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Fungal pretreatment of non-sterile miscanthus for enhanced enzymatic hydrolysis

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HIGHLIGHTS

- Successful fungal pretreatment of non-sterile miscanthus by white rot fungus.
- Inoculation with at least 30% pre-colonized miscanthus was crucial for the success.
- Enzymatic digestibility and glucose yield was 3–4 times higher than raw miscanthus.
- Comparable glucose yield to that of fungal pretreated miscanthus with sterilization.

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ABSTRACT

Miscanthus was pretreated with the fungus *Ceriporiopsis subvermispora* under non-sterile conditions, using sterile miscanthus that had been previously colonized with the fungus as the inoculum. Inoculum ratios equal to or greater than 30% yielded a successful pretreatment, enhancing the enzymatic digestibility of miscanthus by 3- to 4-fold over that of raw miscanthus, which was comparable with the fungal pretreatment under sterile conditions. This enhanced digestibility was linearly correlated with lignin degradation. Although cellulose loss of up to 13% was observed for the successful non-sterile pretreatments, the final glucose yield was 3–4 times higher than that of raw miscanthus and comparable to that of the sterile pretreated miscanthus. A time course study showed that maximum glucose yield can be achieved with a pretreatment time of 21 days.

1. Introduction

The increasing need for alternative renewable sources of energy has prompted the development of biomass-based biorefineries with the potential of replacing a significant portion of the petroleum that is currently used for the production of fuels and chemicals (Cherubini, 2010). Biorefineries' success depends on the availability of renewable, consistent, high volume feedstock supplies, which can be provided by dedicated energy crops, such as switchgrass or miscanthus (U.S. Department of Energy, 2011). Miscanthus is a tall perennial grass that has high annual biomass yields, low nutrients and water requirements, and the ability to adapt to a wide variety of climate and soil conditions (Jones and Walsh, 2001). Therefore, miscanthus has been identified as one of the most promising energy crops (Heaton et al., 2010).

Miscanthus, a lignocellulosic biomass, is mainly composed of cellulose, hemicellulose, and lignin. Biological transformation of lignocellulosics into bioenergy utilizes only the sugars present in the cellulose (and sometimes in the hemicellulose) for the production of biofuels, while the presence of lignin is undesirable (Zeng et al., 2014). Cellulose and hemicellulose are polysaccharides composed of 5- and 6-carbon sugars that can be fermented by microorganisms for the production of biofuels and bioproducts. Lignin is an aromatic heteropolymer that protects the polysaccharides from microbial degradation, besides giving support and stiffness to the plant structure (Hendriks and Zeeman, 2009).

Miscanthus has a recalcitrant structure and pretreatment is required to enhance the accessibility of cellulose to hydrolytic enzymes for sugar release (Brosse, 2009). Commonly used pretreatment methods often employ strong chemicals and/or high temperatures, and require large amounts of water (Kim, 2013). Alternatively, biological pretreatment exploits the ligninolytic capacity of some microorganisms (fungi and bacteria) to reduce the recalcitrance of the lignocellulose (Chen et al., 2010). For fungal

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pretreatment, rotting fungi, such as *Ceriporiopsis subvermispora*, are grown in the lignocellulosic biomass to breakdown lignin, resulting in a feedstock that is easier to digest (Wan and Li, 2012). Compared to other pretreatment methods, fungal pretreatment can be operated at room temperature with minimal use of water and chemicals, and without generation of waste streams (Saritha et al., 2012).

Fungal pretreatment is usually performed via solid-state cultivation of the fungus in the feedstock. But because fungi cannot use lignin as a sole carbon and energy source, parts of the cellulose and/or hemicellulose are also degraded during this process, which is detrimental for the purpose of pretreatment (Sánchez, 2009). *C. subvermispora* is a white rot basidiomycetes that degrades lignin selectively over cellulose. It has been used to successfully pretreat lignocellulosic biomass, resulting in significant lignin degradation and enhanced enzymatic digestibility. Although some parts of the hemicellulose are also degraded, most of the cellulose remains intact (Wan and Li, 2011). Glucose yields between 24% and 69% have been obtained for fungal pretreatment with *C. subvermispora* of feedstocks such as hardwood, corn stover, and switchgrass, but this pretreatment has been unsuccessful with other feedstocks such as soybean and wheat straw (Cianchetta et al., 2014; Ge et al., 2015; Salvachúa et al., 2011; Wan and Li, 2010, 2011).

