

**FINAL TECHNICAL REPORT ON  
CARBONIC ACID PRETREATMENT OF BIOMASS  
DOE CONTRACT DE-FC36-01GO11070, A000**

May 31, 2003

Covering the period from 8-1-2001 to 2-28-2003

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## EXECUTIVE SUMMARY

This project sought to address six objectives, outlined below. The objectives were met through the completion of ten tasks.

1) Solidify the theoretical understanding of the binary CO<sub>2</sub>/H<sub>2</sub>O system at reaction temperatures and pressures. The thermodynamics of pH prediction have been improved to include a more rigorous treatment of non-ideal gas phases. However it was found that experimental attempts to confirm theoretical pH predictions were still off by a factor of about 1.8 pH units. Arrhenius experiments were carried out and the activation energy for carbonic acid appears to be substantially similar to sulfuric acid. Titration experiments have not yet confirmed or quantified the buffering or acid suppression effects of carbonic acid on biomass.

2) Modify the carbonic acid pretreatment severity function to include the effect of endogenous acid formation and carbonate buffering, if necessary. It was found that the existing severity functions serve adequately to account for endogenous acid production and carbonate effects.

3) Quantify the production of soluble carbohydrates at different reaction conditions and severity. Results show that carbonic acid has little effect on increasing soluble carbohydrate concentrations for pretreated aspen wood, compared to pretreatment with water alone. This appears to be connected to the release of endogenous acids by the substrate. A less acidic substrate such as corn stover would derive benefit from the use of carbonic acid.

4) Quantify the production of microbial inhibitors at selected reaction conditions and severity. It was found that the release of inhibitors was correlated to reaction severity and that carbonic acid did not appear to increase or decrease inhibition compared to pretreatment with water alone.

5) Assess the reactivity to enzymatic hydrolysis of material pretreated at selected reaction conditions and severity. Enzymatic hydrolysis rates increased with severity, but no advantage was detected for the use of carbonic acid compared to water alone.

6) Determine optimal conditions for carbonic acid pretreatment of aspen wood. Optimal severities appeared to be in the mid range tested. ASPEN-Plus modeling and economic analysis of the process indicate that the process could be cost competitive with sulfuric acid if the concentration of solids in the pretreatment is maintained very high (~50%). Lower solids concentrations result in larger reactors that become expensive to construct for high pressure applications.

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## PROJECT SUMMARY

**Task 1a:** Xylan and Xylose were hydrolysed in 1%  $\text{H}_2\text{SO}_4$  at 121 °C for varying reaction times. Samples were analyzed with high performance anion exchange (HPAE) and an ultra-violet spectrophotometer. Peak areas for xylan oligomers were integrated by completing a mass balance on samples of varying degrees of hydrolysis. This yielded an appropriate calibration for peaks representing oligomer concentrations, and confirmed theoretical expectations that the area of oligomer peaks was proportional to the molar concentration of the oligomer species. The effect of pressure on hydrolysis was tested by superpressurizing reactors with nitrogen—no pressure effect on hydrolysis was detected. Experiments were conducted to confirm pH predictions of  $\text{CO}_2$  by comparing results to dilute  $\text{H}_2\text{SO}_4$ . Hydrolysis was done by subjecting a 1g/L xylan solution to  $\text{H}_2\text{SO}_4$  and/or  $\text{CO}_2$  or  $\text{N}_2$  at 190°C or 200°C for 16 minutes.

**Task 1b** Experiments were conducted to develop an Arrhenius rate constant for sulfuric acid and  $\text{CO}_2$ . These experiments consisted of hydrolyzing xylan at varying temperatures and initial pressures of  $\text{CO}_2$ , while maintaining a constant pH of 3.4. Comparative results were generated using  $\text{H}_2\text{SO}_4$ . To characterize the species in the hydrolysate, samples were analyzed by pH, HPAE and UV absorbency in a spectrophotometer.

**Task 1c:** Repeat experiments of aspen samples were hydrolyzed and titrated against a standardized NaOH solution to determine molar concentration of acid species in the hydrolysate. In some experiments it appeared that the acid concentration of hydrolysate of reactions with  $\text{CO}_2$  was significantly lower than the hydrolysate of reactions of wood and water alone. This confirms earlier work that consistently measured a higher pH in carbonic acid pretreated hydrolysates, compared to water-only pretreatment. However, in other titration experiments, the opposite result was observed, with the carbonic acid system having more accumulated acids present.

**Task 2:** A data set of pretreatment results on aspen using carbonic acid had been completed prior to the start of this project. Experiments continued to increase the reliability of the results and to achieve uniform performance between laboratory personnel. These objectives were achieved and reduced the scatter and uncertainty in the results.

Experiments were also carried out to determine whether the rate of mass transfer of  $\text{CO}_2$  into or out of solution exerts an effect on the rate of hydrolysis. It was found that extra  $\text{CO}_2$  could be dissolved into solution by allowing more time for the dissolution to occur, but that when the reactor was heated up to reaction temperatures there was no apparent effect on hydrolysis rates or extent.

**Task 3:** It was found that the severity function developed by Overend and Chornet adequately described the action of time and temperature on the pretreatment of aspen wood but not pure xylan. For aspen wood, no significant difference was detected between carbonic acid and water systems, thus the effect of the carbonic acid was negligible and did not need to be incorporated into the severity function. On xylan, however, it was necessary to take into account the action of the carbonic acid, and it was found that the severity function proposed by van Walsum did so adequately. Published literature results for the dilute acid pretreatment of softwood were used for comparing the fit of the severity function and the combined severity function for lower pH systems. It was found that the literature data had considerable

scatter, but that the combined severity function did appear to offer a more predictive capability than the regular severity function, which does not take into account the pH of the system.

**Task 4:** Preliminary experiments using a 150 mL reactor were conducted using water and a range of CO<sub>2</sub> pressures and reaction temperatures to evaluate the reactor performance and to determine when the pressure (and hence temperature) inside the reactor reaches steady-state. This revealed optimal reaction conditions and will minimize variation between experimental results generated from small (15 mL) and larger (150 mL) reactors. The data also provided an accurate determination of the time required for the reactor to reach reaction temperature.

**Task 5:** The newly constructed 150 mL reactor was used in reactions that replicated the conditions of the 15 mL reactor. 1.0 grams of aspen wood, 80 ml of de-ionized water were reacted with and without CO<sub>2</sub> at 800 psig. Reaction temperatures were 180°C, 200°C, and 220°C with reaction times of 8, 16, and 32 minutes. The reactor was preheated in a sand bath set to a temperature 40°C above reaction temperature for two minutes. This allowed the reactor to quickly reach reaction temperature, as determined and reported in the previous progress report. The 150ml reactor successfully delivered the expected 10-fold increase in hydrolysate compared to the 15ml reactor. Results of the pH and UV analysis of the hydrolysate were consistent with those yielded by the 15ml reactor.

**Task 6:** Inhibition tests measured the rate of sugar consumption by *Saccharomyces cerevisiae* growing in batch culture of hydrolysate. It was found that inhibition of the yeast culture increased with severity of pretreatment above a mid level severity. Below this severity, little to no inhibition was observed. No difference was observed between the inhibition of hydrolysates prepared either with or without the presence of CO<sub>2</sub>. To conduct the experiments, serum vials were charged with 21g/L of sterile growth medium containing 20ml of pretreatment hydrolysate. The vials were inoculated with 0.2ml of freshly grown cell broth and incubated. Glucose concentrations over time were determined via glucose assay (Infinity Glucose Reagent) and the HPAE when available.

**Task 7:** Enzyme digestibility tests measured enzymatic hydrolysis rates of pretreated solids by cellulase enzymes (Novozyme 188 and Iogen cellulase). It was found that more severe pretreatments enhanced enzymatic digestibility. The addition of pressurized CO<sub>2</sub> to the pretreatment system did not significantly increase enzymatic hydrolysis rates compared to water-alone pretreatment. To conduct the experiments, serum vials were charged with a pH 5.0 buffer, preservative, enzyme and pretreated solid sample estimated to have 2g/L cellulose (calculated from dry weight of the solid residue) and incubated in a 40°C shaker bath. Glucose concentrations over time were determined via glucose assay and the HPAE when available.

**Tasks 8+ 9:** Pretreatment costs for carbonic acid pretreatment are driven by the high cost of pretreatment reactors capable of containing the pressures used. This makes the cost of the carbonic acid system highly sensitive to reactor volume and thus the concentration of solids in the reactor. The cost of the reactor can be reduced by raising the concentration of the solids in the pretreatment reactor, which in turn reduces the size of the pretreatment reactor and thus diminishes the cost differential between dilute acid and carbonic acid equipment costs. If the solids concentration is put very high, and equal to that used in the NREL model, equipment costs become comparable to those for dilute sulfuric acid pretreatment. Cost of compressing CO<sub>2</sub> is relatively low compared to the equipment cost for the high pressure reactor vessel.

About 50% of the total operating cost is due to the heat demand of the process. This is unavoidable due to difficulties in process heat recovery.

Unless the concentration of solids in the pretreatment reactor can be increased, use of carbonic acid for pretreatment proved to be more expensive than using sulfuric acid. This is mainly due to the use of high pressures such as 2000 psi in the pretreatment reactor.

**Task 10:** Work from this project has been presented at four international meetings: the ACS annual meeting in Orlando Florida, April 7 – 11, 2002, the 24<sup>th</sup> Symposium on Biotechnology for Production of Fuels and Chemicals in Gatlinburg, TN, April 28 - May 1, 2002, the 12th European Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection in Amsterdam, NL, 17-21 June 2002, and the 25<sup>th</sup> Symposium on Biotechnology for Production of Fuels and Chemicals in Breckenridge, CO, May 3- 7, 2003. A brief paper was published in the proceedings of the Amsterdam conference and papers were submitted for peer-reviewed publication in the conference proceedings of the 25th symposium.

## TASK 1a) Determine accuracy of theoretical pH predictions

Vanessa Castleberry, G. Peter van Walsum

### Summary:

Xylan and Xylose were hydrolysed in 1% H<sub>2</sub>SO<sub>4</sub> at 121 °C for varying reaction times. Samples were analyzed with high performance anion exchange (HPAE) and an ultra-violet spectrophotometer. Peak areas for xylan oligomers were integrated by completing a mass balance on samples of varying degrees of hydrolysis. This yielded an appropriate calibration for peaks representing oligomer concentrations, and confirmed theoretical expectations that the area of oligomer peaks was proportional to the molar concentration of the oligomer species. The effect of pressure on hydrolysis was tested by superpressurizing reactors with nitrogen—no pressure effect on hydrolysis was detected. Experiments were conducted to confirm pH predictions of CO<sub>2</sub> by comparing results to dilute H<sub>2</sub>SO<sub>4</sub>. Hydrolysis was done by subjecting a 1g/L xylan solution to H<sub>2</sub>SO<sub>4</sub> and/or CO<sub>2</sub> or N<sub>2</sub> at 190°C or 200°C for 16 minutes.

### Materials and Methods

Apparatus and Materials: DX500 Chromatography System (consists of Dionex GP50 gradient pump, ED40 electrochemical detector, AS3500 autosampler), Xylan (sigma), xylose (Sigma), 15 mL 316 stainless steel reactor vessels, glass serum vials, Techne SBL-2D fluidized aluminum oxide sand baths, Pharmco sulfuric acid at 95.0 to 98.0%, 18 ohm high resistance water, deionized water, spigot-attached vacuum apparatus with tubing, ultra pure helium gas, Eppendorf micro pipettes (various sizes), DU Series 500 Beckman Spectrophotometer, Eppendorf centrifuge 5417C, and centrifuge tubes. Fisher Scientific AR15 pH meter by Accumet Research; Ohaus Explorer digital scale Item #12140 (d=0.1mg); Pyrex glass beakers, graduated cylinders, burettes and flasks;

### Procedures:

*Solution preparation:* A 1-g/L xylan solution was prepared with 0.25-g of dried xylan into 250 mL of DI water. For hydrolysis in 1% H<sub>2</sub>SO<sub>4</sub>, 18 M acid was added to the xylan solution to give a 1% solution. The acidified xylan was placed on an active stir plate with a magnetic stir bar in solution. For hydrolysis with varying pH conditions, 18 M H<sub>2</sub>SO<sub>4</sub> was added to a 1 g/L xylan solution in varying amounts. The xylan solution was placed on an active stir plate with a magnetic stir bar in solution. It was then pipetted into the steel reactor vessels in 10-mL aliquots. The acid solution, in a range of pHs, would next be added to the reactor vessel in 1.1mL aliquots.

*Hydrolysis:* For experiments at 121C, the acidified xylan solution was pipetted into the glass reactor vessels in 10-mL aliquots. The vial was sealed with a rubber stopper and an aluminum crimping ring. Each vial was placed in the sand bath for the desired time. For reactions at higher temperature, 316 stainless steel reactors were used, pressurized with CO<sub>2</sub> if necessary, and placed in a sand bath (190°C or 200°C) for 16 minutes. The samples were frozen for a later time to be analyzed by HPAE. Samples were run in duplicate.

*Analysis:* The samples were analyzed for polymer distribution using the HPAE. The HPAE consists of a pump, a separation column, and a detector. Each prepared sample was pumped through the column to distinguish the amount of hydrolysis occurring at each time interval. To prepare for the run, a 500mmol NaOH solution was made and degassed for use as an eluent. Each of the samples was diluted by 1 in 20 with deionized water. A set of standards was prepared using a 1g/L xylose solution. The stock xylose solution was diluted by 1:20, 1:25, 1:33, 1:50 and 1:100 to obtain standards at 50 mg/L, 40mg/L, 30 mg/L, 20 mg/L and 10 mg/L, respectively. The acidified xylose samples were further analyzed using a DU Series 500 Spectrophotometer to observe absorbency in the ultra-violet light range.

*pH prediction:* Most of the reactions carried out for testing the pH of carbonic acid at elevated temperature were done at 190 C for 16 minutes. This gives a log(severity) value of 3.85, where the severity function in its log form is calculated as per equation (1), first defined by Overend and Chornet (1987):

$$\log(R_O) = \log_{10} \left( t \times \exp \left( (T-100) / 14.75 \right) \right) \quad (1)$$

Where  $R_O$  is the severity,  $t$  is the reaction time expressed in minutes and  $T$  is the temperature expressed in degrees Celcius. The combined severity values for the experimental conditions, with the CO<sub>2</sub> at an initial, room temperature pressure of 800 psi is 0.27. The combined severity function was defined by Chum et al. (1990) as:

$$CS = \log(R_O) - \text{pH} \quad (2)$$

Where  $CS$  signifies the combined severity. In applying the combined severity factor to the carbonic acid system, van Walsum (2001) suggested the following equation, which estimates the value of the solution pH from the temperature and the partial pressure of the CO<sub>2</sub>:

$$CS_{P_{CO_2}} = \log(R_O) - 8.00 \times T^2 + 0.00209 \times T - 0.216 \times \ln(P_{CO_2}) + 3.92 \quad (3)$$

Where  $CS_{P_{CO_2}}$  is the combined severity determined from the partial pressure of CO<sub>2</sub> and  $P_{CO_2}$  is the partial pressure of CO<sub>2</sub> in atmospheres.

The use of the term “partial pressure” under these conditions is somewhat misleading, since the temperature and pressure range of the pretreatment put CO<sub>2</sub> into its supercritical phase, while the water vapor is still present in the gas phase. Thus, equation (3) pertains more precisely to the fugacity of the CO<sub>2</sub> in the non-liquid phase. For a component in solution, fugacity and partial pressure are related by

$$\phi_i = f_i / y_i P \quad (4)$$

Where  $\phi_i$  is the fugacity coefficient of component  $i$ ,  $f_i$  is the fugacity,  $y_i$  is the molar fraction and  $P$  the overall pressure of the system (Smith and Van Ness, 1975). For a binary  $i,j$  gas mixture, the multicomponent Virial equation simplifies to:

$$\ln \phi_i = \frac{P}{RT} (B_{ii} + y_j^2 \delta_{ij}) \quad \text{with } \delta_{ij} = 2B_{ij} - B_{ii} - B_{jj} \quad (5)$$

Where  $R$  is the universal gas constant,  $T$  is the temperature in degrees Kelvin,  $y_j$  is the molar fraction of the solvent in the gas phase and  $B_{ij}$  is the second virial coefficient, which can be expressed by equation (6) (Smith and Van Ness, 1975)

$$B_{ij} = \frac{RT_{cij}(B^0 + \omega_{ij}B^1)}{P_{cij}} \quad (6)$$

In which  $\omega$  is the acentric factor. The parameters  $\omega_{ij}$ ,  $T_{cij}$ , and  $P_{cij}$  were calculated with mixing rules presented in Smith and Van Ness (1975), as follows:

$$\omega_{ij} = (\omega_i + \omega_j) / 2 \quad (7)$$

$$T_{cij} = (T_{ci} T_{cj})^{1/2} \quad (8)$$

$$P_{cij} = (Z_{cij} R T_{cij}) / V_{cij} \quad (9)$$

In which  $Z$  is the compressibility.  $Z_{cij}$  and  $V_{cij}$  are determined by:

$$Z_{cij} = (Z_{ci} + Z_{cj}) / 2 \quad (10)$$

$$V_{cij} = ((V_{ci}^{1/3} + V_{cj}^{1/3}) / 2)^3 \quad (11)$$

$B^0$  and  $B^1$  were calculated as proposed in Smith and Van Ness (1975), as follows:

$$B^0 = 0.083 - 0.422 / T_r^{1.6} \quad (12)$$

$$B^1 = 0.139 - 0.172 / T_r^{4.2} \quad (13)$$

These equations can be solved iteratively starting with the known temperature, total pressure and assumed values for molar fractions. Initial estimates for molar fractions were obtained by assuming ideal gas behavior, which yielded convergent results. Results from these calculations for several reaction conditions are summarized in table 1a.1.

Table 1a.1. Results of thermodynamic determination of carbonic acid pH at elevated temperatures and pressures.

Temperature C	Total pressure (psia)	$y_{CO_2}$	Z	$\phi$	$f_{CO_2}$ (psia)	pH
180	1941	.925	.850	.861	1606	3.54
190					1686	3.58
200	2165	.896	.854	.864	1766	3.62
210					1845	3.67
220	2416	.86	.872	.880	1924	3.71

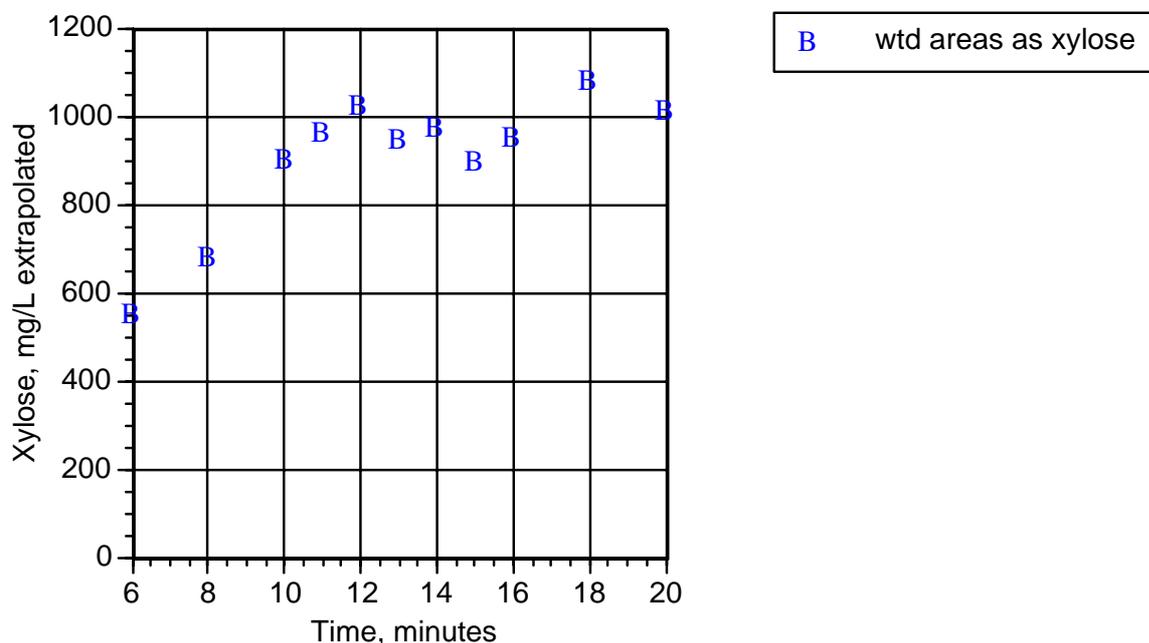
Previously reported [van Walsum, 2001] calculations for the pH of high temperature carbonic acid, which did not incorporate the fugacity coefficient calculations shown above, had predicted a pH of 3.56 at 190 C. This result is in very near agreement with the more thermodynamically correct value calculated in table 1a.1, and indicates that simplified assumptions about the gas phase behavior of CO<sub>2</sub> may still be viable under these conditions.

## Results

### Oligomer quantification

Figure 1a.1 shows results from xylan hydrolysis at 121 C in 1% sulfuric acid. It can be seen that at about time 10 minutes, all of the 1 g/L xylan originally present has been hydrolyzed to oligomers small enough to be quantified by the HPAE. The relative stability of total oligomer concentration at times above 10 minutes demonstrates that the quantification of oligomers is relatively robust, since for all these cases the mass balance comes close to closure. At times less than 10 minutes, insufficient hydrolysis had occurred and large DP oligomers were not being detected.

