

DEVELOPMENT OF ALTERNATE PRETREATMENT AND BIOMASS FRACTIONATION PROCESSES

Subcontract No. XAW-4-14320-01

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Final Technical Progress Report
5. March 1996

Biomass pretreatments using a 0.73% (w/v) sulfuric acid solution at 160-180°C for 10-30 minutes have become the standard for preparing lignocellulosic materials for enzymatic conversion and fermentation. This report presents data on the use of dilute phosphoric acid and oxalic acid in pretreatment of corn stover and switchgrass. A report on the such pretreatments of hybrid poplar was submitted in 1994.

Sulfuric acid becomes gypsum following neutralization with lime; using phosphoric acid should not present similar disposal problems. After neutralizing phosphoric acid with ammonia, and if necessary removing furfural by steam stripping, the spent liquor from the pretreatment could be used as a fermentation nutrient. Oxalic acid could similarly ease environmental concerns because as an organic acid, it could be disposed by burning.

The pretreatments used in this study are indicated in Table I. There were 22 different pretreatments for switchgrass and 28 for corn stover. Results of aqueous phosphoric acid pretreatments, organosolv phosphoric acid pretreatments using 70 percent methanol, two organosolv phosphoric acid pretreatments using 70 percent ethanol, and oxalic acid organosolv pretreatments are presented.

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Sections B and C contain tabulations of yield, lignin, xylan and cellulose compositional data, and enzymatic hydrolysis data on pretreated solids.

Table AI. Biomass Pretreatment Conditions

Switchgrass

Aqueous phosphoric acid

0.05 molar (0.49% w/v solution)

170°C 20, 40, 60 min

180°C 10, 20, 30 min

0.025 molar (0.25% w/v solution)

190°C 10, 20, 30 min

Organosolv phosphoric acid (70% v/v methanol)

0.05 molar (0.49% w/v solution)

170°C 20, 40, 60 min

180°C 10, 20, 30 min

0.025 molar (0.25% w/v solution)

190°C 10, 20, 30 min

0.010 molar (0.10% w/v solution)

190°C 40 min

Organosolv oxalic acid (70% v/v methanol)

0.1 molar (0.90% w/v solution)

165°C 30 min and 175°C 20 min

0.05 molar (0.45% w/v solution)

165°C 30 min and 175°C 20 min

Corn stover

Aqueous phosphoric acid

0.05 molar (0.49% w/v solution)

160°C 20, 40, 60 min

170°C 20, 40, 60 min

180°C 10, 20, 30 min

0.025 molar (0.25% w/v solution)

190°C 10, 20, 30 min

Organosolv phosphoric acid (70% v/v methanol)

0.05 molar (0.49% w/v solution)

160°C 20, 40, 60 min

170°C 20, 40, 60 min

180°C 10, 20, 30 min

0.025 molar (0.25% w/v solution)

190°C 10, 20, 30 min

0.010 molar (0.10% w/v solution)

190°C 40 min

Organosolv oxalic acid (70% v/v methanol)

0.1 molar (0.90% w/v solution)

165°C 30 min and 175°C 20 min

0.05 molar (0.45% w/v solution)

165°C 30 min and 175°C 20 min

A. Methodology

For all of the pretreatments, a solids loading of one part biomass material to nine parts liquid by weight was used. Prior to addition of acid in the aqueous pretreatments, the reactor containing the biomass and most of the liquid was heated to the pretreatment temperature. At time zero, acid addition was accomplished with an injection device using nitrogen over-pressure. Sufficient partially diluted acid was added so that the acid concentration in the liquid phase in the reactor was at the desired final dilute concentration. The reaction was terminated by rapid cooling of the reactor by removal from the heating unit and immediate immersion in cold water. For the organosolv pretreatments the procedure was modified because of the high pressures obtained at temperature. It was not possible to pressure inject the acid solutions and consequently the acid was added initially before heating to temperature.

The actual amount of biomass was 100 grams oven-dried equivalent weight, and the liquid phase was 900 grams including the acid. For the sulfuric acid control pretreatments a 35 ml solution of 18.77 percent by weight sulfuric acid was injected. The 900 grams of liquid included the water in the air dried biomass, the 35 ml of injected dilute acid, and one rinse water of 35 ml for the injection apparatus. A 0.05 M phosphoric acid solution was 0.49 percent by weight of acid. To achieve this amount, a 35 ml solution of 12.60 percent phosphoric acid was injected, and there was one 35 ml rinse of the injection apparatus. For the organosolv pretreatments the required amount of phosphoric or oxalic acid was dissolved directly into the 70 percent (v/v) methanolic or ethanolic solution prior to adding the biomass.

The apparatus used for the pretreatment was a two liter Parr pressure reactor with stirrer. The material of construction for all contact parts was the alloy Carpenter 20 Cb3, otherwise it was stainless steel Type 316. The injection apparatus was identical to that developed by Dr. Mike Himmel of NREL. The time to temperature was approximately 40 minutes (rate of heating 4°C/min). Because of the apparatus used for the pretreatment, it was not possible to determine the actual energy consumed during the pretreatment.

After the pretreatment the cooled reactor was emptied and the liquor filtered from the lignocellulosic substrate. Additional liquor was removed from the substrate by squeezing it with a thin rubber sheet while it was still under water aspirator vacuum in the filter funnel. Volume and pH of the filtrate was determined. The filtrate was used for sugar and furfural content analyses by HPLC and for fermentation to ethyl alcohol. The lignocellulosic substrate was washed to raise the pH of the wash water to 4.5 or higher, the excess liquid was squeezed out, the moist material weighed, and small samples taken to determine moisture content and yield. Another small sample was taken for Klason and acid soluble lignin determinations, and for hydrolysis for sugar content analysis. The remaining residue was used for the enzyme hydrolysis and in six cases, which are described below, simultaneous saccharification and fermentation (SSF) evaluation.

The organosolv pretreatments made fermentation of the spent liquor difficult and the furfural analysis impossible. The methanol in the organosolv spent liquor could not adequately be removed by vacuum distillation, and a modification of the experimental plan was instituted after inhibition of the *Pichia stipitis* was observed in the first round of experiments. Two organosolv pretreatments using 70 percent ethanol were done in order to evaluate the fermentation in the presence of residual amounts of the less toxic alcohol.

The pretreatment liquor contained hydrolysis products of hemicellulose: glucose, mannose, xylose and arabinose. These were quantitated using Biorad HPX-87H column elution with 0.008 N sulfuric acid at 65°C using an NREL Hewlett Packard HPLC with our Waters Associates differential refractive index detector and Gilson autosampler injector. Quantitation was based on standard curves for each monosaccharide component. Degradation of the monosaccharides was represented by furfural and hydroxymethylfurfural, but these could not be quantitated due to small quantities present and wide peak widths that resulted by extending the time of the same chromatographic analysis to approximately 60 minutes. Every sample was analyzed in duplicate, and in cases of poor agreement between these results, in sufficient replication to obtain a coefficient of variance of less than 12 percent.

The criteria for evaluation of the best pretreatment conditions was based on lignin free yield, enzyme hydrolysis of the pretreated residue and a severity parameter, which was developed during the earlier subcontract period. The severity parameter was a function of pretreatment conditions that correlated well with SSF ethanol yields [Lidia A. Brito. Dilute phosphoric and oxalic acids as pretreatments for woody biomass prior to enzymatic hydrolysis. PhD Dissertation. Colorado State University, 1994].

The culture of *Pichia stipitis* strain NRRL Y-11515 was obtained from Dr. C. P. Kurtzman at the National Center for Agricultural Research in Peoria, IL. The protocol for fermentation of the five-carbon sugars in the pretreatment liquor was developed by NREL. Methanol (or ethanol), furfural and hydroxymethylfurfural were stripped from the organosolv pretreatment liquors by vacuum distillation using a Buchi rotary evaporator. Analysis of the fermentation products was by HPLC using Biorad HPX-87H column elution with 0.008 N sulfuric acid at 65°C on the Hewlett Packard Model 10848 HPLC on loan from Dr. David Johnson at NREL. This instrument has been equipped with a Waters Associates Model R401 differential refractive index detector (a loaner from the Department of Biology at CSU) and a Gilson Model 234 autosampler that has been purchased specifically for this project. Quantitation was based on external standard quantitation with standards of ethanol, xylose, xylobiose, acetic acid, succinic acid, lactic acid and glycerol.

Evaluation of the pretreated solids involved a dry weight determination, an HPLC analysis of a neutralized two-stage acid hydrolysate of the solids, an HPLC analysis of an enzyme hydrolysate of the solids, and an SSF fermentation, which was evaluated by HPLC analyses. Determination of the dry weight of the pretreated solids was conducted in duplicate according to NREL Chemical Analysis & Testing Procedure Standard No. 001.

Two stage sulfuric acid hydrolysis of pretreated solids for determination of carbohydrates described in NREL Chemical Analysis & Testing Procedure Standard No. 002 was followed. The hydrolysates were neutralized with calcium carbonate to a pH of 6 or slightly higher. Rather than filtering the whole quantity of hydrolysate, as described, an aliquot of 20 mL was centrifuged and then filtered through 0.45 μ membrane filters (Gilman) in preparation for HPLC analysis. The solutions were analyzed using Biorad HPX-87H column elution with 0.008 N sulfuric acid at 65°C. Quantitation was based on standard curves for each monosaccharide component, glucose, mannose, xylose and arabinose.

Enzyme hydrolysis of pretreated solids protocol described in NREL Chemical Analysis & Testing Procedure Standard No. 008, Revision #3 was followed. Triplicate enzyme digests of each pretreated sample were studied. Samples were taken from closed shaking flasks after 0, 1, 3, 6, and 24 hours. These samples were filtered aseptically into presterilized autosampler vials using 0.45 μ membrane filters (Gilman) and analyzed using Biorad HPX-87H column elution with 0.008 N sulfuric acid at 65°C. Quantitation was based on standard curves for each monosaccharide component, glucose, mannose, xylose and arabinose.

The SSF protocol was described in NREL Chemical Analysis & Testing Procedure Standard No. 008, Revision #3. Yeast, *Saccharomyces cerevisiae* D₅A, culture preservation for long-term storage in glycerol at -70°C and inocula were prepared according to the directions in the same. Triplicate fermentations of each pretreated sample were conducted if sufficient pretreated material was available. Samples were taken from the shaking flasks fitted with gas traps after 24, 48, 72, 96, 120, 144 and 168 hours. These samples were filtered aseptically into presterilized autosampler vials 0.45 μ membrane filters (Gilman) and analyzed by HPLC using Biorad HPX-87H column elution with 0.008 N sulfuric acid at 65°C on the Hewlett Packard HPLC.

B. Switchgrass Pretreatments

Composition of pretreated switchgrass solids, in terms of Klason lignin, soluble lignin, xylan and cellulose are recorded in Table BI for aqueous phosphoric acid pretreatment solids, in Table BII for organosolv phosphoric acid pretreatment solids and in Table BIII for oxalic acid pretreatment solids.

A striking difference in data in Table BI, BII and BIII is the very great hemicellulose content remaining in the organosolv phosphoric acid pretreated solids. Table BII shows an average of 25 percent xylan compared to approximately 6 percent xylan in aqueous- and 8 percent in oxalic acid organosolv pretreated materials. These difference are reflected in the Klason lignin free yields, about which more will be said below.

The phosphoric acid pretreated switchgrass samples were subjected to enzymatic hydrolysis based on the cellulose contents reported in Tables BI, BII and BIII. Graphs relating the hydrolysis kinetics of the switchgrass samples to that of alpha-cellulose are shown in Figures B1 through B7. The data in Figures B1 and B2 indicate that the net hydrolysis of each sample at each time point should be reduced by approximately 1.2 g/L glucose. A control containing the YP medium and enzyme was not chromatographed for this experiment, but was conducted at a later date. A component of either the YP medium or the enzyme preparation apparently coelutes with glucose using the Biorad HPX-87H column. A peak corresponding to the elution time of xylose was resolved, but did not change during the course the hydrolyses; this may also represent a medium component.

By taking into account the 1.2 g/L glucose discussed above, the enzyme hydrolysis yields of all aqueous pretreatment solids were approximately the same, irregardless of the pretreatment temperature. Longer pretreatment times at all three temperatures appear to give the highest yields following cellulase hydrolysis. Hydrolysis of pretreated materials characteristically has been greater than alpha-cellulose, which was incorporated into each graph as a point of reference.

Enzyme hydrolysis yields of organosolv pretreated solids were less than those using aqueous pretreatment switchgrass solids. Unlike with the aqueous pretreatment solids, there was an inverse relationship between pretreatment temperature and glucose yield with the organosolv pretreatment solids. This observation was verified using oxalic acid in organosolv pretreatments; the greatest enzyme conversion was obtained using 0.1 M oxalic acid at 165°C. In the aqueous pretreatments of switchgrass, composition of the solid products did not vary greatly. Originally one cellulose value was in question: that was the 29.0 percent cellulose for the 0.025M H₃PO₄ for 30 min at 190°C pretreatment in Table BI. The two-stage acid procedure was repeated on this sample. The cellulose content was found to be 61.2 ±10.9 percent. The three 190°C solids samples were inadvertently stored in the refrigerator for several months before the two-stage acid hydrolysis procedure was conducted; this may account for the spurious result.

The SSF analysis was conducted on two of the switchgrass pretreated solids which had yielded the greatest yields of glucose from enzyme hydrolysis. Another factor used in selection of pretreated materials for the limited number of SSF's contracted during this study was the Klason-free lignin yields. These results, graphed as a function of pretreatment time in Figure B8 and B9, are taken from the Tables BI, BII and BIII.

The aqueous pretreatments of switchgrass using dilute phosphoric acid are shown in Figure B8. The lignin-free yield varied considerably between 52 and 68 percent with no particular trends as a function of pretreatment conditions. The lignin content of the pretreated materials were independent of conditions and averaged 40 percent (dw basis). Since the two relationships were not proportional, the variability in solubilization of hemicellulose appears highly dependent on acid concentration, pretreatment time and temperature.

The organosolv phosphoric acid pretreatments conducted for 10 and 20 minutes at 190°C using 0.025 M acid and at 180°C using 0.05 M acid gave Klason-lignin free yields greater than 70 percent (Figure B9). As above, the organosolv pretreatments of switchgrass demonstrated great dependence of yield and lignin content on pretreatment conditions. Shorter pretreatment times under the more severe temperatures produced greater yields (~75%) and lesser lignin contents (~25%) than did longer times at the same temperatures. The less severe 170°C pretreatments produced materials that followed the same trends.

Similarly, the lignin content plots vs treatment time for these same samples were less than all other pretreated switchgrass samples (Figure B8 and B9). One additional sample that was processed as an addendum to the original contract exhibited the lowest lignin content; that was 0.010 M phosphoric acid pretreated using ethanol organosolv for 40 minutes at 190°C. These data points are marked with a star on Figure B9.

Oxalic acid pretreatment of cornstover (Figure B10) produced considerably lower lignin-free yields compared to that parameter for phosphoric acid pretreated switchgrass (Figure B9) under the same conditions. However, the lignin content relationships were reversed for the two pretreatments that were conducted in 70% methanol.

Table BI. Characteristics of pretreated switchgrass using aqueous phosphoric acid under various conditions.

Pretreatment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellu- lose w/w %
0.05 M H ₃ PO ₄ 60 min 170°C	52.8	37.3 ± 0.2	2.8 ± 0.1	7.0 ± 0.5	52.4 ± 0.2
0.05 M H ₃ PO ₄ 40 min 170°C	66.7	35.1 ± 0.2	1.9 ± 0.1	7.3	49.6
0.05 M H ₃ PO ₄ 20 min 170°C	62.0	36.6 ± 0.0	2.5 ± 0.2	6.1 ± 0.3	52.2 ± 0.0
0.05 M H ₃ PO ₄ 30 min 180°C	58.4	38.3 ± 0.3	2.4 ± 0.1	7.9 ± 0.3	50.8 ± 1.6
0.05 M H ₃ PO ₄ 20 min 180°C	51.7	38.4 ± 0.2	3.2 ± 0.2	5.4 ± 0.1	50.0 ± 3.7
0.05 M H ₃ PO ₄ 10 min 180°C	66.6	34.2 ± 0.1	1.9 ± 0.1	5.1 ± 0.8	53.2 ± 0.4
0.025M H ₃ PO ₄ 30 min 190°C	62.3	39.4 ± 0.0	2.2 ± 0.1	1.0 ± 0.3	61.2 ± 10.9
0.025M H ₃ PO ₄ 20 min 190°C	55.7	38.4 ± 0.1	2.1 ± 0.1	3.0 ± 0.1	53.7 ± 0.4
0.025M H ₃ PO ₄ 10 min 190°C	57.3	34.6 ± 0.8	2.7 ± 0.2	6.8 ± 0.1	54.3 ± 0.6

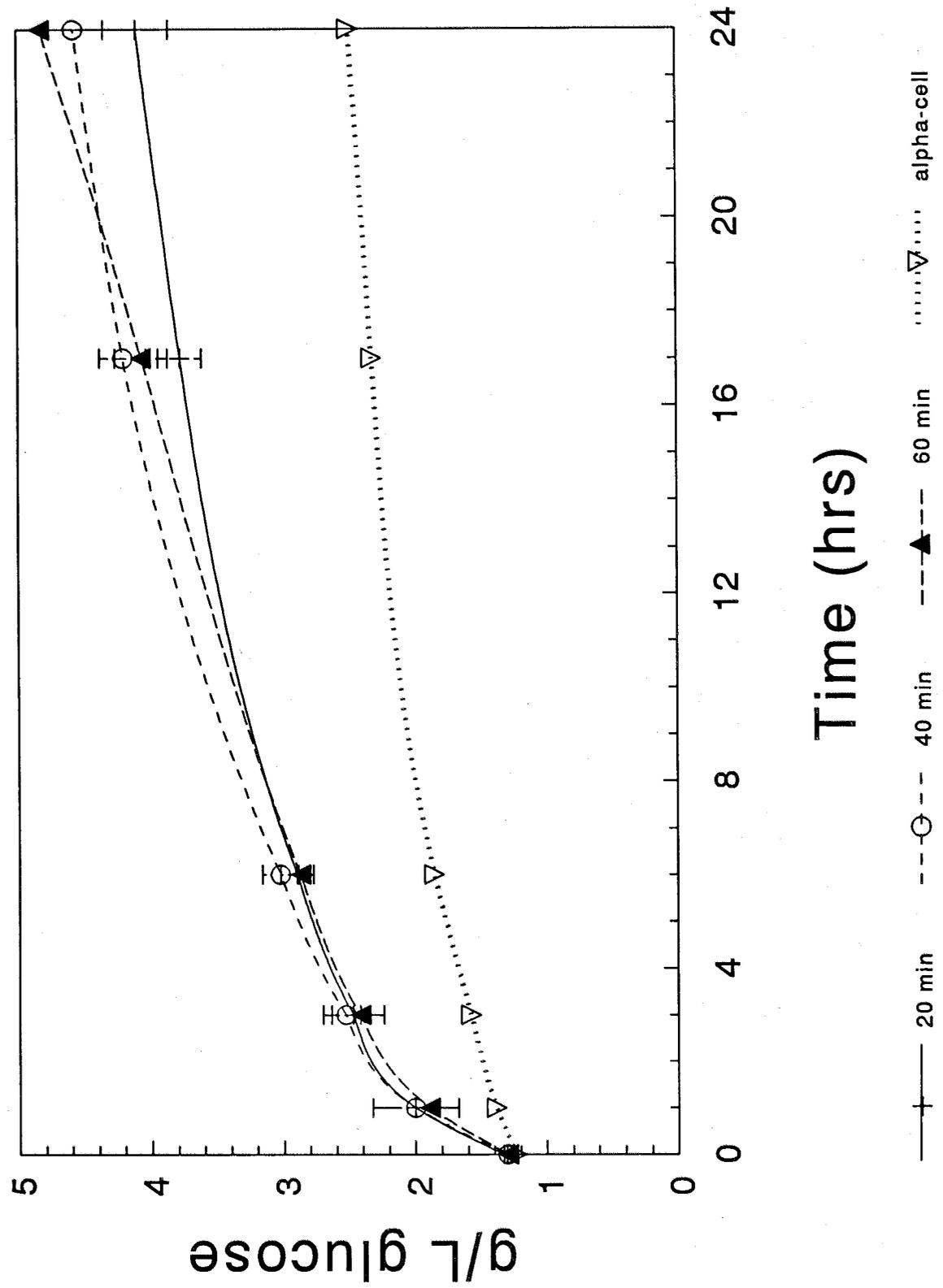
Table BII. Characteristics of pretreated switchgrass using organosolv phosphoric acid under various conditions.

