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Process Development Studies on The Bioconversion of Cellulose and Production of Ethanol

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PROCESS DEVELOPMENT STUDIES ON THE BIOCONVERSION OF CELLULOSE AND PRODUCTION OF ETHANOL

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Charles R. Wilke, Principal Investigator

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I. RAW MATERIALS AND PROCESS EVALUATION

A. Analysis of Purdue University Corn Stover

Approximately 10 pounds of corn stover, 1977 crop, grown in Tippecanoe County, Indiana, was Wiley milled to 2 mm. The composition was determined and the results are shown in Table 1. In Table 2 are the results of presumably similar material (Ref. 2, Table 1, Page 3). In that table, the pentosans and polyuronides are listed together so that a meaningful comparison cannot be made. With the other components, the differences are greater than one would expect from cumulative analytical error, so that further exchange of information concerning the analytical methods employed seems desirable.

B. Analysis and Enzymatic Hydrolysis of Original Materials

The initial studies on the trees, Populus tristis, sycamore, and sweetgum have been completed. The composition of the whole trees (without leaves) are shown in Table 3. The trees as received contained about 35% moisture. They were chipped to approximately one-half inch pieces, air dried to about 10% moisture and then Wiley milled to 2 mm particles. Samples of the 2 mm milled trees were subjected to enzymatic hydrolysis as performed previously for agricultural residues (LBL-6859, Jan. 1977), and the results are shown in Table 4. As can be seen, the wood residues are not readily hydrolyzed by cellulase enzyme. Additionally, wood samples with their original moisture content (35w%) were also subjected to enzymatic hydrolysis with essentially the same results as observed with the air dried samples. Undried wood does not appear to be more susceptible to hydrolysis than the dried material.

(a) Dilute Acid Pretreatment

Pretreatment of the wood residues with 0.9w% sulfuric acid at 100°C for 5 1/2 hours improved the yields of sugar produced by enzymatic hydrolysis by about a factor of two, but the yields are still too low for consideration as an

Table 1

COMPOSITION OF INDIANA CORN STOVER

(In w%, Based on Dry Weight of 0.25 to 0.35 mm Fraction)

GLUCAN	33.2
XYLAN	19.3
ARABINAN	3.7
OTHER CARBOHYDRATE	2.4
LIGNIN	13.7
PROTEIN	4.2
ASH	5.3
AZEOTROPIC BENZENE ALCOHOL EXTRACTIVE	4.7
ACID INSOLUBLES (ORGANIC)	1.2

Table 2

TYPICAL COMPOSITION OF CORN RESIDUE FROM THE PURDUE LABORATORY OF RENEWABLE RESOURCES ENGINEERING PROGRESS REPORT OF FEB. 19, 1978, PAGE 3, TABLE 1.

% COMPONENT	CORN RESIDUE
ASH	7
LIGNIN	10.5
PENTOSANS POLYURONIDES XYLANS, ARABANS	34.8
α-CELLULOSE	37.7
TOTAL	97

Table 3

COMPOSITION OF WHOLE TREE BIOMASS

(in w%, based on dry wt. of .250 to .350 mm fraction)

	GLUCAN	XYLAN	ARABINAN	MANNAN	OTHER CARBO-HYDRATE	LIGNIN	ACID SOLUBLE LIGNIN	ASH	AZEOTROPIC BZ/EtoH EXT.	ACID INSOLUBLES (ORGANIC)
POPULUS TRISTIS #1	36	11	2	3.0	4	20	10	1	6	2
SYCAMORE	45.1	13.4	1	1	-	21.5	-	.9	3.6	.3
SWEETGUM	42	17.2	1.5	2.8	-	19.1	4	1.2	2.4	3.1

Table 4

ENZYME HYDROLYSIS OF ORIGINAL BIOMASS

BASIS: 100 lbs of Original Wiley Milled Material

	GLUCOSE	POLYMERIC GLUCOSE	XYLOSE	ARABINOSE	% CARBOHYDRATE CONVERSION
POPULUS TRISTIS	5.3	-	0.43	-	9.1
SYCAMORE	3.7	-	1.3	-	7.4
SWEET GUM	2.3	0.6	0.7	0.7	6.0

economic process. The yields of sugar in the extraction liquors and the enzymatic hydrolyzates are shown in Tables 5 and 6, respectively.

(b) Ferric Sulfate Pretreatment

One of the methods tried to improve the yields of sugars with enzymatic hydrolysis was based on the work reported by J.E. Marion and A. Wissing (1). They observed that the "hemicellulose fraction" of wood used for railraod ties was considerably depleted in the vicinity of the rusting nails and spikes. Marion and Wissing clearly point out that no delignification by oxidation or solubilization of lignin was observed in their studies, only a decrease in pentosan content. Though this natural action requires many months, it was suggested by Dr. Marion that this treatment with ferric ions might enhance enzymatic hydrolysis.