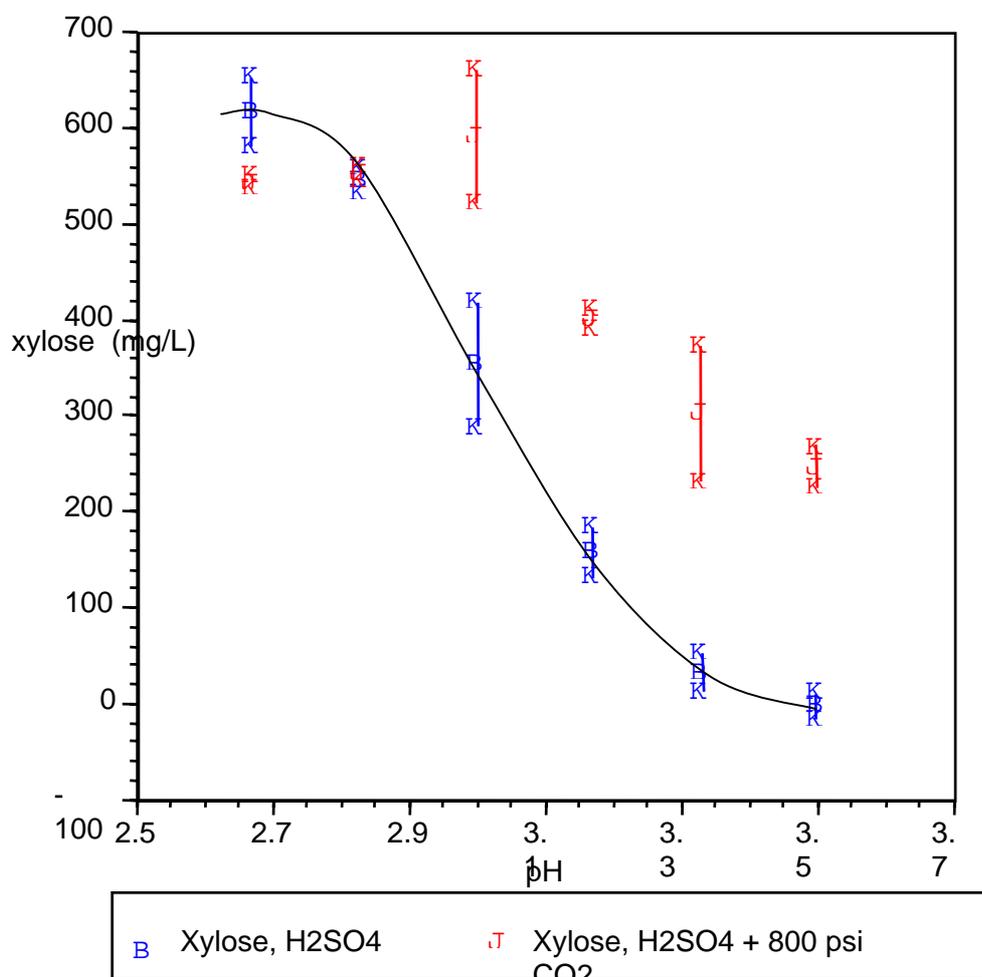
**Figure 1a.1 Total xylose and xylan oligomers released in 1% H<sub>2</sub>SO<sub>4</sub> hydrolysis of 1 g/L xylan at 121 C.**



*pH confirmation*

Figure 1a.2 shows results of dilute sulfuric acid and carbonic acid hydrolysis of 1 g/L xylan at 190 C. The figure shows the effect of combining carbonic and sulfuric acids for hydrolysis of xylan. In this experiment it can be seen that down to a pH of about 3.2, carbonic acid is able to supplement the hydrolysis of the sulfuric acid. Below pH 3.2 the carbonic acid can no longer contribute to enhancing hydrolysis. It appears that carbonic acid on its own has an effective pH of about 3.4 Compared to the theoretical value of 3.58, this appears to show minor deviation from theoretically expected results. Van Walsum[2001] found similar deviation results, where the observed hydrolysis was equivalent to sulfuric acid at 3.37 and the predicted pH, using idealized gas calculations, had been 3.56 .

**Figure 1a.2: Xylose accumulation in varying pH of H<sub>2</sub>SO<sub>4</sub> and CO<sub>2</sub> with varying pH of H<sub>2</sub>SO<sub>4</sub>**



### Discussion & Conclusions

It was found that oligomer quantification based on a correlation of peak area to molar concentration of oligomers served to close a mass balance on partially hydrolyzed xylan solutions. This enabled quantification of xylan solubilization in cases of incomplete hydrolysis.

In the varying pH experiments, CO<sub>2</sub> at 800 psig most closely approximated H<sub>2</sub>SO<sub>4</sub> at a pH of 3.4 and appeared to be able to assert some hydrolytic activity in the presence of other acids down to a pH of 3.2. Predictions of the pH of carbonic acid appear to have over estimated the effective pH by approximately 0.18 pH units. These results are in agreement with previously published findings.

### References

Van Walsum, G. Peter, 2001. Severity function Describing the Hydrolysis of Xylan Using Carbonic Acid.

Smith, J.M, and van Ness, H.C. 1975. Introduction to Chemical Engineering Thermodynamics, 3rd ed'n. McGraw Hill Book Company.

## TASK 1b) Arrhenius equation for H<sub>2</sub>CO<sub>3</sub>

Vanessa Castleberry, G. Peter van Walsum

### Summary:

Experiments were conducted to develop an Arrhenius rate constant for sulfuric acid and CO<sub>2</sub>. These experiments consisted of hydrolyzing xylan at varying temperatures and initial pressures of CO<sub>2</sub>, while maintaining a constant pH of 3.4. Comparative results were generated using H<sub>2</sub>SO<sub>4</sub>. To characterize the species in the hydrolysate, samples were analyzed by pH, HPAE and UV absorbency in a spectrophotometer.

### Materials and Methods:

Apparatus and Materials: DX500 Chromatography System (consists of Dionex GP50 gradient pump, ED40 electrochemical detector, AS3500 autosampler), Xylan (sigma), xylose (Sigma), 15 mL 316 stainless steel reactor vessels, glass serum vials, Techne SBL-2D fluidized aluminum oxide sand baths, Pharmco sulfuric acid at 95.0 to 98.0%, 18 ohm high resistance water, deionized water, spigot-attached vacuum apparatus with tubing, ultra pure helium gas, Eppendorf micro pipettes (various sizes), DU Series 500 Beckman Spectrophotometer, Eppendorf centrifuge 5417C, and centrifuge tubes. Fisher Scientific AR15 pH meter by Accumet Research; Ohaus Explorer digital scale Item #12140 (d=0.1mg); Pyrex glass beakers, graduated cylinders, burettes and flasks;

### Procedures:

*Solution preparation:* A new 1-g/L xylan solution was prepared with 2g of dried xylan into 2 L of DI water. For hydrolysis in 1% H<sub>2</sub>SO<sub>4</sub>, 18 M acid was added to the xylan solution in varying amounts to observe responses to a range of pHs side by side with CO<sub>2</sub>. The xylan was placed on an active stir plate with a magnetic stir bar in solution.

*Hydrolysis:* The solution was pipetted into 150 mL, 316 stainless steel reactor vessels in 10-mL aliquots. To maintain a constant pH for the acids over the temperature range, the temperature dependence of the acid dissociations was taken into account when preparing solutions for use at different temperatures. This resulted in the lower temperature solutions being reacted at lower pressures of CO<sub>2</sub> and lower concentrations of sulfuric acid. The reactor was closed, and if dictated, pressurized, and placed in a sand bath (170°C (210°C preheat), 180°C (220°C preheat), or 190°C (230°C preheat) for 14.5 minutes. Samples were preheated for 3 minutes in the 150 mL reactor. The samples were refrigerated until HPAE analysis was run. Samples were run in duplicate.

*Analysis:* The samples were analyzed using the HPAE. To prepare for the run, a 500mmol NaOH solution was made and degassed for use as an eluent. Each of the samples was diluted by 1 in 20 with deionized water. A set of standards was prepared using a 100mg/L-xylose solution. The xylose solution was diluted by 1:2, 1:2.5, 1:3.3, 1:5 and 1:10 to obtain standards at 50 mg/L, 40mg/L, 30 mg/L, 20 mg/L and 10 mg/L. Each prepared sample was pumped through the column to distinguish the amount of hydrolysis occurring at each time interval.

The acidified xylose samples were further analyzed using a DU Series 500 Spectrophotometer to observe absorbency at 275 nm.

**Results:**

Results from the hydrolysis experiments are presented in figures 1b.1 and 1b.2, which show results for the carbonic and sulfuric acid systems, respectively. The slopes of the plots indicate activation energies of 11.9 kJ/mol for the sulfuric acid system and 12.6 kJ/mol for the carbonic acid system. It is not clear whether this difference is significant in terms of identifying a difference in the effective hydrolysis activity of the two acids. The  $R^2$  values for the slopes of these plots are .988 and .976 respectively, which demonstrates a relatively good fit to the data in each case, but does not instill confidence in the significance of the effect.

**Figure 1b.1 Plot of 1/T versus ln(K) for carbonic acid hydrolysis of xylan**

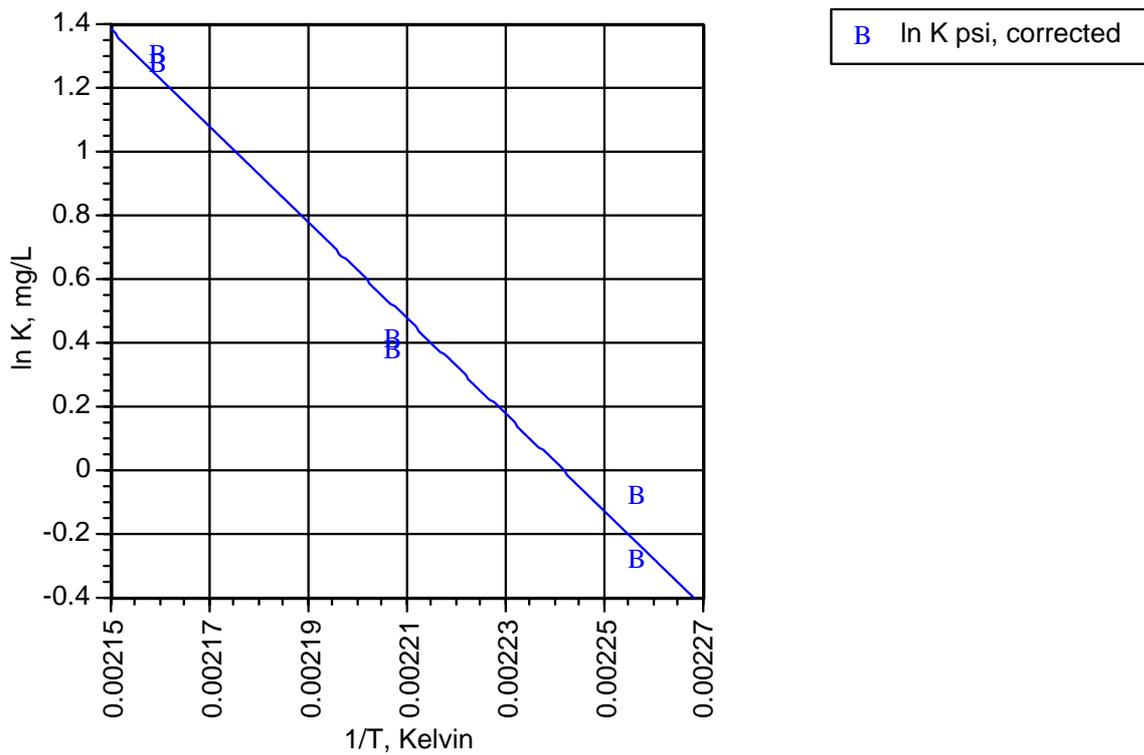
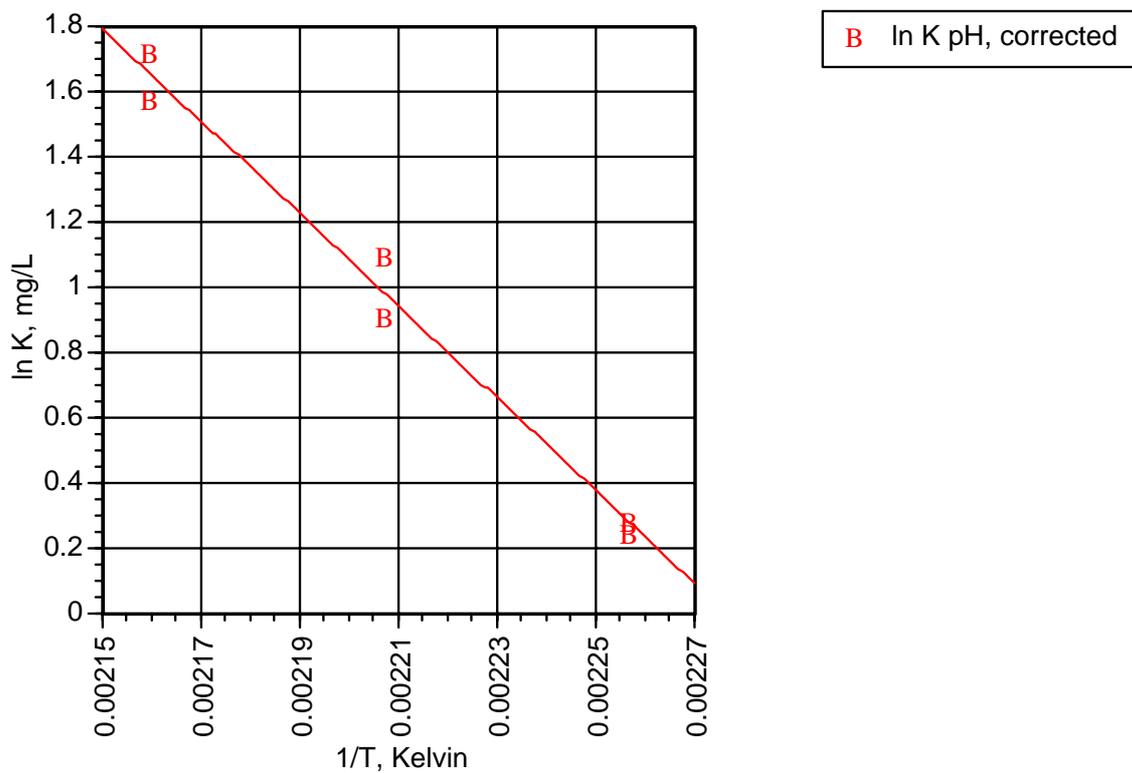


Figure 1b.2 Plot of  $1/T$  versus  $\ln(K)$  for sulfuric acid hydrolysis of xylan



### Discussion and Conclusions

It appeared relatively straight forward to calculate the activation energy for both sulfuric and carbonic acids. There does not appear to be any significant effect on hydrolysis that could be attributed to the type of acid used. Temperature effects on the acid dissociations were taken into account when preparing the samples to achieve a constant pH at each temperature.

## TASK 1c) Buffering Capacity of H<sub>2</sub>CO<sub>3</sub>

Vanessa Castleberry, G. Peter van Walsum

### Summary:

Repeat experiments of aspen samples were hydrolyzed and titrated against a standardized NaOH solution to determine molar concentration of acid species in the hydrolysate. In some experiments it appeared that the acid concentration of hydrolysate of reactions with CO<sub>2</sub> was significantly lower than the hydrolysate of reactions of wood and water alone. This confirms earlier work that consistently measured a higher pH in carbonic acid pretreated hydrolysates, compared to water-only pretreatment. However, in other titration experiments, the opposite result was observed, with the carbonic acid system having more accumulated acids present.

### Introduction

During previous experimentation using aspen wood, the hydrolysate pH of reactions with CO<sub>2</sub> was higher than reactions without. Since acetic acid may likely be the major contributor of H<sup>+</sup> ions into solution, it is hypothesized that the presence of CO<sub>2</sub>/carbonic acid may somehow reduce the release of acetyl groups, or in some other way reduce the activity of acid species in solution. This experiment attempted to close the “acid balance” by titrating hydrolysates and comparing titration results to analytical determination of acetic and formic acids in solution.

### Materials + Methods

Apparatus and Materials: DX500 Chromatography System (consists of Dionex GP50 gradient pump, ED40 electrochemical detector, AS3500 autosampler), xylose (Sigma), 15 mL 316 stainless steel reactor vessels, Techne SBL-2D fluidized aluminum oxide sand baths, J. T. Baker Glacial Acetic acid, Pharmco sulfuric acid at 95.0 to 98.0%, Mallinckrodt AR<sup>®</sup> sodium hydroxide solution at 50% w/w, Tetrabutylammonium hydroxide solution, 40% w/w; Heptafluorobutyric acid solution, 99%; 18 ohm high resistance water, deionized water, Fisher heated stirring plate, magnetic stir bars, glass beakers, Erlenmeyer flasks, Kjeldahl flasks, spigot-attached vacuum apparatus with tubing, ultra pure helium gas, Eppendorf micro pipettes (various sizes), DU Series 500 Beckman Spectrophotometer, Eppendorf centrifuge 5417C, and centrifuge tubes. Fisher Scientific AR15 pH meter by Accumet Research; Swagelock 150ml stainless steel reactor; Ohaus Explorer digital scale Item #12140 (d=0.1mg); Pyrex glass beakers, graduated cylinders, burettes and flasks; Aspen wood; Aspen hydrolysates; Aspen solids.

### Procedures:

*Hydrolysis:* A sand bath was heated to 190 °C, another 230 °C. Aspen was ground and sifted, using the particles of 1 mm. 1.0 g of aspen was weighed and placed in 150-mL stainless steel reactor, along with 80 mL of water. Using 3 reactors, simultaneously, all samples at each parameter were generated at once. Depending on the sample, the reactor was charged either with water and wood or with water, wood and 800 psig of CO<sub>2</sub>. The filled reactors were placed in the 230°C sand bath for a 3-minute preheat. Upon completion of preheat time, the reactors were transferred to 190°C sand bath for 15 minutes of reaction time. Reactions were quenched in ice bath after 15 minutes at target temperature.

*Titration:* ~2 L of ultra pure water were brought to boil for 20-30 minutes. Remove from heat. The water was stoppered to cool and transferred to polyethylene bottles that were tightly

closed. A titrant of ~0.01 M NaOH was mixed up, using newly bottled water. The titrant was stored in a tightly closed polyethylene container. KHP (Potassium Hydrogen Phthalate) was weighed and dried in a 100°C oven overnight. The container was transferred to a desiccator and allowed it to cool. The # of moles in 20 mL of the newly made NaOH solution was calculated and, in the same number of moles of KHP the weight was calculated. That amount was weighed into a 125 mL flask. Enough degassed water was added to dissolve it (~20 mL). Fresh NaOH was poured into a 25 mL burette. The pH probe was placed into the KHP solution and titrated against the NaOH solution. The mV (potential) and pH were recorded after each mL of NaOH was added. Using the derivative of the end point, the exact concentration of NaOH solution was calculated. The exact molarity of the NaOH solution was  $9.91954 \times 10^{-3}$ . Each hydrolysate titration was repeated 4 times and the average was used to derive an end point. For each titration, 25ml of hydrolysate was placed in a 150ml beaker which was then placed on a stir plate. The pH probe was placed in the beaker as well. The 0.1M NaOH was added incrementally (between 0.1ml and 1.0)ml and the pH was measured and recorded at each increment. The data will be plotted and evaluated to determine the number and concentrations of acids present in the hydrolysate.

### **Results:**

Figures 1c.1 to 1c.7 show results from different experiments doing titrations on aspen wood hydrolysates. Figures 1c.1, 1c.4, 1c.6 show results of water pretreated aspen wood, figures 1c.2, 1c.5, 1c.7 carbonic acid pretreated aspen wood. Figure 1c.3 shows the calibration titration using KHP. It can be seen that in the experiment that generated figures 1c.1 and 1c.2, the wood hydrolysate has less overall acid than the carbonic acid hydrolysate, indicated by the lower volume of NaOH solution needed to reach the inflection point in the titration curve. Figures 1c.4 and 1c.5 show little difference between the two titrations, while 1c.6 and 1c.7 show both qualitative and quantitative differences: the CO<sub>2</sub> system has a much more gradually buffered-looking titration curve, indicating a variety of acid species.