Sterilization of the feedstock, a costly step, is usually required for fungal pretreatment, because the indigenous microorganisms may outcompete the introduced fungi. *C. subvermispora* has been reported to be especially sensitive to the presence of other microorganisms (Akin et al., 1995). A promising alternative strategy is to grow the fungus on sterile feedstock first, and then use the colonized feedstock as an inoculum for fungal pretreatment of non-sterile feedstock (Zhao et al., 2014). However, this strategy has only been studied in woody biomass, and it is not clear how it would behave in herbaceous crops, such as miscanthus. White rot fungi naturally colonizes wood, but it has been shown that its growth in herbaceous biomass can be more difficult (Vasco-Correa and Li, 2015; Wan and Li, 2011), probably because of differences in the cell wall structure and composition. However, herbaceous biomass has more potential as a bioenergy feedstock because it is less recalcitrant and grows faster than wood, producing higher biomass yields (Wan and Li, 2011). It is unknown how much previously colonized feedstock would be needed as an inoculum for successful pretreatment of herbaceous biomass, how the moisture content of the feedstock–inoculum mixture would affect this process, and how this strategy would affect the pretreatment time. Therefore, systematic research on using this strategy for fungal pretreatment of non-sterile herbaceous crops is needed. In this study, fungal pretreatment of non-sterile miscanthus, using miscanthus colonized with *C. subvermispora* as the inoculum, was evaluated. Effects of inoculum ratio and moisture content on glucose yield, enzymatic digestibility, lignin degradation, and sugar loss were investigated. Also, a time course study of the fungal pretreatment process was performed, monitoring the degradation of different components during 28 days and effects on the glucose yield.

2. Methods

2.1. Feedstock collection and storage

Miscanthus × *giganteus* (hereafter miscanthus) was harvested from a field in Ashtabula, OH, in spring 2013. The moisture content was around 6% and its composition is shown in Table 1. The feedstock was milled to pass through a 12 mm screen using a hammer mill (The C.S Bell Co., Tiffin, OH, USA) and stored under dry conditions.

Table 1
Characteristics of miscanthus.

	Raw miscanthus	Inoculum (fungal colonized miscanthus)
Total solids (%)	93.8 ± 0.5	47.9 ± 0.5
Extractives[a] (%)	8.9 ± 0.2	15.33 ± 0.1
Cellulose[a] (%)	38.0 ± 0.2	43.6 ± 0.9
Hemicellulose[a] (%)	18.5 ± 0.4	17.4 ± 0.3
Lignin[a] (%)	20.9 ± 0.2	17.1 ± 0.2

[a] Based on total solids.

2.2. Inoculum preparation

Ceriporiopsis subversmipora (ATCC 96608) was obtained from the American Type Culture Collection (Manassas, VA, USA) and kept on 2% malt extract agar at 4 °C. Sterile miscanthus colonized with *C. subversmipora* was used as the inoculum for the fungal pretreatment experiments. To prepare this inoculum, 130 g (dry basis) of raw miscanthus were placed in 2 L reactors, which were supplemented with deionized water to adjust moisture to 60%, and then covered with cotton. Reactors were autoclaved (121 °C, 15 min) and cooled down. Then, *C. subversmipora* mycelium, which was grown in 50 ml of 2% malt extract liquid medium (7 days, 28 °C, static conditions), was added to each reactor and then incubated for 28 days at 28 °C. At the end, the fungal-colonized miscanthus was taken out of the flask and mixed well before using it as inoculum for the successive fungal pretreatment of non-sterile miscanthus. Composition of the inoculum is shown in Table 1.

2.3. Fungal pretreatment of non-sterile miscanthus

Non-sterile raw miscanthus and inoculum (fungal-colonized miscanthus) were mixed in different proportions and added to 1 L reactors. Deionized water was added to adjust moisture content and reactors were incubated at 28 °C. First, a full factorial design with three replicates was performed, with the following factors: inoculum ratio (0%, 10%, 20%, 30%, 40%, and 50%, dry basis) and moisture content (60% and 75%). Reactors were incubated for 28 days. The inoculum ratio 0% (no inoculation) was considered to be the negative control. The positive control was sterile miscanthus inoculated with mycelium (as described in Section 2.2), which was incubated along with the treatments. At the end of the 28 days, samples were taken out of the reactors and mixed well. Part of the fresh material was used for enzymatic hydrolysis and the rest was dried at 40 °C in a convection oven for 24 h, then milled and passed through a 1 mm screen (Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) for subsequent cellulose, hemicellulose, and lignin content determination.

Second, an experiment to evaluate the degradation over time of components of miscanthus during fungal pretreatment was performed at 50% inoculum ratio and 60% moisture content. Reactors were prepared in the same manner as in the first experiment and stopped at different times (0, 7, 14, 21, and 28 days). At the end of the specific incubation time, samples were processed as in the first experiment, for subsequent enzymatic hydrolysis, and cellulose, hemicellulose, and lignin content determination.

2.4. Enzymatic hydrolysis

Enzymatic digestibility of raw and pretreated miscanthus was determined according to Selig et al., using the enzyme mix Cellic CTec2 (Novozymes, Bagsværd, Denmark), at a cellulase concentration of 10 FPU/g dry substrate (Selig et al., 2008). About 2.5 g (dry basis) of feedstock were added to 250 ml flasks along with citrate