

BIOCHEMICAL CONVERSION PROGRAM
ANNUAL REVIEW MEETING

at the

SOLAR ENERGY RESEARCH INSTITUTE

October 13-15, 1987

B02322 3402
Biofuels Information Center

On the Effect of Thermo-Chemical Pretreatment on the Enzymatic Hydrolysis of Lignocellulosic Substrates I

by

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for presentation at the
Biochemical Conversion / Alcohol Fuels Program Review
October 13-15, 1987
Golden, CO

Abstract

A kinetics study¹² has shown that the effect of pretreatment cannot be explained in terms of increased pore volume alone. Furthermore, knowledge of the variation in surface area as the enzymatic hydrolysis proceeds is needed to develop a kinetics model. A project has recently been initiated in order to determine the necessary data and refine the kinetics model.

Recently determined pore size data for Avicel and Japanese rice straw are included.

Introduction

Recent studies ^{1,2} have shown a large increase in the pore volume available to 50-90 Å solute molecules when biomass is subjected to either 'steam explosion' or mild acid hydrolysis. Using the data from these studies, Grethlein ³ and Grethlein and Converse ⁴ have presented a correlation between the enzymatic hydrolysis yield obtained in the first two hours and the surface area available to 51 Å diameter molecules, as estimated from the pore volume measurements carried out by the method of Stone and Scallan ⁵.

Theory

In a recent paper ^{1,2} the ability of an adsorption model to represent the effect of pretreatment on the hydrolysis behavior observed by Grous ¹ was examined. An adsorption model, rather than a homogeneous model, was chosen since in order to represent the pretreatment effect, and this pretreatment results in a large increase in both the surface area and the reaction rate. A unique feature of this work was the fact that the initial value of the surface area was based on pore volume measurements.

In this work, it is assumed that the rate of hydrolysis of the substrate is proportional to the concentration of adsorbed enzyme.

$$dS/dt = k_1 E_a \quad [1]$$

Following Wald ⁶, adsorption equilibrium of the Langmuir type is assumed.

$$E_a = k_2 AE / (E + k_3) \quad [2]$$

The above equation expresses the assumption that the concentration of adsorption sites is proportional to the surface area available to the enzyme. Conservation of enzyme yields the following expression.

$$E_t = E + E_a \quad [3]$$

As the substrate is consumed and the surface recedes, the relationship between the area and the remaining substrate, $A(S)$, depends on the nature of the surface. For a planer surface, $A(S)$ remains constant until the substrate is exhausted. For expanding cylindrical pores, A increases as S decreases until the pores begin to combine whereupon A declines. This gives rise to a maximum in the reaction rate ⁷. For objects reacting at their external surfaces, A decreases as S decreases; for example, in the case of a shrinking sphere, $A = kS^{0.67}$. When there is a distribution of particle and pore sizes, and reaction on both external surfaces and internal pores, as we assume to occur in the present case, $A(S)$ can be quite complex.

The following expression is used for $A(S)$.

$$A = A_0 (S/S_0 - S_i) / (1 - S_i)^p \quad [4]$$

where:

- S_0 = initial substrate concentration,
- p = constant, to be determined experimentally
- S_i = the fraction of the substrate that is unavailable.

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In order to determine the concentration of the adsorbed enzyme, Eqs. [2] and [3] can be combined to yield

$$E_a = (-b - \sqrt{(b^2 - 4c)})/2 \quad [5]$$

$$\text{where: } b = -E_t - k_3 - k_2A \quad [6]$$

$$c = k_2AE_t \quad [7]$$

Eq. [1] can be integrated numerically in accord with the following algorithm:

1. S_{new} from Eq. [1]
 2. A_{new} from Eq. [4]
 3. $E_{a, \text{new}}$ from Eq. [5]
 4. return to step 1
- [8]

Experimental

The glucose yield data and corresponding experimental conditions used in this modelling study were taken from Grous^{1,8}. Grous pretreated poplar wood chips by steam explosion for various times and temperatures as summarized in Table 1 and Ref. 12. The pretreated solids were hydrolyzed with crude cellulase from *Trichoderma reesei* and *Aspergillus niger* as described previously^{1,8}. The surface area is estimated from the pore volume measurement reported by Grous^{1,8} by using the following relationship from Stone and Scallan⁵.

$$A_T(d_k) = \sum_{j=k}^m (2/d_j) * (v(d_j) - v(d_{j+1})) \quad [9]$$

The above relationship is based on the assumption that the pores are bounded by parallel planes separated by a distance equal to the diameter of the solute probe molecule. In calculating the pore volume it is assumed that the largest probe molecule is too large to enter any of the pores; hence external surface area is neglected.

