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1972

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This paper summarizes the studies being carried out at Natick Laboratories on pollution abatement by enzymatic conversion of waste cellulose to useful products. Cellulose is the major component of card board boxes, kraft paper, paper bags, correspondence paper, and newsprint, or any other product of wood pulp or cotton. Millions of tons of waste paper products are generated in the United States every month and a large part originates within facilities of the Department of Defense (15). With current emphasis on separating and recycling our solid wastes it is apparent that large quantities of crude cellulose will be available. Some of it will be recycled as paper products, much that is not suitable for such recycling will be a challenge for new technologies.

One approach to the useful disposal of waste cellulose has been the direct conversion of cellulose to animal protein by ruminant feeding, or to single cell protein by growing bacteria or fungi on cellulose (1). Our approach has been a different one. Cellulose can be quantitatively converted to glucose by acid or enzymatic hydrolysis. Glucose, a nearly universal growth substrate, can be consumed by man, fed to animals as molasses, converted to single cell protein such as yeast, or used as a raw material for fermentation to alcohol, amino acids, drugs, or other useful chemicals. Acid hydrolysis is technically feasible, and has been utilized under conditions of wartime shortage to produce alcohols or food yeasts from wood wastes. At Natick we believe that enzymatic hydrolysis will be simpler and more economical. This paper outlines the sequence of events in our progress towards a successful system.

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1. Selection of Trichoderma viride as the enzyme source.

Many fungi readily grow on and consume cellulose. The first step in such growth must be hydrolysis of the cellulose, but the glucose produced is consumed by the fungus and the final products are cells, CO_2 and other metabolites. To obtain glucose as a product the hydrolysis must be by cell free enzymes. Our first requirement is the production of large quantities of cellulase capable of hydrolyzing insoluble cellulose. The enzymes used by the cellulolytic fungi to hydrolyze cellulose are not always found in the medium. Culture filtrates from most cellulolytic fungi showed only a limited hydrolysis of native cellulose although soluble derivatives such as carboxymethyl cellulose were readily hydrolyzed. Apparently those enzyme components required for attack on insoluble cellulose were unstable. T. viride however produced a complete and stable cellulase complex, and filtrates from this organism were capable of rapid hydrolysis of purified or crude cellulose (9,11). These tests (Table 1) were run at 50°C. All preparations showed less hydrolysis at 25°C. Although T. viride is a mesophile the enzymes are stable and show optimum activity at 50°C.

2. Production of cellulase in quantity.

T. viride grows rapidly on simple media (Table 2) with no requirement for added growth factors. Cultural conditions have been optimized for production of the enzyme in shake flasks or submerged fermentation (7,8,11, 14). For routine enzyme production a 10 liter culture is inoculated with the spores from a 10 ml potato dextrose agar slant and grown for 10-12 days at 28°C with slow agitation (100 RPM) and aeration (0.2 Vol/Vol/min). The culture is harvested by pouring it through a glass wool filter. Mycelium and residual solids are discarded and the clear straw colored filtrate is ready to use as enzyme after pH adjustment. If desired the enzyme can be concentrated by ultrafiltration using a membrane with 20,000-30,000 molecular weight cut off (13) or precipitated to a white powder with ammonium sulfate or 66% acetone. The enzyme is stable for years at refrigerator temperature and is remarkably resistant to chemical inhibition (10). Activity is optimum at pH 4.0-5.0, 50°C, and can be assayed by production of glucose from various cellulose substrates. For predicting the activity on waste cellulose materials the assay on filter paper which measures the entire cellulase complex is preferred (11).

