mM H_2O_2 after 0, 15, 30, and 45 min. The control tube had no added H_2O_2. Samples were withdrawn during the incubation, stored at 4 °C and later assayed for total phenolic compounds changes. Finally, after 60 min, samples were taken every 15 min for bioassay with *L. casei* for evaluating detoxification.

Samples Analysis. The level of LiP activity was determined using the veratryl alcohol assay (*20*), which contained 200 μL of culture supernatant, 2 mM veratryl alcohol, and 0.4 mM H_2O_2 in 50 mM sodium tartrate buffer (pH 2.5). Oxidation of veratryl alcohol was measured as the increase in absorbance at 310 nm ($\epsilon = 9300\ M^{-1}\ cm^{-1}$). The reaction was monitored for 300 s at 37 °C using a DU 640 UV−vis Beckman spectrophotometer equipped with kinetics Soft-Pac 517033 for measuring enzyme activity (Beckman Instruments, Inc., Fullerton, CA). One unit of LiP activity was defined as 1 μmol of veratryl alcohol oxidized per minute at 39 °C and reported as units per liter.

The level of MnP activity was determined by monitoring the oxidation of Mn^{2+} to Mn^{3+} (*24*). The assay mixture contained 200 μL of culture supernatant, 0.1 M sodium tartrate (pH 5.0), 0.1 mM H_2O_2, and 0.1 mM $MnSO_4$. The product, Mn^{3+}, forms a transiently stable complex with tartaric acid, showing a characteristic absorbance at 238 nm ($\epsilon = 6500\ M^{-1}\ cm^{-1}$). Reactions were initiated by the addition of H_2O_2. The reaction was monitored for 300 s at 37 °C using a DU 640 UV−vis Beckman spectrophotometer equipped with kinetics Soft-Pac 517033 for measuring enzyme activity. One unit of MnP activity was defined as 1 μmol of Mn^{2+} oxidized per minute at 39 °C and was reported as units per liter.

The level of laccase, another *Ph. chrysosporium* lignin-degrading enzyme, was determined using ABTS as substrate (*25*). The reaction mixture contained 200 μL of culture supernatant and 2 mM in 0.1 M sodium citrate buffer (pH 3.0). Oxidation of ABTS was monitored by measuring the absorbance increase at 420 nm ($\epsilon = 36000\ M^{-1}\ cm^{-1}$). The reaction was monitored for 300 s at 37 °C using a DU 640 UV−vis Beckman spectrophotometer equipped with kinetics Soft-Pac 517033 for measuring enzyme activity. One unit of laccase activity was defined as 1 μmol of ABTS oxidized per minute at 39 °C and was reported as units per liter.

The concentration of soluble protein in each sample was determined according to the method of Lowry (*26*) after 0, 3, and 6 days for each batch. The reaction mixture was determined spectrophotometrically at 750 nm by using a Spectronic 20 (Milton Roy, Rochester, NY). Bovine serum albumin was used as a standard for extracellular protein determinations (50−400 ug/mL; $r = 0.999$).

For GC-MS analysis, the samples were prepared and analyzed as described in Khiyami (*8*). Briefly, 25 mL from detoxified and non-detoxified Dcs and Dst samples was acidified to pH 4.0 with HCl and then extracted three times with ethyl acetate, three times with ethyl acetate/acetone 2:1, and three times with ethyl ether. The pooled organic phases were dehydrated with anhydrous sodium sulfate, and the solvents were removed on a rotary evaporator. The dried extract was resuspended in 25 mL of ethyl acetate and filtered via syringe filtration unit with 0.45-μm cellulose acetate plus filters (GE Osmonics, Trevose, PA). Sample analysis was performed using an Agilent 6890 series GC system with Agilent autosampler 7683 series injector and equipped with micromass a GCT mass spectrometer and column of JW DB.5 MS (30 m by 0.25 mm by 0.25 μm, Agilent Technologies, Palo Alto, CA). The filtered ethyl acetate sample without derivatization was injected at a split ratio of 1 to 100. Five compounds known to be present in Dcs samples, including phenol, 2-methoxy-5-methylphenol, 2,6-dimethoxyphenol, 1,2,4-trimethoxybenzene, and 1,6-anhydro-β-D-glucopyranose (levoglucosan), and two compounds present in Dst, including 2(5*H*)-furanone and 1,6-anhydro-β-D-glucopyranose (levoglucosan), were used as standards. Levoglucosan is only partially dissolved in ethyl acetate. Thus, for levoglucosan GC-MS quantitative analysis, 25 mL of zero time and fungal-treated Dcs and Dst was dehydrated, and then the dried extract was resuspended in 25 mL of methanol.

