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Solid-Substrate Fermentation of Corn Fiber by *Phanerochaete chrysosporium* and Subsequent Fermentation of Hydrolysate into Ethanol

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The goal of this study was to develop a fungal process for ethanol production from corn fiber. Laboratory-scale solid-substrate fermentation was performed using the white-rot fungus *Phanerochaete chrysosporium* in 1 L polypropylene bottles as reactors via incubation at 37 °C for up to 3 days. Extracellular enzymes produced in situ by *P. chrysosporium* degraded lignin and enhanced saccharification of polysaccharides in corn fiber. The percentage biomass weight loss and Klason lignin reduction were 34 and 41%, respectively. Anaerobic incubation at 37 °C following 2 day incubation reduced the fungal sugar consumption and enhanced the in situ cellulolytic enzyme activities. Two days of aerobic solid-substrate fermentation of corn fiber with *P. chrysosporium*, followed by anaerobic static submerged-culture fermentation resulted in 1.7 g of ethanol/100 g of corn fiber in 6 days, whereas yeast (*Saccharomyces cerevisiae*) cocultured with *P. chrysosporium* demonstrated enhanced ethanol production of 3 g of ethanol/100 g of corn fiber. Specific enzyme activity assays suggested starch and hemi/cellulose contribution of fermentable sugar.

KEYWORDS: Lignocellulosic biomass; corn fiber; solid-substrate fermentation; simultaneous saccharification and fermentation; ethanol; white-rot fungi; *Phanerochaete chrysosporium*; *Saccharomyces cerevisiae*

INTRODUCTION

The United States produced over 6 billion gallons of ethanol from corn in 2007. The production of ethanol is projected to reach 12 billion gallons by 2010 (*1*). Along with this huge ethanol production, the dry-grind and wet-milling corn ethanol industries also produce a huge amount of low-value coproducts such as distiller's dried grains (DDG) and corn fiber, respectively. Wet-milled corn fiber contains large amounts of cellulose, hemicellulose, and residual starch, which together represent >75% of dry weight as complex polysaccharides (*2*). Cellulosic corn fiber has the potential to produce an additional 13% ethanol per bushel of corn (*3*). Conversion of lignocellulosic biomass into ethanol involves several steps such as pretreatment, hydrolysis, fermentation, and separation/purification (*4*).

Pretreatment of plant biomass is an important step in the economical conversion of cellulosic material into ethanol (*5*). Physical, chemical, and biological pretreatments involve the use of mechanical milling, pressurized steam, acids, alkali, ammonia, or enzymes in a separate or combined process to break down the heterogenic and crystalline lignocellulosic fiber matrix and to facilitate the conversion of the fiber components (e.g., cellulose and hemicellulose) into sugars for ethanol production (*4*). New enzyme production technologies aim to reduce the cost of the cellulose-to-ethanol conversion process (*6*) and, therefore, enzymatic hydrolysis of cellulosic feedstock has a potential niche over costly and environmentally unfriendly physical−chemical processes. To date, this is still an expensive option as some pretreatment is still required and the enzymes, produced by specialist enzyme suppliers, are costly.

Numerous indigenous microbes are reportedly capable of degrading cellulose and hemicellulose to their monomers, for example, hexoses and pentoses (*7*). Wood-rot fungi such as

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white- and brown-rot fungi have been examined for their cellulolytic abilities. *Trichoderma reesei* has been studied widely for its cellulase activity (*8*). White-rot fungi produce lignin-degrading enzymes that degrade the lignin to carbon dioxide and water, exposing the hemicellulose and cellulose in the wood matrix (*9, 10*). Likewise, brown-rot fungi have the ability to modify the lignin structure in the wood matrix (*11*). Most of the information available on fungal biodegradation of lignin has been derived from studies on the white-rot fungus *Phanerochaete chrysosporium*, which degrades lignin by secreting different peroxidases (*12*).

