

A Model for Continuous Enzymatic Saccharification of Cellulose with Simultaneous Removal of Glucose Syrup*

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Summary

Cellulase of *Trichoderma viride* was concentrated in various molecular cutoff membranes, and flux rates and retention of activity were studied under ultra-filtration conditions. Little or no cellulase was discharged through the membranes tested. The concentrated (5-8-fold) enzymes were used to saccharify finely ground substrate (Solka Floe) in stirred tank (STR) and membrane reactors (MR). A pressure filtration vessel provided with a membrane for simultaneous removal of low molecular weight products (glucose) from the reacting system (cellulose-cellulase) is designated as a membrane reactor. Continuous digestion of dense cellulose suspension in the membrane reactor was achieved. Using PM-30 (Amicon) membrane reasonably high mass flux values (9.7-23.3 gals/ft²-day) were obtained in separating glucose from a digest of 30% cellulose suspension. Abcor membrane (HFA 300) was equally effective and necessitated less care in handling. Nearly 14% glucose concentration has been achieved in less than 50 hrs in STR by digesting a 30% cellulose suspension. Based on experimental data a model system is proposed for the continuous steady state saccharification of ground substrate in which there is continuous removal of concentrated glucose syrup, and a feedback of enzyme.

INTRODUCTION

Previous studies on enzymatic saccharification of cellulose^{2,3} have demonstrated the possibility of continuous conversion of the substrate

* Presented at the ACS Symposium on Industrial Microbial Enzymes, New York, N. Y., U.S.A., September 7-12, 1969.

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into a product containing 5% glucose over a hold-up period of 40 hours. A higher concentration of glucose has also been obtained by reacting vacuum concentrated cellulase with finely ground and heated cellulose over a period of several days.⁴

Two important factors seem to be responsible for not making the results of cellulase research attractive for commercial utilization. These are (a) the low level of activity of the cellulase available up to now and (b) the resistant nature of the cellulytic substrate. Until a new and useful approach is discovered which will enable the cellulosic fibrils to rapidly open up to the subsequent hydrolytic cleavage, mechanical rupture of the fiber and extension of reactive surface by milling appears to be the only possible method to render cellulose to rapid hydrolysis.

There has been a tremendous interest at present⁵ in the application of the new molecular sieve membranes to the purification and concentration of enzymes, proteins and biological materials^{8,9} and in the separation of microsolutives.¹⁰ However, studies on cellulase concentration on molecular sieves and their effects on the accelerated kinetic pattern of the cellulose-cellulase system have yet to be reported. Because of the availability of several kinds of polymeric membranes with a wide range of molecular cutoff capabilities for separating microsolutives from aqueous solutions and dispersions, most works refer to flux rates through such membranes in relation to the molecular sizes and membrane affinity of the solutes retained. Theories and various applications of ultrafiltration techniques as a potential approach toward complex separation problems involving macromolecules have been developed.⁶ Use of molecular sieve membranes in the concentration of *Trichoderma viride* culture filtrate has, therefore, been extensively studied. The high permeation rates of these membranes permitted separation of low molecular products from the reaction mixtures having high solid contents. Results of these studies provide a novel and fascinating approach toward rapid and complete hydrolysis of cellulose into glucose and, at the same time, recovery of the products from the reaction mixture free of either substrate or enzyme. Perhaps for the first time, an approach is made to re-use the enzyme separated from the products, on fresh and insoluble substrate, thus making use of its catalytic capabilities to the maximum extent.

MATERIALS AND METHODS

Substrate and Its Preparation

Solka Floc-SW40A (Brown Co., Berlin, N. H.) a kind of wood (spruce) pulp used in earlier studies^{5,6} was employed in the present investigation. The material was dry milled in a laboratory porcelain pot mill or in a Sweco (Southwestern Engineering Co., Inc., Los Angeles, Calif.) vibroenergy mill model FM-1 HA 1 cft. The dry milled product was screened down to several fractions, -270, -325, -400, and -500, using Tyler standard screens which corresponded to particle size of less than 53, 44, 37 and 25 microns, respectively. In a few cases the -500 mesh product was heated at 200°C in a running porcelain pot mill for 25 mins, cooled and stored in air tight containers to keep dry. Very fine pulverization of a few samples of substrate was carried out in 2" disc 304 stainless steel Sturtevant Micronizer Fluid Energy Mill (Sturtevant Mill Company, Dorchester, Mass.) with a feed rate of $\frac{1}{2}$ lb per hour of a 72-hour pot milled fine Solka Floc ($<12\mu$) using air at 30 cfm and at 80 psi injection pressure. This operation brought the <12 micron feed down to <4.6 micron in a single run. Measurement of particle sizes of these fine materials was done with a Fisher Sub-sieve Sizer (Sl. No. 319, U. S. Pat. No. 2261802, Fisher Scientific Company, Pittsburgh, Pa.).

Enzyme and Its Assay

Cellulase enzyme employed in the studies described here was obtained from a *Trichoderma viride* (Tv) mutant (QM 9123) produced by irradiation of the parent strain (Tv QM 6a) in high energy electron beam Linear Accelerator.⁷ The method of producing culture filtrates from this strain has been described.¹ Assay procedures using Filter Paper (FP) and Carboxy-methylcellulose (CMC) described earlier² have been followed in the present work.

Reducing Sugars

Estimation of total reducing sugars (RS) as glucose has been based on the DNS method.²

Slurry Reactor

Five (or three) liter fermenter vessels (New Brunswick Scientific Co., New Brunswick, N. J.) with operating volumes of 3.0 and 1.5

liters, respectively, were used as saccharification units for batch, semi- and fully continuous studies. The reactor was kept in a 50°C (± 0.5) water bath and the reacting slurry inside was kept under agitation by means of motor driven stainless steel propeller agitators at speeds between 250–350 rpm. Thick suspensions of cellulose in enzyme (10–30% v/v) were studied in these units in both batch and continuous manner. Cole Parmer Masterflex peristaltic pumps were used for transporting enzymes into and slurries out of the system. For continuous reactions, finely ground substrate was supplied to the reactor by means of a mechanical solid feeding device at pre-set rates. The solid feeder (BIF Omega Disc Feeder Model No. 2201, New York Air Brake Co., Providence, R. I.) delivery port is connected with the reactor (5.1) by means of a stainless steel funnel and a flexible socket. A bag of dessicant suspended under the cover of the vessel kept the cellulose dry. The delivery port of the feeder drops the fine solids on a hemispherical stainless steel screen with large openings and provided with procelain balls ($\frac{1}{2}$ " dia.). The balls are agitated by vibration of the stainless steel funnel and allow the ground cellulose to drop into the reactor at a predetermined rate. This, in turn is rigidly connected with the neck of the funnel of equal diameter. The entire component consisting of the S.S. screen, the S.S. funnel and the flexible socket is kept under a steady vibration (about 150 cy/min) in order to allow an easy and rapid transport of the fine cellulose powder from the feeder into the reactor without any buildup at any point. The rates of solid delivery from the feeder are calibrated against the digital scale setting of the unit.

