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Continuous Enzymatic Saccharification of Cellulose with Culture Filtrates of *Trichoderma viride* QM 6a.*

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Summary

Continuous saccharification of Solka Floc (cellulose pulp) in single and four-vessel stirred-tank reactor systems has been possible employing enzymes obtained directly from submerged fermentation of *Trichoderma viride* QM 6a. Studies on the effect of modification of the solid substrate, enzyme stability, substrate concentration, and the influence of reducing sugar concentration on the rate of hydrolysis are reported. While susceptibility of substrate to digestion is not affected by heating alone, it is strikingly increased by heating plus grinding, or by grinding following heating. Batch and steady state continuous saccharification experiments have yielded more than 5% reducing sugar in the effluent with a dilution rate of 0.025 hr^{-1} at 50°C , at a substrate level of 10%. An average glucose concentration of 3.4% has been obtained in the effluent of a continuous saccharification using 5% substrate at the same dilution rate and temperature.

INTRODUCTION

Biological degradation of cellulose is perhaps the biggest mass hydrolytic reaction taking place in nature, continuously contributing about 95 billion tons of carbon to the atmosphere annually. Life on the planet would have ceased in the absence of this invisible, but gigantic, process. Because of the insoluble nature of cellulose and its inability to permeate cells, the process is slow. In spite of its natural abundance, a very insignificant portion of the estimated 190 billion metric tons of available cellulose has been exploited for com-

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mercial purposes. A large number of rumen and soil microorganisms have been found to possess the capability of degrading native cellulose, and converting it into a wide range of products from hydrocarbons to carbohydrates. Hydrolytic breakdown of the polymer cuts down the chain length and yields reducing sugars as its end products through a split at the β -1,4-glucosidic linkages. The sluggish rate of hydrolytic breakdown of cellulose in fungal systems is related to the slow diffusion of enzymes into the substrate. The fast rate of hydrolysis of the polymer in mineral acids is apparently due to the ability of the relatively small acid molecules to penetrate into the extended amorphous surface. An associated feature of acid hydrolysis is depolymerization at the initial stage of attack without appreciable weight loss. The enzyme-catalyzed process shows a smaller decrease in the degree of polymerization, and maintains a good stoichiometric relationship between the loss in substrate weight and the increase in the reducing sugars (RS) produced. The enzymes are confronted with the complex problem of an increase in substrate resistance following the initial fast rate process. This period varies from a few hours to a few days, depending on the enzyme strength and the nature and amount of impurities associated with the substrate. Thereafter, even a complete replacement of the enzyme by a fresh batch does not improve the situation to any appreciable extent. It is, however, interesting to observe that ground and heat-treated cellulose, which apparently loses its natural crystalline character almost completely, increases its enzyme susceptibility several-fold over the raw cellulose as reported by Katz and Reese.¹ Extended reactive surface exposed due to milling provides only a partial explanation. Most likely, the combined effects of large exposed surface and oxidative changes, brought about by heat treatment, increases activation and the rate of hydration of the substrate associated with an increase in solubility.

Over the last three decades, many communications have appeared dealing with the action of cell free enzymes on cellulosic materials. These reports establish one fact, e.g., that the mechanism of cellulase attack on the substrates is a highly complicated process. This may be due to the involvement of a number of enzyme systems which very likely attack the substrate in sequence, or because of the nature of polymeric structure of cellulose, necessitating swelling or opening up before a progressive breakdown of the substrate takes place. Perhaps a combination of both the possibilities represents the complicated nature of the system.

Several reports reveal information relating to the products of enzymatic hydrolysis of cellulose by Levinson et al.,² the influence of cellulose structure on cellulase action by Walseth and Cowling,^{3,4} cellobiose induction of cellulase by Mandels et al.,⁵ resolution of *Trichoderma viride* cellulolytic complex by Ogawa et al.,⁶ and Toyama et al.,⁷ inhibition of cellulases by Mandels et al.,⁸ physical factors affecting enzymatic hydrolysis of cellulose by Lee⁹ and components of *Trichoderma viride* cellulases by Selby et al.¹⁰ Mandels and Reese¹¹ and Halliwell¹² reported on complete hydrolysis of cellulose at a very low substrate concentration in cellulases derived from *Trichoderma viride* and *T. koningii*; but very little information is available as to the possibility of cellulose yielding large amounts of sugar on saccharification. The only short communication¹ on the subject points out this possibility, using finely pulverized and heat treated cellulose (spruce pulp). The special conditions employed in the studies, namely, highly concentrated enzyme preparation, addition of β -glucosidase to reduce cellobiose inhibition, small amounts of reaction samples and long incubation period, etc. are difficult to adapt to large scale experiments for economic considerations.

