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Evaluation of Potential Fungal Species for the *in situ* Simultaneous Saccharification and Fermentation (SSF) of Cellulosic Material

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ABSTRACT

Three fungal species were evaluated for their abilities to saccharify pure cellulose. The three species chosen represented three major wood-rot molds; brown rot (*Gloeophyllum trabeum*), white rot (*Phanerochaete chrysosporium*) and soft rot (*Trichoderma reesei*). After solid state fermentation of the fungi on the filter paper for four days, the saccharified cellulose was then fermented to ethanol by using *Saccharomyces cerevisiae*. The efficiency of the fungal species in saccharifying the filter paper was compared against a low dose (25 FPU/g cellulose) of a commercial cellulase. Total sugar, cellobiose and glucose were monitored during the fermentation period, along with ethanol, acetic acid and lactic acid. Results indicated that the most efficient fungal species in saccharifying the filter paper was *T. reesei* with 5.13 g/100 g filter paper of ethanol being produced at days 5, followed by *P. chrysosporium* at 1.79 g/100 g filter paper. No ethanol was detected for the filter paper treated with *G. trabeum* throughout the five day fermentation stage. Acetic acid was only produced in the sample treated with *T. reesei* and the commercial enzyme, with concentration 0.95 and 2.57 g/100 g filter paper, respectively at day 5. Lactic acid production was not detected for all the fungal treated filter paper after day 5. Our study indicated that there is potential in utilizing *in situ* enzymatic saccharification of biomass by using *T. reesei* and *P. chrysosporium* that may lead to an economical simultaneous saccharification and fermentation process for the production of fuel ethanol.

Keywords: *Phanerochaete chrysosporium*, *Trichoderma reesei*, *Gloeophyllum trabeum*, *Saccharomyces cerevisiae*, simultaneous saccharification and fermentation (SSF), cellulase

INTRODUCTION

Lignocellulosic materials from biomass such as agricultural crop residues and other energy crops is the most abundant and renewable biopolymer on Earth (Zhang 2008; Fukuda *et al.*, 2009). Made of 75–80% cellulose and hemicellulose, they are low cost feedstocks for various industrial purposes that can be used in the production of chemicals and fuel ethanol, which is a good substitute for gasoline in internal combustion engines (Adsul *et al.*, 2005; Ahamed and Vermette, 2008; Ling *et al.*, 2009). However, the production of fuel grade ethanol from lignocellulosic materials as an alternative or additives for fossil fuels is still expensive. According to Alkasrawi *et al.* (2003), recent economical calculations showed that the production cost of fuel ethanol from lignocellulosic biomass would be higher than the price of gasoline. Thus, additional cost reductions are necessary to achieve economic competitiveness against the existing conventional fuels.

Currently, the most promising platform for the bioconversion of lignocellulosics to ethanol is based on the enzymatic hydrolysis of biomass using cellulase and hemicellulase enzymes via simultaneous saccharification and fermentation (SSF) process, first reported in 1976 by Gauss and colleagues (Ahamed and Vermette, 2008; Olofsson *et al.*, 2008). SSF is a technology that has gained a lot of interest, as it is both logistically and economically favorable in terms of higher final ethanol yield (Ohgren *et al.*, 2007; Tomás-Pejó *et al.*, 2009). Furthermore, this type of process has lower energy consumption when compared to the closely related separate hydrolysis and fermentation (SHF) (Olofsson *et al.*, 2008). However, the drawback of SSF is the high enzyme concentrations that are required for significant hydrolysis of cellulose and hemicellulose (Alkasrawi *et al.*, 2003; Linde *et al.*, 2007). According to Ahamed and Vermette (2008), cellulase production is the most expensive step during ethanol production from cellulosic biomass, accounting for approximately 40% of the total cost. Therefore, because the high cost of cellulase

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enzyme production and enzyme loading is a major economical factor in the overall ethanol production cost, it is imperative to find methods of reducing the enzyme loading and increasing the hydrolysis of cellulose to fermentable sugars (Gregg *et al.*, 1998; Adsul *et al.*, 2005).

Another challenge in making the bioconversion of lignocellulosics to ethanol more feasible is the pretreatments that are needed to be performed on the feedstocks prior to enzymatic hydrolysis (Silverstein *et al.*, 2007; Zhu *et al.*, 2008). The problems with many current pretreatments technologies are the generations of toxic by-products that can hinder the bio-mechanisms of the cellulolytic and hemicellulolytic enzymes, and may also inhibit downstream alcoholic fermentation (Ortega *et al.*, 2001; Keating *et al.*, 2006). Furthermore, these practices are environmentally detrimental and energy intensive (Chundawat *et al.*, 2007). Therefore, it is imperative to develop means of direct enzymatic hydrolysis of lignocellulosics that do not sacrifice ethanol production. One possible solution is to use lignolytic, cellulolytic and hemicellulolytic organisms, such as fungi, to perform enzymatic saccharifications that will liberate fermentable sugars from the biomass.

