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Evaluation of Using Three Trichoderma Species for Increasing Cellulose Activity in Feed Stock for the production of Bio Fuels

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Abstract

Biomass from plant sources is a significant source of liquid fuels. The literature with studies concerning the importance of fungi on the degradation of biomass is ample. Very few studies document the bioengineering of fungi specifically for purposes of increasing the activity of cellulase for pretreatment of biological waste. The aim of this study is to begin to evaluate organisms engineered for high cellulase activity for purposes of pre-processing cellulosic based waste. Three soft rot species, *Trichoderma viride*, *Trichoderma reesei*, and *Trichoderma longibrachiatum* were selected for this study. Pretreatment of filter paper was conducted to enhance cellulase production. The filter paper was incubated in various concentrations of HCl and NaOH which have been recognized as cellulase enhancers. Commercial cellulases from three Trichoderma species were purchased. Protein samples were prepared for SDS Page and silver staining to verify the presence of cellulases from these Trichoderma species. The results indicated that cellulase was present with a predicted cellulase protein size of 50 kDa. A filter paper assay was conducted to measure total cellulase activity. The amount of glucose released from 50 mg of filter paper was calculated. Assay incubation time was 60 mins at 50° C. Absorbance was read at 540 nm. The results will provide valuable information for pretreatment options for the biomass currently used by Company X as well as reduce the initial cost for processing feedstocks.

Keywords: Trichoderma, Cellulase, Fungi, SDS Page, Soft Rot

1.0 Introduction

It has previously been documented that biomass from plant sources are available in great abundance and is a significant source of liquid fuels and valuable chemicals. When these non-edible biomasses fail to exhibit economic use, it is then deemed as waste product. This waste is desired for conversion into liquid fuels, and valuable chemicals, due to the fact that it is less expensive and does not compete with human and animal food sources (O'Connor *et al*., 2011).

Company X Inc. is a next-generation, fast paced, renewable fuels company based in Texas. The operations of Company X result in economic growth in rural regions, as well as the reduction of greenhouse gas emission by over 80% (Company X, 2013). The non-edible biomass utilized by Company X contains large amounts of compacted lignin, which shields hemi-cellulose and crystalline cellulose, making it less susceptible to enzymatic and chemical conversion (Ryu *et al*., 2011). For this particular experiment, our aim is to evaluate organisms engineered for high cellulase activity for purposes of pre-processing cellulosic based waste. Currently, our lab is thought to have the ability to alter fungi by increasing cellulase activity for the pretreatment of hardwood used by Company X. Three Trichoderma species were selected for further study based on literature, commercial availability, and ability to degrade the same Southern Yellow Pine hardwood that Company X uses. It is believed that chemically manipulating and modifying potential fungi for increased cellulase activity will result in decreased time, production cost, and energy

needed in Company X processing.

2.0 Materials and Methods

Parameters were changed, such as pretreating substrate to enhance desired activity, to increase cellulase production from commercial enzymes. *T. reesei, T. viride*, and *T. longibrachiatum* samples were prepared for SDS Page by mixing a 1:2 dilution of enzyme/ sample buffer in a 0.2 ml micro- centrifuge tube. Proteins were denatured at 95° C for 5 minutes and placed on ice for 5 minutes. Samples were electrophorized on a 4-20 % Tris- HCL, 0.02% NaN3 gel, in 1x SDS running buffer (25 mM Tris- HCL, 200 mM Glycine and 0.1% (w/v) SDS) for 1 hour on 100 V.

Silver staining was used as the general detection method for proteins. A Bio-Rad Silver Stain kit (Bio-Rad) was used and manufacturer's instructions were followed.

Experimental reagents were prepared according to the standard FPA published by International Union of Pure and Applied Chemistry. The sample was incubated at 95° C for 5 minutes. Thirty-six ìl of each sample was transferred to a microplate along with 160 ìl of H2O. Samples were measured at an absorbance of 540 nm on an iMark microplate reader (Bio-Rad). All experiments were conducted in triplicate.

The effects of NaOH and HCl on cellulase production were observed by applying various concentrations of each reagent to Whatman No.1 filter paper as a chemical pretreatment. The filter paper was soaked in the following concentrations of NaOH; 1%, 2%, 3%, and 4%. As a separate experiment, the filter paper was soaked in the following concentrations of HCl; 1%, 2%, 3%, and 4%. The filter paper along with the reagents was incubated at room temperature for 2 hours (Irfan *et al*., 2010). The filter paper assay was next conducted as previously described.

3.0 Results

Protein samples were prepared for SDS Page and silver staining to verify the presence of cellulase proteins. Figure 1 depicts a protein gel analysis of the cellulase enzymes from *T. longibrachiatum*, *T. reesei*, and *T. viride*. The presence of the cellulases was indicated on the gel with bands showing a predicted protein size of 50 kDa (lanes 3, 5, 7). The image was taken with the BioRad gel doc EZ Imager.

