PILOT SCALE PRODUCTION OF CELLULASE AND ENZYMATIC HYDROLYSIS OF WASTE CELLULOSE

Robert K. Andren
and
John M. Nystrom

The cognizance of cellulose as a renewable chemical resource is evidenced by the growing number of recent conferences and symposia held to consider the utilization of this valuable material. Processes for cellulose conversion are extremely attractive in that they offer potential means of changing a substance which comprises a large fraction of the world's solid waste into useful products. The utility of cellulose is greatly increased if it is first hydrolyzed to glucose, which can be used in human or animal foods as a microbial substrate to produce a variety of materials or directly as a chemical feedstock. Enzymatic saccharification offers the advantage of being carried out at moderate reaction conditions and is specific for cellulose, eliminating the problem of producing unwanted by-products or reversion compounds. The technical feasibility of the enzymatic process has been shown in the laboratory (*1* to *5*); however, its economic practicality will be decided based on data obtained by pilot level experimentation.

The development of a process technology for large scale production of cellulase is a major objective of the Natick pilot program. Much is known about the production of cellulase via submerged fermentation of *Trichoderma viride* (*2,6,7,8*), but most of the developed technology, until recently (*9,10*), applied solely to laboratory scale operation. Transferral and refinement of this technology from laboratory to pilot scale has been one of the first steps towards a commercial process.

Current studies have led to an established pilot scale process for production of *T. viride* cellulase. Process changes are currently being evaluated in an effort to increase enzyme yields and fermentor productivities. Initial results were extremely encouraging, with more sophisticated processing yielding a doubling and tripling of productivity and yield, respectively.

Food Sciences Laboratory, U.S. Army Natick Development Center, Natick, Massachusetts 01760.

Batch saccharifications of a wide variety of cellulosic substrates have been carried out using *Trichoderma* cellulase. This has included both the pure cellulosics—cotton, Avicel (microcrystalline cellulose), Solka Floc (bleached sulfite pulp)—and the more complex agricultural, municipal, and industrial wastes (*4,11,12*).

As a model of the cellulosic fraction of municipal refuse, newspaper has received considerable study as a saccharification substrate. Using laboratory ball milled newspaper, 4 to 7% glucose solutions have been produced in 1 to 3 l reactors at 50°C after 24 hr (*13*). Under similar conditions, with the substrate industrially milled, 6% syrups were produced. Ball milling is used to increase the reactivity of the substrate by reducing the high degree of crystallinity often associated with natural cellulose. Delignification with mild caustic at elevated temperatures also causes a marked increase in susceptibility.

Toyama (*14*) has reported on the saccharification of delignified cellulosic materials using both pure enzyme preparations and solid cultures (*Koji*). He has also reported on autosaccharification of such solid cultures. Sugar syrups up to 21% have been obtained with rice hulls and bagasse. Our studies have shown some waste materials to be very reactive as produced, thus eliminating the cost of further pretreatment. Hydrolysis of corrugated cardboard and sawdust have yielded 8 and 11% sugars, respectively. Saccharification of hydropulped waste documents produced 5% sugars, while 3.5% syrups resulted from the enzymatic hydrolysis of a glassine paper mill sludge (*11*).

The production of sugars via the enzymatic route becomes most economical and efficient when carried out in a continuous reactor system. Several systems have been studied using a pure cellulose, Solka Floc, as the substrate with up to 14% syrups being produced (*4, 5*).

With the complex waste cellulosic substrates, constant re-

moval of noncellulosics is necessary to prevent their buildup within the reactor. The product slurry must then be filtered to remove the unreacted solids for recycle or further treatment for enzyme recovery. This report discusses some of the problems involved when dealing with ball milled newspaper as a substrate. Series of batch and continuous runs have been carried out at various enzyme and substrate concentrations to provide data suitable for the economic evaluation of enzymatically hydrolyzing newspaper. The direction of our future studies at the Natick Development Center will depend on these results.

EXPERIMENTAL METHODS

PRODUCTION OF ENZYME

Process Flow Diagram. Figure 1 shows the pilot plant process flow scheme for production of cellulase. In order to properly evaluate the range of parameters affecting enzyme production, a highly instrumented concept was adopted in the design of the pre pilot plant. A two-vessel system was used for production of enzyme in a batch process. A 30 l fermentor (22.5 l working volume) was used to produce a 7.8% inoculum to a 400 l (280 l working volume) production fermentor. Major raw materials were cellulose, nutrient salts, and water.