The surface area available to the 90 Å probe molecule was used as the initially available surface area in this current study. Previous correlations^{3,4} are based on the area available to the 51 Å probe, however, the choice is somewhat arbitrary since the enzyme is estimated to be ellipsoidal, 30 by 180 Å⁹. In the current data there is some inconsistency in the 51 Å data and it therefore is more convenient to use the 90 Å area.

Results

In the shrinking sphere model (used by Wald⁶), $p = 0.67$ and $S_i = 0$. As shown in Figure 1, even though there are three constants to adjust, it was not possible to obtain a good fit to the experimental data. It tends to underestimate the initial rate and overestimate the rate during the latter portions of the run. If the parameters are adjusted to fit one region, the fit in the other region becomes worse.

As shown in Figure 2, with $p=2$ and $S_i=0$, the model fits the data well. Although it is possible to fit the data with several different values of k_3 by adjusting the other k 's, k_3 is set = 3 to

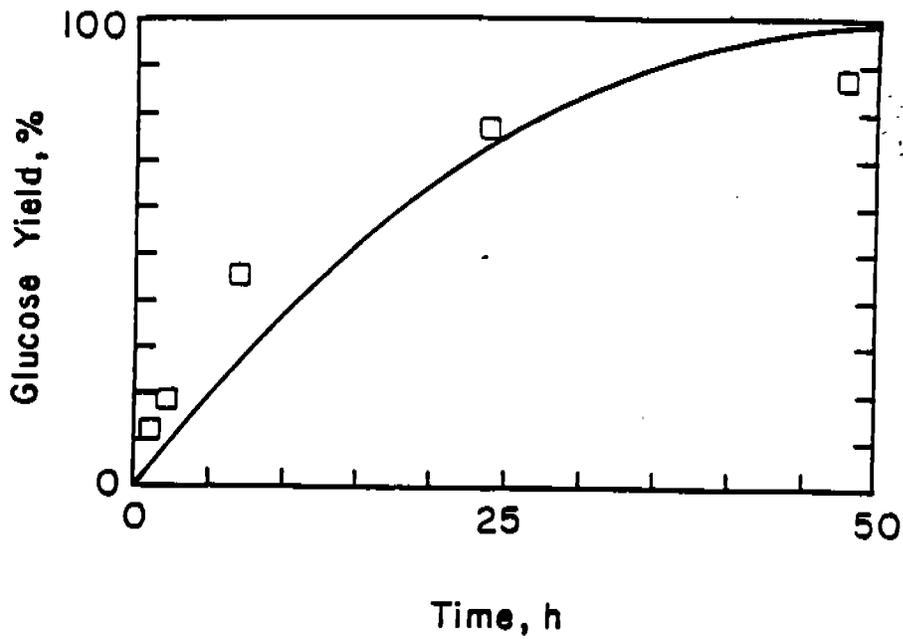


Figure 1: Glucose yield, % vs. time, h. The line represents the shrinking sphere ($p=0.67$) model. See Table 1 for explanation of symbols and parameter values.