Many fungal groups have been known to be able to degrade the main components of lignocellulosics, such as cellulose, hemicellulose and lignin (Arantes and Milagres, 2006; Sanchez, 2009; Shrestha *et al.*, 2009; Rasmussen *et al.*, 2010). The first of this group, the filamentous molds are well documented for their highly efficient cellulolytic and hemicellulolytic enzyme systems for the complete hydrolysis of biomass into its monomeric sugar components. The extracellular cellulolytic system of this fungus group composed of 60–80% cellobiohydrolases or exoglucanases, 20–36% of endoglucanases and 1% of β -glucosidases that act synergistically (Ahamed and Vermette, 2008).

The next fungal group, the white-rots, have been studied extensively for their abilities to efficiently degrade and depolymerize major plant cell wall components, especially the more recalcitrant lignin, making it extensively used in the study of lignin biodegradation and other biotechnological applications, such as biobleaching and pulp mill effluents treatment (Wymelenberg *et al.*, 2005; Kersten and Cullen, 2007; Ravalason *et al.*, 2008). White rots effectively perform all these processes because they secrete several varieties of lignin degrading proteins, such as lignin peroxidases (LiPs), manganese peroxidases (MnPs) and other low redox-potential peroxidases, in addition to expressing multiple cellulases and hemicellulase (Suzuki *et al.*, 2008).

The third fungal group consists of the brown-rots. These saprophytic fungi are major forest biomass degraders that contribute significantly to the soil fertility in the ecosystem (Kerem *et al.*, 1999; Cohen *et al.*, 2005). Brown-rot fungi also cause the most destructive type of decay in wooden structures, although their biodegradation mechanisms are still relatively unknown (Kerem *et al.*, 1999; Schilling *et al.*, 2009). Fungi from this group appear to produce some cellulases, but a larger part of the cellulose degradation seems to be non-enzymatic,

involving low molecular weight catalysts such as chelating peptides and radicals (Henriksson *et al.*, 1999; Cohen *et al.*, 2005).

In this study, we evaluated three fungal species that represent the three major wood-rot; brown-rot (*Gloeophyllum trabeum*), white-rot (*Phanerochaete chrysosporium*) and soft-rot (*Trichoderma reesei*), for their abilities to enzymatically saccharify filter paper via *in situ*. The efficiencies of their enzyme activities are measure via the release of cellobiose, glucose and the end fermentation products in the form of ethanol and organic acids. To perform fermentation, *Saccharomyces cerevisiae* was used to maximize the conversion of the saccharification products.

MATERIALS AND METHODS

Microorganisms stocks and culture preparation

All fungal cultures used in this study were obtained from American Type Culture Collection (Rockville, MD). The *Gloeophyllum trabeum* (ATCC 11539), *Phanerochaete chrysosporium* (ATCC 24725), *Trichoderma reesei* (ATCC 13631) and *Saccharomyces cerevisiae* (ATCC 24859) cultures were revived onto potato dextrose broth (PDB) (Difco, Becton Dickinson and Co., Sparks, MD) at 24 °C with shaking at 120 rpm overnight (Shrestha *et al.*, 2009). For long term storages, the stock cultures were aliquoted in Yeast Malt (YM) extract broth (glucose, 10.0 g/L; peptone, 5.0 g/L; yeast extract, 3.0 g/L; and malt extract, 3.0 g/L) (Difco) supplemented with 20% (v/v) glycerol, at -80 °C in an ultralow-temperature freezer (So-Low Environmental Equipment Co., Inc., Cincinnati, OH) (Shrestha *et al.*, 2008; Shrestha *et al.*, 2009).

Seed cultures from spore suspension of *G. trabeum*, *P. chrysosporium* and *T. reesei* were prepared in 1 L YM broth and incubated at 30 °C, agitated at 150 rpm. After a 7-day of incubation period, the mycelial pellets were separated from the broth via centrifugation (Sorvall-RC3B Plus centrifuge, Thermo Fisher Scientific, Wilmington, DE) at 7,277g for 20 min in a sterilized 1 L polypropylene centrifuge bottle (Nalgene, Nalge Nunc, Rochester, NY) (Shrestha *et al.*, 2008). Next, the mycelial pellets were rinsed with a solution containing 50 mM Phosphate buffer (pH 4.5-4.8), 0.5% (NH₄)₂SO₄ and basal salt solution (0.25 g KH₂PO₄, 0.063 g MgSO₄·7H₂O, 0.013 g CaCl₂·2H₂O, in 1 L water) and 1.25 mL of premix trace element solution (3.0 g MgSO₄·7H₂O, 0.5 g MnSO₄·H₂O, 1.0 g NaCl, 0.1 g FeSO₄·7H₂O, 0.181 g CoSO₄·7H₂O, 0.082 g CaCl₂·2H₂O, 0.1 g ZnSO₄, 0.01 g CuSO₄·5H₂O, 0.01 g Al₂(SO₄)₃·2H₂O, 0.01 g H₃BO₃, and 0.01 g NaMoO₄) in 1 L of deionized water (Shrestha *et al.*, 2009). The mycelial pellets were once more separated from the broth via centrifugation at 7,277g for 20 min in a sterilized 1 L polypropylene centrifuge bottle. The final mycelial mat collected was mixed with an equal volume of the same solution mixture.