The instrumentation for monitoring and controlling the vessels included temperature, pressure, weight, *p*H, dissolved oxygen, power, agitation rate, sparge, and inlet and exit gas analysis of oxygen and carbon dioxide. Foam was controlled by employment of draft tubes and by addition of surfactants. Both vessels were also equipped with mechanical foam breakers.

Culture and Media. Trichoderma viride was chosen as the best organism on the basis of the extensive work of Mandels et al. (*2,6,7,8*). A mutant strain QM 9414 was selected because of its increased enzyme productivity over the parent or wild strain QM 6A.

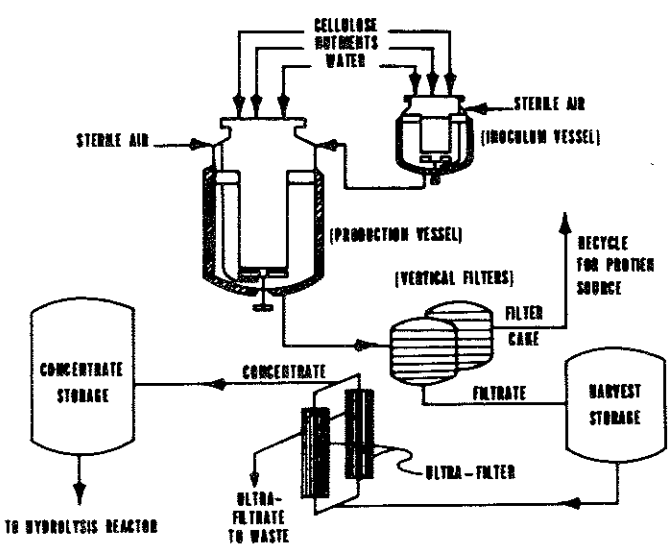


Fig. 1. Pilot plant process for cellulase production.

The media for production of the enzyme consisted of the standard mineral salts described by Mandels and Weber (*2*) with 1% cellulose (SW 40: Brown Company, Berlin, NH), 0.075% proteose peptone, and a variable amount (0 to 0.2%) of Tween 80 (Atlas Chemical Industries, polyoxyethylene sorbitan mono-oleate). In addition, 65 p.p.m. of polypropylene glycol (M.W. 2 000) was added as an antifoam. The inoculum vessel (30 l) contained the same medium, but no Tween 80 was added. Both vessels and media were sterilized at 121°C for 60 min.

The inoculum for the 30 l vessel was prepared in 1 l Fernbach Shake Flasks using the mineral salts medium with 0.5% cellulose (SW 40), 0.05% proteose peptone, and 0.2% Tween 80. Cultures were inoculated with a spore suspension and incubated at 30°C on a reciprocating shaker for 4 days. When the media in the 30 l fermentor reached the appropriate cell density and enzyme strength, it was transferred to the production (400 l) fermentor.

At the completion of the fermentation, the culture from the production vessel was harvested through a vertical tank filter containing 80 × 70 mesh screens. The mycelia bridge the screen, forming the precoat. The broth or filtrate is a clear straw colored liquid resembling a noncarbonated beer. The mycelia or filter cake can be partially recycled as a source of protein in the fermentation.

The clear filtrate or broth was assayed for enzyme, then concentrated via an ultrafiltration unit (Romicon HF2SSS, HF22-20-PM10 cartridge) and stored in a large refrigerated tank until used in the hydrolysis reactor.

Analytical Methods. Enzyme activity was measured in International Units using the standard assay described by Mandels et al. (*15, 16*).

The solids dry weight was determined by freeze drying the filter cake obtained during the harvesting. No attempt was made to distinguish between mycelia and undigested cellulose. The main purpose of the measurement was to calculate the filtration area required for harvesting and the quality (percent moisture) and quantity of the cake as function of the fermentation conditions.

HYDROLYSIS OF NEWSPAPER

Process Flow Diagram. Figure 2 shows the process flow scheme for the pilot hydrolysis system. It was built around a 250 l stainless steel bioreactor currently being operated at a 100 l working volume. A botton driven pumping impeller draws the contents over the sides and down through a Waldhof draught tube to promote effective mixing. Capabilities were provided for control and monitoring of vessel temperature, reactor *p*H, agitation speed, power input to the reaction slurry, and vessel weight. When operating in a batch mode, the vessel was sealed, and the toluene was added periodically to prevent microbial growth. Samples were withdrawn through an air activated rapid sampling valve.