The phenolic compounds were extracted as described above for GC-MS analysis, and then the dried extract was resuspended in 50 mM phosphate buffer (pH 6.0). The phenolics assay is based on the oxidation of phenolate ion in which ferric ions are reduced to the ferrous state, detected by the formation of the Prussian blue complex $[Fe_4[Fe(CN)_6]_3]$ with a potassium ferricyanide-containing reagent (*27*). The reagents of the assay were ferric chloride reagent, which was prepared by mixing a 0.1 M solution of ferric chloride ($FeCl_3$) in 0.1 M hydrochloric acid, and potassium ferricyanide reagent [0.008 M $K_3Fe(CN)_6$ in deionized water] (*27*). The assay mixture contained 25 mL of deionized water, 250 μL of sample, 3 mL of ferric chloride reagent, and 3 mL of potassium ferricyanide reagent. The sample absorbance was measured at 720 nm. The phenolic contents in the samples were expressed as syringic acid equivalents (10 μg mL^{-1} gives A_{760} of 0.377/mL) (*28*). Total phenolic assay was performed on Dcs samples and not Dst samples, which contained no lignin. Furfurals found in Dst were capable of being oxidized by the reagents of the total phenolic assay, which will reduce the reagents causing a color change and appearing as a phenolic (*27*).

For bioassay, all samples were collected, centrifuged at 10000*g* for 20 min at 20 °C, and then filter sterilized with a 0.45-μm PTFE filter membrane. The bioassay was performed as described by Khiyami (*8*). Briefly, the assay was carried out in a final volume of 10 mL by mixing 2:1 (v/v) of zero time or fungal-treated Dcs or Dst and concentrated MRS medium. The MRS medium alone was used as a positive control, whereas MRS medium with zero time Dcs or Dst was used as a negative control. The assay tubes were inoculated with 100 μL (A_{620} of 0.5) of *L. casei* cultures and incubated at 37 °C as a static culture for 24 h. *L. casei* was selected for the bioassay because it is a homofermentative bacterium that produces L-lactic acid and it has the potential for use by industry (*29*). The control was incubated under the same conditions with zero time Dcs or Dst. The samples were analyzed for cell concentration by measuring the absorbance at 620 nm using a Spectronic 20 and by measuring lactic acid production by using a Hewlett-Packard (San Fernando, CA) 1100 high-pressure liquid chromatograph (HPLC) equipped with water model 2410 refractive index detector, column heater, autosampler, and computer controller. Lactic acid was separated on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.12 N sulfuric acid as the mobile phase at a flow rate of 0.8 mL/min with a 20-μL injection volume and a 65 °C column temperature (*29*).

RESULTS

Fungal Detoxification of Dcs and Dst. To study the effect of ligninolytic enzymes of *Ph. chrysosporium* to detoxify Dcs