Present studies are aimed at developing a reasonably stable model reaction system to saccharify treated cellulose, using culture filtrates obtained directly from submerged fermentation of *Trichoderma viride* (Tv) QM 6a. It also provides information on the nature of treatment given to the substrate, the enzyme stability, optimum temperature and pH of saccharification, and a possible kinetic model involving solid substrate and enzyme solution.

MATERIALS AND METHODS

The Substrate

Solka Floc, a kind of wood (spruce) pulp widely used as filter aid (Brown Co., Berlin, N.H.) has been employed as a basic cellulosic substrate for saccharification studies. In order to increase susceptibility of the substrate towards enzyme action, the material was milled dry in a laboratory porcelain pot mill, or with a Sweco (South-Western Engineering Company, Los Angeles, Calif.) vibro-energy mill. The dry milled product was screened down to -270 U.S. standard screen ($< 53 \mu$). As heat treatment of the substrate was found to further improve susceptibility, the Solka Floc was heated to 200°C for 25 min, either prior to milling or immediately after milling.

The following abbreviations are used in the text to designate specific materials employed in various experiments: SF, Solka Floc without any treatment; SF-M, Milled (pot) Solka Floc; SF-H, Heated Solka Floc, SF-HM, Heated and milled Solka Floc; SF-MH, Milled and heated Solka Floc; SF-MHD, milled, heated, and digested Solka Floc; Sweco 70, Solka Floc milled in Sweco Vibro-Energy Mill for 70 hrs.

Substrate Modification

The basic cellulosic material Solka Floc was subjected to several kinds of treatment, e.g., heating at constant temperatures for a fixed time, heating followed by milling over a given period of time, and grinding followed by heating operation. Heat treatment of cellulose was considered useful, in view of a definite indication of increased susceptibility towards acid saccharification based on two Russian reports. Krupnova and Sharkov^{13,14} used heated and milled cotton to convert it into a readily hydrolyzable state. The combined effects of increased surface due to size reduction and oxidative treatment of Sweco 70 are illustrated in Table I. The ability of heat treated (210°C for 4 hr) cotton to increase reducing sugar yield in acid hydrolysis (10% H₂SO₄) by a factor of two was also reported by Sharkov et al.¹⁵ The basic cellulosic substrate was therefore subjected to heat treatment at temperatures between 50 and 250°C for 25 min. Most

TABLE I
Effect of Heat Treatment of Finely Pulverized Solka Floc
(Sweco 70) on Enzymatic Hydrolysis^a

Substrate, 100 mg/ml	Reducing sugar produced, mg/ml		Increase in sugar, %	
	in 2.75 hr	in 7.0 hr	in 2.75 hr	in 7.0 hr
Sweco 70	22.5	43	—	—
Sweco 70 heated to				
200°C for 25 min	38	50	70.6	14
200°C for 40 min	27	51	22.7	16
200°C for 60 min	29	50	31.8	14
200°C for 120 min	21.5	42	-2.2	2

^aCellulase activity = 111 C_z units/ml; temperature, 50°C; pH, 4.8.

of the rate studies were, however, carried out with ground Solka Floc pre- or post-heated with milling time between 24 and 48 hr. In order to ascertain the nature of changes occurring while heating Solka Floc, the raw and milled material was subjected to heat exposure in air and in the presence of N₂ gas (O₂ free) at various temperatures. Effects of these treatments on the enzymatic hydrolysis are illustrated in Table II. It reveals a distinct difference between oxidative and nonoxidative heating. Evidently, the increase in the substrate susceptibility to enzymes is due both to exposure of inner surface of cel-

TABLE II
Effects of Various Treatments of Substrates on the Production
of Reducing Sugars^a