During continuous operation, concentrated enzyme was added by a small (New Brunswick Model DF-42) sterilizable diaphragm pump. Flow was regulated by both stroke adjust-

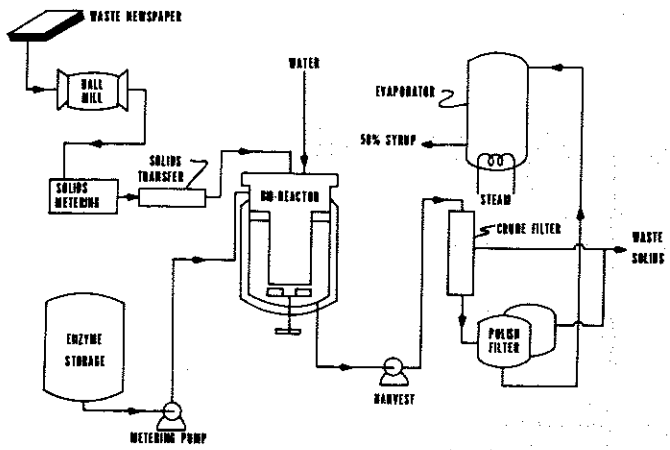


Fig. 2. Pilot plant process for newspaper hydrolysis.

ment and speed control. Newspaper used as the feed was industrially ball milled (Brown Company, Berlin, NH) to -200 mesh. Accurate metering of the substrate was accomplished with a Acrison Model 403 gravemetric feeder. The feed auger discharged into the hopper of a screw type flexible conveyor system which emptied at the top of the vessel. Newspaper dropped into the center of the draught tube to mix with the contents of the reactor. A continuous operating mode was effected by maintaining the vessel weight constant. When an increase caused by addition of reactants was detected, the weight/level controller activated a harvest pump to remove enough hydrolyzate to return the system to its original weight. A pulsafeeder hydraulically actuated diaphragm pump was used for this purpose.

Ball milled newspaper has proven to be a somewhat difficult material to work with on a pilot scale. A very fine powder, it is extremely hygroscopic. As it picks up moisture, it tends to agglomerate and build up on any surfaces which it contacts. This has often caused problems with the solids feeding apparatus, resulting in the necessity of lagging the system with heating tape to keep the substrate dry. On a larger scale, this should be less of a problem.

A proven practical method for removal of unreacted solids from the product stream has not yet been developed. The partially hydrolyzed substrate particles are very small and difficult to remove from the syrups. Settling rates are low, and conventional filters tend to blind. By allowing the mixture to drain slowly through a 10 μ filter bag, most of the suspended solids could be removed, but this remains an area warranting further investigation. Toluene continues in use as a preservative during and after sugar production, though other methods remain under study.

Reaction Conditions. All of the batch and continuous runs, except one, were carried out at 50°C and pH = 4.8. Enzyme and substrate concentrations were varied during the batch tests as shown in Table 1. During continuous operation, the system was run in a batch mode for 24 hr to allow a buildup of sugars prior to activation of the feed and harvest streams. All continuous runs were carried out at 50°C with a 15% solids feed. Table 2 lists the operating parameters and holding times. Two holding times were sufficient in all cases for steady state to be reached.

Analytical Techniques. Syrups produced were analyzed for total reducing sugars by a DNS procedure (*17*) and the individual components—glucose, cellobiose, and xylose—by high pressure liquid chromatography (*18*). The chromatograms indicate the presence of higher order sugars which have not yet been identified, but these are felt to be present only in very

TABLE 1. BATCH HYDROLYSIS OF INDUSTRIALLY BALL MILLED NEWSPAPER

RUN	INITIAL SUBSTRATE CONC. % SOLIDS	ENZYME CONC. UNITS/ML	TEMP °C	PH	GLUCOSE CONC. (24 HOURS) MG/ML	CELLOBIOSE CONC. (24 HOURS) MG/ML	XYLOSE CONC. (24 HOURS) MG/ML	TOTAL LC SUGARS (24 HOURS) MG/ML	TOTAL REDUCING SUGARS (24 HOURS) MG/ML
B-1	9.9	.27	50	4.8	6	7	2	15	19
B-2	12.8	.25	50	4.8	5	6	2	13	
B-3	7.0	.24	50	4.8	4	4.5	1	9.5	
B-4	14.9	1.05	55	5.3	21	15	5	41	38
C-1 (Batch Part)	9.9	.28	50	4.8	3.5	5	1	9.5	
C-2 (Batch Part)	15.0	.62	50	4.8	18	11	4	33	29
C-3 (Batch Part)	14.5	1.2	50	4.8	32.5	15.5	6.5	54.5	60
C-4 (Batch Part)	14.5	1.5	50	4.8	37	16.5	7	60.5	