Molecular Sieve Membranes

Diaflow ultrafiltration membranes are specific molecular weight cutoff filters developed from synthetic hydrated polymers designed for rapid concentration, purification, and fractionation of biologically active molecules including living cells like bacteria and viruses. All membrane transport processes have the common features of (a) transport of mass by the action of free energy driving force, and (b) the capacity to alter mixture composition on account of the ability of the membrane barrier to pass one component more rapidly than another despite equality of driving potential. This unique character of polymeric film separation differentiates this process from most common separation operations. The membranes used, obtained from

Amicon Corp., Lexington, Mass., and Abcor, Inc., Cambridge, Mass., represented a total range of 2000–50,000 molecular weights. Molecules of a given size and dimension are retained, whereas those below the membrane cutoff level are passed. The membranes of 2.75" and 5.25", i.d., specified by Amicon as UM 2, UM 10, PM 30 and XM 50 membranes and representing molecular weight cutoff values of 2000, 10,000, 30,000 and 50,000, respectively, were used in the studies. Other membranes used in the studies were designated by Abcor as HFA 200 and HFA 300 representing a true cutoff value of 20,000 and 30,000 molecular weights, respectively. These membranes are fabricated from cellulose acetate. Under the conditions of operation (except UM 10 which was found to be very sensitive to damage) all the membranes showed no sign of deterioration even after long use. Ultrafiltration rates are functions of solid contents, apparent viscosity, total soluble sugars, and density of the slurry system. Because each system contained merthiolate as preservative, the membranes were not exposed to microbial contamination. Membranes were not sterilized before or after use.

Membrane Reactors

The membrane reactor is essentially an ultrafiltration cell with heat transfer controlled and temperature maintained at 50°C either by wrapping heating tape around the cell (Amicon Model 400) or by placing the entire filtration unit (Amicon Model 2000) in a water bath. In each case a margin of about $\frac{1}{16}$ " is maintained between the membrane and the stirring bar. Both batch filtration and diaflow ultrafiltration of various systems involving cellulase, cellulose-cellulase suspensions, and dilute and concentrated glucose solutions were carried out under constant pressure of 42–44 lbs. per square inch and pre-set volumes.

EXPERIMENTAL

Concentration of Tv Cellulase in Molecular Sieve Membranes

Several initial tests on diaflow ultrafiltration of de-ionized water, mineral Tv medium with and without Tween 80 and Tv culture filtrates were carried out in order to ascertain flow characteristics and suitabilities of these membranes for cellulase concentration. These membranes include molecular cutoff values of 10,000, 20,000 and

30,000. Ultrafiltration rates of various fluids through these membranes are illustrated in Figure 1. Subsequently, the membranes were tested in respect to their flux rates for glucose solutions of various concentrations (Figs. 2 and 3). These tests were conducted in the Amicon Ultrafiltration (UF) Model 400 cell using membranes of 75

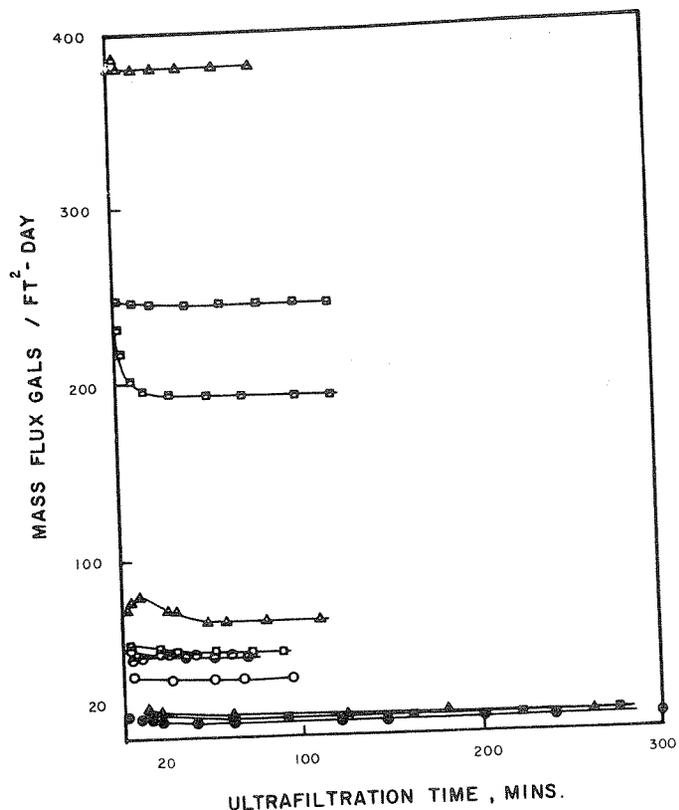


Fig. 1. Characteristics of UM (10,000 cut off), HFA 200 (20,000 cut off) and PM 30 (30,000 cut off) membranes in respect to various fluids. UM 10 [(⊙) deionized water, (○) Tv medium, (○) Tv medium + 0.1% Tween 80, (●) Tv cellulase (0.74 FP)]. HFA 200 [(⊙) deionized water, (⊙) Tv medium, (□) Tv medium + 0.1% Tween 80, (■) Tv cellulase (0.72 FP)]. PM 30 [(▲) Tv medium, (△) Tv medium + 0.1% Tween 80, (▲) Tv cellulase (0.78 FP)].

Note: 400 ml of the fluids were ultrafiltered through the membrane (76mm) cell containing initially 100 ml at constant volume at 42-44 lbs. sq. in.

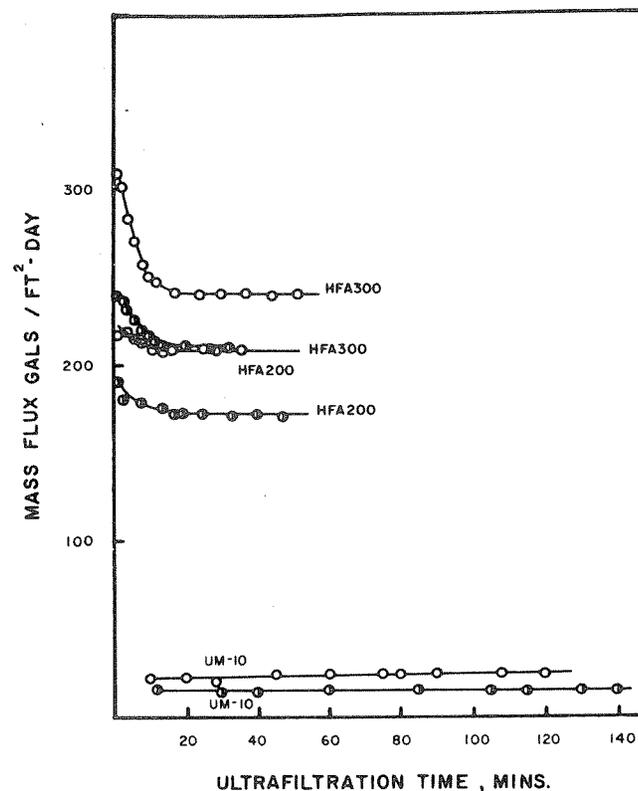


Fig. 2. Ultrafiltration of various concentrations of glucose through 10,000, 20,000 and 30,000 cutoff membranes. (○) 5% glucose solution. (●) 10% glucose solution. Membranes tested: UM 10 (10,000), HFA 200 (20,000), HFA 300 (30,000).