Pretreat- ment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellu- lose w/w %
0.05 M H ₃ PO ₄ 60 min 170°C	52.9	32.1 ± 0.3	2.2 ± 0.3	17.4 ± 0.0	60.2 ± 0.6
0.05 M H ₃ PO ₄ 40 min 170°C	56.7	30.5 ± 0.1	2.1 ± 0.3	17.0 ± 0.0	59.5 ± 0.1
0.05 M H ₃ PO ₄ 20 min 170°C	59.6	26.8 ± 0.4	1.5 ± 0.3	22.4 ± 0.1	55.7 ± 0.8
0.05 M H ₃ PO ₄ 30 min 180°C	65.0	26.3 ± 0.8	4.3 ± 0.0	25.6 ± 0.1	52.5 ± 0.1
0.05 M H ₃ PO ₄ 20 min 180°C	71.0	24.2 ± 1.0	2.9 ± 0.1	29.1 ± 0.4	49.2 ± 0.9
0.05 M H ₃ PO ₄ 10 min 180°C	74.4	23.8 ± 0.1	2.5 ± 0.1	31.6 ± 0.0	47.5 ± 1.7
0.025M H ₃ PO ₄ 30 min 190°C	63.1	28.2 ± 0.3	2.2 ± 0.3	24.7 ± 0.3	54.9 ± 0.4
0.025M H ₃ PO ₄ 20 min 190°C	67.5	24.5 ± 1.4	4.7 ± 0.2	26.2 ± 1.2	50.4 ± 1.6
0.025M H ₃ PO ₄ 10 min 190°C	76.2	24.2 ± 0.4	4.6 ± 0.2	30.9 ± 0.1	48.0 ± 0.1

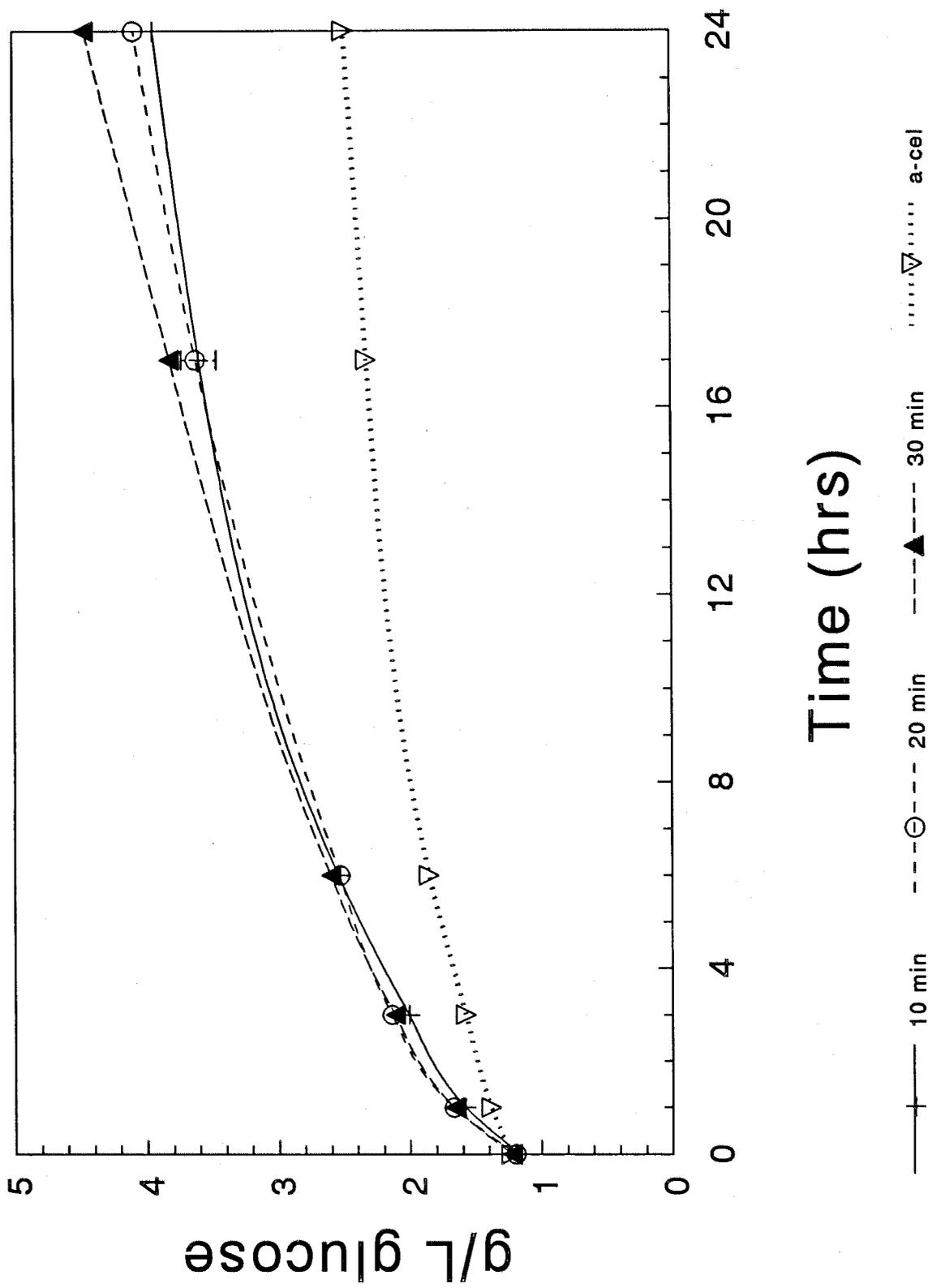
Table BIII. Characteristics of pretreated switchgrass using organosolv oxalic acid under various conditions.

Pretreatment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellulose w/w %
0.1 M C ₂ H ₂ O ₄ 30 min 165°C	51.5	31.1 ± 0.3	3.1 ± 0.3	9.8 ± 0.1	68.0 ± 0.8
0.1 M C ₂ H ₂ O ₄ 20 min 175°C	51.2	33.0 ± 0.1	3.0 ± 0.3	9.7 ± 0.7	68.5 ± 1.3
0.05 M C ₂ H ₂ O ₄ 30 min 165°C	56.7	33.2 ± 0.4	4.9 ± 0.3	7.0 ± 0.2	66.8 ± 0.7
0.05 M C ₂ H ₂ O ₄ 20 min 175°C	53.0	30.4 ± 0.3	4.2 ± 0.3	6.6 ± 0.1	70.3 ± 1.8

