2015

The Impact of Electron Beam Pretreatment on the Fermentation of Wood-based Sugars

Charlene Grabowski

The Impact of Electron Beam Pretreatment on the Fermentation of Wood-based Sugars

by

Charlene Grabowski
Candidate for Bachelor of Science
Department of Chemistry
With Honors

May 2015

APPROVED

Thesis Project Advisor: ______________________________
Arthur J. Stipanovic, Ph.D

Second Reader: ______________________________
William M. Shields, Ph.D

Honors Director: ______________________________
William M. Shields, Ph.D.

Date: ______________________________

ABSTRACT

Lignocellulosic biomass has the potential to become an integral feedstock in the production of alternative fuels. The presence of a recalcitrant matrix of polymeric materials consisting of cellulose, hemicellulose, and lignin complicates the retrieval of valuable constituent sugars. Effective and efficient pretreatment is required to reduce this recalcitrance. Electron beam (EB) pretreatment is an option which has been shown to enhance the liberation of sugars from hardwood biomass and enhance the fermentation of wood-derived sugars, as opposed to other pretreatment options which create inhibitory by-products. In this study, untreated white pine extract was shown to inhibit ethanol production, and EB pretreatment was seen to mitigate this effect. Sugar yields from the saccharification of white pine treated with EB were much higher than untreated samples, with samples treated with high dosages approaching 40% conversion of cellulose after 24 hours. Additionally, the presence of EB pretreated biomass was seen to inhibit the growth of *P. stipitis* under fermentation conditions. The enhancement effect previously seen in hardwoods was explored with respect to soluble ion concentration and lignin content, and a link was established suggesting a higher content of both components enhance fermentation.

TABLE OF CONTENTS

List of Figures

List of Tables

1. INTRODUCTION

1.A. BACKGROUND

With the necessity to provide an alternative to fossil fuels becoming increasingly urgent, the fermentation of sugars extracted from woody biomass has been a popular topic among renewable energy experts in recent years. The issue is an interdisciplinary concern, with broad environmental, political, and economic implications. Major environmental concerns about current fossil fuel use are the expulsion of greenhouse gas emissions upon combustion and the sustainability of fuel sources. Cellulosic ethanol reduces harmful emissions of greenhouse gases up to 85% compared to petroleum, and sources are inherently renewable.[1] The EPA's Renewable Fuel Standard (RFS) mandates a minimum of 36 billion gallons of cellulosic ethanol to be produced and used by the year 2022 in an effort to reduce the dependence on fossil fuels in America. The EPA also sets and enforces limitations on fossil fuel production, processing, and use. Biomass-derived fuels also see economic advantage compared to fossil fuel recovery and refining, with some specialized pretreatments requiring little energy input.[2]

Ethanol production from woody biomass does not come without limitations. A major obstacle in the process is the complex nature of the material, being composed of cellulose, hemicellulose, lignin, and inorganic materials, bound by a complicated physical and chemical matrix; collectively, the resistance of wood to conversion is termed recalcitrance.[2] This recalcitrance evolved for the protection of the plant from biological degradation.[3] Recalcitrance is a physical and chemical barrier which hinders the retrieval of the sugars required for the fermentation to bioethanol.[2] This creates the need for effective and efficient pretreatment techniques to reduce recalcitrance prior to saccharification (conversion of polysaccharides to constituent sugars) and fermentation of the sugars to ethanol.

1.B. Biomass Pretreatment and Fermentation

Various pretreatment options are being studied with respect to their efficiency, efficacy, and cost-benefit analysis due to the fact that lignocellulosic ethanol is inherently more expensive and labor-intensive to produce than grain-derived ethanol.[4] However, since pretreatment must be vigorous, many options create by-products which may be inhibitory to fermentation and/or saccharification. The effect of high energy electron beam (EB) pretreatment has been studied by many groups, and EB pretreatment has repeatedly been shown to enhance the rate and yield of glucose release from cellulosic substrates by enzymes.[5,6,7]

EB pretreatment uses accelerated beams of electrons to locally irradiate woody material in order to disrupt the structure of cell wall polymers (lignin, cellulose, hemicellulose) by producing free radicals, inducing cross-linking or chain scission, decrystallization, and/or decreasing the degree of polymerization.[8] The EB pretreatment method is a cost-effective, efficient, and clean technique. During EB pretreatment, no chemicals are used as solvents or stabilizers, and it thus requires no waste management. EB pretreatment also requires no special temperature, pressure adjustments, or atmospheric, and after pretreatment, no chemical workup is required. EB accelerators are currently commercially available for sterilization, curing, and other purposes.[9] These advantages, along with its effectiveness in reducing recalcitrance establish EB as a valuable pretreatment option.

Other pretreatment methods used in this study are biodelignification and hot water extraction (HWE). Biodelignification is the use of living organisms to begin to break down lignin polymers. This technique is used because it does not require any additional chemicals, is a low-energy process, and produces no hazardous waste; however, using this technique is very time consuming.[10] HWE is the use of high pressure to maintain liquid water at high temperatures (120-200°C) to quickly hydrolyze hemicellulose, and produces few inhibitory by-products.[8]

Fermentation is the metabolic process by which sugars are converted to products such as acids, gases, and/or alcohol. In the field of biofuels, the most commonly studied fermentation process is the conversion of glucose liberated from cellulose to ethanol. However, because other sugars contribute to a significant amount of biomass, conversion of these sugars to value-added products must likewise be investigated. Hemicellulose, which constitutes 25-35% of woody biomass, contains a large portion of xylose, a fermentable five-carbon sugar. A few studies have investigated the potential of utilizing xylose-fermenting organisms, such as *Pichia stipitis*, to advance the full exploitation of biomass polymers. Co-cultures with *Saccharomyces cerevisiae* have been successfully created to simultaneously ferment both glucose and xylose to ethanol.[11] *P. stipitis* has been seen to have the ability to produce significant quantities of ethanol, e.g. on a g/L scale.[12]A few studies have explored the effect of pretreatment options such as acid and base hydrolyses on the fermentation of xylose by *P. stipitis*, with acid hydrolysis causing significant inhibition and base hydrolyzed biomass providing ethanol yields close to maximum conversion.[13,14] Currently, there are no publications on the fermentation of electron beam pretreated biomass-derived sugars by *P. stipitis*.