S. cerevisiae culture inoculum for the fermentation stage was prepared by growing the stock culture in sterile 50 mL YM broth, in 250-mL Erlenmeyer flasks at 32 °C (120 rpm). After harvesting the yeast cells in 50 mL

conical centrifuge tubes (BD Falcon, BD, Franklin Lakes, NJ) at 2,852 g for 10 min (Beckman J2-21centrifuge, Beckman Coulter Inc., Brea, CA), the cell concentration was adjusted with sterile YM broth to 10^7 - 10^8 CFU/mL as determined turbidometrically at 600 nm (Nguyen *et al.*, 2009).

Filter paper compositional analysis

The compositional analysis of the filter paper used in this study was performed in triplicate via complete enzymatic analysis as described by Selig *et al.* (2008), with minor modifications. Filter paper strips (0.1 g) were soaked in 50.0 mL 0.1 M citrate buffer (pH 4.8) and 1.38 mL (60 FPU/mL) of Spezyme CP (Genencor, Rochester, NY) in a 250 mL flask. Distilled water and 1.0 mL of a 2% sodium azide solution, as microbial inhibitor, was added to bring the total volume in each flask to 100.0 mL. The flask was incubated in an incubator shaker at 50 °C for 5 days for complete hydrolysis of the filter paper.

Solid state fermentation for enzyme induction

Prior to the addition of fungal inoculum for enzyme induction, 2.0 g of shredded filter paper with 5 mL buffered basal salt solution was sterilized at 121 °C for 1 h in loosely mouth covered polypropylene bottles. Then, 2 mL of harvested fungal mycelia in 100 mM phosphate buffer (pH 4.5-4.8), 0.5% (NH₄)₂SO₄ and basal salt solution was added and mixed well using glass marbles. Solid state fermentation was then performed for 4 days at 37 °C, in a humidified incubator, for the production of cellulases and hemicellulases.

Determination of total protein concentration and enzyme activities

Sample aliquots of 1.5 mL were taken from the medium washed fungal grown filter paper (Whatman No. 1, Whatman Inc., Clifton, NJ) at day 4 of solid substrate fermentation for each of the three fungal species treated filter paper. The supernatant was centrifuged at 1,118 g for 5 min (MiniSpin Plus, Eppendorf, Hauppauge, NY) and filtered through a 0.2 µm nylon syringe filter (VWR International, Batavia, IL), and was used to perform total protein analysis and enzyme activities assay.

Protein production by *P. chrysosporium* and *G. trabeum* grown on the filter paper was measured via the NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). This system measures a loading of 2 µL sample size and calculates the protein concentration (mg/mL) from the protein's absorbance at 280 nm (A₂₈₀). A separate fermentation broth from the filter paper control bottle with no fungal culture was used as the blank reading.

The commercial cellulase enzyme (Spezyme CP) was kindly provided by Genencor International (Palo Alto, CA). The cellulase activity was measured using the filter paper activity (FPase) assay, expressed in filter paper units (FPU/mL) according to the standard procedure of the

National Renewable Energy Laboratory (NREL) (Adney and Baker, 2008). This procedure measures the release of reducing sugar produced in 60 min from a mixture of enzyme solution (0.5 mL) and of citrate buffer (0.05 M, pH 4.8, 1 mL) in the presence of 50 mg Whatman No. 1 filter paper (1 x 6 cm strip) and incubated at 50 °C. The released sugars were analyzed by the dinitrosalicylic (DNS) acid reducing sugar assay. One unit of enzyme activity was defined as the amount of enzyme releasing 2.0 mg reducing sugar from 50 mg of filter paper in 60 min has been designated as the intercept for calculating filter paper cellulase units (FPU) by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). All samples were analyzed in triplicate and mean values were calculated.

Simultaneous saccharification and fermentation (SSF)

SSF reactions were carried out in 250 mL polypropylene bottles with batch cultures of 100 mL final volume, consisting of 25 mL 4X Yeast Extract Broth (1.8 g yeast extract, 0.07 g CaCl₂·2H₂O, 0.45 g of KH₂PO₄, 1.2 g (NH₄)₂SO₄ and 0.3 g MgSO₄·7H₂O per liter of deionized water) (Shrestha *et al.*, 2009) buffered basal medium (pH 4.5-4.8) (50 mM Phosphate Buffer + Basal Salt Solution) (Shrestha *et al.*, 2009). For the sample set that was treated with the commercial cellulase enzyme, 25 FPU of Spezyme CP/g cellulose was added. The flasks were then aseptically inoculated with *S. cerevisiae* suspension. Batch culture SSF was performed under static condition for 5 days at 37 °C. All experiments were performed in triplicates.