Enzyme Hydrolysis of Switchgrass 0.05M H₃PO₄ Pretreated 170°C

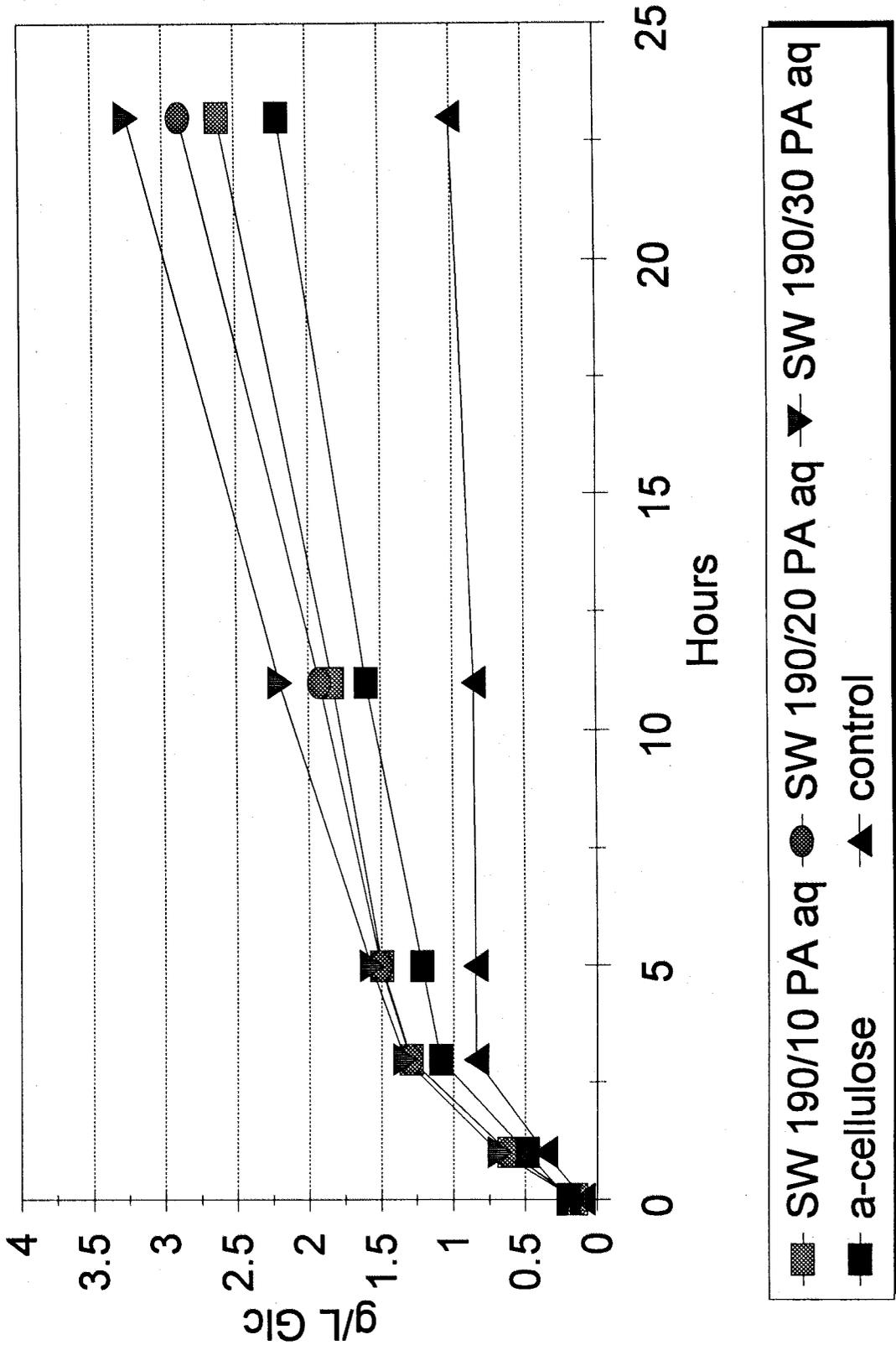


Enzyme Hydrolysis of Switchgrass 0.05M H₃PO₄ Pretreated 180°C



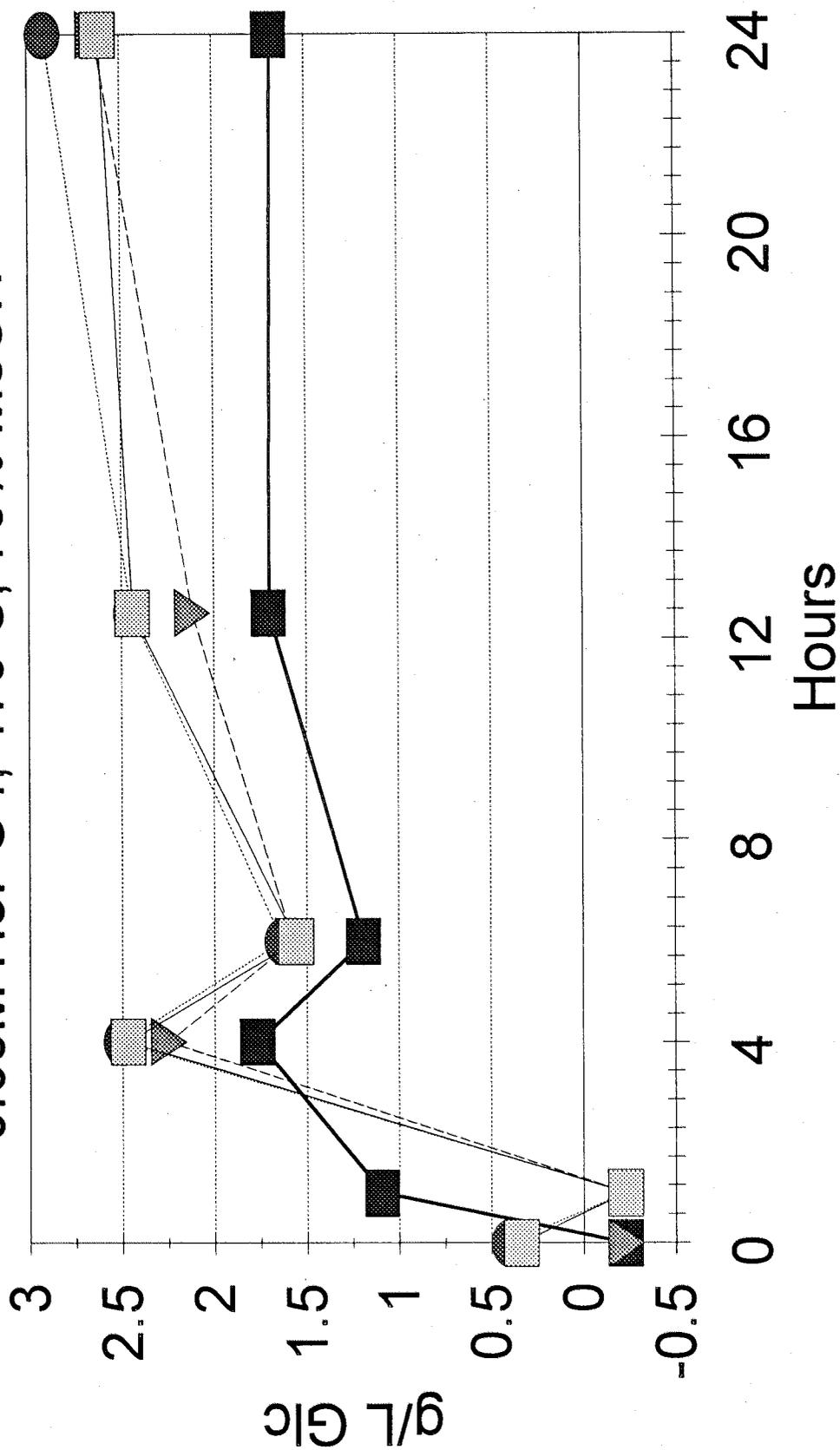
Switchgrass Enzyme Hydrolysis

0.025 H₃PO₄, Aqueous



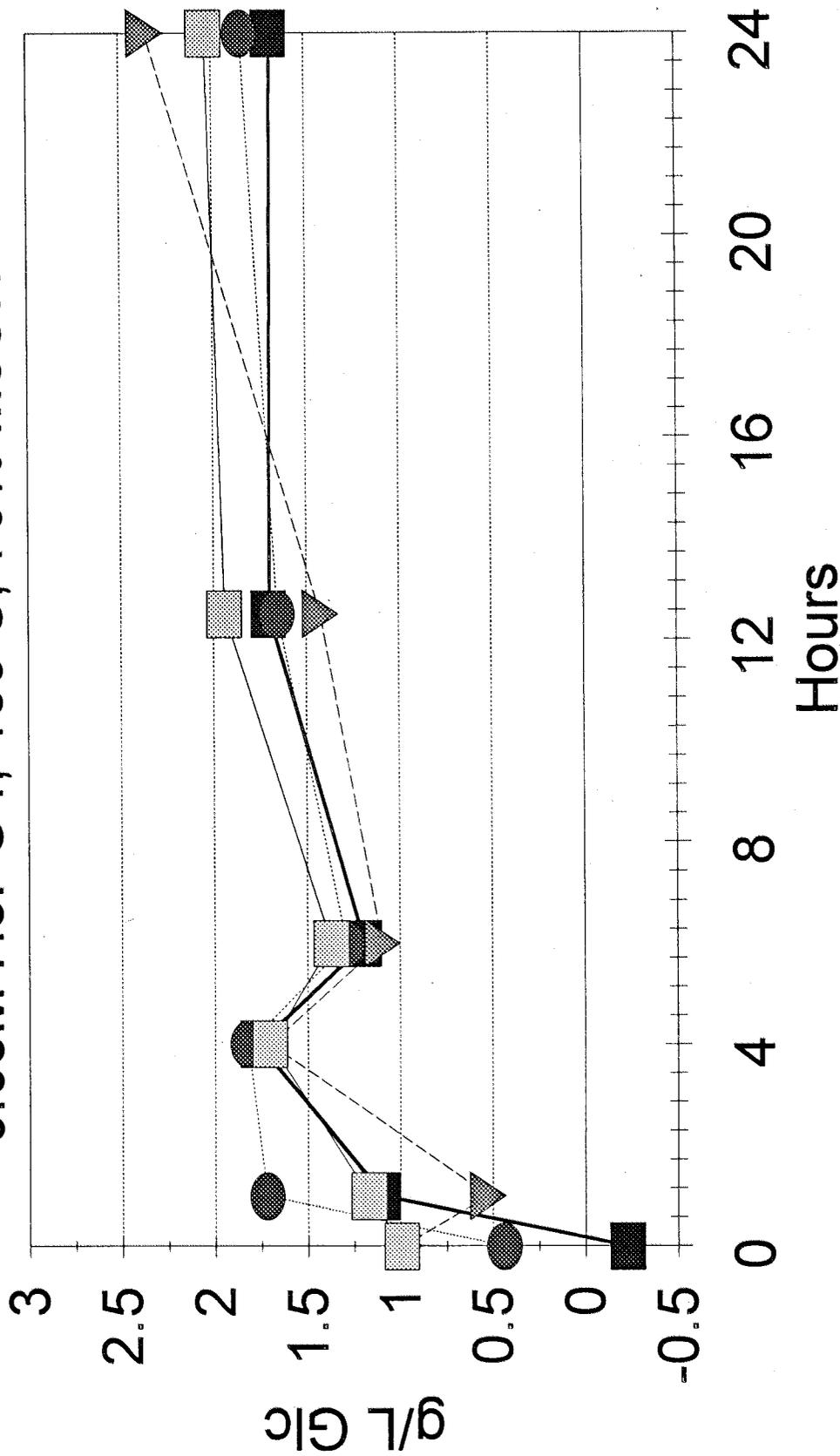
Switchgrass Enzyme Hydrolysis

0.05M H₃PO₄, 170 C, 70% MeOH



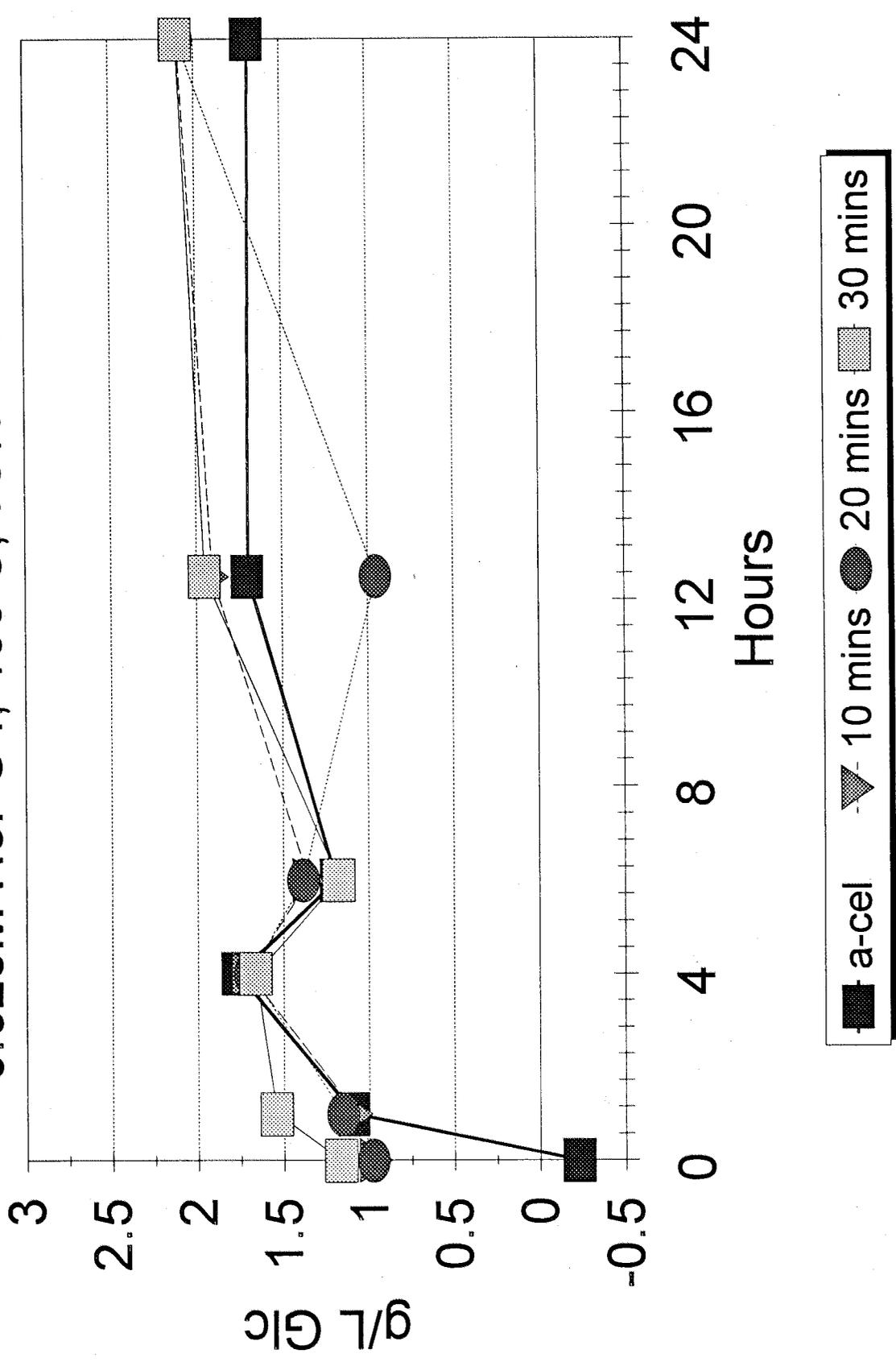
Switchgrass Enzyme Hydrolysis

0.05M H₃PO₄, 180 C, 70% MeOH



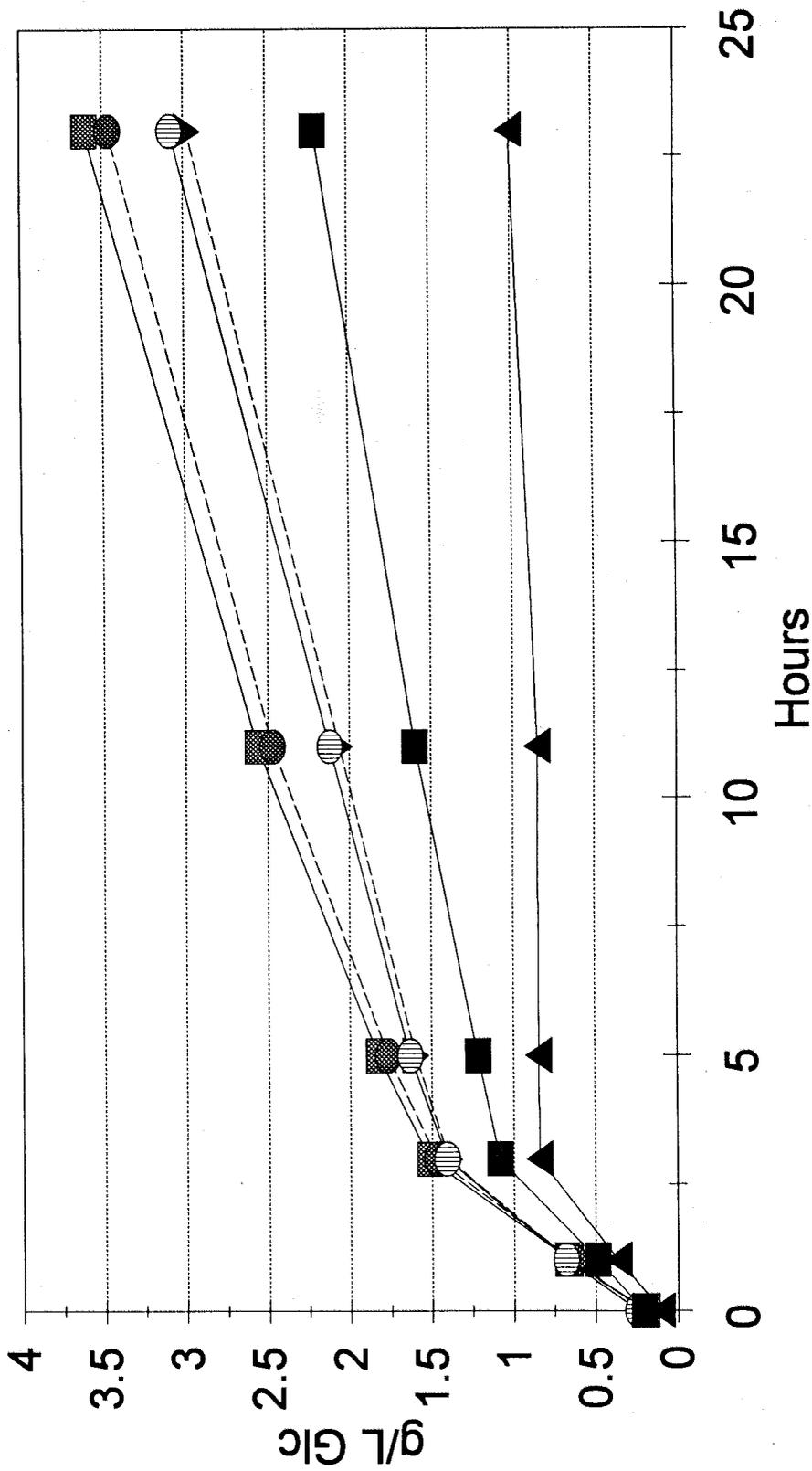
Switchgrass Enzyme Hydrolysis

0.025M H₃PO₄, 190 C, 70% MeOH



Switchgrass Enzyme Hydrolysis

Oxalic Acid, Organic



SW 165/30 0.1M oa
 SW 175/20 0.1M oa
 SW 165/30 0.05M oa
 a-cellulose
 control

Figure B8. Aqueous pretreatments of switchgrass using dilute phosphoric acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.

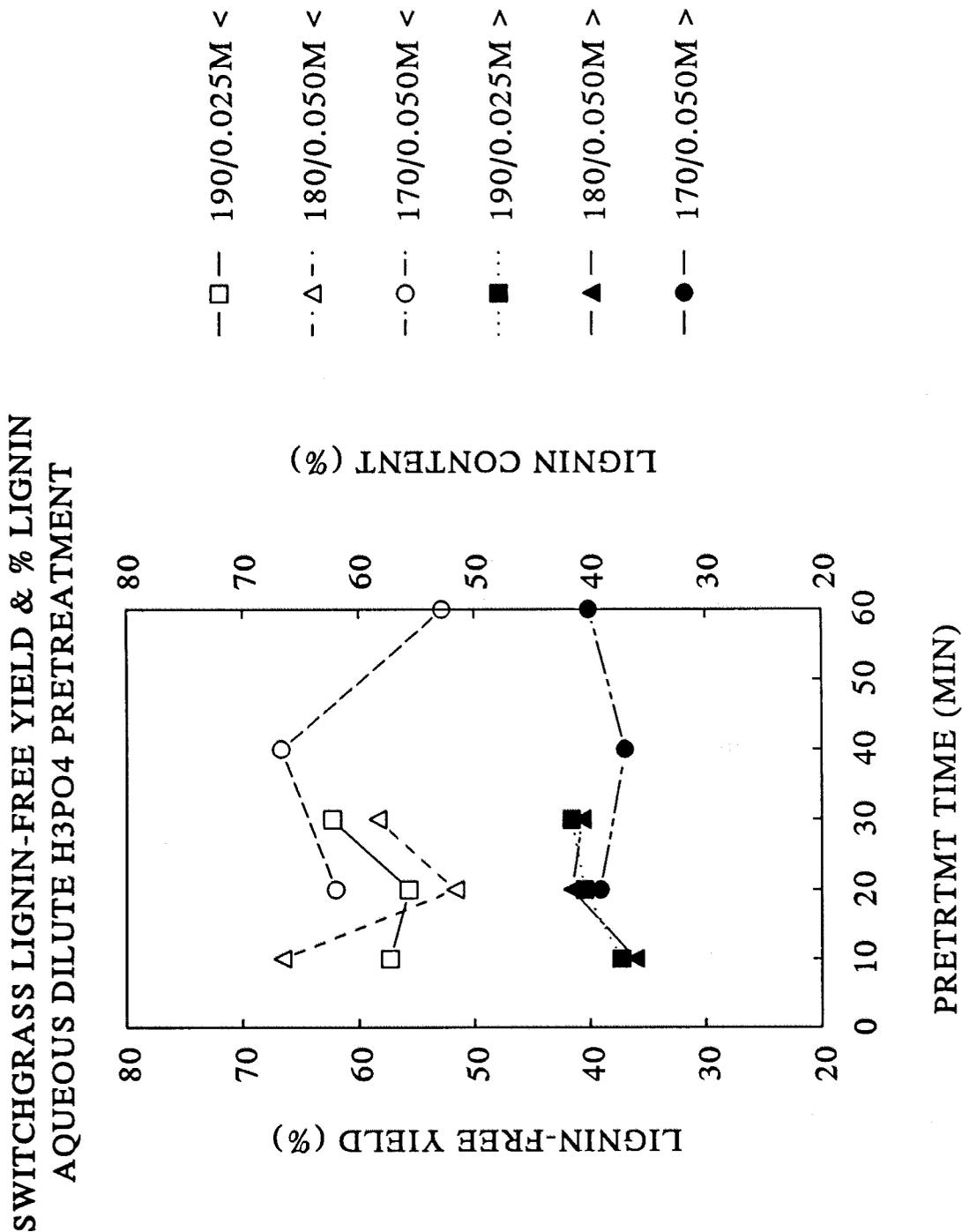


Figure B9. Organosolv pretreatments of switchgrass using dilute phosphoric acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.

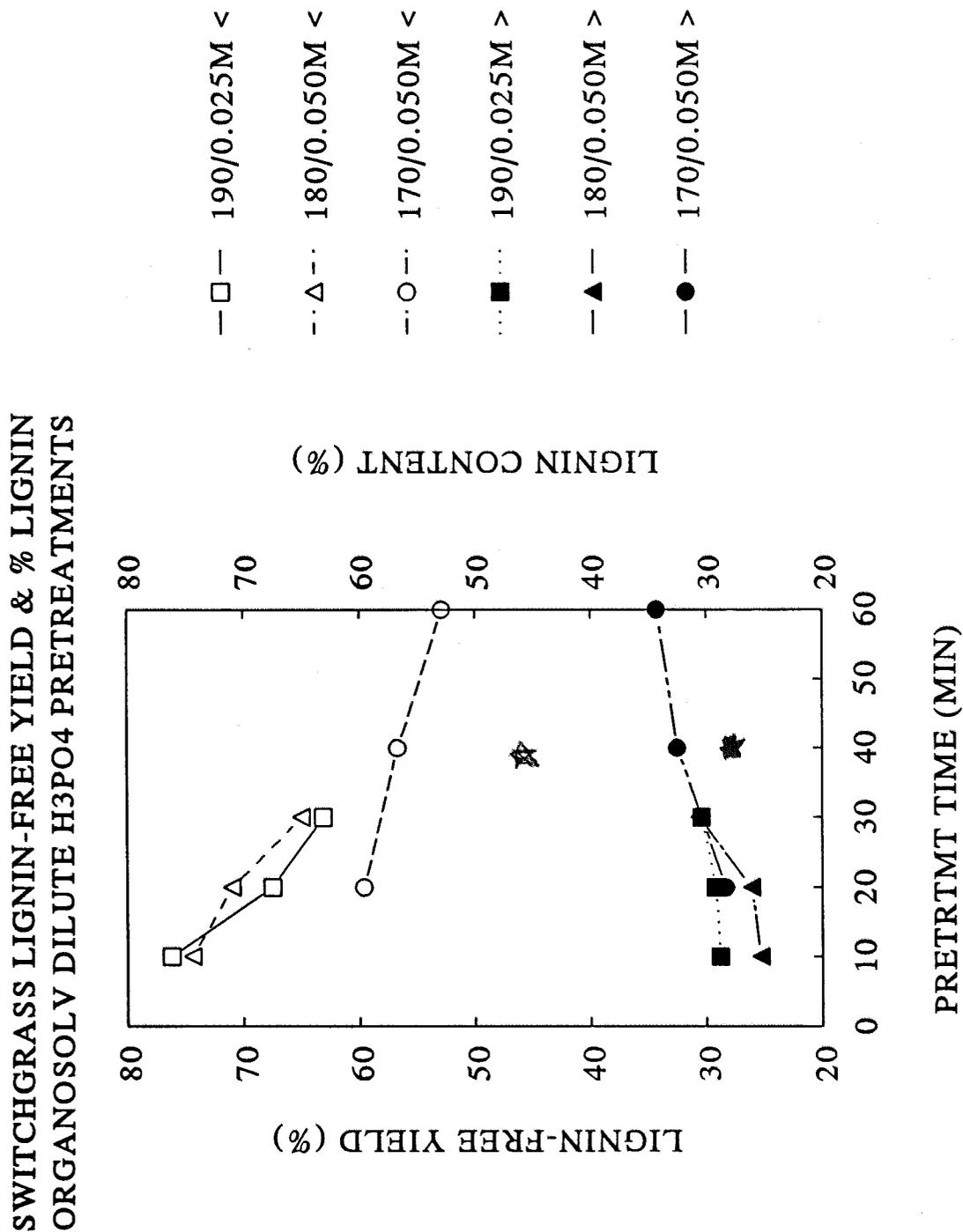
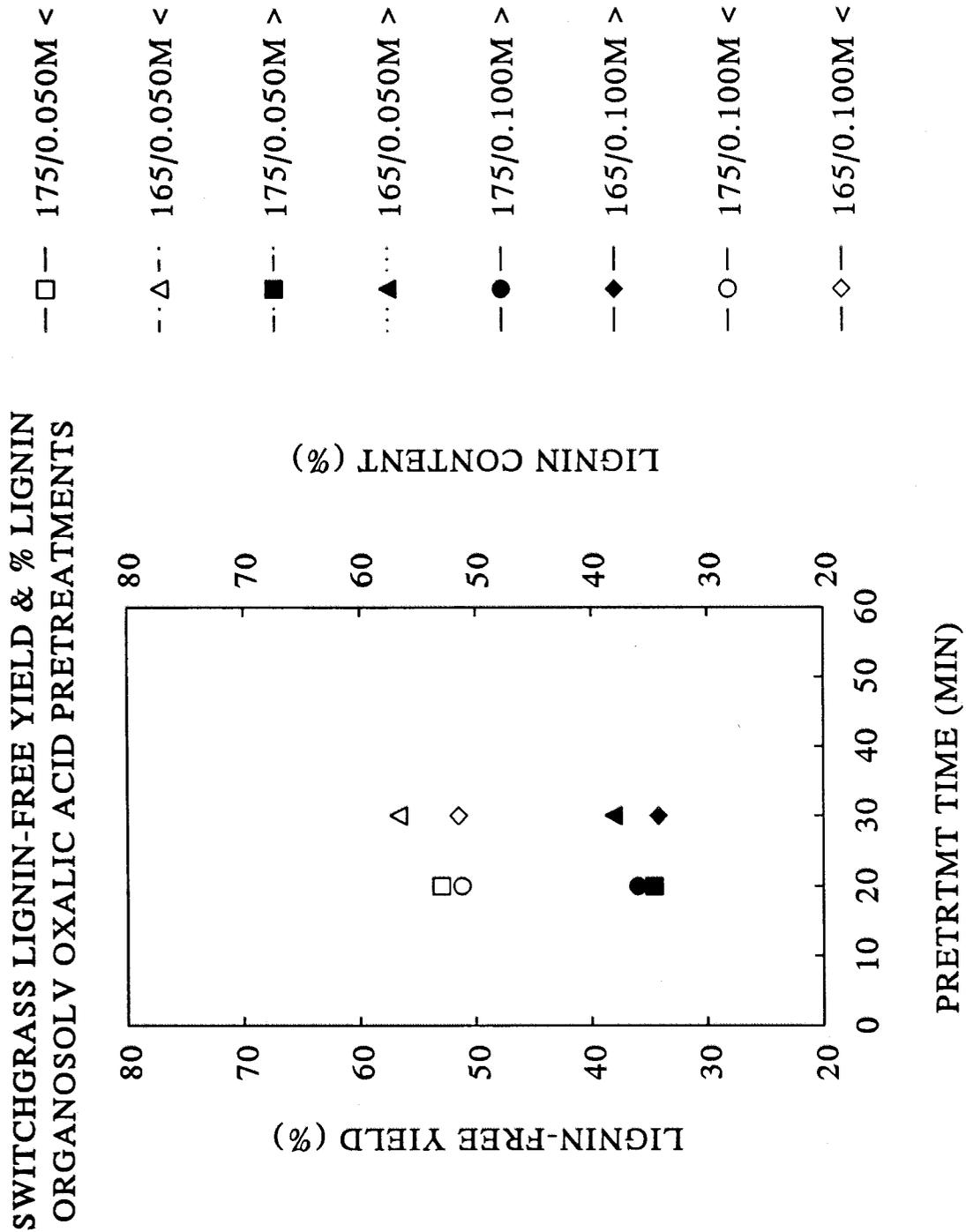