1.C. PRECURSORY RESEARCH AND PROJECT OBJECTIVES

Previous studies in this lab have shown electron beam (EB) irradiation to be an effective pretreatment in reducing recalcitrance in maple wood. This research has also shown that EB pretreatment enhances the rate and yield of the conversion of wood-based polymers to sugars.[8] A consistent increase in sugar conversion by cellulase enzymes has been observed, as seen in **Figure 1.1** EB radiation, being a high energy process, is known to cause bond cleavage in chemicals and produce free radicals, as well as breaking down cellulose and lowering its crystallinity.[15, 16] These chemical changes can be the cause or partial cause of this effect.

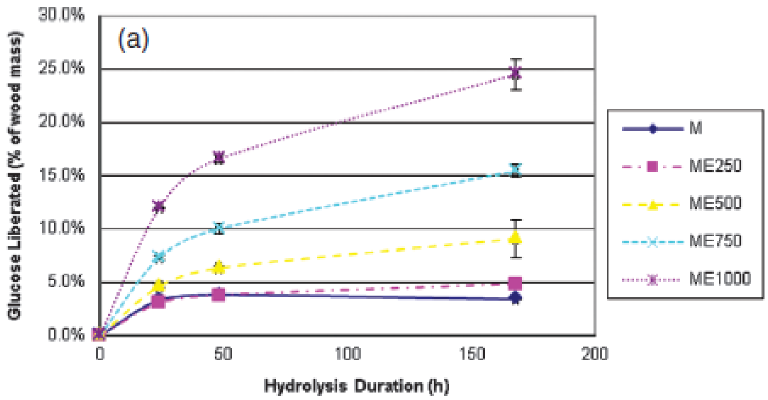


Figure 1.1 The effect of EB pretreatment on the conversion of biomass (maple wood) to sugars. M is the control, and ME is maple wood pretreated with EB, followed by the dosage in kGy.[8]

In currently unpublished results, a significant (up to 40%) enhancement is seen in the fermentation of glucose with the presence of extract from HWE of untreated and EB-pretreated hardwoods. Conversely, the presence of extract from untreated white pine, a softwood, inhibited fermentation of glucose.

In this study, the impact of EB pretreatment on the rate of saccharification of EB pretreated white pine is investigated, as well as glucose fermentation in the presence of EB-pretreated white pine extract. Additionally, the impact of the presence of EB pretreated maple wood on the rate of xylose fermentation is measured. Finally, because the exact cause of the previously observed hardwood extract fermentation enhancement effect is still unknown, enhancement with respect to soluble metal ion concentration and lignin content is explored.

2. METHODS

2.A. MATERIALS

Debarked wood chips from sugar maple (*Acer saccharum*) and white pine (*Pinus strobus*) from SUNY-ESF properties in New York were kindly provided by the SUNY-ESF Department of Paper and Bioprocess Engineering. Samples were approximately 30 mm x 30 mm x 5 mm and milled using a Thomas ® Wiley Mill with a 30-mesh sieve after electron beam pretreatment.

Yeast nitrogenous base without amino acids was purchased from Sigma Aldrich. *Pichia stipitis* (ATCC 58785) freeze-dried culture was purchased from ATCC, *Saccharomyces cerevisiae* (Ethanol Red) was kindly donated by Fermentis. Xylose (manufactured by Tokyo Kasei) was purchased from the ESF chemical stockroom and D-glucose (manufactured by Amresco) purchased from Fischer Scientific. Cellic® CTec2 cellulases were generously donated by Novozymes.

2.B. Pretreatment

High energy electron beam irradiation was performed by the NEO Beam Facility at Kent State University in Kent, OH. Wood samples (100 g each) were placed in polyethylene bags and irradiated in 250 kGy (J/kg) dosages to achieve 250, 500, 750, and 1000 kGy treatments. Irradiation was performed in air.

After electron beam pretreatment and milling, hot water extraction (HWE) was performed. For each batch, 27 g of each dosage (including untreated) was placed in 0.3 L of deionized water and then heated to 121°C at 15 psi for 40 minutes in an autoclave. After hot water extraction, solids were filtered out through a 0.45 μm PVDF filter, and the extracted "tea" was lyophilized.

The nomenclature used in this report will reference the pretreatment methods in the following manner: **E#** indicates the dosage of EB radiation in kGy, **M** represents sugar maple wood, **WP** represents white pine wood, **HWE** represents hot water extraction. For example, 250-M-HWE describes a maple sample which was irradiated with a 250 kGy dosage and hot water extracted, and 0-WP-HWE represents an non-EB pretreated white pine sample which was hot water extracted.

2.C. Compositional Characterization Methods

Near-infrared (NIR) spectroscopy was used as the main method of characterization in these studies to predict sugar and ethanol concentrations in fermentation broths. A Bruker MPA Multi-Purpose FT-NIR Analyzer was used to take measurements, and the software OPUS v6.5 was used to analyze spectra. Liquid fermentation broth was filtered through a 0.22 um cellulose acetate syringe filter to fill a 1 mL glass vial prior to recording NIR spectra of the solution.