Total sugars assays

Sample aliquots of 1.8 mL were collected aseptically from each bottle every 24 h. The sample mixtures were centrifuged and filtered through a 0.2 µm nylon syringe filter. The filtered supernatants were tested for total sugars via the phenol-sulfuric (Crawford and Pometto, 1988) method. The total sugar determination was determined via the phenol sulfuric carbohydrate test at 490 nm (SpectraMax Plus384, Molecular Devices, Inc., Sunnyvale, CA, USA) with glucose standards. The equivalent sugar concentration (g/L) was determined based on a standard glucose solution curve that was generated prior to the assays.

High Pressure Liquid Chromatography (HPLC) analyses

Filtered sample aliquots were tested for cellulose, glucose and fermentation products (ethanol, acetic acid, lactic acid) were analyzed by using a Waters High Pressure Liquid Chromatography (Millipore Corp., Milford, MA) equipped with a Waters Model 401 refractive index (RI) detector, column heater, autosampler and computer controller. The separation and analysis of ethanol and other fermentation constituents was done on a Bio-Rad Aminex HPX-87H column (300.0 x 7.8 mm) (Bio-Rad

Chemical Division, Richmond, CA) using 0.012 N H₂SO₄ as a mobile phase with a flow rate of 0.6 mL/min, a 20 µL injection volume and a column temperature of 65.0 °C (Ramos, 2003; Liu *et al.*, 2008; Shrestha *et al.*, 2009). Percentages of theoretical ethanol yields (TEY) were calculated based on a theoretical ethanol yield of 56.8 g per 100 g of cellulose (Doran and Ingram, 1993).

$$\text{Theoretical ethanol yield (\%)} = \frac{\text{Ethanol produced (g)}}{\text{Initial cellulose (g)} \times 0.568} \times 100$$

Statistical analyses

The experimental data were analyzed statistically using the statistical software, JMP 8.0 (SAS Institute Inc., Cary, NC). The data (n=3) on ethanol production were fitted to non-linear polynomial (2nd degree) models. Error bars were determined based on the standard deviation from the mean values. Student's t-test for significant differences were also performed for all final data set to determine multiple comparisons of the ethanol production based on the different fungal treatments. A *p*-value of less than 0.05 was considered significantly different.

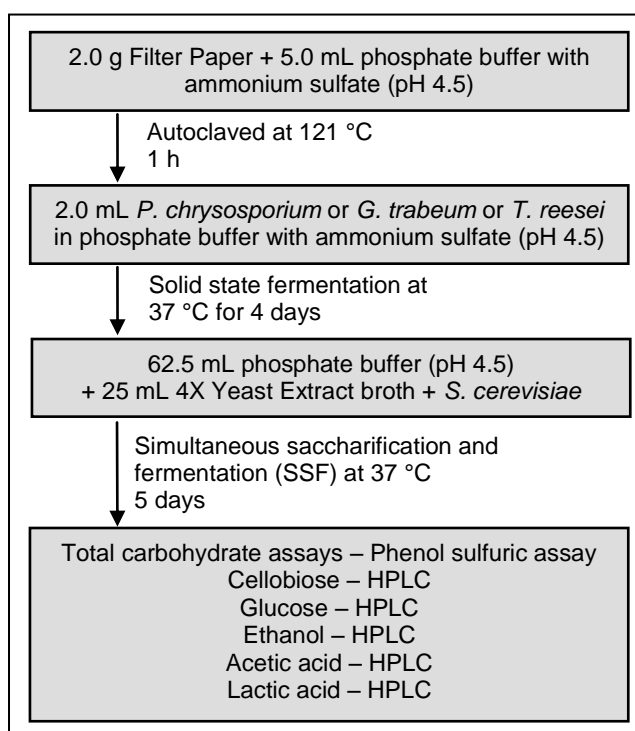
RESULTS AND DISCUSSION

Cellulose degrading microorganisms hydrolyze cellulose using complicated consortia of different enzymes that work individually, but synergistically on the cellulose, converting it to cellobiose and glucose (Henriksson *et al.*, 1999). This group of enzymes is produced by a wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles (Bhat and Bhat, 1997). However, only few of these microorganisms produce a complete cellulase complex and significant levels of extracellular cellulase capable of efficient depolymerization and solubilizing lignocellulosic biomass (Ahamed and Vermette, 2008).

These cellulolytic enzymes are inducible enzyme systems (Suto and Tomita, 2001; Ling *et al.*, 2009). The induction process hypothesizes that basal levels of cellulase that is constitutively produced by fungi first hydrolyses cellulose to soluble oligosaccharides or their derivative sugars that is then absorbed into the cells, ultimately becoming the actual inducers (Lynd *et al.*, 2002; Ling *et al.*, 2009). In the case of *Trichoderma*, the conidial bound cellobiohydrolase hydrolyses the cellulose chains, liberating cellobiose and cellobiono-1,5-lactone (CBL) that are then taken up by the mycelia and promote further cellulase expressions (Bhat and Bhat, 1997; Suto and Tomita, 2001).

