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ORIGINAL RESEARCH

Exploring filamentous fungi depolymerization of corn stover in the context bioenergy queuing operations

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Abstract

Recalcitrance of lignocellulosic feedstocks to depolymerization is a significant barrier for bioenergy production approaches that require conversion of monomeric carbohydrates to renewable energy sources. This study assesses how low-cost modifications in the supply chain can be transformed into targeted pretreatments in the context of the entire bioenergy supply chain. This research aims to overcome the physiochemical barriers in corn stover that necessitate increased severity in downstream conversion in terms of chemical loading, temperature, and residence time. Corn stover samples were inoculated with a selective (*Ceriporiopsis subvermispora*) and nonselective (*Phanerochaete chrysosporium*) lignin-degrading filamentous fungal strains, then stored aerobically to determine the working envelope for fungal pretreatment to achieve lignin degradation. Dry matter loss and gross chemical makeup of corn stover varied by the length of treatment (2 and 4 weeks) and by the moisture content of the treated corn stover samples (40% and 60%, wet basis). Dry matter loss in *P. chrysosporium* inoculated biomass was elevated compared to *C. subvermispora* inoculated biomass; however, treatment also induced additional chemical composition changes suggestive of depolymerization. These results highlight that fungal treatment approaches must balance the loss of convertible material with the potential for reduction in recalcitrance. Techno-economic assessment (TEA) of fungal pretreatment in a short-term queuing system indicated the viability of this approach compared to conventional queuing operations. Total queuing system cost was estimated at $1.65/ton of biomass stored. After applying the credit of $1.48/ton from energy savings in the conversion phase using fungal pretreated biomass, the total system cost was $0.80 lower than traditional biomass queuing approach. While the TEA results suggested that treating biomass with *C. subvermispora* is the most economically viable storage method in the designed fungal-assisted queuing system, future research should focus on additional fungal depolymerization such as those observed in the *P. chrysosporium* inoculated biomass.

KEYWORDS
corn stover, filamentous fungi, queuing piles, supply chains, techno-economic assessment

1 | INTRODUCTION

Producing renewable liquid vehicle fuels from agricultural residues that would otherwise be discarded is a significant opportunity to reduce the carbon footprint of the transportation sector (U.S. Department of Energy & Bioenergy Technologies Office, 2016). Agricultural residues in the United States are presently available in quantities of >130 million dry tons and could be used for bioenergy (Langholtz et al., 2016). Whereas corn grain can be easily broken down mechanically and through enzymatic activity prior to fermentation to ethanol, the heterogeneity of lignocellulosic material is a challenge for bioenergy production due to the inherent molecular and structural complexity. The matrix of cellulose microfibrils, hemicellulose, and lignin provides strength to the plant cell walls such that plants can resist environmental challenges in the field (Cosgrove, 2005; Cosgrove & Jarvis, 2012). However, this intricately woven matrix results in a feedstock that is recalcitrant to physical and biological deconstruction to carbohydrate monomers that can readily be fermented to fuels (Himmel et al., 2008). To counter this recalcitrance, extensive focus has been devoted to exploring the mechanical, thermal, or chemical inputs required to deconstruct lignocellulosic material in the context of a biorefinery. Mechanical preprocessing through milling is used initially (Tumuluru et al., 2014), and advances in fractional milling result in a narrow particle size distribution tailored to biorefinery quality needs (Tumuluru & Yancey, 2018). Chemical treatments combined with heat further reduce recalcitrance by liberating hemicellulose in the case of acid pretreatment (Torget et al., 1991) or lignin in the case of alkali pretreatment (Katahira et al., 2016). Steam explosion (Saddler et al., 1993) and ammonia fiber explosion (AFEX) (Gollapalli et al., 2002) result in defibrillation and result in acetyl groups from hemicellulose. Enzymatic digestion with glycosidases typically follows these treatments to further hydrolyze carbohydrates into monomeric forms readily used by fermentative organisms (Bhutto et al., 2017).

Filamentous fungi are well-known degraders of lignocellulosic biomass, thriving in aerobic, moist environments. The enzymes excreted by filamentous fungi, including laccase, lignin and manganese peroxidase, and others, allow them to degrade lignin and ultimately make cellulose more accessible to enzymatic attack (Ander & Eriksson, 1977; Oliveira Rodrigues et al., 2020), and filamentous fungi have characterized for their role in bioenergy conversion, biopulping, and biobleaching to depolymerize lignocellulosic feedstocks (Chu et al., 2021; Gu et al., 2021; Millati et al., 2011; Singh & Singh, 2014). These enzymes can oxidatively depolymerize lignin building blocks of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units or cleave the acid bridges, such as ferulic acid or hydroxycinnamic acid, that link lignin to hemicellulose (Harris et al., 2008; Marriott et al., 2016). The pulp and paper industry has employed lignin degradation in a short-term "seasoning" step prior to processing, and this is accomplished primarily through the use of white-rot fungi that produce laccase and peroxidases that cleave lignin bonds and expose hemicellulose and cellulose (Gutiérrez et al., 2001). The associated lignin degradation accomplished by filamentous fungi during storage effectively pretreats the biomass leading higher yields in conversion of carbohydrate monomers to biofuels (Bjurman & Viitanen, 1996; Liu et al., 2014; Millati et al., 2011; Rouches et al., 2016).

The primary challenge with utilizing filamentous fungi for pretreatment is based on the selectivity of the strain for lignin versus cellulose breakdown. Specific and nonspecific lignin-degrading fungi have been classified based on their enzymatic response to degrading the complex lignocellulosic biomass structure. Nonspecific lignin degraders must utilize cellulose as a carbon source and therefore possess cellulytic enzymes (Su et al., 2018) and can result in significant total loss of dry matter during lignin degradation. Specific lignin-degrading fungi generally leave cellulose intact but have slower reaction times, which can increase the cost of the treatment if fungal pretreatment is the primary or only pretreatment approach. Many studies simply focus on enzymatic hydrolysis after storage treatments, and sugar yields of <50% are commonly reported (Bjurman & Viitanen, 1996; Liu et al., 2014; Millati et al., 2011; Rouches et al., 2016; Shirkavand et al., 2016).