Table 1. Production of Cellulase by Celluloytic Fungi Grown on Cellulose

A. Thermophiles - Culture Filtrates Grown at 37°C.

Organism	QM No.	Cellulase units/ml
Humicola grisea	228	2.3
Hormiscium sp.	1192	1.5
Chaetomium thermophile	9381	0
Sporotrichum thermophile	9382	2.9
Thermoascus aurantiacus	9383	2.2

B. Mesophiles - Culture Filtrates Grown at 28°C

Organism	QM No.	Cellulase units/ml	Cellulase units/mg protein
Trichoderma viride	6a	15.	22.
Trichoderma viride mutant	9123	29.	23.
Trichoderma viride mutant	9414	41.	31.
Trichoderma viride mutant	9136	0	-
Fusarium solani	21d	3.2	-
Gliocladium sp.	365	4.2	-
Pestalotiopsis westerdijkii	381	1.9	-
Humicola grisea	542	2.0	-
Stilbum sp.	833	1.9	-
Graphium sp.	9380	1.7	-
Myrothecium verrucaria	460	1.0	1.6
Basidiomycete	806	3.5	3.3
Chrysosporium pruinosum	826	3.0	2.7
Streptomyces sp.	B814	3.0	4.0
Aspergillus niger *		2.0	1.6
Trametes sanguinea		3.5	2.2
Poria (SEAB)		6.0	3.7
Helix pomatia (snail)		0.5	0.6

*Commercial cellulase preparation at 5 mg/ml.

Shake flask cultures grown on salts with 0.5% cellulose. One cellulase unit produces 0.5 mg glucose from Whatman No. 1 filter paper in one hour at 50°C. Protein by phenol method (11).

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Table 2. Trichoderma viride Medium for Cellulase Production

$(NH_4)_2SO_4$	1.4 g/liter	Fe $SO_4 \cdot 7H_2O$	5.0 mg/liter
KH_2PO_4	2.0 g/liter	Mn $SO_4 \cdot H_2O$	1.6 mg/liter
Urea	0.3 g/liter	Zn $SO_4 \cdot 7H_2O$	1.4 mg/liter
$CaCl_2$	0.3 g/liter	Co Cl_2	2.0 mg/liter
$MgSO_4$	0.3 g/liter	Proteose peptone	0.075%
Cellulose	0.75%	Tween 80	0.2%

Initial pH 5.0-6.0

3. Improving the strain by mutation.

Once the organism has been selected and the cultural conditions optimized the best hope of achieving large increases in enzyme yield is by mutation of the strain. Irradiation of conidia of QM6a with high energy electrons from the Natick Laboratories linear accelerator has resulted in a mutant strain QM9123 (dose 0.05 megarads) that produced twice as much cellulase as QM6a (Figure 1). Another strain QM9136 (dose 0.05 megarads) produced no cellulase. Further treatment of strain QM9123 has yielded mutant strain QM9414 (dose 0.10 megarads) that produced even higher levels of cellulase (Table 1). Except for cellulase production these mutant strains closely resembled their parent. The mutants also produced higher levels of protein, most of which was rapidly secreted into the medium (Figure 1). The mutations appear to be stable. Other mutagenic treatments, cobalt 60 gamma irradiation, ultra violet irradiation, and chemical treatment with N-methyl-N-nitro-N-nitroso-guanidine are also being used and the mutation program continues.

4. Pretreatment of the substrate to increase reactivity.

The major problems in optimizing the saccharification process are due to the fact that cellulose is insoluble so that the reaction rate depends on the available surface. Cellulose can be solubilized by introducing substituent groups such as carboxymethyl, hydroxyethyl, or sulfate, and these soluble derivatives were readily hydrolyzed by cellulases from T. viride or other organisms, provided the degree of substitution was below 1.0 (11) but this is not a practical solution to the problem. Many cellulose wastes contain lignin which does not inhibit cellulase, but may limit access of the enzyme to the cellulose. Available surface can be increased by delignification and by swelling in acid or alkali but these treatments reduce bulk density so that suspensions above 4-5% are too thick to stir or pump. More concentrated suspensions are desired so that sugar solutions of reasonable concentration may be attained. The most satisfactory treatment is shredding or grinding followed by ball milling. Ball milling increases both available surface and bulk density so that 20-30% suspensions can be handled. Heating pure cellulose to 200°C in air before or during ball milling increased the efficiency of the process and the initial reactivity of the product (2,3,6). However such heating increases costs and results in formation of some oxidized cellulose that is resistant to hydrolysis. Syrups derived from heated cellulose were very dark colored. When crude cellulose is the substrate, heating might well cause undesirable interactions between the cellulose and impurities. Overall it is preferable to ball mill at or near room temperature, particularly for cellulose wastes and for continuous processes.

