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Estimation of Treatment Time for Microbial Preprocessing of Biomass

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Abstract Biochemical conversion of lignocellulosic biomass to ethanol involves size reduction, preprocessing, pretreatment, enzyme hydrolysis, and fermentation. In recent years, microbial preprocessing has been gaining attention as a means to produce labile biomass for lessening the requirement of pretreatment severity. However, loss of sugars due to microbial consumption is a major consequence, suggesting its minimization through optimization of nutrients, temperature, and preprocessing time. In this work, we emphasized estimation of fungal preprocessing time, at which higher sugar yields can be achieved after preprocessing and enzyme hydrolysis. The estimation is based on the enzymatic activity profile obtained by treating switchgrass with *Phanerochaete chrysosporium* for 28 days. Enzyme assays were conducted once in every 7 days for 28 days, for activities of phenol oxidase, peroxidase, β-glucosidase, β-xylosidase, and cellobiohydrolase. We found no activity for phenol oxidase and peroxidase, but the greatest activities for cellulases on the seventh day. We then treated switchgrass for 7 days with *P. chrysosporium* and observed that the preprocessed switchgrass had higher glucan (39%), xylan (17.5%), and total sugar yields (25.5%) than the unpreprocessed switchgrass (34%, 37.5%, and 20.5%, respectively, $p<0.05$). This verifies the utility of using enzyme assays for initial estimation of preprocessing time to enhance sugar yields.

Keywords Microbial preprocessing · Fungal preprocessing · Preprocessing time · Enzyme activity assays · *Phanerochaete chrysosporium* · Partial cellulose degradation

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Introduction

With increasing demands for fuel and environmental concerns, biofuels have gained new importance [1]. In spite of advances in fermentation technology, commercialization of ethanol from lignocellulosic biomass is still hindered by the recalcitrance of biomass. Various pretreatment processes can reduce the recalcitrance of biomass; however, obstacles to hydrolysis and fermentation remain due to the production of degradation products during pretreatment, as a result of greater pretreatment severity [2]. Although washing and other chemical methods of treating the degradation products can alleviate inhibition, significant water and chemical usage combined with capital equipment and energy contribute to higher costs [3]. Microbial preprocessing of biomass before pretreatment may potentially lessen the requirements of pretreatment severity. Solid-state fungal treatment is one such technique involving less aggressive treatment with simpler processing parameters and equipment. It also offers a simpler reactor system with minimum downstream processing [4] and brings the preprocessing technology closer to the farms that are the source of agricultural residues. A solid state fungal system producing cellulases may potentially deconstruct the biomass, improving digestibility and thus ethanol production.

Phanerochaete chrysosporium is a white rot fungus known to degrade different synthetic chemicals, most of which are recalcitrant to biodegradation [5]. *P. chrysosporium* is a potential lignin degrading fungus with ability to partially breakdown lignin carbohydrate complexes [6]. Although it produces lignin peroxidase and manganese-dependent peroxidase [7] and laccase [8] to break lignin, it also produces multiple endoglucanases which exhibit endo-exo synergism with cellobiohydrolases. β-glucosidase obtained from *P. chrysosporium* can also cleave hemicellulose to produce xylose, mannose, and arabinose due to its non-specificity [9]. Besides the lignin-degrading capability of *P. chrysosporium*, its potential for partial cellulose degradation can be explored to recover sugars during the prolonged storage of biomass.

Although most of the work related to using *P. chrysosporium* for producing higher cellulosic materials from biomass has shown significant lignin degradation, considerable cellulose losses have been reported. Corn stover treated for 29 days using *P. chrysosporium* showed reduced viscosity, but showed no improvement in enzyme digestibility [6]. *P. chrysosporium*-treated cotton stalks also showed similar results of reduced digestibility over a 14-day solid-state treatment in spite of lignin degradation [10]. It is apparent from these previous works that, although prolonged treatment time resulted in lignin degradation, loss in sugars was observed due to microbial consumption. Thus a method to determine an appropriate treatment time is critical, for exploiting the capacity of the fungus to partially degrade cellulose and hemicellulose complexes, to minimize the sugar consumption and produce higher sugar yields.