Long- and short-term storage of lignocellulosic material is required to provide a year round supply of seasonally available biomass sources, and short-term storage at a biorefinery gate allows for a readily available supply of feedstock to feed a reactor (Wendt & Zhao, 2020). Innovative approaches to reduce biomass recalcitrance during storage have the potential to reduce the energy required for bioconversion and improve the sustainability of bioenergy systems. This study aims to investigate filamentous fungi-assisted approaches to reduce recalcitrance. Success of this approach has the potential to transform short-term storage in a queuing operation into

a preprocessing step that adds value to the material by reducing the energy required to convert lignocellulosic biomass into fuel, in a step that currently is a net cost to the operation.

To date, a cost competitive filamentous fungi-assisted supply chain resulting in complete hydrolysis of cellulose and hemicellulose to sugar monomers has not been developed, which is an impediment to converting the sugars to fuels and/or chemical precursors. Techno-economic analysis of utilizing filamentous fungi pretreatment systems alone suggests that the slow reaction times of the approach renders them more costly compared to chemical-based pretreatment systems (Baral & Shah, 2017; Vasco-Correa & Shah, 2019). Vasco-Correa and Shah modeled that a 5-day residence time fungal pretreatment occurring at a biorefinery in a packed bed, aerated bioreactor was at least fourfold more costly than conventional pretreatment systems due to the high capital cost of the bioreactor (Vasco-Correa & Shah, 2019). Baral and Shah modeled fungal pretreatment was three times more costly than steam explosion, sulfuric acid, and ammonia fiber counterparts due to the long residence time of 23 days increasing capital costs and corresponding high losses of carbohydrates requiring twice as much feedstock for fungal pretreatment approach (Baral & Shah, 2017). Similarly, sterilization of biomass during pretreatment operations can be required to remove competing microorganisms during fermentation. However, the literature is not universal in the conclusion that fungal pretreatment is more costly, and numerous studies consider how higher biofuel yields (Gui et al., 2014; Salvachúa et al., 2011) and reduction of inhibitors (Ishola & Taherzadeh, 2014; Kuhar et al., 2008) can increase economic viability of the approach beyond just increased enzymatic hydrolysis yields.

This study investigates a fungal pretreatment approach to initial recalcitrance reduction in corn stover. Success of this approach has the potential to transform short-term storage at a preprocessing gate, a step that currently is a net cost to the operation, to a value-added operation to the material by reducing the energy required to convert lignocellulosic biomass into fuel. The goal of this study was to explore operating window of fungal treatment on corn stover, specifically balancing dry matter loss with observed compositional changes with the goal of evaluating the impacts of these structural changes on downstream deconstruction. Strain selectivity for lignin degradation, time, and moisture content was screened to understand the ranges of viable conditions promoting depolymerization in sterilized corn stover. A techno-economic analysis was then used to understand how fungal treatment might complement existing queuing operations and an acid-based pretreatment approach to hemicellulose monomerization.

2 | METHODS AND MATERIALS

2.1 | Filamentous fungi cultivation

Experiments were performed using *Phanerochaete chrysosporium* (NRRL 6370), a nonselective lignin degrader, and *Ceriporiopsis subvermispora* (ATCC 96608), a selective lignin degrader, using methodology described in Saha et al., (2016) The cultures were first grown on Yeast Mold Agar (BD Difco™, Franklin Lakes, NJ). Fungal cultures were grown on Yeast Mold Broth (BD Difco™, Franklin Lakes, NJ) with shaking at 50 rpm at 28°C. *C. subvermispora* was grown for 6 days until it reached a density of 2.3 mg dry weight/ml. *P. chrysosporium* fungus was grown for 7 days where it reached a density of 8.4 mg dry weight/ml. The cultures were pelleted by centrifugation at 7300 g, resuspended with 25 ml of a 10-mM phosphate buffer, and homogenized in a Waring blender. The cultures were pelleted once more, resuspended in 65 ml of sterile tap water. Dry fungal weight was calculated based on OD_{600} measurements and correlated to the fungal slurry weight after drying overnight at 105°C.

2.2 | Corn stover source and storage procedure for screening experiment

Two-pass harvested corn stover was sourced from Boone, IA, in 2015 and is available as reference material through Biomass Feedstock National User Facility Library at Idaho National Laboratory (https://bfnuf.inl.gov). Corn stover was size reduced to pass through a 6-mm screen with a Wiley Mill Model 4 (Thomas Scientific, Swedesboro, NJ). 20 g (dry weight equivalent, dw) samples of corn stover were added to 500 ml flasks at 40% and 60% moisture (wet basis, wb) autoclaved at 121°C for 60 min to sterilize the corn stover, and flasks were allowed to cool to room temperature. The corn stover was inoculated with the fungal culture by pipetting 5 ml of either a 1.78 mg/ml loading of *C. subvermispora* or a 2.39 mg/ml loading of *P. chrysosporium*, or sterilized water to the flask. Similar mass-based approaches to loading are reported elsewhere (Saha et al., 2016). Flasks were gently rotated by hand to ensure sufficient distribution of the fungal culture and water. Loose-fitting lids were placed over flasks to allow for oxygen infiltration while preventing moisture loss to the atmosphere. Six reactors were prepared for each condition, and the flasks were stored at room temperature in the dark for 2 and 4 weeks.

Initial moisture content of the corn stover was assessed using a sample collected at three intervals during flask loading. Moisture content was determined by the weight of moisture lost after drying overnight at 105°C according to Equation 1:

$$\%\ Moisture\,(wb) = \frac{g\,Biomass_{wet} - g\,Biomass_{dry}}{g\,Biomass_{wet}} \times 100 \quad (1)$$

After 2 or 4 weeks of storage, three flasks for each treatment were emptied into a plastic bag, homogenized by manual mixing, and sampled for moisture content ($n = 2$). All moisture contents are reported on a wet basis. Dry matter loss was calculated according to Equation 2 using the dry weight basis of biomass:

$$\%\ \text{Dry Matter Loss} = \frac{\text{g Biomass}_{\text{pre storage}} - \text{g Biomass}_{\text{post storage}}}{\text{g Biomass}_{\text{pre storage}}} \times 100 \quad (2)$$

After drying to stability at 40°C, all replicates for a condition (treatment, moisture content, and time) were combined into a single composite sample for analysis and size reduced to pass through a 2-mm screen using a Thomas Model 4 Wiley® Mill (Thomas Scientific, Swedesboro, NJ).