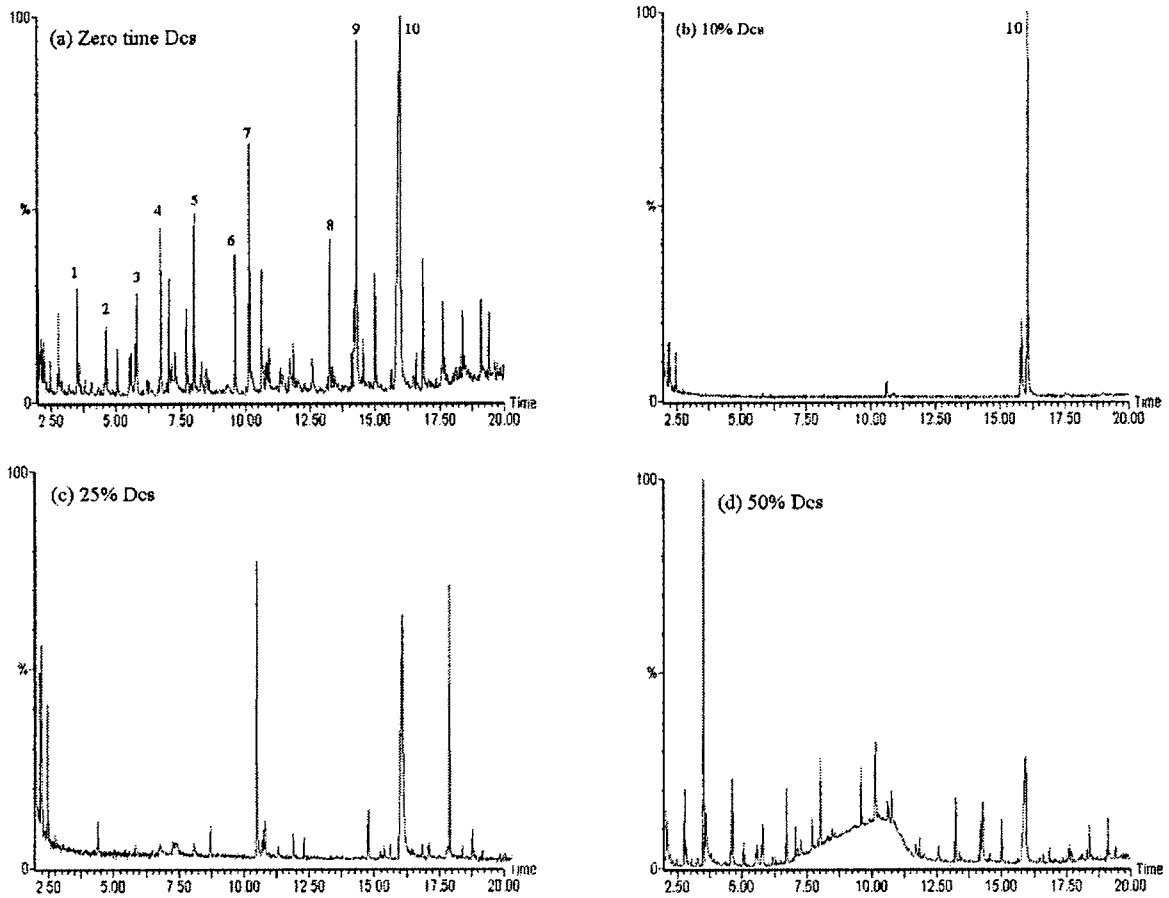

Figure 2. Chromatographic profiles of non-detoxified Dcs (**a**) [phenol (peak 1), 1–2-furanyl (peak 2), 2-furancarboxaldehyde (peak 3), 2-hydroxy-3-methyl-2-cyclopenten (peak 4), 2-methoxyphenol (peak 5), 4-ethylphenol (peak 6), 2,6-dimethoxyphenol (peak 7), 1,2,4-trimethoxybenzene (peak 8), 2-methoxy-5-methylphenol (peak 9), and 1,6-anhydro-β-D-glucopyranose (peak 10)] and Dcs after detoxification by *Ph. chrysosporium* 10% (v/v) (**b**), 25% (v/v) (**c**), and 50% (v/v) (**d**).

and Dst, different concentrations of 10, 25, and 50% (v/v) of Dcs and Dst were added to the culture broth on day 4 so as not to inhibit fungal growth and MnP production. *Ph. chrysosporium* was able to continue growing for 10 additional days at all three concentrations of Dcs and Dst as determined by dry biomass weight measurement, although growth was slowest with 50% (v/v) Dcs and Dst. The average dry mycelium dry weights (replicate of three) were 1.7, 1.6, and 1.0 g/L with 10, 25, and 50% (v/v) Dcs, respectively, 1.6, 1.8, and 0.8 g/L with 10, 25, and 50% (v/v) Dst, respectively, and 2.2 g/L with control flasks on day 14 (no Dcs or Dst addition).

GC-MS Analysis. The analysis of zero time Dcs and Dst samples showed many major peaks as described previously (*8*). In the zero time corn stover Dcs, phenol, 1−2-furanyl, 2-furancarboxaldehyde, 2-hydroxy-3-methylcyclopentene, 2-methoxyphenol, 4-ethylphenol, 2,6-dimethoxyphenol, 1,2,4-trimethoxybenzene, 2-methoxy-5-methylphenol, and 1,6-anhydro-β-D-glucopyranose were identified (*8*). In the zero time corn starch Dst, 2-furancarboxaldehyde, 2(5*H*)-furanone, 1,3-cyclopentanedione, 5-methyl-2-furancarboxldehyde, 2-hydroxy-3-methylcyclopentene, 3,6-dianhydro-β-D-glucoyranose, furyl hydroxymethyl ketone, 3,6-dianhydro-D-glucopyranose, 5-hydroxymethyl-2-furancarboxaldehyde, and 1,6-anhydro-β-D-glucopyranose were identified (*8*).

The fungal-treated Dcs and Dst samples showed a decrease in some peaks. The decrease was more noticeable in 10 and 25% (v/v) Dcs and Dst (**Figures 2** and **3**) compared to the decrease in 50% (v/v) Dcs and Dst. The decrease quantities of some compounds in Dcs and Dst fungal-treated samples are recorded in **Table 1**.

Extracellular Proteins. In 10, 25, and 50% (v/v) Dcs the extracellular protein concentration reached a maximum at day 4 at 17, 12, and 15 mg/L, respectively, and decreased gradually to 1.1, 0.8, and 1.0 mg/L by day 14, respectively (**Figure 4**). When 10% (v/v) Dst was added to the medium, the extracellular protein level reached a maximum on day 4 with 15 mg/L and remained constant until day 7, when it decreased to 0.1 mg/L. With 25 and 50% (v/v) Dst, the extracellular protein level reached a maximum on days 6 and 8 at 13 and 15 mg/L, respectively, and then decreased gradually to 0.3 and 0.6 mg/L by day 14 (**Figure 4**). The control flasks (no Dcs or Dst) continue 18 mg/L extracellular protein.

Ligninolytic Activity during Detoxification. The activity of ligninolytic enzymes of *Ph. chrysosporium* in the shake flask culture media with different concentrations of Dcs and Dst were monitored over 14 days. Laccase activity was not detected. In culture media with 10, 25, and 50% (v/v) Dcs or Dst (**Figure 5**) the MnP activity reached a peak on day 6 in a medium containing 10, 25, or 50% (v/v) Dcs, whereas MnP reached a peak on day 4 with 25% (v/v) Dst (**Figure 5**).