In the present study, we used enzyme profiling as a method to estimate an appropriate treatment time for preprocessing switchgrass. We treated switchgrass under solid-state conditions with *P. chrysosporium* (in triplicate) for 28 days and assayed for activities of phenol oxidase (laccase), peroxidase, β-glucosidase, β-xylosidase, and cellobiohydrolase. Assays were conducted for every 7 days of incubation starting with the initial day, to obtain a profile of activities against time, for the 28-day period. A time point on the plot of enzyme activity against time with highest activities on the plot was chosen to be an appropriate treatment time. In a subsequent experiment, we treated switchgrass for the time period obtained from the previous step. The treated samples were analyzed for glucose and total sugar yields, showing an increase in total glucose yield and total sugar yield, thus validating the method of using enzyme profiling for estimation of treatment time.

Materials and Methods

Propagation of Inoculum

P. chrysosporium (strain BKM-F-1767) was obtained from USDA Forest Products Laboratory (Madison, WI, USA) and was propagated onto potato dextrose agar (PDA) plates of 90 mm×12 mm size and allowed to grow at 37 °C for 7 days [6]. Stock cultures were stored for a week at 4 °C, and the culture was maintained by periodically transferring to fresh PDA plates. Prior to the treatments, *P. chrysosporium* was grown for 7 days on PDA plates, and a spore suspension was prepared by scraping the spores aseptically from three plates into 60 ml sterile water, ensuring uniform spore distribution in the liquid by vortexing. A 5-ml sample of this suspension was used as inoculum for each treatment flask.

Preparation of Switchgrass and Solid-state Treatment with *P. chrysosporium*

Seeds of switchgrass (*Panicum virgatum* L. var.) were purchased from Sharp's Bros. Seed Co., Clinton, MO, USA. Switchgrass, consisting of entire, green, leafy, shoots bearing expanded infructescenses, was harvested from 2- and 3-year populations established in Waller labs, University of Mississippi, it was air dried and ground to 3 mm mesh size. The ground samples were stored under dry conditions. These were further dried at 35 °C for 2 days prior to experimentation in a convection incubator. Switchgrass (10 g) was weighed, placed in a 250-ml flask, and autoclaved (121 °C, 30 min). Sterilized water (50 mL) was added to maintain approximately 80% moisture and 5 mL of supplemental growth media ($NaNO_3$—3 g, KCl—0.5 g, $MgSO_4.7H_2O$—0.5 g, $FeSO_4.7H_2O$—0.5 g, KH_2PO_4—1.0 g, Glucose—20 g in 1 L solution) [11], sterilized separately, was added in addition to 20 μL of tetracycline (20 mg/mL in ethanol) to minimize bacterial contamination in the flasks. Treatment flasks received 5 mL of *P. chrysosporium* suspension while other flasks, supplemented with 5 mL of additional sterilized water and no *P. chrysosporium*, were used as controls. Flasks, three treated (with *P. chrysosporium*) and three controls (without *P. chrysosporium*), were allowed to incubate at 37 °C under solid-state conditions for 7 days.

Enzyme Activity Assays

Flasks (three treated and three controls) were incubated at 37 °C under solid-state conditions for 28 days and were sampled for phenol oxidase, peroxidase, β-glucosidase, β-xylosidase, and cellobiohydrolase activities for every 7 days during the 28-day period of treatment. The substrate used for phenoloxidase and peroxidase tests is 5 mM L-3,4-dihydroxyphenylalanine (L-dopa), and those for β-glucosidase, β-xylosidase, and cellobiohydrolase tests are 5 mM pNP-β-glucopyronoside, 5 mM pNP-β-xylopyranoside, and 5 mM pNP-cellobioside, respectively.

A known amount (approximately a gram) of sample was taken in a test tube and diluted to 5 mL by addition of water and mixed well, 150 μL of supernatant was incubated with 150 μL of substrate solution (and 15 μL of 0.3% H_2O_2, only for peroxidase assay) for a noted time, and the mixture was analyzed spectrophotometrically at 460 and 410 μm for L-dopa assays and cellulose assays, respectively. The units of activity are defined as micromoles of the substrate reacted with the enzyme in 1 ml of sample per hour of incubation (U/ml) [12].