Another advantage of ball milling is that it decreases the crystallinity of the cellulose particles thereby increasing reactivity. A comparison of hydrolysis of various materials (Table 3) shows the importance of the physical nature of the cellulose. Cellulose pulp was readily hydrolyzed, but rice hulls and bagasse were scarcely saccharified unless they were first milled or extracted with alkali. Ball milling increased the saccharification of absorbent cotton from 9 to 55% and of cellulose pulp from 27 to 80%. Microcrystalline cellulose (Avicel) was much more resistant than ball milled pulp of the same particle size.

Table 3. Hydrolysis of Cellulose Materials by T. viride cellulase

"Cellulose" 10%	Glucose mg/ml 4 hr	24 hr	48 hr	Saccharification % 48 hr
Absorbent Cotton	-	7.	10.	9.
Cotton, Ball Milled	29.	54.	61.	55.
Avicel PH 102 30μ	14.	30.	36.	32.
Avicel PH 101 90μ	12.	31.	40.	36.
Avicel PH 105 400 mesh (30μ)	15.	32.	42.	38.
Solka Floc (cellulose pulp)	15.	26.	30.	27.
Solka Floc Ball Milled 270 mesh	37.	73.	85.	76.
Solka Floc Ball Milled 350 mesh	39.	83.	86.	77.
Solka Floc Ball Milled 400 mesh	41.	75.	86.	77.
Solka Floc Ball Milled 500 mesh	35.	81.	92.	83.
Rice Hulls	0.8	2.	2.	2.
Rice Hulls Crushed	1.5	4.	4.	4.
Rice Hulls Ball Milled	15.	23.	27.	24.
Bagasse	3.6	6.	7.	6.
Bagasse - Alkali Treated	26.	55.	56.	51.
Bagasse Ball Milled	32.	47.	53.	48.
Newspaper Shredded	-	28.	33.	30.
Newspaper Ball Milled	22.	37.	46.	42.
Corrugated Fiberboard Shredded	-	36.	52.	47.
Corrugated Fiberboard Ball Milled	42.	73.	87.	78.
Paper Mill Waste, Coarse Pulp	24.	43.	47.	42.
Paper Mill Waste, 48 Mesh Pulp	22.	36.	41.	37.
Waste Paper Mill Sludge	22.	38.	39.	35.
Computer Printout Shredded	-	47.	57.	51.
Computer Printout Ball Milled	41.	77.	86.	77.
Milk Carton Shredded	-	46.	57.	51.
Milk Carton Ball Milled	14.	46.	59.	53.

QM9123 cellulase (2 mg protein/ml) pH 4.7 50°C

5. Hydrolysis of pure cellulose in model systems.

We investigated the hydrolysis of pure cellulose in stirred tank reactors of 1-3 liters capacity. Experiments with batch digestion gave results similar to those in shake flasks (Table 3). Continuous digests in single and multistage reactors were carried out by Ghose and Kostick (2,3). After a period of batch digestion to build glucose concentration to the desired level a continuous feed of 10% milled cellulose slurry in unconcentrated M6a filtrate was begun and digest was removed at an equal rate. With a 40 hour retention time, the digest contained 5% glucose and 5% undigested cellulose (3). Higher glucose concentrations in the effluent could be achieved by using higher cellulose concentrations and more active enzyme preparations. Yeasts grew readily on the autoclaved digest (11). This process required large quantities of enzyme and only 50% of the cellulose was saccharified.