Substrate, 50 mg/ml	Nature of Treatment	Reducing sugar produced, mg/ml			
		2.5 hr	4.5 hr	7.0 hr	23.5 hr
Solka Floc (SF)	None	6.6	8.5	8.5	17.0
Solka Floc (SF)	Soaked with N-HCl for 5 min in 5% susp. fil- tered, washed and dried at 30°C and heated to 200°C for 25 min	1.85	1.87	2.35	4.2
Solka Floc (SF)	Milled for 48 hr (82% < 149 μ)	8.7	12.7	14.5	22.2
Solka Floc (SF)	Milled and heated to 200°C for 25 min in air (82% < 149 μ)	16.0	21.0	21.0	33
Solka Floc (SF)	Milled and heated to 200°C for 25 min in N ₂	7.8	11.0	13.0	25.5
Sweco 70 ^b	None	11.2	13.0	15.0	31.0
Sweco 70	Heated to 200°C for 25 min in air	17.1	20.0	21.0	34.0
Sweco 70	Heated in 200°C for 25 min in N ₂	10.2	13.2	15.5	28.5

^a Cellulase activity = 60 C_z units/ml; Temperature, 50°C; pH, 4.8; reaction mixture in 10 ml test tubes.

^b 94% < 53 μ.

lulose to enzymes because of size reduction, and to oxidative modification of the material by heat treatment. Milling of Solka Floe was performed in a 2.5 gallon porcelain pot mill using assorted glazed porcelain balls of 1 in. (2.65 kg) and $\frac{1}{2}$ in. (1.2 kg) over a period of time. Sweco 70 used in several experiments was a dry pulverized product of Sweco Vibro-Energy Mill received from Southwestern Engineering Company, Los Angeles, Calif.

Screen Analysis of the Substrates

The milled cellulose was screen-analyzed in a Ro-Tap Testing Sieve Shaker (The W. S. Tyler Company, Cleveland, Ohio) using a series of 40, 60, 100, 120, 170, 200, and 270 mesh U.S. standard screens, sifting for 40 min, plus an additional 5 min after the first weighing to check the reproducibility of weights of the screened fractions. Both the weights checked very well. The same procedure was followed in the case of SF, SF-HM, SF-MH, and Sweco 70 samples, all of which were subsequently tested. The particle size distribution of various raw, heated and milled Solka Floe is illustrated in Table III and Figure 1. These values suggest that fine grinding of cellulose increases the per cent of small particles at the cost of larger size particles ($< 73 \mu > 53 \mu$) only with increased milling time. The order of heating and milling is important. When heating precedes milling, the particle size is smaller than when milling precedes heating.

TABLE III
Screen Analysis of Solka Floe and Heated Solka Floe

Material	Particle size distribution, %		
	$> 149 \mu$	$< 149 \mu$	$< 53 \mu$
SF	19.4	80.5	11.6
SF-H, 200°C for 25 min	23.7	74.8	11.9
SF-HM, 200°C for 25 min, and pot milled for 25 hrs	4.9	95.9	76.0
SF-MH milled before heating	8.6	91.9	54.9
Sweco 70	0.9	99.0	93.8
Sweco 70-H, 200°C for 25 min	1.1	99.3	93.8

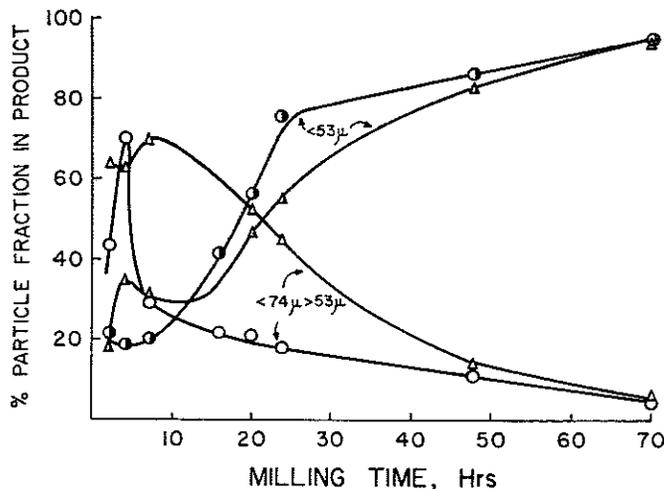


Fig. 1. Effect of pre- and post-heating of Solka Floc on particle size distribution due to milling. Heating at 200°C for 25 min in forced draft air oven. Pre-heated: (\circ) $< 74 \mu > 53 \mu$; (\bullet) $< 53 \mu$; postheated: (Δ) $< 74 \mu > 53 \mu$, (∇) $< 53 \mu$.