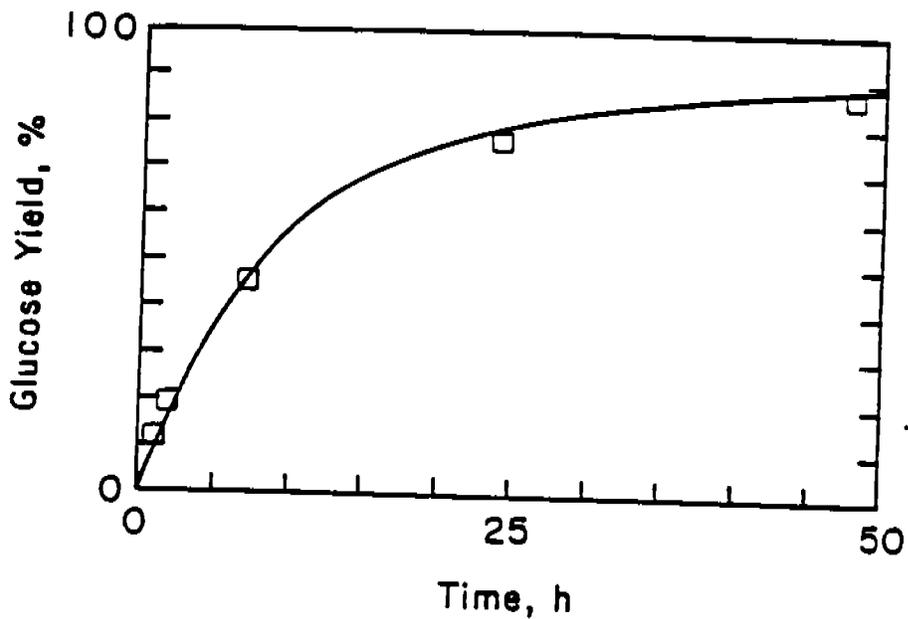


Figure 2: Glucose yield, % vs. time, h. The line represents the model in which the area, A, is proportional to the square of the remaining substrate. See Table 1 for explanation of symbols and parameter values.

Converse

agree with the value found by Wald⁶. Since it was not possible to represent the data with $p < 2$ it appears that the available surface area falls off rapidly as the substrate is consumed.

Consider the following question: With the values of k_1 , k_2 , k_3 , p and S_i set by fitting the data from this one run (substrate pretreated at 205 C for 5 minutes), is it possible to represent the behavior of substrates pretreated at other conditions by varying only the initial value of the available area, as calculated from pore volume measurements? Figure 3 demonstrates that a good fit is not obtained.

As shown in Figure 4, it is possible to fit the data. However, for the untreated and the 2 min pretreatment it is necessary to vary S_i in order to limit the conversion at long times. Furthermore, for the 20 min pretreatment, it is necessary to increase k_2 , the density of active sites, in order to fit the high initial rates.

Discussion

Certain aspects generally found in hydrolysis models are not found in this study. For example, two types of substrate, amorphous and crystalline, have been assumed by Wald⁶ and others. While it would be possible to fit the behavior of the untreated substrate with this feature, fitting the high initial rates of the pretreated substrates could be accomplished only by assuming an increase in the amorphous content due to pretreatment whereas Tanahashi *et al*¹⁰, Taniguchi *et al*¹¹ and Grethlein³ report an increase or no change in cellulose crystallinity with steam explosion. Product inhibition has also been neglected because the hydrolysis runs were made with excess cellobiase present (Grous^{1,8}) in order to reduce product inhibition due to cellobiase. Furthermore, in this study the problem is to explain the high rates that go along with high conversion, not a slowing down of the rate when the conversion is high, as would be the case with product inhibition.

In the model we were forced to resort to assuming that a fraction of the surface area was unavailable. Others¹³⁻¹⁵ have demonstrated the redistribution of lignin as a result of steam explosion, and Wong *et. al.*¹⁵ have shown that this effect can be reversed by washing with NaOH. Hence, it appears justified to include such a factor in a kinetics model.

Conclusions

1. It is possible to model the effect of pretreatment with a six-parameter (k_1 , k_2 , k_3 , A_0 , S_i , and p) adsorption model, with A_0 estimated from pore volume measurements, without changing k_1 , k_2 , or p .
2. For untreated and mildly pretreated substrate, it is necessary to assume that a fraction of the substrate, S_i , remains unavailable.
3. For the more severely pretreated substrates it is necessary to increase k_2 , i.e., assume that the density of active adsorption sites is increase.
4. More accurate model discrimination is needed and can be achieved by measuring the fraction of adsorbed enzyme and surface area as the hydrolysis proceeds since these quantities are also predicted.