Figure B10. Organosolv pretreatments of switchgrass using dilute oxalic acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.



C. Cornstover Pretreatments

The pretreatment solids compositional data for cornstover are given in Tables CI, CII and CIII. The data in Table CI exhibit consistent yields and lignin contents within each treatment group. Generally, cellulose and xylan content decrease with treatment time. Data reported in Table CI for the 160°C 0.05M phosphoric acid pretreatments was different than first reported in the Eleventh Monthly Technical Progress Report. The two-stage acid hydrolysis analysis was repeated because differences were greater than acceptable. These probably were a result of HPLC glucose/xylose resolution differences.

The organosolv pretreatment compositional and yield data in Tables CII and CIII present no particular trends either within pretreatment groups. However, comparisons between pretreatment groups indicate that Klason lignin, soluble lignin, xylan and cellulose contents of oxalic acid pretreated solids were less than the averages for either aqueous or organosolv phosphoric acid derived materials.

These data were used to set up the enzymatic hydrolysis evaluations for all of these pretreatments. These data are presented in Figures C1 through C8. The greatest enzyme hydrolysis glucose yield from the aqueous phosphoric acid pretreatment series is seen in Figure C3 using 0.025 M acid at 190°C; all three treatment times were equivalent yielding 3.1 g/L glucose. In comparison, the organosolv phosphoric acid pretreatments yielded approximately 3.5 g/L glucose, while that obtained from the organosolv oxalic acid pretreatments were slightly greater.

In the cornstover series of experiments, the HPLC was standardized using cellobiose as well as glucose. Consequently, yields may be calculated as a proviso of the NREL Chemical Analysis & Testing Procedure Standard No. 008, Revision #3. Figure C9 is a composite of the yield plots from all of the cornstover enzyme hydrolysis analysis. Cornstover organosolv oxalic acid pretreated materials exhibited the greatest yields.

Aqueous pretreatments of cornstover produced data for which both lignin-free yields and lignin contents were relatively independent of pretreatment conditions (Figure C10). The average yields using cornstover (~67%) were greater than those using switchgrass (~60%) and the percent lignin contents of pretreated cornstover (~44%) were also greater than those of pretreated switchgrass (~40%) for the same set of pretreatment conditions.

Organosolv pretreatments of cornstover also produced lignin-free yields and lignin contents that were relatively independent of pretreatment conditions (Figure C11). Again the respective yields and lignin contents were ~67% and ~44%. There were noticeable trends in lower values for these parameters and short treatment times at 190°C.

Oxalic acid pretreatment of cornstover (Figure C12) produced similar lignin-free yields compared to that parameter for phosphoric acid pretreated cornstover (Figure C11) under the same conditions. However, the lignin contents for the oxalic acid pretreated materials were

lower than the phosphoric acid pretreated cornstover using these these two organosolv pretreatments that were conducted in 70% methanol.

One additional sample exhibited low lignin content; that was 0.01M phosphoric acid pretreated using ethanol organosolv for 40 minutes at 190°C. These data points are marked with a star on Figure C10.

Table CI. Characteristics of pretreated cornstover using aqueous phosphoric acid under various conditions.

Pretreatment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellulose w/w %
0.05 M H ₃ PO ₄ 60 min 160°C	65.5	38.9 ± 0.3	4.7 ± 0.1	4.9 ± 0.2	50.1 ± 0.7
0.05 M H ₃ PO ₄ 40 min 160°C	63.6	39.8 ± 0.3	5.2 ± 0.2	4.6 ± 0.1	48.9 ± 0.4
0.05 M H ₃ PO ₄ 20 min 160°C	66.0	37.8 ± 0.3	4.8 ± 0.2	7.0 ± 0.5	46.7 ± 2.8
0.05 M H ₃ PO ₄ 60 min 170°C	64.4	40.1 ± 1.0	5.0 ± 0.1	5.9 ± 1.2	59.9 ± 6.3
0.05 M H ₃ PO ₄ 40 min 170°C	67.6	39.2 ± 1.2	4.9 ± 0.1	5.7 ± 0.2	60.6 ± 1.2
0.05 M H ₃ PO ₄ 20 min 170°C	69.1	37.9 ± 0.3	4.9 ± 0.4	8.0 ± 0.1	65.2 ± 0.8
0.05 M H ₃ PO ₄ 30 min 180°C	67.3	40.7 ± 1.2	5.0 ± 0.5	4.8 ± 0.0	67.3 ± 0.5
0.05 M H ₃ PO ₄ 20 min 180°C	69.3	40.1 ± 0.2	4.9 ± 0.5	5.3 ± 0.1	56.6 ± 1.2
0.05 M H ₃ PO ₄ 10 min 180°C	66.8	37.7 ± 0.5	4.5 ± 0.1	8.9 ± 2.6	85.1 ± 2.3
0.025M H ₃ PO ₄ 30 min 190°C	64.7	41.9 ± 0.3	5.2 ± 0.1	4.4 ± 0.3	57.0 ± 4.9
0.025M H ₃ PO ₄ 20 min 190°C	63.6	39.5 ± 0.5	4.8 ± 0.2	5.4 ± 0.3	70.7 ± 3.5
0.025M H ₃ PO ₄ 10 min 190°C	64.6	40.4 ± 0.1	5.1 ± 0.0	7.1 ± 1.7	65.1 ± 4.0

Table CII. Characteristics of pretreated cornstover using organosolv phosphoric acid under various conditions.

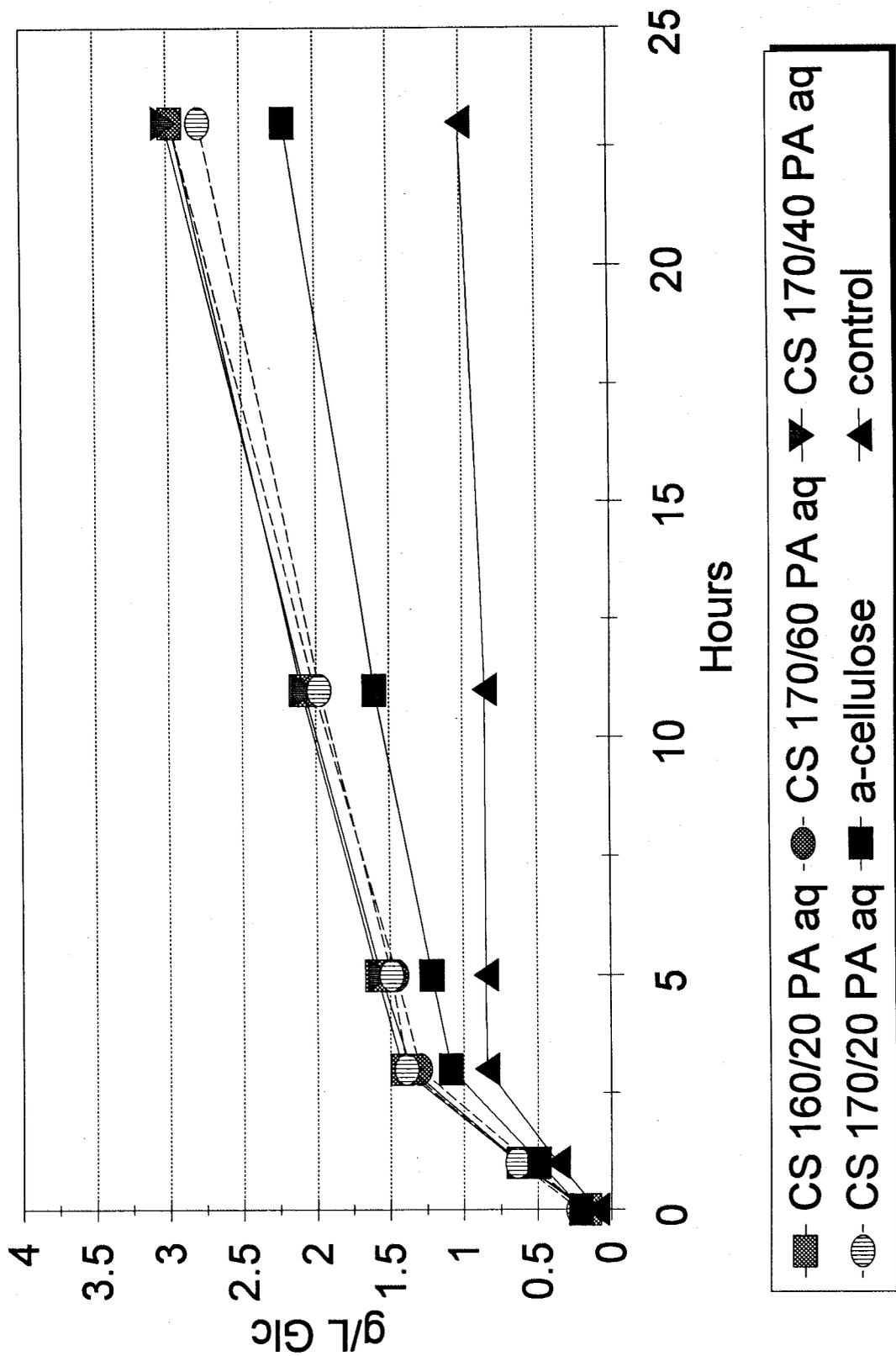
Pretreatment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellulose w/w %
0.05 M H ₃ PO ₄ 60 min 160°C	65.0	37.4 ± 0.3	4.8 ± 0.3	8.5 ± 0.4	66.5 ± 0.1
0.05 M H ₃ PO ₄ 40 min 160°C	65.3	38.0 ± 0.1	4.9 ± 0.3	7.5 ± 0.6	55.1
0.05 M H ₃ PO ₄ 20 min 160°C	65.9	39.6 ± 0.4	5.0 ± 0.3	7.9 ± 0.6	62.7 ± 0.9
0.05 M H ₃ PO ₄ 60 min 170°C	66.3	41.0 ± 0.3	5.1 ± 0.3	7.1 ± 0.9	64.2 ± 17.1
0.05 M H ₃ PO ₄ 40 min 170°C	64.9	39.5 ± 0.1	4.9 ± 0.3	8.2 ± 0.2	64.6 ± 3.1
0.05 M H ₃ PO ₄ 20 min 170°C	64.3	38.7 ± 0.4	5.1 ± 0.3	8.8 ± 0.1	59.5 ± 0.8
0.05 M H ₃ PO ₄ 30 min 180°C	62.4	40.1 ± 0.1	5.1 ± 0.1	6.5 ± 0.2	66.6 ± 0.8
0.05 M H ₃ PO ₄ 20 min 180°C	65.0	39.8 ± 0.1	4.8 ± 0.1	7.3 ± 0.0	61.8 ± 2.4
0.05 M H ₃ PO ₄ 10 min 180°C	65.3	39.5 ± 0.1	5.2 ± 0.1	8.2 ± 0.1	64.9 ± 1.5
0.025M H ₃ PO ₄ 30 min 190°C	64.9	39.9 ± 0.3	5.1 ± 0.3	7.1 ± 3.1	66.2 ± 5.2
0.025M H ₃ PO ₄ 20 min 190°C	62.6	36.3 ± 1.4	4.7 ± 0.2	10.1 ± 2.4	69.8 ± 5.0
0.025M H ₃ PO ₄ 10 min 190°C	60.6	35.3 ± 0.4	4.0 ± 0.2	8.2 ± 2.1	64.2 ± 13.1

Table CIII. Characteristics of pretreated cornstover using organosolv oxalic acid under various conditions.

Pretreatment Conditions	Yield w/w %	Klason Lignin w/w %	Soluble Lignin w/w %	Xylan w/w %	Cellulose w/w %
0.1 M C ₂ H ₂ O ₄ 30 min 165°C	60.6	35.0 ± 0.3	3.8 ± 0.3	6.8 ± 0.0	66.0 ± 0.4
0.1 M C ₂ H ₂ O ₄ 20 min 175°C	60.0	36.7 ± 0.1	4.1 ± 0.3	6.7 ± 0.0	55.4 ± 4.6
0.05 M C ₂ H ₂ O ₄ 30 min 165°C	66.2	36.1 ± 0.4	4.1 ± 0.3	7.2 ± 0.4	54.3 ± 4.0
0.05 M C ₂ H ₂ O ₄ 20 min 175°C	65.6	36.2 ± 0.3	4.5 ± 0.3	5.6 ± 0.3	55.2 ± 1.8