Note: 400 ml of the glucose solutions were ultrafiltered through the membrane (76 mm) cell containing initially 100 ml at constant volume at 42-44 lbs/sq. in.

mm diameter. Generally, 400 ml of the specific fluid under test was taken in a stainless steel pressure supply reservoir and 100 ml in the UF cell with the membrane in position at 42-44 lbs/sq in. N₂ gas pressure.

The results of these experiments show the effect of a small amount of surface active agent (Tween 80) in the Tv medium on the UF rate. For UM 10 membranes the flow rate of Tv medium containing

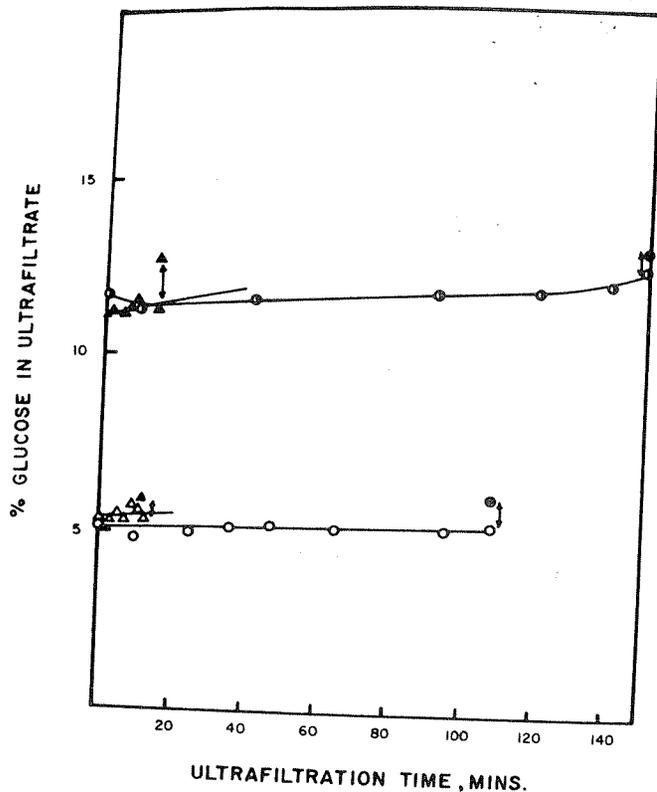


Fig. 3. Rate of glucose removal in ultrafiltration through 10,000 and 30,000 cutoff membranes. (○) 5.12% initial glucose concentration on UM 10 membrane. (△) 5.3% initial glucose concentration on HFA 300 membrane. (●) 11.8% initial glucose concentration on UM 10 membrane. (▲) 11.12% initial glucose concentration of HFA 300 membrane. (●) Designate concentration of films respectively.

Note: 400 ml of the glucose solutions were ultrafiltered through the membrane (76 mm) cell containing initially 100 ml at constant volume at 42-44 lbs/sq. in.

0.1% Tween 80 is about 70% that of Tv medium without Tween 80. For HFA and PM 30 membranes Tween reduces the rate to 25% that of the control. The capabilities of higher molecular cutoff membranes for high flux rates appear to be considerably reduced in

the presence of strong surface active agents which apparently block the membrane pores, and interferes with the rejection mechanism. In the case of lower cutoff range membranes the interference is less pronounced. This is perhaps due to the fact that Tween 80, having a molecular weight of 1200 gets into the pore channels of the membranes much more easily and remains adsorbed there. However, the main reason for a greater difference in the mass flux in the higher cut off membranes relative to the lower ones may be attributed to the structural features of these films. This property may widely affect the adsorption coefficients of the films toward specific surface active agents like the Tweens. Further, since the flow rate varies as the square of the channel diameter, decrease of the channel diameter by half reduces the flux by a factor of four. Recovery of cellulase activity achieved by HFA 200 and HFA 300 membranes are shown (Fig. 4). Although the enzymes were concentrated five fold, and no activity was detected in the effluents, the % recovery as measured by either FP activity of C_x units is far less than 100% (Table I). It also includes recovery data for UM-10 and PM-30 membranes. The zero values of activity in the filtrate indicate no passage of cellulase through the membranes.

TABLE I
Recovery of Cellulase Activity in Membrane Concentrated Enzyme*

Membrane type	Membrane cutoff	Initial activity		Final activity		Activity in filtrate		% recovery	
		FPx 100	Cx	FPx 100	Cx	FP	Cx	FP	Cx
UM 10	10,000	74	36	164	120	—	—	44.3	56.7
HFA 200	20,000	72	32	168	80	—	—	46.7	50
PM 30	30,000	84	48	168	100	—	—	40	41.7
HFA 300	30,000	160	41	270	104	—	—	33.7	50.7

* 5-Fold concentration achieved in ultrafiltration system at 42-44 lbs/sq. in. cell pressure.

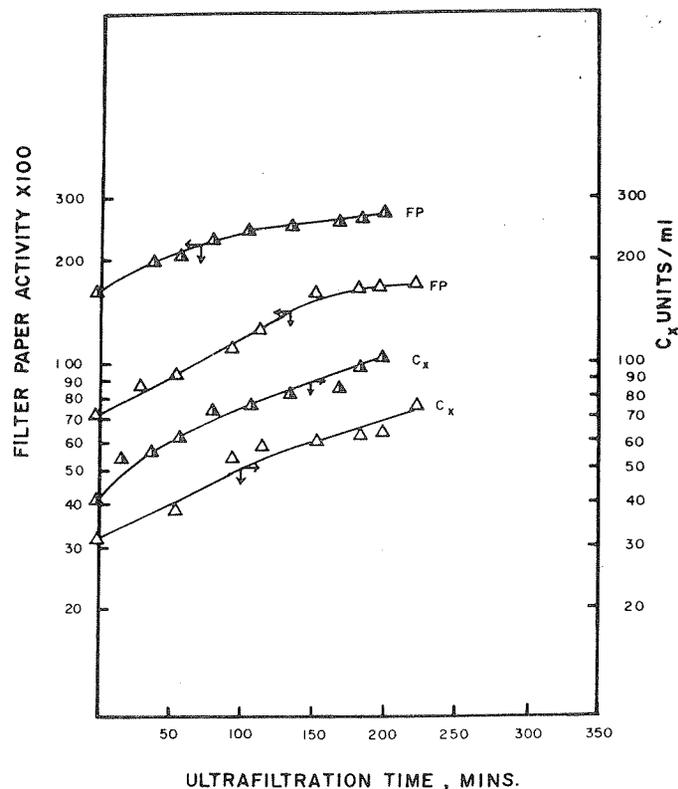


Fig. 4. Concentration of cellulase activity by ultrafiltration through HFA 200 and HFA 300 Membranes. (Δ) HFA 200 membrane. (\blacktriangle) HFA 300 membrane.