The ratio of iron oxide to wood was estimated from their work. A 11.6w% suspension of 2 mm Wiley milled Populus tristis (100 grams) was boiled and stirred in a solution of 1.2 w% (0.03M) ferric sulfate (pH = 2.1), made by dissolving 16.3 grams of (73.2 w% assayed) ferric sulfate per liter of solution. The sugar production in the liquor was tracked via H.P.L.C., sampled every 30 minutes, and appeared to increase in approximately two hour steps. No further change occurred after six hours of boiling. On the basis of 100 lbs of Populus, the ferric sulfate liquor contained 1 pound of mannose, 0.6 lbs of glucose and the washed residue was 87.6 lbs. The enzymatic yield of sugar based on 87.6 lbs of this treated wood, in a 5w% suspension in cellulase, was 4 pounds of glucose and 1.3 pounds of xylose, which is not a material improvement versus the untreated Populus. It is interesting to note that none of the pentosan as pentose was extracted into the ferric sulfate liquor, contrary to the results of Marion and Wissing. Boiling 2 mm Wiley milled Populus for 5 1/2 hours in a dilute sulfuric acid solution (pH = 2.1) released about 1.6 lbs of xylose per 100 lbs of wood. It was concluded, therefore, that pretreatment with ferric

Table 5

ACID EXTRACTION OF WOOD

BASIS: 100 Lb of Original Material

	GLUCOSE	XYLOSE	ARABINOSE	LBS OF RESIDUE FOR ENZYMATIC HYDROLYSIS
POPULUS TRISTIS #1	1.2	3.4	0.44	80.2
SYCAMORE	1.6	4.2	0.3	79.6
SWEET GUM	0.12	5.1	0.31	78.9

Table 6

ENZYME HYDROLYSIS OF ACID TREATED WOOD

BASIS: 100 Lb. of Original Wood

	GLUCOSE	XYLOSE	LBS. OF RESIDUE	% CARBOHYDRATE CONVERSION TO SUGAR INCLUDING ACID LIQUOR
POPULUS TRISTIS #1	5.4	0.85	71.8	18.6
SYCAMORE	5.6	1.4	71.9	19.9
SWEET GUM	4.1	1.0	73.2	16.4

solutions does not offer significant benefit.

(c) Sulfur Trioxide Pretreatment

Since these wood residues appear to be quite resistant to enzymatic hydrolysis, it was thought that a pretreatment by gaseous sulfur trioxide at 50 mm pressure or less (only the monomer exists at this pressure at 20°C) might preferentially sulfonate the lignin and thereby open the cell structure for enzymatic hydrolysis. A simple apparatus was arranged to flow, by gravity, sulfur trioxide onto a 10 gram sample of 2 mm Wiley milled Populus evenly distributed over the bottom of a six liter conical flask. The stopper contained a valved inlet tube and a vacuum/pressure gage. The reaction flask was evacuated and filled to 400 mm of dry nitrogen. This pressure of nitrogen was found to be sufficient to dilute the amounts of sulfur trioxide portions used and thereby decrease the rate of reaction on the wood.

The sulfur trioxide was obtained by vacuum distillation at 40°C from 30% fuming sulfuric acid into a valved glass trap at -78°C. (Dry ice/trichloroethylene slush bath.) The SO_3 was then added to the reaction flask in about 0.15 gram portions, determined by the decrease in weight of the supply trap. This amount of sulfur trioxide was equivalent to about 10 mm pressure in the reaction flask. After about 3 hours, 2.27 grams (0.0283 mols) SO_3 was introduced and when visible existence of free sulfur trioxide disappeared, water was added to the reaction flask. The brown slurry was quantitatively transferred to a 500 ml screw cap conical flask and autoclaved at 121°C for 1 hour to effect secondary hydrolysis. Analysis of the liquor showed very little sugar present, although the cellulose was attacked considerably. Relative large amounts of glucuronic acid and another substance, possibly a sulfonated furoic acid, was formed. It is possible that these compounds arose from a dehydration of cellulose before hydration to the sugar and the consequent formation of sulfuric acid even though the wood did contain some moisture.

Approximately 25% of the lignin was solublized, presumably by sulfonation, and dissolved upon addition of water to a dark brown solution. Approximately 15% of the sulfur trioxide was consumed or lost. The low pressure hygroscopic reaction, with and without stirring, was difficult to maintain. If further work with this method were to be done more sophisticated equipment would be required.