A pre-developed model was used to monitor the conversion of glucose to ethanol after 0, 24, 48, 96, and 128 hours of fermentation. In the study of xylose conversion to ethanol, a new model was developed by taking the NIR spectra of a series of samples (n = 70) with a range of xylose:ethanol ratios (0 – 16% w/v and v/v, respectively). The model was then compiled using the OPUS software by cross-validation.

Thermogravimetric analysis was used to analyze cellulose, hemicellulose, and lignin content in biodelignified willow samples. A TGA Q5000 series Thermogravimetric Analyzer from TA Instruments was used with TA Instruments Universal Analysis.

Optical density was measured with a Thermo Scientific Genesys 20 at 600 nm and a 10x dilution in DI water. Measurements were taken in duplicate.

2.D Fermentation in the Presence of Irradiated biomass

Ethanol Red (Fermentis) *Saccharomyces cerevisiae* was used as the fermentative microorganism in glucose fermentation studies. Fermentation broths were prepared by adding 20 mL of 50% (wt/v) glucose to 40 mL of sterile yeast media (YM) stock solution (final YM concentration of 6.7 g/L). Freeze-dried biomass tea extracts were dissolved in 15 mL of YM and added to fermentation broths for experimental conditions. For control conditions, tea extracts were replaced with an additional 15 mL of YM.

Seed cultures were prepared with 36 mg of Ethanol Red yeast spores, 20 mL of 50% (wt/v) glucose, and 55 mL of YM grown aerobically for one day prior to inoculating fermentation broths. All fermentations were run in triplicate.

2.E. *P. stipitis* Culturing and Fermentation of Xylose

A freeze-dried culture of *P. stipitis* was multiplied in order to maintain a viable stock of the microorganism. The freeze-dried culture was rehydrated in 5 mL of sterile water for 15-20 hours at room temperature. Seed cultures were grown with 8 mL of either 50% (wt/v) glucose or 50% (wt/v) xylose with 55 mL of sterile YM. Seed cultures were inoculated with 7 drops of rehydrated yeast culture, and left undisturbed for three days at 27°C in aerobic conditions. Seed cultures not used in fermentation tests were then centrifuged at 3700 RPM for 10 minutes and the pellet suspended in a sterile 10% solution of skim milk, then frozen, lyophilized and stored at 3°C for later use.

To utilize lyophilized cultures, the sample was rehydrated in 5 mL of water for 15-20 hours, then centrifuged to remove the remaining skim milk. The yeast pellet was then suspended again in 5 mL of water and used to inoculate a seed culture.

Fermentations were conducted in the same manner above for glucose fermentation, but with a 10% w/t xylose concentration. Experiments were run in duplicate.

2.F. Enzymatic Hydrolysis of Softwood Biomass

Milled white pine biomass samples were subjected to enzymatic hydrolysis by Cellic® CTec2 enzymes which contain both cellulase and hemicellulase enzymes.

Wood samples (2.00 g ± 0.02 g) were placed in centrifuge tubes with 40 mL of water and 0.25 g of enzymes (based on dosage guidelines of 30% mass of cellulose present in sample). Samples were buffered with 0.1 M citrate buffer and pH adjusted to 5.0-5.5. Samples were placed on a rotating wheel to ensure adequate mixing and kept at 47°C. Experiments were run in duplicate.

3. Results and Discussion

3.A. Investigation of Enhancing Component

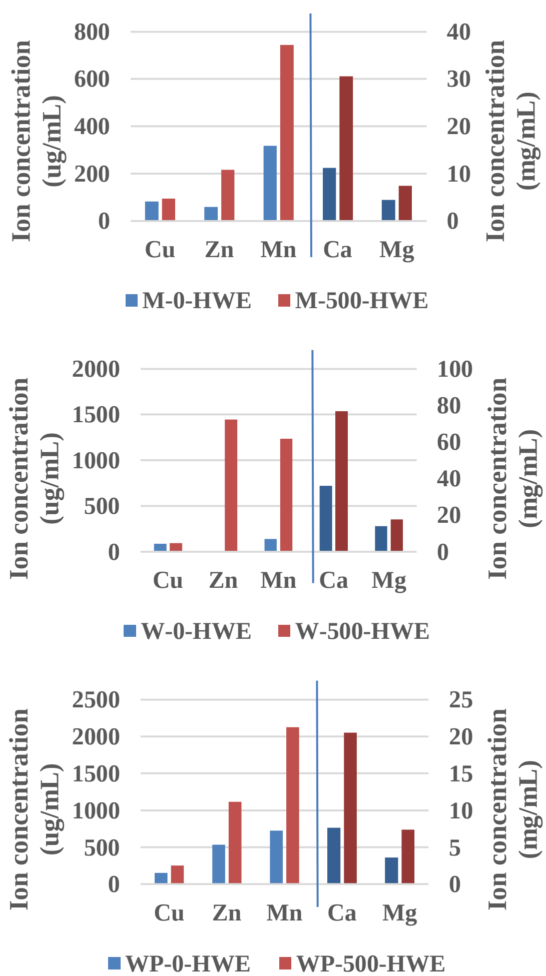


Figure 3.1. Divalent ion concentrations in untreated and EB-pretreated biomass samples. Ions to the right of the vertical line are on an mg/mL scale.