Note: 400 ml of the Tv culture filtrate were ultrafiltered through the membrane (76 mm) cell containing initially 100 ml at constant volume at 42–44 lbs/sq. in.

Effect of Substrate Particles Size in Dense Suspensions on Initial Reaction Rates

For insoluble substrate like cellulose, the extent of surface exposed to enzyme action is a more important rate controlling parameter than concentration (w/v). Fine grinding exposes an extensive surface and decreases the crystallinity. This point has been discussed³ on the basis of x-ray diffraction scans of dry milled Solka Floc sam-

ples. However, in view of the subsequent studies made on concentrated cellulase-finely ground cellulose systems, some data on the extent of conversion possible in the first 24 hours were necessary. The four different types of cellulose employed in the studies represent (a) Sokla Floc ($> 80\%$ of $< 149\mu$), (b) laboratory pot milled SF ($< 12\mu$), (c) Sturtevant Micronizer milled SF ($< 4.7\mu$), and (d) the latter heated SF-MH ($< 4.7\mu$), all in 10% consistency (5 ml enzyme containing 0.5g of the substrate). Tv QM 6a culture filtrate used in the experiments was previously concentrated (8-fold) over UM 2 and it represented a cellulase activity of 380 Cx units/ml. The rate profiles (Fig. 5) show a tremendous difference in the rate of saccharification on account of the difference in particle size and the mechanical and thermal effects on the breaking up of H-bonds of cellulose microfibrils. At the third hour of contact the % sugar produced from SF is about 42% of SF-M ($< 12\mu$), 32% of SF-M ($< 4.7\mu$), and about 26% of SF-MH ($< 4.7\mu$), while the corresponding values at the 48th hour are 69%, 62% and 56%, respectively. The SF particles were also found to be totally disintegrated and dispersed beyond 3 hours of contact with the highly active enzyme system. Comparing the slopes of the initial conversion rates for the three different fine substrates (b, c and d) it is evident that they follow first order reaction because of the predominance of principally reactive cellulose for its hydrolytic conversion into glucose.

Continuous Saccharification in Stirred Tank Reactors with Independent Substrate and Enzyme Streams Entering the Reactors

In both of the earlier communications^{2,3} techniques of continuous saccharification experimental systems were presented and their results discussed. Even though the feed slurry in those experiments was kept at 1–2°C, there was an appreciable initial reaction resulting in an output of about 0.5% glucose in the feed stream before entering the reactors. Although this small amount of glucose does not affect the reaction rate to any noticeable extent, the dry and highly active surface of the substrate particles is lost due to the cellulase being adsorbed on its surface at a lower temperature. Further, Tv submerged culture filtrates have, generally, been found to lose some of their activity while in storage in the presence of merthiolate and separation of sediments has been noticed.

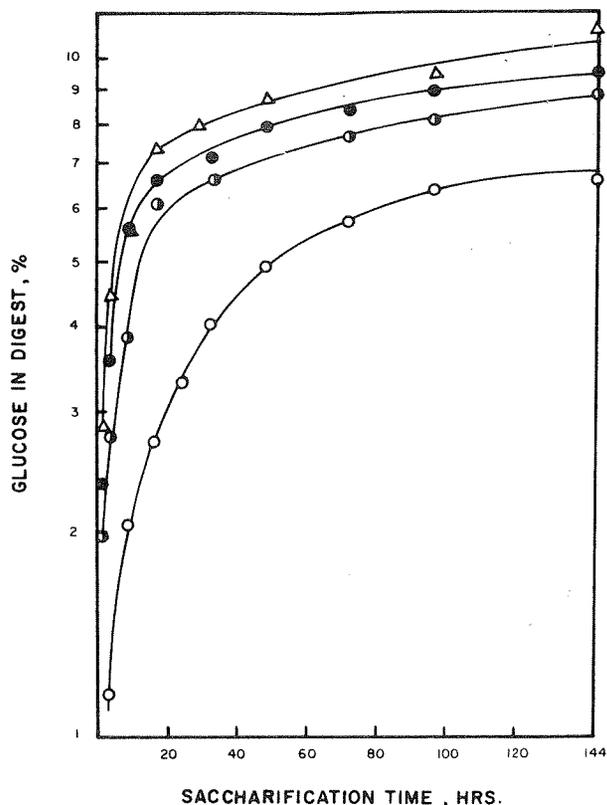


Fig. 5. Effect of substrate particle size in dense suspensions on initial reaction rates. (○) SF (> 80% of < 149 μ); (◐) SF-M (< 12 μ); (●) SF-M (< 4.6 μ); (Δ) SF-MH (< 4.6 μ).

Note: Saccharification tests were carried out in 5 ml test tubes using membrane (2000 cutoff) concentrated cellulase (380C_x units/ml) at 50°C.

In order, therefore, to allow dry and active substrate particles to come in contact with the enzyme solution only at the reaction temperature (50°C), solid milled substrate and Tv culture filtrate (QM 9123) were allowed to enter the reactor as independent streams in the study of a series of continuous saccharification reactions. Feeding of solid substrate was made by a Disc Feeder (described before) at different digital settings. A 10% (v/v) substrate (300 gms of -37 μ