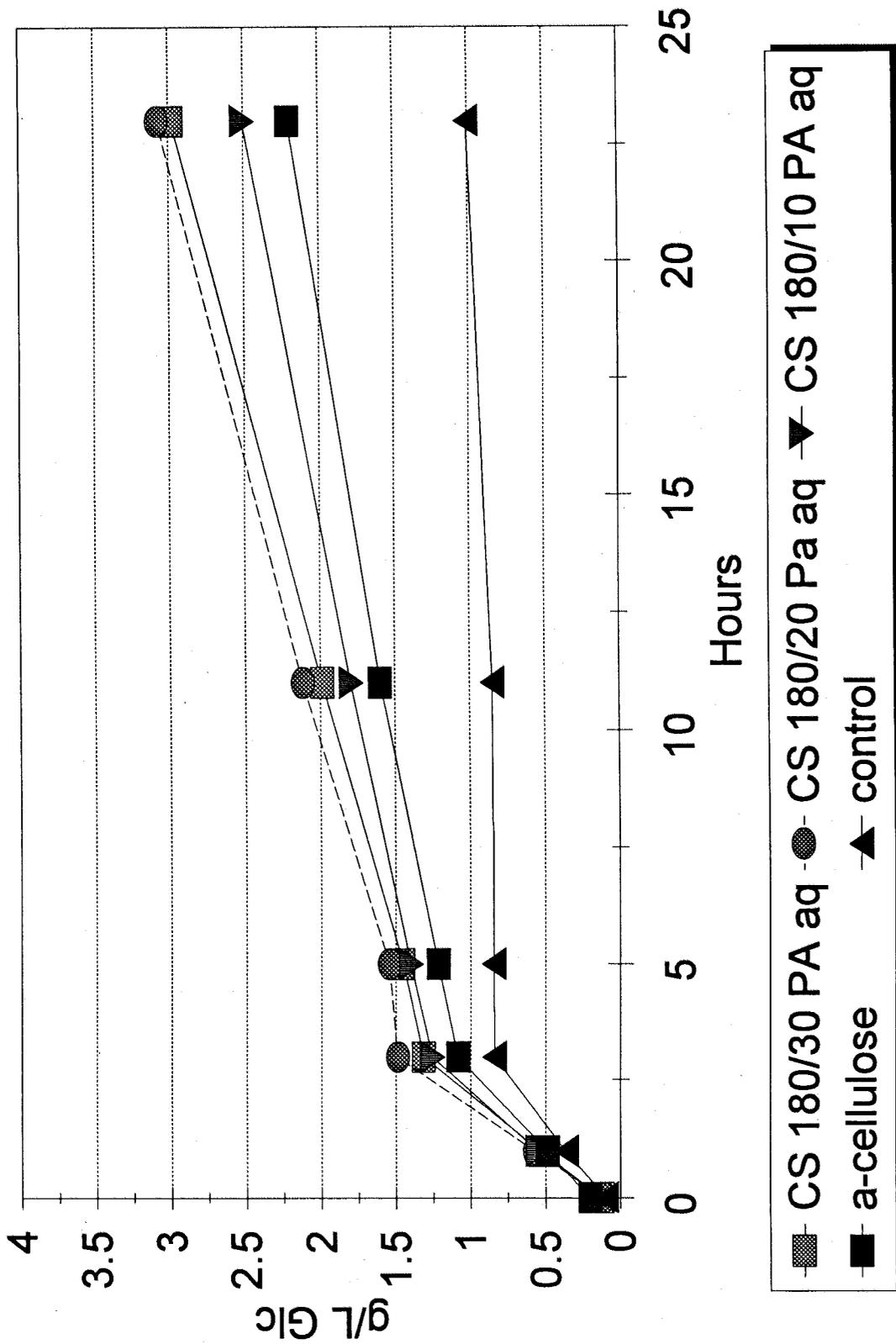
Cornstover Enzyme Hydrolysis

0.05 H₃PO₄, Aqueous



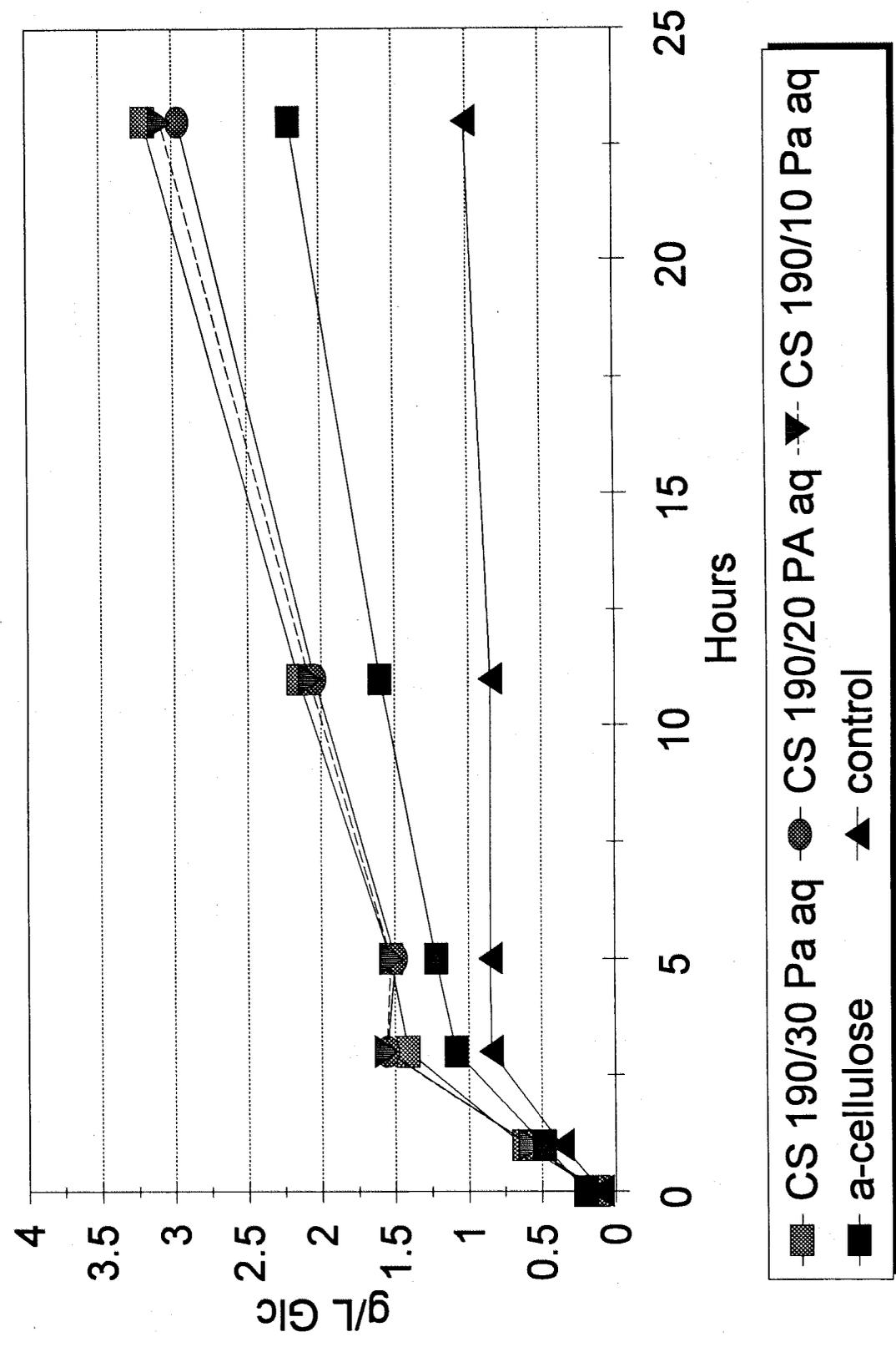
Cornstover Enzyme Hydrolysis

0.05 H₃PO₄, Aqueous



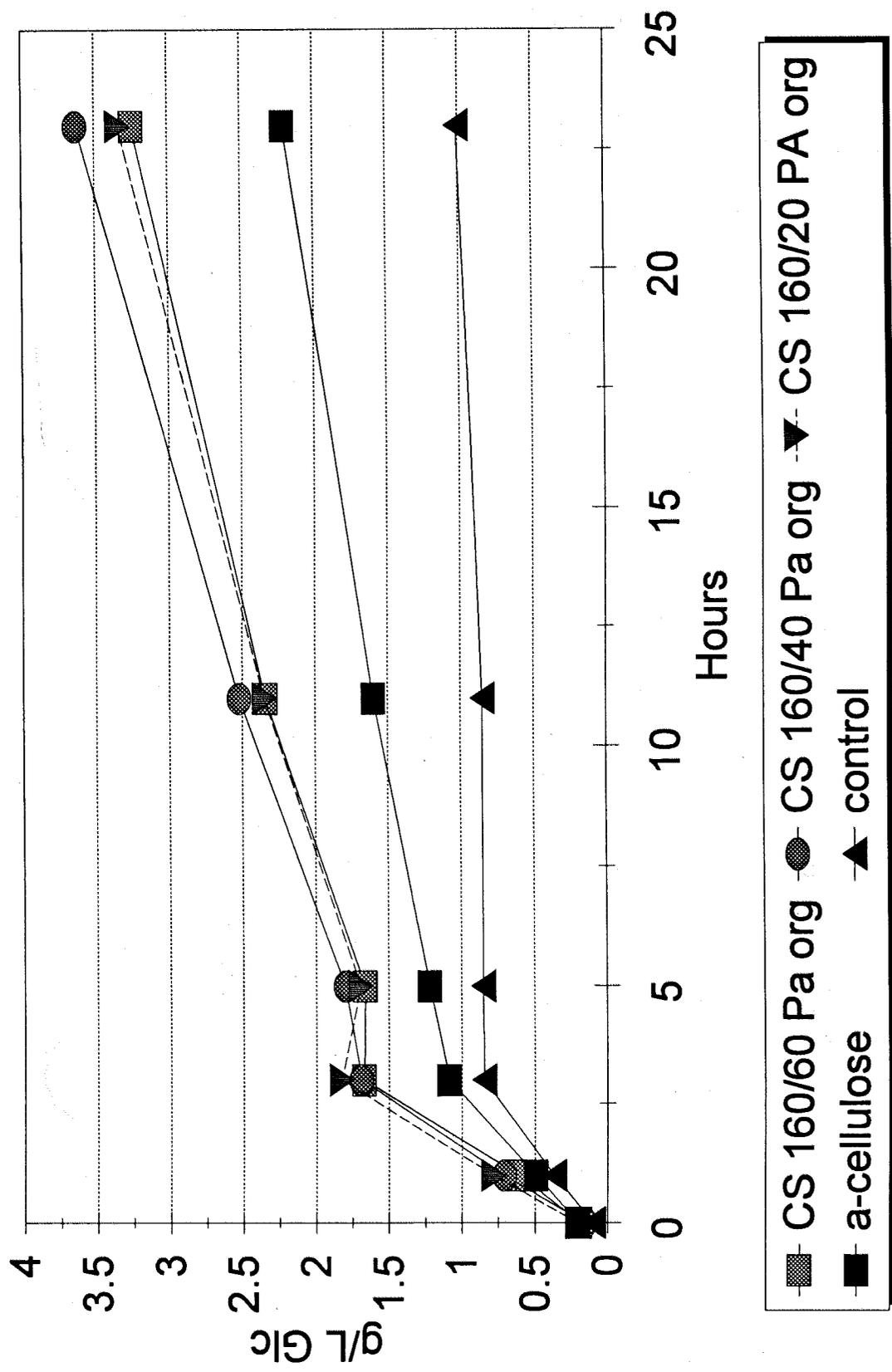
Constover Enzyme Hydrolysis

0.025 H₃PO₄, Aqueous



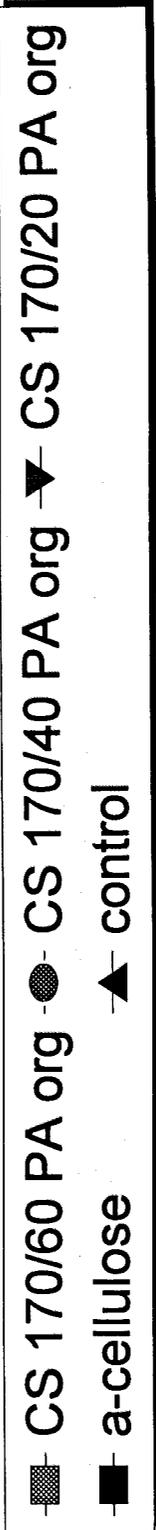
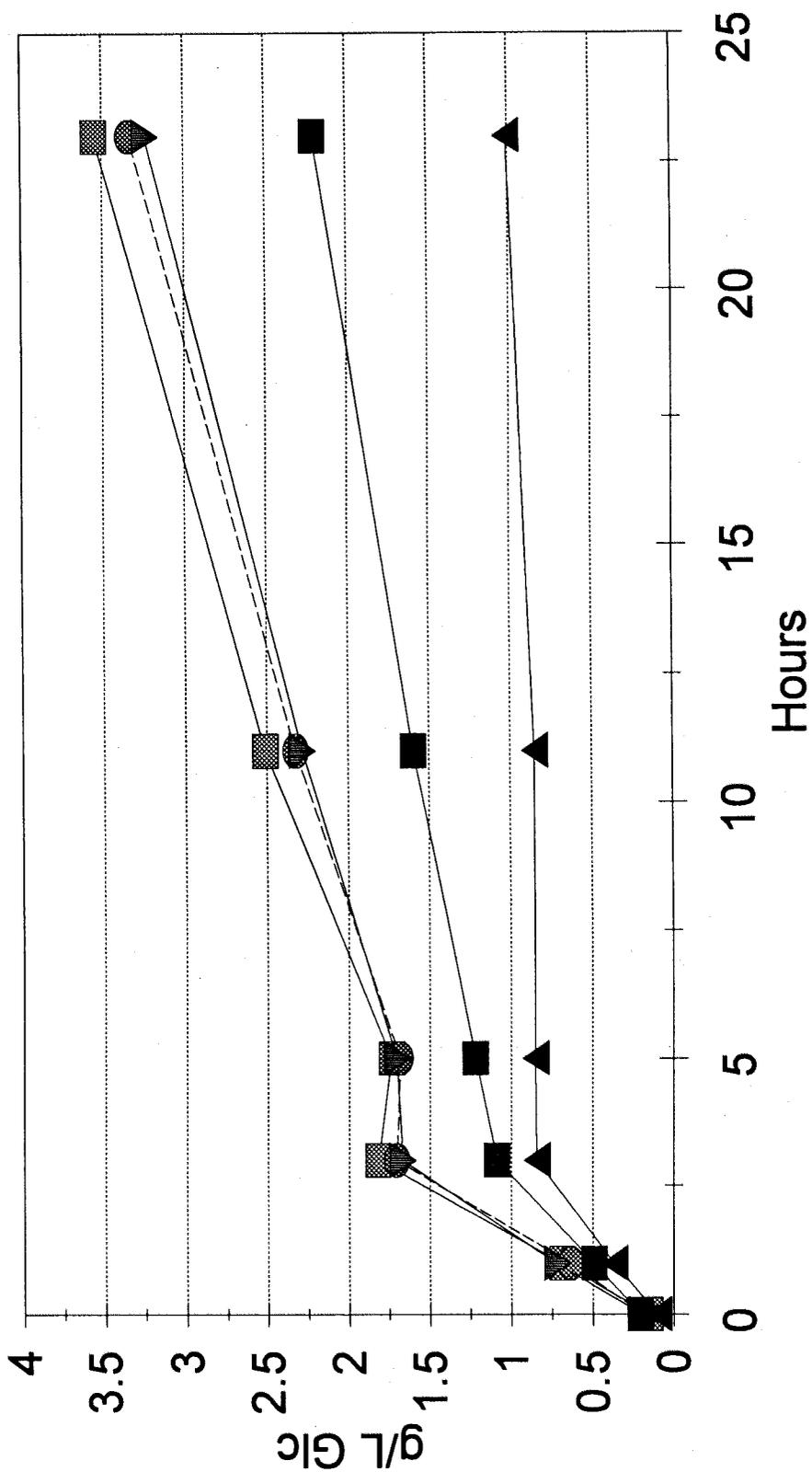
Cornstover Enzyme Hydrolysis