The overall cost of the enzymatic hydrolysis can be reduced further by coupling the enzyme production with the hydrolysis of lignocellulose materials into a single step such as solid-substrate fermentation. Pandey et al. (*13*) reported solid-substrate fermentation as a promising fermentation technique for in situ production of ligninolytic and cellulolytic enzymes. Several studies explored ligninolytic and cellulolytic enzyme activities in solid-substrate fermentation using *P. chrysosporium* under various substrates and operating conditions (*14, 15*). This technique has both technological and economic merits over suspended-growth fermentation for the enzymatic hydrolysis of lignocellulosic biomass (*16*). This research envisaged the evaluation of the effectiveness of *P. chrysosporium* in saccharification of corn fiber in solid-substrate fermentation and the subsequent simultaneous saccharification and fermentation of hydrolysates into ethanol using *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Fungus Culture. *P. chrysosporium* (ATCC 24725) and *S. cerevisiae* (ATCC 24859) cultures were obtained from American Type Culture Collection (Rockville, MD). The fungus culture was revived by inoculating it in potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD) and was incubated with shaking at 24 °C. Stock cultures were stored in cryogenic vials (Nalgene, Nalge Nunc, International, Rochester, NY) and preserved in an ultralow-temperature freezer (−80 °C, So-Low, Cincinnati, OH).

The frozen stock culture was thawed and poured aseptically into 1 L of sterilized yeast mold (YM) broth (Difco, Becton Dickinson and Co.). The seed culture was incubated with shaking at 150 rp*m* and 30 or 37 °C for rejuvenation. *P. chrysosporium* mycelia grew into pellets of 2−3 mm size in 3−5 days.

Substrate for Solid-Substrate Fermentation. The substrate for the fermentation experiments was corn fiber obtained from wet corn milling plants (ADM, Decatur, IL). Fiber from corn wet milling had been processed through hot water steeping and sulfur dioxide (SO_2) treatment. Abbas et al. (*2*) reported this corn fiber composition: 35% (w/w) hemicellulose, 18% (w/w) cellulose, 17% (w/w) starch, 11% (w/w) protein, 6% (w/w) ash, 6% (w/w) galactan, 3% (w/w) oil, 1% (w/w) mannan, and 3% (w/w) other materials. The Klason lignin content of the fiber according to a modified Klason procedure (*17*) was 18% (w/w) of dry weight. The corn fiber was, therefore, rich in polysaccharides (hemicelluloses, cellulose, and starch). Sterilization of the fiber was done by autoclaving at 121 °C for 75 min during the preparation of culture bottles.

Experimental Setup. A series of batch fermentation experiments with corn fiber were conducted using *P. chrysosporium*. Culture bottles with fungal mycelia growing on corn fiber at 37 °C served as solid-substrate fermentation bioreactors. The fermentation was carried out in 1 L polypropylene bottles (Nalgene, Nalge Nunc). Control bottles contained corn fiber, marbles, and culture media but no fungi. The following studies were done: (a) determination of sugar release and biomass weight loss in both culture and control bottles during fermentation; (b) fungal delignification of Klason lignin in corn fiber with application of various chemicals such as hydrogen peroxide, manganese sulfate, and veratryl alcohol; and (c) saccharification and fermentation of corn fiber to ethanol in anaerobic conditions.

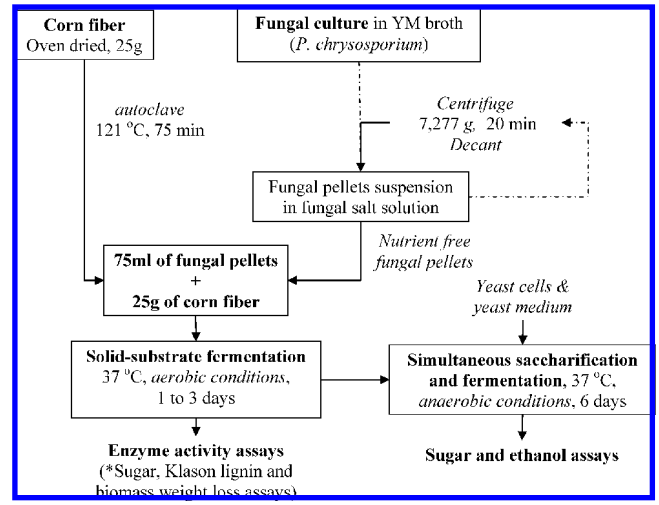

Figure 1. Overall process describing the steps for substrate and fungal culture (*P. chrysosporium*) preparation followed by solid-substrate fermentation. Simultaneous saccharification and fermentation of hydrolysate from solid-substrate fermentation was performed using *S. cerevisiae*. * Solid-substrate fermentation of corn fiber using *P. chrysosporium* was performed and studied for several weeks in the beginning of the research.