SF-M) suspension^{3,2,1} was prepared using 3.0l of Tv culture filtrate (pH 5.2, FP activity 1.8) in a pyrex STR. Following 47 hours of batch run 1750 ml of the digesting slurry was removed and an equal volume of enzyme plus 150 gms of the substrate were added into the reactor. At the 47th hour the sugar content was checked as 4.09%. The batch system was allowed to run 48 hours more and its glucose and pH were checked as 4.38 and 5.2, respectively. At this point feeding of substrate at 5.07 gms/hr (digital setting 15.0), and enzyme at 100 ml/hr, and removal of saccharified slurry at 100 ml/hr was started. Steady flow of input and output streams were maintained at the specified rates during the next 79 hours and the effluent sugar contents gave a range of 3.87-4.03%. After 174th hour the solid feeder contents were quickly removed and replaced by a batch (1156g) of dry SF-MH (-37 μ). Input and output streams were immediately resumed and the process continued for the next 90 hrs. All the reaction parameters were checked as before. In the next half hour the solid feed rate was changed to 13.2 g/hr (digital setting 30) and the process continued keeping the enzyme feed rate as before. Between 198th and 263rd hours, the glucose contents of the reaction mixture ranged between 4.06-4.23%. Following the increased feed rate of solid substrate, the continuous reaction was further continued during the next 120.5 hours; and the glucose contents of the streams gave values ranging between 4.29-4.67%. Beyond the total reaction period of 385 hours comprising 95 hours of batch run and 290 hours of continuous run, the saccharification was continued again as a batch system without any input or output streams during a further period of 48 hours. The sugar content of the system showed a progressive rise from 4.64% at the 383rd hr. to 5.48% at the 407th hour, to 6.07% at the 431st hour. Effluent samples were collected in 10 ml volume and after centrifugation the supernatant was used for sugar determination and the solids repeatedly washed to remove soluble fractions, dried for 3 hours at 105°C and dry weights determined. These values gave amounts of unreacted cellulose (S₁) and percent conversion values of cellulose to sugars were calculated as a ratio of S₀ - S₁ to the actual initial concentration (S₀) of cellulose. Results, excluding the initial period (95 hours) of batch saccharification, are presented (Fig. 6) for glucose content of the slurry, percentage conversion of cellulose to glucose and pH level of the system.

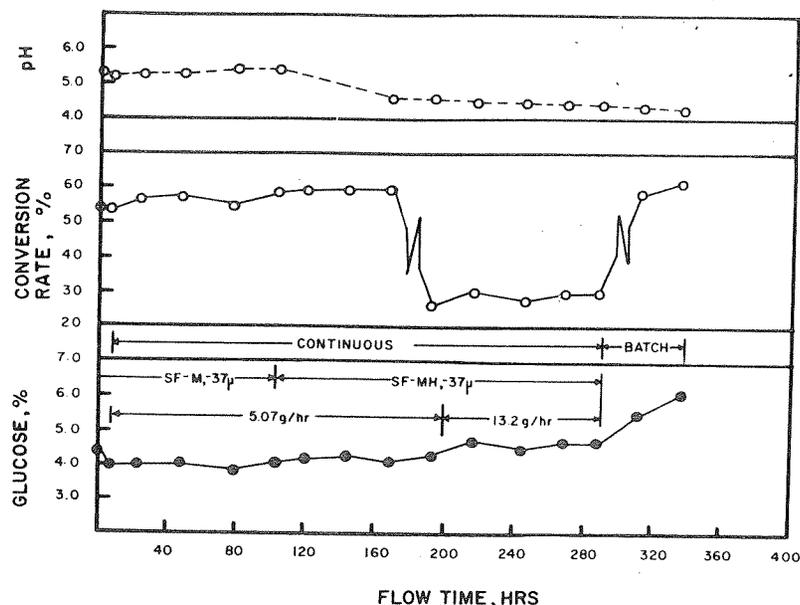


Fig. 6. Continuous saccharification in stirred tank reactor with independent substrate and enzyme streams entering the reactor. Initial suspension strength = 30%; feed consistency 5.07% of -37μ size SF-M substrate up to 198th hour, followed by 13.2% suspension in the next 90 hours of continuous phase. In the first 102 hours of continuous phase feed was SF-M (-37μ) and in the next 186 hours feeding was done with SF-HM (-37μ). Reaction temperature = 50°C . Cellulose = 1.8 FP activity.

Continuous Saccharification in Membrane Reactors

Polymeric molecular sieve membranes have been used in several areas of separation, concentration, purification dialysis and reverse osmosis studies. But no work on the separation of reaction products from a dense solid suspension with a continuously diminishing particle size has been reported. The cellulose-cellulase system is such a typical, but even more interesting case because certain membranes are capable of rejecting the entire amount of cellulase present in the system while the low molecular weight reaction products pass out as long as the membrane channels are not plugged. Such membranes also show good flux rates for aqueous solutions of glucose for fairly high concentrations (Fig. 3).

A batch of 1050 ml of a 30% milled Solka Floc ($<37\mu$) suspension in membrane (UM 10) concentrated (8-fold) cellulase (FP-3.6) was allowed to run at 50°C for 46 hours 500 ml of the digest was then transferred into an Amicon 2000 model ultrafiltration unit furnished with a UM-10 membrane, the cell pressurized with N_2 at 44 lbs/sq. in. and the contents flushed with deionized water under agitation. The remainder of the digest was stored in a refrigerator at 2°C . The initial glucose content of the digest was 14.06% which was brought down to a value of 2.95% through flushing over a period of 520 minutes. The operation of flushing was repeated under exactly the same conditions using HFA 200 and PM 30 membranes in order to evaluate the effectiveness of the three membranes with respect to their ultrafiltration and glucose separation rates under dense suspension conditions. The results (Fig. 7) reflect the membrane characteristics—generally higher flux rates and greater speed of sugar removal with higher molecular cutoff values. Penetration of glucose and perhaps cellobiose seems to be better accomplished through HFA 200 membranes than the other two films. It is clear that separation of reaction products of comparatively low molecular weights from a highly dense cellulose-cellulase system free of enzyme and substrate is possible under agitated condition without damage to the membrane. Subsequently, a continuous saccharification system (membrane reactor) was set up using a model 400 Amicon cell as the reactor vessel which is furnished with a HFA 200 membrane (76mm diameter). A flexible heating tape ($\frac{1}{2}$ " wide, 110V, 480 watts) was wrapped around the polycarbonate cylinder. The input was controlled by a Honeywell Thermister Sensor with a temperature probe connected with the reactor contents which was kept at 50°C . 200 ml of a 20% milled cellulose ($<37\mu$) -cellulase (FP-2.16) suspension which was predigested at 50° for 46 hours was pumped into the membrane cell and kept under agitation. The reactor was then pressurized up to 46 lbs/sq in. and was connected with a prepressurized (50 lbs/sq in.) agitated reservoir containing a 10% aqueous suspension (0.098 gm/ml) of the same substrate. As in a normal ultrafiltration system the reservoir (substrate slurry supply vessel) and the membrane cell (the reactor) were connected with an N_2 cylinder and desired pressures were maintained in the two units. Flow of substrate slurry into the reactor continued at the same rate as the aqueous solution of reaction products (sugars) was discharged