2.3 | Scanning electron microscopy (SEM)

Dried corn stover pieces representing stalk were selected and prepared for SEM imaging using a protocol adapted for confocal imaging (Gierlinger et al., 2012). Polyethylene glycol (PEG) 2000 chips (Sigma Aldrich, St. Louis, MO) were heated at 60°C until completely melted. Samples were soaked in water to rehydrate at 60°C and then submerged in a 50% polyethylene glycol (PEG) 2000 solution in a closed container at 60°C until they were permeated with the solution. Once samples sank to the bottom of the container, the lid was removed to allow for water evaporation. After approximately half of the volume of the solution was evaporated, the samples were submerged in 100% PEG. A pan of water was placed at 60°C with the samples in 100% PEG to prevent the PEG from becoming dry and crumbly upon hardening. The samples were removed from the PEG, placed on a microscope slide, and allowed to harden to the slide at room temperature overnight. Samples were sectioned with a scalpel and soaked in water to remove residual PEG, and then, they were dried to a microscope slide at room temperature overnight. The sections were removed from the microscope slides with a scalpel, mounted to SEM mounts with double-sided copper tape, and sputter coated with gold. Sections were imaged using a JEOL JSM-6610LV (Peabody, MA) scanning electron microscope.

2.4 | Compositional analysis

Chemical compositional analysis on all samples was analyzed using duplicate samples according to standard Laboratory Analytical Procedures (Sluiter et al., 2010; Sluiter & Sluiter, 2011). Briefly, corn stover was extracted at 100°C using water and ethanol with an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA) (Sluiter et al., 2008). Remaining biomass was subject to a two-stage acid hydrolysis to solubilize structural carbohydrates (Sluiter et al., 2008). Liquors were analyzed for monomeric carbohydrates using high-performance liquid chromatography and a refractive index detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 × 7.8 mm, Hercules, CA) (Sluiter et al., 2008). Acid-soluble lignin was analyzed using a Varian Cary 50 ultraviolet-visible spectrophotometer (Agilent, Santa Clara, CA). Acid insoluble lignin and structural ash were determined gravimetrically on the remaining solids. Protein content was calculated as a function of total nitrogen, and total was determined gravimetrically (Sluiter et al., 2008).

2.5 | Techno-economic assumptions

A queuing system was designed based on a stacking and reclaiming queuing approach commonly used at pulp and paper mills in the United States with on-site storage in piles used to maintain consistent supply (McDonald & Twaddle, 2000). All unit operations from a traditional two-pass corn stover harvest and collection approach were preserved including on-farm storage, transportation, preprocessing at a depot, and delivery to a biorefinery (Roni et al., 2020). Queuing operations for baled biomass at a depot colocated with a biorefinery are reported at \$0.97/ton biomass (\$0.88/US ton biomass) (Roni et al., 2020). Capital and annual operating costs were calculated for a Bruks COSR stacker reclaiming system using the Biomass Logistics Model (BLM) framework developed at Idaho National Laboratory (Roni et al., 2019). Model parameters were shown in Table 1. Equipment costs were calculated using American Society of Agricultural and Biological Engineers (ASABE) standard calculations and represented in terms of \$/dry ton biomass and similar to reported elsewhere (Wendt et al., 2021). Installed price was obtained by vendor quotes. Annual discounted salvage value was calculated according to Equation 3:

$$Discounted\ salvage\ value = \frac{Purchase\ price \times Salvage\ rate}{(1 + Interest\ rate)^{lifetime(yr)}} \quad (3)$$

TABLE 1 Capital and annual operating costs of a stacking reclaiming queuing pile

	Bruks COSR stacker and reclaimer	Inoculant delivery pump
Fuel type	Electricity	Electricity
Installed purchase price	$5,000,000.00	$3080.00
Salvage rate	0.1	0
Machine life (years)	35	15
Interest rate	8%	8%
Insurance and tax	2%	2%
Maintenance (% of annual value)	10%	10%
Energy usage (kWh)	1475	1
Energy unit cost ($/kWh)	$0.07	$0.07
Labor rate ($/h)	$33.00	$33.00
Operators required	1	0
Discounted salvage value	$33,817.27	$0.00
Capital recovery	$63.73	$0.00
Insurance and tax cost	$11.90	$0.01
Maintenance and repair	$6.37	$0.00
Energy cost	$95.88	$0.07
Labor cost	$33.00	$0.00
Throughput (wet tons/hr)	458.41	229.21
Moisture Content In (%)	60%	60%
Moisture Content Out (%)	60%	60%
Throughput (dry tons/hr)	183.37	91.68
Hourly cost	$210.88	$0.07
Units	1	4
Inoculum cost ($/ton biomass)		$0.50
Unit operation cost ($/ton biomass)	$1.15	$0.503
Total cost ($/ton biomass)	$1.65	

Note: Processing parameters associated with a 2000 metric ton/day biorefinery. Costs listed on a 2016 basis in U.S. dollars.

Capital recovery was calculated according to Equation 4:

$$\text{Capital recovery} = \frac{\left[(\text{Purchase price} - \text{Discounted salvage value}) * (\text{Interest rate}/(1-(1+\text{Interest rate})^{-\text{lifetime(yr)}} + (\text{Discounted salvage value} * \text{Interest rate})\right]}{\text{Operating hrs per year}} \quad (4)$$

Ownership costs included interest and depreciation, insurance, and taxes.

The commercially available stacker reclaimer chosen can build up to eight separate piles, which allows for the appropriate residence time, and then, it reclaims biomass and delivers it to a conveyor that feeds the biorefinery. The biorefinery was assumed to operate 350 days a year and 24 h a day, which can process 2000 dry tons of biomass daily. Moisture content of 60% was assumed and a storage pile density of 240 wet kg/m^3 (14.98 wet lb/ft^3). Multiple dry matter loss levels were considered based on preprocessing costs at depot of a baseline of $26.25/ton biomass (Roni et al., 2020). Fungal inoculant was assumed to be applied during stacking using a pump for storage piles described previously (Wendt et al., 2018) and assigned a cost of $0.50/ton biomass based on previous estimates (Scott et al., 1998). Residence times of 7 and 14 days were modeled, such that the stacker reclaimer system was able to handle the pile volume with associated, modeled residence time. All reported costs are presented in 2016 dollars.