The Enzyme

Culture filtrates directly obtained from 10 liter batch or semicontinuous submerged fermentation of *Trichoderma viride* (Tv) QM 6a were used in the saccharification studies after adjustment of pH ($\sim 3 \rightarrow 5.0$). The basic composition of the medium used in the *Trichoderma viride* fermentation for every 10 liter batch was in grams per liter: $(\text{NH}_4)_2\text{SO}_4$, 1.4; KH_2PO_4 , 2.042; $(\text{HN}_2)_2\text{CO}$, 0.3; CaCl_2 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; proteose peptone (No. 2, Difco), 0.5; Solka Floc (SW40A, Brown Co., New Hampshire, USA), 10.0; and trace metals in milligram per liter: Fe^{++} , 1.0; Mn^{++} , 0.8; Zn^{++} , 0.5; and Co^{++} , 0.5; pH adjusted to 5.0 [with K_2HPO_4 (*M*) or citric acid (*N*)]. The prepared medium (10 liter) was sterilized under slow agitation in a New Brunswick sterilizer for 30 min at 120°C , cooled to room temperature, inoculated with 100 ml of a 6–10 day old shake flask culture and incubated in water bath at 29°C for a week under constant aeration rate of 0.1–0.3 vvm of sterile air. When the culture was ready for use in the saccharification experiments, both the terminal pH (~ 3) and enzyme strength (~ 1.0 filter paper activity) were used as end points of completion of fermentation. The semicontinuous culture filtrates

used in the studies were obtained from Dr. Mary Mandels of the same laboratory. The enzyme concentrations expressed in terms of filter paper activity of the batch and semicontinuous culture filtrates used in the studies varied generally between 0.75–1.26 and 0.9–1.86, respectively. In most cases several harvests obtained from the semicontinuous fermentations were mixed together to bring the activity to about 1.0 in order to be able to use enzymes of uniform activity in the various comparative experiments. Following harvests, the culture fluids were filtered off on glass wool to remove the suspended mycelium, pH adjusted to 4.8–5.0 and stored at 2°C. The filtrates appeared sparkling clear with a faint yellow-green coloration and an agreeable smell.

Enzyme Assay

Assays of enzyme activity of all samples received from fermenters were performed immediately after each harvest. The assay of enzyme strength in these cases is based on the Filter Paper Activity method discussed by Mandels and Weber.¹⁶ In experiments dealing with the evaluation of the effect of pH and temperature on enzyme stability, the same assay method has been followed. The method essentially consists of suspending a 50 mg strip of Whatman No. 1 filter paper measuring 1 × 6 cm in a mixture containing 1.0 ml of 0.05M citrate buffer (pH = 4.5) and 1.0 ml of filtered undiluted enzyme solution and incubating the mixture for 1 hr at 50°C followed by the estimation of reducing sugar produced in the sample by the standard dinitrosalicylic (DNS) method. The cellulase is expressed as *Filter Paper (FP) activity* in milligrams of glucose produced in the above tests. In the studies connected with the effects of pH on cellulase activity and adsorption on substrates, which are discussed later, the carboxymethylcellulose (CMC) assay method¹¹ was used. It consists of incubating 0.5 ml CMC solution with a mixture of 0.5 ml of filtered and properly diluted enzyme solution at 40°C for 30 min followed by estimation of reducing sugar produced in the sample by the standard DNS method. The number of C_x units per milliliter equals the inverse of the dilution that gives 0.5 mg of glucose in the test.

Reducing Sugars

Estimation of total reducing sugars (RS) has been based on the dinitrosalicylic acid (DNS) method proposed by Sumner and

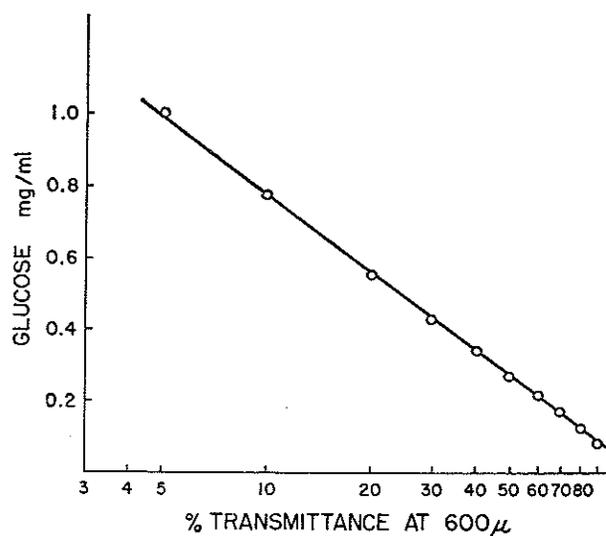


Fig. 2. Standard curve for estimation of reducing sugars as glucose by DNS method.