(d) Nitric Oxide--Oxygen Pretreatment

This project is concerned with the contacting of wheat straw with gaseous NO and O_2 at room temperature followed by aqueous extraction of the treated solids after removal of unreacted gases. The solids maintained in an approximately 10% wt suspension with water at 100°C, resulting in extraction and acid hydrolysis of the hemicellulose fraction. Enzymatic hydrolysis is then employed for further conversion of the cellulosic fraction to glucose and other sugars.

The kinetics of formation and destruction of xylose during the extraction stage were investigated. The formation kinetics follow closely the equation $\frac{dX}{dt} = k_1(a-X)[H^+]$. Where: a is the initial amount of xylose present as xylan and X is the amount of xylose present at time, t, $[H^+]$ is the hydrogen ion concentration and k_1 is the reaction rate constant. The degradation kinetics of xylose were well represented by the equation: $\frac{-dC_x}{dt} = k_2 C_x [H^+]$, where C_x is the xylose concentration in solution and k_2 is the degradation rate constant.

Recycling the extraction liquor was found to enhance the rate of xylose formation. The reason for this is not known since the pH of the liquor remains essentially constant upon recycling. A possible explanation is that the effective acidity is greater than that measured by the pH electrode.

The effect of using pure oxygen versus air in the mixture with NO was

investigated. The results obtained were found to be nearly the same for the two cases.

A series of NO_x reactions on straw were conducted at 80°C rather than at room temperature. The overall sugar yield obtained after enzymatic hydrolysis was found to be very low. For this reason, experimentation at 80°C has been discontinued.

C. Sulfuric Acid Hydrolysis

(a) Hydrolysis with 72w% Sulfuric Acid

A series of preliminary experiments on Populus tristis were performed with 72w% sulfuric acid solution. The experiments were similar to the scheme employed for the analytical determination of cellulosic residues (LBL-5967). In this analytical scheme a large excess of sulfuric acid is used for the solvation step (10 ml of 24.1 $\underline{N}$ H_2SO_4 equivalent to 0.121 mols sulfuric acid per gram of sample). These experiments were performed to establish the minimum amount of sulfuric acid required for solvation and secondary hydrolysis of this wood.

1.071 gram samples (1 gram, 100% dry) of 2 mm Wiley milled Populus were packed into 17 mm diameter glass tubes. Increasing amounts of 24.1 $\underline{N}$ H_2SO_4 (72 w/w%) were added, so that a range of approximately 0.6 gram to 3.5 grams of 100% sulfuric acid per gram of wood was obtained. As observed in the analytical scheme, an indication of solvation of the wood at room temperature was that the mixture blackened and liquefied. In the analytical scheme, with a large excess of acid, solvation is complete in 15 to 20 minutes. Whereas, in the present experiments with minimal amounts of liquid present, the solvation process is considerably longer at 30°C. When using 2 mm particles, the solvation time is shortened by about 50%, placing the wood under vacuum before the acid is added. The acid is then sucked in and disperses more rapidly. The results of these experiments are shown in Table 7. Included are the duplicate solvation times

Table 7

SUMMARY
ACID HYDROLYSIS OF 2 MM POPULUS[a]

24.1N H_2SO_4 (CC)	100% H_2SO_4 (GM)	CONTACT TIME HRS.		SUGAR FOUND (GM)	GLUCOSE FOUND (GM)	GM H_2SO_4[c] PER GM		SUGAR CONVERSION (%)
		RUN	DUPLICATE (b)			SUGAR	GLUCOSE	
.5	0.590	24	>60	.095	.0128	6.21	46.1	15.1
1.0	1.181	24	>60	.215	.0707	5.49	16.7	34.1
1.5	1.772	24	∿48	.402	.242	4.40	7.32	63.8
1.75	2.066	24	∿30	.523	.324	3.95	6.37	83.0
2.0	2.362	12	12	.588	.374	4.01	6.31	93.3
2.25	2.657	12	12	.610	.395	4.36	6.73	96.8
2.5	2.952	4	4	.591	.384	4.99	7.69	93.8
2.75	3.247	1	.5	.592	.384	5.48	8.46	94.0
3.0	3.543	1	.5	.584	.380	6.06	9.32	92.7

(a) 1 gram samples (dry) of Populus tristis with 40% glucose (equivalent) and 63% total sugar (equivalent).
(b) Time when mix appeared to be totally black
(c) GM H_2SO_4 used per GM sugar or glucose produced

required for the complete formation of the black slurry, which in some cases were unreasonably long, and which did not produce significant improvement in sugars yields. The time of the completion of the secondary hydrolysis was variable at 125°C (autoclave) depending on the acid concentration obtained on dilution of the black slurry with water. Autoclaving was carried out for 40 to 60 minutes at acid concentrations of 0.8 to 1.4 N. The kinetics of the secondary acid hydrolysis are well known and will not be discussed here. As can be seen in Table 7 about 2.36 grams of 100% sulfuric acid per gram of Populus will produce, on secondary hydrolysis, about 0.59 gram of sugar (determined via H.P.L.C.) of which 0.37 gram is glucose. This is a 93% conversion of the carbohydrate to sugar and a similar conversion of the glucan to glucose. On the basis of a pound of total sugars or of glucose produced, the sulfuric acid requirements are 4 and 6.3 pounds, respectively. Because of the high acid requirement this method does not appear economically promising.