We chose filter paper as the cellulosic starting material because of its high cellulose and low impurities content (www.whatman.com). From the results of the total enzymatic analysis done on the filter paper, the content of the filter paper was approximately 98.0% cellulose. Because of its high cellulose purity, filter paper contains

no lignin or other inhibitory compound that may inhibit the fermentation of the glucose released into ethanol, or interfere with other analyses. It was also used in previous fungal enzyme induction studies (van Wyk, 1999), largely due to its Crystallinity index (CrI) of 0.45, that is within the range of susceptible cellulosic substrates of 0.4-0.7 like other pretreated biomass, and its degree of polymerization (DP) of 750-2800 that is also very close to conventional pretreated cellulosic substrates of 400-1000 (Zhang *et al.*, 2006). In fact, it is the material chosen by NREL for standardized method of cellulase activities measurement (Decker *et al.*, 2003; Adney and Baker, 2008).



^a n.d. – non detected

Figure 1: Flow-chart of process outlining the steps for solid state fermentation of *P. chrysosporium* or *G. trabeum* or *T. reesei* on filter paper, followed by SSF using *S. cerevisiae* as the fermenting organisms.

The general outline of our study is shown in Figure 1. While many studies have been done on *P. chrysosporium*, *G. trabeum* and *T. reesei* to produce various cellulases, hemicellulases and lignolytic enzymes, and their direct cellulose hydrolysis activities, only few have reported their coupled applications in SSF (van Wyk, 1999; Decker *et al.*, 2003; Cohen *et al.*, 2005; Shrestha *et al.*, 2008; Shrestha *et al.*, 2009; Rasmussen *et al.*, 2010). Therefore, our study was extended to further examine the efficiencies of the respective fungal species and their enzymatic mechanisms on high cellulose feedstock, such as filter paper, in the presence of *S. cerevisiae*, as the fermenting organism. To achieve this, we performed SSF on the filter paper and measured the final fermentation products via

HPLC. This technology combines continuous enzymatic hydrolysis of cellulose with the simultaneous fermentation of the sugars released to ethanol via a chosen fermenting microorganisms (i.e. the yeast *S. cerevisiae*), in a single reactor (Ballesteros *et al.*, 2004). We also prepared a separate sample set that was added with the commercial cellulase enzyme, Spezyme CP, at a low dose of 25 FPU/g cellulose, as a control. The combination of Spezyme CP and *S. cerevisiae* yielded 47.91 g/100 g filter paper of ethanol (84.35% theoretical).

Table 1: Enzyme activity and total protein assays (n=3)

	Protein Assay (mg/mL) ^a	Enzyme Assay (FPU/mL) ^b
<i>P. chrysosporium</i>	10.52 ± 0.78	0.76 ± 0.01
<i>T. reesei</i>	10.67 ± 0.10	1.76 ± 0.03
<i>G. trabeum</i>	10.04 ± 0.19	1.52 ± 0.02

^a Protein was determined by NanoDrop™ 1000 Spectrophotometer.

^b Filter paper unit activities (FPase) based on the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper, at 4% conversion, in 1 h (units FPU/mL)

From Table 1, the results of the total protein assay using the NanoDrop™ 1000 spectrophotometer showed that the highest protein concentration of 10.67 mg/mL was produced in the sample treated with *T. reesei*, followed by

10.52 mg/mL in the sample treated with *P. chrysosporium* while in the sample treated with *G. trabeum*, the concentration was at 10.04 mg/mL. We then determined the enzyme activities based on the filter paper units (FPU), as described previously (Ghose, 1987; Adney and Baker, 2008). The result from FPase assays from the induction experiments indicated that cellulase activities were highest in the sample treated with *T. reesei*, at 1.76 FPU/mL. The sample treated with *G. trabeum* had a lower protein activities value of 1.52 FPU/mL and the sample treated with *P. chrysosporium* had the lowest FPase activities of 0.76 FPU/mL. This trend is expected as *T. reesei* have been known to produce high concentration of potent cellulases (Jovanovic *et al.*, 2009), and in fact, this fungus serves as a reference organism for cellulose degradation studies and for the mass production of cellulases and hemicellulases for various applications (Martinez *et al.*, 2008).

During the five-day SSF period, total sugar production was recorded. From Figure 2, residual total sugar remained at a very steady level for all the samples treated with the three different fungi. The concentration was at 0.678 g of total sugar per 100 g of filter paper at day 0 and by day 5, the concentration ranged from 0.663–1.692 g of total sugar per 100 g of filter paper. The total sugar profile for the Spezyme control showed a sharp increase in day 1, followed by a sharp decrease in day 2.