Somers¹⁷ using 1 ml of a properly diluted sample of clear (centrifuged or filtered) hydrolysate with 3 ml of DNS solution, heating the mixture for 5 min at 100°C, cooling for 5 min under tap water and measuring the transmittance of the developed color in a Bausch and Lomb Spectronic 20 Photo-electric Colorimeter at 600 μ . Dilutions of samples were adjusted such that percentages transmittance values fall between 20 and 80%. The sugar values were read from the glucose calibration curve (Fig. 2).

EXPERIMENTS AND RESULTS

Effect of Temperature on Losses of Cellulose Activity Due to Inactivation and Adsorption

Before the saccharification rate studies were started, it was necessary to find out the effects of temperatures at which the Tv cellulase is generally reported to be very stable, on the pattern of enzyme loss by adsorption, direct inactivation or both. The studies were conducted at 40, 50, and 60°C employing an enzyme solution having 68 C_x (CMC) units/ml. The substrate studied was the basic cellulose,

Solka Floc, at 5% suspension and Fuller's earth as the agent for adsorption. Ten-ml samples were used and these were tested for residual activities at 0, 10, and 60 min. The results are illustrated in Figure 3.

The results show that small enzyme losses (up to 10%) are difficult to detect. Enzyme alone does not appear to lose any activity at 50°C for 1 hr. At 60°C, the loss due to inactivation alone increases from 6% at 0 time to 13% at 10 min and to 25% at 1 hr. However, loss due to adsorption on Fuller's earth at 60°C rises at a much faster rate, namely 13% on immediate contact, 38% after 10 min and 42% after 1 hr. Because of continued interaction between the substrate and the enzyme molecules, effective loss of enzyme activity in terms of inactivation and adsorption is apparently lower than adsorption on Fuller's earth. The net losses due to both the effects at 50°C remain almost constant near 13% up to 1 hr of contact.

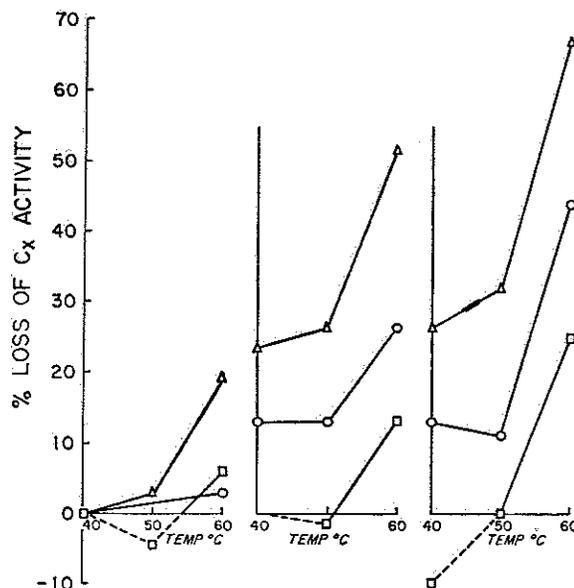


Fig. 3. Inactivation and adsorption of TV cellulase at 40, 50, and 60°C. Initial activity of enzyme = 68 C_x units/ml; Substrate, 5% Solka Floc; Adsorbent, 5% Fuller's earth; pH, 4.5. (—□—) enzyme alone; (—○—) enzyme plus substrate; (—△—) enzyme plus adsorbent. (a) at 0 time; (b) after 10 min contact; (c) after 1 hr contact.

Effect of pH on Adsorption, Activity and Inactivation of Tv Cellulase

In all microbiological and enzyme systems involving growth, enzyme biosynthesis and product formation, pH plays a key role. In the case of *Trichoderma viride* the pH range 2.8–3.2 has been found to be the most effective for enzyme elaboration while the cellulase activity for saccharification is most pronounced at pH values between 4.0 and 5.0. As a separate series of studies on the temperature effects on enzyme loss has been made, it was considered necessary to isolate these factors from the pH effects on activity loss and the optimum pH. A broad pH range (3–6.5) was used in the studies in presence of substrate (5%) with enzyme alone as control. The contact time up to 4 hr was studied. Adjustment of pH values was made with citric acid and tripotassium phosphate. The loss of C_x activity (Fig. 4) of enzyme alone remains below 10% between pH 4.0 and 5.0 at 50°C in 4 hr. Outside this range there appears a marked loss in the activity (about 94% at pH 3 and 37% at pH 6.5). In contact with substrate, the loss of activity remains almost constant at 18% between pH 3.5–5.5 at 50°C over a 4 hr period. For best enzyme activity and reducing sugar yield, a pH range of 4.8–5.0 appears to be nearly optimum.