Composition Analysis and Enzyme Hydrolysis

Samples, before and after treatment, were analyzed for the glucan and xylan compositions. Commercial enzymes, Novozyme 188 (10 FPU/0.5 g glucan) and celluclast (15 FPU/0.5 g glucan), were used for 72-h enzyme hydrolysis of samples using laboratory analytical procedure (LAP) from National Renewable Energy Laboratory (NREL).

Analysis

Activities of phenol oxidase, peroxidase, β-glucosidase, β-xylosidase, and cellobiohydrolase were determined at day 0, 7, 14, 21, and 28 of the 28-day solid-state microbial treatment. Flasks at the end of the treatment were washed with 25 mL of water heated up to 50 °C, and the switchgrass was filtered and stored in plastic bags. To estimate overall changes, yields in washate (for free sugars) and solid phases of the treated substrate were accounted for. Samples, before and after treatment, were analyzed for the glucan and xylan compositions. Commercial enzymes, Novozyme 188 (10 FPU/0.5 g glucan) and celluclast (15 FPU/0.5 g glucan), were used for 72-h enzyme hydrolysis of samples using LAP from NREL.

The samples from compositional analysis were analyzed using HPLC with an Aminex HPX-87P column at 85 °C, using deionized water as mobile phase and refractive index detector at 50 °C. Analysis of enzyme hydrolysis samples was done using an Aminex HPX-87H column at 65 °C, 0.05 N H_2SO_4 as mobile phase, and a refractive index detector at 50 °C. Calculations were performed as follows:

$$\text{Glucose yield} = \frac{\text{Concentration of glucose}}{\text{Amount of sample}} \times \text{Volume of liquid} \times 100$$

$$\text{Total glucose yield} = \text{Glucose yield}_{\text{Enzyme hydrolysis}} + \text{Glucose yield}_{\text{Washate}}$$

$$\text{Sugar yield} = \frac{\text{Total sugar concentration}}{\text{Amount of sample}} \times \text{Volume of liquid} \times 100$$

$$\text{Total sugar yield} = \text{Sugar yield}_{\text{Enzyme hydrolysis}} + \text{Sugar yield}_{\text{Washate}}$$

Results and Discussion

Enzyme Activity Profiles and Estimation of Treatment Period

Cellobiohydrolase, β-glucosidase, and β-xylosidase activities increased from the initial day until the seventh day and then decreased over the 28-day period of incubation (Fig. 1). β-glucosidase activity decreased gradually until 21 days, but increased again on the 28th day. β-xylosidase and cellobiohydrolase activities decreased steeply until the 21st day and increased slightly on the 28th day. β-glucosidase activity was significant throughout the 28-day period compared to β-xylosidase and cellobiohydrolase activities, with the highest

activity of 5.5 U/mL on the seventh day of incubation. Deconstruction of cellulose network results from production of enzymes for release of free sugars necessary for metabolic growth of *P. chrysosporium* [13]. However, evidence from earlier works [6, 14] suggests that with long preprocessing time, degradation of lignin was accompanied by considerable loss in cellulose and glucose yield due to the sugar consumption by the fungi. Thus a time point (7 days from Fig. 1) with highest activity from the enzyme profiles (Fig. 1) can be an appropriate treatment time due to greater rate of sugar release compared to the rate of sugar consumed.

Activities of phenol oxidase and peroxidase were not observed during this period which could be because of their very low concentrations. Besides, white rot fungi do not use lignin as growth substrate [15] leading to utilization of the initial glucose and a partial degradation of cellulose without the release of lignin degrading enzymes.

Composition Analysis

Glucan % and xylan % were determined for the samples before and after fungal treatment. The samples include treated samples (with *P. chrysosporium*), control samples (without *P. chrysosporium*) and raw switchgrass sample. Glucan and xylan composition of the preprocessed (with *P. chrysosporium*) and control samples (without *P. chrysosporium*) are higher ($p<0.005$) than the unprocessed (raw) switchgrass (Fig. 2). This supports that a higher proportion of non-cellulosic part of the switchgrass underwent degradation [14]. An increase in % glucan composition for the treated samples resulted from the shorter treatment time (7 days) derived from the enzyme activity profiles. Thus activity profiles seem to give a good estimation of treatment time for obtaining increased glucan % and xylan %, in contrast with the samples having decreased glucan % with 14 day treatment work by Shi et al. [10]. Higher glucan composition also results in lesser biomass loading in the enzyme hydrolysis stage than untreated substrate for an equivalent glucan weight, thus reducing the operational costs of hydrolysis reactors.