The maximum MnP levels were 23, 13, and 15 units/L in culture media with 10, 25, and 50% (v/v) Dcs and 21, 14, and 9 units/L in culture media with 10, 25, and 50% (v/v) Dst, respectively. The levels of MnP were decreased in the culture media with all concentrations of Dcs and Dst by the end of

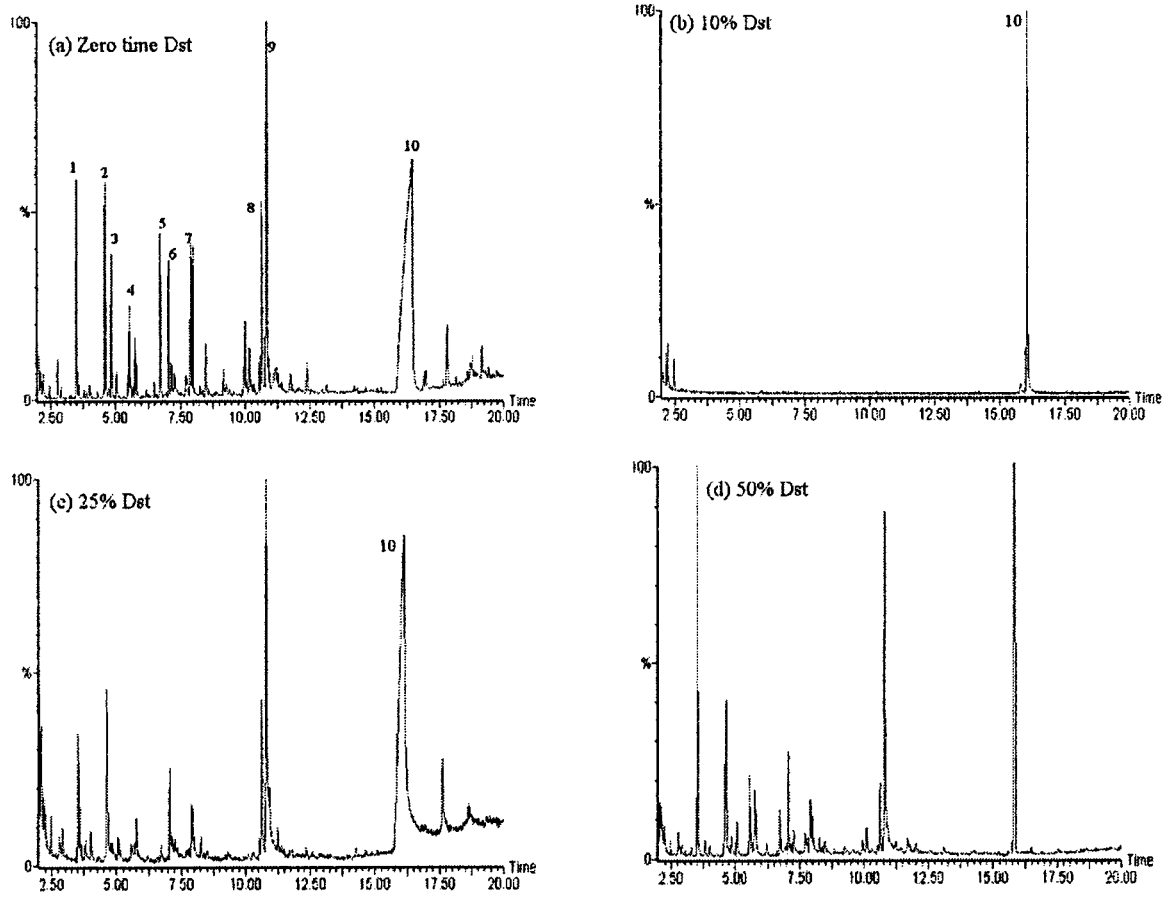

Figure 3. Chromatographic profiles of non-detoxified Dst (**a**) [(peak 1) 2-furancarboxaldehyde, (peak 2) 2(5*H*)-furanone, (peak 3) 1,3-cyclopentanedione, (peak 4) 5-methyl-2-furancarboxldehyde, (peak 5) 2-hydroxy-3-methyl-2-cyclopentene, (peak 6) 3,6-dianhydro-β-D-glucoyranose, (peak 7) furyl hydroxymethyl ketone, (peak 8) 3,6-dianhydro-D-glucopyranose, (peak 9) 5-hydroxymethyl-2-furancarboxaldehyde, (peak 10) 1,6-anhydro-β-D-glucopyranose] and Dst after detoxification by *Ph. chrysosporium* 10% (v/v) (**b**), 25% (v/v) (**c**), and 50% (v/v) (**d**).

Table 1. GC-MS Quantitative Analysis Results of Some Compounds in Different Concentrations of Diluted Corn Stover Pyrolysis Liquors (Dcs) and Diluted Corn Starch Pyrolysis Liquors (Dst) before and after *Ph. chrysosporium* Treatment[a]

percentage diluted pyrolysis liquors with and without fungal treatment	compounds in mM concn in Dcs					compounds in mM concn in Dst	
	phenol	2-methoxy-5-methylphenol	2,6-dimethoxy-phenol	1,2,4-trimethoxy-benzene	levoglucosan	2(5*H*)-furanone	levoglucosan
10% (v/v)	0.25	0.94	1.1	0.056	50	4.4	120
10% (v/v) fungal treated	0.0	0.0	0.0	0.0	ND[b]	0.0	ND
25% (v/v)	0.64	2.3	2.6	0.14	110	11	240
25% (v/v) fungal treated	0.0	0.0	0.0	0.0	110	6.2	220
50% (v/v)	19	4.7	5.3	0.28	240	22	400
50% (v/v) fungal treated	1.3	0.0	0.013	0.0	230	7.9	400

[a] The quantitative analysis was determined according to pure standard for each compounds. [b] Not determined.

study. In control flasks (no Dcs or Dst), the MnP reached a peak on day six with 31 and 7 units/L on day 14 (**Figure 5**).