TABLE 2. CONTINUOUS HYDROLYSIS OF INDUSTRIALLY BALL MILLED NEWSPAPER

SUBSTRATE FEED CONC. % SOLIDS	ENZYME FEED CONC UNITS/ML	HOLDING TIME HOURS	PRODUCT GLUCOSE CONC. MG/ML	PRODUCT CELLOBIOSE CONC. MG/ML	PRODUCT XYLOSE CONC. MG/ML	PRODUCT TOTAL LC SUGARS MG/ML	PRODUCT DNS SUGARS MG/ML	PERCENT CONVERSION
14.8	.6	8	11.8	9.1	2.5	23.4	23.8	14
14.8	.6	16	13.6	10.0	3.2	26.8	26.7	16
14.8	.6	24	16.3	10.1	3.6	30	28.2	18
14.7	1.2	48	27	10	7	47		29
14.5	1.5	24	23	4	9	36		23
11.1	1.5	12	17	4	9	30		25

Temp = 50°C
pH = 4.8

small quantities. The product stream was also analyzed for suspended solids concentration.

RESULTS

PRODUCTION OF ENZYME

Inoculum. Initial development work was carried out to establish a reproducible fermentation profile for production of a consistant inoculum (*9*). The results of these runs indicated that the optimum conditions for an inoculum were defined by size, culture age, and enzyme strength. The standardized conditions for inoculum production were as follows:

Temperature	29° to 30°C
*p*H	uncontrolled
Aeration	0.1 to 0.15 VVM
Minimum D.O.	15% of saturation
$k_L a$ (Max)	60 mmole oxygen/l-hr-atm
Time	25 hr

If the inoculum were either too young or too small, a serious lag was experienced in the production vessel. Under normal conditions, one would like to transfer during the most rapid growth period well before the culture becomes stressed or approaches a stationary phase. However, in the case of the *T. viride* fermentation, the concentration of enzyme in the inoculum was also important.

The level of enzyme controls or limits the amount of sugar within the broth. A very low level prior to transfer will initially starve the culture in the production stage. In this case, shifts in metabolism became apparent. If the soluble carbohydrate level was low, the organism began to consume the more readily available protein within the media. Changes in respiratory quotient and rate of *p*H change were noticeable, indicating deamination and consumption of the more available protein. The ultimate result was a rather large lag in the growth curve and a serious reduction in fermentor productivity.

If the enzyme level was too high, the resulting soluble carbohydrate level was high. This usually occurred when the culture was quite old and well past the stationary phase (some cell lysis had probably begun). Cellular metabolism was not geared to rapid growth, and therefore initial growth was slow even though the soluble sugar level was high. The result was another long lag period. To minimize the lag, a 25 hr old 10% inoculum was found to be best (*9, 10*).

In the production stage, the operating parameters believed to have the greatest effect on enzyme production were investigated. The level of dissolved oxygen, minimum *p*H, and the surfactant, Tween 80, were all studied.

Fermentation Profile (Production Phase). During the course of the fermentation, a number of parameters were monitored as shown in Figure 3. Changes in respiration activity were indicated in the carbon dioxide production and oxygen utilization curves. The *p*H curve was characteristic of the *T. viride* fermentation with an initial fall to a controlled level followed by an extensive period at a low *p*H value. The rise in *p*H, in this case at the 70 hr mark, indicated the beginning of the end of the fermentation.

Control of *p*H was accomplished by addition of 2.5N sodium hydroxide, with the rate of addition carefully monitored and used to predict the final upturn in *p*H. Enzyme

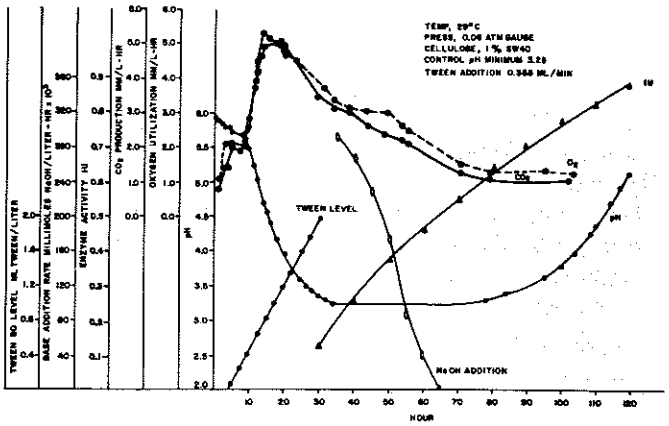


Fig. 3. T. viride fermentation run #20–400 liter.

production was continuous during the entire fermentation, with the maximum overall productivity (IU/ml-hr) usually occurring between 80 and 100 hr.