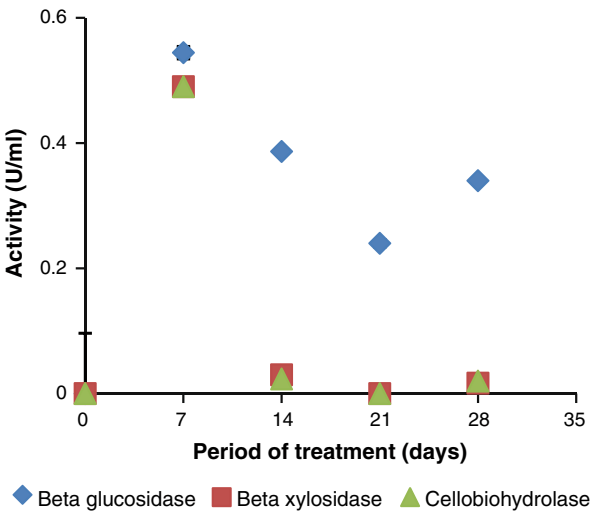

Fig. 1 Profile of β-glucosidase (*diamond*), β-xylosidase (*square*), and cellobiohydrolase (*triangle*) activities during the 28 days treatment of switchgrass with *P. chrysosporium*

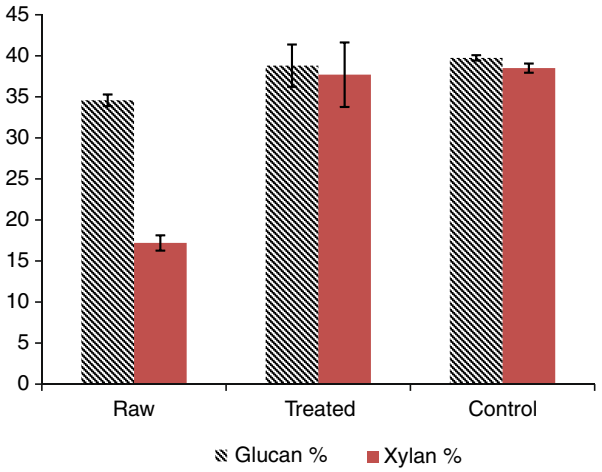

Fig. 2 Composition (in terms of glucan % and xylan %) of raw, treated, (preprocessed with *P. chrysosporium*), and control (preprocessed without *P. chrysosporium*) samples. The *error bars* represent 95% confidence interval