Additional experiments were conducted with more dilute sulfuric acid (but with constant mols of sulfuric acid per gram of wood and consequently more liquid for wetting the wood) to determine the minimum concentration of sulfuric acid for solvolysis and the expected longer contact time. The minimum concentration for solvolysis of Populus is 21.1 N sulfuric (64w/w%). With less concentration, regardless of contact time, the sulfuric acid acts only as a pentosan hydrolyzer. For example, 3 ml of 16.16N sulfuric acid (0.024 mol) was added to 1.071 gram (1 gm dry) of packed 2 mm Wiley milled Populus. This is more than enough liquid to wet the wood, whether it is evacuated or not. It required nearly 23 hours to turn black, but it did not liquefy. A duplicate at 48 hours gave essentially the same result. The sludge was diluted to 0.91 N acid concentration and autoclaved at 122°C for 45 minutes. The sugar yield was 111 milligrams of

xylose and 27.3 mg of glucose, or approximately 22% conversion of the 630 mg sugar equivalent available. On this basis 17 pounds of sulfuric acid would be required per pound of sugar produced, and 87 pounds would be required per pound of glucose produced.

(b) Hydrolysis with Sulfuric Acid Dispersed in Kerosene

Experiments using varying amounts of sulfuric acid (but considerably less than the minimum mols per gram determined previously) suspended in kerosene also resulted in essentially a pentosan-pentose extraction pretreatment. The purpose was to see if the sulfuric acid could be spread by dispersion as an emulsion in a cheap recoverable solvent followed by acid hydrolysis or enzymatic hydrolysis, whichever was suitable. 107.12 grams (100 gm-dry) of 2 mm Populus was added to a vigorously agitated emulsion of 65.42 grams of 24.1 $\underline{N}$ H_2SO_4 in 1 liter of kerosene resulting in a 10.4 w% suspension. The runs were stirred, for 6 or 26 hours at 30°C, and the kerosene and water layers were separated. The solid phase, with kerosene interspersed, was diluted with water and then autoclaved at 125°C for 1 hour. The resultant liquor contained mainly the pentoses. A conversion of about 14% was obtained. Extracting the kerosene (for recycle) from the unreacted wood (approximately 3/4 of the original amount) was difficult because of the existence of three phases which resulted in nearly 5% of the separated kerosene remaining adsorbed in unreacted wood. The kerosene in the solid interferes with subsequent enzymatic hydrolysis.

(c) Effect of Particle Size on Acid Hydrolysis

Experiments were performed to see what effect the size of particle of Populus had on solvolysis by moderately concentrated sulfuric acid. The requirement for using less than 3 pounds of sulfuric acid per pound of wood was maintained. Therefore, 3 ml of 17.0 $\underline{N}$ H_2SO_4 (0.026 mol of acid per gram of wood) were added to tubes containing previously sized, 1 mm, 1-2 mm, 2-4 mm and

4-6 mm dense packed and evacuated Populus alba. These samples required a minimum of thirty hours and up to 60 hours to turn black and partially liquefy. On dilution with water to 0.95 N acid and autoclaving at 122°C for 40 minutes, to effect secondary hydrolysis, all gave essentially equal pentosan-pentose extractions. It is expected from these results that the size (up to 6 mm) of the particles does not materially affect the sugar yields, but it does effect the time for solvation or partial solvation depending on the acid concentration. Experiments in which the sulfuric acid was added without previous evacuation of the wood required over a week for partial liquefacation of the particles. This is compounded by the fact that 17 N acid does not perform the desired solvolysis.

(d) Methanol Extraction of Sulfuric Acid Solvolyzed Wood.

Because of the large acid requirement for wood hydrolysis a method for its recovery and reuse would be desirable As one such possibility, the concept of extracting the acid with a recoverable solvent such as methanol was tried. This idea is not new, and has been proposed as part of a process (2). Because of the limited information available on this method a series of experiments were conducted to evaluate the use of methanol.