The total system cost of $1.65/dry ton was calculated based on hourly throughput of storing 183.37 dry tons of material. Due to the dry matter loss during the storage

process, preserved biomass weight will be less than 183.37 dry tons per hour, which caused increases in the total per dry ton storage cost. This is also true for all the operations in the depot before the storage process such as biomass handling and comminution. In order to reflect the cost of dry matter loss, dry matter loss cost was estimated using equation below (Oliveira Rodrigues et al., 2020):

$$\text{Cost}_{\text{DML}} = \text{DML} * (\text{Total Cost})/(1 - \text{DML}) \quad (5)$$

where Cost_{DML} is the cost of dry matter loss, DML is the dry matter loss measure in %, Total Cost is the total cost to process and store the biomass at the colocated depot in $/dry ton.

Cost saving for reduced temperature requirements was calculated based on the design of the dilute acid pretreatment operation as reported in 2015 by Davis et al., (2015). This is a biochemical approach to creating fuels from carbohydrate streams originating from lignocellulosic biomass, and a 160°C dilute acid pretreatment is used to liberate hemicellulose from biomass. The design utilizes high pressure steam (Stream 220; flow rate = 23,888 kg/h) and hot process water (Stream 250; Flow rate = 304,369 kg/h) to maintain the temperature. Dry biomass is fed to the reactor at a rate of 2000 tons/day (83,333 kg/hr). Pretreatment data (data not shown) from the present study assessed the impact on sugar yield from by reducing the temperature of reaction from 160°C to 130°C. Previous studies have shown that pretreatment at this temperature allows for differences in feedstock reactivity to be more readily discerned than at higher temperatures, such as 150–160°C (Wolfrum et al., 2013). While Wolfrum et al. explored the varying reactivity of different feedstocks in pretreatment, in this study, it was reasoned that the lower pretreatment temperature would offer a similar advantage to the present study and enable that decreased recalcitrance to be more readily identified. Therefore, the following equation was used to account for energy savings realized by reducing the temperature of process water from 160°C to 130°C, where Cp is specific heat, q is change in energy, m is mass, and Δ T is temperature change:

$$Cp = \frac{q}{(m\,x\,\Delta\,T)} \quad (6)$$

The Cp of water at 130°C of 4.26 kJ/(kg) and a Δ T = 30°C were used to calculate the energy savings per kg of process water, and the flow rate of process water was used to estimate total savings per hour. Total cost per ton savings were calculated based on the biomass flow rate of 83,333 kg/h and a natural gas cost of $3.36/MMBTU, consistent with the 2019 Herbaceous Feedstock State of Technology case developed at Idaho National Laboratory (Roni et al., 2020).

3 | RESULTS AND DISCUSSION

This study aimed to assess the impact of fungal pretreatment in corn stover in terms of compositional, structural, and convertibility changes to gain a fundamental understanding of this potential treatment to reduce biomass recalcitrance in the context of a biorefinery. Dry matter losses and compositional changes were used as a guide to understand the working envelope of a selective (*C. subvermispora*) and nonselective (*P. chrysosporium*) lignin degrader.

3.1 | Storage induced losses due a selective and nonselective lignin degrader

Loss of dry matter is an important consideration for a biorefinery for economic reasons and sustainability concerns surrounding carbon retention, and it is also an indicator of microbial activity and quality changes as a function of moisture. Screening studies were performed to understand the range of viable conditions (moisture content, strain, and duration) for fungal growth on sterilized corn stover. Dry matter loss was assessed in triplicate reactors (Table 2). In the corn stover stored without an inoculant, no measurable loss occurred after 2 weeks at 40% moisture, but in the experiment conducted using 60% moisture samples resulted in over 2% loss. Results were similar after 4 weeks. These low values are likely due to sterilization of the corn stover prior to the beginning of the experiment, and Smith et al. showed that >30% dry matter loss can occur in corn stover stored aerobically at similar moisture contents (Smith et al., 2020). The *C. subvermispora* treatment resulted in a similar loss profile to the moisture control after 4 weeks of storage at 40% moisture. These similarities between the control and *C. subvermispora* treatment indicate no statistical difference as a function of dry matter loss.

TABLE 2 Dry matter loss fungal-treated corn stover after storage for 2 or 4 weeks

	Dry matter loss (%, dry basis)	
Experiment	**2 weeks**	**4 weeks**
Control, 40% moisture	NA	0.1 ± 0.2
Control, 60% moisture	2.7 ± 2.4	1.4 ± 0.1
C. subvermispora, 40% moisture	0.5 ± 0.3	1.2 ± 0.4
C. subvermispora, 60% moisture	3.3 ± 1.6	2.6 ± 1.7
P. chrysosporium, 40% moisture	4.6 ± 0.7	11.9 ± 0.6
P. chrysosporium, 60% moisture	10.7 ± 1.0	21.0 ± 0.1

Note: NA: Dry matter loss for this treatment was negligible.

Phanerochaete chrysosporium resulted in a significantly different dry matter loss profile. This is expected given that this strain is a nonselective lignin degrader and depolymerizes cellulose while also releasing the enzymes necessary to oxidize lignin. Losses at 40% moisture were 4.5% of total dry matter after 2 weeks and 12% after 4 weeks. In the 60% moisture corn stover, the losses were accelerated, with 11% and 21% total dry matter loss after 2 and 4 weeks, respectively. Visible fungal mycelia were present in the *P. chrysosporium* inoculated corn stover after just 2 weeks in storage while changes in the moisture control and *C. subvermispora* treatment were less evident. Figure S1, S2, and S3 in the Supplemental Information illustrate this vast change in fungal growth. *P. chrysosporium* inoculated corn stover stored at moisture contents of 40% and 60% also show visible differences with the naked eye, with more hyphae present.