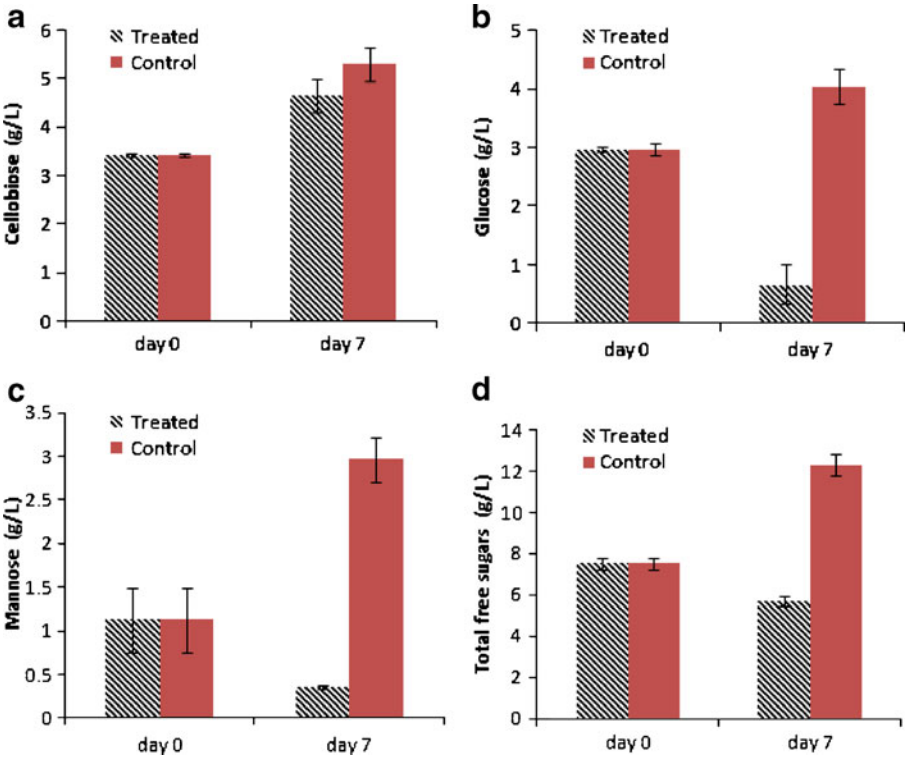

Fig. 3 Concentration of free sugars from the washate. **a** Cellobiose, **b** glucose, **c** mannose, and **d** concentration of total free sugars released after 7-day fungal conditioning of switchgrass. The *error bars* represent 95% confidence interval

Free Sugar Concentration

Figure 3 displays the concentration of free sugars present in the liquid phase from the day 0 to day 7 of the fungal treatment. Cellobiose, glucose, and mannose were the three free sugars present in significant amounts in all the samples. Glucose and mannose concentrations in the treated samples decreased with respect to both the 0 day samples and controls indicating the utilization of glucose and mannose by *P. chrysosporium*. Controls had higher free sugar concentrations than the treated samples, confirming the consumption of sugars by the fungus. However, cellobiose concentration increased during the 7-day treatment (Fig. 3a) which is a reflection of the cellobiohydrolase activity that releases cellobiose from cellulose (Fig. 1). Moreover, cellobiose concentration in the treated samples could be lower than that in the controls, indicating its conversion to glucose due to β-glucosidase activity. Total free sugar concentration (in washate) of the treated sample was lower than the initial day and the control samples (Fig. 3d), further confirming the monomeric sugar consumption by *P. chrysosporium* for metabolic growth.

Glucose and Total Sugar Yield

Glucose yield from enzyme hydrolysis for treated samples was lower than the raw and controls samples (Fig. 4a). Enzyme hydrolysis glucose yield of the control was similar to that of the raw sample and higher than that of the treated sample. However, the total glucose yield of treated samples is significantly higher ($p<0.05$) than the raw samples (Fig. 4b) considering the glucose equivalent of cellobiose in the free sugars. Also, the controls had

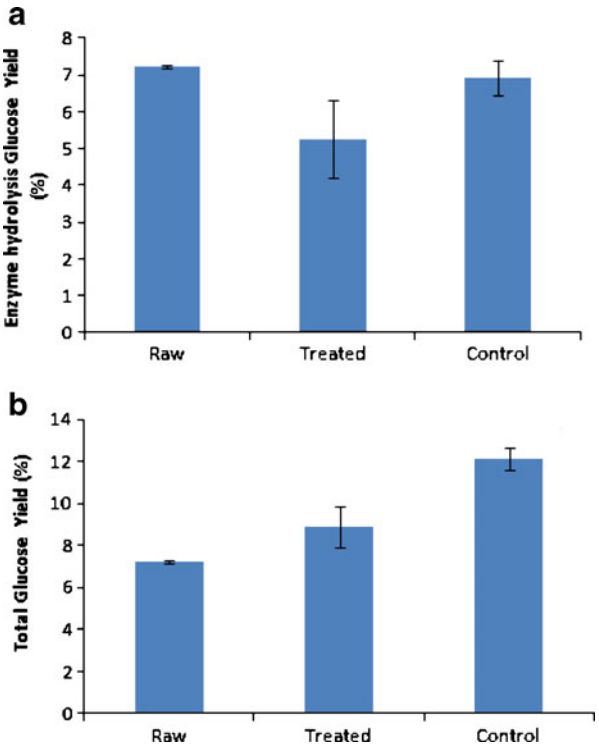

Fig. 4 **a** Glucose yield (%) of raw, treated, and control samples of switchgrass. **b** Total glucose yield (%) of raw, treated, and control samples of switchgrass after 72-h enzyme hydrolysis. The *error bars* represent 95% confidence interval