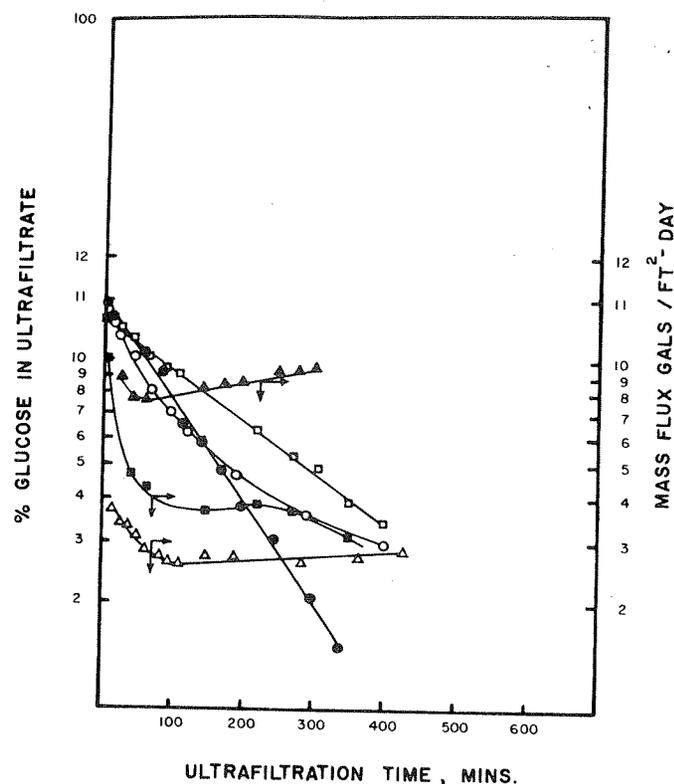


Fig. 7. Recovery of sugars from dense suspensions of digested cellulose in concentrated cellulose. UM 10 [(○) % glucose; (△) mass flux]. PM 30 [(●) % glucose; (▲) mass flux]. HFA 200 [(□) % glucose; (■) mass flux].

Note: 500 ml of digest (cellulose-sugar-enzyme-water reacted at 50°C) were flushed with distilled water over different membranes at room temperature.

by the cell. Soon equilibrium conditions were attained for both flow and reaction rate. The results on sugar level and flux rates are presented (Fig. 8). From the plot of glucose concentration in the effluent the average value is around 7.5% and based on equal input and output rates, the average rate of removal of sugars from the membrane reactor is 0.074 g/min. The average percentage conversion of 76.6% is, therefore, fairly high. After an operation of over 8 hours with almost steady flux rates the process was stopped and the reactor opened (490th minute). Analyses showed a total

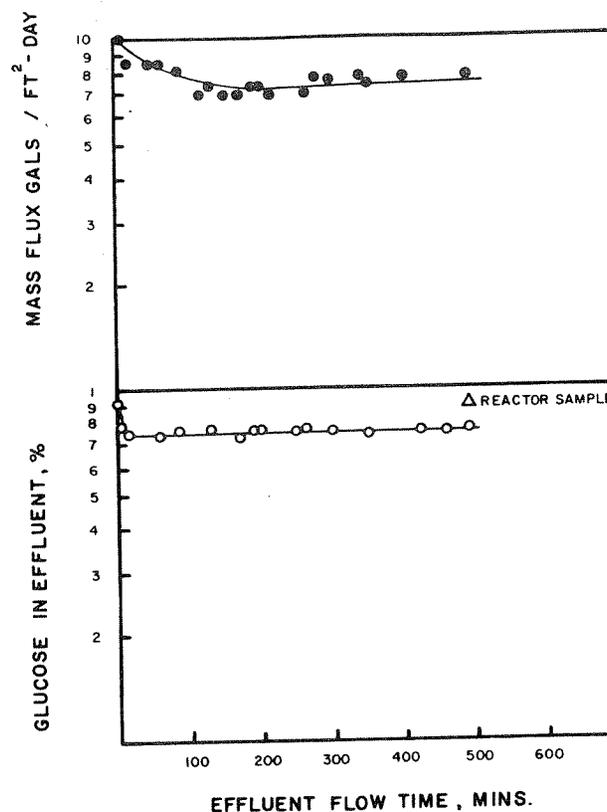


Fig. 8. Continuous saccharification in membrane reactor.

Note: Membrane used = HFA 300 (76 mm) reaction temperature 50°C; reaction volume 200 ml; average mass flux = 0.993 ml/min; suspension strength = 0.098 gm/ml.

sugar 8.93%; the corresponding effluent sample at this point showed a sugar value of 7.64%.

A Model System for Cellulose Saccharification in Stirred Tank Reactor and Removal of Glucose Syrup in Membrane Cell

The possibilities of concentrating cellulase, ultrafiltration of high concentrations of glucose solutions, separation of sugars (glucose-cellobiose) from dense digest of cellulose-cellulase slurries through

polymeric membranes of different types having been established, a model was developed combining STR of dense cellulose-cellulase system and separation of reaction products from the circuit. The concept is based on coordination of rapid rate of reaction of a high solid suspension in concentrated enzyme with rapid rate of removal of products from the system soon after they are formed, free of substrate and enzymes and feedback of the concentrated enzyme into the system. The major components of the system (Fig. 9) are the STR, slurry transport and membrane cell for removal of saccharification products from the circuit.

Trichoderma viride culture (QM 9123) filtrate (2.96 FP) was concentrated 8-fold (14.4 FP) through a UM 10 membrane and pH adjusted to 4.85. A dense cellulose slurry containing 900 gms milled Solka Flocc ($<25\mu$) and 3000 ml of concentrated cellulase was prepared and the reaction was started in a glass ST reactor at 50°C . Following 48 hours of batch saccharification, 1500 ml of the reacting

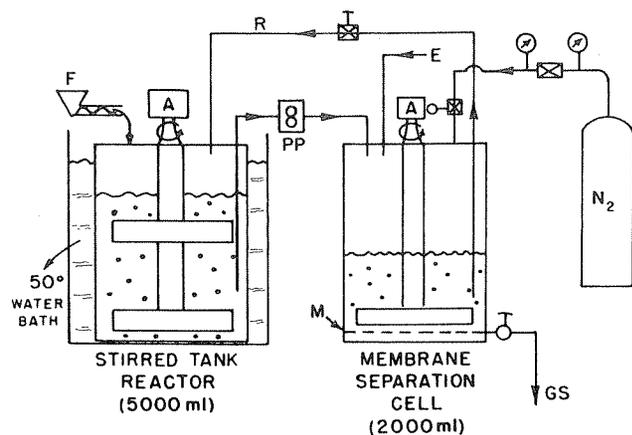


Fig. 9. Combined system for cellulose saccharification in STR and removal of glucose in membrane cell.