Figure 1c.1 Titration of aspen hydrolysate without CO<sub>2</sub>

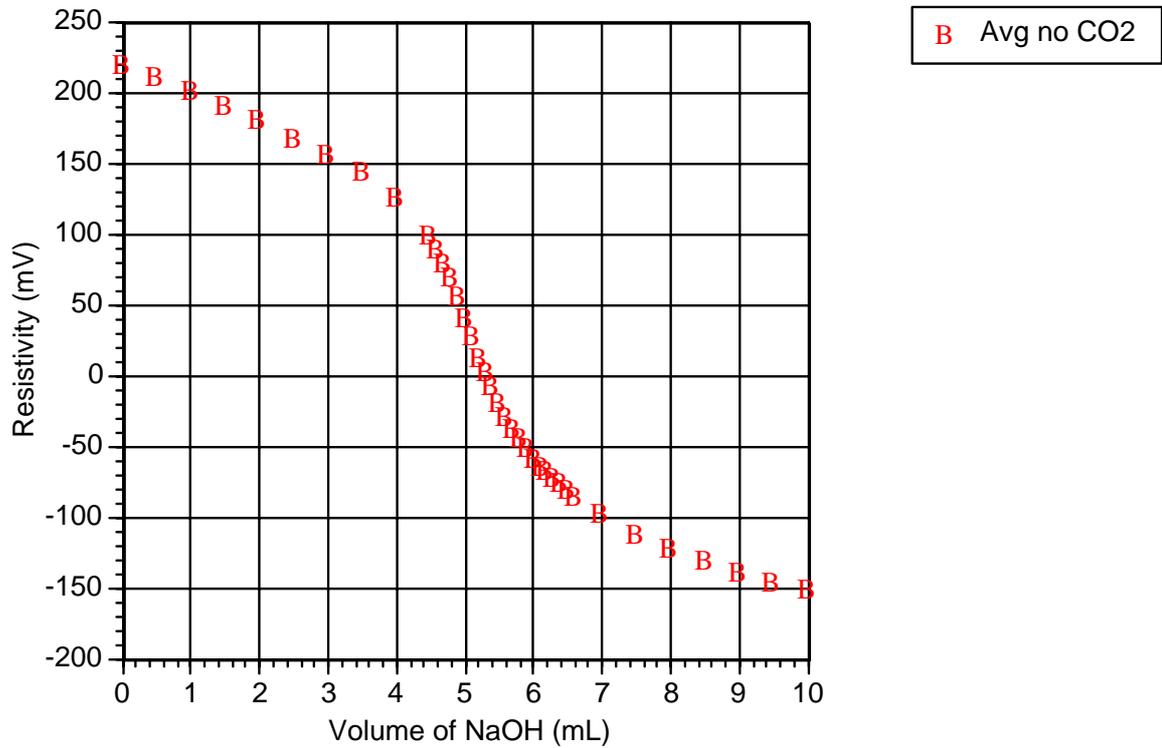


Figure 1c.2 Titration of aspen hydrolysate with CO<sub>2</sub>

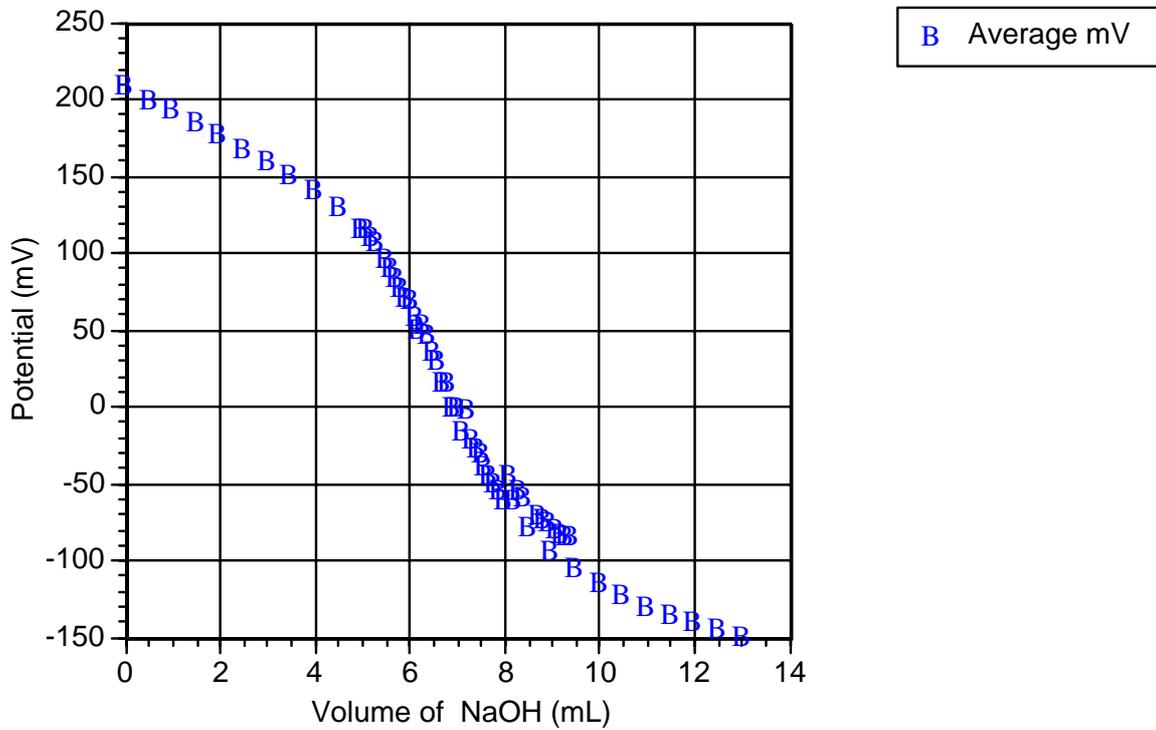


Figure 1c.3: KHP Standard Titration curve

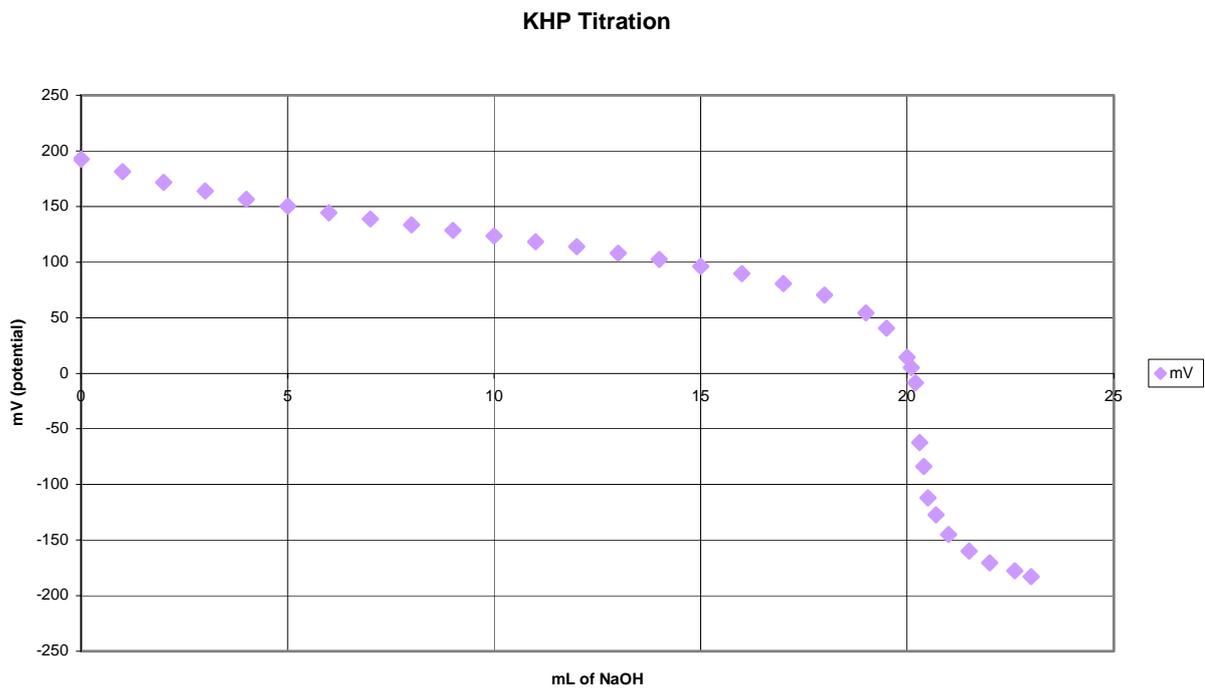


Figure 1c.4: Titration Curve of Water Pretreated Aspen

Average Titration of Aspen without CO2

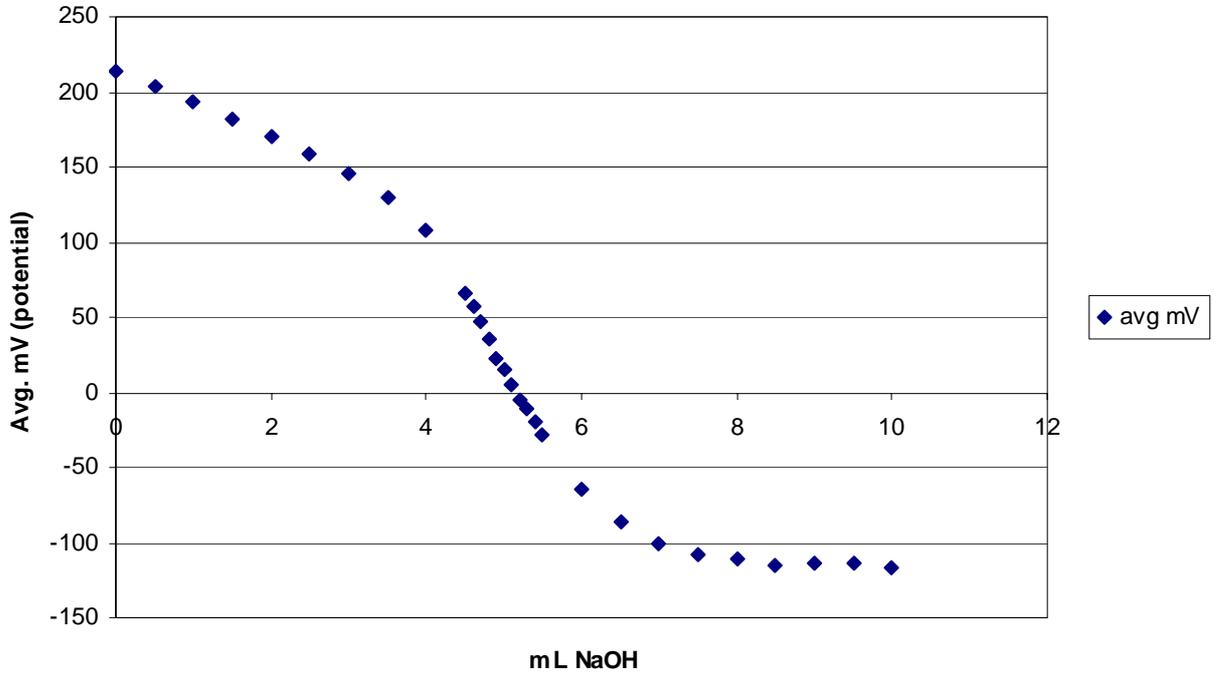
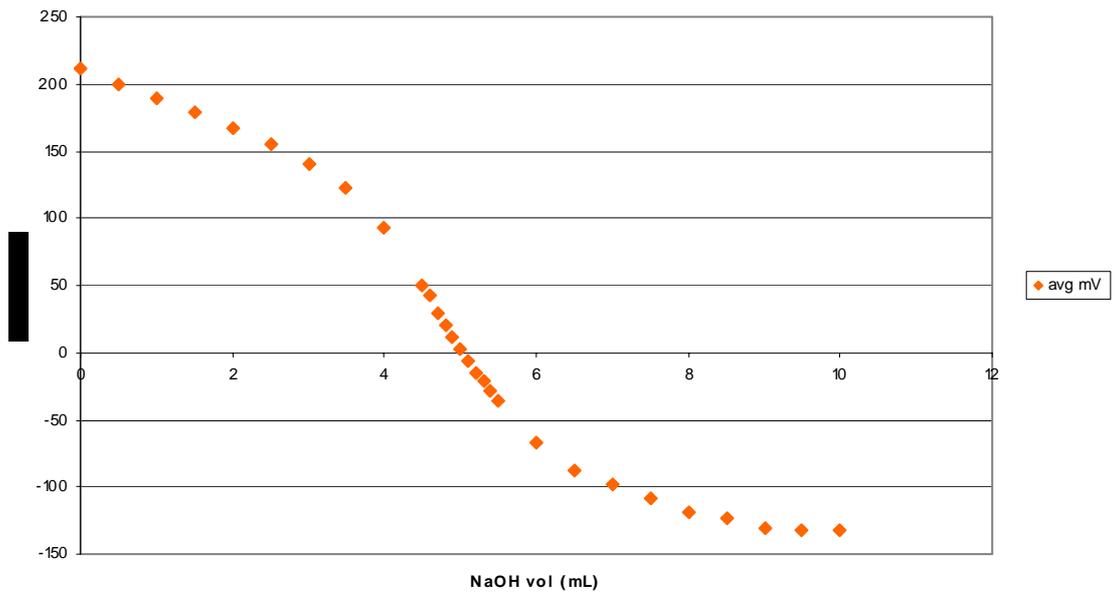
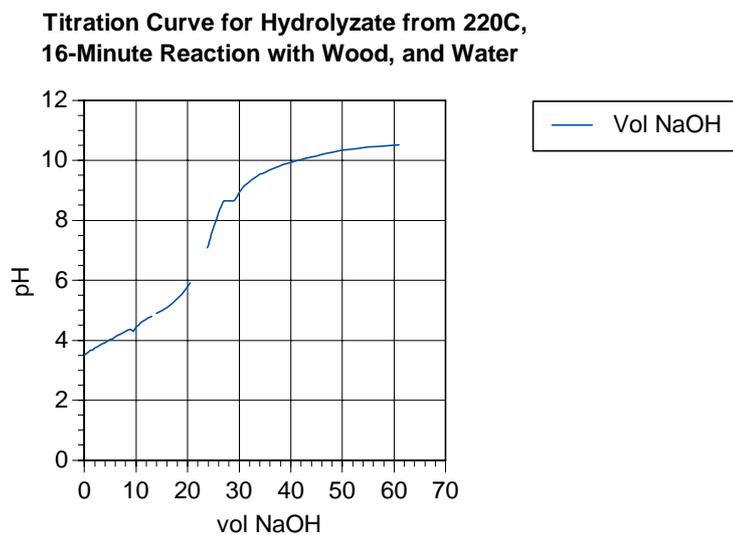


Figure 1c.5 Titration of Carbonic Acid Pretreated Aspenwood

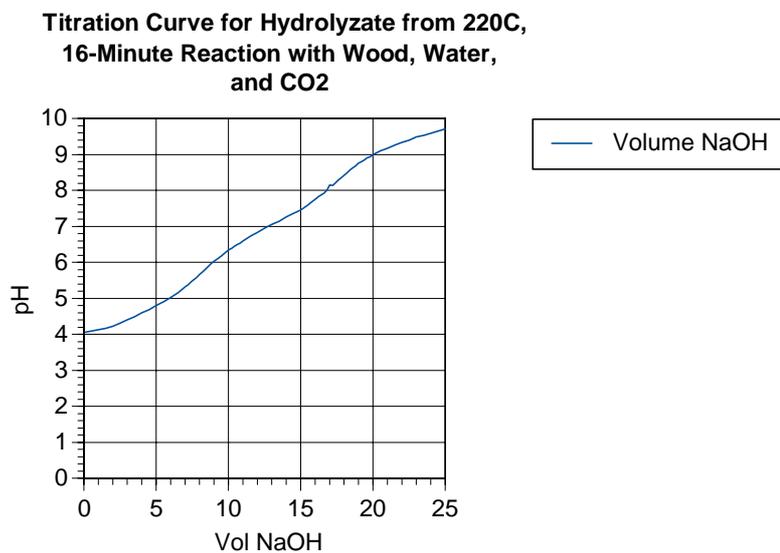
Titration Average of Hydrolysate with CO2



**Figure 2c.6**  
**pH Titration Results for Neutralization of Hydrolysate from Liquid Hot Water Pretreatment**



**Figure 2c.7**  
**pH Titration Results for Neutralization of Hydrolysate from Carbonic Acid Pretreatment**



## **Discussion and Conclusions**

The curious, and previously reported [1] result of carbonic acid having a net effect of increasing the final aspen wood hydrolysate pH has been confirmed for most experiments, but this result is not consistent with all of the titration studies undertaken. At this point there are inconsistent results in the titration results and so no conclusions can be drawn. Further work is being done to increase the reproducibility of these results.

## **TASK 2 Perform laboratory experiments on the pretreatment of raw biomass**

John Lam, Robert McWilliams, Connaly Miller, G. Peter van Walsum

### **Summary**

A data set of pretreatment results on aspen using carbonic acid had been completed prior to the start of this project [1]. Experiments continued to increase the reliability of the results and to achieve uniform performance between laboratory personnel. These objectives were achieved and reduced the scatter and uncertainty in the results.

Experiments were also carried out to determine whether the rate of mass transfer of CO<sub>2</sub> into or out of solution exerts an effect on the rate of hydrolysis. It was found that extra CO<sub>2</sub> could be dissolved into solution by allowing more time for the dissolution to occur, but that when the reactor was heated up to reaction temperatures there was no apparent effect on hydrolysis rates or extent.

### **Introduction**

Previously published results showed a large degree of scatter in the data for Carbonic acid pretreatment of aspen wood. Further experiments were carried out to improve the data set and increase confidence in the results. With the increased number of data, a Q test at 99% certainty was used to eliminate outlier points and thus tighten up the results.

Through our studies of H<sub>2</sub>CO<sub>3</sub> pretreatment of biomass, it has become apparent that there may be kinetic limitations affecting the consistency of hydrolysis results. A set of experiments was carried out to determine if the kinetics of solubilization of the CO<sub>2</sub> was affecting hydrolysis. A reactor was charged with a xylan solution and CO<sub>2</sub> at 800 psi. The pressurized reactor was then allowed to sit for a set period of time to allow mass transfer of the CO<sub>2</sub> into solution prior to heating and initiation of the reaction.

### **Materials and Methods**

Apparatus and materials: A 15 mL reactor, 150 mL reactor, sand bath, a coffee grinder, raw aspen wood, a sieve, a balance, deionized water, pH meter, a tank of carbon dioxide (lab grade), beakers, graduated cylinders, glass vials, and a spectrophotometer with quartz cuvettes.

#### Procedures:

*Feedstock preparation:* Raw pieces of aspen wood are placed into a coffee grinder. After the coffee grinder turned the wood into fine chips, the chips are placed into a sieve and shaken. The desired size of the wood was 1 mm, and was collected for storage.

*Aspen wood Pretreatment:* The wood was taken to a balance and 0.1 grams is weighed. The 0.1 grams of wood was placed into a 15 mL reactor along with 8 mL of deionized water. For reactions involving carbon dioxide, 800 psi of carbon dioxide was added through a 1/8 inch stainless steel tube. The reactors are closed and placed in a sandbath. Some reactors were reacted at 180 Celsius and some at 200 Celsius. The amount of time that each reactor was placed in the sandbath was 8 minutes, 16 minutes, and 32 minutes. For repetition of older

results, no pre-heat time was used to shorten temperature transients. This was because some of the earlier reported results were generated without pre-heat times.

*Post reaction pH:* After the reaction, the reactor was taken out and the hydrolysate emptied into a container such as a beaker. The pH was quickly measured. A magnetic stirrer bar was placed into the beaker and the hydrolysate was stirred in order to accelerate de-gassing. The pH was measured every 5 minutes until constant pH was attained.

*UV-absorption:* 30 microliters of the hydrolysate was placed into a micro-centrifuge tube and centrifuged for 3 minutes at 12,000 rpm. The hydrolysate was then pipetted into a quartz cuvette with 2970 microliters of water. The cuvette was placed into a spectrophotometer and the ultraviolet absorbance at 270 nm was measured.

*Carbohydrates:* Were measured by HPAE-PAD using a Dionex GP50 gradient pump, an ED40 amperometric detector and a CarboPac column running an eluent of dilute NaOH.

*Mass transfer experiments:* Reactors were charged with aspen wood or xylan and water. The reactors were then pressurized with CO<sub>2</sub> and then treated in one of three methods: 1) the reactors were sealed and immediately immersed in the sand bath to initiate reaction; 2) the reactors were sealed and allowed to sit idle at room temperature for a specific period of time: 15 minutes to 24 hours prior to reaction--this allowed the solution to become saturated with CO<sub>2</sub> before reaction; and 3) the reactors were kept pressurized with CO<sub>2</sub> for an hour before sealing the reactor, which allowed even more CO<sub>2</sub> to go into solution, after which the reaction was then reacted. Conditions 1 and 2 were intended to compare the effects of saturation prior to reaction, while condition 3 allowed an even higher degree of saturation with CO<sub>2</sub>. Hydrolysates were analyzed for pH, absorbance and xylose concentration.

## Results

Tables 2.1 and 2.2 lists previous (McWilliams and van Walsum[1]) and newly generated (this study) results for aspen wood pretreatment experiments. As can be seen from the table, in most cases mean values from previous experiments have been confirmed and the error about the mean reduced. These same data are also plotted in figures 2.1 – 2.14. On most of these figures it can be seen that the more recent results show less variation. Figure 2.15 shows the combined, averaged results from McWilliams and van Walsum[1] and the additional points generated in this study.

Results from experiments investigating the effect of dissolving time on xylan hydrolysis are found in tables 2.3 and 2.4. Figures 2.16 and 2.17 show the same data plotted versus dissolving time. Figure 2.18 shows xylose concentrations measured in response to three different conditions for dissolving time: no extra time for dissolution, three hours for dissolving, and 800 psi of CO<sub>2</sub> pressure maintained during one hour prior to sealing the reactor. These three conditions represent progressively stronger conditions for dissolving CO<sub>2</sub> prior to reaction. Although there appears to be a downward trend in the xylose concentration as solubilization intensity increases, the scatter in the repeats of each condition suggest that this may be arbitrary and not related to the degree of pre-reaction solubilization of CO<sub>2</sub>. If the trend is valid, then it appears to function in the opposite, and less easily explained, direction than that indicated by the apparent trend in UV absorption.

Figure 2.19 shows results from increasing CO<sub>2</sub> pressure applied to the reactor when hydrolyzing xylan. It can be seen that up to an initial pressure of 200 psi the additional CO<sub>2</sub> offers advantages for hydrolysis. Above 200 psi the benefits of extra CO<sub>2</sub> diminish.

**Table 2.1. Comparison of Previous (McWilliams) and Recent (Lam) pH and UV Absorbance of Aspen Wood Hydrolysate Pretreated with Hot Carbonic Acid.**

	McWilliams	Lam		McWilliams	Lam
H <sub>2</sub> O, CO <sub>2</sub> , 180 C, 16 min	pH	pH		UV abs	UV abs
1	4.89	4.03	1	0.134	0.118
2	3.54	4.39	2	0.243	0.147
3	3.89	3.76	3	0.133	0.127
4	4.04	4.04	4	0.132	0.175
5		3.79	5		0.186
mean	4.090	4.002		0.1605	0.1506
std deviation	0.496	0.226		0.048	0.026
% std dev/mean	12.1	5.7		29.7	17.5
H <sub>2</sub> O, CO <sub>2</sub> , 180 C, 32 min					
1	4.34	3.7	1	0.197	0.197
2	3.75	3.97	2	0.325	0.213
3	3.9	4.17	3	0.144	0.152
4	4.42	3.68	4	0.122	0.216
5		3.93			
mean	4.103	3.890		0.1970	0.1945
std deviation	0.284	0.183		0.079	0.026
% std dev/mean	6.9	4.7		40.0	13.2
H <sub>2</sub> O, CO <sub>2</sub> , 200 C, 16 min					
1	5.13	3.74	1	0.24	0.316
2	3.78	3.69	2	0.451	0.221
3	4.01	3.79	3	0.304	0.237
4		3.75	4		0.228
mean	4.307	3.743		0.3317	0.2505
std deviation	0.590	0.036		0.088	0.038
% std dev/mean	13.7	1.0		26.6	15.3

**Table 2.2. Comparison of Previous (McWilliams) and Recent (Lam) pH and UV Absorbance of Aspen Wood Hydrolysate Pretreated with Liquid Hot Water.**

	McWilliams	Lam		McWilliams	Lam
H2O, 180 C, 8 min	<b>pH</b>	<b>pH</b>		<b>UV abs</b>	<b>UV abs</b>
1	4.29	3.76	1	0.087	0.122
2	3.29	4.04	2	0.135	0.13
3	4.25	3.79	3	0.076	0.085
4	4.02	3.98	4	0.169	0.12
mean	3.963	3.893		0.1168	0.1143
std deviation	0.402	0.120		0.037	0.017
% std dev/mean	10.1	3.1		32.1	15.1
H2O, 180 C, 16 min					
1	3.95	3.75	1	0.153	0.171
2	3.22	3.61	2	0.185	0.189
3	3.56	3.52	3	0.168	0.22
4	3.78	3.63	4	0.139	0.166
mean	3.628	3.628		0.1613	0.1865
std deviation	0.273	0.082		0.017	0.021
% std dev/mean	7.5	2.3		10.6	11.3
H2O, 180 C, 32 min					
1	3.85	3.34	1	0.193	0.197
2	3.34	3.35	2	0.443	0.213
3	3.56	3.67	3	0.19	0.152
4	3.65	3.68	4	0.179	0.216
mean	3.600	3.510		0.2513	0.1945
std deviation	0.183	0.165		0.111	0.026
% std dev/mean	5.1	4.7		44.1	13.2
H2O, CO2, 180 C, 8 min					
1	4.7	4.17	1	0.091	0.111
2	3.62	4.36	2	0.456	0.114
3	4.23	4.36	3	0.099	0.106
4	4.29	4.74	4	0.092	0.025
5		5.16	5		0.107
mean	4.047	4.655		0.2157	0.0880
std deviation	0.303	0.330		0.170	0.037
% std dev/mean	7.5	7.1		78.8	41.5