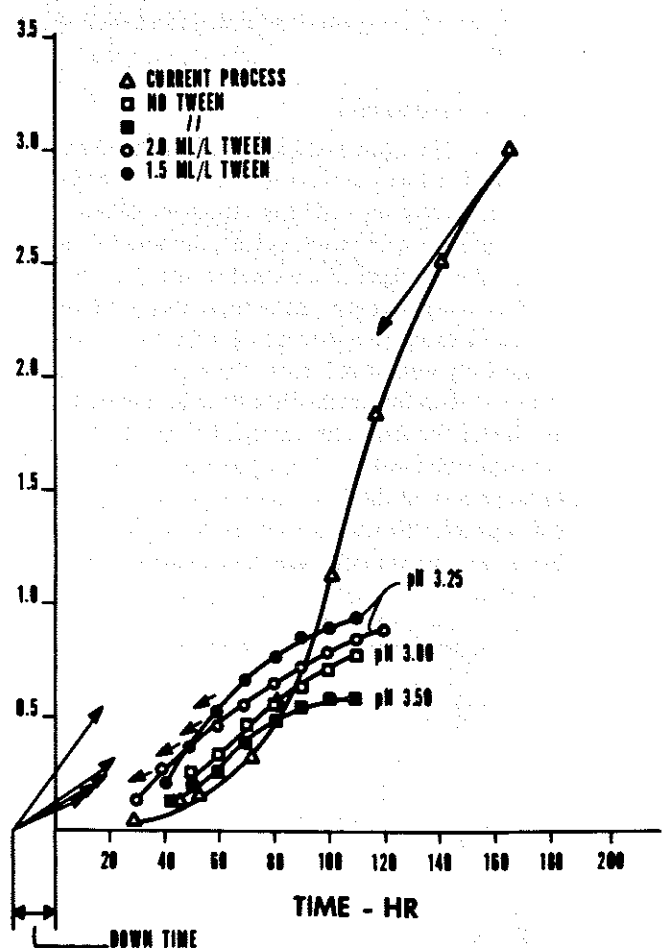
Dissolved Oxygen Level. The effect of maintaining the dissolved oxygen level above 10% saturation was minimal or nonexistent. Below this level, some deleterious effect was observed on both microbial growth (reflected in respiration activity) and enzyme production (lower overall yields). The oxygen transfer requirements were quite small, with oxygen utilization never exceeding 6.0 mmole/l/hr.

Minimum pH Level. During the course of the fermentation ammonium sulfate is consumed as a primary nitrogen source. As ammonia is taken up, hydrogen ions are released continually driving the *p*H lower, to an observed value of about 2.7 with the current medium. Growth at this level was extremely slow, and enzyme inactivation (probably via acid proteolysis) occurred at a high rate. To minimize this, *p*H control was employed to limit the minimum value. In general, the lower the minimum *p*H the higher the ultimate yield of enzyme but the longer the fermentation. In order to find an optimum, *p*H control was tried at several levels. As *p*H decreased between the control levels, 3.5 to 3.0 productivity increased 17% and yield increased 34%.

Effect of Surfactant Tween 80. To investigate the effect of a surfactant on the production of enzyme, an intermediate value of minimum *p*H (3.25) was chosen. Tween 80 was selected because of its proven beneficial effect (*2*). To minimize the foam enhancing properties of this surfactant, controlled addition during the fermentation was necessary. The addition was tried at two different rates and was accomplished by an automatic pulse of a solenoid admitting a calibrated quantity of sterile Tween per pulse. Two different levels of this surfactant were also tried. In both cases, Tween 80 enhanced both the yield and productivity. The greatest effect occurred at the lower concentration, amounting to a 31% increase in fermentation productivity over the previous high. Because of the expense of Tween 80, its effect on the overall economics is questionable.

In addition, there was considerable risk associated with its foam enhancing properties, even when it was added in a stepwise manner. There was a noticeable increase in foam level which would ultimately decreased the working volume of larger scale fermentors and affect the overall economic picture.

Overall Productivity. Figure 4 summarizes the development work affecting the production of enzyme. Productivity was calculated assuming a 15 hr down time or turn around time for a vessel and the appropriate values determined by projecting the tangent to the production curve from that negative time point.