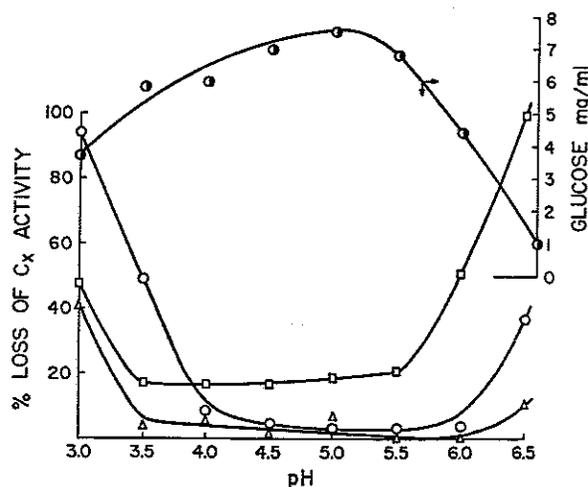


Fig. 4. Effect of pH on adsorption, activity, and inactivation of TV cellulase at 50°C. (—●—) RS, mg/ml after 4 hr; (—□—) enzyme plus substrate, 4 hr; (—△—) enzyme plus substrate, 0 hr; (—○—) enzyme alone, 4 hr. Solka Floe concentration, 50 mg/ml; cellulase activity, 115 C_x units/ml.

Effects of Enzyme Concentration on Saccharification of Cellulose

Earlier reports^{11,18,19} suggest an association of two distinct groups of enzyme systems (C_1 and C_x) in the hydrolytic breakdown of cellulose. The native form is transformed first into reactive cellulose catalyzed by C_1 which is not hydrolytic. The next step, hydrolytic breakdown, is only possible by what is termed the C_x factor, a complex of β -1,4-glucanases^{11,20} which results in the appearance of reducing sugars in the hydrolysate. This combined or synergistic effect of two widely separated enzyme systems enables native cellulose to be saccharified into sugars. In the case of a soluble substituted cellulose (like CMC) C_1 obviously does not have any role to play and C_x alone is involved in the attack on the β -1,4-glucosidic linkage. Because the activity of C_1 is measured by the hydrolysis of cotton in conjunction with C_x , it is dependent on the presence of C_x . It was therefore interesting to find out to what extent cellulase concentration plays a role on the rate of hydrolysis and reducing sugar yield, and if there is any significant difference between these rates over an extended period of time.

The Tv enzyme filtrates were concentrated in laboratory vacuum evaporator at 40°C to about one-fourth the original volume. Different dilutions were prepared from this sample with distilled water. Corresponding C_x activities of each sample were assayed as 184 and 77 units/ml respectively. The substrate used was Sweco 70 at 10% concentration and tests were carried out at 50°C over a period of 50 hr at pH 4.8 (Fig. 5). In order to evaluate the initial loss of enzymes taking place in various concentrations, C_x assays of samples without substrates were run and checked against controls. The more concentrated enzyme lost about 13% and the less concentrated sample (77 C_x units/ml) lost about 42% of their original activities in 2 hr. Figure 6 presents batch saccharification data of cellulose (Sweco 70) cellulase systems at 50°C under various conditions of enzyme concentration, both in agitated reactions and in test tubes. These data bring out two distinctive features of the reaction between solid substrates and aqueous enzyme solution. The highly heterogeneous nature of the suspension increases its initial rate of hydrolysis due to agitation bringing the system close to homogeneous even at a lower enzyme concentration. Second, at the same substrate level, increased concentrations of enzymes increase the rate of hydrolysis