Substrate Quantification and Pretreatment. The fermentation process employed was modified from that of Crawford and Pometto (*17*). The moisture content of the fiber was about 7% (w/w). The corn fiber was oven-dried at 80 °C for 4 days followed by desiccation prior to use. Dried corn fiber (25 g) was weighed and put in 1 L polypropylene bottles. Five marbles were added to each bottle. These marbles were added for distribution of corn fiber and fungal biomass along the inner wall of the bottle during gentle mixing and shaking. Five milliliters of deionized water was added to the bottle. The water was added to create steam within the plant biomass during autoclaving of the substrate. Many bottles were prepared in the manner as described above. The bottles were then loosely capped and autoclaved at 121 °C for 1 h. The autoclave was exhausted rapidly. The bottle caps were replaced with blue autoclave cloth (Propper Steri-Wrap III, Propper Manufacturing Co. Inc., Long Island City, NY) and then resterilized for 15 min at 121 °C. The autoclave cloth served two purposes during solid-substrate fermentation: permeating exchange of air and keeping contaminants from reaching inside the bottles.

Fungal Inoculum Preparation and Solid-Substrate Fermentation. The overall process is illustrated as a flowchart (**Figure 1**). The fungal stock culture was rejuvenated in YM broth and formed visible pellets in 3−5 days. The pellets in YM broth were aseptically transferred to a sterilized 1 L polypropylene centrifuge bottle. The bottle was centrifuged at 7277*g* for 20 min. The supernatant was decanted, and the centrifuge bottle was aseptically filled to the top with nitrogen-free basal salt solution (*18*), then mixed by shaking. This process (centrifugation and decantation) was repeated to rinse the fungal pellets and minimize nutrient transfer from YM broth to the substrate (corn fiber) during inoculation of fungal pellets into the fermentation bottles. Once the complex nutrient from YM broth was washed and rinsed, fungal pellets (in basal salt solution) were transferred to a sterile 2 L Erlenmeyer flask. Approximately 1 L of fungal pellets slurry was prepared by adding sterile basal medium. A programmed peristaltic pump was used to dispense 75 mL of fungal pellets slurry into the 1 L sterile polypropylene bottles (containing 25 g of sterile corn fiber and 5 marbles). For control bottles (containing 25 g of sterile corn fiber and 5 marbles) 75 mL of sterile basal salt solution was added. The bottles were recapped with the autoclave cloth.

The bottles were rolled next for about 50 revolutions forward and backward on their sides. The marbles in the bottles helped to mix and disperse the corn fiber and fungi uniformly over the inner surface of the bottles. The bottles were placed in a humidified incubator at 37 °C. Moist air in the incubator was maintained by placing water troughs at the bottom and passing air into the water.

Incubation under Anaerobic Conditions. Anaerobic incubation of the fermentation bottles was aimed to maximize sugar production and minimize the consumption of sugar by *P. chrysosporium*. Anaerobic incubation of these bottles was performed by adding 300 mL of sterile 0.2 M acetate buffer (pH 4.73) and maintaining the tightly capped bottles at 37 °C in a water bath for 2 days. Control and culture bottles incubated for 5, 7, 9, and 11 days were treated anaerobically in buffer solution as described earlier.

Simultaneous Saccharification and Fermentation of Hydrolysate to Ethanol. The biomass in control and culture bottles was washed to the bottom by adding 200 mL of sterile deionized water. Then 100 mL of sterile triple-strength yeast culture medium (*19*) without glucose was added to the bottles aseptically. The pH was manually adjusted to 5.5. Each bottle was inoculated aseptically with 2 mL of freeze-dried culture of *S. cerevisiae* (5.6×10^8 cells/mL). The bottles were loosely capped (to create anaerobic conditions but allow CO_2 release) and incubated at 37 °C.

Analytical Methods. In one set of experiments, culture and control solid-substrate fermentation bottles were kept in a humidified incubator for 3 weeks. Studies on biomass weight loss and sugars were done on weekly basis ($n = 4$). Klason lignin assay was performed on biomass residue from 2-week-old solid-substrate fermentation ($n = 1$). Biomass weight loss and sugar assays were also performed for solid-substrate fermentation bottles that had undergone anaerobic buffer treatment as described under Incubation under Anaerobic Conditions. At a later phase of the experiments, the solid-substrate fermentation of corn fiber, using *P. chrysosporium*, was performed for up to 3 days only. These bottles were then subjected to simultaneous saccharification and fermentation in anaerobic conditions for 6 days. Ethanol and reducing sugar levels were determined on alternate days ($n = 2$). Enzyme activity assay was also performed on cell-free extracts from 2-day-old solid-substrate fermentation bottles.