Cross sections of the parenchyma cells and vascular bundles in the pith fraction of the stalk were visually assessed for structural impacts using SEM. Figure 1 shows SEM micrographs of corn stover before and after 2 weeks of *P. chrysosporium* treatment at 40% and 60% moisture. The structural variability masks many small changes with few visually discernable changes at the 100× magnification levels. However, a 500× magnification of the stover stored at 60% moisture reveals fungal hyphae attached to the secondary cell wall within the cell lumen. Fungal hyphae have also been reported to penetrate pine cell lumens (Blanchette et al., 1997). This result suggests that fungal hyphae may have a greater cellular impact at the elevated moisture content. Similarly, Asgher et al. varied moisture content of corn cobs inoculated with *P. chrysosporium* and found an optimal moisture content for laccase activity occurred between 60% and 80% moisture, with approximately half the activity occurring at 40% moisture. SEM micrographs also show slight physical changes visually, where the 40% and 60% moisture samples appear to have increased cell wall tearing and breakage at 100× magnification levels. Overall, the changes observed in SEM micrographs can aid in interpreting other metrics of degradation, as discussed in the following section.

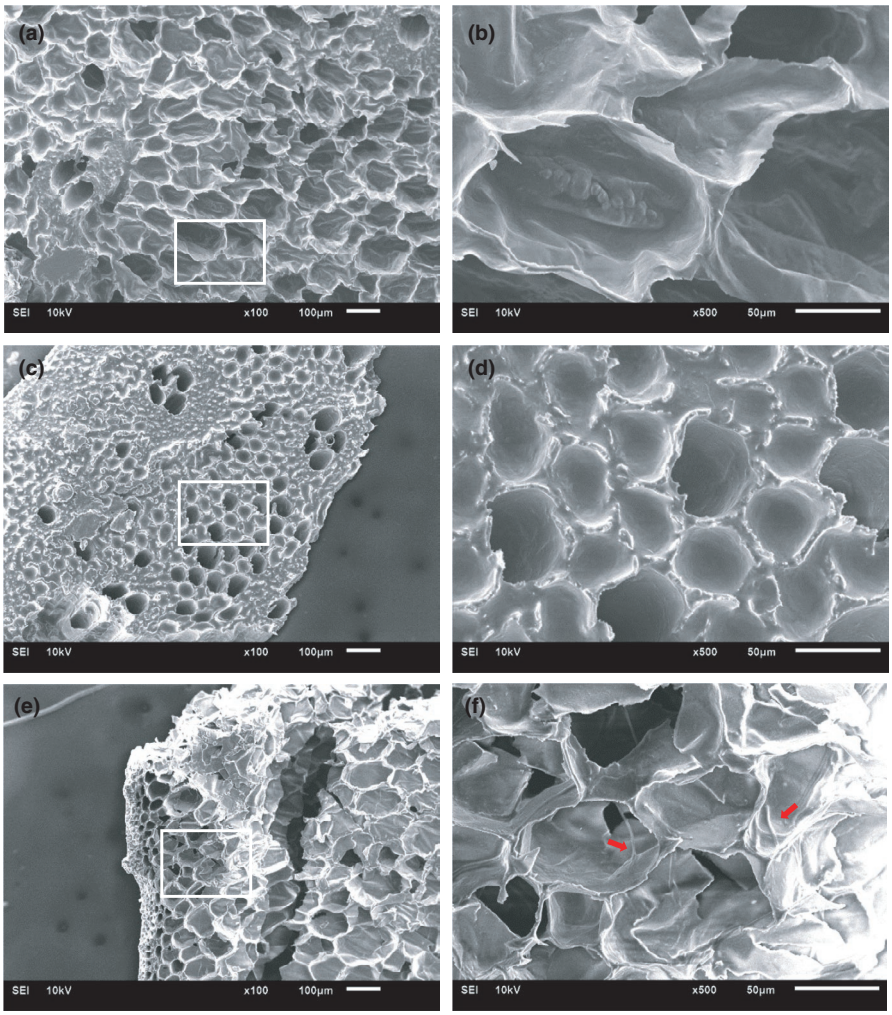

FIGURE 1 SEM micrographs of corn stover (a), (b): unstored; (c), (d): stored 2 weeks with *P. chrysosporium* at 40% moisture; (e), (f): stored 2 weeks with *P. chrysosporium* at 60% moisture. Red arrows indicate fungal hyphae attached to cell lumen

3.2 | Compositional changes provide insight into fungal mechanism

Corn stover was assessed for changes in chemical composition as a result of storage, time, moisture content, and fungal strain characterized by selective and nonselective lignin degradation. All treatments were assessed for their ability to change the soluble and structural composition of the biomass (e.g., soluble and structural carbohydrates, acetate, lignin, and extractives). The objective was to evaluate structural components cleaved during degradation with the aim of assessing the abundance and relative proportions of these components to provide insight into mechanisms of potential recalcitrance reduction.

The unstored corn stover served as a basis for comparison, and the control that only had moisture added was used to understand the impact of moisture content and residence time as a function of compositional changes. Compositional changes in the primary components of glucan, xylan, lignin, and water extractives are shown in Figure 2. Results represent the chemical composition corrected for dry matter lost during storage. Compositional analysis of the final stored mater, uncorrected for dry matter loss, is reported in the Supplemental Information, Tables S1 and S2, with the dry matter loss-weighted composition presented in Tables S3 and S4.

Few compositional changes were observed in the moisture control, likely because the corn stover was sterilized prior to storage such that the native microflora had been eliminated. However, structural galactan and arabinan decreased by a relative 11%–15% decrease after 4 weeks of storage at both moisture contents. Galactan decreased from 1.14% to 1.02% and 0.97%, respectively, at 40% and 60% moisture conditions; arabinan decreased from 2.55% to 2.26% and 2.13%, respectively, at 40% and 60% moisture conditions. A corresponding slight increase in soluble arabinan occurred from 0.19% in the unstored to 0.36% and 0.26% after 4 weeks in 40% and 60% moisture conditions, respectively. While these changes are small, they are greater than error seen in the analytical control included in all compositional analyses. However, changes in galactan were not observed, suggesting those carbohydrates were consumed by microbial activity. Protein content also decreased at a similar rate over 4 weeks to structural galactan and arabinan, beginning at 3.17% and existing storage at 2.71% and 2.74% at 40% and 60% moisture, respectively; this trend supports the hypothesis that minor microbial activity was present and consuming protein to support cell growth. Lastly, slight amounts of xylan solubilization were observed at 40% and 60% moisture over 4 weeks. The corn stover entered storage with 23.58% xylan and decreased to 23.38% and 22.94% at 40% and 60% moisture, respectively. A corresponding increase in soluble xylan was observed from 0.35% to 0.49% and 0.51% at 40% and 60% moisture, respectively. These results indicate that, despite sterilization, there was minor microbial activity that began to depolymerize hemicellulose components over 4 weeks of storage, an important consideration when comparing to more severe fungal treatments.