59.4 ml (97.15 gm) of 24.1 N sulfuric acid (equivalent to 7.02 grams or .0716 mols per gram of wood) was added to a flask containing 10.71 grams (10 gm-dry) of 2 mm Wiley milled Populus resulting in a 9.92 w/w% suspension and swirled occasionaly. The mixture turned black and liquefied in one hour at 30°C. Then 527 ml (416 gms) of anhydrous methyl alcohol were added to the black slurry which was previously cooled in an ice bath to prevent the boiling and spattering that otherwise occurs on dilution of moderately concentrated sulfuric acid solutions. The mixture was vigorously shaken until room temperature was attained (approximately 10 minutes) and then centrifuged at 10,000 RPM to separate the

solids. The measured supernate and methyl alcohol washes were retained to determine several parameters and a solvent material balance. To determine how much hydrogen ion and sulfate ion were left in solution, a sample was taken and (on the assumption no acid or sulfate ion was consumed or lost) an equivalent amount of freshly prepared barium hydroxide solution was added. The excess hydroxide was quickly back titrated with standard hydrochloric acid to a pH of 5.5, the onset point of a buffer formation (presumably from a wood acid). There was the expected recovery of 98.9% of the hydrogen ion. The solution with the brown solid (previously acid soluble lignin) plus the $BaSO_4$ precipitate was digested on a steam bath in accordance with the well known barium or sulfate ion determination. It is not a recommended procedure because it is tedious and requires great care in coagulating the fine barium sulfate precipitate and minimizing the occlusion of the slight excess of barium ion. The results of the gravimeric determination, including controls, showed that 4.6% of the sulfate ion is consumed per gram of wood treated. This consumption would be taken into account in any solvation process utilizing 72 w/w% sulfuric acid.

In the process of determining the sulfate ion consumption, the acid soluble lignin component in Populus tristis was found to be 10.5 w% based on the dry wood. This confirms the value seen previously with our analytical scheme.

Since it has been proposed that the sulfuric acid be recycled to new substrate after the methanol removal (2), the methanol/sulfuric acid supernate was checked for presence of sugars.

Analysis by H.P.L.C. showed a concentration of 2.03 mg of sugars per ml in liquor with a distribution of 35.1% xylose, 14.0% mannose and 50.9% glucose. Based on the liquors and washes, 1.19 grams of sugar were extracted by the methanol from 10 grams of wood containing a carbohydrate equivalent of 6.3 grams of sugar. This 18.9% of the total sugars available are present in the methyl alcohol-sulfuric acid extract. These sugars would interfere with atmospheric

pressure evaporation to remove the methanol and might therefore require a vacuum recovery system. Also, the ultimate effect of these soluble sugars on reuse of the acid is uncertain since presumably the sugars and their decomposition products plus other products would accumulate in the acid with repeated recycling.

An assessment was also made of possible methanol loss in the process. A sample of the original methanol/acid liquor was distilled including a control to check recovery. Analysis by gas chromatography on the distilled fractions showed only a 92.6% recovery of the methanol used on the sulfuric acid. At this time, the cause of this loss by this method is unknown. This loss was not seen with a control added to cooled sulfuric acid and then distilled. Since the methanol-sulfuric acid liquor contained appreciable sugar, work with methanol was discontinued on Populus. The sugar in similar liquors will be discussed further in this report with respect to corn stover.

(e) Summary of the Acid Hydrolysis Results

A brief summary of some of the observations on 2 mm Wiley milled Populus tristis solvation follows:

1) The minimum sulfuric acid concentration for solvolysis with a consequently longer contact time of 48 hours, at room temperature, is 21.1 N compared to 24.1 N for 12 hours contact time.

2) 0.0241 mols of sulfuric acid per gram of wood are required for maximum conversion of carbohydrate to sugar, with a minimum of 6.3 gram of pure sulfuric acid per gram of glucose produced.

3) The solvation contact time, at room temperature, appears to be directly proportional to the particle size (up to 6 mm).

4) Sugar appears in the solvolysis liquor under the experimental conditions.

5) About 4.6% of the sulfate is consumed per gram of wood treated.

6) Methanol extraction of wood-acid mixtures removes appreciable sugars as well as sulfuric acid.

D. Study of the Purdue Processing Scheme

Since sugars were observed in the methyl alcohol-sulfuric acid Populus liquor, it was decided to see if this also occurred in the treatment of corn stover. Experiments on 100 gram and later on 5 gram scale duplicating the acid hydrolysis process on Indiana corn stover shown by the Purdue laboratory in the April 1978, "Biomass Newsletter" were singularly unsuccessful. First, it would appear that the process flow diagram shown in the progress report (2), and again in above newsletter is for bagasse and not corn stover as indicated at the very top of the figure. If the typical compositions listed in their table are correct, and the column headings are not interchanged, then their Figure 19 is for bagasse. Therefore assumptions, based on our experience were made to establish an experimental scheme. By inspection of the Purdue scheme (pentsan/pentose extraction) step, it would appear to use these conditions per 100 grams of substrate:

a) 21.7 w% of suspension of substrate

b) 0.0645 mol sulfuric acid

c) 0.182 M (1.9%) sulfuric acid exclusive of dilution by substrate water content

d) 21.5 grams (27 ml) of methyl alcohol

e) heat 121°C for 50 minutes, autoclave?