Effect of *p*H and Tween 80 on Enzyme Productivity

Run #	*p*H	Tween level	Max. enzyme yield	Max. productivity*
17	3.5		0.58 IU	5.2×10^{-3} IU/ml-hr
18	3.0		0.78	6.1×10^{-3}
19	3.25	1.5 ml/l	0.93	8.0×10^{-3}
20	3.25	2.0	0.89	7.0×10^{-3}
23†			3.00	17.1×10^{-3}

T. viride (QM 9414), temperature 29°C, 0.06 ATM gauge
Dissolved oxygen >15% of saturation

*Maximum productivity assumes 15 hr down time for each fermentor.
†Yields using enriched medium (paper in preparation).

Fig. 4. Enzyme activity IU/ML.

Work is in progress to produce higher enzyme yields in enriched media after the procedure of Sternberg (*20*). Initial results are encouraging and have led to an increase in process efficiency. Figure 4 very dramatically shows this increase in process efficiency. We believe the limit of possible enhancement has yet to be reached. The possibility of another tripling of the enzyme yield and an additional four to fivefold increase in productivity seem well within reach with the appropriate processing strategies. It is hoped this work to develop a usable enzyme production technology will lead to a process which can supply a consistently high quality enzyme preparation at a price not seriously affecting the economics of a cellulose hydrolysis process.

HYDROLYSIS OF NEWSPAPER

Batch Studies. The results of a series of batch runs and the initial batch parts of four continuous runs are summarized in Table 1. The concentrations of three sugars measured chromatographically, glucose, cellobiose, xylose, the sum of the three, and the total reducing sugars are indicated after a 24 hr hydrolysis. At low enzyme strengths, little sugar was produced because the noncellulosics adsorbed a major fraction of the enzyme, effectively removing it from the reaction mixture. In runs C-2 and C-3 there was little difference in rate of sugar production during the first 4 hr, though C-3 had twice the enzyme concentration as C-2. It may be that there was sufficient enzyme present at the lower level to saccharify the readily hydrolyzable cellulose present early in the run. However, as the more resistant substrate began to predominate in the reaction mixture, a higher concentration of enzyme was required to produce additional sugars.

Figures 5 and 6 show sugar production as a function of hydrolysis time for the runs carried out at the higher enzyme strengths. Xylose was produced by hydrolysis of xylan by hemicellulases present in the enzyme broth. As the curves indicate, conversion was rapid with concentrations leveling off shortly after the start of the run. Mannose, the monomer of another C-6 polysaccharide, mannan, has not been detected in the hydrolyzates. Cellobiose was initially produced at a greater rate than glucose. However, usually after 10 to 15 hr into a run, its rate of conversion into glucose exceeded its rate of production, and, after reaching a maximum, the concentration of cellobiose gradually decreased.

Previous studies indicate 50°C and a *p*H value of 4.8 to be optimum for sugar production. Increasing the reaction temperature had two effects. Reaction rate tended to increase, but so did the rate of enzyme denaturization. Run B-4 at 55°C demonstrates this, as sugar production was lower than would be expected. Since enzyme cost has been shown to greatly affect the total process cost (*10, 19*), retention of enzyme activity becomes a high priority. In this case, it may be more economical to run at lower temperatures. Though reaction rates will be lower, enzyme will be conserved.

Control of *p*H was maintained through addition of either nitric acid or sodium hydroxide to the vessel. As the hydrolysis progressed, the mixture was driven toward the acid side as indicated by the continuous addition of base. This is shown in Figure 7, where the amount of base added closely parallels