The selective lignin degrader, *C. subvermispora*, incurred only minor compositional changes between 2 and 4 weeks, corresponding to the low dry matter loss levels observed in this treatment. This fungal strain exhibited enhanced xylan solubilization compared to the uninoculated moisture control. The most notable structural xylan decrease was exhibited at 60% moisture, entering storage at 23.58% and exiting at 21.63% and 21.55% after 2 and 4 weeks, respectively. Hemicellulases have been documented as one of the many enzymes produced by *C. subvermispora* (Sethuraman et al., 1998). However, the arabinan solubilization exhibited in the uninoculated control was not observed, and structural arabinan was enriched due to the loss of other components. Glucan content was within the same relative percentage change as the uninoculated moisture control, consistent with other reports that *C. subvermispora* targets lignin not cellulose. However, protein content decreased throughout the storage period compared to the controls, entering storage at 3.17% and exiting at 1.82% and 1.64% after 4 weeks of storage at 40% and 60% moisture, respectively. These findings suggest that *C. subvermispora* struggled with growth but was degrading the corn stover minimally to access the protein and hemicellulose, and slight oxidation of the more soluble lignin fraction occurred. Similar results have been reported elsewhere (Fernandez-Fueyo et al., 2012).

Slight lignin solubilization was exhibited as a function of *C. subvermispora* inoculation in comparison to the native and uninoculated, stored corn stover controls. The amount of lignin that was solubilized during acid hydrolysis tended to decrease in comparison to the acid insoluble, which tended to increase due to loss of other components. This is consistent with the known laccase and peroxidase activity that is expressed in *C. subvermispora* to support depolymerization of phenolic lignin (citation in comment above). Total lignin concentration remained constant, suggesting it degraded at a rate consistent with dry matter loss. Losses of 7.7% and 13.1% have been observed in 65% moisture content corn stover inoculated with *C. subvermispora*, with losses of greater than 50% lignin by 15 days with only 3.5% and 14.7% losses in cellulose and hemicellulose, respectively (Huang et al., 2019). However, the chemical composition of this corn stover was more concentrated in hemicellulose (33.0% vs. 27.3% in this study) and reduced in lignin (10.6% vs. 17.7% in this study), which is suggestive of a younger, less lignified corn plant that may be inherently more susceptible to fungal attack.

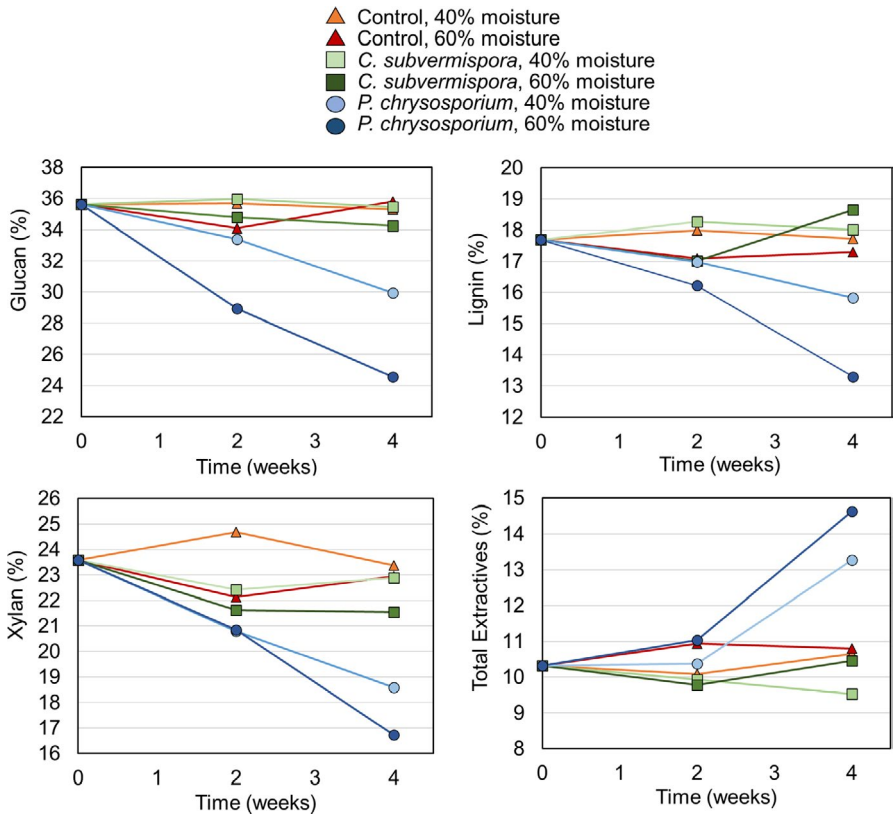

FIGURE 2 Compositional changes adjusted for dry matter loss as a function of time in screening study in corn stover inoculated with *C. subvermispora* and *P. chrysosporium*

Less mature corn plants also have a higher concentration in free sugars (Pordesimo et al., 2005), which provide a nutrient source to encourage all microbial growth including fungal. Further studies are warranted to investigate the initial growth requirements of *C. subvermispora* on corn stover as a function of incoming attributes including free sugars, standing age, and resulting structural features including lignin and carbohydrate distributions.