3.A.i. Soluble Ion Concentrations after Electron Beam Pretreatment

Because of the complex physical and chemical changes caused by EB pretreatment, it is suspected that soluble ions may be more easily released after irradiation. The presence of these ions may be responsible for improvement in yeast growth, as many metabolic processes require metal cofactors for their requisite enzymes

Inductively coupled plasma optical emissions spectroscopy

(ICP-OES) was used to determine the concentration of Cu^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+} ions within biomass samples (sugar maple, willow, and white pine) with or without EB pretreatment. In all cases, ion concentration increased with EB pretreatment (**Figure 3.1**). The magnitude by which ion concentration increased varied between species (**Table 3.2**). In some cases, such as Zn^{2+} in willow, concentration increased over 10,000%, while only increasing 100-200% in the other species. However, measurements were only taken on one sample each, so these results are not statistically significant.

These ions were likely enzyme cofactors in the plant cells when living, and can be released into solution when the biomass is placed in an aqueous suspension. Because these ions are found in the aqueous "tea" extract after hot water extraction, their presence within experimental fermentation broths might be responsible for some of the observed ethanol production rate enhancement versus control, as studies have shown that these ions' presence at certain concentrations enhances yeast growth.[17,18]

	Percent Increase (%)				
Biomass Sample	Cu^{2+}	Zn^{2+}	Mn^{2+}	Ca^{2+}	Mg^{2+}
Sugar Maple	1.4×10^{1}	2.6×10^{2}	1.3×10^{2}	1.7×10^{2}	6.7×10^{1}
Willow	9.5×10^{0}	1.4×10^{5}	7.8×10^{2}	1.1×10^{2}	2.5×10^{1}
White Pine	6.4×10^{1}	1.1×10^{2}	1.9×10^{2}	1.7×10^{2}	1.0×10^{2}

Table 3.2. Percent (%) increase in ion concentration after EB pretreatment

A test fermentation was performed with the addition of metal ions at the measured concentrations of untreated willow. A 25% enhancement in initial rate of ethanol production was observed, as well as a slight increase in concentration of ethanol produced (**Figure 3.3**). In this study, the fermentations were errantly prepared at a sub-optimal YM concentration, so the observed enhancement in Figure 3.3 is potentially related to replacing missing ion concentration. A subsequent study was performed likewise to that shown in **Figure 3.3** but with the correct YM concentration, and only a 4% increase in initial fermentation rate versus control was observed. This study should be repeated in triplicate to verify its results.

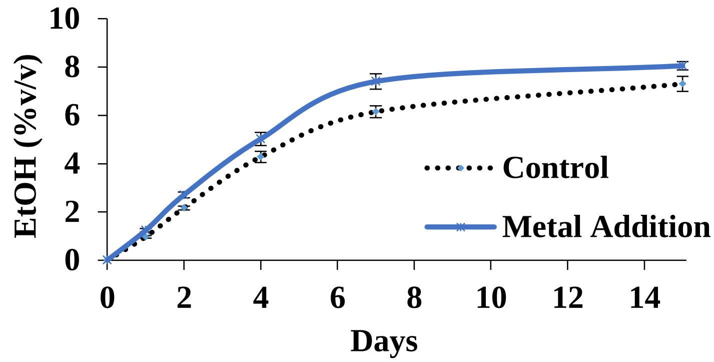


Figure 3.3. Ethanol production (% v/v) over 14 days in the presence of additional soluble ions (± 1 SD)

Based on these results, it is possible that a portion of the ethanol enhancement effect from the presence of biomass extracts is due to the release of metal ions, but results are still inconclusive as to how substantial this ion effect might be. EB pretreatment results in greater ion concentrations, but the fermentation enhancement effect is not generally greater for EB-pretreated samples. The effect of the addition of ions at the concentrations measured for EB-pretreated maple should be investigated more thoroughly.

3.A.2 Effect of Lignin Composition

Lignin's complex architecture of aromatic alcohols experiences extensive chemical changes, such as bond cleavages and cross-linkages, with EB pretreatment.[18] It is hypothesized that breakdown products may have an impact on fermentation of glucose to ethanol. Fermentation in the presence of biodelignified willow was performed to investigate the impact of lignin degradation on the fermentation enhancement effect.

Fermentation of glucose in the presence of EB-pretreated, biodelignified willow showed little variation in initial rate of fermentation between EB dosages, though enhancement of fermentation rate with respect to the control is still seen in all samples. Non-biodelignified samples showed the strongest enhancement, with a rate of ethanol production nearly 30% higher than control. This suggests that lignin modification is responsible for a reduction in enhancement. Interestingly, the effect of the lignin modification appears to be mitigated with higher dosages of EB pretreatment (**Figure 3.4**).

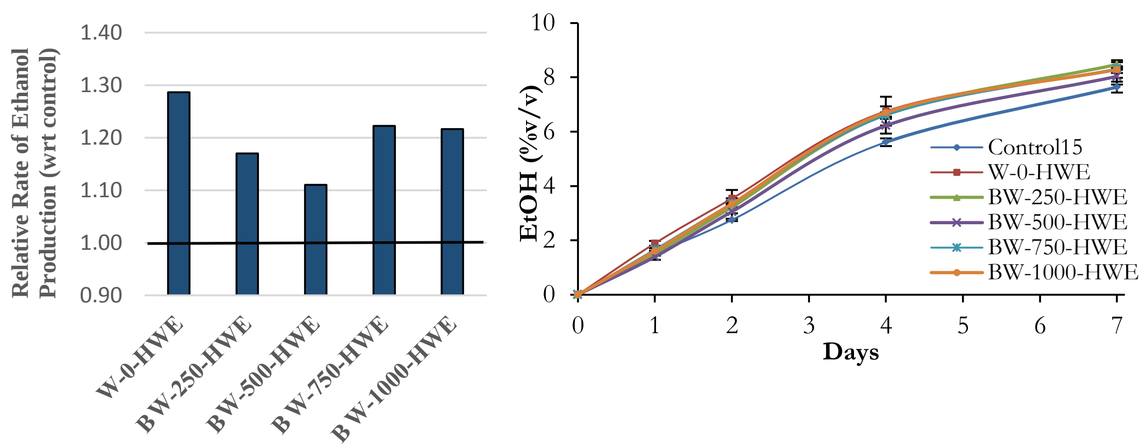


Figure 3.4. (Left) Initial rate (through 2 days) of ethanol production in the presence of EB-pretreated, biodelignified willow samples. (Right) Ethanol production (% v/v) curve through 7 days (±1 SD). "B" signifies biodelignification.