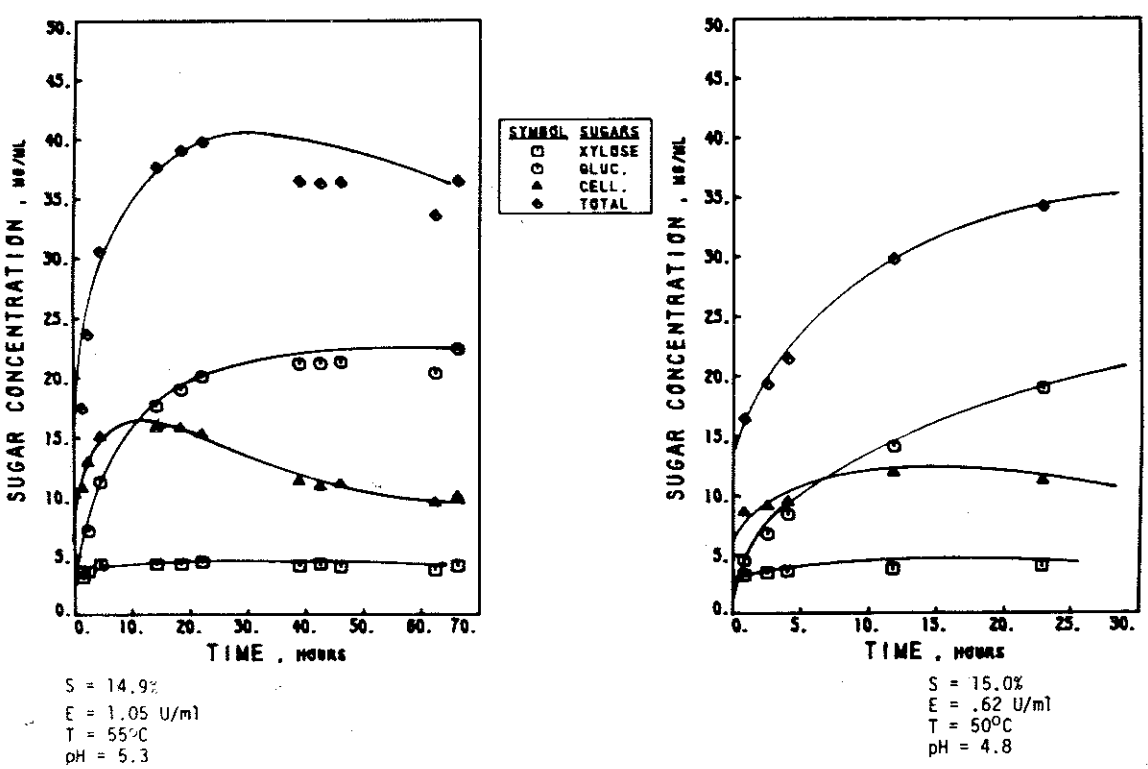


Fig. 5. Composition of sugars produced during hydrolysis of industrially ball milled newspaper.

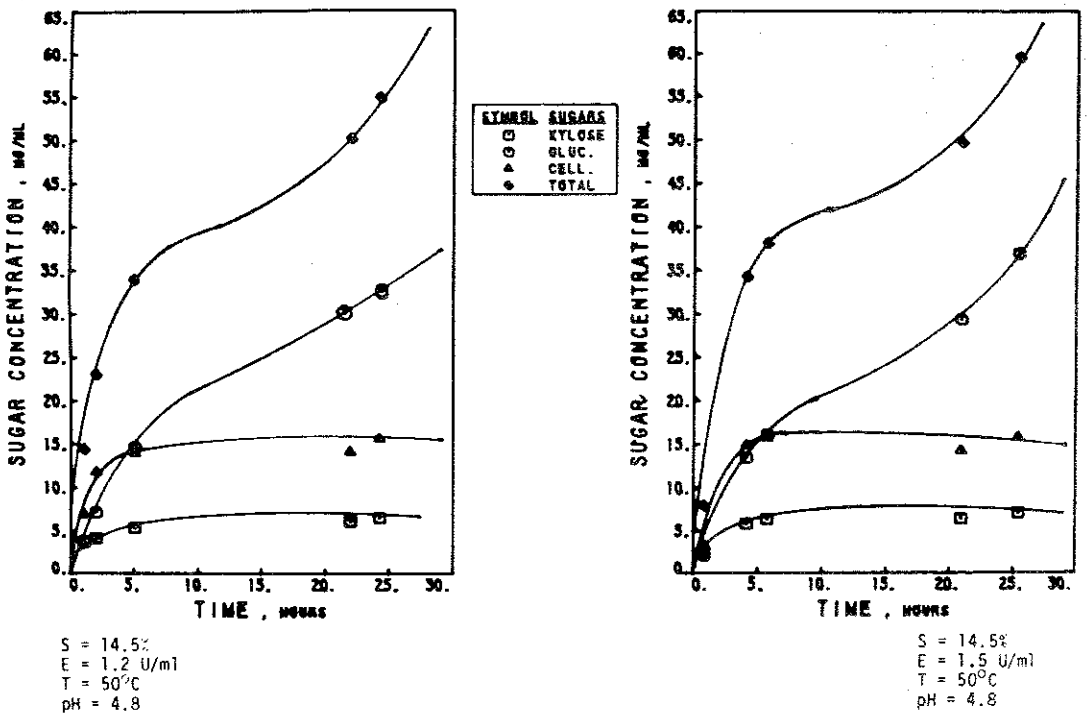


Fig. 6. Composition of sugars produced during hydrolysis of industrially ball milled newspaper.