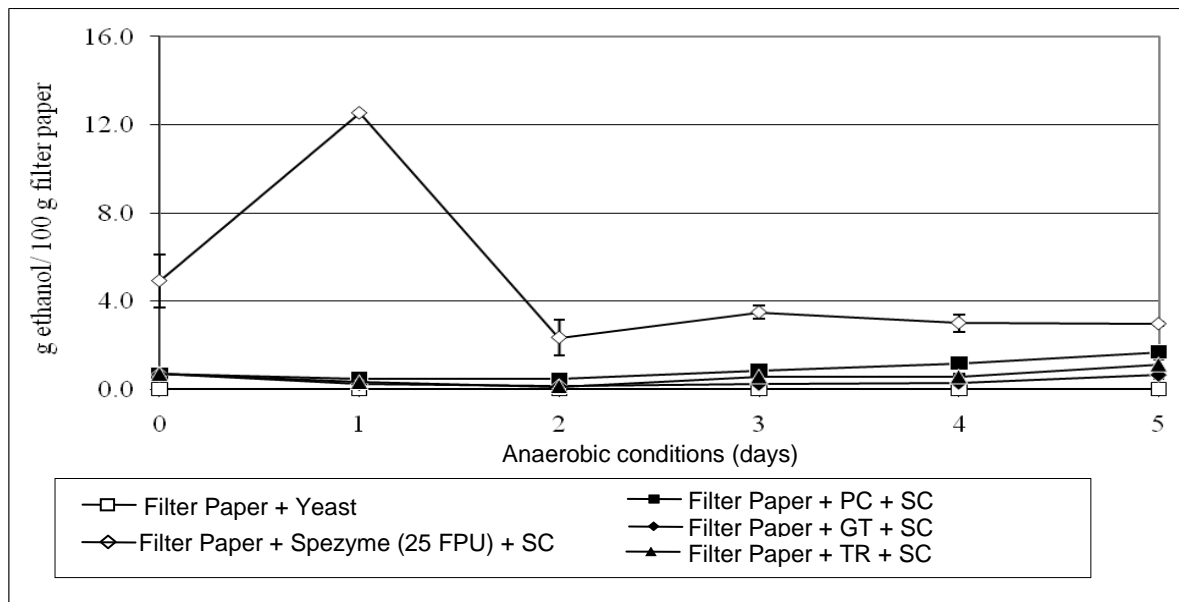
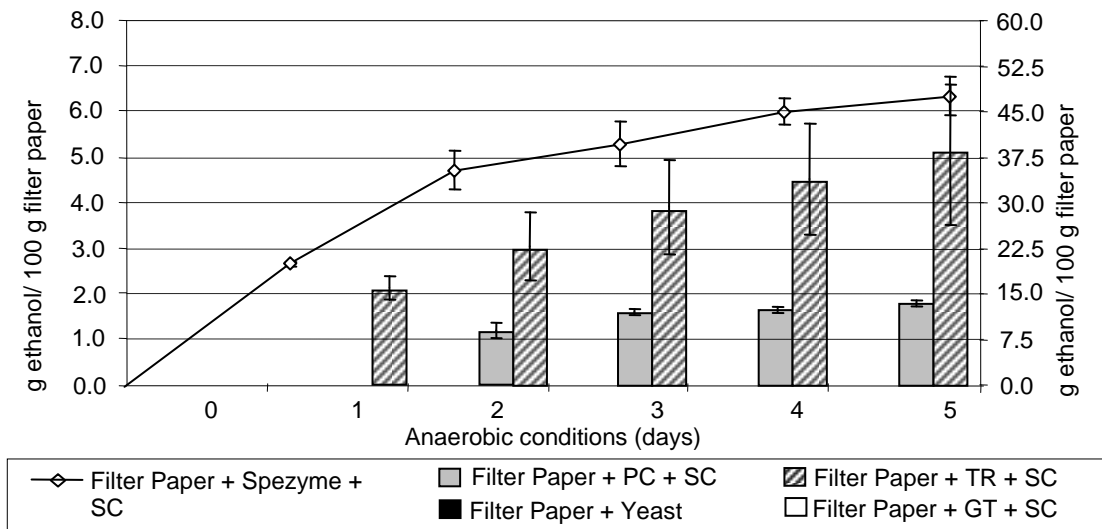


Figure 2: Time course of total sugar production, as determined via the phenol-sulfuric method. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisiae*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium*, *G. trabeum* or *T. reesei*).



1

Figure 3: Time course of ethanol production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisiae*. Left y-axis represents the bar charts, Right y-axis represents the line regression. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium*, *G. trabeum* or *T. reesei*).

From Figure 3, ethanol production was highest for the filter paper inoculated with *T. reesei*. Ethanol production was in steady increments even during the final day of experiment at day 5, with the concentration values of 5.13 g/100 g filter paper, corresponding to 9.03% TEY (Table 2). The filter paper inoculated with *P. chrysosporium* was at 1.79 g/ 100 g filter paper (3.15 % TEY). In comparing the results of the ethanol production at day 5, the FPU values between *T. reesei* and *P. chrysosporium* treated filter paper reflects the final ethanol concentration. Higher enzymatic activities in *T. reesei* resulted in more ethanol production, and in fact the difference of approximately 286%. Another explanation to the lower ethanol yield in the sample treated with *P. chrysosporium* is the possibility incomplete hydrolysis of the cellulose to glucose. Statistic analyses validated the significance of these results (Figure 4).