Sugar Assays. Three hundred milliliters of deionized water was added to culture bottles to wash down the fiber and fungal mycelia to the bottom of the bottle. The bottles were kept in a steaming cabinet (~100 °C) for 1 h. The contents were then poured into 0.5 L polypropylene centrifuge bottles. The bottles were centrifuged at 17696*g* for 20 min, and the supernatants were filtered through predried and preweighed Whatman no. 54 filter papers. The filtrates were then analyzed for free reducing sugar according to the modified Somogyi−Nelson carbohydrate assay (*17*) via absorbance at 500 nm with a glucose standard. The samples were also tested for total sugar via phenol sulfuric carbohydrate test (*17*) via absorbance at 490 nm with a glucose standard. The absorbance readings of samples were achieved using a spectrophotometer (Spectronic 20 Genesys, Thermo Electron, Cambridge, U.K.). The absorbance readings were converted into equivalent sugar concentration using a standard glucose solution curve.

Klason Lignin Assay. Effects of hydrogen peroxide (*20*), manganese sulfate, and veratryl alcohol (3,4-dimethoxybenzyl alcohol) (*21*) on delignification and sugar release were examined by adding 10 mL of different concentrations of these chemicals aseptically to 2-week-old cultures in bottles. H_2O_2 is a natural substrate of both lignin peroxidase and manganese peroxidase. Manganese is a natural substrate of manganese peroxidase. Veratryl alcohol is a metabolite produced at the same time as lignin peroxidase by *P. chrysosporium*. The concentrations and volume of chemicals added were 0.4, 4, and 40 mM hydrogen peroxide and 3, 30, and 300 mM veratryl alcohol and manganese sulfate. The content inside these bottles was analyzed for Klason lignin degradation 1 week after the addition of these chemicals.

The lignin content was determined by using a modified Klason lignin assay (*17*), which measures lignin as the acid-insoluble fraction of lignocellulosic material (Klason lignin) after hydrolysis by strong acid (H_2SO_4) and heat. The residue on the filter paper was thoroughly rinsed with deionized water and dried in the oven at 80 °C for 2 days. The Klason lignin was determined as the weight of dry residue collected on the filter paper.

Biomass Weight Loss. Following the filtration and sugar-harvesting steps, the centrifuge bottles containing wet fiber were dried in an oven at 80 °C for 4 days. After cooling in a desiccator, the dry weight of the bottles was determined. Biomass weight loss was calculated as the difference in weights of the initial and final biomass.

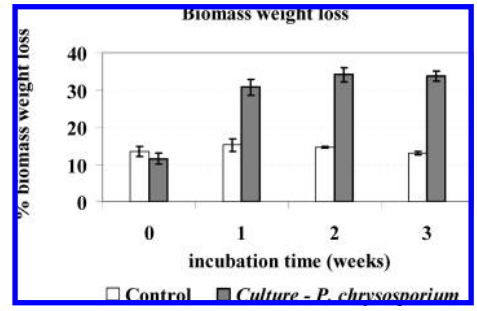

Figure 2. Biomass weight loss is compared between solid-substrate fermentation of corn fiber using *P. chrysosporium* (residual corn fiber with fungal biomass) and controls (no fungal inoculation) ($n = 4$).

Ethanol Assay. Samples were taken at intervals of 48 h and analyzed for ethanol and reducing sugar (described under Sugar Assays). Ethanol concentrations were measured by using a Waters high-pressure liquid chromatograph (Millipore Corp., Milford, MA) equipped with a Waters model 401 refractive index detector, column heater, autosampler, and computer controller. The separation of ethanol, glucose, and other broth constituents was done using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) with 0.012 N sulfuric acid as mobile phase at a flow rate of 0.8 mL/min, injection volume of 20 μL, and a column temperature of 65 °C.

Enzyme Activity Assays. Cell-free culture extracts from 2-day solid-substrate fermentation bottles (control and culture) were collected. Determination of specific enzyme activities of five enzymes (α-amylase, glucoamylase, xylanase, endocellulase, and exocellulase) over corn starch, xylan, carboxymethyl cellulose (CMC), and Sigmacell 20 (cellulose) was performed by slightly modifying the procedures of Abd El-Nasser et al. (*22*) and Lee et al. (*23*). Mixture of 0.25 mL of cell-free extract, 0.25 mL of acetate buffer (0.06M, pH 5), and 0.25 mL of individual substrate (concentration = 3 g/L) were prepared in micro-centrifuge vials, and then the vials were placed in a water bath at 37 °C. The pH and temperature conditions were similar to conditions of laboratory fermentation bottles. Reducing sugar was determined, via the modified Somogyi−Nelson carbohydrate assay (*17*), for samples at 0, 15, 30, and 60 min intervals (at 0 and 24 h for the exocellulase test) from individual microcentrifuge tubes. The whole experiment was run in duplicate. Specific enzyme activities were determined as micrograms of reducing sugar released per minute per milligram of protein. Crude protein in the culture and control extract was determined via a modified Lowry's method against standard bovine serum albumin (BSA) proteins.