Figure 2.1

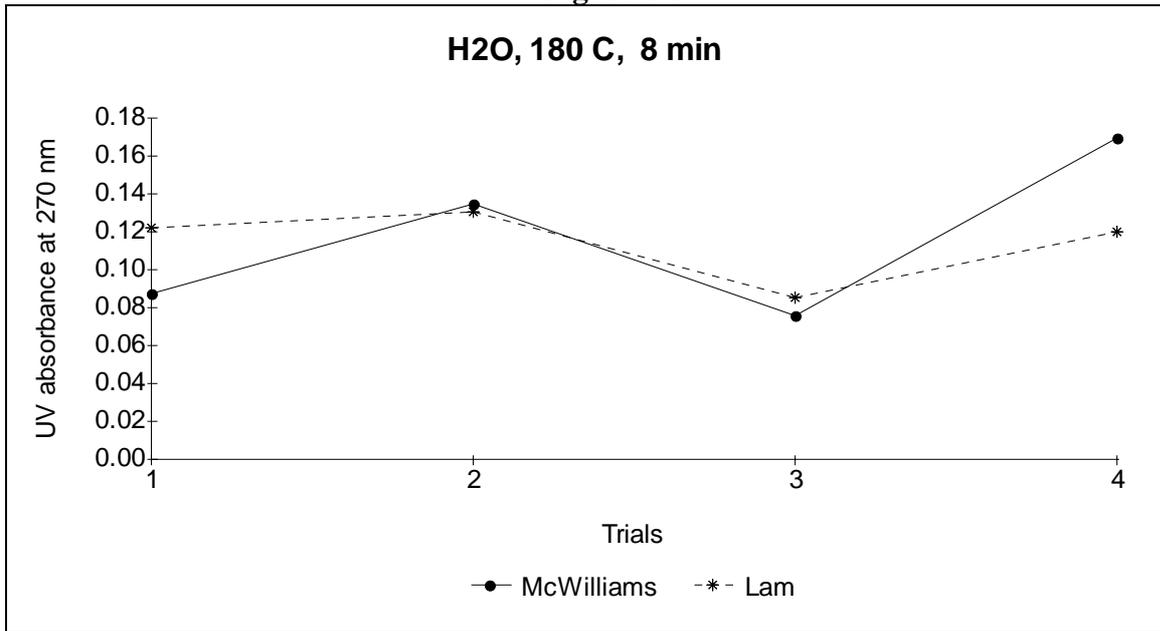


Figure 2.2

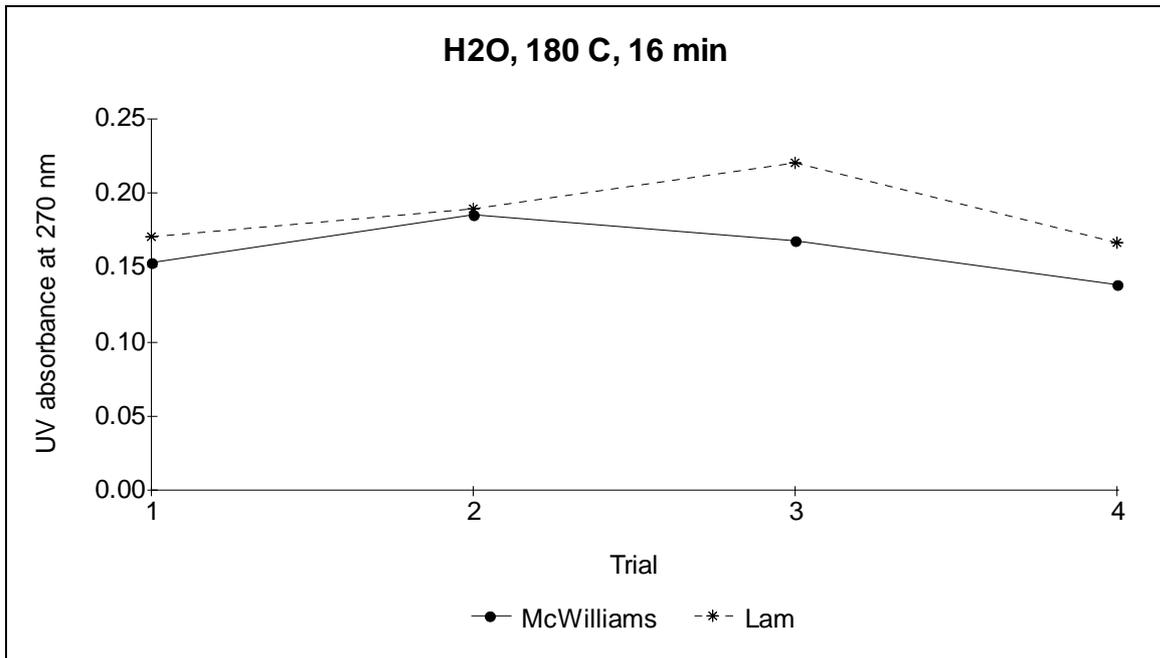


Figure 2.3

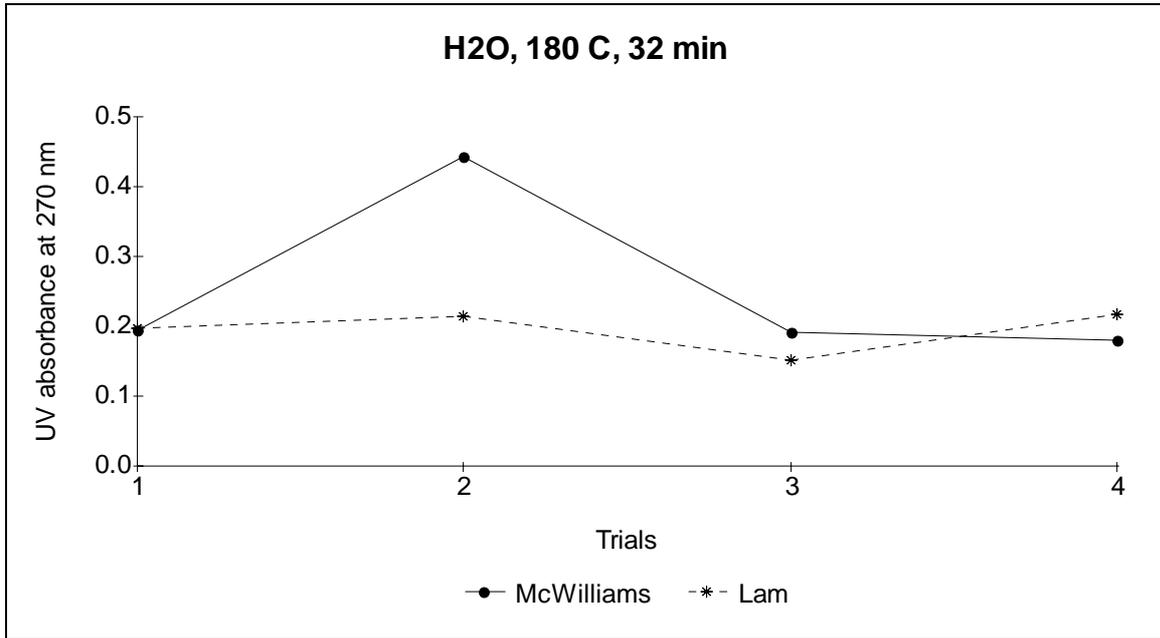


Figure 2.4

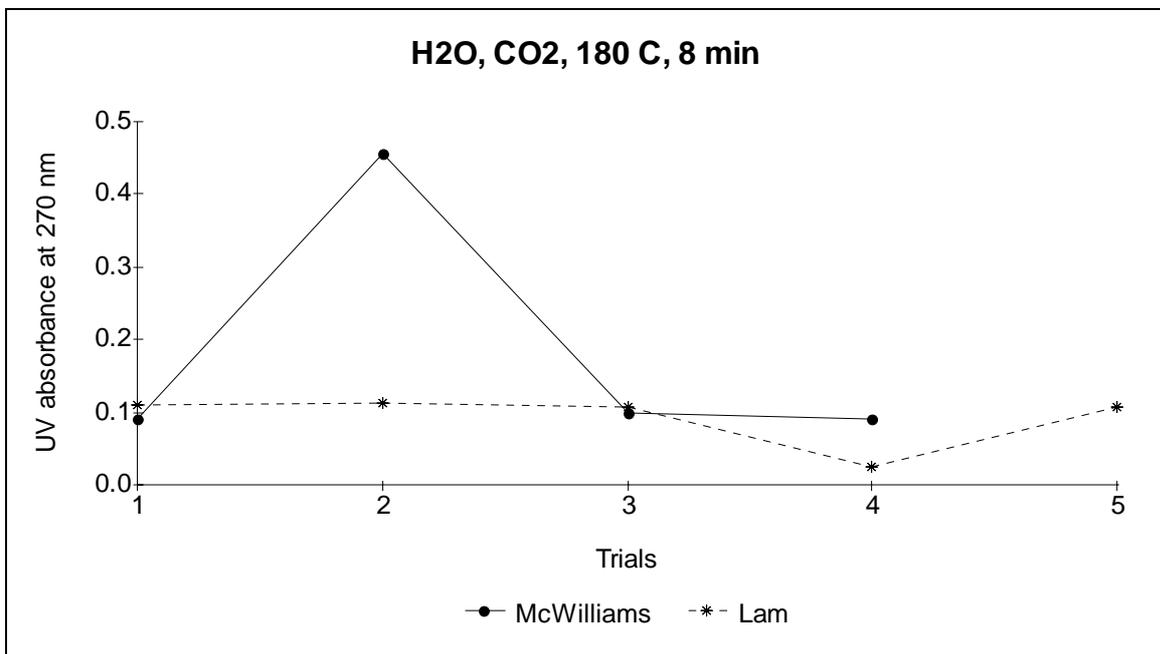


Figure 2.5

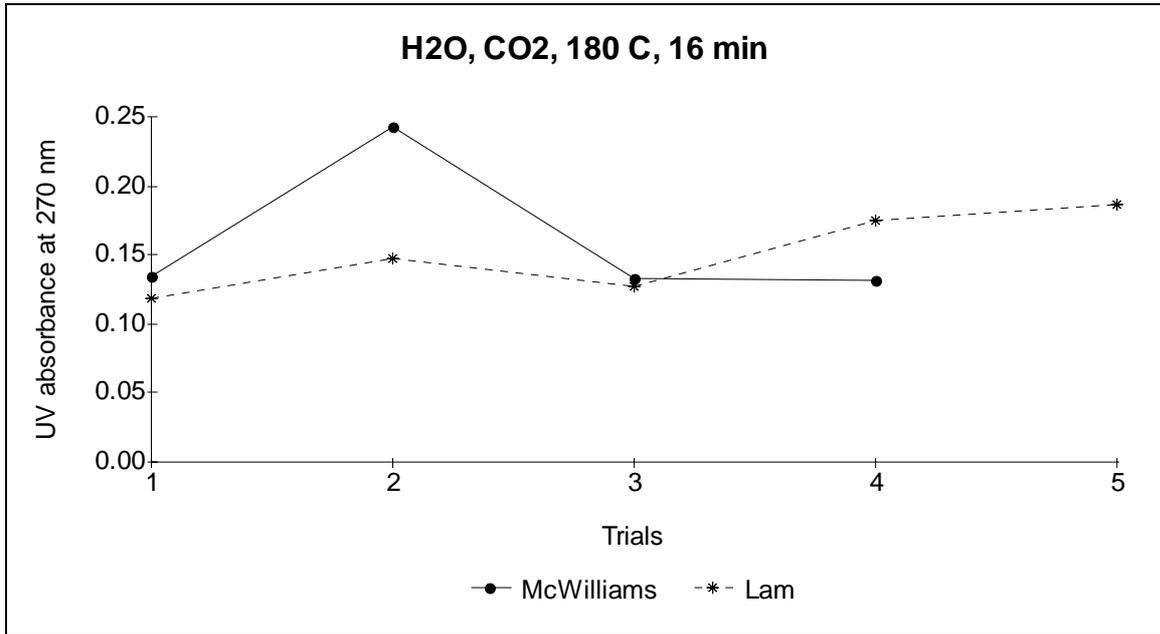


Figure 2.6

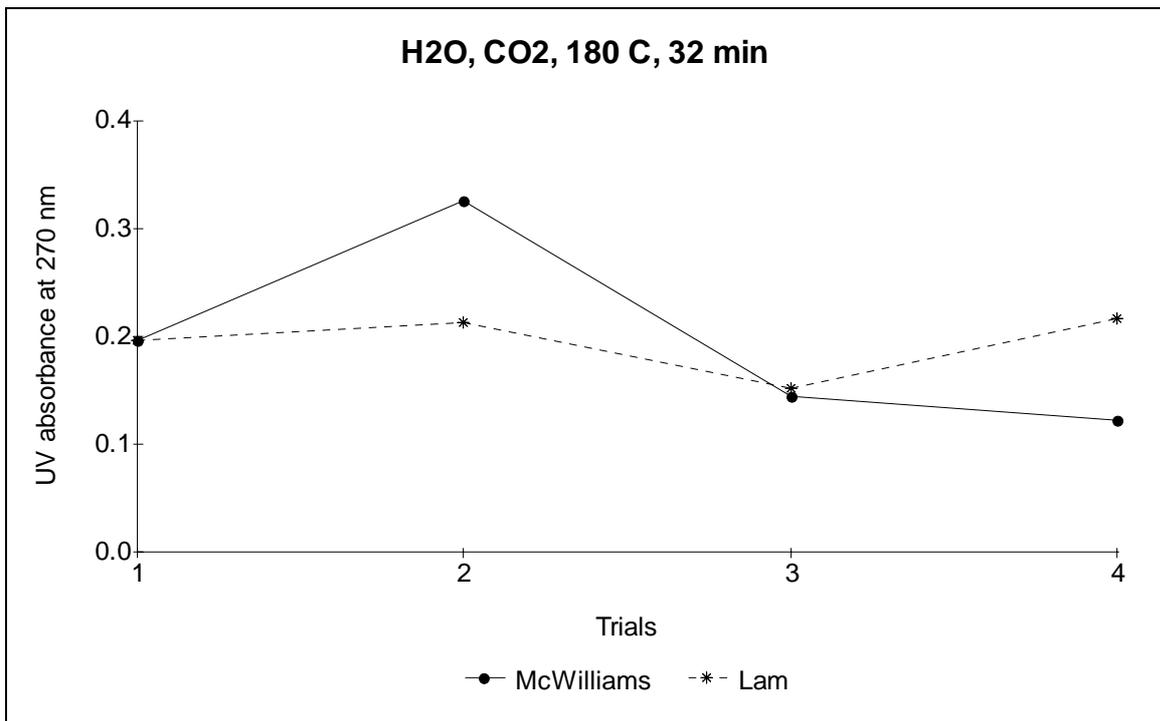
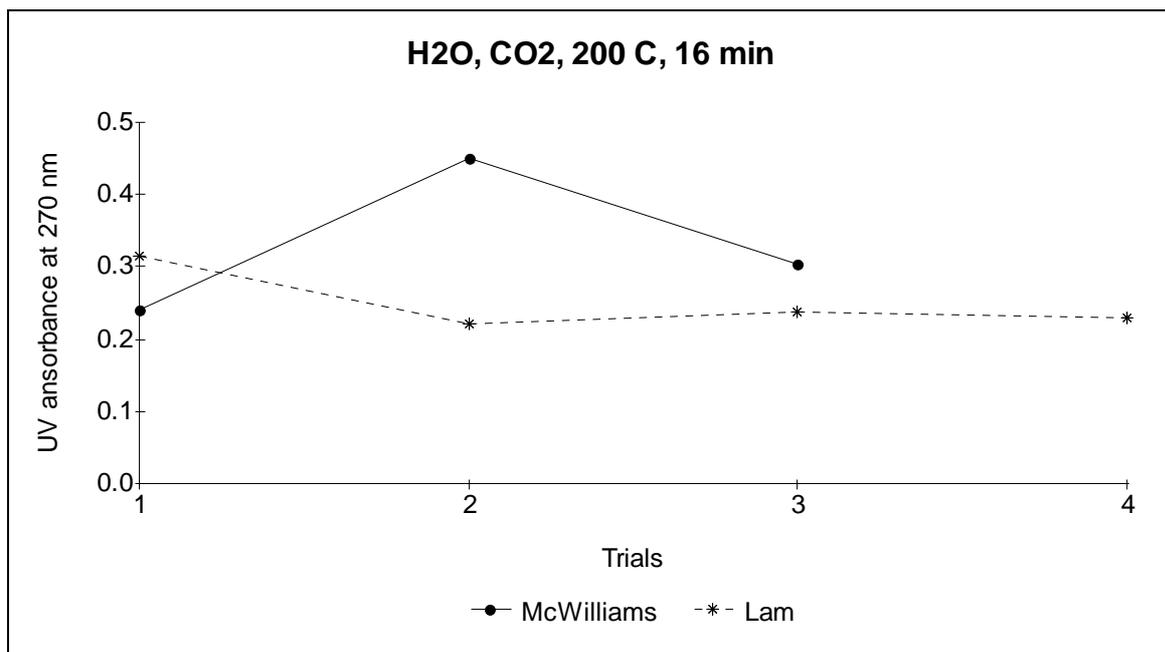
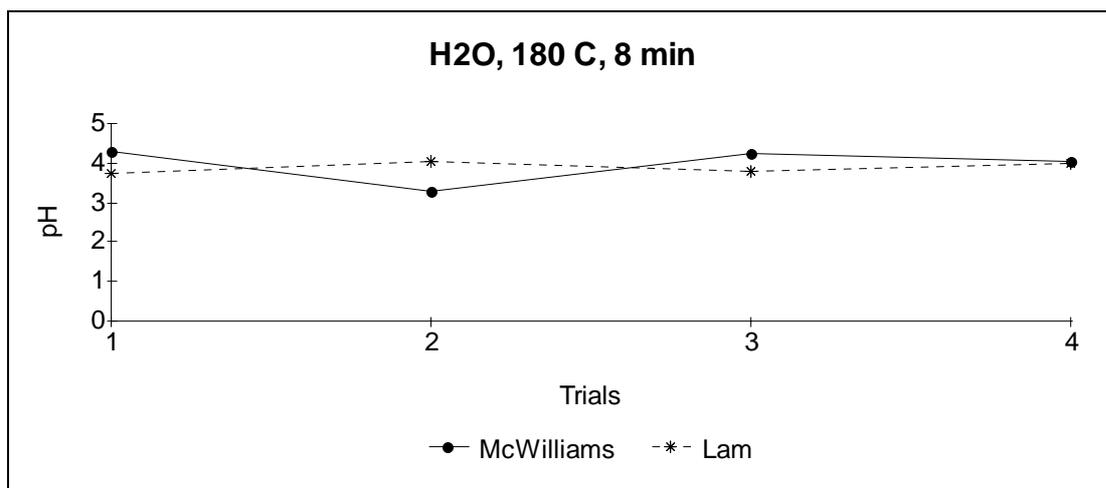