An ingenious system was designed by Ghose and Kostick (4) whereby the enzyme and undigested cellulose could be retained in the reactor and the glucose syrup removed through an ultrafiltration membrane of 10,000-30,000 MW cut off. Water, make up enzyme, and dry milled cellulose were added to the reaction vessel to maintain a steady state. With 30% cellulose in the reactor, glucose syrups of 14% were attained. The advantages of this system were (a) the enzyme was retained in the reaction vessel for further use, (b) the undigested cellulose was also retained so that a high percent conversion was achieved, (c) removal of products increased the rate of the reaction, and (d) the glucose was removed as a clear aqueous syrup free of cellulose and protein and containing over 80% reducing sugar on a dry weight basis. However the use of membranes and pressure filtration raised questions as to the economics of the process.

A major improvement in the economic potential resulted from the development of the adsorption reactor (13, Figure 2). The cellulase enzyme is strongly adsorbed by milled cellulose at pH and temperatures optimum for saccharification. As long as cellulose was maintained at 10-20%, 90% of the enzyme was insolubilized. As cellulose was digested the adsorbed enzyme was released and readsorbed on fresh cellulose particles. Glucose syrups could be

removed from the reactor by a coarse filter eliminating the pressure filtration required with membranes. Dry cellulose and buffer or dilute make up enzyme were continually added. When no make up enzyme was added glucose syrups of 5-14% could be continuously removed from 10-20% cellulose reactors for about 4 volume replacements (13) giving yields of glucose in excess of those attained in batch reactions (Figure 2). Dry cellulose was replenished in the batch reactions but there was no removal of syrup or addition of buffer. The advantages of the adsorption reaction were greater for higher cellulose concentrations and more active enzyme preparations. When diluted enzyme (0.1 mg protein/ml) was used in the feed, glucose in the effluent of a 20% cellulose reactor was maintained at 6-10% for 7 weeks (data not shown).

6. Hydrolysis of waste cellulose.

A number of waste materials were successfully hydrolyzed in shake flasks (Table 3). Although shredded paper, milk cartons and corrugated fiberboard at 10% did not form slurries until after 4 hours when considerable saccharification had occurred, they were hydrolyzed. Bagasse and rice hulls required ball milling or alkali extraction to render them wettable and susceptible. Paper mill wastes were hydrolyzed without further treatment. Since these waste materials are not pure cellulose, 100% saccharification is usually not attainable.

Shredded corrugated fiberboard was hydrolyzed in a column of about 2 liters volume (Figure 3), using an initial charge of 200 g suspended in 3 liters of concentrated cellulase (2.4 mg protein/ml) at pH 4.8, 50 C. Elution was with 6 liters of one tenth strength enzyme. Each day the elute was collected through a filter containing fresh substrate equal to the weight of glucose removed the previous day. This was to adsorb any enzyme released by digestion on the column. This substrate plus enzyme was added to the column. A total of 190 g of fiberboard was so added as digestion occurred. From day 5 to 16 500 ml of effluent per day was collected, averaging 2.8% to 48% saccharification of the 390 g of fiberboard used.

Table 4. Hydrolysis of Milled Newspaper in Stirred Reactors

Enzyme Protein mg/ml	Newspaper %	Temp C	Glucose 1 hr %	4 hr %	24 hr %	48 hr %	Saccharification %
0.06	5	50	0.3	0.6	1.6	2.0	36
0.2	5	45	0.5	1.2	2.1	-	39
0.2	5	50	0.7	1.3	2.1	-	39
0.7	5	50	1.0	2.0	2.8	-	50
0.9	5	45	0.6	1.4	2.5	-	45
0.9	5	50	1.3	1.9	2.2	-	40
0.06	10	38	0.3	0.4	1.0	1.2	11
0.06	10	50	0.3	0.6	1.7	2.0	18
0.06	10	60	0.4	0.8	1.4	1.5	14
1.0	10	50	2.1	3.1	5.5	7.3	66
1.6	10	45	2.0	3.6	5.4	6.5	59
1.6	10	50	2.3	4.2	6.4	6.3	57
0.6	15	45	0.7	1.7	2.8	-	17
0.6	15	50	0.7	1.9	3.2	-	19
0.8	15	45	1.5	2.8	5.3	7.7	46
0.8	15	50	0.8	2.8	6.1	6.3	38
1.8	15	50	3.2	6.0	8.6	10.0	60
0.06	20	50	0.4	0.9	1.5	3.1	14