Corn stover inoculated with the nonselective lignin degrader, *P. chrysosporium*, exhibited more marked changes than either the moisture controls or the *C. subvermispora* treatments. Total extractives in the *P. chrysosporium*-treated stover were elevated to 10.37% and 11.04% after 2 weeks of storage at 40% and 60% moisture, respectively; this was increased to 13.28% and 14.64% after 4 weeks of storage. The greatest relative component changes, up to 300%, observed in the *P. chrysosporium* treatments were in soluble xylan and arabinan, which increased at similar rates suggesting arabinoxylan degradation. The primary hemicellulose in corn stover is arabinoxylan, a β,1-4 xylan backbone with arabinan substitutions along the chain. Likewise, structural xylan decreases from 23.58% to 16.72% in the 4-week, 60% moisture corn stover suggests cleavage of glyosidic bonds occurred in hemicellulose. However, this loss in protective hemicellulose likely exposed the cellulose and made it more susceptible to attack by *P. chrysosporium*. Cellulose degradation was evident by structural glucan decreases from 35.61% to 33.40% and 29.94% at 40% moisture and 28.94% and 24.54% at 60% moisture. Soluble glucose was unchanged, further suggesting consumption by the fungi to support respiration and lignin degradation. Lignin content in the 40% moisture *P. chrysosporium* treatment began at 17.69% but was reduced to 16.98% and 15.83% after 2 and 4 weeks of storage, respectively, but the highest decrease in lignin was evident at 60% moisture with reduction to 15.83% and 13.29% after 2 and 4 weeks of storage. *P. chrysosporium* inoculation also resulted in a higher relative decrease in the acid soluble lignin and ethanol extractives than what occurred during *C. subvermispora* treatment. These results are consistent with the reaction mechanisms of *P. chrysosporium* when actively degrading corn stover and the combination of cellulases, hemicellulases, and lignin-degrading enzymes expressed. Adav et al., (2012) characterized over 60 cellulases expressed in the fungi when corn stover was used as a growth substrate, with cellulase as a dominant contributor. This is consistent with the elevated glucan degradation that occurred during the first 2 weeks of storage compared to xylan and lignin degradation. Thirty-two actively produced hemicellulases were also characterized by Adav et al., and these play a critical role in exposing the cellulose for attack. They also found upregulation of cellobiose dehydrogenase in *P. chrysosporium*, which oxidizes cellobiose to produce the hydrogen peroxide that can further degrade lignin through the Fenton reactions that form highly reactive hydroxyl radicals.

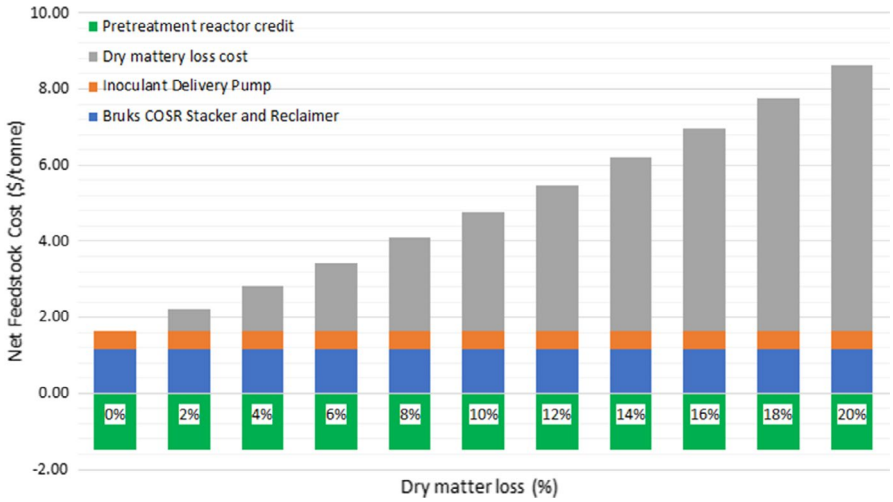

FIGURE 3 Net cost of the fungal-mediated queuing costs and corresponding cost offsets as a function of dry matter loss

In summary, the screening study results suggest that the combination of cellulases, hemicellulases, and lignin-degrading enzymes were in action in *P. chrysosporium* treatment of corn stover. Similar trends in dry matter loss and associated compositional changes have been observed in *P. chrysosporium* after 15 and 30 days of storage at 75% moisture, with high cellulase activity occurring compared to other strains assessed (Ding et al., 2019). *C. subvermispora*-related impacts were minor, but hemicellulase activity indicated the fungi had begun to depolymerize the corn stover. One key finding from the screening study indicates the importance of moisture content to support fungal depolymerization, with 60% moisture conditions exhibiting enhanced solubilization in this study. A higher moisture content is also aligned with outdoor storage of fungi in creating an additional barrier to uncontrolled combustion.

Cellulose and hemicellulose preservation is also a metric of importance when considering adopting a fungal pretreatment into a biorefinery model. Total structural carbohydrate levels of 59% or greater are required in biochemical conversion designs (Davis et al., 2018), and all treatments except the *P. chrysosporium* 4-week samples met that specification. Failing to meet the 59% carbohydrate target will either result in lower biofuel conversion yield or higher feedstock cost because more biomass will be needed to maintain the same conversion yield. The enhanced solubilization of hemicellulose components suggest that *P. chrysosporium* treatment may be complimentary to biorefinery-based treatments that utilize acid pretreatments to depolymerize hemicellulose and isolate cellulose that is then carried into fermentation. While corn stover was the feedstock chosen to study in this work, the results would also be applicable to other lignocellulosic material including switchgrass, sorghum, or woody biomass. Additionally, the dry matter loss that was experienced in this study will vary based on the natural recalcitrance of each biomass type, and this is due to both chemical factors (degree of polymerization, carbohydrate composition, lignin subunit ratio, etc.) and physical factors (surface area, surface volume, etc.) (Zoghlami & Paës, 2019).

3.3 | Techno-economic implications of a combined fungal and chemical pretreatment

Typical biomass pretreatments necessitate a combination of high temperature, pressure, and acidic or alkaline conditions to ensure cellulose accessibility for downstream enzymatic conversion to monomers. Conversion systems require yields ideally near 100%; consequently, high severity pretreatment conditions are typically required. This can result in over-pretreatment of less recalcitrant biomass tissues, such as leaves, resulting in the formation of byproducts well known to inhibit microbial fermentation. Therefore, a combination of fungal pretreatment, nonbiological pretreatment, enzymatic hydrolysis, and conversion of monomers to fuels is likely necessary for a fully integrated biomass conversion process.