Current Results

Figure 5 displays recent pore size measurements done in our laboratory on Avicel and Japanese rice straw. The experimental method is presented in Appendix A. Notice that in Avicel, the pore

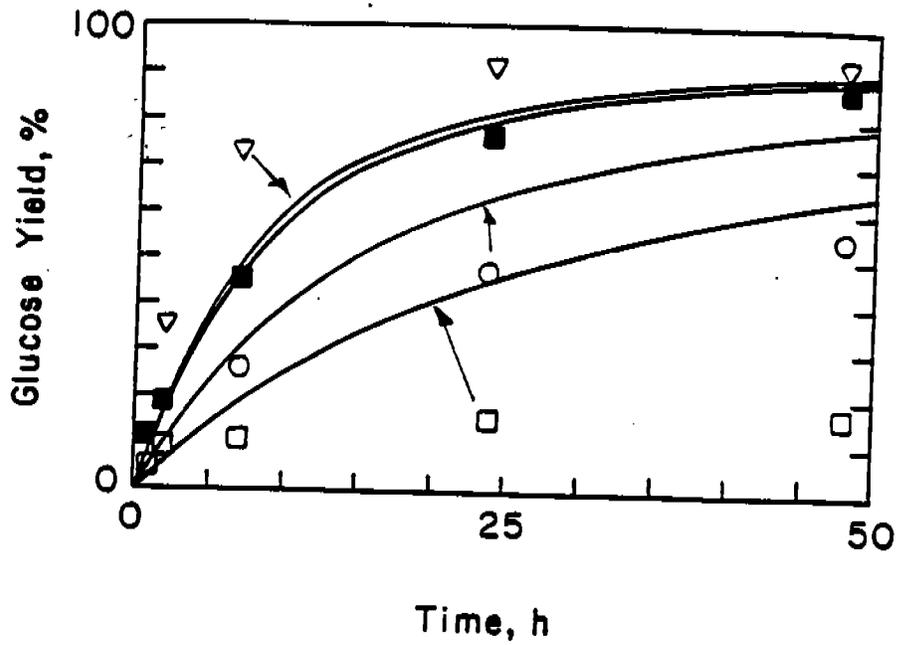


Figure 3: Glucose yield, % vs. time, h, demonstrates the inability of the model to represent the change in pretreatment conditions on the basis of measured initial available area alone. See Table 1 for explanation of symbols and parameter values.

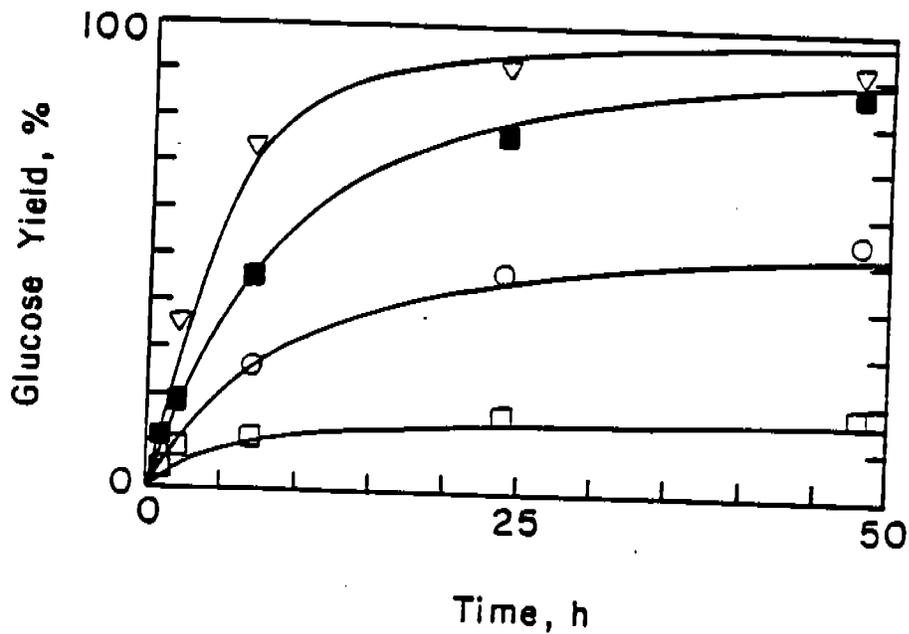


Figure 4: Glucose yield, % vs. time, h, demonstrates the ability of the model to represent the effect of changing the pretreatment conditions when S_1 and k_2 are varied. See Table 1 for explanation of symbols and parameters values.