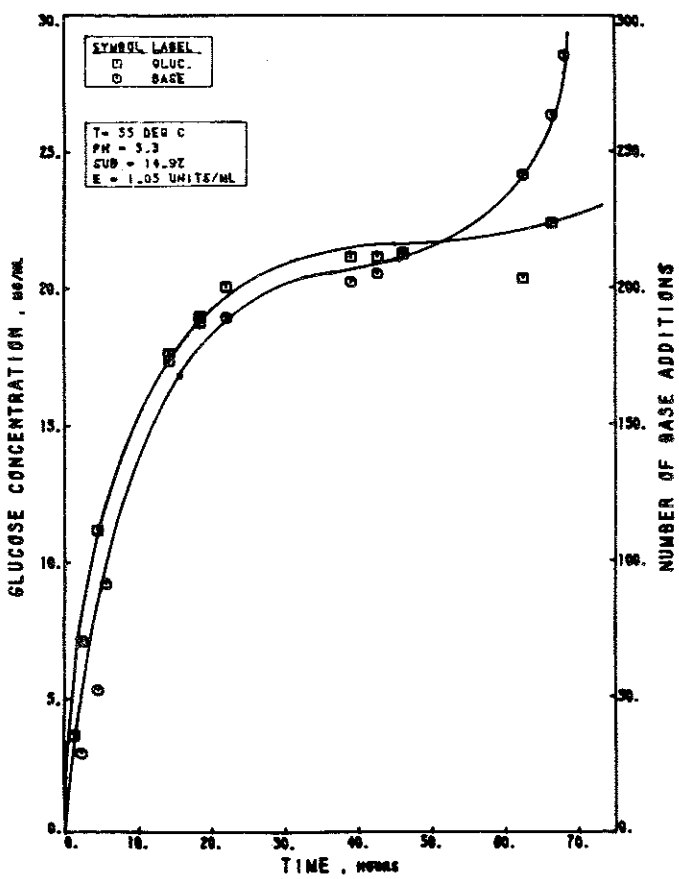


Fig. 7. Hydrolysis of industrially balled milled newspaper. Run 84.

sugar production. A satisfactory explanation of this phenomena has not yet been determined.

Continuous Studies. Run in the continuous stirred tank reactor (CSTR) mode, production of sugars was accomplished in a series of experiments with the results shown in Table 2. Newspaper represented approximately 15% of the feed during all of the runs. Variations were made in both enzyme concentration and residence time in the reactor. As seen in the data using 0.6 units/ml enzyme, tripling of the residence time resulted in only about a 25% increase in sugar production. Maximum cellobiose production occurred at a holding time between 16 and 24 hr. For longer holding times, its concentration decreased as the equilibrium shifted. A 20% conversion of the feed substrate took place with a residence time of 24 hr. By doubling the holding time and the enzyme strength, the figure was increased to 29% (40 to 50% of the cellulose present).

A 24 hr residence time with a 15% feed and an increase in enzyme strength from 0.6 to 1.5 units/ml resulted in only a 20% increase in sugars. Higher conversions at lower residence times were possible by lowering the substrates feed concentration.

Process Factors. Total process costs are dependent on substrate and enzyme costs, capital equipment costs, costs for filtration and purification of syrups, and the price to dispose of unreacted solids. However, the most important single hydrolysis cost is that of pretreatment of the substrate. Physical treatments have been investigated, and ball milling has been found to result in a substrate most susceptible to enzymatic

attack (*15, 20*). There are two main drawbacks, however. It is very expensive, and the noncellulosics remain in the substrate. Lignin is the major noncellulosic component present in materials of wood origin. A complex amorphous polymeric substance, it is very high in phenolic content. As the technology is developed for breaking the structure and economically recovering the phenols, lignin may well acquire considerable value.

It is possible to increase substrate reactivity by chemical means. Mild caustic treatment at high temperatures has converted cellulosic materials such as rice hulls and bagasse into substrates which are very susceptible to direct microbial digestion. Wood pulp which had undergone both kraft and sulfite pulping was very readily saccharified enzymatically. These processes delignify the substrate and severely disrupt the crystalline structure of the cellulose. Besides the obvious benefit of producing a highly reactive substrate, a stream is produced which may prove to be an excellent source of lignous materials.

In view of this, it is felt that a delignified substrate will eventually prove to be the most practical material for enzymatic conversion to sugars. Being high in cellulose, it will result in a more efficient use of reactor volume and potentially higher sugar concentrations. Costly enzyme would be conserved for reuse, since losses by adsorption on noncellulosics would be minimized. Costs for filtration and recovery of the sugars would also be lower, as would those for disposal of the unreacted residues. Thus the emphasis of the pilot studies being carried out at Natick will be shifted to saccharification of chemical wood pulps. In this way we feel that the most economical process for the enzymatic hydrolysis of cellulose can be achieved.

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