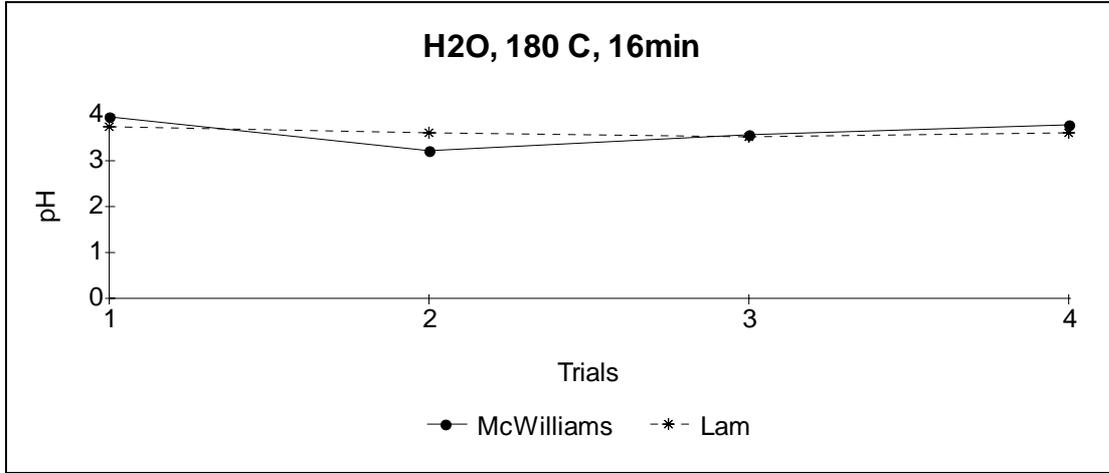
Figure 2.7



**Figure 2.8**



**Figure 2.9**



**Figure 2.10**

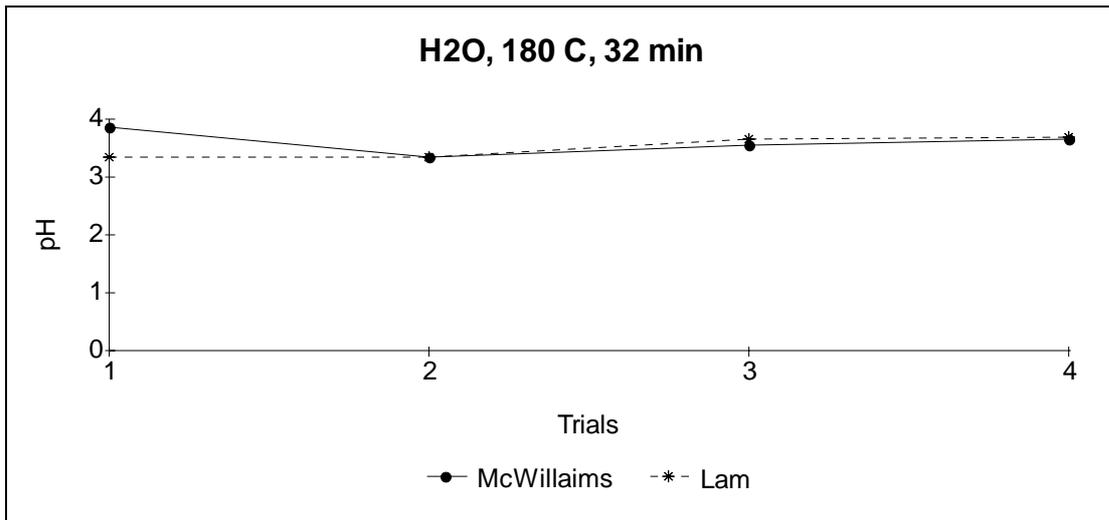


Figure 2.11

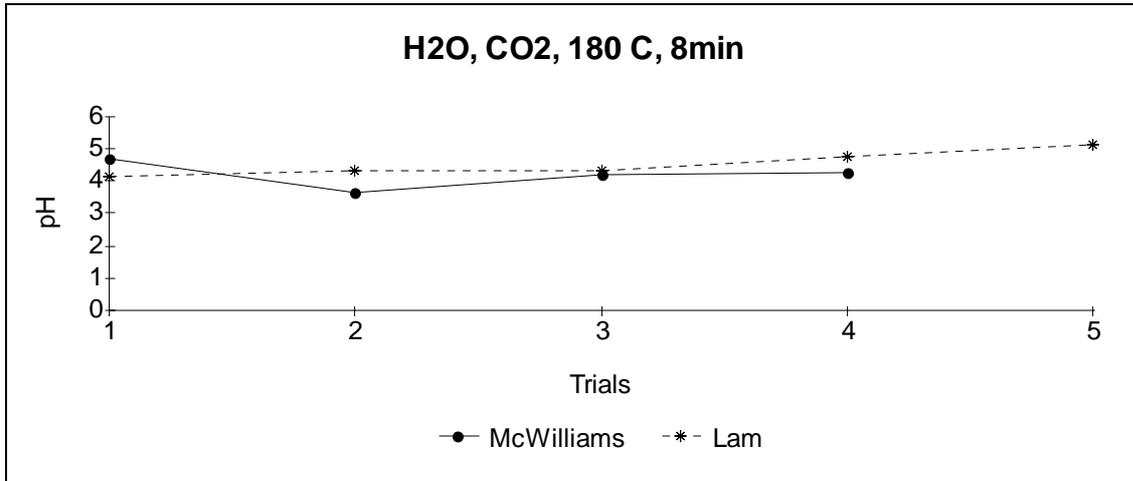


Figure 2.12

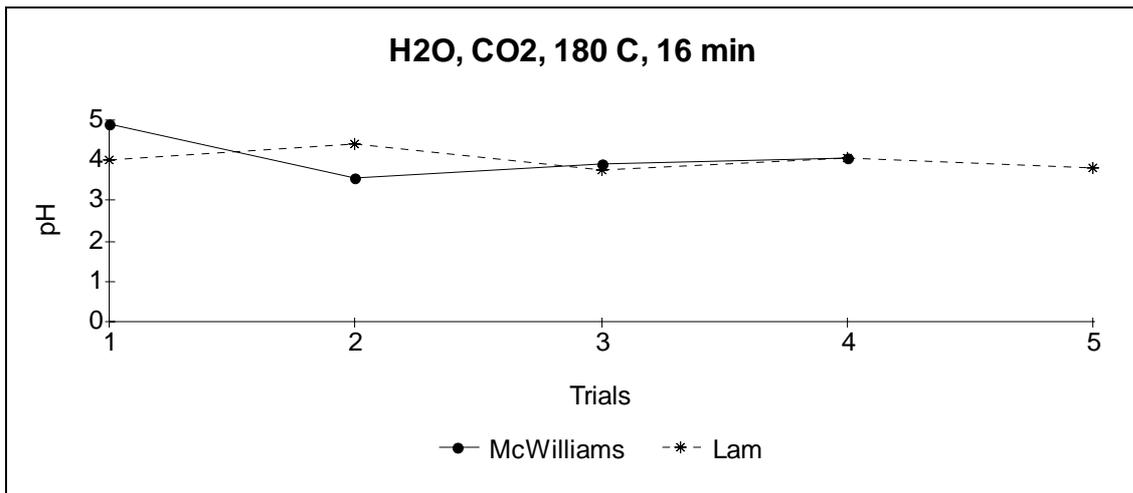


Figure 2.13

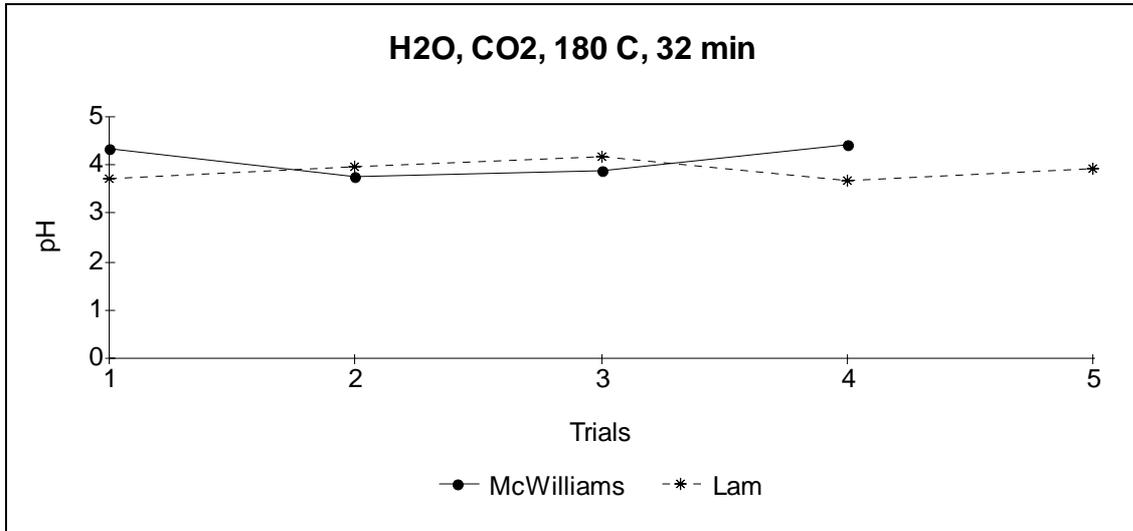
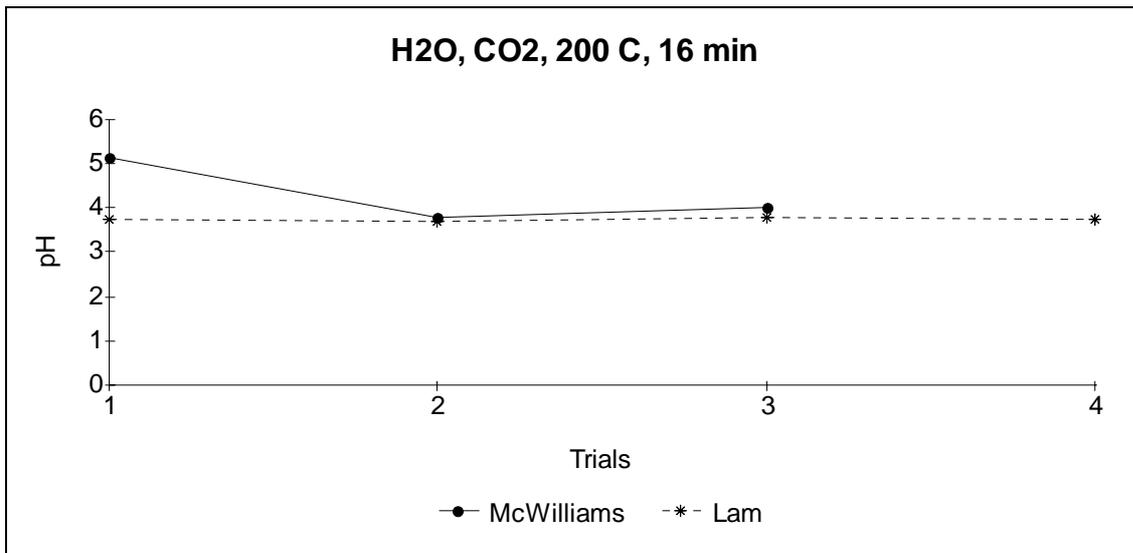
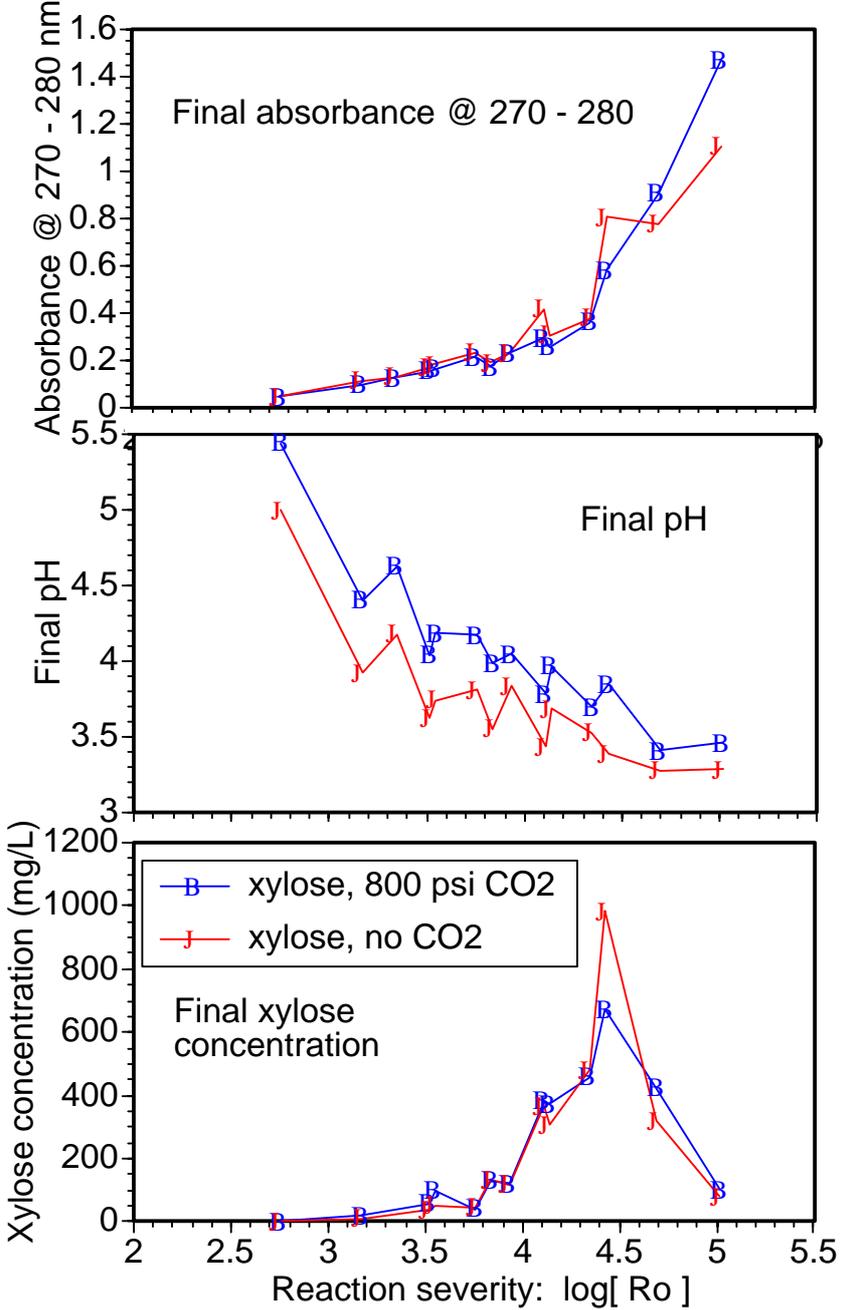


Figure 2.14



**Figure 2.15 Response of UV absorbance, final pH and xylose concentrations versus reaction severity for Carbonic Acid Pretreatment of Aspen Wood: combined results from McWilliams and van Walsum [1] and this study**



**Table 2.3 pH and Absorbance Results for Varying  
Pre-reaction Solubilization Time**

Sample	Time waited	Post reaction pH	pH	UV absorbance
1	0	4.86	5.94	0.069
2	0	5.21	6.42	0.166
3	0	4.91	6.08	0.093
4	0	4.76	5.71	0.1
5	0	5.54	6.46	0.085

Sample	Time waited	Post reaction pH	pH final	UV absorbance
1	15 minutes	5.57	5.78	0.065
2	15 minutes	5.11	5.81	0.12
3	15 minutes	5.02	6.71	0.132
4	15 minutes	5.5	6.12	0.166

Sample	Time waited	Post reaction pH	pH final	UV absorbance
1	30 minutes	4.86	6.26	0.13
2	30 minutes	4.91	6.77	0.102

Sample	Time waited	Post reaction pH	pH final	UV absorbance
1	5 hours	5.51	6.44	0.166

Sample	Time waited	Post reaction pH	pH final	UV absorbance
1	24 hours	5.24	6.35	0.141
2	24 hours	5.32	6.4	0.126
3	24 hours	5.14	6.11	0.109
4	24 hours	5.35	6.65	0.161

Figure 2.16 pH values versus Solubilization time

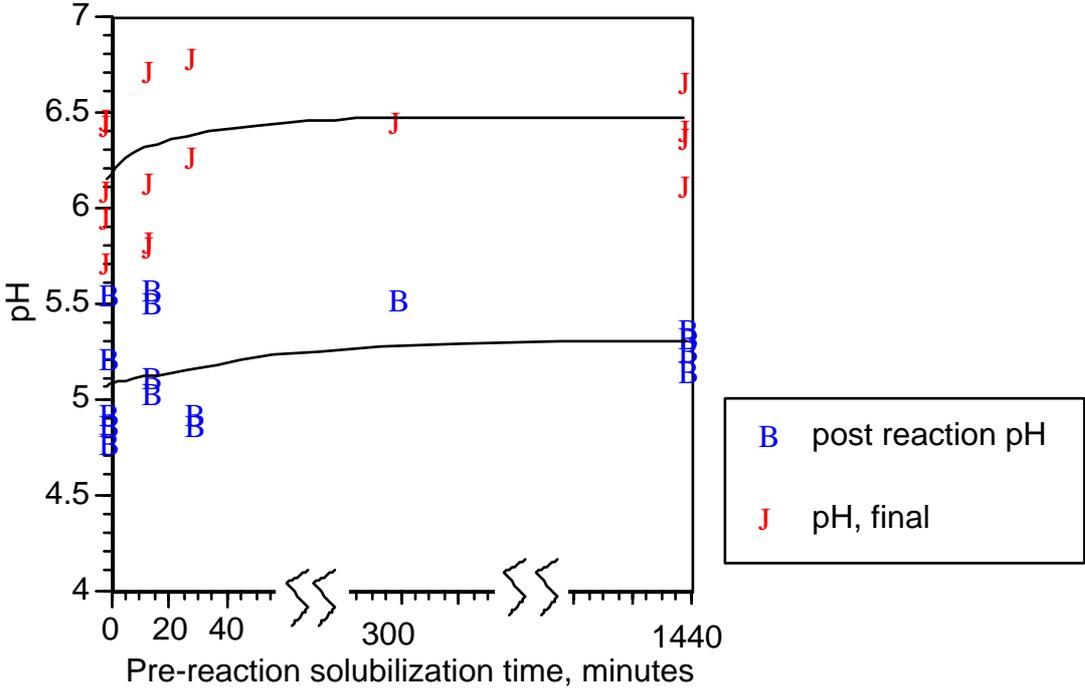
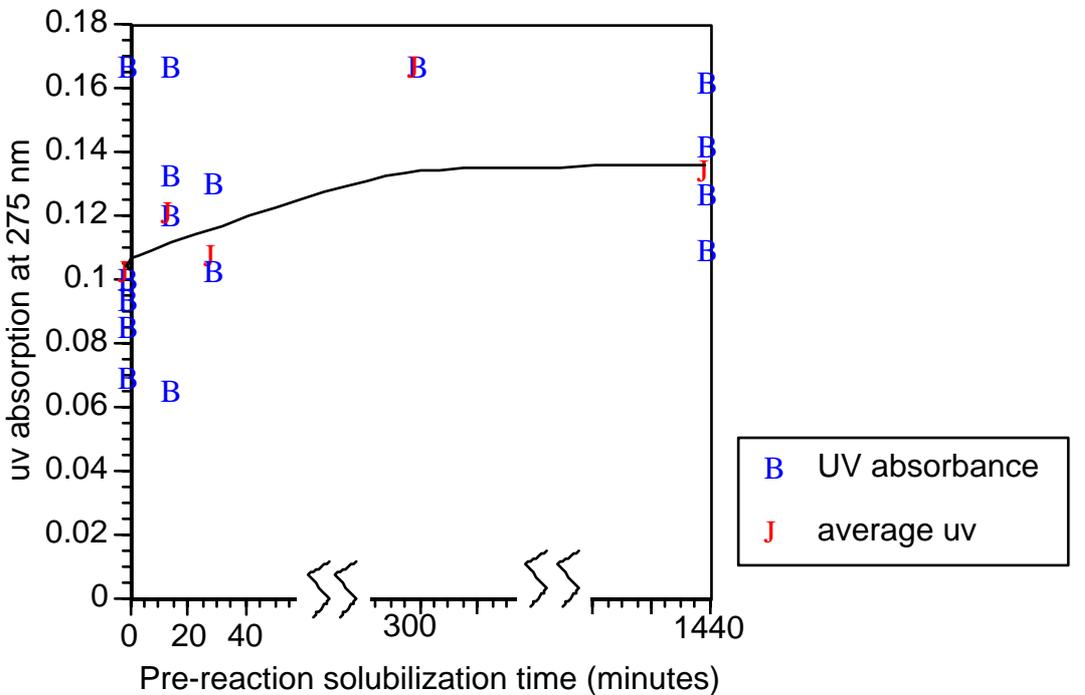
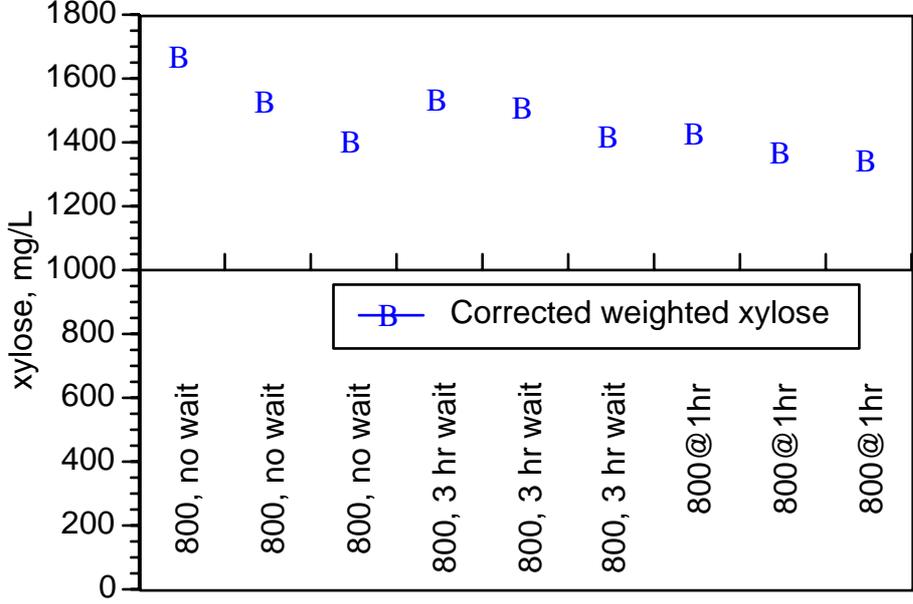


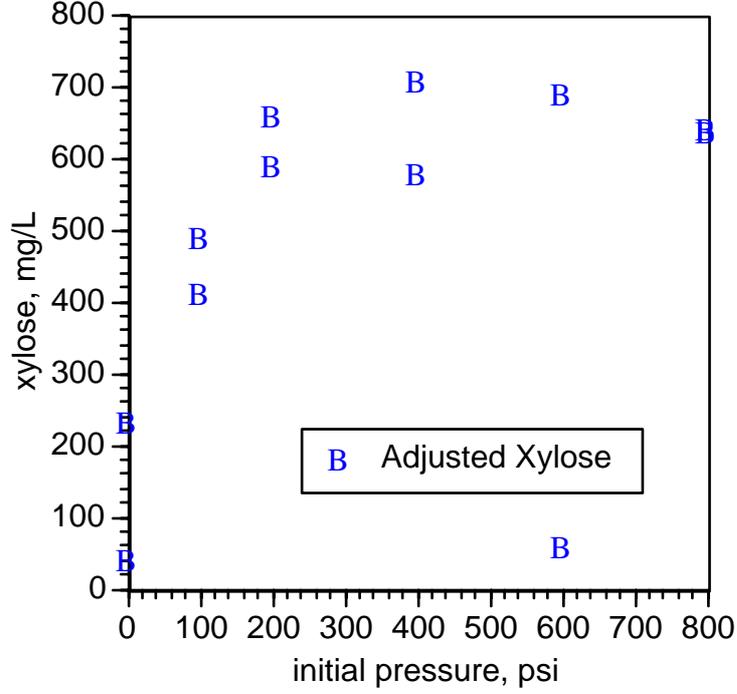
Figure 2.17 UV absorption vs Solubilization time



**Figure 2.18 Effect on xylose concentration of increasing CO<sub>2</sub> solubilization prior to reaction.**



**Figure 2.19: Response of xylose accumulation to increasing initial CO<sub>2</sub> pressure.**



## Discussion

Tables 2.1 and 2.2 show how previous results have been confirmed and the accuracy of the experiment improved. This improvement is encouraging, since the final results include experiments carried out by three different researchers, over a period of more than one year—illustrating good reliability for the methods. Of the original data collection, the UV absorbance data were the most erratic. Repeat experiments have improved upon the pH data and have in some cases dramatically improved the UV absorption data. In most cases, the large error reported in the initial findings were due to extreme outlying points. Statistically, it is now possible to justify dropping these severe outlying points with a high degree of certainty.

For the mass transfer experiments, scatter in the data do prevent drawing any firm conclusions at this point. There does appear to be a mild trends associated with longer periods of time allowed for solubilization of the CO<sub>2</sub>, but these trends appear to move in opposite directions for UV absorbance and xylose production, and thus are likely figments of the scatter in the data. Issues that still ought to be addressed with respect to this investigation are the amount of time needed to achieve equilibrium concentrations and whether or not super saturation of the solution is occurring in the reaction, since this could allow more acidic conditions than predicted by thermodynamic predictions of the equilibrium state of the system.

Results in figure 2.19, plotting xylose production versus CO<sub>2</sub> pressure are similar to those published by van Walsum in 2001[2], although van Walsum found a more continuous response to increased pressure throughout the range. It is possible that the quantity of xylose released in this more recent study was reaching a maximum due to completion of hydrolysis, as opposed to saturation with acid. However, if the observed result is more robust than this, it implies that carbonic acid pretreatment may be effective at lower pressures than currently used for most investigations. Because high pressures are expensive to implement in process conditions, this point deserves further exploration.

## Conclusion

It appears that increased availability of CO<sub>2</sub> increases hydrolysis, but that saturation of the system prior to reaction has relatively little effect on hydrolysis. This is likely caused by the CO<sub>2</sub> coming out of solution as the reactor is heated up to reaction temperature. There may be a leveling off of hydrolysis enhancement at about 200 psi initial pressure of CO<sub>2</sub>. This result should be confirmed because of its strong implications for reducing processing costs associated with high pressures.

## References

- [1] Robert C. McWilliams, G. Peter van Walsum, 2002. Comparison of Aspen Wood Hydrolysates Produced by Pretreatment with Liquid Hot Water and Carbonic Acid. Accepted for publication in *Applied Biochemistry and Biotechnology*
- [2] Van Walsum, G. Peter, 2001. Severity Function Describing the Hydrolysis of Xylan Using Carbonic Acid. *Applied Biochemistry and Biotechnology*, **91-93**: 317 - 329

### **TASK 3 Compare the results between hydrolysis of purified xylan and raw biomass.**

John Lam, Robert McWilliams, G. Peter van Walsum

#### **Summary**

It was found that the severity function developed by Overend and Chornet [2] adequately described the action of time and temperature on the pretreatment of aspen wood but not pure xylan. For aspen wood, no significant difference was detected between carbonic acid and water systems, thus the effect of the carbonic acid was negligible and did not need to be incorporated into the severity function. On xylan, however, it was necessary to take into account the action of the carbonic acid, and it was found that the severity function proposed by van Walsum [3] did so adequately. Published literature results for the dilute acid pretreatment of softwood [4,5] were used for comparing the fit of the severity function and the combined severity function for lower pH systems. It was found that the literature data had considerable scatter, but that the combined severity function did appear to offer a more predictive capability than the regular severity function, which does not take into account the pH of the system.

#### **Results:**

Figure 3.1 (same as 2.15, above) shows how absorbance, final pH and xylose yield respond to varying reaction severity. In this case, the severity is calculated without taking into account the pH of the system. Because the results from the liquid hot water system mirror those of the carbonic acid system, it appears that the pH of the carbonic acid has no significant effect on the degree of hydrolysis. Thus, in the case of aspen wood, it appears that the simple severity function is adequate to characterize the extent of reaction.

The same result was not found with xylan. Figure 3.2 (same as 2.19 above) shows how the extent of hydrolysis for xylan is strongly influenced by the pressure of CO<sub>2</sub> in the reactor. In this case, the combined severity function proposed by van Walsum [3] was used to replot the data presented in figure 3.2. In figure 3.3 it can be seen that the combined severity function does indeed offer a better characterization of the system than does the simple severity function, which for these data would have a single value.