Statistical Analyses. The statistical tool, SAS, was used for statistical validation of the results obtained from the various data sets. Mixed models (compound symmetry for within-bottle correlation) were fitted for reducing sugar and ethanol data during simultaneous saccharification and fermentation. A two-factor fixed effects model with interactions was fitted to the biomass weight-loss data and reducing sugar data profile for control and culture bottles during solid-substrate fermentation. Student's *t* test was performed on enzyme assay data sets for control and culture bottles.

RESULTS AND DISCUSSION

Biomass Weight Loss during Solid-Substrate Fermentation. In this study, the term *biomass* refers to both fungal biomass and residual corn fiber in solid-substrate fermentation. The difference between total dry biomass weight following sugar harvesting and the initial fiber weight (25 g) was regarded as biomass weight loss. In control bottles, the biomass weight loss remained fairly constant between 10 and 15% (**Figure 2**) throughout the experimental period (3 weeks), whereas the biomass weight loss during aerobic solid-substrate fermentation with *P. chrysosporium* increased from 11.5% at time zero to 34.1% in successive weeks; there was a significant difference between weekly control and culture biomass weight loss data

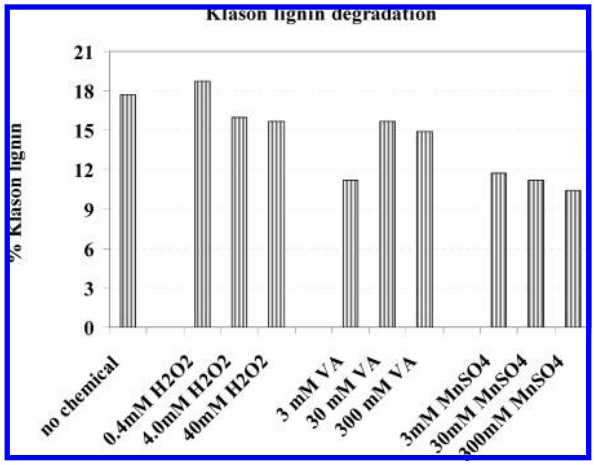

Figure 3. Effect of chemicals [hydrogen peroxide, veratryl alcohol (VA), and manganese sulfate] in lignin degradation for 2-week *P. chrysosporium* culture bottles ($n = 1$).

($p < 0.0001$). It also had statistical significance when interaction in bottles (fungal addition to fiber and sampling week) was considered ($p < 0.0001$). In addition, the biomass weight loss data were not statistically different within weekly control ($p = 0.734$) or culture ($p = 0.222$) samples as suggested by respective p values. One possible explanation for the control weight loss (10−15%) could be the release of high-temperature water-soluble fractions from the corn fiber during steam treatment after fermentation. All fungal water-soluble fractions after steam treatment contained significantly more carbohydrates, proteins, organic acids, and other soluble materials than the controls and, therefore, higher biomass weight loss in the case of culture bottles.

Effect of Chemicals on Lignin Degradation during Solid-Substrate Fermentation. There was a significant difference in Klason lignin percentages between the bottles with different chemicals (**Figure 3**). The maximum Klason lignin reductions were 41, 37, and 11%, respectively, for 300 mM $MnSO_4$, 3 mM veratryl alcohol, and 40 mM hydrogen peroxide.

P. chrysosporium produces both manganese peroxidase (MnP) and lignin peroxidase (LiP) enzymes that catalyze lignin degradation (*23, 24*). The addition of H_2O_2, veratryl alcohol, and $MnSO_4$ was found to enhance the activities of lignin-degrading enzymes (*20, 21*). These chemicals enhanced the enzymes needed to catalyze the ligninolytic activities. Veratryl alcohol is believed to protect the LiP from excess H_2O_2 in situ. Excess H_2O_2 oxidizes the LiP enzyme system II to LiP compound III. Ward et al. (*25*) reported that LiP compound III would not revert back to the native LiP. The exogenous H_2O_2 addition during fermentation could have oxidized the LiP II to LiP III. This would reduce the enzyme-catalyzed lignin degradation reaction. On the other hand, veratryl alcohol helps to bring LiP III back to its native state. Additionally, the easily diffusible manganese ion (Mn^{3+}) formed by oxidation of Mn^{2+} catalyzed the oxidative lignin degradation reaction. Manganese serves as substrate for the manganese peroxidase. The presence of manganese is important for the expression of extracellular manganese peroxidase (*26*). Thus, more Klason lignin reduction was observed with veratryl alcohol and $MnSO_4$ amendment than with H_2O_2. These results suggest lignin peroxidase production for corn fiber, even with its low (2%) lignin content. For this strain of *P. chrysosporium*, laccase activity was not observed previously by Khiyami et al. (*21*), so we decided not to test for it.