- A = Agitator
 PP = Peristaltic pump for transporting cellulose-cellulase slurry from STR into the Membrane cell
 GS = Glucose Syrup free from cellulase and substrate
 F = Finely milled cellulose
 E = Aq. solution of cellulase in water or in Tv medium
 R = Digest returning into reactor after glucose separator
 M = Mol. sieve membrane

slurry was transferred into a 2000 ml (150 mm diameter) membrane cell furnished with a HFA 300 membrane and the rest of the slurry was allowed to continue reaction in the reactor. The cell was pressurized up to 45 lbs/sq in. with N_2 gas from a cylinder and ultra-filtration of the slurry started by bringing into the cell a stream of dilute cellulase solution from a pressure supply vessel at a rate equal to the rate of filtration. The process was continued for 5 hours at room temperature (27°C) during which the slurry volume inside the cell remained constant. This process of dilution and permeation brought the total sugar (as glucose) of the slurry from 14.21 to 6.1%; during the same period the reactor slurry increased its sugar content from 14.21 to 15.32%. The washed slurry was now transported back into the reactor and 95 gms of fresh cellulose (dry, milled, $<25\mu$) was added into the reactor. The saccharification and product removal cycles were continued over a period of 10 days repeating the steps. Table II gives a summary of the material balance of the system. The amount of total sugar (957.4 gm) removed from the system over a period of 10 days (243 hours) with consecutive substrate feeding and product removal represented a yield of 54.3% sugar.

TABLE II
 Material Balance in the Model System for
 Cellulose Saccharification and Glucose Removal

Reaction time, hrs	Input		Glucose output			
	Solid cellulose gms	Water in cellulase ml	In digest initial %	In effluent average %	Removed GMS	Removed %
0.0	900	3000	—	—	—	—
53.0	95	1135	14.21	9.59	108.8	21.87
75.0	125	2065	12.56	7.46	154.1	35.02
100.50	135	2020	13.40	6.74	136.2	29.04
172.25	100	2050	14.21	7.76	159.2	32.00
194.75	120	2000	13.71	7.26	145.1	30.24
220.25	100	1975	11.85	6.40	126.5	30.49
243.00	—	2025	12.10	6.30	127.5	30.11
Totals	1575				957.4	76.85

Note: Water in cellulase (input) = water in glucose solution (output) conversion level = 71.3%.

However, only 43% of the reaction volume was handled for sugar removal while the remainder was left in the reactor. This figure (54.3%), therefore, represents percentage removal of glucose from the system as a whole (3500 ml). The overall conversion of cellulose into glucose is 71.14%. This means that about 17% of the converted cellulose (sugars) remains in the system without removal. Paper chromatograms of the effluent from the membrane cell show that about 70% of the sugars is glucose and most of the rest is cellobiose with a very small amount of xylobiose. No difference in the monomer/dimer ratio has been noticed in either the reactor samples, or in the membrane cell samples or even in the membrane effluent samples. This suggests that the two sugars are passed out of the system in the same ratio as they appear in the digest. The effects of initial glucose contents of the digests on the mass flux values of its permeation through HFA 300 are also presented (Table III). The loss of the enzyme, in the system, if any, can be assumed to be due to a possible denaturation of the protein. The permeation rate is affected principally by the solid concentration of the system rather than the initial sugar content or the total amount of sugar removed. Table IV gives a summary of average (after leveling off) mass flux of various fluids ultrafiltered through molecular sieve membranes.

TABLE III
Effect of Initial Glucose Concentration on Flux Rate

% total sugar as glucose		Total ultrafiltration period, mins	Range of mass flux gals/ft ² -day
Initial	Final		
14.21	6.10	300	11.61-7.32
12.56	3.36	300	21.31-11.64
13.40	2.77	465	19.37-8.58
14.31	3.6	405	19.37-9.80
13.71	3.24	345	25.18-11.21
11.85	2.95	445	21.30-8.58
12.13	2.72	349	25.18-11.23

Note: Initial substrate concentration 30% (-25 μ); cellulase activity 14.4FP; molecular sieve membrane used = HFA 300; saccharification at 50°C; membrane separation at 27°C.

TABLE IV
Summary of Average Mass Flux of Several Substances Filtering Through Molecular Sieve Membrane

	Av. mass flux, gals/ft ² -day			
	UM 10	HFA 200	HFA 300	PM 30
Deionized water	46.9	247.9	—	377.7
TV medium	47.0	199.7	—	293.1
TV medium +0.1% tween 80	32.9	47.3	—	74.6
5% glucose solution	23.5	210.9	252.4	—
10% glucose solution	15.7	176.9	216.0	—
20% glucose solution	—	117.9	139.3	296.1
Cellulase	7.11	11.5	14.2	10.2
Digest	2.9	3.9	4.7	8.4
14.06% sugar				
17.33% cellulose				

DISCUSSIONS

When cellulase of *Trichoderma viride* is concentrated by ultrafiltration on polymeric molecular sieve membrane with a cut off value of 30,000 none of the enzyme is found in the effluent; yet the recovery of activity in the concentrate is far less than 100%. This may be due to inadequacies of the enzyme assay procedure, but it is also likely that some inactivation may take place.

Four different kinds of molecular sieve membranes employed in the studies showed different mass permeation rates for cellulase in gallons/ft²-day (Table IV). Mass rates for water, Tv medium (without Tween 80, cellulose or peptone), Tv medium with 0.1% Tween 80, and glucose solutions of different concentrations were also tested in these membranes. The results bring out a very significant observation related to the use of such membranes in cellulase research. Presence of Tween 80 at 0.1% in mineral Tv medium cuts down the membrane mass flux by several-fold. This effect is much more pronounced in the case of high molecular cutoff films than for lower values. It is suggested that the highly surface active Tween 80 (molecular weight 1200) enters into the channels of the porous structure of the polymer films. Consequently, the detergent mole-

cules quickly cut down the effective passage for diffusion of the solutes at a relatively higher rate in the case of higher cutoff membranes than those which provide a less porous structure. Considerable difference in the permeation rates has been observed between HFA 300, HFA 200 and UM 10 in the ultrafiltration of glucose solution (Fig. 2). Further, the difference of these rates for UM 10 between 5% and 10% glucose solution is much smaller than either for HFA 200 or HFA 300. In the concentration of cellulase PM 30 film seems to follow a progressive slowdown of rate greater than either UM 10 or HFA 200. Similarly, HFA 300 starts off at a fairly high initial mass flux, but ends up with a third of the initial rates of either of the three other films. This and PM 30 would seem to be most suitable for our system. These films have also proven very effective (Fig. 7) in the separation of sugars (glucose and cellobiose) from digests composed of a dense suspension of cellulose in cellulase and large amounts of sugars (14–15%). As long as the concentration polarization is cut down by a continuous mechanical displacement of the slurry from the skin side of the film, fairly high mass flux values are possible. Films HFA 200, HFA 300, PM 30 show no signs of damage even after repeated use; UM 10 membranes appear to be more sensitive to handling. A membrane cell was converted into a continuous reactor which maintained steady saccharification and simultaneous removal of products at a high conversion of cellulose (>76%). Successful performance of such a reaction system in a membrane cell is an extreme example of how a dense suspension of solid discrete particles continuously undergoing reduction in sizes does not affect the performance of the films, both in terms of selectivity and mass permeation. Because of the rapid transport of the reaction products through the film the level of conversion on the skin side can be maintained fairly high.