Pore Size Measurements

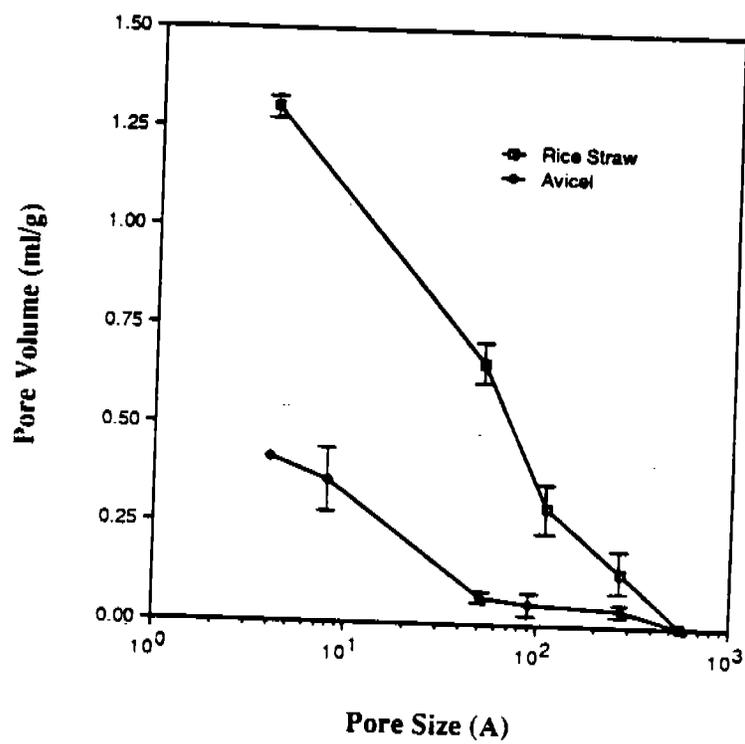


Figure 5: Pore Volume Distribution.

volume accessible to molecules greater than 5 nm in diameter is very small. We believe that this is the main cause of its low rate of hydrolysis.

Future Work

We are now beginning a project, jointly supported by SERI and NSF, in which the surface area and adsorbed enzyme will be measured during the enzymatic hydrolysis, and an attempt will be made to relate the effect of pretreatment to hemicellulose hydrolysis kinetics. In order to facilitate the pore size measurements, we will attempt to develop an improved method of measurement. This work is undertaken in cooperation with Prof. Matsuno's laboratory at Kyoto University.

Nomenclature

| | |
|------------|--|
| A | = available surface area, m^2/g |
| A_0 | = initial value of A |
| $A_T(d_T)$ | = total area available to molecules of size, d_T , m^2/g |
| b | = defined by Eq. [10] |
| c | = defined by Eq. [11] |
| d_j | = diameter of the j^{th} solute probe, μ |
| E | = concentration of free enzyme, g/L |
| E_a | = concentration of adsorbed enzyme, g/L |
| E_t | = concentration of all enzyme, g/L |
| k_1 | = reaction rate constant, h^{-1} |
| k_2 | = adsorption constant, g^2/m^2-L , see Eq. [2] |
| k_3 | = adsorption constant, g/L , see Eq. [2] |
| m | = the index of the largest solute probe |
| n | = number of particles in shrinking sphere model |
| p | = parameter in Eq. [8a] |
| r | = radius in shrinking sphere model, cm |
| S | = substrate concentration, g/L |
| S_i | = fraction of the substrate that is unavailable |
| S_0 | = initial substrate concentration |
| t | = time, h |
| $v(d_i)$ | = volume available to molecules of size d_i , cc/g |
| π | = pi |
| ρ | = solid density, $g/$ |

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Table 1: Parameter Values

$S_0 = 20$ g/L through out; $E_t = 0.925$ g/L throughout

| Fig. No. | Sym bol | Pre. Temp.C | Time Min | k_1 | k_2 | k_3 | S_i | P | A_i |
|----------|---------|-------------|----------|-------|-------|-------|-------|---|-------|
| 1 | □ | 205 | 5 | 2 | 5e-6 | 6 | - | - | 31.6 |
| 2 | □ | 205 | 5 | 1.8 | 7e-4 | 3 | 0 | 2 | 31.6 |
| 3 | ○ | 205 | 2 | 1.8 | 7e-4 | 3 | 0 | 2 | 13.6 |
| 3 | ■ | 205 | 5 | 1.8 | 7e-4 | 3 | 0 | 2 | 31.6 |
| 3 | ▽ | 205 | 20 | 1.8 | 7e-4 | 3 | 0 | 2 | 35.3 |
| 3 | □ | Untreated | | 1.8 | 7e-4 | 3 | 0 | 2 | 6.5 |
| 4 | ○ | 205 | 2 | 1.8 | 7e-4 | 3 | 0.4 | 2 | 13.6 |
| 4 | ■ | 205 | 5 | 1.8 | 7e-4 | 3 | 0 | 2 | 31.6 |
| 4 | ▽ | 205 | 20 | 1.8 | 3e-3 | 3 | 0 | 2 | 35.3 |
| 4 | □ | Untreated | | 1.8 | 7e-4 | 3 | 0.83 | 2 | 6.5 |