The yield of pentosans is approximately 100% based on the Purdue analysis of bagasse. Presumably the results would be the same with corn stover. The experiments with Indiana corn on the 100 gram and 5 gram scale, amounts proportioned as above, but conditions otherwise the same, resulted in the yields shown in Table 8. The 100 gram scale treatment yielded 70.95 g of residue. The reproducibility is poor. Two of the reasons for this poor reproducibility are: (1) with large samples, mixing is critical and consequently acid homogeneity is difficult to attain with over 15 weight percent suspensions. (2) Reaction

Table 8

SUGAR YIELD (LBS) IN 1.9% SULFURIC ACID PRETREATMENT LIQUOR ON INDIANA CORN STOVER

BASIS: 100 LBS CORN
(Treatement Residue = 71 lbs.)

SUGAR COMPONENT IN ACID LIQUOR	100 GM SCALE (20.3% SUSPENSION)	5 GM SCALE (20.3% SUSPENSION)
XYLOSE	6.9	11.2
ARABINOSE	4.2	3.5
GLUCOSE	0.6	1.3
OTHER (MAINLY FRUCTOSE)	1.3	1.6
% PENTOSE CONVERSION (vs. 26.1 LBS AVAILABLE)	4.3	5.6

times at temperatures over 100°C are critical. Sugars yields, in 50 minutes, very considerably when it is assumed that the temperature of a non-agitated, and non-thermally uniform reaction mixture has attained 121°C. A plot of results of pretreatments on corn stover shows an inverse relationship, perhaps coincidental, between pounds of sugar produced per 100 pounds of corn stover and weight percent suspension of substrate in sulfuric acid. As data are accumulated by other tests, we may expect to see a trend of decreasing sugar produced with increased substrate loading partially offset by increased reaction times.

It becomes increasingly difficult to proportion amounts since in the Purdue scheme different and less solid is being treated in the sulfuric acid solvolysis step. In the Purdue scheme it would appear, based on 51.5 M of solids that the following conditions were used on a per gram basis:

a) 30.36 w% suspension

b) used 1.54 grams of sulfuric acid equivalent to 15.66 millimols,

c) a total water in solid and added water of 0.758 grams,

d) 3.5 grams (4.4 ml) methanol added to dilute the solvolysis liquor,

e) tumbling time not indicated

Ten grams (dry) of acid pretreated corn and the above reagents were tumbled (175 RPM) with a laboratory ball mill tumbler for six hours at which time all the mass was converted to extremely hard black balls of various sizes.

The sugar contents of the methanol-acid liquors are shown in Table 9. Reproducibility is poor, and any comment would be speculative. What cannot be ignored is the sugar content in the methanol-sulfuric acid liquor. At least 14 pounds to nearly 22 pounds of sugars are present from this corn stover. It has been previously noted that nearly 19 lbs of sugars were observed on a similar treatment on Populus tristis.

Table 9

SUGAR (LBS) 67w% SULFURIC ACID SOLVOLYSIS LIQUOR AFTER TUMBLING WITH ACID PRETREATED INDIANA CORN STOVER

BASIS: 100 LBS of ORIGINAL

(Treatment Residue = 46.33 lbs)

SUGAR COMPONENT IN METHANOL-ACID LIQUOR	10 GRAM SCALE (30.4% SUSPENSION)	5 GRAM SCALE (30.4% SUSPENSION)
GLUCOSE	4.7	1.05
OTHER (FRUCTOSE)	-	6.8
XYLOSE	7.5	12.4
ARABINOSE	1.9	1.4
LBS SUGARS (FROM 71 LBS TREATED)	14.1	21.7

The treated residue obtained from the methanol-acid mix was 6.53 grams, which on the basis of 71 lbs tumbled would be 46.33 lbs of the original 100 lbs.

Finally, the secondary acid hydrolysis was performed on the solvation residue. The Purdue scheme appears to use these conditions on a per gram basis:

a) 18.3% substrate suspension,

b) 0.0219 gram or 0.224 millimols sulfuric acid,

c) 4.059 gram water (added and in solids),

d) 0.075 grams (.094 ml) methyl alcohol,

e) heated to 125°C, 15 minutes.