0.05 H₃PO₄, Organic



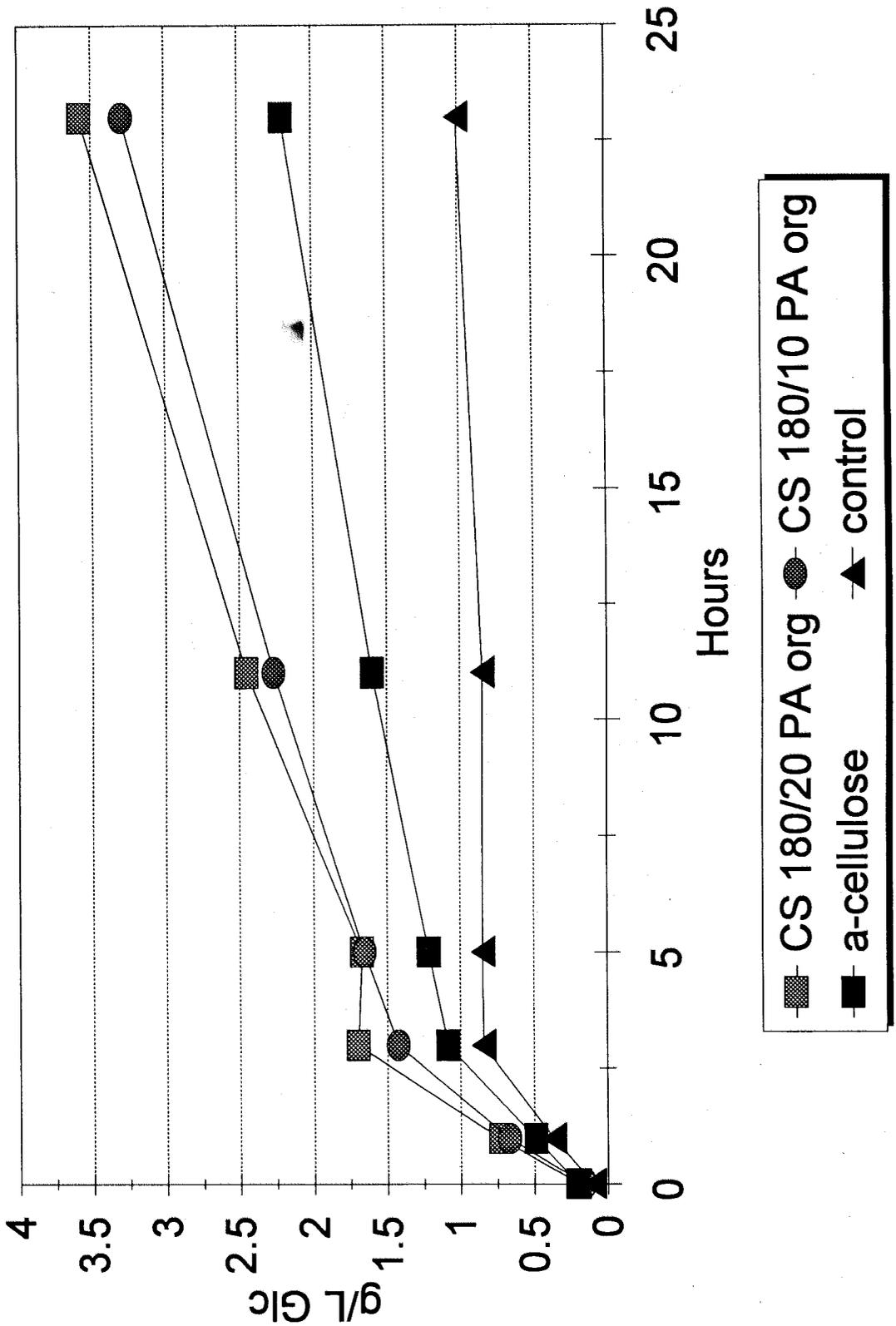
Cornstover Enzyme Hydrolysis

0.05 H₃PO₄, Organic



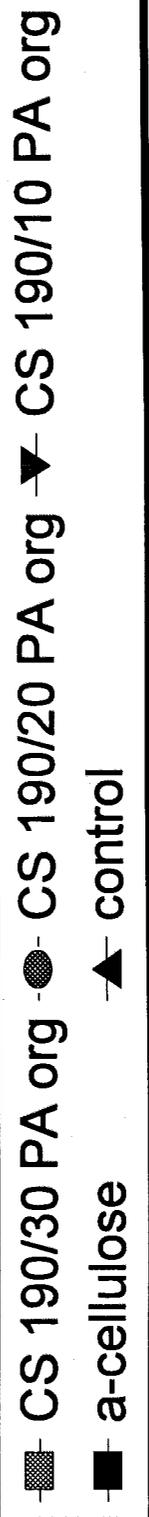
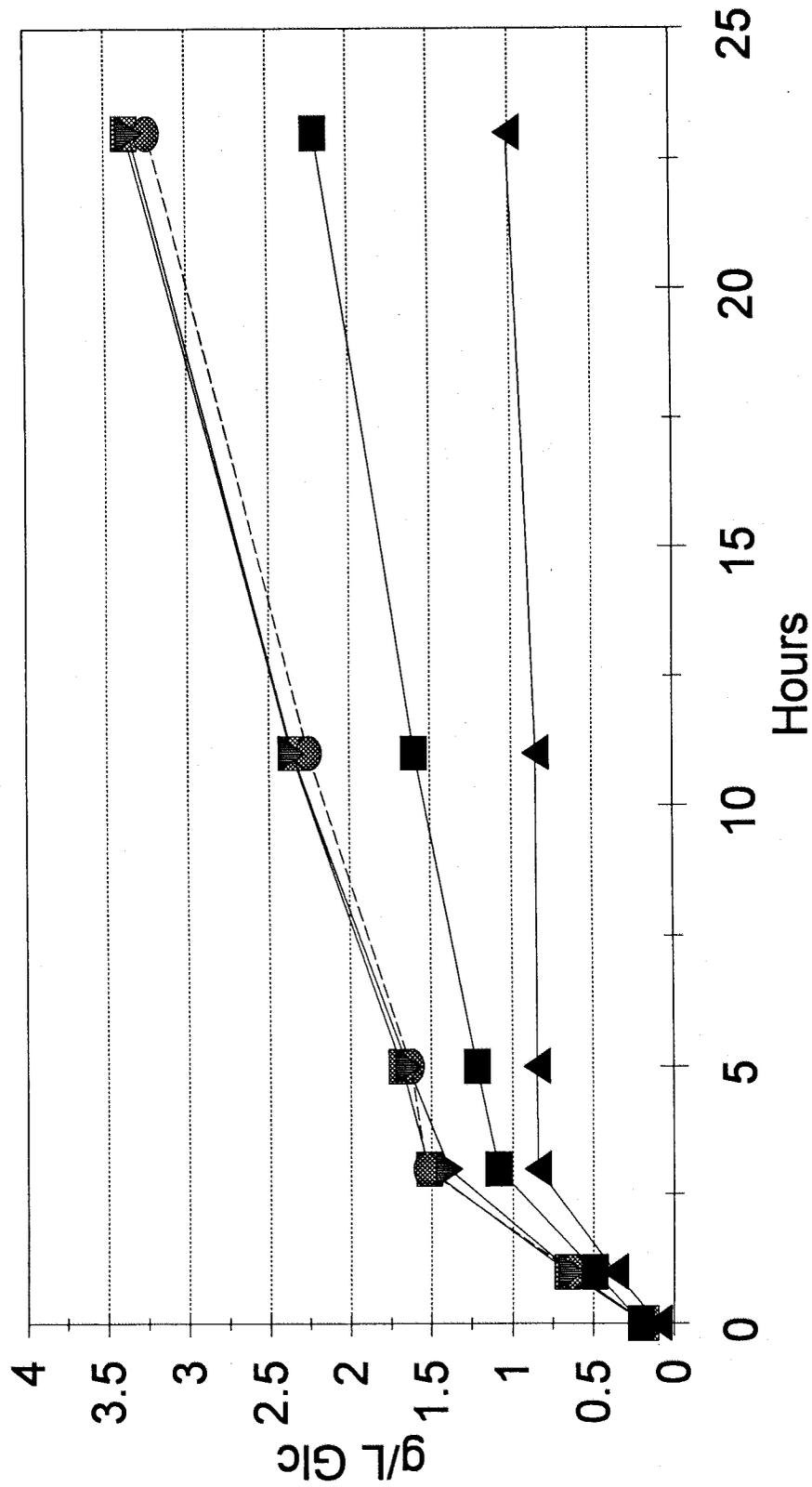
Cornstover Enzyme Hydrolysis

0.05 H₃PO₄, Organic



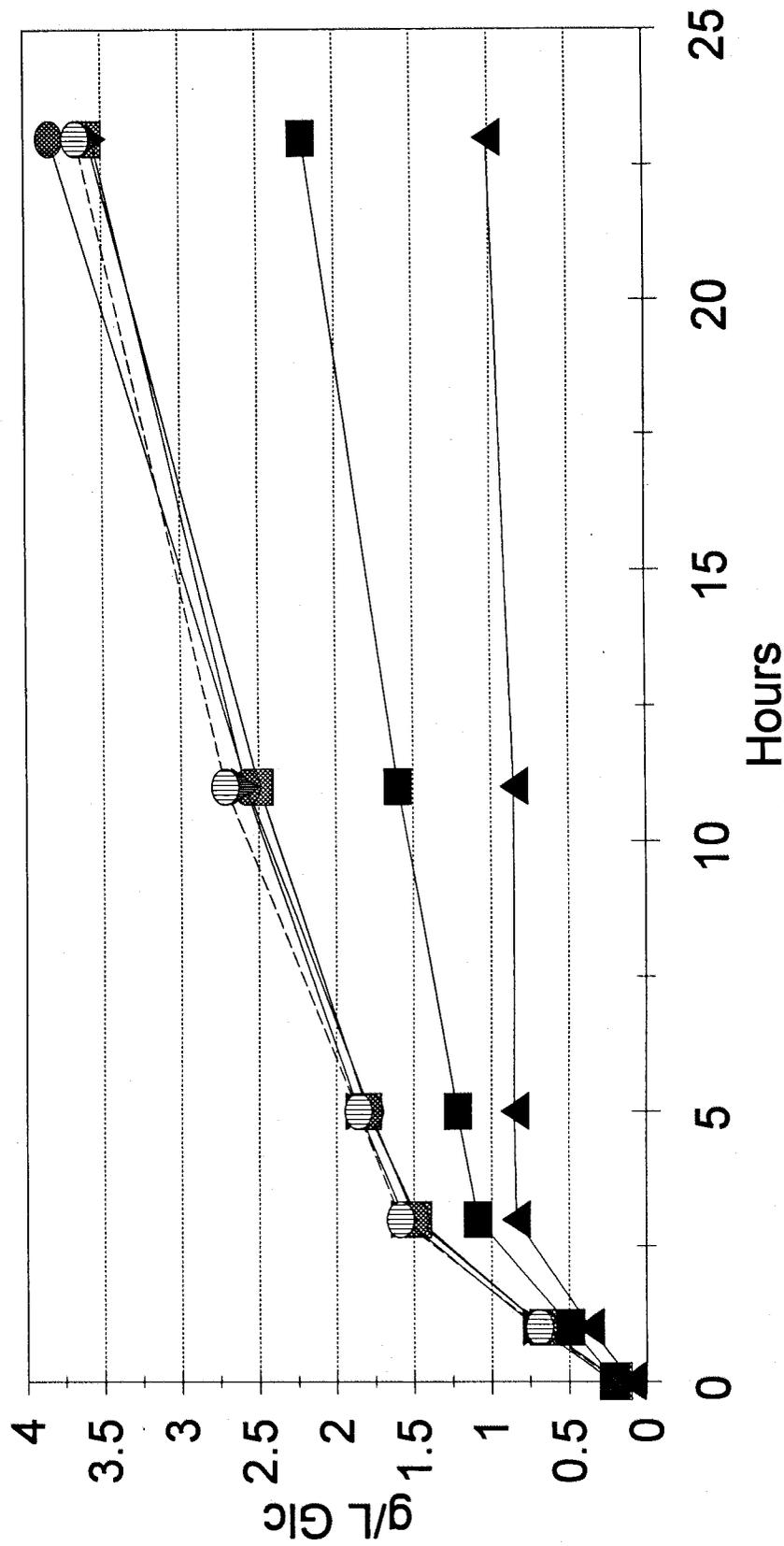
Cornstover Enzyme Hydrolysis

0.025 H₃PO₄, Organic



Cornstover Enzyme Hydrolysis

Oxalic Acid, Organic



CS 165/30 0.1M oa
 CS 175/20 0.1M oa
 CS 165/30 0.05M oa
 CS 175/20 0.05M oa
 a-cellulose
 control

Figure C10. Aqueous pretreatments of cornstover using dilute phosphoric acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.

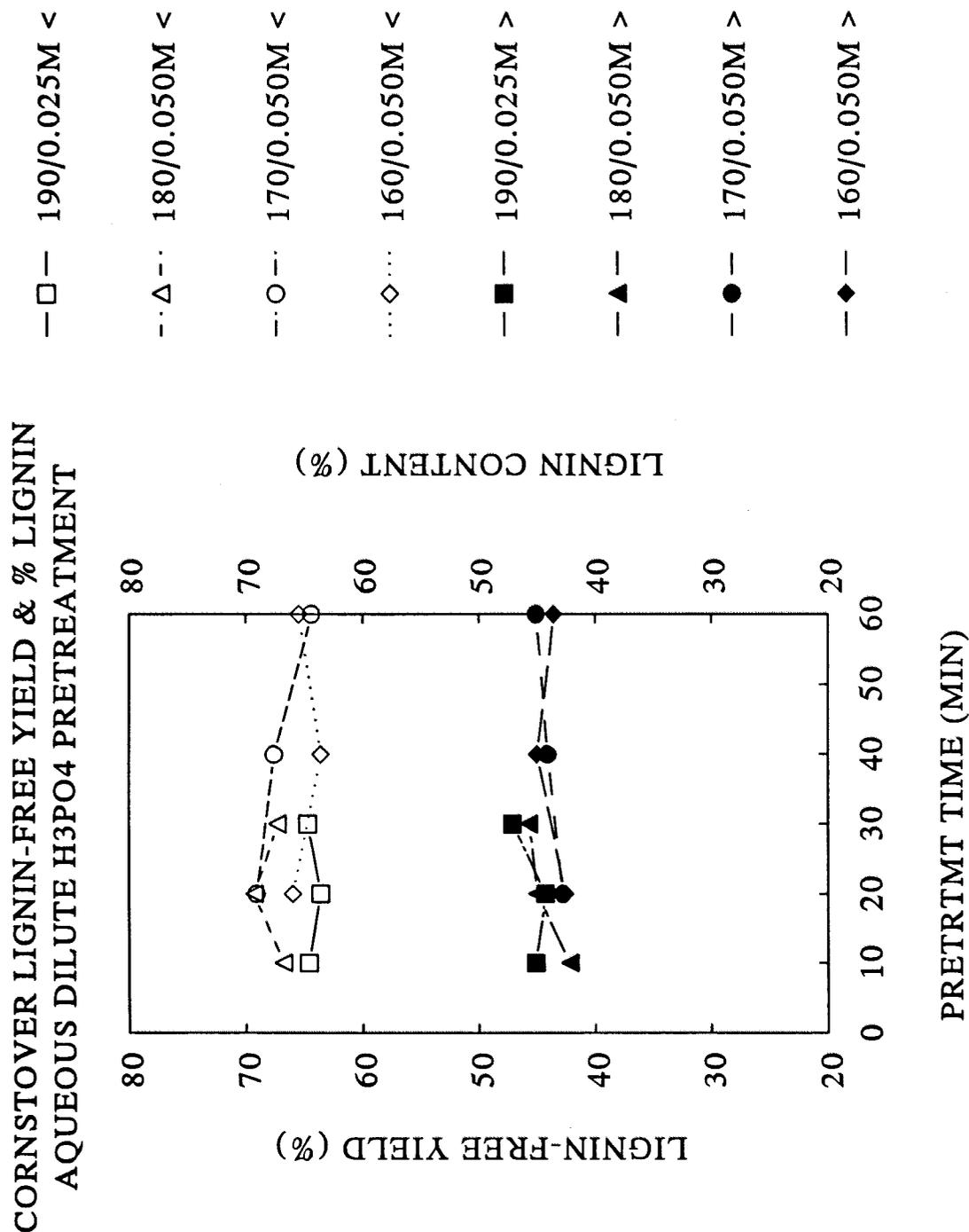


Figure C11. Organosolv pretreatments of cornstover using dilute phosphoric acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.

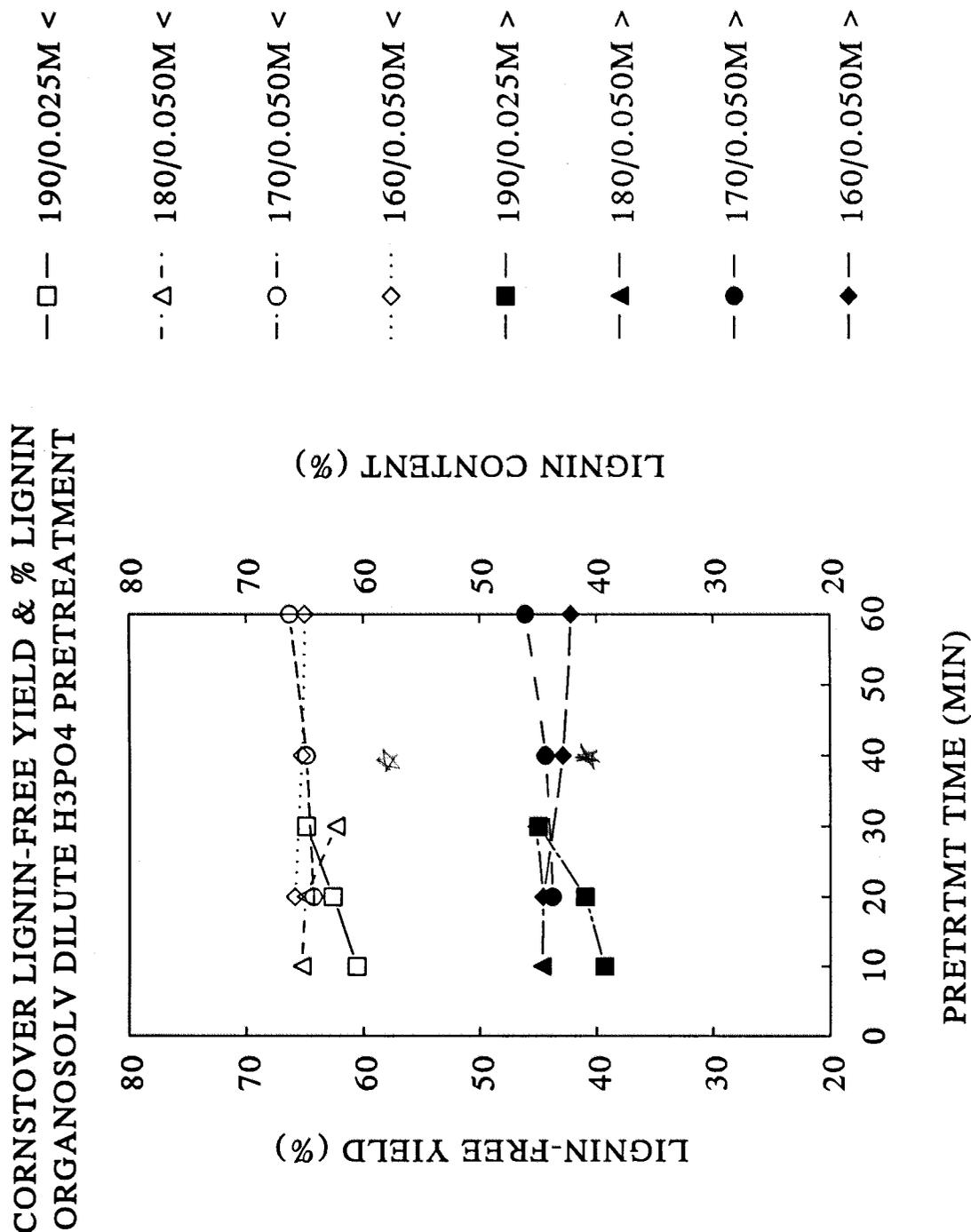
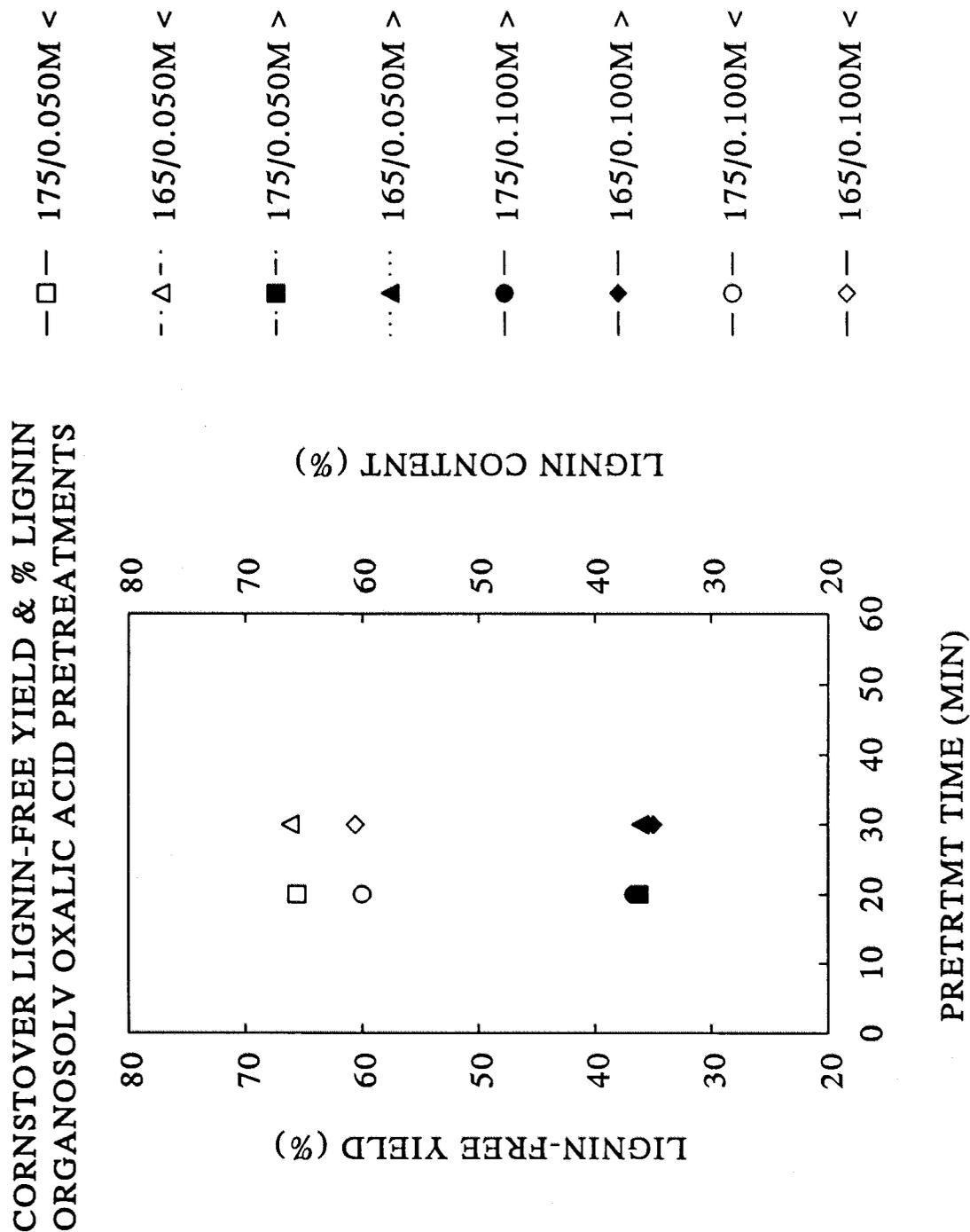


Figure C12. Organosolv pretreatments of cornstover using dilute oxalic acid: Relationships of pretreatment conditions of lignin-free yields and lignin contents.