Reactor Volume 1 Liter Stirred 60 RPM pH 4.8

Milled newspaper was digested by cellulase in stirred tank reactors (Table 4) with 11-66% saccharification in 24-48 hr yielding glucose syrups of 1-10% concentrations. Ink, lignin, and other impurities did not cause apparent problems. The residue after hydrolysis was a black sticky material that dried to a hard unwettable cake. Continuous hydrolysis of milled newspaper was also carried out in a stirred tank reactor (Figure 4). The initial charge was 10% milled newspaper in cellulase (0.7 mg protein/ml) and dry newspaper was continuously fed to maintain the concentration near 10%. After 24 hours enzyme feed was begun and digest removed at an equal rate. With a retention of 24 hours 4% glucose was maintained in the effluent.

DISCUSSION

Enzymatic saccharification of waste cellulose as an aid to pollution control is technically feasible and the economic outlook is hopeful. The cellulase produced by Trichoderma viride is very active and remarkably stable. It is not inhibited by biocides and impurities in waste materials. The enzyme yield has been increased by mutation. The broth from a submerged fermentation can be used directly as enzyme with no processing other than filtration and pH adjustment. Pretreatment of the substrate by delignification and ball milling yields maximum reactivity. Immobilization of the enzyme by adsorption on pure milled cellulose permits a continuous process with marked economy of enzyme utilization. Reactors and columns have been operated continuously at 50°C for several weeks with minimal enzyme replenishments. A variety of waste cellulose materials including paper and card board cartons were readily saccharified.

Cellulose is our most abundant organic compound. Net annual production world wide is estimated at 10^{11} tons. Municipal waste in the United States is about 500 pounds per person per year and 40-60% of this is cellulose. Our immediate objective is alleviation of pollution. Our long range objective must be to consider waste cellulose as a resource which we can not use up because it is annually replenished. If enzymatic conversion of cellulose is adapted for waste disposal large quantities of glucose will be produced. Although glucose from cellulose is a very attractive substrate for production of single cell protein, we believe that of greater military interest is the probability that cellulose will eventually have to replace hydrocarbons and coal as a source of organic chemicals and fuel (5).

In the future nuclear energy and solar energy will presumably be converted to electricity which will run our industry and our households. But we shall still require fuel for the internal combustion engine as a free moving power source. The United States as a major consumer and importer of fossil fuels will feel this need sooner and more acutely than other nations, particularly if war comes. A realistic source of such fuel is cellulose. One way to convert cellulose to a clean burning fuel is by enzymatic conversion to glucose followed by fermentation to ethanol.

It is true that today it is cheaper to produce ethanol and other chemicals from hydrocarbons than by fermentation. Such economics are unrealistic, especially since the costs of pollution are not included, and can not long continue. The main reserves of oil are concentrated in a small and politically delicate area in the Middle East. Since 1970 collective pressure applied by the oil producers particularly the Teheran and Tripoli deals of 1971 have forced prices up steeply (16). This trend will surely continue.

Acknowledgement

We are grateful to Dr. Elwyn Reese whose research on cellulase provided the foundation for our studies, to Dr. J. Fred Oesterling and Dr. Durwood Rowley who have always supported and encouraged our work, and to Mr. John Kostick for technical assistance. Some of the data in Table 4 was collected by Mr. Randy Partridge and Mr. George Oliver of Worcester Polytechnic Institute while working in our laboratory on a student project.