6.53 grams of recovered solvolysis residue with proportioned amounts of the above reagents were heated to 125°C for 15 minutes. The results of analysis of the acid hydrolyzate are shown in Table 10. The sugar in solution was quite low. The washed, centrifuged, and dried residue amounted to 5.20 grams, or a 78.6% recovery. On the basis of an original 100 lbs, the final residue would be about 36.4 lbs.

With this process the overall sugar yield appears to be between 35 to 49% on corn stover.

E. Economic Analysis of Corn Stover Processing

A preliminary process design and economic assessment for the production of sugars and ethanol from corn stover has been completed (3) In this design a dilute acid pretreatment of corn stover is followed by enzymatic hydrolysis of residual solids. Designs are based on the production of 31,480 gallons per day of 95% ethanol. The studies indicate that with the process as presently developed, 23 gallons of ethanol can be obtained per ton of corn stover at a processing cost of about $1.80 per gallon exclusive of by-product credits and corn stover costs. The analysis shows the cost of ethanol to be highly dependent upon (1) the cost of the biomass, (2) the extent of conversion to glucose,

Table 10

SUGAR (LBS) IN SECONDARY ACID HYDROLYZATE OF SOLVATED INDIANA CORN STOVER. BASIS: 100 LBS OF ORIGINAL MATERIAL (TREATMENT RESIDUE = 36.4 LBS).

COMPONENT IN ACID LIQUOR	6.5 GM SCALE (18.4% SUSPENSION)
GLUCOSE	0.5
OTHER (MAINLY FRUCTOSE)	0.1
XYLOSE	0.07
LBS, SUGAR (OVER ALL 3 TREATMENTS)	23
% OVER ALL CONVERSION (vs. 65.7 LBS. AVAILABLE	35

(3) enzyme production and recovery cost and (4) potential utilization of xylose. Significant cost reduction appears possible through further research in these areas.

F. Fermentability of Enzymatic Hydrolyzate

Preliminary experiments were conducted to determine the fermentability of enzymatic hydrolyzates of corn stover by S. cerevisiae. It was found that the fermentability decreased as the hydrolyzate was concentrated to more than 3-fold by evaporation at 100°C. It was observed, however, that when the hydrolyzate was concentrated by vacuum evaporation, glucose was completely fermented. However, a somewhat slower rate of fermentation was observed with the glucose in the concentrated hydrolyzate relative to a pure glucose in the defined medium. Preliminary evidences indicate that these decreases in fermentability and rate of fermentation are probably due to inhibition by the increased concentration of some metabolic by-products of T. viride present in the enzyme solution. Further studies are being conducted for identification and elimination of these inhibitory substances.

II. ENZYME FERMENTATION STUDIES

A. Pilot Plant Process Development and Design Studies

a) Cellulase Production: Batch Cultures

Shake-flask studies indicate that decreasing the peptone and Tween 80 levels by half results in increased enzyme levels. Keeping the growth temperature at 30°C for the first 24 hours of incubation and then decreasing the temperature to 28°C for the balance of the fermentation also stimulates enzyme production

The above optimized parameters were utilized in batch-fermentor studies where three other variables were controlled and optimized: The G/N ratio was increased from 8.0 to 10.1. The substrate concentration was increased from 2%

to 2.5%, and the pH was allowed to fall to 3.3 and then controlled to prevent the occurrence of lower values.

The manipulations resulted in an increase of cellulase activity from 5.6 FPA to 7.9 FPA. Work is continuing with the objective of increasing the present enzyme activity by at least a factor of two.

(b) Continuous Cultures

It was noticed in previous work (4)(5) that increasing the cell density or substrate concentration did not proportionally increase the enzyme productivity. With the help of environmental manipulations encouraging results have been obtained and efforts are still in progress to increase enzyme productivity by increasing cell density. The difference in approach between present and past workers can be seen in Table 11.

(c) Cellulase Induction

Inducers are being sought which will enhance cellulase production. Sorbital and pectin have shown promising preliminary results. These carbohydrates will be examined in greater detail.

III. HYDROLYSIS REACTOR DEVELOPMENT

A. Process Design and Optimization of the Hydrolysis Reactor System

Previous designs, although useful in their own right, have made no attempt to optimize the process. Residence time for hydrolysis has been set at 40 hours with a 5.0 w% solids suspension and a Filter Paper Activity of 3.5. Analysis of these variables shows that the sugar cost is highly dependent on the cost of stover, the conversion obtained in hydrolysis,and the enzyme recovery and production costs.