D. *Pichia stipitus* Fermentations

In this section are presented the results from fermentations using *Pichia stipitus* of the pretreatment liquor samples from two organosolv pretreatments using 70 percent ethanol are compared with those using 70 percent methanol and aqueous pretreatments.

The pretreatment of cornstover and switchgrass were each conducted at 190°C for 40 minutes using 0.010 M phosphoric acid. The more dilute acid concentration was selected because of the effectiveness of the methanol organosolv pretreatments conducted at 190°C using 0.025 and 0.05 M phosphoric acid. Lignin free yields approached 70 percent were using switchgrass under these two conditions; using 0.01 M phosphoric acid in ethanol with switchgrass reduced the lignin-free yield to 45 percent. Lignin-free yields of 55 to 60 percent were obtained using cornstover under the severe conditions (methanol organosolv pretreatments conducted at 190°C using 0.025 and 0.05 M phosphoric acid); the same was obtained using the more mild and economical 190°C for 40 minutes in 0.010 M phosphoric acid and 70 percent ethanol.

The ethanol yields from the cornstover pretreated in ethanol organosolv at 190°C for 40 min (Table DI) were less than those from the methanol pretreatments (Table DII). Fermentations of 80 percent amendments produced lower yields and productivities than did 40 percent amendments. Switchgrass pretreatment liquors did not ferment as well as did those produced under identical conditions from cornstover (Table DIII).

As reported in the 12th Monthly Technical Progress Report for switchgrass, the *P. stipitus* fermentations of organosolv pretreatment liquors of the cornstover also gave unreliable results because of the unknown effect of residual methanol on the yeast. Without a doing a study of methanol toxicity of *P. stipitus*, the validity of any results from fermentation of methanol-containing organosolv liquors is in question. It is recommended that no further fermentations of methanol-containing organosolv liquors be conducted until the toxicity to the fermenting microorganism is determined. The *P. stipitus* fermentations of aqueous pretreatment liquors of the cornstover

Table DI. Fermentation yields and volumetric productivities of pretreatment liquors from **cornstover** and **switchgrass** ethanol organosolv phosphoric acid under various conditions.

Pretreatment conditions	Yield	Productivity	Cell Mass	Yield	Productivity	Cell Mass
	$\frac{\text{g etoh}}{\text{g sugar}}$	$\frac{\text{g etoh}}{\text{liter hr}}$	$\frac{\text{g cell dw}}{\text{liter}}$	$\frac{\text{g etoh}}{\text{g sugar}}$	$\frac{\text{g etoh}}{\text{liter hr}}$	$\frac{\text{g cell dw}}{\text{liter}}$
	40 percent amendment			80 percent amendment		
0.01 M H ₃ PO ₄ 70% ethanol 40 min 190°C switchgrass	0.229	0.088	9.60	0.104	.054	15.71
0.01 M H ₃ PO ₄ 70% ethanol 40 min 190°C cornstover	0.339	0.121	6.44	0.145	.052	12.53
3 percent xylose (no amendment)	0.296	0.111	11.30			
3 percent xylose (no amendment)	0.354	0.132	9.72			

Table DII. Fermentation yields and volumetric productivities of pretreatment liquors from **cornstover** using aqueous and organosolv phosphoric acid under various conditions.

Pretreatment Conditions	Yield	Productivity	Cell Mass	Yield	Productivity	Cell Mass
	<u>g etoh</u> g sugar	<u>g etoh</u> liter hr	<u>g cell dw</u> liter	<u>g etoh</u> g sugar	<u>g etoh</u> liter hr	<u>g cell dw</u> liter
	40 percent amendment			80 percent amendment		
0.05 M H ₃ PO ₄ 70% methanol 20 min 160°C	0.22	0.16	4.91	0.00	0.00	1.66
0.05 M H ₃ PO ₄ 70% methanol 40 min 160°C	0.33	0.23	6.00	0.01	0.00	3.00
3 percent xylose (no amendment)	0.32	0.21	6.47			
0.05 M H ₃ PO ₄ 40 min 160°C	0.51	0.36	nd	0.44	0.38	nd
0.05 M H ₃ PO ₄ 60 min 160°C	0.47	0.35	nd	0.23	0.21	nd
3 percent xylose (no amendment)	0.47	0.33	nd			

nd = not determined

Table DIII. Fermentation yields and volumetric productivities of pretreatment liquors from **switchgrass** using aqueous phosphoric acid under various conditions.

Pretreatment Conditions	Yield	Productivity	Cell growth	Yield	Productivity	Cell growth
	$\frac{\text{g etoh}}{\text{g sugar}}$	$\frac{\text{g etoh}}{\text{liter hr}}$	$\frac{\text{g dw}}{\text{liter}}$	$\frac{\text{g etoh}}{\text{g sugar}}$	$\frac{\text{g etoh}}{\text{liter hr}}$	$\frac{\text{g dw}}{\text{liter}}$
	40 percent amendment			80 percent amendment		
0.05 M H ₃ PO ₄ 60 min 170°C	0.32	0.28	4.67 ±0.16	0.30	0.13	5.02 ±0.21
0.05 M H ₃ PO ₄ 40 min 170°C	0.34	0.29	4.93 ±0.19	---	---	7.36 ±2.83
0.05 M H ₃ PO ₄ 20 min 170°C	0.37	0.32	5.34 ±0.11	0.34	0.30	6.17 ±0.40
0.05 M H ₃ PO ₄ 30 min 180°C	0.32	0.28	4.12 ±0.04	0.30	0.13	4.40 ±0.20
0.05 M H ₃ PO ₄ 20 min 180°C	0.33	0.29	5.11 ±0.47	0.30	0.13	4.97 ±0.20
0.05 M H ₃ PO ₄ 10 min 180°C	0.35	0.29	5.12 ±0.01	0.35	0.32	6.35 ±0.49
0.025M H ₃ PO ₄ 30 min 190°C	0.34	0.14	4.85 ±0.57	0.02	0.02	0.80 ±0.17
0.025M H ₃ PO ₄ 20 min 190°C	0.31	0.28	4.81 ±0.31	0.09	0.04	2.34 ±1.48
0.025M H ₃ PO ₄ 10 min 190°C	0.33	0.28	5.56 ±0.13	0.34	0.29	6.50 ±0.05
3 percent xylose (no amendment)	0.70	1.94	4.51 ±0.98			

E. Simultaneous Saccharification and Fermentations

E.1. Switchgrass

The SSF analysis was conducted on two of the switchgrass pretreated solids which had yielded the greatest yields of glucose from enzyme hydrolysis. Another factor used in selection of pretreated materials for the limited number of SSF's conducted during this study was the Klason-free lignin yields. These results, graphed as a function of pretreatment time in Figures B8 and B9, are taken from the Tables BI, BII and BIII.

The two switchgrass pretreated solids selected for SSF were:

0.100 M organosolv oxalic acid pretreatment at 165°C for 30 minutes, and

0.025 M aqueous phosphoric acid pretreatment at 190°C for 30 minutes.

The two respective samples had yielded 3.6 and 3.3 g/L glucose in the 24 hour enzyme hydrolysis experiment.

The data for the two SSF fermentations are averaged and plotted in Figure E1. The data for a control without substrate and a control with alpha-cellulose are also included. The usual increase in cellobiose is seen during the first 24 hours of the fermentation in the case of the oxalic acid pretreated switchgrass. However, the increase in cellobiose is not transitory in the case of the fermentation of the aqueous phosphoric acid pretreatment. Cellobiose accumulation was prolonged until 48 hours (Figure E1a) and in fact the glucose accumulated through the 72 hour sample (Figure E1b). The only conclusion from this anomaly is that the yeast fermentation was slow to start in these duplicated samples. There was no noticeable difference in the yeast cell density, morphology or contamination at the end of the fermentation when compared to others run at the same time. The ethanol content data (Figure E1d) and theoretical conversion data (Figure E1e) indicate a biphasic fermentation. Perhaps this pretreated material had a toxic component that inhibited the initial fermentation noted in other SSFs conducted at the same time. As stated above (section B), these aqueous pretreated sample were excessively wet and were refrigerated for several months before the discovery that they had not be analyzed.

The ultimate yield of ethanol from the oxalic acid pretreated switchgrass (82 percent of theoretical) was not as great as that from the SSF of alpha-cellulose (Figure E1e).

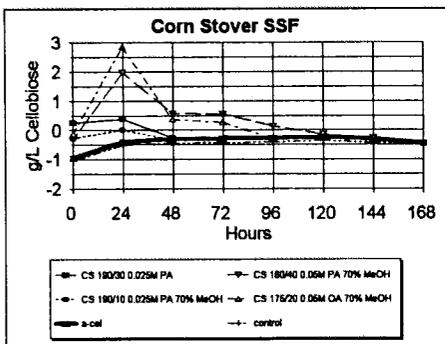


Figure E1a. Change in the content of cellobiose in SSFs of pretreated switchgrass materials.

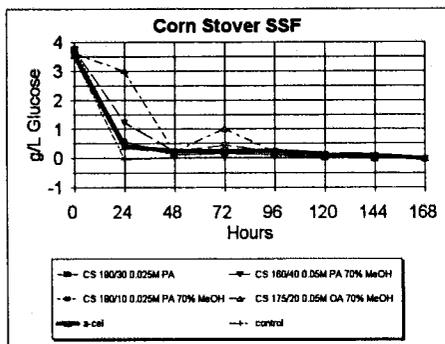


Figure E1b. Change in the content of glucose in SSFs of pretreated switchgrass materials.

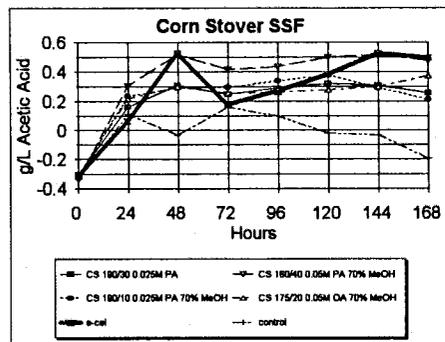


Figure E1c. Change in the content of acetic acid in SSFs of pretreated switchgrass materials.

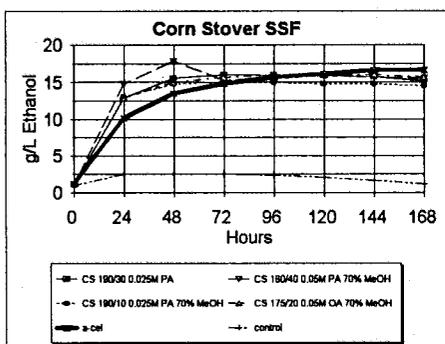


Figure E1d. Change in the content of ethanol in SSFs of pretreated switchgrass materials.

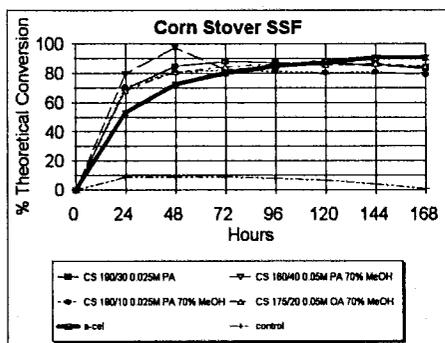


Figure E1e. Change in the theoretical conversion of cellulose to ethanol in SSFs of pretreated switchgrass materials.

E.2. Cornstover

The SSF analysis was conducted on four of the cornstover pretreated solids which had yielded the greatest yields of glucose from enzyme hydrolysis. Another factor used in selection of pretreated materials for the limited number of SSF's contracted during this study was the Klason-free lignin yields. All of the oxalic acid pretreatments and the organosolv phosphoric acid pretreatments conducted for 10 and 20 minutes gave Klason-lignin free yields greater than 59 percent, and were grouped separately from all other data. Similarly, the lignin content plots vs treatment time for these same samples were less than all other pretreated cornstover samples.

The four cornstover pretreated solids selected for SSF were:

- 0.025 M aqueous phosphoric acid pretreatment at 190°C for 30 minutes,
- 0.050 M organosolv phosphoric acid pretreatment at 160°C for 40 minutes,
- 0.025 M organosolv phosphoric acid pretreatment at 190°C for 10 minutes, and
- 0.050 M organosolv oxalic acid pretreatment at 175°C for 20 minutes.

The four respective samples had yielded 3.2, 3.7, 3.4, and 3.7 g/L glucose in the 24 hour enzyme hydrolysis experiment.

The kinetics of the SSFs of these four pretreated cornstover samples was as anticipated. The ultimate yields of ethanol from the pretreated switchgrass samples were between 80 and 90 percent of theoretical, which were not as great as that from the SSF of alpha-cellulose (Figure E2e).

E.3. Ethanol Organosolv Pretreated Switchgrass and Cornstover

Although not a part of the subcontract are reported data from an SSF experiment using the two organosolv pretreatments using 70 percent ethanol. Because these pretreatments using 0.01 M phosphoric acid were apparently effective in terms of the lignin removal (cf. Section B), the SSFs were conducted to compare with the data presented above. In Figure E3 are presented the percent theoretical ethanol yield data from alpha-cellulose, cornstover and switchgrass samples from the first four samples. The HPLC ceased operation at this point, but the data obtained were sufficient to show very rapid fermentation and bioconversion yields of 98 percent from switchgrass and 80 percent from cornstover, which were greater than that obtained using alpha-cellulose.

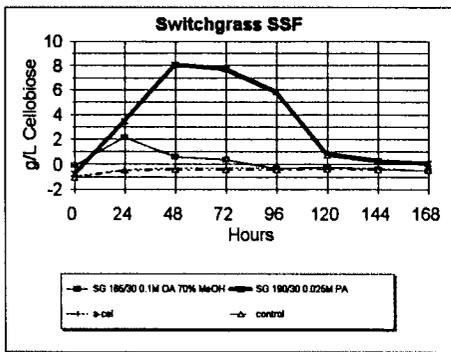


Figure E2a. Change in the content of cellobiose in SSFs of pretreated cornstover materials.

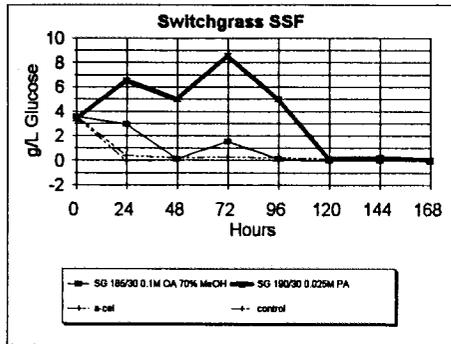


Figure E2b. Change in the content of glucose in SSFs of pretreated cornstover materials.

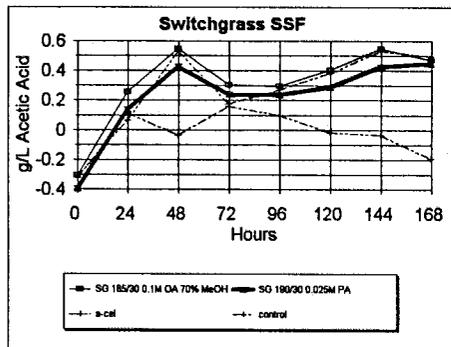


Figure E2c. Change in the content of acetic acid in SSFs of pretreated cornstover materials.

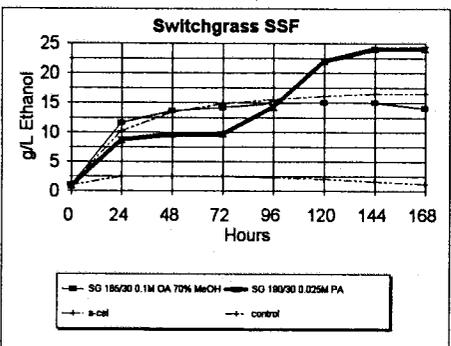


Figure E2d. Change in the content of ethanol in SSFs of pretreated cornstover materials.