The hot water extract of extractive-free red pine reaction wood (compression wood) was also studied to investigate the effect of lignin presence on fermentation. Reaction wood is created in response to gravity and creates a lean in the wood. Compression wood is the biomass located on the inner curve of the leaning branch, and is reported to have a higher content of lignin than regular sapwood (see

Appendix). Though red pine is a softwood, enhancement with respect to the control was seen in both samples, suggesting extractives, or non-cell wall components, may be responsible for inhibition seen in fermentation in the presence of softwood. Additionally, fermentation rate was higher in the presence of compression wood than sapwood (**Figure 3.5**). Since compression wood inherently has a higher content of lignin than sapwood, lignin composition may be the reason for this enhancement. This also suggests that non-cell wall component "extractives" may be responsible for the inhibition of fermentation previously seen in the presence of white pine.

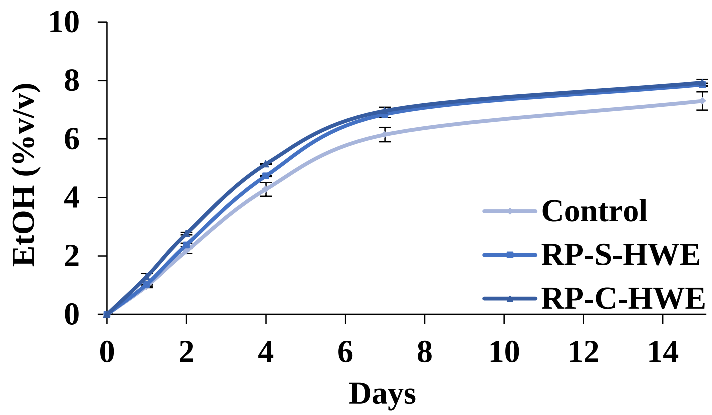
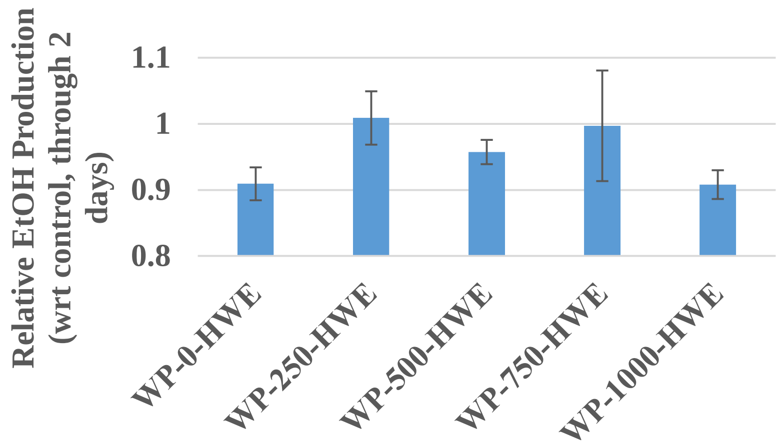


Figure 3.5. Ethanol production (% v/v) in the presence of extractive-free red pine compression (C) and sap (S) wood.

3.B. Softwood Saccharification and Fermentation

An inhibition of fermentation was previously seen in the presence of non-EB pretreated white pine extract. Here, we investigate the effect EB pretreated white pine has on fermentation. While no significant enhancement was seen, a mitigation of the inhibition seen in the presence of untreated white pine was noticed with small or moderate radiation dosages, such as 250 or 750 kGy (**Figure 3.6**). This could be a result of the release of ions seen when white pine is irradiated, or soluble lignin degradation products.

Figure 3.6. Relative ethanol production (wrt control) through two days in the presence of untreated and EB-pretreated white pine.



The saccharification of EB-pretreated white pine biomass yielded results similar in trend to EB-pretreated hardwood. Conversion of cellulose to glucose increased with increasing dosage of EB. A significant conversion was attained after just 24 hours in all cases, approaching 40% in 1000 kGy samples and 17% in untreated biomass, but stagnated after this time (**Figure 3.7**). This is most likely due to the failure to add anti-microbial agents to samples, resulting in glucose consumption by contaminating organisms. Regardless, a promising increase in the liberation of glucose after EB-pretreatment was seen. This is consistent with a previous publication of the effect of EB-