A current project involves the use of a modified Michaelis-Menten equation describing the hydrolysis kinetics in an attempt to qualitatively determine the most cost-effective process. The effects of residence time, substrate

Table 11

CONTROLLED VARIABLE	CURRENT PROCESS		PAST PROCESS	
	Growth Stage (I)	Induction stage (II)	Growth stage (I)	Induction stage (II)
Temperature	28°C	28°C	30°C	30°C
pH	4	3.3	4.8	4.8
Inlet Sub. Concentration	17.5 $g.l^{-1}$		15. $g.l^{-1}$	
Dilution Rate	0.017-0.018 h^{-1}		0.02 h^{-1}	
FPA		5.75		2.5

concentration, and Filter Paper Activity have been explored, and a new process design has evolved. Details of this design and the approach taken are presented in a report now in preparation.

IV. UTILIZATION OF HEMICELLULOSE SUGARS

(a) Enzymatic Hydrolysis of Xylan and Xylanase Production

In our continuing studies of the production of xylanase from wheat bran by Streptomyces xylophagus it was noted that the xylan component is completely utilized. After four hours of fermentation, 40% of the xylan was consumed, while after 72 hours none remained. The main goal now is to increase the rate of enzyme production. Approaches to be investigated include: addition of an inducer, increasing cell density with the aid of complementary nutrients, improving mass transfer in the system, or by changing the control variables.

Work is also in progress on another strain Chaetomium trilaterale IAM 8042 obtained from Japan, which is known to produce xylanase.

(b) Fermentation of Xylose

In the previous report it was noted that growth of the mold Fusarium oxysporium with xylose to produce ethanol and CO_2 was slow. Three approaches were proposed to speed the fermentation rate: (1) Increase the sugar concentration in hopes of producing a yeast-like form with a faster growth rate. (2) Shear the growing mycelium to create more growing points. (3) Grow the organism with glucose to see if a faster growth rate is possible. If the organism grows faster with glucose, one could expect to select xylose-fermenting mutants with growth rates approximately those on glucose.

It was found that: (1) the mold grew well at xylose concentrations of up to 20%. There was no inhibition of growth at this high sugar concentration, but no formation of yeast-like cells took place either. (2) Shearing the mycelial

culture intermittently increased the growth rate by a factor of two. This enhancement was considered insufficient. (3) The mold grew twice as fast with glucose as with xylose. This difference was not considered large enough to warrant a mutant-selection program.

A comprehensive literature search to identify other attractive xylose-fermenting microorganisms have been completed. The most promising candidate is a facultatively anaerobic bacterium, Bacillus macerans. This organism produces ethanol, acetone, CO_2 and some acids from xylose. A crude temperature optimization has been completed, and preliminary experiments indicate that this organism grows significantly faster than F. oxysporum. Work is currently in progress to measure product yields and optimize fermentation conditions. This organism has also been described as fermenting xylans and hemicellulose, so it may be feasible to ferment these polymers directly in untreated biomass eliminating a pretreatment step.

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3. Enhancement of Enzymatic Hydrolysis of Cellulose by Coupling with Ethanol Fermentation, with Takeshi Harima) Report in preparation.

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5. Process Design and Optimization of Cellulose Hydrolysis (with R. Lindsey) LBL-7864 (in Press).

APPENDIX

CELLULOSE BIOCONVERSION AND PILOT PLANT STUDIES

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CELLULOSE BIOCONVERSION TO SUGARS AND ETHANOL

BERKELEY PROGRAM--July 1978

Evaluation of Potential Raw Materials (Agricultural Residues)

(a) Analysis of composition--carbohydrates, lignin, etc.
(b) Hemicellulose extraction--dilute acid pretreatment
(c) Enzymatic hydrolysis of original and pretreated materials
(d) Chemical hydrolysis
(e) Process design and economic evaluation studies

Cellulase Production

(a) Multistage fermentation with *Trichoderma viride*
(b) Computative Evaluation of *T. viride* mutants and alternative organisms

Hydrolysis Kinetics (*T. viride* cellulase)

(a) Kinetic models for product inhibition multi-science mixed enzyme systems

Enzyme Adsorption-Desorption

(a) Adsorption of C_1, C_x and β-glucosidase activities on cellulose
(b) Enzyme fractionation by adsorption on cellulose
(c) Enzyme desoprtion--effect of additives

Mixed or Supplementary Enzyme System Development

(a) β-glucosidase from *Aspergillus phoenicis* (NSF project)
(b) C_2 complex from *T. koningii*
(c) Xylanase from *Streptomyces xylophagus*

Chemical Pretreatment

(a) NO_x pretreatment

Enzymatic and Microbial Delignification

(a) Study of *Phanarochaete chrysosporium* (NSF Project)

Cellulose and Glucose Fermentation to Ethanol

(a) Vacuum and cell recycle system development
(b) Media optimization
(c) Evaluation of hydrolyzates for fermentability and ethanol yield

Xylose Fermentation to Ethanol

(a) *Fusarium oxysporum* (f. sp. lini)
(b) *Bacillus macerans*

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