A standard DNS curve showing the release of glucose during FPA was conducted to determine the dilution of enzyme that releases approximately 0.128 mg of glucose (Figure 2). Absorbances obtained from various amounts of glucose were measured with a BioRad Microplate Absorbance Reader. Various amounts of *T. reesei* cellulase were used; 0.5 mg, 0.2 mg, 0.66 mg, and 0.05 mg. There was a linear increase in absorbance as the glucose standard amounts increased.

T. reesei released a glucose amount of 3.87 mg from 0.5 mg of cellulase. An amount of 1.2 mg of glucose was released from 0.2 mg of cellulase. Likewise, 0.66 mg and 0.127 mg of glucose was released from 0.1 mg and 0.05 mg of cellulase respectfully. Glucose amounts from the DNS curve were plotted against cellulase concentrations to determine which concentration would produce approximately 0.128 mg of glucose (Figure 3). A cellulase amount of 0.05 mg produced 0.127 mg of glucose.

Pretreatment of cellulosic substrate was conducted to enhance cellulase production. Pretreatment of filter paper with various concentrations of HCl and NaOH resulted in greater FPU Activity when compared to filter paper without HCl/ NaOH; therefore, increasing cellulase (Figure 4). Filter paper without chemical pretreatment resulted in 0.99 FPU/ml. Filter paper that was pretreated with 1%, 2%, 3%, and 4% HCl resulted in 1.51 FPU/ml, 1.36 FPU/ml, 1.56 FPU/ml, and 1.46 FPU/ml respectfully. Filter paper that was pretreated with 1%, 2%, 3%, and 4% NaOH resulted in 1.75 FPU/ml, 2.08 FPU/ml, 2.36 FPU/ml, and 1.56 FPU/ml respectfully. Both HCl and NaOH enhanced cellulase activity at 3%; with NaOH producing a greater cellulase yield than HCl.

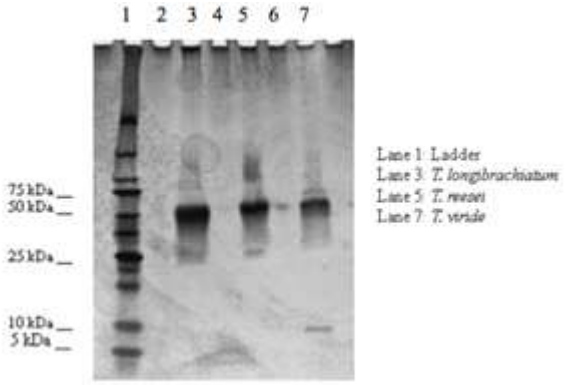

Figure 1: SDS Page of Trichoderma cellulases. Samples were subject to electrophoresis on a 4-20% Tris-HCl gel.

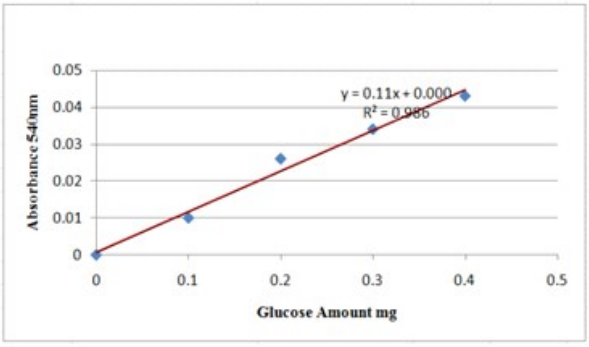

Figure 2: Standard DNS Curve for Measuring the Release of Glucose from *T. reesei* Cellulase during FPA. The image shows the release of glucose standards during FPA. The equation given was used to determine the amount of *T. reesei* cellulase that will release approximately 0.128 mg of glucose. Absorbances obtained with various amounts of glucose were measured with a BioRad Microplate Absorbance Reader.

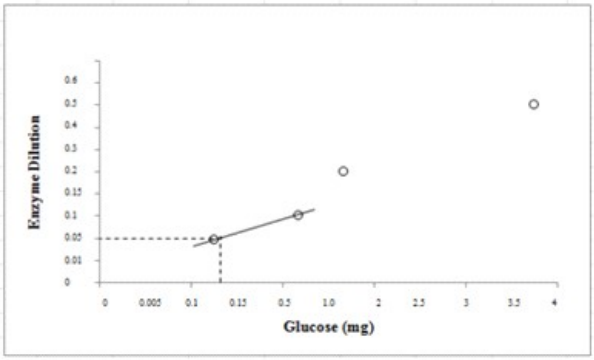

Figure 3: Determination of the Concentration of Cellulase from *T. reesei* that will Release Approximately 0.128 mg of Glucose. Glucose amounts obtained from the DNS curve were plotted against enzyme concentrations to determine the enzyme concentration that will release approximately 0.128 mg of glucose. Samples were from *T. reesei* cellulase.