REFERENCES

1. Callihan, C. D. 1970. Chemical Engineering. 160-164.

2. Ghose, T. K. 1969. Biotechnology Bioengineering 11, 239-261.

3. Ghose, T. K., and J. Kostick. 1969. Advances in Chemistry 95, 415-446.

4. Ghose, T. K., and J. Kostick. 1970. Biotechnology Bioengineering 12, 921-946.

5. Hubbert, M. K. 1971. Scientific American 225, Sept. 61-70.

6. Katz, M., and E. T. Reese. 1968. Applied Microbiol. 16, 419-420.

7. Mandels, M., and E. T. Reese. 1957. J. Bact. 73, 269-278.

8. Mandels, M., and E. T. Reese. 1960. J. Bact. 79, 816-826.

9. Mandels, M., and E. T. Reese. 1964. Developments in Industrial Microbiology 5, 5-20.

10. Mandels, M. and E. T. Reese. 1965. Ann. Rev. Phytopathology 3, 85-102.

11. Mandels, M., and J. Weber. 1969. Advances in Chemistry Series 95, 391-414.

12. Mandels, M., J. Weber, and R. Parizek. 1971. Applied Microbiol. 21, 152-154.

13. Mandels, M., J. Kostick, and R. Parizek. 1971. J. Polymer Science Part C. 36, 445-459.

14. Reese, E. T. and A. Maguire. 1971. Developments in Industrial Microbiology 12, 212-224.

15. Shapley, D. 1971. Science 175, 45-46.

16. Smith, W. D. 1972. New York Times Financial Section, page 1. Sunday Feb 27.

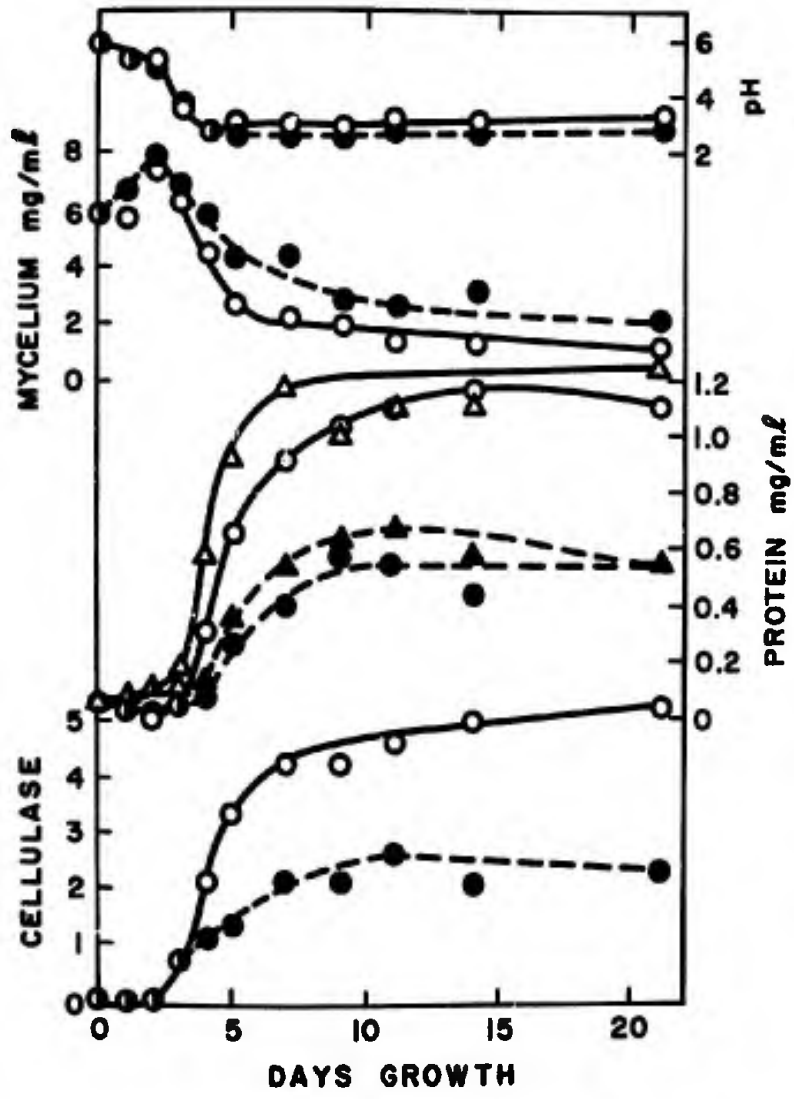

Figure 1. Growth of Trichoderma viride on cellulose

●-----● ▲-----▲ QM6a
o——o Δ——Δ QM9123

Protein soluble ●, o, total ▲, Δ

Mycelial weight includes undigested cellulose.