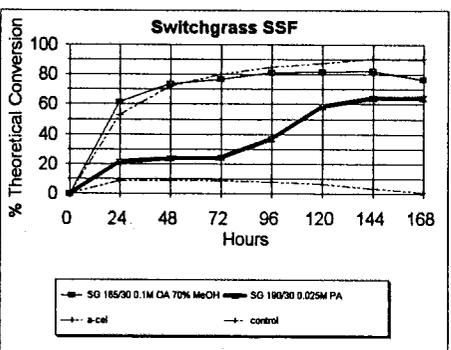
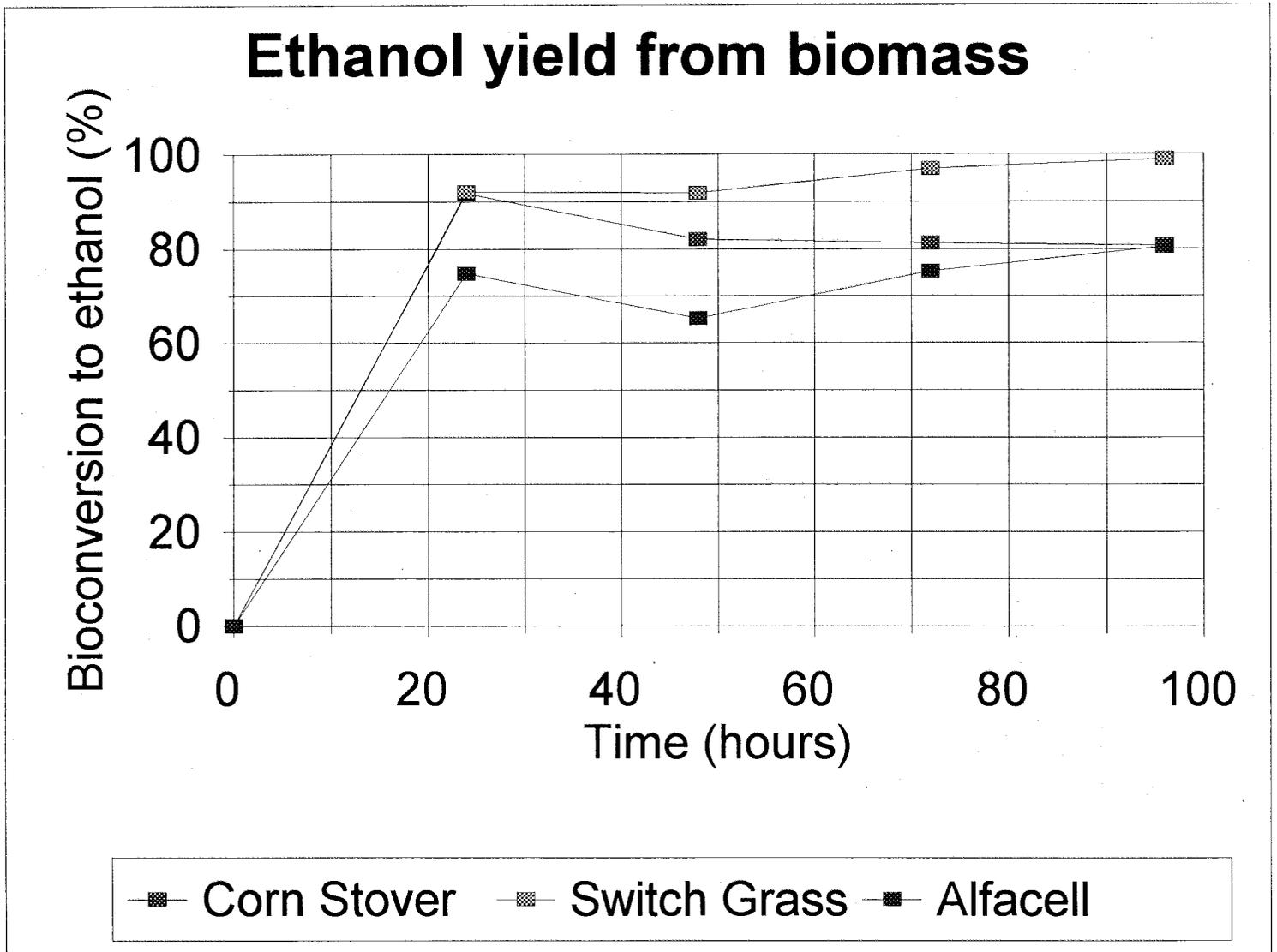


Figure E2e. Change in the theoretical conversion of cellulose to ethanol in SSFs of pretreated cornstover materials.

Figure E3. Results of SSF of 0.01M phosphoric acid pretreated cornstover and switchgrass.



F. Conclusions and Analysis

The cost of producing ethanol from biomass can be divided into three areas of expenditure: pretreatment costs, fermentation costs, and other costs. Pretreatment costs include the cost of milling the biomass, reagents for the pretreatment, maintenance of the equipment, power and water, and cost of neutralization and disposal of waste. The fermentation costs include enzymes, nutrient supplements, yeast maintenance and scale-up, and waste disposal. Other costs include purchase and transport of the biomass, storage, labor, plant utilities, distillation of the ethanol, and administration. The following cost evaluation on ethanol production is based on the cost to treat one dry ton (U.S.) of biomass containing 50% cellulose and 20% xylose (w/w). All purchase prices for chemicals were taken from recent issues of the Weekly Chemical Reporter unless otherwise stated.

Pretreatment

We have studied two main pretreatment methods, sulfuric acid (0.73% w/w) and phosphoric acid (0.05 and 0.025 M). The pretreatments were done using a 10% solids loading. Bulk sulfuric acid (96%) is \$86.20/ton. Bulk phosphoric acid (85%) is \$680.00/ton.

H₂SO₄ cost:

$$\frac{0.073 \text{ tons } 100 \% \text{H}_2\text{SO}_4}{10 \text{ tons } \text{Pretx}} \times \frac{100 \text{ tons } 96 \% \text{H}_2\text{SO}_4}{96 \text{ tons } 100 \% \text{H}_2\text{SO}_4} \times \frac{10 \text{ tons } \text{Pretx}}{1 \text{ ton } \text{biomass}} \times \frac{\$86.20}{\text{ton } 96 \% \text{H}_2\text{SO}_4} = \frac{\$6.55}{\text{ton } \text{biomass}}$$



H₂SO₄ neutralization cost:

$$\frac{0.073 \text{ ton } 100 \% \text{H}_2\text{SO}_4}{\text{ton } \text{biomass}} \times \frac{907185 \text{ g } \text{H}_2\text{SO}_4}{\text{ton } \text{H}_2\text{SO}_4} \times \frac{1 \text{ mole } \text{H}_2\text{SO}_4}{98 \text{ g } \text{H}_2\text{SO}_4} \times \frac{1 \text{ mole } \text{CaCO}_3}{1 \text{ mole } \text{H}_2\text{SO}_4} \times \frac{100 \text{ g } \text{CaCO}_3}{\text{mole } \text{CaCO}_3} \times \frac{1 \text{ ton } \text{CaCO}_3}{907185 \text{ g } \text{CaCO}_3} \times \frac{\$70.00}{\text{ton } \text{CaCO}_3} = \frac{\$5.21}{\text{ton } \text{biomass}}$$

CaSO₄ disposal cost:

Disposal costs are based on \$20.00/cubic yard quoted by Larimer County Landfill. It is assumed that one cubic yard of wet CaSO₄ weighs approximately one ton. This does not include the cost of hauling the material to the landfill, but this cost may be diminished by pumping to a local facility.

$$\frac{0.073 \text{ ton } 100 \% \text{H}_2\text{SO}_4}{\text{ton } \text{biomass}} \times \frac{907185 \text{ g } \text{H}_2\text{SO}_4}{\text{ton } \text{H}_2\text{SO}_4} \times \frac{1 \text{ mole } \text{H}_2\text{SO}_4}{98 \text{ g } \text{H}_2\text{SO}_4} \times \frac{1 \text{ mole } \text{CaSO}_4}{1 \text{ mole } \text{H}_2\text{SO}_4} \times \frac{136 \text{ g } \text{CaSO}_4}{\text{mole } \text{CaSO}_4} \times \frac{1 \text{ ton } \text{CaSO}_4}{907185 \text{ g } \text{CaSO}_4} \times \frac{\$20.00}{\text{ton } \text{CaSO}_4} = \frac{\$2.03}{\text{ton } \text{biomass}}$$

H₃PO₄ cost:

$$\frac{0.05 \text{ Mole } H_3PO_4}{1 \ell} \times \frac{98 \text{ g}}{\text{Mole}} = \frac{4.9 \text{ g}}{\ell} = 0.49 \% H_3PO_4$$

$$\frac{0.049 \text{ tons } 100 \% H_3PO_4}{10 \text{ tons } \text{Pretx}} \times \frac{100 \text{ tons } 85 \% H_3PO_4}{85 \text{ tons } 100 \% H_3PO_4} \times \frac{10 \text{ tons } \text{Pretx}}{1 \text{ ton } \text{biomass}} \times \frac{\$680.00}{\text{ton } 85 \% H_3PO_4} = \frac{\$39.20}{\text{ton } \text{biomass}}$$

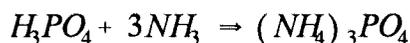
$$\frac{0.025 \text{ Moles } H_3PO_4}{1 \ell} \times \frac{98 \text{ g}}{\text{Mole}} = \frac{2.45 \text{ g}}{\ell} = 0.245 \% H_3PO_4$$

$$\frac{0.0245 \text{ tons } 100 \% H_3PO_4}{10 \text{ tons } \text{Pretx}} \times \frac{100 \text{ tons } 85 \% H_3PO_4}{85 \text{ tons } 100 \% H_3PO_4} \times \frac{10 \text{ tons } \text{Pretx}}{1 \text{ ton } \text{biomass}} \times \frac{\$680.00}{\text{ton } 85 \% H_3PO_4} = \frac{\$19.60}{\text{ton } \text{biomass}}$$

$$\frac{0.01 \text{ Mole } H_3PO_4}{1 \ell} \times \frac{98 \text{ g}}{\text{Mole}} = \frac{0.98 \text{ g}}{\ell} = 0.01 \% H_3PO_4$$

$$\frac{0.01 \text{ tons } 100 \% H_3PO_4}{10 \text{ tons } \text{Pretx}} \times \frac{100 \text{ tons } 85 \% H_3PO_4}{85 \text{ tons } 100 \% H_3PO_4} \times \frac{10 \text{ tons } \text{Pretx}}{1 \text{ ton } \text{biomass}} \times \frac{\$680.00}{\text{ton } 85 \% H_3PO_4} = \frac{\$8.00}{\text{ton } \text{biomass}}$$

H₃PO₄ neutralization cost:



$$\frac{0.049 \text{ tons } H_3PO_4}{10 \text{ tons } \text{Pretx}} \times \frac{10 \text{ tons } \text{Pretx}}{1 \text{ ton } \text{biomass}} \times \frac{907185 \text{ g } H_3PO_4}{\text{ton } H_3PO_4} \times \frac{1 \text{ mole } H_3PO_4}{98 \text{ g } H_3PO_4} \times$$

$$\frac{3 \text{ moles } NH_3}{1 \text{ mole } H_3PO_4} \times \frac{17 \text{ g } NH_3}{1 \text{ mole } NH_3} \times \frac{1 \text{ ton } NH_3}{907185 \text{ g } NH_3} \times \frac{\$200.00}{\text{ton } NH_3} = \frac{\$5.10}{\text{ton } \text{biomass}} \text{ for } 0.05 \text{ M } H_3PO_4$$

$$= \frac{\$2.55}{\text{ton } \text{biomass}} \text{ for } 0.025 \text{ M } H_3PO_4$$

$$= \frac{\$1.02}{\text{ton } \text{biomass}} \text{ for } 0.01 \text{ M } H_3PO_4$$

In addition to the pretreatment costs, fermentation costs must also be considered. These costs include enzymes, medium components and supplements, inoculum scale-up and maintenance, and waste disposal. Only those elements of the system that are impacted by the alternate pretreatment are considered.

Enzyme Cost: (Based on bulk lot quotations from Novo)

$$\frac{1000 \text{ lbs cellulose}}{1 \text{ ton BM}} \times \frac{454 \text{ g cellulose}}{1 \text{ lb cellulose}} \times \frac{25 \text{ IU enzyme}}{\text{g cellulose}} \times \frac{1 \text{ l enzyme}}{80,000 \text{ IU}} \times \frac{\$12.00}{\text{l enzyme}} = \frac{\$1702.50}{\text{ton BM}} @ \frac{25 \text{ IU}}{\text{g cellulose}}$$

$$\frac{1000 \text{ lbs cellulose}}{1 \text{ ton BM}} \times \frac{454 \text{ g cellulose}}{1 \text{ lb cellulose}} \times \frac{5 \text{ IU enzyme}}{\text{g cellulose}} \times \frac{1 \text{ l enzyme}}{80,000 \text{ IU}} \times \frac{\$12.00}{\text{l enzyme}} = \frac{\$340.50}{\text{ton BM}} @ \frac{5 \text{ IU}}{\text{g cellulose}}$$

***In vivo* Enzyme Cost: (Based on composting costs)**

Dr. Robert Tengerdy is currently producing 5 IU cellulase/g DW on spent biomass using solid state production by *Gliocladium spp.*

$$\frac{1000 \text{ lbs cell}}{\text{ton BM}} \times \frac{454 \text{ g cell}}{1 \text{ lb cell}} \times \frac{25 \text{ IU cellulase}}{\text{g cell}} \times \frac{1 \text{ kg DWBM}}{5000 \text{ IU cellulase}} \times \frac{1 \text{ MTDWBM}}{1000 \text{ kg DWBM}} \times \frac{\$24.00}{\text{MTDWBM}} = \frac{\$54.48}{\text{ton BM}} @ \frac{25 \text{ IU}}{\text{g cellulase}}$$

$$\frac{\text{cell}}{\text{cell}} \times \frac{5 \text{ IU cellulase}}{\text{g cell}} \times \frac{1 \text{ kg DWBM}}{5000 \text{ IU cellulase}} \times \frac{1}{10} = \frac{\$10.90}{\text{ton BM}} @ \frac{5 \text{ IU}}{\text{g cellulase}}$$

(NH₄)₂HPO₄ Addition (cellulose stream):

Media used for yeast grown in batch culture typically contains 3.0 g/L (NH₄)₂SO₄, or 0.82 g NH₄⁺/L. Phosphate is typically supplied as KH₂PO₄ at 3.0 g/L, or 2.1 g PO₄⁻³/L (1). If (NH₄)₂HPO₄ is used to supply these nutrients, the following is obtained:

$$\frac{10 \text{ tons Pretx}}{1 \text{ ton BM}} \times \frac{907185 \text{ g Pretx}}{1 \text{ ton Pretx}} \times \frac{1 \text{ L Pretx}}{1000 \text{ g Pretx}} \times \frac{3 \text{ g (NH}_4\text{)}_2\text{SO}_4}{\text{L Pretx}} \times \frac{0.27 \text{ g NH}_4^+}{\text{g (NH}_4\text{)}_2\text{SO}_4} = \frac{7348 \text{ g NH}_4^+}{\text{ton BM}}$$

$$\frac{7348 \text{ g NH}_4^+}{1 \text{ ton BM}} \times \frac{1.0 \text{ g (NH}_4\text{)}_2\text{HPO}_4}{0.27 \text{ g NH}_4^+} \times \frac{1 \text{ ton (NH}_4\text{)}_2\text{HPO}_4}{907185 \text{ g (NH}_4\text{)}_2\text{HPO}_4} \times \frac{\$148.00}{\text{ton (NH}_4\text{)}_2\text{HPO}_4} = \frac{\$4.44}{\text{ton BM}} \text{ for NH}_4^+ \text{ requirements}$$

$$\frac{10 \text{ tons Pretx}}{1 \text{ ton BM}} \times \frac{907185 \text{ g Pretx}}{1 \text{ ton Pretx}} \times \frac{1 \text{ L Pretx}}{1000 \text{ g Pretx}} \times \frac{3 \text{ g KH}_2\text{PO}_4}{\text{L Pretx}} \times \frac{0.70 \text{ g PO}_4^{-3}}{\text{g KH}_2\text{PO}_4} = \frac{19051 \text{ g PO}_4^{-3}}{\text{ton BM}}$$

$$\frac{19051 \text{ g PO}_4^{-3}}{1 \text{ ton BM}} \times \frac{1.0 \text{ g (NH}_4\text{)}_2\text{HPO}_4}{0.72 \text{ g PO}_4^{-3}} \times \frac{1 \text{ ton (NH}_4\text{)}_2\text{HPO}_4}{907185 \text{ g (NH}_4\text{)}_2\text{HPO}_4} \times \frac{\$148.00}{\text{ton (NH}_4\text{)}_2\text{HPO}_4} = \frac{\$4.32}{\text{ton BM}} \text{ for PO}_4^{-3} \text{ requirements}$$

(NH₄)₂HPO₄ Addition (xylose stream):

The calculations below assume the same nutrient requirements for *Pichia stipitis* with NH₄⁺ being limiting (see above), and a xylose concentration of 30 g/L in the fermentation.

H₂SO₄ pretreatment-

$$\frac{400 \text{ lbs xylan}}{1 \text{ ton BM}} \times \frac{1.14 \text{ lbs xylose}}{1 \text{ lb xylan}} \times \frac{45.4 \text{ g xylose}}{1 \text{ lb xylose}} \times \frac{1.0 \text{ L broth}}{30.0 \text{ g xylose}} \times \frac{3.0 \text{ g } (NH_4)_2SO_4}{1 \text{ L broth}} \times \frac{0.27 \text{ g } NH_4^+}{1 \text{ g } (NH_4)_2SO_4} \times \frac{1.0 \text{ g } (NH_4)_2HPQ}{0.27 \text{ g } NH_4^+} \times \frac{1 \text{ ton } (NH_4)_2HPQ}{907185 \text{ g } (NH_4)_2HPQ} \times \frac{\$148.00}{\text{ton } (NH_4)_2HPQ} = \frac{\$3.38}{\text{ton BM}}$$

H₃PO₄ pretreatment-

Neutralizing H₃PO₄ with ammonia results in enough ammonium phosphate to meet the yeast's requirements.

$$\frac{0.049 \text{ tons } H_3PO_4}{10 \text{ tons Pretx}} \times \frac{10 \text{ tons Pretx}}{1 \text{ ton biomass}} \times \frac{907185 \text{ g } H_3PO_4}{\text{ton } H_3PO_4} \times \frac{1 \text{ mole } H_3PO_4}{98 \text{ g } H_3PO_4} \times \frac{3 \text{ moles } NH_3}{1 \text{ mole } H_3PO_4} \times \frac{17 \text{ g } NH_3}{\text{mole } NH_3} = \frac{23133 \text{ g } NH_3}{\text{ton biomass}} \text{ for } 0.05 \text{ M } H_3PO_4$$

$$= \frac{11803 \text{ g } NH_3}{\text{ton biomass}} \text{ for } 0.025 \text{ M } H_3PO_4$$

$$= \frac{4721 \text{ g } NH_3}{\text{ton biomass}} \text{ for } 0.01 \text{ M } H_3PO_4$$

SUMMARY

Cost of pretreatment of one ton dry biomass not including enzyme costs:

		0.73% H ₂ SO ₄	0.05 M H ₃ PO ₄	0.025 M H ₃ PO ₄	0.01 M H ₃ PO ₄
Pretreatment	Acid cost	\$6.55	\$39.20	\$19.60	\$7.84
	Neutralization	5.21	5.10	2.55	1.02
	Disposal	2.03	0.00	0.00	0.00
<i>subtotal</i>		<i>\$13.79</i>	<i>\$44.30</i>	<i>\$22.15</i>	<i>\$8.86</i>
Cellulose Ferm.	(NH ₄) ₂ HPO ₄	\$3.62	\$3.62	\$3.62	\$3.62
Xylose Ferm.	(NH ₄) ₂ HPO ₄	3.62	0.00	0.00	0.00
<i>subtotal</i>		<i>\$7.24</i>	<i>\$3.62</i>	<i>\$3.62</i>	<i>\$3.62</i>
Total		\$21.03	\$47.92	\$25.77	\$12.48

References

1. D. R. Berry. 1989. Growth of yeast. *In* Fermentation process development of industrial organisms. Ed. J. O. Neway. Marcel Dekker, Inc. New York.