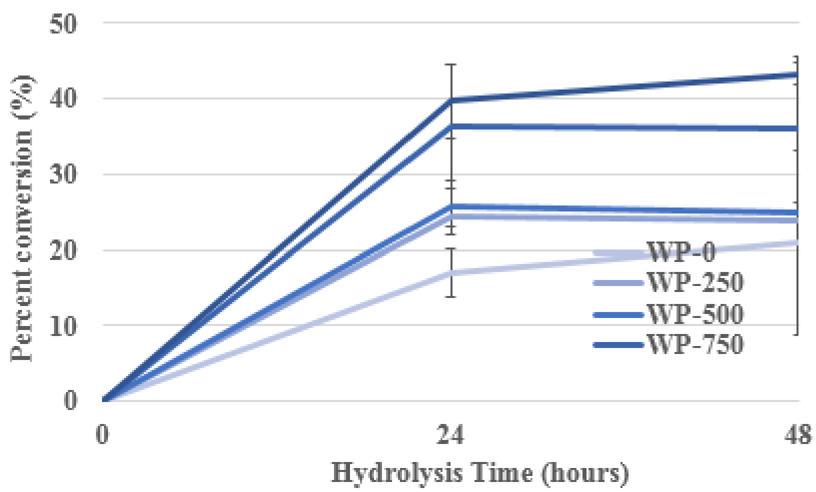


Figure 3.7 Percent conversion of white pine cellulose by enzymatic hydrolysis over 48 hours.

pretreatment of spruce, another softwood, which showed an enhancement in sugar liberation after EB-pretreatment.[20]

3.C. Xylose Modelling and Fermentation

An NIR model was successfully created to predict xylose and ethanol concentration during fermentation by *P. stipitis* (**Figure 3.8**). Xylose is predicted with an R^2 of 0.9538, root mean square error of cross validation (RMSECV) of 1.03, and residual predictive deviation (RPD) of 4.65. Ethanol is predicted with an R^2 of 0.9564, RMSECV of 0.852, and RPD of 4.79. This demonstrates that accurate

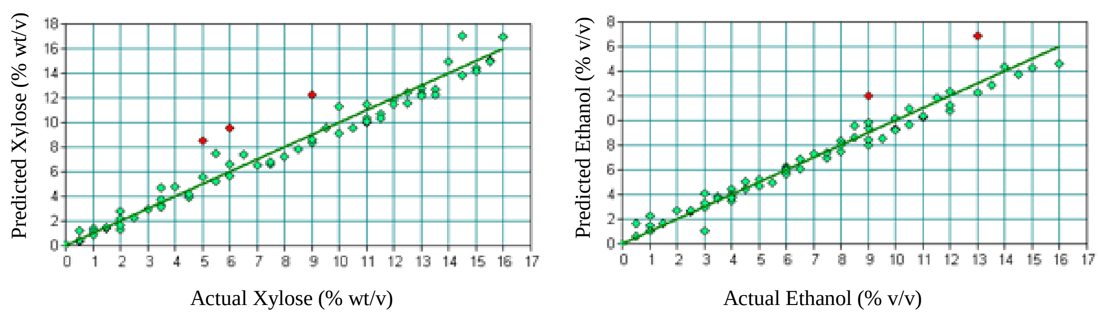


Figure 3.8. Cross-validation model of xylose (left) and ethanol (right) prediction. Outliers are highlighted in red.

prediction of xylose and ethanol concentration in solution can be achieved.

Trial experiments were run to elucidate conditions under which fermentation is possible. In some cases, fermentation appeared to occur when xylose concentration is 55 g/L under oxygen-limited conditions, even when the pH is not adjusted to ideal range (5.0-5.5). Approximately 0.75 mL (or 10% v/v) of ethanol was produced under these conditions. In other cases, under identical conditions, no ethanol was produced.

Several attempts at fermenting xylose by *P. stipitis* in the presence of biomass extracts (maple and white pine) were made but none were successful. During these experiments no ethanol was produced, even in the control samples. However, OD600 increased and pH declined over time in these samples. This implies there was some metabolic activity by the yeast, but the ethanol end product was not reached. Since results were inconsistent between batches and controls, it is still unclear why these results were attained. It is possible that the purchased yeast culture was not completely viable, or the culturing method used was not successful. More success is likely if seed cultures are grown to high levels of cell density, such as an OD600 of greater than 1.0.

While no viable fermentation results were attained, preliminary OD600 measurements suggest the presence of maple wood may have an inhibitory effect on the growth of *P. stipitis*. This would be consistent with the study of the effect of certain lignin degradation products on xylose fermentation by *P. stipitis* which show an inhibitory effect.[21]

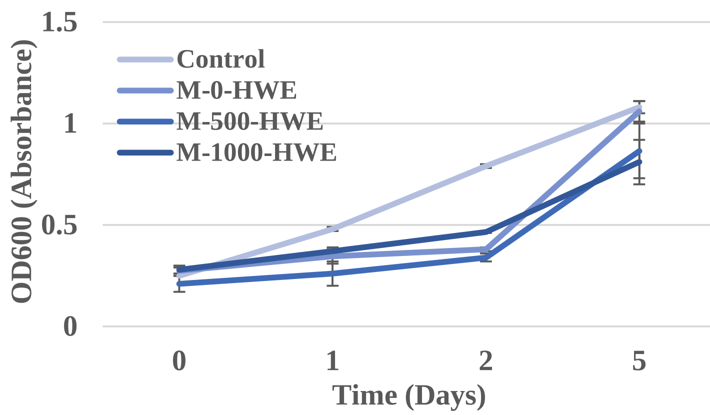


Figure 3.9. Optical density (±1 SD) of xylose fermentation broths in the presence of maple wood extracts.

4. Conclusions

Significant progress has been made on exploring the effect the presence of electron beam pretreated biomass has on the fermentation of wood-based sugars. It has been established that electron beam pretreatment causes the release of soluble ions, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}, in variable quantities, and the addition of these ions to control fermentation conditions yields an enhancement in rate and quantity of ethanol production. Additionally, fermentation in the presence of EB-pretreated biodelignified willow samples showed an enhancement in initial rate of fermentation but of lower magnitude than willow that has not undergone EB pretreatment and biodelignification. This suggests that lignin modification brings a reduction in enhancement.