The LiP activity was detected on day 4 in culture media with 10, 25, and 50% Dcs or Dst (v/v) (**Figure 6**). For corn stovers LiP activity reached a maximum on day 6 with 10 and 25% (v/v) Dcs and on day 8 with 50% (v/v) Dcs. For corn starch, LiP reached a maximum on day 4 with 10% Dst (v/v) and on day 6 with 25 and 50% Dst (v/v). With 50% (v/v) Dst the LiP maintained 8 units/L for three consecutive days from days 6 to 10 (**Figure 6**). The LiP activity was not significantly different between Dcs or Dst at any concentration (~9 units/L); however, in control flasks (no Dcs or Dst addition) the LiP activity reached a peak on day 6 with 19 and 4 units/L on day14 (**Figure 6**).

Decrease of Phenolic Compounds in Dcs by *Ph. chrysosporium*. *Ph. chrysosporium* was able to degrade most of the phenolic compounds in 10 and 25% (v/v) Dcs. The phenols began to decrease gradually on day 5 (**Figure 7**). *Ph. chrysosporium* culture almost completely biodegraded the phenolic compounds in 10 and 25% (v/v) Dcs. However, in 50% (v/v) Dcs, the phenolic compounds concentration started to decrease on day 4 until day 8. After day 8, the decrease slowed, and phenols persisted in medium on day 14 (**Figure 7**). The concentrated ligninolytic enzymes (28 and 43 units/L LiP and MnP, respectively) reduced the total phenolics in 10% (v/v) Dcs from 0.1 to 0.01 mg/mL after 60 min of incubation at 39 °C (**Figure 8**).

Bioassay. The 10% (v/v) Dcs and Dst incubated with *Ph. chrysosporium* demonstrated the significant loss of Dcs and Dst toxicity to *L. casei* (**Table 2**). The A_{620} values were 3.5 and 3.2 and the lactic acid productions were 10.4 and 9.5 g/L for 10%

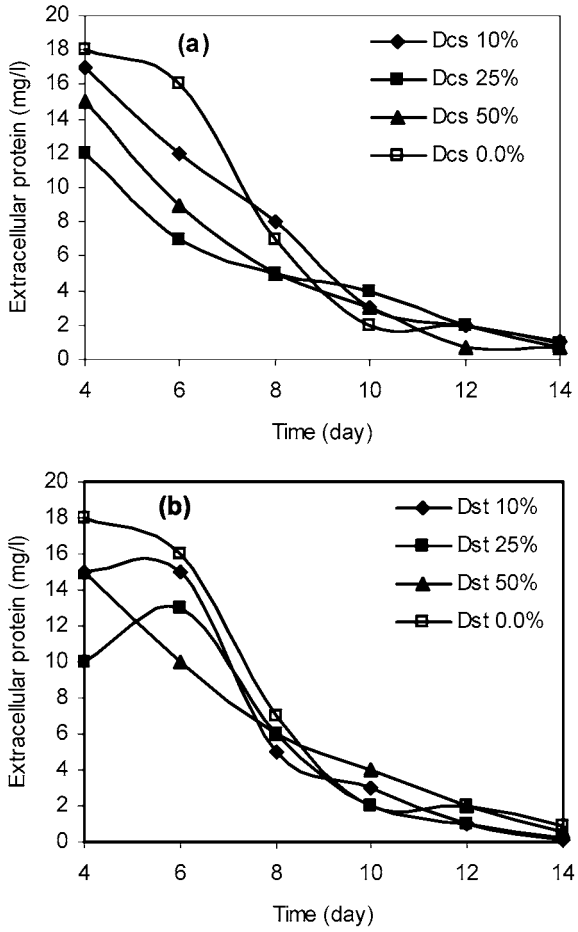

Figure 4. Extracellular proteins produced during the detoxification by *Ph. chrysosporium* to 0, 10, 25, and 50% (v/v) of (**a**) diluted corn stover (Dcs) (average standard deviations were 0.29, 0.42, and 0.29, respectively) or (**b**) diluted corn starch (Dst) (average standard deviations were 0.18, 0.3, and 0.22, respectively), which were added on day 4 ($n = 3$).