Table 2: Cellulose conversion and theoretical ethanol yield at day 5 (n=3)

	Cellulose Conversion (g / 100 g filter paper)	Theoretical ethanol yield (%)
<i>P. chrysosporium</i>	1.79	3.15
<i>T. reesei</i>	5.13	9.03
<i>G. trabeum</i>	n.d. ^a	n.d. ^a
Spezyme (25 FPU/g cellulose)	47.91	84.35

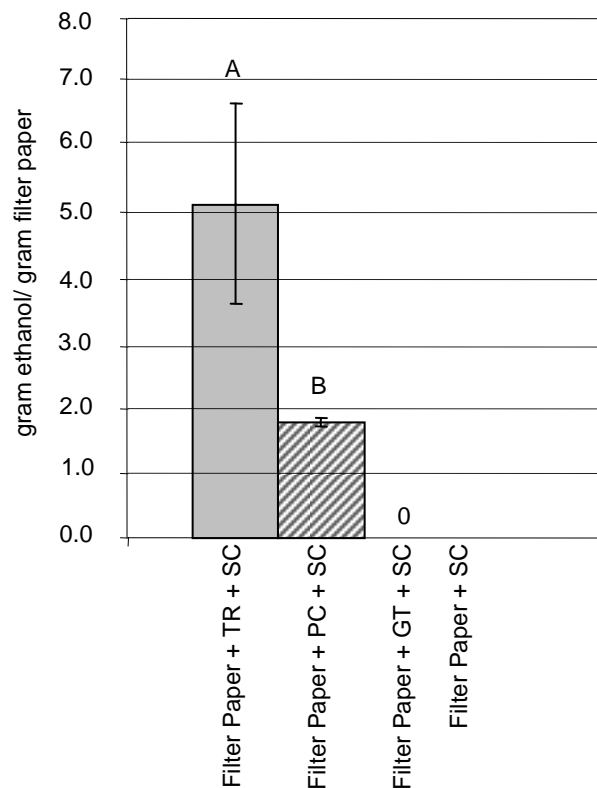


Figure 4: Maximum ethanol yields of different fungal treatments conditions. Letters on top of the columns indicate significant differences (Student's t test, $\alpha=0.05$).

Unlike the previous two fungi, the data (Table 2, Figure 3 and Figure 5) showed that none of the samples inoculated with *G. trabeum* produced any fermentation products (ethanol, acetic acid and lactic acid), suggesting that *G. trabeum* may not be an effective fungus for the use

in the hydrolysis of pure cellulose, albeit to its highly documented potent cellulolytic enzyme systems on other substrates (Cohen *et al.*, 2005; Daniel *et al.*, 2007). There are several possible explanations to these observations.

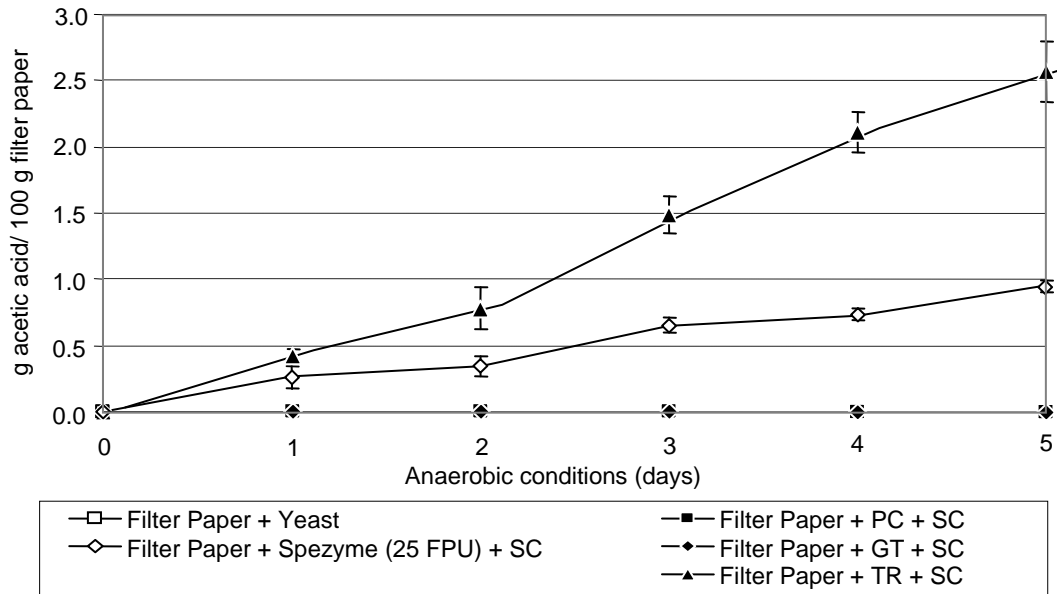


Figure 5: Time course of acetic acid production. The data points represent the averages of three independent experiments (n=3). Note: PC – *P. chrysosporium*, TR – *T. reesei*, GT – *G. trabeum*, SC – *S. cerevisiae*. Time zero is after 4 days of solid state fermentation with a specific fungus (*P. chrysosporium*, *G. trabeum* or *T. reesei*).