Fermentation in the presence of EB pretreated white pine showed a mitigation of the inhibition seen in the presence of untreated white pine. Saccharification of white pine cellulose showed a significant increase in conversion to sugars with increasing radiation dosage.

An NIR model was successfully compiled to predict xylose and ethanol concentrations. Xylose fermentation in the presence of biomass extract was not successful. An inhibition in *P. stipitis* growth was seen in the presence of biomass samples.

5. ACKNOWLEDGEMENTS

This work was supported by NYSERDA, agreement 43681. Electron beam pretreatment was performed by Kent State University NEO Beam Facility. Thank you to ESF Analytical and Technical Services for ICP-OES analysis and ESF Nomura Research Group for the use of their instrumentation. I would like to thank Dr. Arthur Stipanovic for allowing me the opportunity to do this work under his supervision, and Scott Bergey for his extensive guidance.

6. SOURCES CITED AND CONSULTED

1. Farrel, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M. (2006). Ethanol can contribute to energy and environmental goals. *Science*, 311(5760), 506-508. doi: 10.1126/science.1121416
2. Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, 316(5827), 804-807. doi: 10.1126/science.1137016
3. Zhao, X., Zhang, L., Liu, D. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. (2012). *Biofuels, Bioproducts & Biorefining*, 6(4), 465-482. doi: 10.1002/bbb.1331
4. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, Ladisch, M. (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, Vol. 96, 673- 686. doi: 10.1016/j.biortech.2004.06.025
5. Bak,J., Ko, J., Han, Y., Lee, B., Choi, I., Kim, K. (2009) Improved enzymatic hydrolysis yield of rice straw using electron beam irradiation pretreatment, *Bioresource Technology*, 100(3), 1285-1290
6. Shin, S.J. & Sung, Y.J. (2008). Improving enzymatic hydrolysis of industrial hemp (Cannabis sativa L.) by electron beam irradiation. *Radiation Physics and Chemistry*, Vol. 77, 1034- 1038. doi: 10.1016/j.radphyschem.2008.05.047
7. Shin, S.J. & Sung, Y.J. (2010). Improving enzymatic saccharification of hybrid poplar by electron beam irradiation. *Journal of Biobased Materials and Bioenergy*, Vol. 4, 23-26.
8. Cheng, K., Barber V.A., Driscoll, M.S., Winter, W.T., Stipanovic, A.J. (2013). Reducing woody biomass recalcitrance by electron beams, biodelignification and hot-water extraction. *Journal of Bioprocess Engineering and Biorefinery*, 2(2), 1-10. doi: 10.1166/jbeb.2013.1048
9. Clough, R. (2001). High-energy radiation and polymers: A review of commercial processes and emerging applications. *Nuclear Instruments and Methods in Physics Research*, Vol 185, 8-33.
10. Chandel, A., Goncalves, B., Strap, J., da Silva, S. (2013). Biodelignification of lignocellulose substrates: An intrinsic and sustainable pretreatment strategy for clean energy production. *Critical Reviews in Biotechnology*, 1-3.

11. De Bari, I., De Canio, P., Cuna, D., Liuzzi, F., Capece, A., Romano, P. (2013). Bioethanol production from mixed sugars by *Scheffersomyces stipitis* free and immobilized cells, and co-cultures with *Saccharomyces cerevisiae. New Biotechnology*, 30(6), 591- 597.
12. Lee, T., Kim, M., Kim, K., Park, K., Ryu, Y., Seo, J. (2002). A parametric study on ethanol production from xylose by *Pichia stipitis*. *Biotechnology and Bioprocess Engineering*, Vol 5, 27-31.
13. Arslan, Y., Eken-Saracoglu, N. (2010). Effects of pretreatment methods for hazelnut shell hydrolysate fermentation with Pichia stipitis to ethanol. *Bioresource Technology,* 101(22).
14. Karagoz, P., Ozkan, M. (2014). Ethanol production from wheat straw by Saccharomyces cerevisiae and Scheffersomyces stipitis co-culture in batch and continuous system. *Bioresource Technology,* Vol. 157, 286-294.
15. Charlesby, A. (1955). The degradation of cellulose by ionizing radiation. *Journal of Polymer Science,* 15(79), 263-270. doi: 10.1002/pol.1955.120157921
16. Driscoll, M., Stipanovic, A., Winter, W., Cheng, K., Manning, M., Spiese, J., Galloway, R., Cleland, M. (2009). Electron beam irradiation of cellulose. *Radiation Physics and Chemistry*, 78(7), 539-542. doi: 10.1016/j.radphyschem.2009.03.080
17. Stehilk-Tomas, V., Zetić, V.G., Stanzer, D., Grba, S., Vahčić. (2004). Zinc, copper, and magnesium enrichment in yeast *Saccharomyces cerevisiae*. *Food Technology and Biotechnology*, 42(2), 115-120.
18. Walker, G.M. (2004). Metals in yeast fermentation processes. *Advances in Applied Microbiology*¸ Vol. 54, 197-229. doi: 10.1016/S0065-2164(04)54008-X
19. Schnabel, T., Huber, H., Grünewald, T. Petutschnigg, A (2015). Changes in mechanical and chemical wood properties by electron beam irradiation. *Applied Surface Science*, Volume 332, 704-709. doi.org: 10.1016/j.apsusc.2015.01.142.
20. Kahn, A.W, Labrie, J.P., McKeown, J. (1985). Effect of electron beam irradiation pretreatment on the enzymatic hydrolysis of softwood. *Biotechnology and Bioengineering*, 28(9), 1449-1453.
21. Zhu, J., Yang, J., Zhu, Y., Zhang, L., Yong, Q., Xu, Y., Li, X., Yu, S. (2014). Cause analysis of the effects of acid-catalyzed steam-exploded corn stover prehydrolyzate on ethanol fermentation by *Pichia stipitis* CBS 5776. *Bioprocess and Biosystems Engineering,* Vol 37, 2215-2222. doi 10.1007/s00449-014-1199-0