Published results were used to assess the usefulness of the combined severity function at lower pH values. Reported values of dry matter remaining after hydrolysis were plotted versus the log of the severity function:  $\log(R_0)$ , and the combined severity function:  $CS = \log(R_0) - \text{pH}$ . Results are shown in figures 3.4 and 3.5. It can be seen that the general linearity of fit is better with the combined severity function, especially at higher severities.

Figure 3.1 Response of UV absorbance, final pH and xylose concentrations versus reaction severity for Carbonic Acid Pretreatment of Aspen Wood: combined results from McWilliams and van Walsum [1] and task 2 of this study.

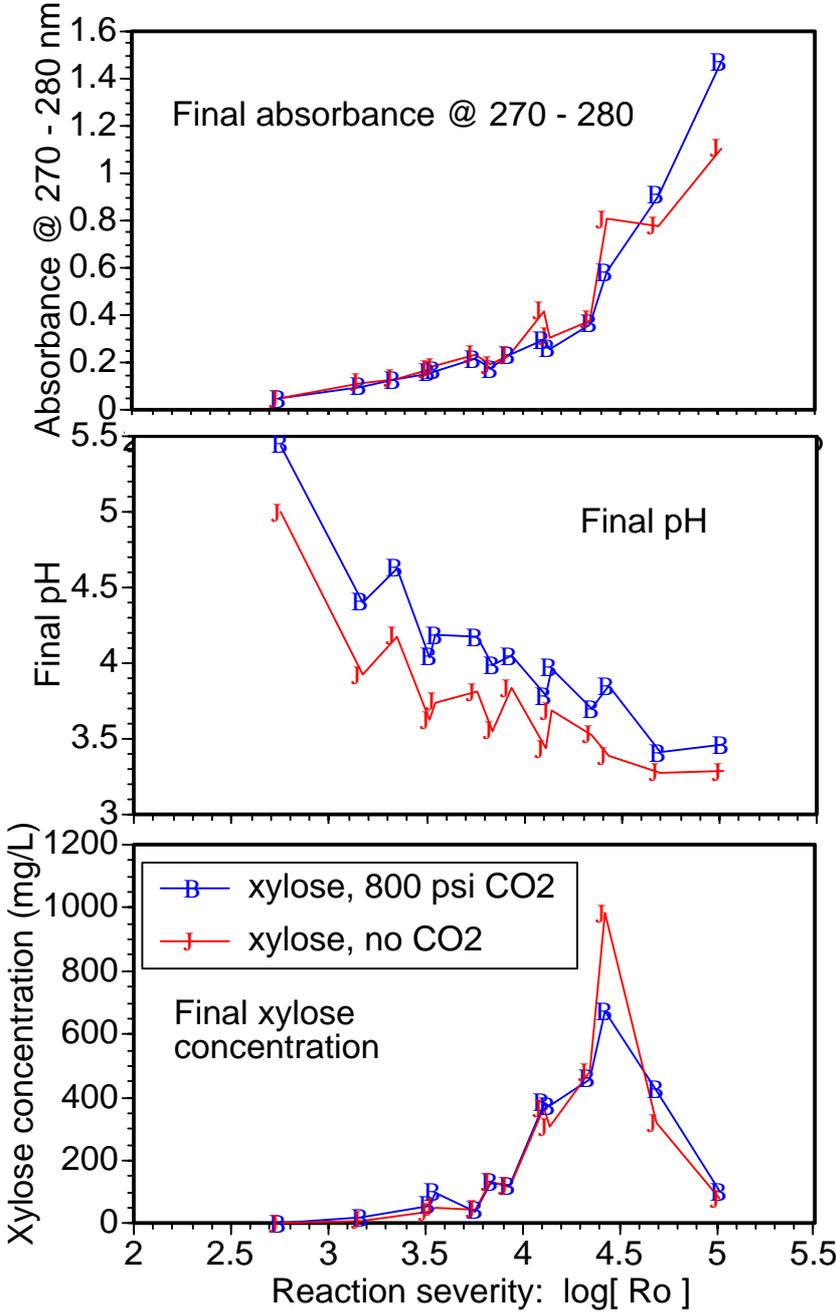


Figure 3.2: Response of xylose accumulation to increasing initial CO<sub>2</sub> pressure.

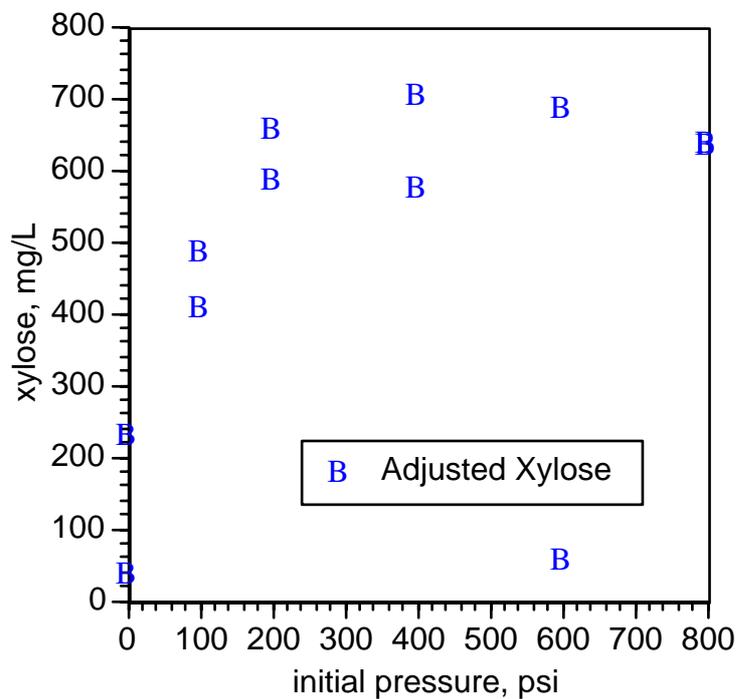


Figure 3.3 Xylose yield from hydrolysis of xylan at 190 C for 16 minutes, with combined severity reflecting varying pressures of CO<sub>2</sub>.

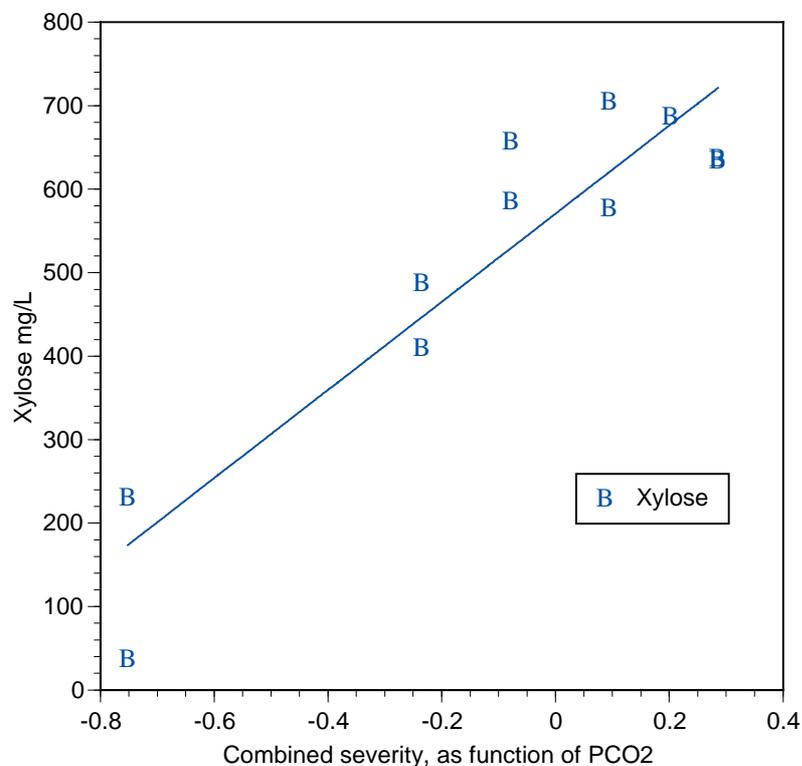


Figure 3.4 Residual dry matter versus combined severity for dilute sulfuric acid hydrolysis of pine wood [4]

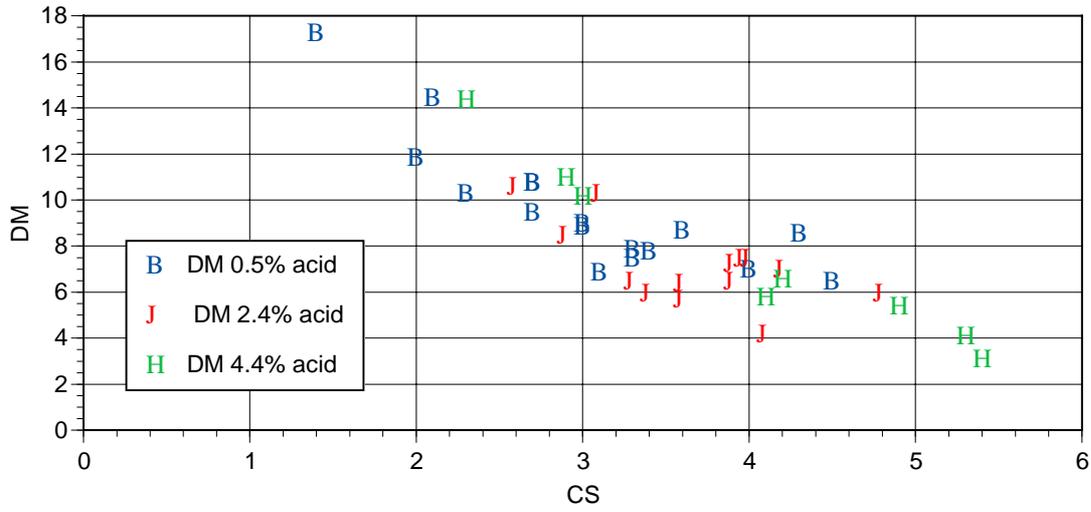
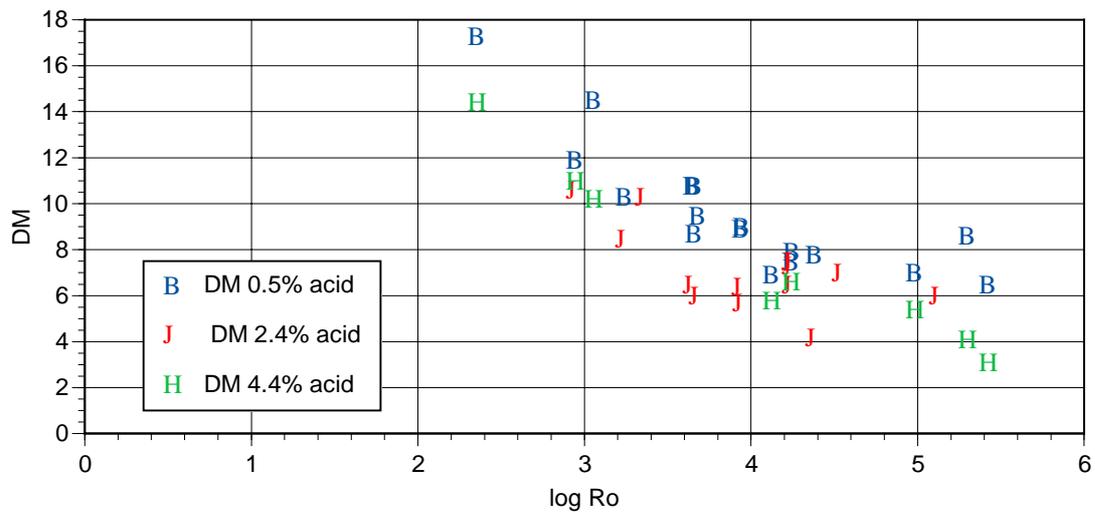


Figure 3.5 Residual dry matter versus log(Ro) for dilute sulfuric acid hydrolysis of pine wood [4]



## Discussion + Conclusion

From this brief investigation of recent and previously published results, it appears that the combined severity function, which is often used to characterize acid-catalyzed pretreatments, does indeed offer a better fit to solubilization data than does the regular severity factor when pretreatment is carried out at a pH lower than that resulting from the endogenous production of acids through thermal degradation. When added acids, such as carbonic acid, result in a pH similar to that of the endogenous acid production, use of the combined severity function appears to offer no improvement over the standard severity function.

## References:

- [1] McWilliams, Robert C. , van Walsum, G. Peter. 2002. Comparison of Aspen Wood Hydrolysates Produced by Pretreatment with Liquid Hot Water and Carbonic Acid. Accepted for publication in *Applied Biochemistry and Biotechnology*
- [2] Overend, R. P., Chornet, E. 1987. Fractionation of lignocellulosics by steam-aqueous pretreatments. *Phil. Trans. R. Soc. Lond* **A321**, 523-536
- [3] van Walsum, G. Peter. 2001. Severity Function Describing the Hydrolysis of Xylan using Carbonic Acid. *Applied Biochemistry and Biotechnology*. **91-93**:317-329.
- [4] Olsson, L. and Hahn-Hagerdal, B., Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme Microb. Technol.*, 1996. **18**: p. 312-331.
- [5] Larsson, S., Palmqvist, E., Hahn-Hagerdal, B., Tengborg, C., et al., The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enz. Microb. Technol.*, 1998. **24**: p. 151-159.

## TASK 4 – Test the performance of a larger capacity reactor

Robert C. McWilliams, G. Peter van Walsum

### Summary

Preliminary experiments using a 150 mL reactor were conducted using water and a range of CO<sub>2</sub> pressures and reaction temperatures to evaluate the reactor performance and to determine when the pressure (and hence temperature) inside the reactor reaches steady-state. This revealed optimal reaction conditions and will minimize variation between experimental results generated from small (15 mL) and larger (150 mL) reactors. The data also provided an accurate determination of the time required for the reactor to reach reaction temperature.

### Introduction

Up to this point, most experimental work with carbonic acid has been done on a small scale. This provided adequate volumes for studies of liquid hydrolysate composition, but does not provide sufficient volume of liquid or solid material to test hydrolysate inhibition or enzymatic hydrolysis rates of solids. Thus, this task served the purpose of testing the performance of a reactor to generate volume of samples for inhibition and enzymatic digestibility tests (task 5).

### Materials + Methods

#### Apparatus and Materials:

150 mL stainless steel reactor (Swagelok Corporation), sand bath (Techne, Oxford UK, model SBL 2D) and temperature controller (Techne model TC-8D); laboratory quality de-ionized water; and carbon dioxide from a pressurized cylinder.

Experimental design: Using a 10-fold scale-up from the 15 mL reactor, 80 mL of de-ionized water was placed in the 150 mL reactor. The reactor was then placed in a sand bath at temperatures of 180°C, 200 °C, 220 °C, and 240 °C. At each temperature, initial CO<sub>2</sub> pressures were 0 psi, 200 psi, 400 psi, and 800 psi. While in the sand bath, internal reactor pressures were measured and recorded at the following time increments: 15 sec, 30 sec, 45 sec, 1 min, 2 min, 3 min, 5 min, 10 min, 20 min, 30 min, 45 min, and 1 hr. Pressures were evaluated to determine steady-state pressures/times at various temperatures and to correlate pressure/time intersections at different temperatures to yield a favorable pre-heat time to allow the larger reactor to be brought rapidly up to reaction temperature before placing the reactor in the sand bath at reaction temperatures.

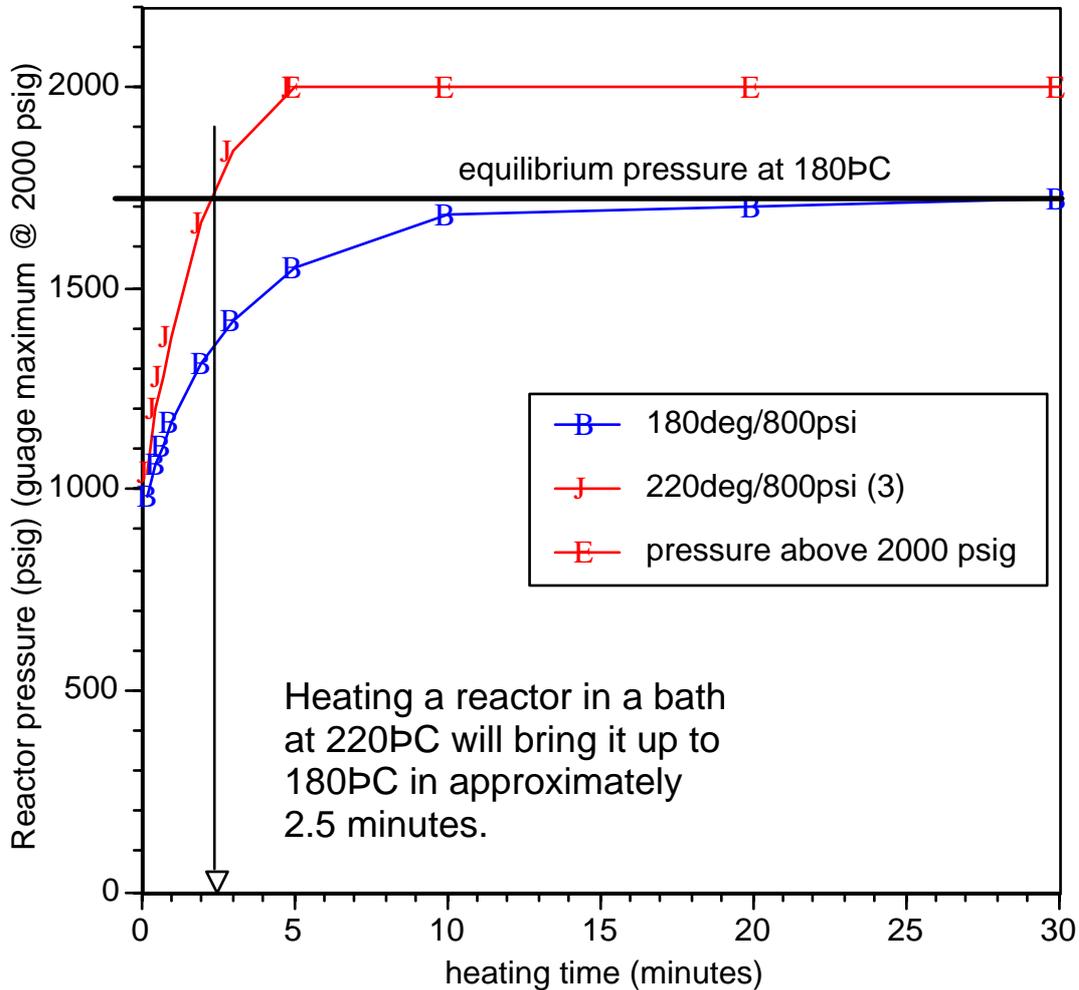
### Results:

Pressures measured at various times are recorded in table 4.1. Arrows in the table indicate the time required to reach the equilibrium pressure at T – 40°C. Figure 4.1 shows pressure/time curves for the reactor at 180 and 220 °C, charged with 800 psi of CO<sub>2</sub>.

**Pressure experiment results**

Temp (C)	CO <sub>2</sub> pres room temp (psig)	P at 15sec	P at 30sec	P at 45sec	P at 60sec	P at 120 sec	P at 3min	P at 5min	P at 10min	P at 20min	P at 30min	P at 45min
180	0	0	0		0	0	0	60	80	100	<b>120</b>	120
180	200	200	220	235	250	260	280	305	360	400	410	420
180	400	460	480	500	520	580	620	670	690	690	690	690
180	800	980	1060	1110	1170	1315	1420	1550	1680	1710	1720	<b>1730</b>
200	0 (1)	80	120	135	150	200	240	300	350	380	380	380
200	0 (2)	0	0	0	0	60	90	140	200	210	210	210
200	0 (3)	0	0	0	0	60	90	150	200	210	210	210
200	200	220	240	265	280	310	340	400	445	460	460	460
200	400	460	500	520	550	620	665	715	760	780	780	780
200	800 (1)	1020	1120	1230	1300	1520	1685	1880	2000+	2000+	2000+	2000+
200	800 (2)	980	1050	1130	1200	1380	1500	1640	1820	1900	1920	<b>1930</b>
200	800 (3)	980	1120	1260		1540	1660	1800	1940	2000	2000	2000
220	0 (1)	0	0	0	0	80	<b>120</b>	200	280	300	300	300
220	0 (2)	0	0	0	0	70	115	190	280	310	320	320
220	200	240	260	280	300	350	400	500		600	600	600
220	400	500	550	585	610	700	770	880	980	1000	1000	1000
220	800 (1)	980	1060	1150	1220	1400	1500	1640	1800	1820	1820	1820
220	800 (2)	1040	1200	1300	1420	1680	1820	2000+	2000+	2000+	2000+	2000+
220	800 (3)	1040	1200	1280	1380	1660	<b>1840</b>	2000+	2000+	2000+	2000+	2000+
240	0	0	0	0	60	140	230	350	410	460	460	460
240	800 (1)	1080	1160	1260	1400	1600	1780	2000	2000+	2000+	2000+	2000+
240	800 (2)	1040	1200	1320	1420	1760	<b>1930</b>	2000+	2000+	2000+	2000+	2000+

**Figure 4.1. Determination of reactor heat up time at reaction temperature + 40°C**



**Discussion:** At 0 psi for all temperatures, the larger reactor had a slower initial heat-up time compared with experiments conducted under the same conditions with a 15ml stainless steel reactor of similar design. Given the larger size of the reactor, this was expected. Typically, at this initial pressure it took 2 minutes for the pressure to begin to increase gradually followed by a more pronounced pressure increase and finally a “leveling-off” as the reactor’s internal pressure reached steady-state. At 200 psi, 400 psi, and 800 psi, initial pressures, there was a gradual initial increase, followed by a more rapid increase between 3 and 5 minutes, then returning to a gradual increase and leveling-off between 5 and 45 minutes. This experiment provided an excellent data set to determine pressure/time intersections of reactions at different temperatures. From this data set it was determined that a 40 degree temperature increase (keeping initial pressure the same) results in reaching reaction pressure of the target reaction temperature in considerably less time. For example, a reaction at 180°C and 800 psi initial CO<sub>2</sub> pressure took 30 minutes to reach a steady-state pressure of 1720 psi. In contrast, a reaction at 220°C and 800 psi took between 2 and 3 minutes to reach the same pressure. Therefore, when conducting reactions at 180°C, preheating the reactor (with the water or water/CO<sub>2</sub>, and wood already inside) at 220°C would allow the reactor to reach steady state much faster and yield more consistent results.

**Conclusion:** The 150 mL reactor design is adequate in design to allow the scale up of previous experiments and to produce a larger volume of product for further evaluation. Results from experiments with water alone were comparable to published steam table data at each temperature [1] and consistent with results for the 15 mL reactor under the same circumstances. The determination of an accurate preheat time and temperature allows the reactor to begin the reaction at steady-state temperature which will provide more consistency and a truer picture of the reaction a the established reaction time.