higher total glucose yield than the treated and raw samples, indicating the contribution of free glucose and the glucose equivalent cellobiose released during the treatment, in increasing the total glucose yields. Sugar yields after 72-h enzyme hydrolysis for raw, treated, and control samples were not significantly different from each other (Fig. 5a). Total sugar yield per gram of switchgrass evaluated for samples with and without treatment shows that the treated samples showed a 24.39% higher total sugar yield than the raw switchgrass (Fig. 5b). Thus the increase in glucose and free sugar yields was majorly contributed by the free sugar release during fungal treatment, a highly desirable effect if optimized.

However, control samples had higher total glucose and total sugar yields than the raw ones because they underwent similar processing as that of the treated samples but without the fungus resulting in water hydrolysis of switch grass and eventually higher sugar yields compared to raw ones.

Control samples had higher total glucose and total sugar yields than the treated samples due to no consumption of sugars in the absence of *P. chrysosporium*. With further optimization, higher sugar yields can be obtained in the treated samples compared to the control samples by minimizing the sugar consumption. In the present work, although treated samples had lower sugar yields than the controls, they had higher sugar yields than the raw samples contrary to works of Keller et al. and Shi et al. due to the shorter treatment time. Thus, validating the technique of using enzyme assays for estimating the treatment time.

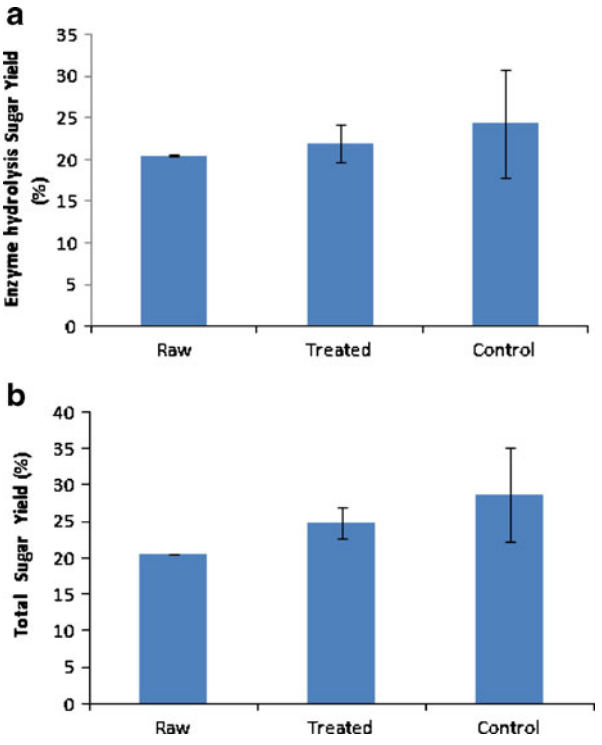

Fig. 5 **a** Sugar yield of raw, treated, and control samples. **b** Total sugar yield (%) of switchgrass for raw, treated, and control samples obtained after 72-h enzyme hydrolysis. The *error bars* represent 95% confidence interval

Conclusions

A profile of β-glucosidase, β-xylosidase, and cellobiohydrolase activities during fungal preprocessing of switchgrass was helpful in estimating an appropriate treatment time of 7 days based on the time of highest activity. Fungal preprocessing of switchgrass for 7 days with *P. chrysosporium* resulted in higher glucose yields and monomeric sugar yields. Glucan and xylan compositions were also higher for the treated sample than the raw sample. Higher sugar yields and glucose yields are attributed to the free sugars released during preprocessing of switchgrass with *P. chrysosporium.* Although improvements in sugar yields and glucose yields due to the fungal treatment were not extremely high, observed improvement supports the concept of using enzyme activity profiles for initial estimation of treatment time. Further work should be pursued in the direction of decreasing treatment time during fungal preprocessing and optimizing it for higher sugar yields.

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