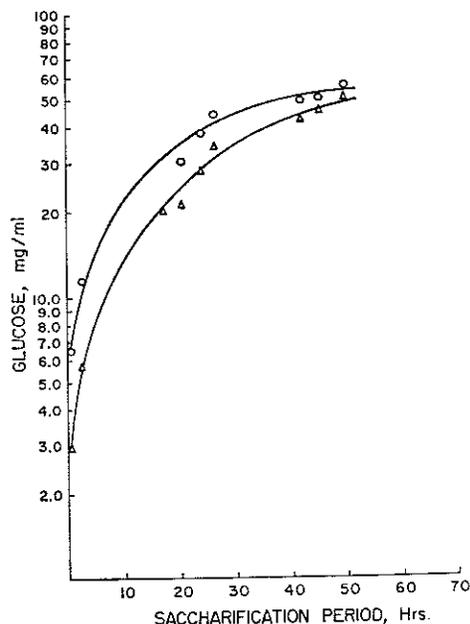


Fig. 5. Effect of enzyme concentration on the rate of saccharification of Sweco 70 at 50°C. Enzymes: (—○—) 184 C_x units/ml; (—△—) 72 C_x units/ml. Substrate: 5% Sweco 70 in tubes without agitation.

considerably. For example, at 0.5 hr the sugar produced from a 10% Sweco 70 suspension is 0.65% in 184 C_x units/ml, 0.29% in 77 C_x units/ml, and 0.21% and 0.20% in the other two samples, providing 32 and 22 C_x units/ml, respectively. At 50 hr the reducing sugar yields were 5.55, 5.0, 4.2, and 3.0% in the respective samples.

Effect of Initial RS Concentration on the Rate of Saccharification and Sugar Yield

With the progress of hydrolysis one of the factors likely to affect the rate is the continued and increasing reducing sugar buildup in a batch system. Experimental tests to ascertain the nature of this effect were conducted in tubes containing 5 ml enzyme-substrate suspension without agitation. The enzyme used was found to correspond to 0.9 FP activity and the substrate studied was SF-HM. Initial sugar concentration used in the study ranged between 11.83

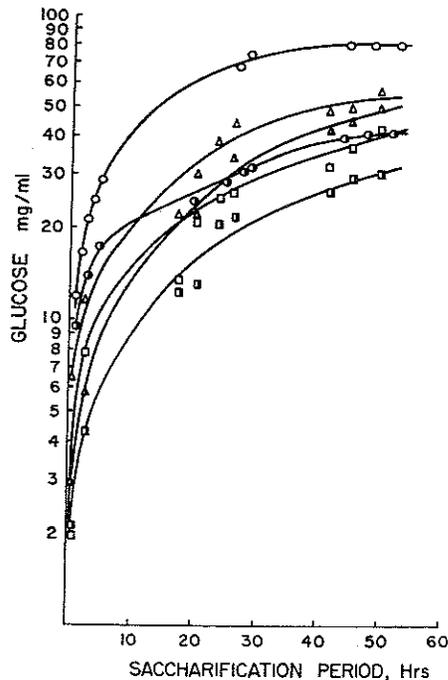


Fig. 6. Batch saccharification of cellulose (Sweco 70) by cellulase systems at 50°C. (—○—) [E] = 87.0 C_x units/ml, [S] = 10% Sweco 70, pH = 4.3, 1.0 liter stirred tank reactor; (—●—) [E] = 88.0 C_x units/ml, [S] = 5% Sweco 70, pH = 4.3, 1.0 liter stirred tank reactor; (—△—) [E] = 184.0 C_x units/ml, [S] = 10% Sweco 70, pH = 4.8, 10 ml tubes not agitated; (—▲—) [E] = 77.0 C_x units/ml, [S] = 10% Sweco 70, pH = 4.8, 10 ml tubes not agitated; (—□—) [E] = 32.0 C_x units/ml, [S] = 10% Sweco 70, pH = 4.8, 10 ml tubes not agitated; (—■—) [E] = 22.0 C_x units/ml, [S] = 10% Sweco 70, pH = 4.8, 10 ml tubes not agitated.

and 64.13 mg/ml. These were tested over a period of 2, 8, 24, and 48 hr contact time at 50°C and pH 5.0 (Fig. 7). It is apparent that the presence of sugar exercises an inhibitory effect on the hydrolysis. The values are similar to those of Katz and Reese¹. The changed slope at 48 hr probably reflects the accumulation of sugar in the digest, and the disappearance of the more readily digested substrate.