Results of the effects of particle size on the initial rates of saccharification of cellulose in concentrated enzyme corroborated the earlier^{2,3} observations that the initial fast rate is a first order reaction. For a more crystalline cellulose with greater percent of inaccessible sites as in Solka Floc the initial rate does not appear to be linear even in concentrated enzyme (Fig. 5). A close study of the results reveals the possibility of maintaining a very high rate of saccharification using fine cellulose and concentrated enzyme in fairly dense suspensions. However, such an approach is justifiable only if it is possible

to remove the products as fast as they are formed and to reuse the enzyme.

Independent feeding of substrate and enzyme into STR for continuous saccharification improves the conversion rate. Equilibrium conversion level, which is similar to those reported in the earlier communication,² is attained and is maintained steadily over a considerable period of time. A reduction in the retention time (to reach a given saccharification rate) is obtained by a combined effect of feeding dry solid substrate and use of more active Tv enzyme (QM 9123).

Based on the data obtained from the foregoing experiments, a model system was developed by combining a stirred reaction of finely milled cellulose in concentrated cellulase with a membrane separation cell (Fig. 10). Its input consisted of fine substrate (dry) and small amounts of dilute Tv culture filtrate. The cellulase content of this input was a very small percentage of the total activity of the system. A high level of conversion could be maintained in the system. The output was a sugar syrup equivalent to 77% of the saccharified cellulose. The system also maintained a high mass flux large sugar concentration, and a high % conversion (71%) of substrate. It also offers an economy of cellulase consumption because of the enzyme feed back, which is not practiced in any of the established batch or continuous enzymatic reaction systems. No build-up of unreacted substrate or denatured proteins (inactivated cellulase) has been observed during the period of 243.0 hours of semicontinuous run.

This concept of continuous re-use of the enzyme which is set free due to removal of products from the system will be best accomplished with unmodified substrate. Such a substrate should be highly reactive but without having any sites toxic or inaccessible to the enzyme. However, the controlling factor for the model to operate successfully is the initial reaction rate of product formation and not the mass flux of product removal. Several possibilities have been demonstrated (Figs. 5, 7, Table III) to build up high glucose in the first 24 hours. The high mass flux values obtained in ultrafiltering dense cellulose-cellulase slurries without causing any damage to the membrane films also hold great promise for the concept. For the production of fairly concentrated glucose (>12%) in the model developed it is necessary either to use a large reactor volume and a

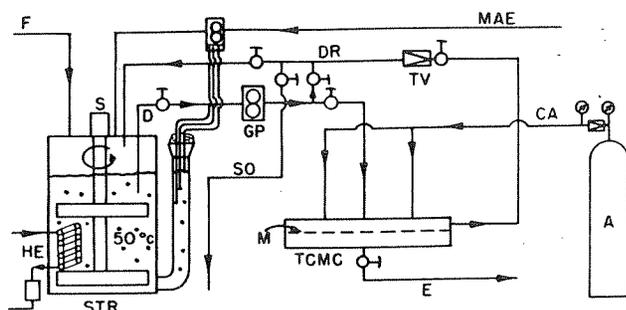


Fig. 10. Model for continuous enzymic saccharification of cellulose with simultaneous removal of glucose syrup.

- S = Agitator.
 GP = Gear pump transporting saccharified cellulose slurry.
 D = Saccharified cellulose slurry transported into the thin channel membrane cell.
 TCMC = Thin channel membrane cell separating sugars from the digest.
 STR = Stirred tank reactor.
 M = 20,000–30,000 cut off molecular sieve membrane.
 HE = Heat exchanged to control reactor temperature.
 E = Glucose syrup free of enzyme or cellulose.
 F = Finely ground cellulose feed.
 DR = Saccharified slurry return from the membrane cell into the reactor.
 MAE = Make-up enzyme (dilute) mixture of cellulase and Tv medium or water.
 SO = 2–4% slurry output to cut down build-up of unreacted cellulose and denatured proteins.
 TU = Throttle valve to control return of digest from the TCMC into the STR.
 A = Compressed air source.
 CA = Compressed air (50 "/>) supply line.

comparatively small separation system, or to attain a high initial reaction rate of a dense suspension.

CONCLUSION

A new concept of rapid enzymatic reaction of insoluble substrate, separation of products and continuous re-use of the enzymes has been established. Application of this concept to the cellulose-cellulase system to produce glucose syrups has been demonstrated. Major advantages of this approach to enzyme involved reactions where

product molecular weights are lower than either the enzymes or the substrates are (a) removal of products from the reaction system free of contaminants, (b) retention and feed back of the enzyme in the system, (c) a high level of conversion of the substrate into products, and (d) a high initial reaction rate. Use of polymeric membranes of suitable molecular cutoff values for concentrating enzymes and separating products from the reacting system was essential in establishing this concept.

There are several possibilities of application of the model to chemical, enzymatic, and microbial reaction systems involving products of lower molecular weights. These products may be removed from the system free of reactants and catalysts. If the membranes are available at a reasonably low price the model can be applied to starch saccharification systems on the same principle which has been clearly demonstrated for cellulose-cellulase system. However, considerable amount of basic engineering data on transport, reaction constants, relative sizes of reaction and separation systems, bleed factors and cellulase inactivations, *etc.* will be collected in the steady state model which is being designed now.

The authors thank the U.S. Army Natick Laboratories for making available the facilities to carry out the studies reported here. The senior author is grateful to the National Academy of Sciences, National Research Council for the Visiting Scientist award made to him and to the Jadavpur University, India for granting leave to enable him to conduct the experimental studies at Natick, Massachusetts. The authors also appreciate the encouragement, cooperation, and friendly suggestions received from Dr. Mary Mandels and Dr. E. T. Reese of the U.S. Army Natick Laboratories in the course of the work. Interest shown by Dr. Hamed M. El-Bisi, Chief, Microbiology Division, of the NLABS is appreciated.

This paper reports research undertaken at the U.S. Army Natick Laboratories and has been assigned No. TP632 in the series of papers approved for publication. The findings in this report are not to be construed as an official Department of the Army position. Citation of trade names in this report does not constitute an official endorsement of approval of the use of such items.

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Received May, 7, 1970