Appendix A

Experimental Procedure:

Dextran Solution

Dextran solutions were prepared by adding weighed Dextran to distilled water to make a 4% (w/w) solution. The solution was heated and stirred until completely dissolved. Once dissolved, the solution was passed through a glass-filter crucible. 0.5 % (w/w) Sodium Azide was added in order to preserve the solution. The Dextran solution was then stored in a tightly capped bottle, and placed in the refrigerator.

Avicel

About 20 grams of Avicel was placed in a 200 ml Erlenmeyer flask and soaked with several volumes of distilled water in order to remove the air from the pores, as indicated by its sinking to the bottom of the flask. The Avicel mixture was filtered through a 5.0 mm Nucleopore filter under vacuum. Several volumes of distilled water were poured over the Avicel in order to wash the sample of any solubles. As soon as the water stopped dripping from the filter, the vacuum was removed. Be sure not to let the solids dry out; the pores may collapse, causing a change in structure.

About 4 grams of wet Avicel was added to each of 11 preweighed plastic bottles and reweighed. About 16 ml of 4% Dextran solution was added to each sample bottle in duplicate. The new weights of the sample bottles were then obtained. The blank solution was made by adding 16 ml of distilled water to container 11. Each bottle was shaken several times, placed in the refrigerator overnight, and shaken periodically.

Ten petri dishes with 5.0 μm Nucleopore filters were labeled and weighed. A millipore filter system was set up using the filter in petri dish 1. The filtrate from bottle 1 was passed through the filter into a test tube, which was removed, sealed, and placed in the refrigerator. One should be very careful not to evaporate or dilute any of the collected filtrate, as this will alter the concentration. The solids remaining in the plastic bottle and cap were then washed onto the filter with distilled water. The solids and the filter were then carefully slid into the petri dish labeled 1. The petri dish was then placed in an oven (65 °C), in order to dry. This process was repeated for samples 2 - 10. Only the filtrate was collected for the blank solution, as the dry weight of solids need not be determined.

The optical rotations of the samples, Dextran stock solutions, and the Blank were recorded using a photoelectric spectropolarimeter. If the filtrate was cloudy, or the percent transmittance was low, the solution was refiltered using a 0.4 μm Nucleopore filter. The petri dishes were removed from the oven and placed in a dessicator. Once at room temperature, each dish was reweighed to determine the dry weight of sample. The inaccessible water was calculated for each probe size used (560, 270, 90, 51, 8, and 4 Angstroms).

Rice Straw

The procedure for Rice Straw was the same as that for Avicel except for a couple variations. Rice Straw was chopped in a blender in order to make a more uniform size distribution. The sample was then soaked in several volumes of distilled water, and placed in a refrigerator. The Rice Straw and water mixture was then placed into a large vacuum flask under a vacuum. The Rice Straw floated to the surface suggesting that there was still air in the pores. Heat and vacuum were alternated until the Rice Straw remained at the bottom of the flask when under a vacuum.

15 ~ 18 grams of wet Rice Straw was added to each preweighed bottle, then reweighed. About 30 ml of 4% Dextran solution was added to each bottle, and the new weights recorded. Each sample was shaken and placed in the refrigerator overnight. The filtrate was collected as described earlier, except that analytical filter paper was used instead of 5.0 μm Nucleopore filters.

The filtrate was then filtered through a 0.4 μm Nucleopore filter in order to remove the cloudiness, and then placed in the refrigerator.

The wet solids were collected into petri dishes and placed in the oven (65 °C). Once dry, the dishes were placed in a dessicator to cool. Once at room temperature, each petri dish was weighed, and the dry weight of sample was calculated. The optical rotations were obtained and the inaccessible water was then calculated for the 560, 270, 110, 51, and 4 Angstrom size probes.