Cellulase = mg glucose from filter paper by 1 ml culture filtrate in 1 hr 50°C

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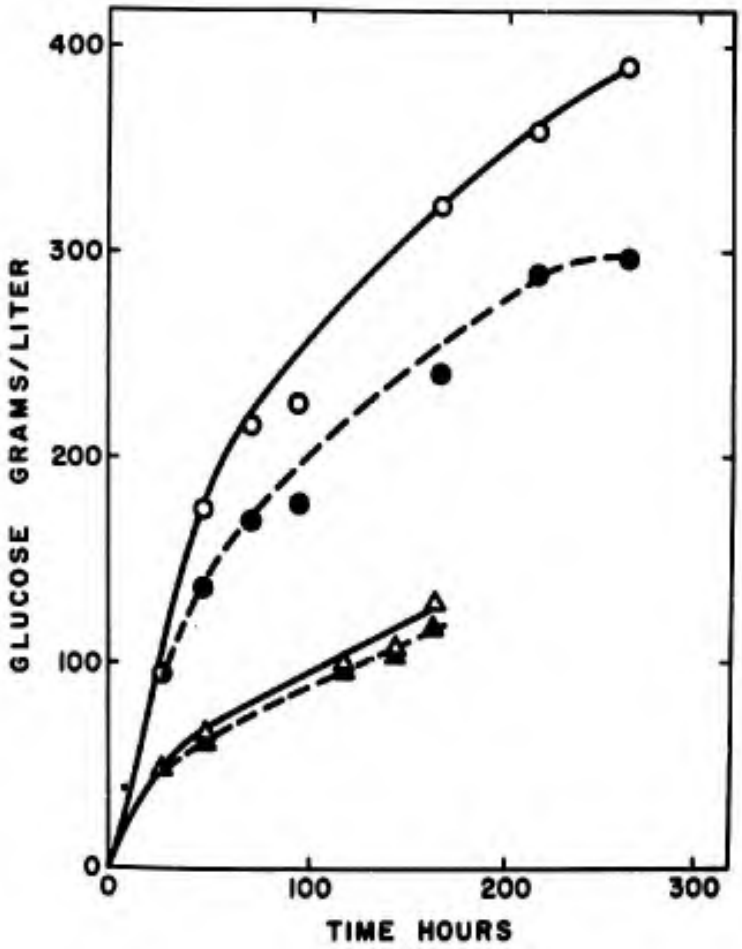

Figure 2. Continuous hydrolysis of pure cellulose in adsorption reactor.

Δ——Δ Continuous 10% milled cellulose in unconcentrated cellulase (0.7 mg protein/ml). Solids retained. No enzyme in feed - 10 ml/liter x hr. Exp. 1.

▲-----▲ Batch digestion - Cellulose replenished Exp. 1.

o——o Continuous 20% milled cellulose in concentrated cellulase (1.8 mg protein/ml) Solids retained. No enzyme in feed- 17 ml/liter x hr. Exp. 2.

●-----● Batch digestion - Cellulose replenished. Exp. 2.