Sugar Release following Solid-Substrate Fermentation. Extracellular enzymes secreted by *P. chrysosporium* are capable of hydrolyzing the polysaccharides into oligo-, tri-, di-, and

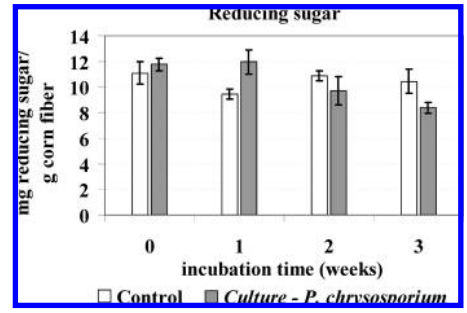

Figure 4. Reducing sugar harvested from solid-substrate fermentation of corn fiber using *P. chrysosporium* and controls ($n = 4$).

monosaccharides. In this study, the reducing sugar in the control bottles varied between 9 and 12 mg/g of initial fiber. In *P. chrysosporium* culture bottles, the reducing sugar decreased gradually from 13 to 9 mg/g of initial fiber during 3 weeks of aerobic solid-substrate fermentation (**Figure 4**). The overall weekly sugar data in control and culture samples were not statistically different ($p = 0.726$), but there were positive (week 1) and negative (weeks 2 and 3) differences in reducing sugar data between culture and control bottles in different weeks. This strongly suggests activity of fungus on corn fiber in the release and consumption of sugars during solid-substrate fermentation. The reducing sugar in culture bottles decreased on a weekly basis ($p = 0.007$). The reducing sugar in control bottles did not change from week 0 to 3 ($p = 0.381$).

Anaerobic conditions resulted in higher concentrations of reducing sugar accumulating in culture bottles. Simultaneous extracellular cellulase and hemicellulase activities and reduced sugar consumption by the fungi under anaerobic conditions was observed. The control bottles did not contain fungi and hence lacked an enzyme system. The reducing sugar yield increased by 140% in 7-day-culture bottles from 10 to 24 mg of reducing sugar/g of initial fiber under anaerobic conditions using 0.2 M acetate buffer (pH 4.7). The 9- and 11-day-culture bottles had less enzyme activity, and hence the release of extra reducing sugars was marginally low.

The accumulation of end-products (e.g., glucose and cellobiose) could suppress the cellulase activity (*27*). The hemicellulase activity of *P. chrysosporium* has been studied by Highley and Dashek (*11*). The hydrolysis of hemicellulose releases both hexoses and pentoses. The reducing sugar measurements depend on the availability of aldose or ketose reducing end, and mono-, di-, tri-, and short-chained carbohydrates have one reducing end each. Simultaneous saccharification and fermentation helps to overcome this inhibition by converting these end-products into ethanol as soon as they are produced (*28, 29*) and thus facilitating continuous cellulase activity. The fermentable portion of the reducing sugar can be determined by yeast fermentation of hydrolysate collected during sugar harvesting. Therefore, simultaneous saccharification and fermentation, with the addition of *S. cerevisiae*, followed the 1−3-day solid-substrate fermentation. *S. cerevisiae* primarily ferments hexoses to ethanol. Genetically modified organisms are available that can ferment both five- and six-carbon sugars from biomass-derived sugars (*3*).