7. Appendix

Thermogravimetric Analysis of Red Pine Compression and Sapwood

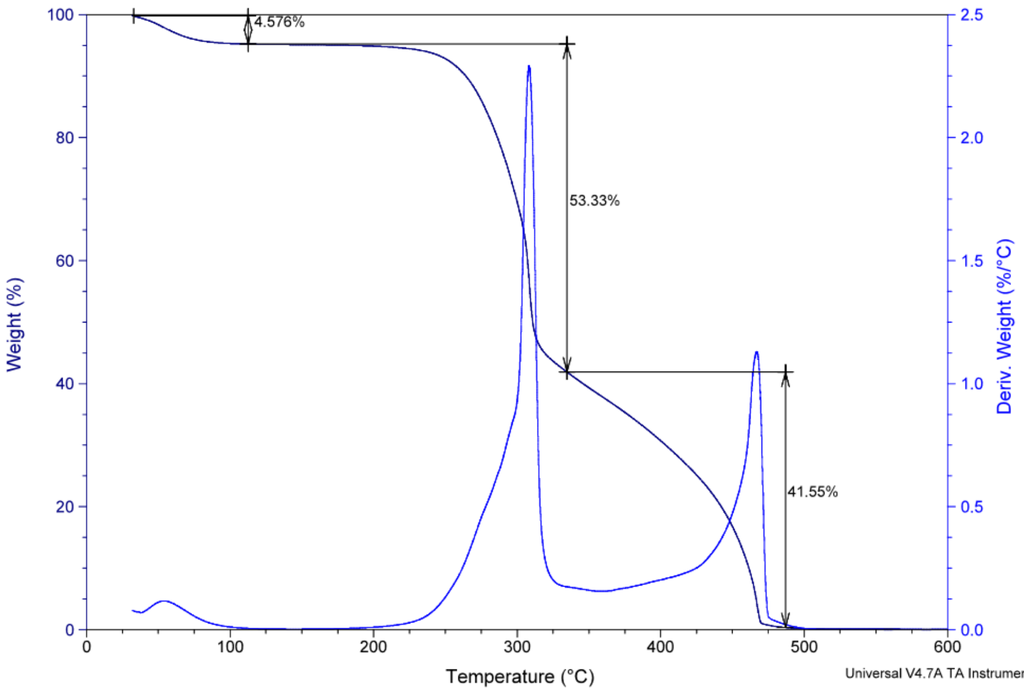


Figure 1. TGA of red pine compression wood. Note: 43.5% lignin composition (correcting for water)

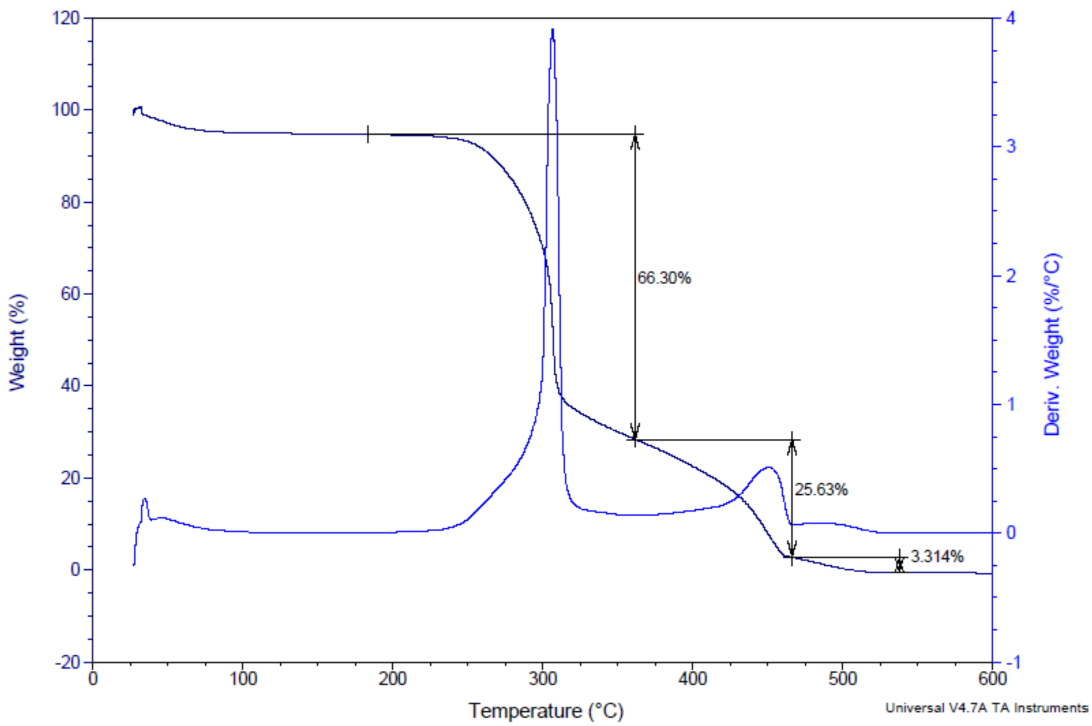


Figure 2. TGA of red pine sapwood. Note: 25.6% lignin content.