Firstly, *G. trabeum* is reported to lack the complete combination of the enzymes needed for efficient cellulose hydrolysis on pure cellulose (Mansfield *et al.*, 1998; Cohen *et al.*, 2002). Unlike the cellulases of *T. reesei*, *G. trabeum* lacks cellobiohydrolases, although endoglucanases were detected (Henriksson *et al.*, 1999). This is an important finding as, in many cases, CBHs are also more efficient on cellulose than EGs (Henriksson *et al.*, 1999). However, brown-rots compensate the lack of processive cellulases by degrading biomass largely through non-enzymatic mechanisms, via a hydroquinone-driven system for the production of extracellular reactive oxygen species (ROS) in an 'enhanced' Fenton system (Paszczynski *et al.*, 1999; Cohen *et al.*, 2002). The Fenton system plays an extremely important role in the early stages of cellulose degradation by brown-rot fungi. However, this reaction only occurs under favorable conditions, catalyzed by a low-molecular-weight peptide, termed Gt factor (Wang and Gao, 2003). According to Xu and Goodell (2001), these conditions must include the presence of iron, hydrogen peroxide, biochelators, oxalate and light. Iron is present in woody biomass as bound iron and ferric hydroxide complexes. However, in our experiment, the absence on iron on the highly cellulose-pure filter paper may have adverse effect on the natural iron dependent hydrolytic processes.

Secondly, another study done by Cohen and colleagues (2005) added that the cellulolytic system of *G.*

trabeum may hydrolyze amorphous cellulose but not crystalline cellulose. However, in the degradation of amorphous cellulose, hydrolysis is partial with the end product being cellotriose instead of glucose, a phenomenon also reported in other microorganisms (Reese *et al.*, 1959; Lejuene *et al.*, 1988). This same observation is supported by our result with the negative glucose reading in all samples inoculated with *G. trabeum*. This is further supported by another related work done by Schilling and colleagues (2009) that observed the difficulties of brown-rot fungi in metabolizing lignin-free microcrystalline cellulose.

The production of other fermentation co-products, such as, acetic acid and lactic acid were also recorded. No lactic acid was produced by any of the samples at the end of the five-day experiments. Acetic acid was only detected in the samples inoculated with *T. reesei* at 2.57 g/100 g filter paper (Figure 5). This trend is supported by other studies that documented high production of acetic acid by *T. reesei* (Chambergo *et al.*, 2002; Shrestha *et al.*, 2009). This is due to the enzymatic actions of the two paralogous genes for aldehyde dehydrogenase (*ALD1* and *ALD2*) capable of converting acetaldehyde to acetate, present in the *T. reesei* genome (Chambergo *et al.*, 2002; Shrestha *et al.*, 2009). Furthermore, other genes such as acetyl esterases are also reported to function in the same manner and interestingly, these genes interact with other cellulases for the production of acetates from other

biomass (Harrison *et al.*, 2002). Acetic acid (0.95 g/100 g filter paper) was also detected in the sample treated with Spezyme CP (Figure 5).

By comparing these three fungal species, our study suggests that the most efficient fungal species in saccharifying pure cellulose was *P. chrysosporium* followed by *T. reesei*, while *G. trabeum* failed to effectively liberate fermentable product. *P. chrysosporium* is worth noted as not only it is the cellulolytic enzymes system efficient, but it offers greater flexibilities when lignocellulosic biomass is the feedstock for ethanol production. This is because *P. chrysosporium* also harbors lignolytic enzyme that may be advantageous in eliminating a major inhibitor in conventional SSF, which is lignin (Ballesteros *et al.*, 2004).

In conclusion, the results from our study of the solid state fermentation of cellulose-rich filter paper for the production of ethanol indicated that the fungal species *P. chrysosporium* and *T. reesei* are potentially useful for this form of application. Further experimentation may be done by inoculating these two species onto more complex feedstocks that are lignin rich, such as switchgrass, corn stover and other perennial grasses, to evaluate their enzymatic efficiencies against more recalcitrant feedstocks and the presence of potential inhibitors (Varga *et al.*, 2004; Wyman *et al.*, 2005). Direct fungal enzymatic saccharification mechanisms for SSF are indeed very promising and can lead to a more environmentally friendlier processing, whereby ethanol producers can skip or minimize the environmentally detrimental pretreatment steps. This will ultimately lead to a more economically sound ethanol production when manufacturers can produce their own enzymes *in situ* to supplement the use of expensive commercial preparations.

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