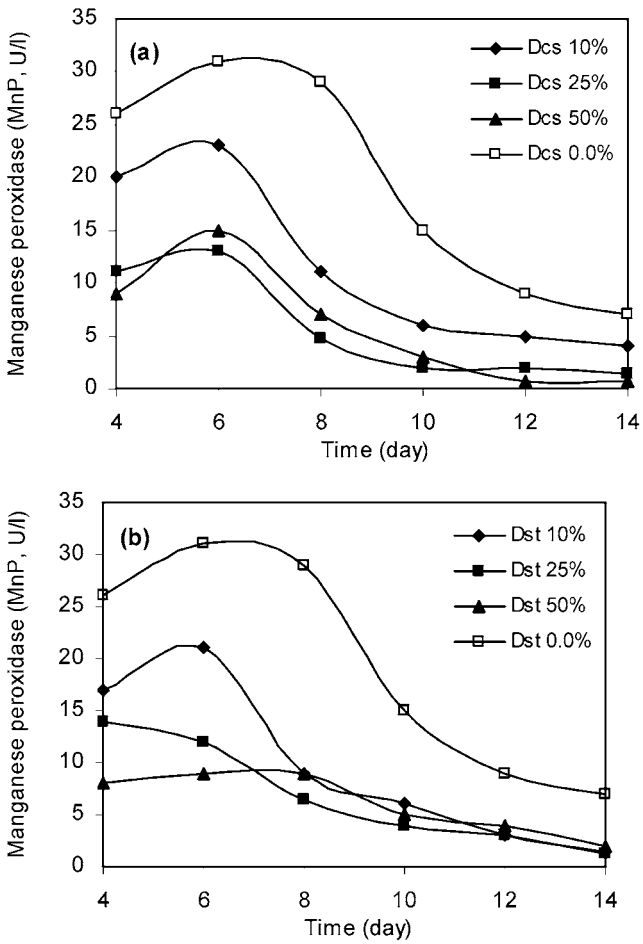

Figure 5. Manganese peroxidase produced by *Ph. chrysosporium* during the detoxification to 0, 10, 25, and 50% (v/v) of (**a**) diluted corn stover (Dcs) (average standard deviations were 0.06, 0.39, and 0.24, respectively) or (**b**) diluted corn starch (Dst) (average standard deviations were 0.14, 0.43, and 0.46, respectively), which were added on day 4 to cultures ($n = 3$).

(v/v) fungal-treated Dcs and Dst, respectively. The positive control, *L. casei* cultures have an A_{620} of 3.8 and ~19 g/L lactic acid production after an 18 h incubation, wheres no growth or lactic acid was detected in the negative control (zero time Dsc or Dst). In fungal-treated 25% (v/v) Dcs and Dst, the bioassay also demonstrated detoxification, but the A_{620} and lactic acid produced were significantly less than that produced with 10% (v/v) detoxified Dcs and Dst. *L. casei* did not grow in fungal-treated 50% (v/v) Dcs or Dst.

Effect of Inhibitor Treatment with *Ph. chrysosporium*. To confirm that the detoxification of Dcs and Dst was the result of *Ph. chrysosporium* ligninolytic enzyme activity, sodium azide or cycloheximide was added to the culture flasks with 10% (v/v) Dcs or Dst. Without enzyme inhibitors LiP activity was ~8 units/L for Dcs and Dst culture flasks. The Dcs and Dst culture flasks amended with 0.5 or 2.5 μM sodium azide on day 2 produced a small amount of mycelium, no LiP activity, and a corresponding negative bioassay, whereas day 4 Dcs and Dst culture flasks amended with 0.5 and 2.5 μM sodium azide produced more mycelia, but there was no LiP activity detected and *L. casei* was again unable to grow in these liquors. Addition of 70 mg of cycloheximide to day 2 culture flasks with 10% (v/v) Dcs or Dst produced a small amount of mycelium, and no LiP activity was detected, whereas 70 mg of cycloheximide addition to day 4 culture flasks produced more mycelia and higher LiP activity (5 units/L), and the bioassay results were A_{620} values of 0.1 and 0.2 and lactic acid production of 2.1 and 2.3 g/L for Dcs and Dst, respectively.

DISCUSSION

This study demonstrates the feasibility of employing *Ph. chrysosporium* to remove toxic compounds from Dcs and Dst pyrolysis liquors instead of the conventional physical/chemical methods (*7*). Most biodegradation studies using *Ph. chrysosporium* have been performed with fungal cultures (*10*, *15*, *30*). These studies suggested that ligninase enzymes are involved in xenobiotic degradation. *Ph. chrysosporium* ligninase enzymes degrade several organopollutants similar to lignin because it is non-stereoselective and nonspecific (*25*, *28*, *31*, *37*). The ligninase-mediated biodegradation of aromatic compounds depends on their structure and the oxidation rates, which increased in the presence of methoxy and hydroxyl groups (*32*). These enzymes effectively removed the microbial inhibitors in Dcs and Dst, but our ability to distinguish the specific compounds degraded was limited (**Figures 2** and **3**). The decrease of toxic compounds as determined by GC-MS (**Figures 2** and **3**) and the bioassay (**Table 2**) were considered good parameters to elucidate the removal of toxic compounds in Dcs and Dst. Confirmation of the phenolic compounds decrease in Dcs was also obtained with total phenolic compounds assays (**Figures 7** and **8**).