Total sugar values were as high as 77 mg/g of initial fiber in 3 days of solid-substrate fermentation with *P. chrysosporium*. The increase in total and reducing sugars in culture bottles, compared to controls, confirmed cellulolytic activities of the fungus. The decreasing pattern, however, suggests that the released sugars were consumed by the fungi during the extended incubation. The difference in total and reducing sugar levels

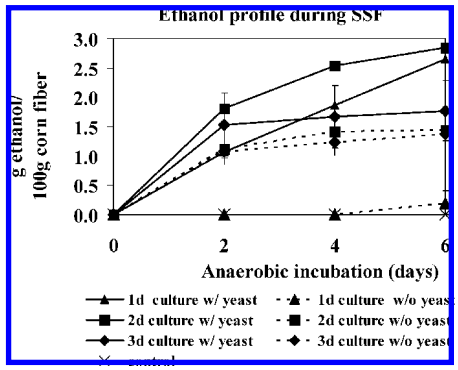

Figure 5. Anaerobic simultaneous saccharification and fermentation ethanol production in *P. chrysosporium* culture bottles incubated for 1, 2, or 3 days with (w/) and without (w/o) yeast *S. cerevisiae* inoculation ($n = 2$). Control is without fungal inoculations.

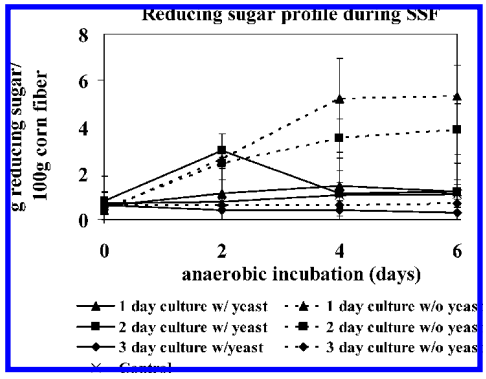

Figure 6. Residual reducing sugars present in culture broth from anaerobic simultaneous saccharification and fermentation for ethanol production in *P. chrysosporium* culture bottles incubated for 1, 2, or 3 days with (w/) and without (w/o) yeast *S. cerevisiae* inoculation ($n = 2$). Control is without fungal inoculations.

revealed that the available soluble sugars had not been completely hydrolyzed. Consistently, the sugar data were highly variable for the control samples.

Ethanol and Reducing Sugar Profile during Simultaneous Saccharification and Fermentation. Simultaneous saccharification and fermentation following the solid-substrate fermentation have the potential to enhance the cellulolytic enzyme activities, and this approach could also minimize the fungal consumption of hydrolyzed sugar. Fiber to ethanol conversion was close to 3 g of ethanol/100 g of fiber in 1 and 2 days of solid-substrate fermentation (in culture bottles) with yeast inoculation. By day 3 of solid-substrate fermentation, the hydrolyzed sugar was believed to be consumed by the fungus, thereby leaving reduced amounts of fermentable sugar. Without yeast culture addition, the fiber to ethanol conversion was 1.7 g of ethanol/100 g of corn fiber during anaerobic incubation following 2 days of solid-substrate fermentation with *P. chrysosporium* (**Figure 5**). Addition of yeast enhanced the net ethanol yield from corn fiber ($p = 0.002$). There was also a strong effect on ethanol levels due to yeast addition following different periods of solid-substrate fermentation along sampling days ($p < 0.0001$). The ethanol data on the second day of simultaneous saccharification and fermentation were not significantly different in bottles preceded by 1, 2, and 3 days of solid-substrate fermentation ($p = 0.141$) but the fourth ($p = 0.066$) and sixth ($p = 0.022$) day ethanol data showed significant difference between these bottles.

Estimation of the mean suggested that 2 days of solid-substrate fermentation of corn fiber was optimal ($p < 0.0001$) prior to simultaneous saccharification and fermentation of hydrolysate to ethanol. Conversion of sugar to ethanol would reduce the substrate inhibition and further increased the availability of fermentable sugars. The fungal enzyme activities had been enhanced in sequential sugar fermentation to ethanol by the yeast. Cell-free extracts were collected from 2-day solid-substrate fermentation bottles for enzyme assays to confirm activities over hemi/cellulose during fermentation.

The reducing sugar profile during simultaneous saccharification and fermentation also showed significant differences between the bottles (along with interactions for sampling days and preceded solid-substrate fermentation) with and without yeast addition ($p < 0.0001$). Without the control data, it was determined that yeast affects sugar reduction as sugar would be converted to ethanol (**Figure 6**). The exact nature of that difference depends on the sampling day and the time of yeast addition (after solid-substrate fermentation). It was also concluded that the reducing sugar data were significantly different ($p = 0.006$) between culture and control bottles following the addition of yeast after 2 days of solid-substrate fermentation. The exact magnitude of this difference also depends on the sampling day as interactions ($p < 0.0001$ were present in our two-factor model.