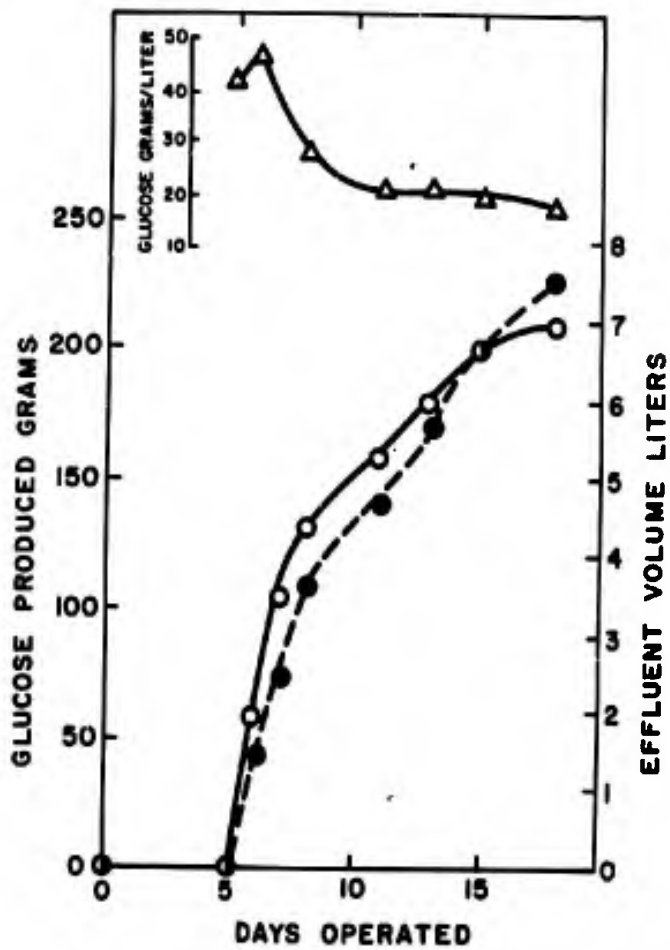

Figure 3. Hydrolysis of shredded corrugated fiberboard in a column

Δ-----Δ Glucose concentration in effluent g/liter

o——o Total glucose yield grams

●-----● Effluent volume liters

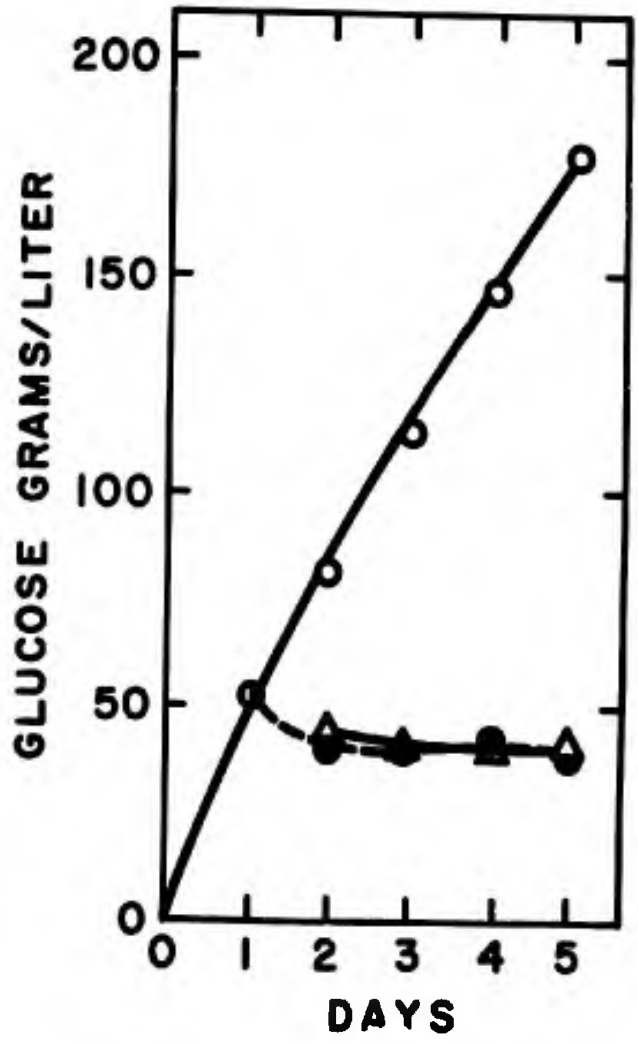

Figure 4. Continuous hydrolysis of newspaper in stirred tank reactor.

Δ——Δ Glucose concentration in effluent g/liter

●-----● Glucose concentration in reactor g/liter

o——o Total glucose yield grams/liter

10% ball milled newspaper in cellulase (0.7 mg protein/ml) Feed 41 ml/liter x hr. Harvest slurry.

END