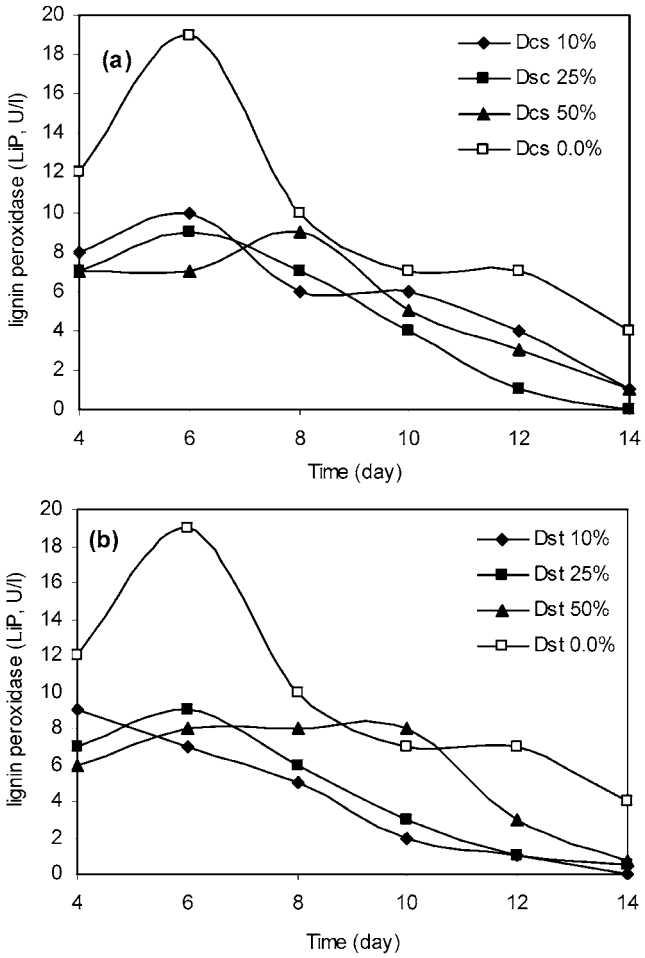

Figure 6. Lignin peroxidase produced by *Ph. chrysosporium* during incubation with 0, 10, 25, and 50% (v/v) of (**a**) diluted corn stover (Dcs) (average standard deviations were 0.09, 0.26, and 0.68, respectively) or (**b**) diluted corn starch (Dst) (average standard deviations were 0.09, 0.31, and 0.57, respectively), which were added on day 4 to cultures ($n = 3$).

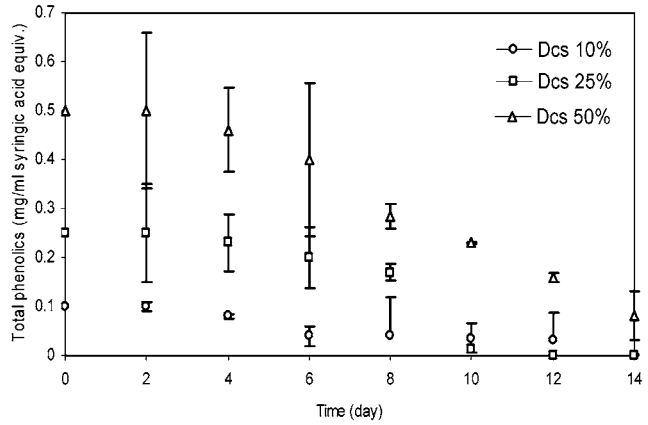

Figure 7. Changes in total phenolic compounds in 10, 25, and 50% (v/v) diluted corn stover (Dcs) during 14 days of incubation with *Ph. chrysosporium.* Each value represents the average of at least two replicates ($n = 2$).

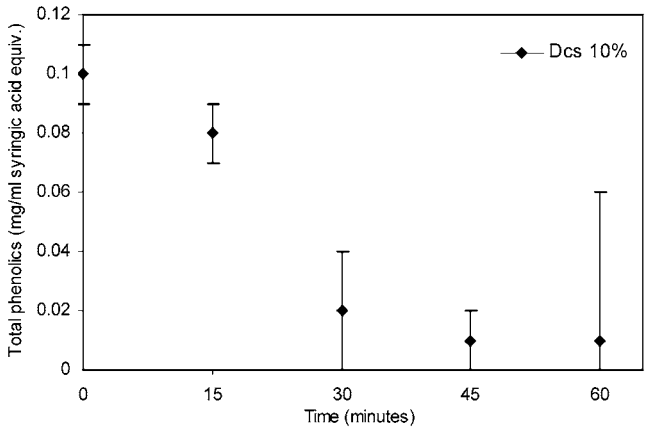

Figure 8. Change in total phenolic compounds in 10% (v/v) diluted corn stover (Dcs) during 60 min of incubation with concentrated *Ph. chrysosporium* enzymes. Each point represents the average of three replicates ($n = 3$).

In 10, 25, and 50% (v/v) Dcs and Dst detoxification experiments, *Ph. chrysosporium* produced 8−10 units/L of LiP and 10−23 units/L of MnP. The ligninolytic enzyme system of *Ph. chrysosporium* culture flasks catalyzed the oxidation of toxic compounds as did ligninolytic enzyme concentrated with 10% (v/v) Dcs and Dst. These results also indicated that the ligninolytic enzymes (LiP and MnP) were produced in sufficient amounts during fungal growth to catalyze the reduction of microbial inhibitors (**Figures 5** and **6**). In fungal-treated 25% (v/v) Dcs and Dst, the toxic compounds were also removed (**Figures 2** and **3**), but in the bioassay the lactic acid production was low compared to 10% (v/v) detoxified Dcs and Dst bioassays (**Table 2**). In fungal-treated 50% (v/v) Dcs and Dst, the incomplete removal of toxic compounds was confirmed by the bioassay with no detectable growth or lactic acid production, as the GC-MS analysis showed (**Figure 2**). To complete the detoxification in 25 and 50% (v/v) Dcs and Dst, increasing the period of incubation might be helpful to remove residual toxic compounds, because the transformations by *Ph. chrysosporium* cultures are slow (*33*).