Enzyme Activity Assay. Table 1 shows the specific enzymatic activities of α-amylase, glucoamylase, xylanase, endocellulase, and exocellulase over starch, xylan, CMC, and Sigmacell 20 (cellulose). The cell-free extract from 2-day-old solid-substrate fermentation bottles, with *P. chrysosporium*, had higher enzyme activities compared to control. All of these specific enzyme activities were statistically significant according to *p* values obtained from the *t* test. Activities over starch, xylan, and CMC were comparatively higher (**Table 1**) compared to that over Sigmacell 20. This strongly suggested that the fermentable sugar for ethanol fermentation was contributed by the starch and hemi/cellulose fraction of the corn fiber. The lower exocellulase activity could have limited overall glucose

Table 1. Specific Enzyme Activities of Different Enzymes Expressed as Micrograms of Product per Minute per Milligram of Protein in 2-Day-Old Solid-Substrate Fermentation Extracts of *P. chrysosporium* and Control ($n = 2$)

specific enzyme activity[a]	substrate[b] (at 3 mg/mL)	*P. chrysosporium*	control	additional activity	*p* value
α-amylase (μg of maltose/mg of protein · min)	corn starch	2.39	0.98	1.42	0.0232
glucoamylase (μg of glucose/mg of protein · min)	corn starch	5.37	2.26	3.11	0.0096
xylanase (μg of xylose/mg of protein · min)	xylan	4.61	0.57	4.04	0.0013
endocellulase (μg of glucose/mg of protein · min)	CMC	3.42	2.26	1.16	0.0579
exocellulase (μg of glucose/mg of protein · min)	Sigmacell 20	0.23	0.08	0.15	0.0289

[a] The unit used for specific enzyme activity was μg of product/min/mg of protein. Crude protein concentrations are 0.93 and 0.75 mg/mL in *P. chrysosporium* and control (without fungus) extracts, respectively. [b] All chemicals were purchased from Sigma Chemical Inc. (St. Louis, MO). Specific enzyme activities were measured as rate of product formation (μg of product/min) per mg of protein from respective substrate when subjected to reaction over an hour (except Sigmacell 20 was 24 h) with culture (*P. chrysosporium*) or control cell-free extract at pH 5 and 37 °C.

availability from cellulose, and thus net fiber to ethanol conversion yield was limited to 3 g of ethanol/100 g of corn fiber.

Corn fiber from a wet-milling plant provided an excellent lignocellulose substrate for saccharification by *P. chrysosporium.* Increase in reducing sugar and total biomass weight loss (up to 35%) and reduction of lignin content (>40%) of the lignocellulose biomass apparently suggest that white-rot fungi, *P. chrysosporium*, were able to degrade the corn fiber during solid-substrate fermentation. Anaerobic incubation of the fermentation bottles enhanced the cellulolytic and/or hemicellulolytic enzyme activity and produced additional sugars. Such treatment can be an important intermediate step during cellulose simultaneous saccharification and fermentation of sugars to ethanol in industrial application. This study also envisaged the concept of simultaneous- saccharification and fermentation processes to enhance the enzymatic hydrolysis, reduce fungal consumption of sugar during saccharification of substrate, and facilitate improved ethanol fermentation. The ethanol yield level was as high as 3 g/100 g of fiber in aerobic solid-substrate fermentation using *P. chrysosporium* and anaerobic fermentation of hydrolysate using *S. cerevisiae*. However, from cellulose (18% of corn fiber) bioconversion alone we would expect 9 g of ethanol/100 g of corn fiber. The current yield, therefore, indicates incomplete bioconversion. Enzyme activity tests confirmed the majority of hexose came from starch, cellulose, and hemicellulose fractions. Solid-substrate fermentation for shorter periods (1−2 days) followed by simultaneous saccharification and fermentation is a promising approach for enhancing the enzyme activities and fiber conversion to ethanol. The research team is currently conducting experiments to optimize solid-substrate fermentation for economical ethanol production from corn fiber and other lignocellulosic biomass.

ABBREVIATIONS USED

ADM, Archer Daniels Midland; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; CSREES, Cooperative State Research, Education, and Extension Service; DDG, distiller's dried grain; IEC, Iowa Energy Center; LiP, lignin peroxidase; MnP, manganese peroxidase; NREL, National Renewable Energy Laboratory; PDB, potato dextrose broth; PP, polypropylene; RFA, Renewable Fuels Association; USDA, U.S. Department of Agriculture; USDOE, U.S. Department of Energy; VA, veratryl alcohol; YM, yeast mold.

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