**References:**

[1] Chemical Rubber Company, Handbook of Chemistry and Physics, 62<sup>nd</sup> Edition, CRC press, Boca Raton, FL, 1982-2, E-17, 18

## TASK 5 – Generate larger samples at selected reaction conditions

Robert C. McWilliams, G. Peter van Walsum

### Summary

The newly constructed 150 mL reactor was used in reactions that replicated the conditions of the 15 mL reactor. 1.0 grams of aspen wood, 80 ml of de-ionized water were reacted with and without CO<sub>2</sub> at 800 psig. Reaction temperatures were 180°C, 200°C, and 220°C with reactions times of 8, 16, and 32 minutes. The reactor was preheated in a sand bath set to a temperature 40°C above reaction temperature for two minutes. This allowed the reactor to quickly reach reaction temperature, as determined and reported in the previous progress report. The 150ml reactor successfully delivered the expected 10-fold increase in hydrolysate compared to the 15ml reactor. Results of the pH and UV analysis of the hydrolysate were consistent with those yielded by the 15ml reactor.

### Introduction

Up to this point, most experimental work with carbonic acid has been done on a small scale. This provided adequate volumes for studies of liquid hydrolysate composition, but does not provide sufficient volume of liquid or solid material to test hydrolysate inhibition or enzymatic hydrolysis rates of solids. This task served the purpose of testing generating volume of samples for inhibition and enzymatic digestibility tests (tasks 6 and 7).

### Materials + Methods

Apparatus and Materials: 150 mL stainless steel reactor (parts supplied by Swagelok Corporation); two sand baths (Techne, Oxford UK, model SBL 2D) and temperature controllers (Techne model TC-8D); laboratory quality de-ionized water; and carbon dioxide from a pressurized cylinder, ground Aspen wood using a domestic coffee grinder, stainless steel sieve (2 mm to 500 micron mesh), pH meter (Acumet AR-15, Fisher Scientific), spectrophotometer (Beckman, DU-600), centrifuge (Eppendorf) laboratory balance (Ohaus, Explorer)

Experimental: Aspen wood chips are ground using a domestic coffee grinder and sifted in the stainless steel sieve. The wood particles are allowed to separate and the particles between 0.5 mm and 1 mm are retained for experimental use. 1 gram samples of the 0.5 – 1 mm wood particles are weighed and placed in the reactor. In addition to the wood, 80 mL of water is added. The reaction temperatures (180, 200, 220°C) and times (8, 16, 32 minutes) selected are based on previous results using the smaller reactor. At each of these sets of conditions, reactions were run using wood and water alone (as previously described) and wood, water, and CO<sub>2</sub> at 800 psi. The reactor was placed in one sand bath set at a temperature 40 degrees Celsius higher than the desired reaction temperature for three minutes. This allowed the reactor to reach steady-state before immersion into the sand bath at reaction temperature. The pH of the hydrolysate was measured. Finally, spectrophotometric measurements of the hydrolysate (1% solutions) were made using the spectrophotometer in scan wavelength mode between 190 nm and 350 nm. Absorption peaks along this spectrum were compared in reactions with and without CO<sub>2</sub> to evaluate the concentration of degradation products that are potentially inhibitory to enzymatic digestion.

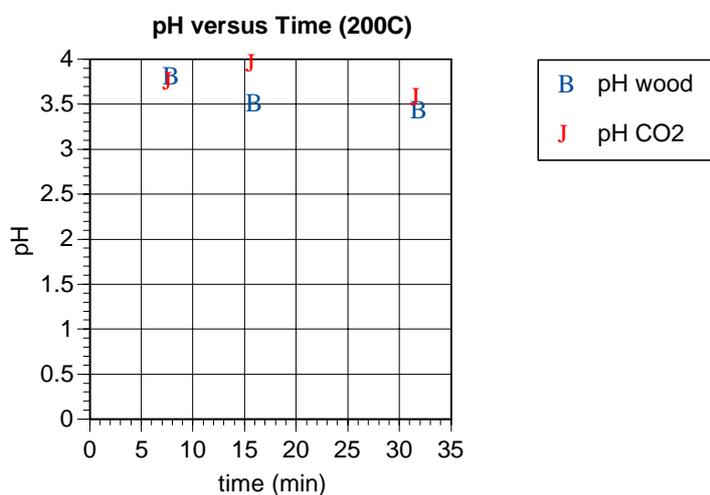
## Results:

Table 5.1 and figures 5.1 and 5.2 illustrate data generated to date using the larger volume reactor.

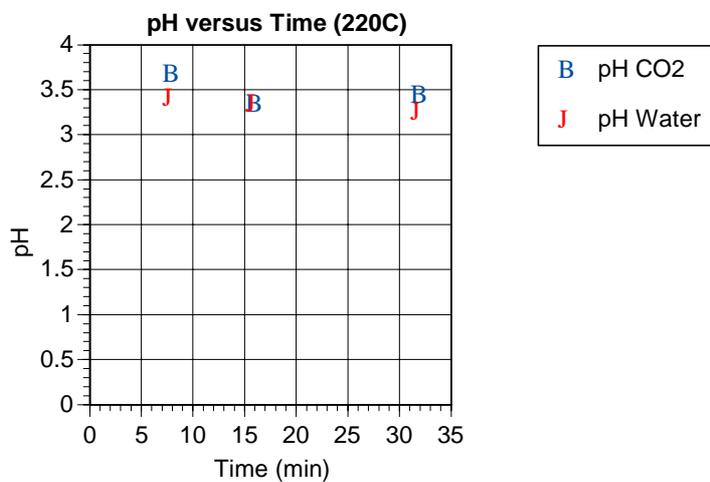
**Table 5.1**

temp	time	pH wood	pH CO2
200C	8	3.81	3.76
200C	16	3.51	3.96
200C	32	3.44	3.58
220C	8	3.68	3.42
220C	16	3.34	3.34
220C	32	3.44	3.26

**Figure 5.1**



**Figure 5.2**



**Discussion and Conclusion:** The 150ml reactor met all performance expectations generating larger volumes of hydrolysate which was consistent with hydrolysate yielded by the smaller reactor. This larger volume of hydrolysate will enable faster analysis of toxicity and an applied understanding of the composition of the hydrolysate.

## Task 6: In vitro determination of inhibition

Damon Yourchisin, G. Peter van Walsum

### Summary

Inhibition tests measured the rate of sugar consumption by *Saccharomyces cerevisiae* growing in batch culture of hydrolysate. It was found that inhibition of the yeast culture increased with severity of pretreatment above a mid level severity. Below this severity, little to no inhibition was observed. No difference was observed between the inhibition of hydrolysates prepared either with or without the presence of CO<sub>2</sub>. To conduct the experiments, serum vials were charged with 21g/L of sterile growth medium containing 20ml of pretreatment hydrolysate. The vials were inoculated with 0.2ml of freshly grown cell broth and incubated. Glucose concentrations over time were determined via glucose assay (Infinity Glucose Reagent) and the HPAE when available.

### Introduction

The organisms used for the fermentation of ethanol are often inhibited by the degradation products produced during pretreatment. Enzymatic hydrolysis can also be impaired by inhibitors produced during pretreatment. Inhibitory compounds originate from: the hydrolysis of extractive components, organic and sugar acids (e.g. acetic, formic, glucuronic, galacturonic) esterified to hemicellulose; solubilized phenolic lignin derivatives; the degradation of solubilized compounds (e.g. furfural, hydroxymethyl furfural); and the release of corrosion products (e.g. metal ions)]. The production of inhibitors has been documented for dilute-acid, steam-explosion, acid-hydrolysis and liquid hot water pretreatments. To date no published studies have looked at inhibition resulting from carbonic acid pretreatment. Treatments to eliminate or reduce inhibitors, such as ion exchange and over-liming, add considerable expense to processing systems.

### Materials + Methods

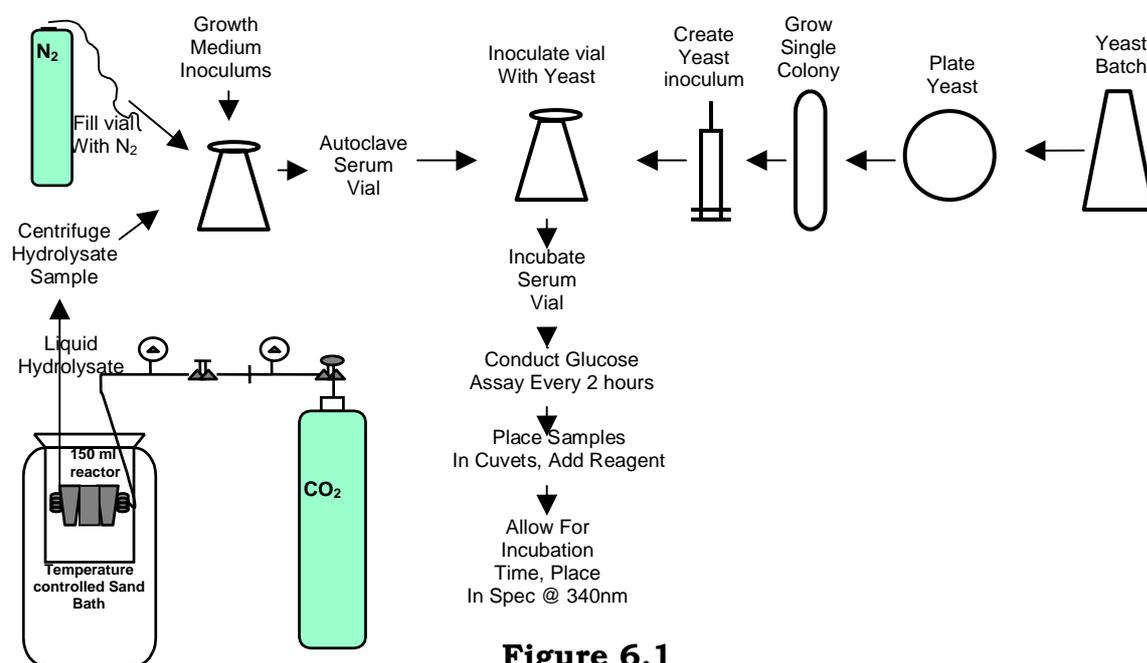
#### Apparatus and materials

Two Techne Fluidised Sand Baths, model SBL-2d, with temperature controllers.  
Beckman DU 520 General Purpose UV/Vis Spectrophotometer (with single cell module)  
Fisher Scientific AR15 pH meter by Accumet Research  
150ml stainless steel immersible reactors  
Eppendorf Reference series auto pipettes  
Ohaus Expolorer digital scale Item #12140 (d=0.1mg)  
Domestic brand coffee grinders  
Fisher Scientific U.S.A. Standard Testing Sieve, ASTM-E-11 Spec #18 (1mm opening)  
Standard laboratory de-ionized quality water  
Standard laboratory grade carbon dioxide  
Vacuum filter with water aspirator  
Schleicher and Schuell Sharkskin 100Cir. 5.5cm Microfilter paper  
Sigma Yeast Malt Agar @ 41g/L  
Sigma Yeast Malt Broth @ 21g/L  
Fleischmann's Active Dry Yeast  
Fisher Scientific Marathon 21000R centrifuge  
Market Forge Sterilmatic STM-E autoclave

Thermolyne MaximixII type 37600 mixer  
 SIGMA Diagnostics - Infinity™ Glucose Reagent (Procedure 17-UV) Kit  
 Glass serum vials (50mL)  
 Aspen wood chips (provided by USDA Forest Products Laboratory in Madison, WI)  
 Sigma D-Glucose Anhydrous

### Experimental Methods

The experiments followed a general methodology regardless of reaction conditions. The general steps included: grinding the feedstock, pretreatment, preparation of hydrolysate, separating the solids from the liquids, microbial culturing, inhibition tests, analysis of the samples. These steps are detailed below and summarized in figure 6.1.



**Figure 6.1**

### Feedstock Preparation

Feedstock was ground and sifted. Particles that passed through a 1mm screen were used, particle sizes of 0.6-1mm. Two samples of 1.25g each of uniform particulate sized feedstock were weighed out and placed in separate 150ml 316 stainless steel reactors, along with 100ml of DI water each. The reactors were filled and emptied by removing a swage connection on one end. One reactor was pressurized with 800 psi of CO<sub>2</sub>. For reactions using CO<sub>2</sub>, a 1/8" and 1/4" stainless steel tubing connection and valve with pressure gage were fitted to the reactor.

### Pretreatment

One sand bath was heated to the desired reaction temperature. The other sand bath was heated to 40<sup>0</sup>C above the desired reaction temperature. The higher temperature sand bath was used for preheating the reaction vessels to better control thermal and pressure equilibrium. A 3 minute pre-heat at reaction temperature plus 40<sup>0</sup>C allowed the reactors to quickly reach a state of constant pressure, thereby reducing the variability of reaction dynamics when compared to a slower heat-up. (27) Both filled reactors were placed into the reaction temperature plus 40<sup>0</sup>C sand bath for 3 minutes as a preheat. They were immediately removed and placed in the reaction temperature sand bath for the desired reaction duration.

The reaction was quenched in an ice bath immediately after the reaction duration time period was complete.

Reaction times consisted of short durations (4-8 minutes), a mid-range duration (16 minutes), and long-range durations (32-64 minutes). Pretreated samples were extracted from the reactors and placed in glass collection bottles for the next step.

#### *Preparation of hydrolysates*

Solids were filtered out from the hydrolysate samples generated using vacuum filter and microfilter paper. The solids remaining on the filter paper were washed with DI water 3 times (12ml DI water total). During the final rinse the vacuum filter was allowed to work for 3 minutes and then shut off to collect solids. The solids were placed in weighing tins in preparation for enzymatic hydrolysis experiments. The liquids were then centrifuged to remove all solids. The original liquid was placed into centrifuge bottles and centrifuged at 4000rpm for 15minutes at 15<sup>0</sup>C. The centrifuge bottles were emptied using a pipette, to ensure no solids were present, into clean collection bottles. The liquid hydrolysate was now prepared for experimentation.

#### *Microbial culturing*

A new batch of yeast agar, yeast broth, and yeast were prepared for each experiment according to the manufacturers directions. Yeast agar and broth were prepared in the following manner: 500ml of water were brought to a boil and 20.5grams of yeast agar or 10.5grams of yeast broth were placed into it. Once all solids were dissolved, the mixtures were autoclaved. After autoclaving, 20ml of the agar were aseptically transferred from the autoclaved flask to a Petri dish and 10ml of the broth were aseptically transferred from the autoclaved flask to a culture tube under the laminar flow hood. Three plates and three culture tubes were created each time to ensure growth and to act as a back-up during each step if contamination occurred. A new batch of bakers yeast was prepared according to manufacturers directions: 2 1/4 teaspoons of yeast was added to 1/4 cup of water at 1000F with 1 teaspoon of glucose. The mixture was let to stand for 10 minutes. Once growth of yeast was confirmed, they were plated for isolation under the laminar flow hood using the fresh yeast agar plates. After plating, the Petri dishes were placed in the incubator for 48 hours at 30<sup>0</sup>C along with the culture tubes of broth (although nothing was in them yet). After 48 hours, the plates and broth culture tubes were removed and put under laminar flow hood in order to aseptically transfer a single yeast colony from the agar plate to the broth culture tube. An isolated colony from the plate was aseptically transferred into the 10ml of broth in the culture tube using a loop. The plates were properly disposed of and the culture tubes were placed into the incubator at 30<sup>0</sup>C for 48 hours.

#### *Preparation of test vials*

Twenty milliliters of the previously generated liquid hydrolysate were placed into serum vials. 20ml was the maximum amount of liquid per vial. The vials contained from 0% to 100% hydrolysate. The remaining 20ml of the less than 100% hydrolysate was made up using DI water. Yeast broth at 21g/L (0.42g) was weighed and placed into each serum vial to serve as a known growth medium. Prior to autoclaving the serum vials N<sub>2</sub> gas was bubbled in them for 30 seconds. Each vial was capped immediately and sealed after removal from the N<sub>2</sub> injector. Once all vials were sealed, they were placed in the autoclave for 20 minutes at 121<sup>0</sup>C. The pH for each of these samples was tested and found to be within the tolerance range of yeast, as shown in table 6.1 below for the most recently performed experiment.

**Table 6.1** pH of hydrolysates prior to yeast cultivation

Exp 138:pH of hydrol+nutrient	
Liquid Samp	pH
T/t/P	
180/8/0	5.69
180/8/800	5.87
200/16/0	5.04
200/16/800	5.05
220/4/0	5.25
220/4/800	5.39
180/64/0	5.01
180/64/800	4.97
0% hydrol	6.31

### *Inhibition test*

Once the vials were cooled they were placed in the incubator to bring them to the same temperature as the yeast broth that would be transferred to them. Once the yeast broth had been incubated for exactly 48 hours, the serum vials were each injected 0.2ml of the vortexed yeast inoculum from the culture tubes using a 1ml syringe. This was performed under the laminar flow hood and each serum vial top was flamed prior to needle insertion. This became time zero (T=0) for the experiment. Samples were taken for the next 12 hours every 2 hours, except for the first 4 hours, T+4 was the second measurement.

### *Glucose Assay*

At each testing period, 0.2ml of sample was removed from the serum vials and placed into a 1ml microcentrifuge tube. The sample was diluted 5:1, so 0.8ml of DI water was added to each centrifuge tube. This was done because the yeast broth contained 10g/l dextrose and the glucose assay was only good for up to 5g/l, but 2g/l was best. Next, 1500 microliters of glucose reagent was placed into a 2ml cuvette for each sample, minimizing the time the glucose reagent is out of the refrigerator as much as possible. Using the spectrophotometer, UV measurements of the reagent only were taken and recorded. This was done by placing each cuvette in the spectrophotometer set at 340nm absorbance and measuring the AUs. Then, 15 microliters of each sample were removed and placed in their respective 2ml cuvettes. The cuvettes were covered and incubated for 14 minutes (ambient room temperature should be between 20<sup>0</sup>C and 22<sup>0</sup>C). Serum vials were immediately placed in the incubator at 30<sup>0</sup>C. After 14 minutes, each cuvette was again placed in the spectrophotometer set at 340nm absorbance and was measured and recorded. The recorded AUs of each cuvette with reagent only were subtracted from the recorded AUs of the cuvettes with sample and reacted reagent. A glucose standard of 2g/l was run at each sampling time in order to compare the samples to a known standard to determine glucose generated throughout the experiment. At T+24 the dilution was only 1:1 for the samples and at T+48 there was no dilution of sample prior to analysis with the assay. The glucose assay was found to be linear up to 3g/L of glucose.

This information was used when calculating theoretical glucose yield of samples prior to experimentation to ensure they would be within the range of the assay's testing limits. It was also used to determine the correct g/L of glucose standard to run as a standard for each experiment, which was chosen to be a constant 2g/L for each experiment. A control was also

used for each yeast inhibition experiment by running a sample that contained 0% hydrolysate but all the other components of the nutrient broth.

## Results

Data were analyzed using the DU 500 Spectrophotometer, pH meter and Infinity Glucose Reagent Assay kit. Two metrics were used, total recovered glucose and reaction speed. The raw numbers were averaged amongst duplicates within experiments and graphed (Absorbance Units versus time, transposed into Glucose yield versus time).

Figure 6.2 shows that there is no effective difference in the yeast growth rates in hydrolysates produced either with or without the presence of CO<sub>2</sub>. These experiments were carried out at the mid point severity of the study, at 200 C and 16 minutes reaction time.

Figure 6.2

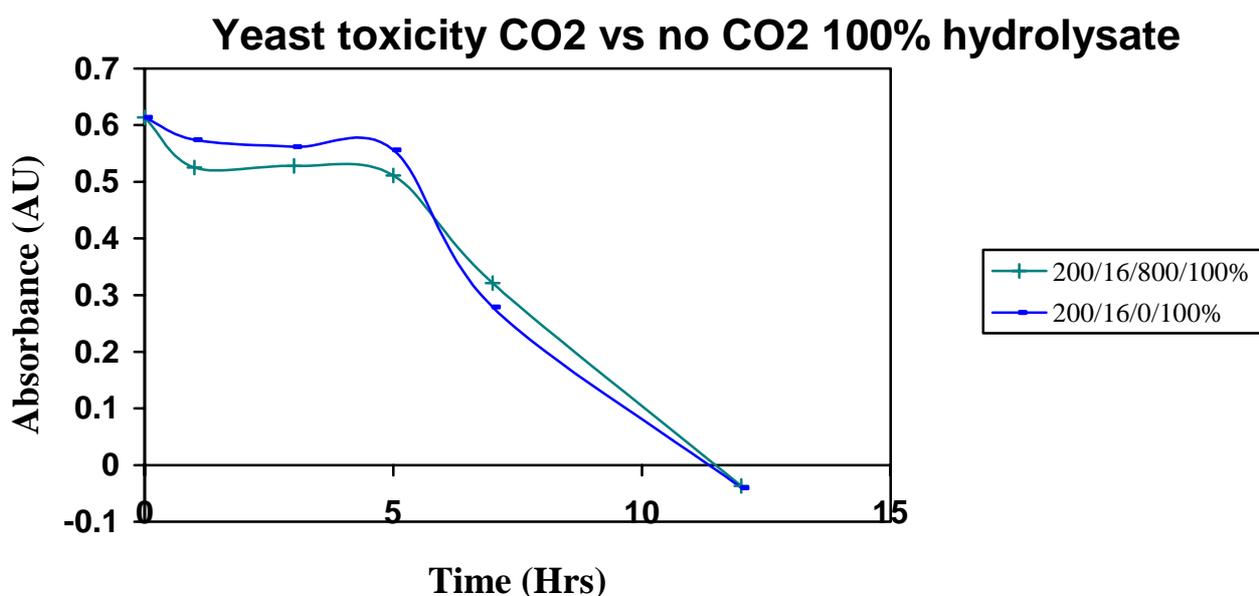
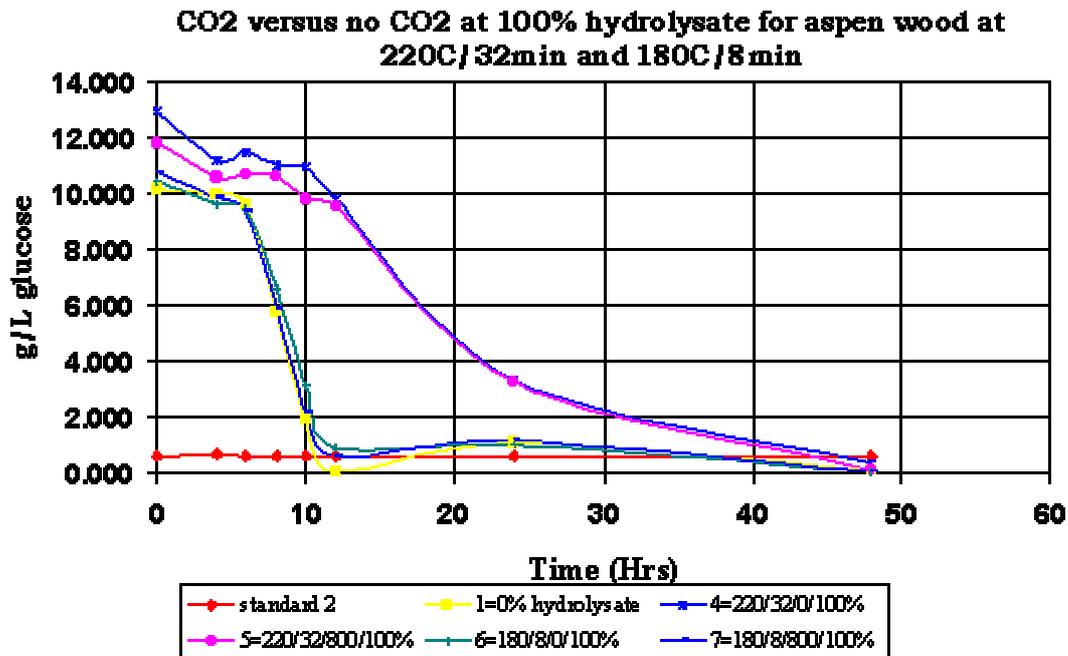


Figure 6.3 below, again shows that there is no difference between samples that contain CO<sub>2</sub> and samples without CO<sub>2</sub>. In this case, a marked difference is evident in the inhibition of the yeast by hydrolysates produced at high ( 220 C, 32 minutes) and low (180 C, 8 minutes) severities.