Table 2. Ability of *L. casei* To Grow on Mixture of MRS Medium and Different Concentrations of Detoxified Diluted Corn Stover Pyrolysis Liquors (Dcs) or Diluted Corn Starch Pyrolysis Liquors (Dst)[a]

	detoxified Dcs (v/v)						detoxified Dst (v/v)					
	10%		25%		50%		10%		25%		50%	
treatment	A_{620}	LA[b] (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)	A_{620}	LA (g/L)
control[c]	ND[d]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ph. chrysosporium	3.5	10.4	0.3	2.7	ND	ND	3.2	9.5	0.5	3.2	ND	ND
concentrated enzyme	1.7	5.3	NA[e]	NA	NA	NA	0.8	4.1	NA	NA	NA	NA

[a] *Ph. chrysosporium* treatment of Dcs and Dst was carried out by whole cell culture flasks or by concentrated enzyme in replicates of three ($n = 3$). [b] Lactic acid production resulted from *L. casei* growth. In MRS medium without amendment, Dcs or Dst (19.0 g/L) of lactic acid is produced. [c] Negative control: a mixture of MRS medium with Dcs or Dst but without fungal or enzyme treatment and then inoculated with *L. casei.* [d] No *L. casei* growth or lactic acid production was detected. [e] Not assayed.

In bioremediation long periods of incubation are not desired; thus, concentrated enzymes will carry out the detoxification in hours, whereas fungus culture treatments will carry it out in weeks (*10*, *33*). The direct oxidation of chemical compounds by purified ligninase has been reported, which seems to implicate the ligninolytic system directly in the detoxification of xenobiotics (*21*, *34*, *35*). In our result, the phenolic compounds in 10% (v/v) Dcs were removed within 60 min with concentrated *Ph. chrysosporium* enzyme (**Figure 8**), which was confirmed by detoxification in bioassay (**Table 2**).

Besides the ability of lignolytic enzymes degradation, there is also evidence to suggest that white-rot fungi possess other mechanisms of xenobiotic degradation that are not reliant on the ligninolytic system. *Ph. chrysosporium* was observed to degrade the polycyclic aromatic hydrocarbon under ligninolytic as well as nonligninolytic conditions, suggesting that the potential of *Ph. chrysosporium* for degradation of certain environmental pollutants is not limited to nutrient starvation conditions (*36*). Therefore, it has been postulated that a plasma membrane potential might be involved in the bioconversion of various xenobiotics (*12*). For example, several redox dyes have been shown to be possibly reduced via such a mechanism, and trinitrotoluene (TNT) may also be reduced by this method (*37*). It been suggested that *Ph. chrysosporium* maintains the pH of its environment at about 4.5 via proton pumping and that bioconversion of chemicals may be linked to this proton gradient (*12*, *14*). Therefore, the addition of sodium azide to *Ph. chrysosporium* media with 10% (v/v) Dcs or Dst on days 2 and 4 confirmed the ligninases' role. Our result showed that the addition of sodium azide on days 2 and 4 of shake flask culture incubation inhibited LiP activity and inhibited toxic compounds decrease. Sodium azide is a potent inhibitor of LiP activity. Inhibition of veratryl alcohol oxidase activity by sodium azide indicated that inhibition was due to the reaction of sodium azide with activated enzyme, compound **I**, rather than binding of sodium azide to the native enzyme (*18*). It is also thought to affect the plasma membrane redox system of the fungus (*14*).

Cycloheximide inhibits protein synthesis. It blocks leucine incorporation into proteins (*19*). Therefore, the addition of the cycloheximide to *Ph. chrysosporium* culture on day 2 or earlier will prevent ligninolytic enzyme production (*19*) and, thus, fungal-treated Dcs or Dst demonstrated toxicity in the bioassay. Both LiP and MnP are produced in idiophase, and cycloheximide addition should stop their production (*22*). Thus, when the cycloheximide was added on day 4 after the enzyme production and excretion, toxic compounds were removed from fungal-treated 10% (v/v) Dcs and Dst. Dosoretz (*38*) demonstrated that addition of cycloheximide on day 6 to *Ph. chrysosporium* cultures resulted in no change of LiP activity.

In conclusion, *Ph. chrysosporium* was able to biodegrade the toxic compounds present in diluted corn stover and diluted corn starch pyrolysis liquors. Extracellular *Ph. chrysosporium* ligninolytic enzymes were responsible for this detoxification. The complete detoxification process needs longer incubation with fungal culture, because the transformations by *Ph. chrysosporium* are slow (*33*, *39*). However, the concentrated enzymes can do in hours what fungi cultures do in weeks. Therefore, industrial fermentor procedures are needed for the large-scale production of these enzymes.

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