The queuing operation allows for short-term storage at the biorefinery gate and mitigates the risk of lack of the hourly feedstock being provided to the biorefinery. Baseline queuing residence times are 3–5 days of feedstock supply, and yet longer residence times are possible. In this study, the modeled residence time was 1–2 weeks. A logistics system was designed to utilize short-term queuing at the depot to perform fungal treatment in storage piles, similar to what has been designed for wood chip piles (Scott et al., 1998). Capital and operating costs for this system were estimated at $1.65/ton biomass, a 1.7-fold increase over the traditional queuing pile costs of $0.97/ton. Experimental results reported elsewhere have indicated that a 30°C

temperature reduction was possible in pretreatment to achieve equivalent xylose yields, which is the primary goal of dilute acid pretreatment (unpublished results). This correlated to an energy savings of 0.44 MMBTU/ton biomass, and thus a cost savings of $1.48/ton was applied to the fungal-assisted queuing system due to reduced natural gas consumption required heating for pretreatment at the biorefinery. After considering the cost savings from using pretreated biomass, the system cost for the fungal-assisted queuing system was about $0.17/dry ton, which was $0.80 lower than the traditional queuing pile cost.

As discussed in the Methods section, dry matter loss will impact total system cost in a negative way. Dry matter loss cost increased proportionally with dry matter loss (Figure 3), and therefore, future research should target opportunities to preserve dry matter loss while maintaining recalcitrance reduction opportunities. Interestingly, overall dry matter loss is not a primary metric reported in literature describing fungal pretreatment for lignocellulose, and this study highlights the importance of the metric for overall cost-effectiveness. This result implied that future research should target reduced dry matter loss levels in queuing or increased recalcitrance reduction, which could be possible with alternative approaches. For example, nonselective lignin degraders combined with ferulic acid esterase active at low moisture contents could further liberate lignin and hemicellulose bonds. Additional fungal strains with unique enzyme complexes could also be explored, and Shirkavand et al., (2016) provide an extensive review of strains employed recently.

Figure 4 presents the total net feedstock costs for the pretreatments assessed in this study with different residence times and moisture contents. As shown in the figure, residence time of 4 weeks generally resulted in higher dry matter loss and thus high total cost than residence time of 2 weeks for all the pretreatment groups. Storing biomass with *C. subvermispora* achieved the lowest total net cost compared to the control treatment and the *P. chrysosporium* treatment in both residence times modeled, which suggested that *C. subvermispora* treatment is an economically viable way to store biomass in the fungal-assisted queuing system. Likewise, controlling dry matter loss in *P. chrysosporium* through limited storage time also reduced excessive costs due to degradation.

One key highlight of this study is that compatibility of this approach with the existing bale-based logistics systems for corn stover since no upstream unit operations are impacted by fungal treatment at the biorefinery gate. In addition, this approach allows for a biorefinery gate to store a larger portion of required biomass and prevent any feedstock delivery-related delays that may occur for a variety of reasons including weather events or shipping and transport delays. Additional flexibility in the biomass feedstock supply chain is warranted given the disruptions that have been observed recently in other supply chains on an international level.

4 | CONCLUSION

Filamentous fungi can effectively depolymerize lignocellulosic biomass through enzymatic action. This study defined the range of viable conditions, including moisture content and residence time, of both a selective and nonselective fungal strain in the context of a queuing pile at a biorefinery reactor throat. This study indicated that enhanced structural depolymerization that occurred with *P. chrysosporium* should be limited to 2 weeks of residence time. Slower growth and degradation of *C. subvermispora* allowed for tolerance of the longer residence time. Furthermore, dry matter loss, structural carbohydrate and lignin changes, and techno-economic analysis were used to suggest potential approaches for further investigation. Increased reduction in recalcitrance, which could possibly be achieved with alternative approaches, has the potential to offset costs associated with material losses and

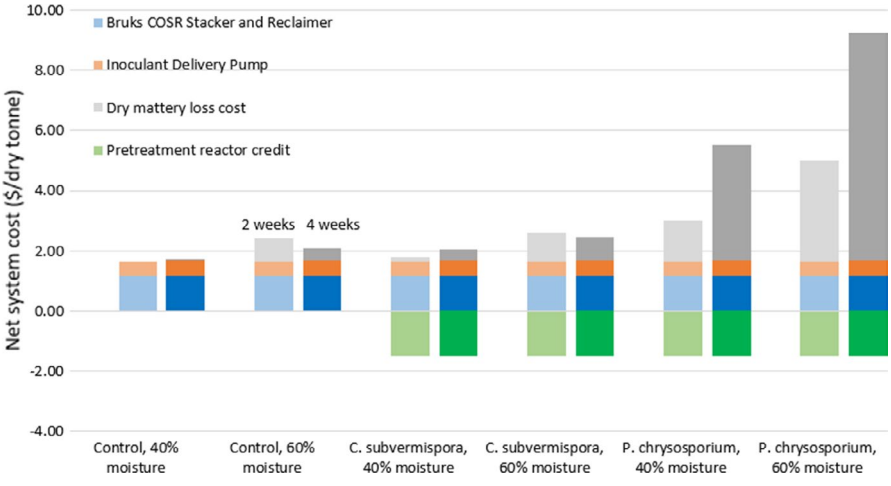

FIGURE 4 Net cost of fungal-mediated queuing costs and corresponding cost offsets in different treatments

should also be explored in future research. Additionally, follow-on research should explore storage performance in larger systems to understand the range of dry matter loss correlated to not only compositional changes but also conversion-related impacts. Opportunities also exist to understand the fungal impact on a mechanistic level by following molecular changes using techniques such as analytical pyrolysis, spectroscopic characterization, X-ray diffraction, and nuclear magnetic resonance. These characterizations may reveal the physicochemical impacts of fungal treatment on specific lignin molecules, hemicellulose linkages, and even cellulose physical state, which could open new pathways for recalcitrance reduction in corn stover.

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CONFLICT OF INTEREST

The authors have no competing interests.

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SUPPORTING INFORMATION

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