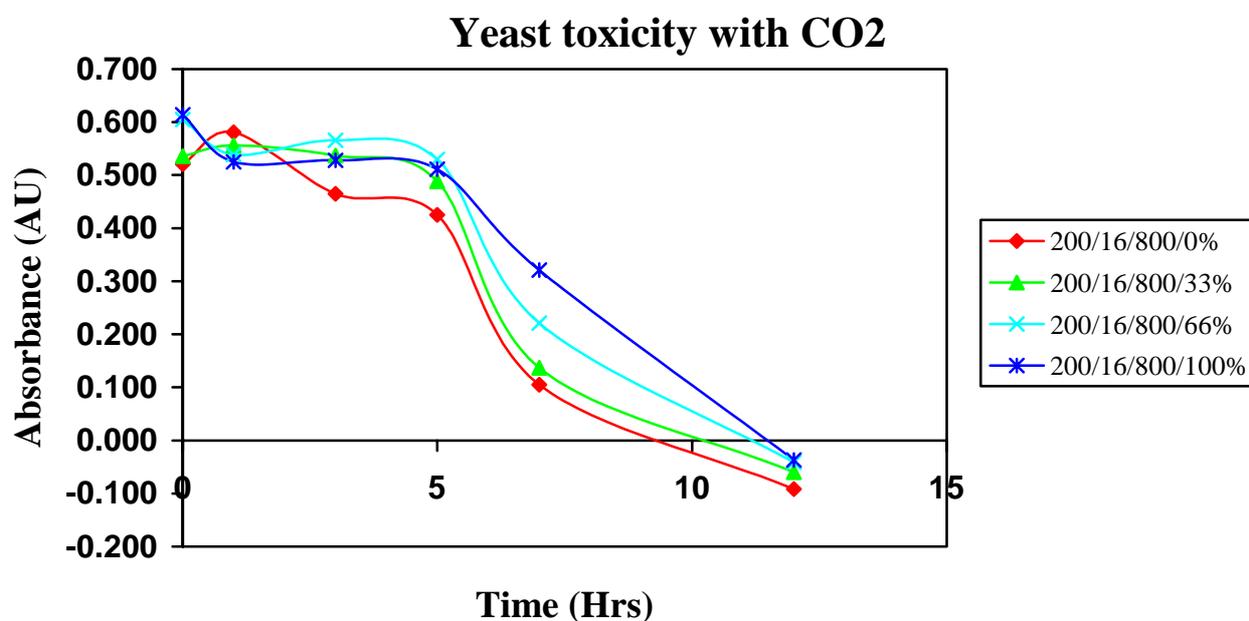
Figure 6.3



At the lowest tested severity parameter of 180<sup>0</sup>C for 8minutes, there was almost no inhibition when compared to the control that was run at each experiment. However, at the highest tested severity parameter of 220<sup>0</sup>C for 32minutes, there was an almost 50% reduction in the speed of glucose consumption by the yeast. This division of inhibition as it correlates to severity is well repeated among the samples.

Figure 6.4 illustrates the effect of dilution of the inhibition by the hydrolysates. It can be seen that diluted hydrolysates are less inhibitory, thus there appears to be a relatively continuous dose-response to the inhibitors.

Figure 6.4



## Discussion

The midpoint pretreatment condition of 200<sup>0</sup>C for 16 minutes could be an optimal condition for reduced microbial inhibition. For severities around the midpoint and below, there was no improvement in the reduction of microbial inhibition. A possible explanation for this result is that at the midpoint and below conditions, there are not enough inhibitory compounds released to effect the hearty yeast organisms. However, the more severe reaction conditions, above the midpoint, are continuing to breakdown the biomass and releasing more and more inhibitory compounds into the hydrolysate, thereby inhibiting the yeast by making their environment more difficult to survive in, but not impossible and not killing them.

## Conclusion

Pretreatment with carbonic acid showed no significant advantage when compared to pretreatment with liquid hot water. Differences between the samples could be attributed to testing error since in some cases the samples with carbonic acid showed reduced inhibition rates and yields and in other cases they showed an increase in inhibition rates and yields.

## **Task 7: Determine enzyme digestibility of pretreated solids**

Damon M. Yourchisin, G. Peter van Walsum

### **Summary**

Enzyme digestibility tests measured enzymatic hydrolysis rates of pretreated solids by cellulase enzymes (Novozyme 188 and Iogen cellulase). It was found that more severe pretreatments enhanced enzymatic digestibility. The addition of pressurized CO<sub>2</sub> to the pretreatment system did not significantly increase enzymatic hydrolysis rates compared to water-alone pretreatment. To conduct the experiments, serum vials were charged with a pH 5.0 buffer, preservative, enzyme and pretreated solid sample estimated to have 2g/L cellulose (calculated from dry weight of the solid residue) and incubated in a 40<sup>0</sup>C shaker bath. Glucose concentrations over time were determined via glucose assay and the HPAE when available.

### **Introduction**

The main purpose of pretreatment is to enhance fiber reactivity to enzymatic hydrolysis. The rate of enzymatic digestion is important because higher rates increase productivity and allows for use of less enzyme. Increased fiber reactivity is accomplished through a variety of mechanisms: solubilization of hemicellulose, removal of lignin, reduction of particle size, alteration of the cellulose characteristics such as degree of polymerization, abundance of cellulose chain ends and crystallinity. Effective pretreatments in general approach or exceed 80 % of theoretical cellulose conversion upon subsequent hydrolysis of a representative hardwood feedstock (e.g. poplar) using moderate (e.g. 10 to 15 FPU/g cellulose) cellulase loadings. Such conversions are achieved in a period on the order of five days, although this is highly feedstock-dependent.

### **Materials + Methods**

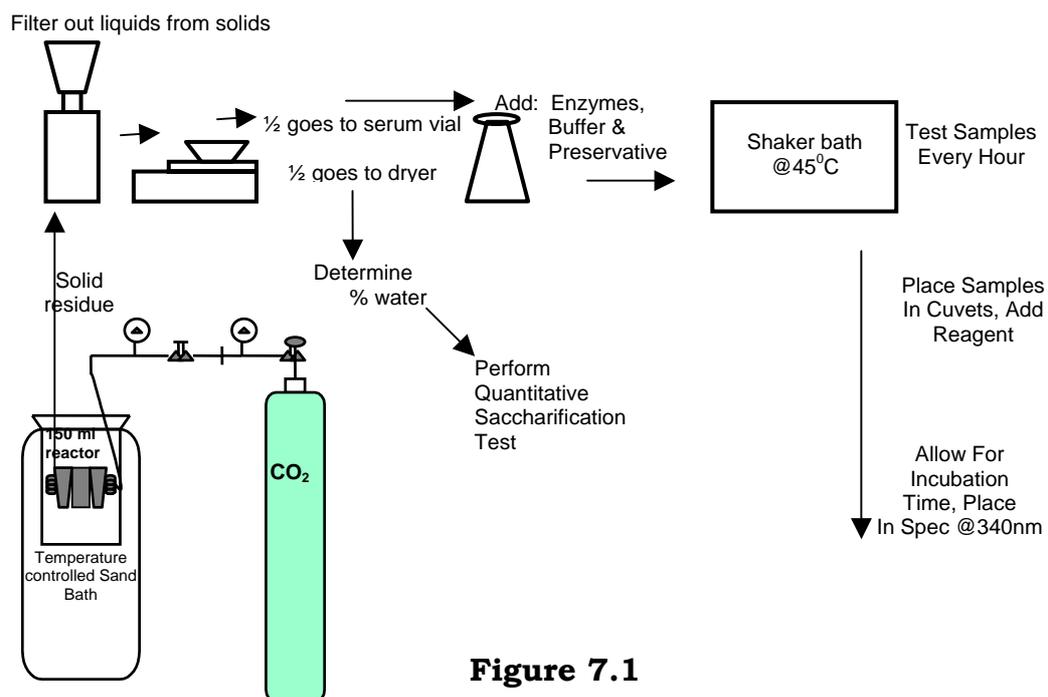
#### Apparatus and materials

Two Techne Fluidised Sand Baths SBL-2d with temperature controllers.  
Beckman DU 520 General Purpose UV/Vis Spectrophotometer (with single cell module)  
150ml stainless steel immersible reactor  
Ohaus Explorer digital scale Item #12140 (d=0.1mg)  
Cole Parmer 2ml disposable plastic cuvettes  
Domestic brand coffee grinders  
Fisher Scientific U.S.A. Standard Testing Sieve, ASTM E-11 Spec #18 (1mm opening)  
Standard laboratory de-ionized quality water  
Standard laboratory grade carbon dioxide  
Vacuum filter with water aspirator  
Schleicher and Schuell Sharkskin 100Cir. 5.5cm Microfilter paper  
Blue M drying oven model OV-18A set at 100<sup>0</sup>C and 40<sup>0</sup>C  
New Brunswick Scientific Reciprocal Water Bath model R76  
Thermolyne MaximixII type 37600 mixer  
SIGMA Diagnostics - Infinity™ Glucose Reagent (Procedure 17-UV) Kit  
Glass serum vials (50mL) and (100mL)  
Novo Nordisk's cellobiase (Novozyme 188L cellulase enzyme)  
Iogen cellobiohydrolase, endoglucanase, and B-glucosidase (Iogen cellulase enzyme)

Citric Acid, 99%  
 Benzoic Acid, 99%  
 Sulfuric Acid, 72%  
 Sodium Hydroxide Solution, 50/50 w/w  
 Aspen wood chips (provided by USDA Forest Products Laboratory in Madison, WI)  
 Aspen wood liquid hydrolysate  
 Avicel pure dried cellulose  
 Sigma D-Glucose Anhydrous

### Experimental Design

Enzyme digestibility of pretreated solids at varying parameters around the midpoint of (200°C/16min/with and without CO<sub>2</sub>) for both aspen wood and corn stover was measured. An assessment of hydrolysis enhancement of pretreatment with carbonic acid (CO<sub>2</sub>) was then made. The general steps included: preparation of the feedstock, pretreatment, separating the solids from the liquids, drying some of the solids and calculating dry weight; preparation of the testing vials, enzymatic hydrolysis tests, performing a glucose assay on the samples. The following procedure is summarized in figure 7.1, below.



**Figure 7.1**

#### *Preparation of feedstock*

This step used the same methods as detailed under task 6.

#### *Pretreatment*

This step used the same methods as detailed under task 6.

#### *Preparation of the solid residue*

The solids were separated from the combined reactor contents using a vacuum filter and microfilter paper. The solids remaining on the filter paper were washed with DI water 3 times (12ml DI water total). During the final rinse the vacuum filter was allowed to work for 3 minutes and then shut off to collect solids. The solids were placed in weighing tins and the weighing tins were then placed in a 100% humidity equilibrium chamber for 72 hours. Once all the samples were at a water content equilibrium, half of each sample was placed into its own pre-weighed weighing tin and weighed. The weight of the tin, and the wet sample were recorded for each sample. The original wet samples were placed back into the equilibrium chamber to ensure their water content would not change prior to experimentation. The weighed wet samples were placed into the dryer oven at 101<sup>0</sup>C for 72 hours. Once the samples were dry, they were weighed again to determine water content of the original samples still in the equilibrium chamber.

#### *Preparation of the testing vials*

Each sample now had a known moisture content. Quantitative saccharification (see below) found the solids to be approximately 63% cellulose. This was used to then determine the amount of sample needed for each testing vial to achieve a final glucose level of less than 2g/L in the post hydrolysate

Next the pH 5.0 buffer solution with preservative was prepared. This was done by mixing citric acid with DI water and adding benzoic acid to the mixture at 0.49%. The mixture was then titrated with sodium hydroxide (NaOH) until the pH was raised to 5.0 and remained constant.

Finally, the amount of enzyme mixture was determined. Enzyme loadings were set to 20 cellulase units per gram of solid sample and 100 units of beta-glucosidase per gram of solid sample.

Vials were prepared so that each sample was done in duplicate. Once the buffer solution and solid samples were added to the vials, they were prepared to begin the test. (29,31,32,33,34)

#### *Enzymatic hydrolysis tests*

The addition of the enzymes was used as time zero (T=0). After a sample of each testing vial was taken at time zero the hydrolysis testing began. Immediately after taking the time zero samples the testing vials were placed in the shaker bath at 40<sup>0</sup>C. The shaker bath oscillator was set to medium (1/2 way on the dial or number 5). The serum vials were then removed for testing every 2 hours for up to 12 hours. They were then tested at 24 hours, 48 hours and 120 hours.

#### *Analysis of the sample*

The enzymatic hydrolysis samples did not have to be diluted, so the sample taken from each serum vial was placed directly into its corresponding cuvette at each sampling hour. Also, the control that was also used for each enzymatic hydrolysis experiment was a sample that contained buffer, preservative and enzymes but no pretreatment sample.

#### *Quantitative saccharification*

This process was done in accordance with NREL LAP-002. Sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) was prepared by adding 99% H<sub>2</sub>SO<sub>4</sub> to water to achieve a 72% H<sub>2</sub>SO<sub>4</sub>. The density of H<sub>2</sub>SO<sub>4</sub> was close to double that of water so a balance was used to determine the amounts needed. The dried samples from the enzymatic hydrolysis tests were used. Samples of approximately 0.4

grams were dispensed into separate test tubes. Duplicates of each sample were again created for each experiment. All masses were immediately recorded once they were close to the 0.4 grams and before the effects of moisture in the air could affect the weights. A standard of pure cellulose (Avicel) was also measured in triplicate with each experiment. It was also dried in the dryer oven for 72 hours to ensure no moisture content when weighed. The tubes with sample in them were placed in a tube rack which was further placed on ice. The 72% H<sub>2</sub>SO<sub>4</sub> mixture was added to each of the tubes creating an acid to sample ratio of 0.01ml/mg. Once all tubes had sufficient H<sub>2</sub>SO<sub>4</sub>, they were placed into the reciprocal water bath set at 30°C and the shaker mechanism set to half way, or number 5 on the dial. The tubes were immersed in the shaker bath for 2 hours. Every 15 minutes they were stirred with glass stirring rods that stayed in each of the tubes to prevent sample loss or cross contamination. The stirring was key to breaking up clumps and ensuring uniform acid penetration to all the sample. While the tubes were in the bath, a set of serum vials corresponding to each test tube was prepared for the next step of the quantitative saccharification test. The 100mL serum vials were placed on ice and water was added to each serum vial. The total water added to each vial was 0.27mL/mg of sample minus 20ml. For example, if sample tube #3 has 400mg of sample, then  $400 \times 0.27 - 20 = 88\text{ml}$  of water were placed into serum vial #3. The 20ml of water subtracted from 0.27mL/mg ratio was used to rinse the contents of the test tubes and stirring rods into the serum vials to ensure complete mass transfer. At the end of the 2 hours, the content of each tube was transferred to their respective serum vials. Each tube and stirring rod was washed with 20ml of water and again transferred to its respective serum vial. Each serum vial was then capped and sealed. They were taken off the ice and placed in the autoclave for 1 hour. Once the autoclaving was complete and the serum vials were cool enough to be handled, they were analyzed.

#### *Analysis of the Quantitative Saccharification Samples*

The glucose analysis method used for the quantitative saccharification samples was the enzymatic glucose assay, as was done with the enzymatic hydrolysis samples and yeast inhibition samples.

## **Results**

Data were analyzed using the DU 500 Spectrophotometer and Infinity Glucose Reagent Assay kit. Two metrics were used, total recovered glucose and reaction speed, after the raw numbers were graphed (Absorbance Units versus time, transposed into Glucose yield versus time).

Figure 7.2, below, shows rapid hydrolysis of all samples pretreated at a similar severity. Note that there is no difference between samples with or without carbonic acid. Also, the graph shows that after 24 hours there was little difference in the yield, even when the testing continued for 120 hours (not shown, but the time it is thought to take for enzymes to achieve about 90% hydrolysis) there was very little difference in the yield.

Figure 7.3, below, shows that reaction severity played a major role in the rate and yield of enzymatic hydrolysis and that as the reaction severity increased so to did the rate and final yield of the enzymatic hydrolysis throughout the range of severities. Again, however, the presence or absence of carbonic acid appears to have played no distinguishing role in rates or extents of enzymatic hydrolysis.

### **Figure 7.2 Enzymatic Hydrolysis of Pretreated Aspen Wood**

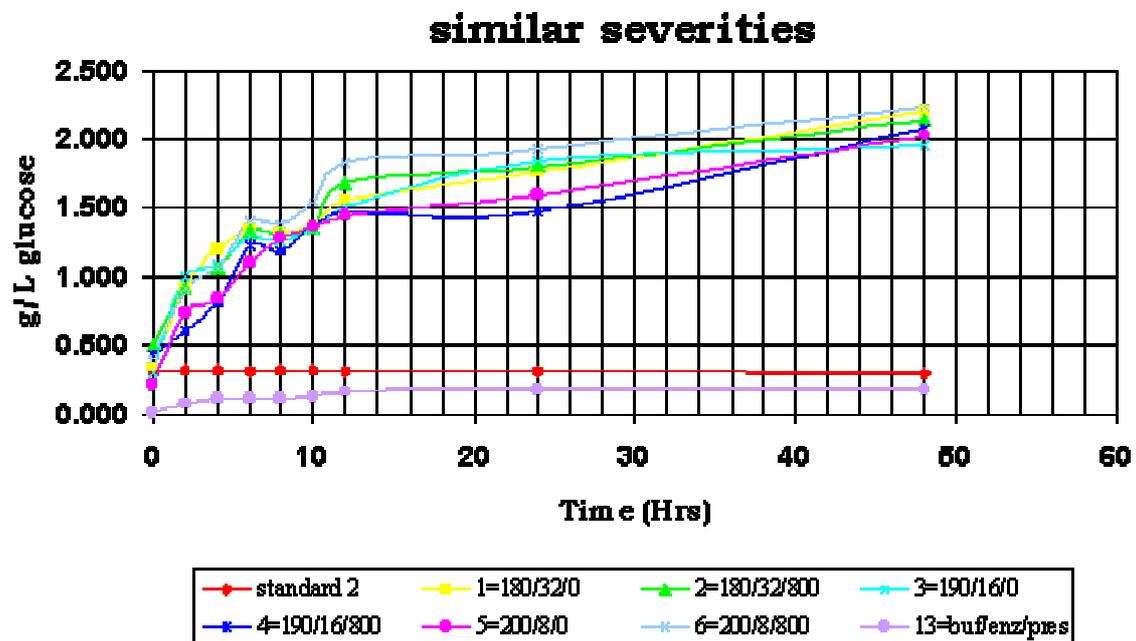
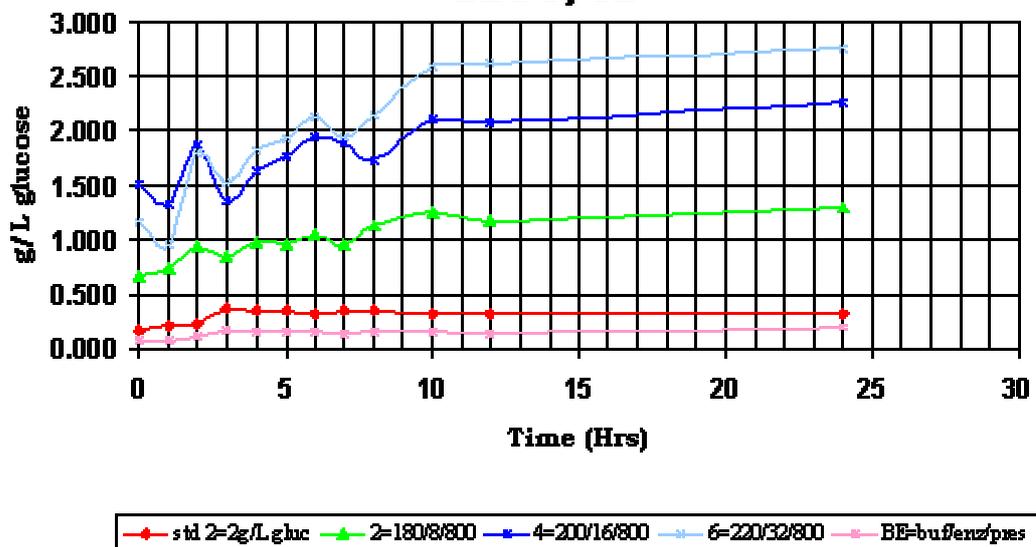


Figure 7.3: Enzymatic hydrolysis of Aspen wood pretreated at varying severity.

**Paramters at 180C/8, 200C/16,  
220C/32**



**Discussion**

The midpoint pretreatment condition of 200<sup>0</sup>C for 16 minutes does not appear to be the optimum condition for enzymatic hydrolysis. It is clear that the optimal severity of 200<sup>0</sup>C for

32minutes that produces the maximum xylose sugars as found by McWilliams (2002) from the pretreatment step is not the optimal severity for enzymatic hydrolysis. The rates and yields of enzymatic hydrolysis continued to increase as the reaction severity of the pretreatment increased. It is possible that the enzymatic hydrolysis rates and yields would continue to increase past the maximum severity that was tested of 220<sup>0</sup>C for 32minutes. A possible explanation for this result is that the more sever reaction conditions are continuing to breakdown the hemicellulose and solubilize the lignin, allowing the enzymes more and easier access to the cellulose

## **Conclusion**

Pretreatment with carbonic acid showed no significant advantage when compared to pretreatment with liquid hot water. Differences between the samples could be attributed to testing error since in some cases the samples with carbonic acid showed improved enzymatic hydrolysis rates and yields and in other cases they showed a decrease in enzymatic hydrolysis rates and yields.

The reaction severity did have a significant effect on the enzymatic hydrolysis yields of the samples. On average, for every 10 fold increase in severity, the enzymatic hydrolysis yield increased by about 30%. More testing would be needed to determine the increase in rate. Also, data points need to be tightened and a standard for slope determination amongst the samples would need to be determined.

## **References**

McWilliams R. C., Van Walsum G. P., 2001, *Comparison of Aspen Wood Hydrolysates Produced by Pretreatment with Liquid Hot Water and Carbonic Acid*, 3-17

McWilliams, R.C. 2002 Master's Thesis, Comparison of Aspen Wood Hydrolysates Produced by Pretreatment with Liquid Hot Water and Carbonic Acid, p1-17