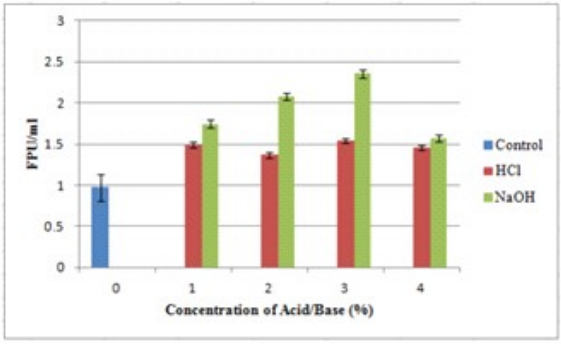

Figure 4: *T. reesei* Cellulase Activity from HCl/ NaOH Pretreated Filter Paper. The figure shows the effects of different concentrations of HCl/ NaOH pretreatment on glucose production from *Trichoderma Reesei* following the FPA.

4.0 Discussion

T. reesei, T. viride, and *T. longibrachiatum,* were chosen based on their ability to degrade cellulose into glucose. Based on literature they produced a FPU/ml of 2.8, 3.18, and 3.86 respectfully, making them excellent degraders of cellulosic waste. For this reason, *T. reesei, T. viride*, and *T. longibrachiatum* are thought to exhibit greater cellulase activity.

Pretreatment of cellulosic substrate was conducted to enhance cellulase production. Pretreatment of filter paper with various concentrations of HCl and NaOH resulted in greater FPU Activity when compared to filter paper without HCl/ NaOH; therefore, increasing cellulase (Figure 4). Filter paper without chemical pretreatment resulted in 0.99 FPU/ml. Filter paper that was pretreated with 1%, 2%, 3%, and 4% HCl resulted in 1.51 FPU/ml, 1.36 FPU/ml, 1.56 FPU/ml, and 1.46 FPU/ml respectfully. Filter paper that was pretreated with 1%, 2%, 3%, and 4% NaOH resulted in 1.75 FPU/ml, 2.08 FPU/ml, 2.36 FPU/ml, and 1.56 FPU/ml respectfully. Both HCl and NaOH enhanced cellulase activity at 3%; with NaOH producing a greater cellulase yield than HCl. Irfan *et al*., 2010, conducted an experiment to compare the effects of different concentrations of HCl/ NaOH on CMCase production from *T. viride*. His results showed that CMCase production had greater yield with 2% HCl and 4% NaOH. Overall, greater amounts of cellulase

were produced with HCl pretreatment. Although our results are slightly different from previous works, our findings were in agreement with Irfan *et al*., concerning the increase of cellulase production from pretreatment of substrate with HCl and NaOH.

The filter paper was incubated in various concentrations of HCl and NaOH because of their recognition as cellulase enhancers, and their ability to delignify the substrate. The larger the amounts of delignification of the samples, the more cellulose can be exposed to microbes, resulting in greater cellulase production. Cellulase enhancement by HCl pretreated filter paper may be due to rapid hydrolysis of the components of the cell wall into free sugars which are available to the organism. Likewise, cellulase enhancement of NaOH treated filter paper may be due to alkaline properties during treatment (Irfan *et al*., 2010).

Although this particular assay is widely used to measure total cellulase, it is very time consuming due to many manual manipulations. This method has been recognized for its complexity and operators error (Xiao *et al*., 2004). Inconsistencies in folding of filter paper, size, shape and placement, may result in errors. In addition, evaporative losses can result in significant error. The method also requires large amounts of reagents and is very laborious. The FPA method requires a long assay time and because exact dilutions are desired, several trials are needed to produce accurate activity measurements.

5.0 Conclusion

The *objective* of this study was to begin the process of altering fungi by enhancing cellulase activity for the pretreatment of hardwood used by Company X. The Trichoderma genus was selected for further study based on literature and the ability to degrade Southern Yellow Pine hardwood. *T. reesei* was selected for the FPA because of its commercial availability. We hypothesized that pretreatment of substrate with various concentrations of HCl/ NaOH would result in increased cellulase production from *Trichoderma reesei*. Our hypothesis was proven precisely, with *T. reesei* cellulase showing greater yield with both 3% HCl and 3% NaOH when compared to non pretreated filter paper. Due to these findings, it is believed that chemically manipulating and modifying *T. reesei* for increased cellulase activity will result in decreased time, production cost and energy needed in Company X processing. Future work will consist of combining a 1:1 dilution of HCl and NaOH to filter paper at the same time, in expectation of enhancing FPU/ml.

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