Batch Saccharification of Sweco 70

Several batch hydrolyses of the substrate were carried out before the semicontinuous or continuous studies were taken up. Most of these rate studies were conducted with modified cellulose (milled and/

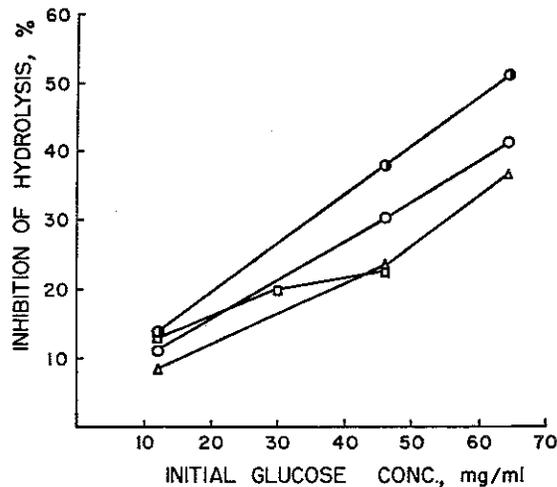


Fig. 7. Effect of initial reducing sugar concentration on hydrolysis of Solka Floc (heated and milled; $< 53 \mu$) at 50°C . (—○—) 2 hr contact; (—●—) 8 hr contact; (—△—) 24 hr contact; (—□—) 48 hr contact; cellulase = 0.9 FP activity, pH = 5.0.

or heat treated), which consistently showed much higher susceptibility than Solka Floc. The other advantage in favor of using modified cellulose was its ability to form a homogeneous suspension with a much higher solid content (nearly 30%) than the Solka Floc which forms a difficult suspension in enzymes even at solid content of 4–5%. The rate experiments were carried out in 0.5–1.0 liter agitated reactors in the presence of 0.0025–0.005% merthiolate as preservative, at or around 5.0. Three of these typical rate curves are plotted in Figure 8. A maximum of about 8.0% total RS (equivalent to 72% substrate conversion) was obtained on a 10% (w/v) suspension of Sweco 70 ($< 10 \mu$ average size) in 45 hr at 50°C . A parallel experiment run with 5% suspension under exactly the same conditions of enzyme strength, reactor volume, and temperature showed an almost equal per cent conversion at the end of 48 hr (about 74%). The enzyme filtrate contained 75–78 C_x units/ml. The reaction mixture was supplemented with 1 mg of merthiolate per liter.

Continuous Saccharification Process

Single Stage: Following the initial batch saccharification studies with various substrates at 50°C with Tv filtrates generally obtained

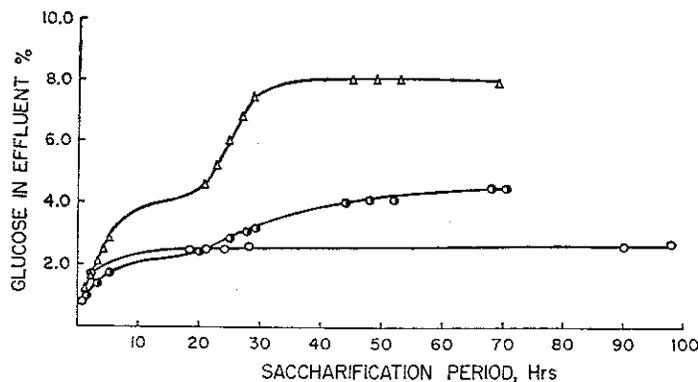


Fig. 8. Batch saccharification of cellulose with Tv filtrates at 50°C. (—Δ—) 10% Sweco 70; (—●—) 5% Sweco 70; (—○—) 5% Solka Floc; cellulase = 75–78 C_x units/ml; pH = 4.8.

from shake flask cultures, a few semicontinuous tests were run in 0.5–1 liter reactors which are reported by Ghose et al.²¹ It was evident from these studies that the reaction mixture of 10% substrate reached a level of about 5% RS at 40 hr with SF-HM and Sweco 70, whose average particle size consisted of 53 μ or less. Generally, higher reducing sugar values were obtained in Sweco 70, which contained more than 93% of 53 μ particles as against a little over 40% in the case of SF-MH, and about 85% for SF-HM, both milled for 48 hr. Because of a limited supply of Sweco 70, most of the continuous studies were based on the latter two materials. One of the major difficulties encountered was in feeding the substrate continuously because of small reactor volumes and necessarily low feed rates. It was considered possible to maintain the feed rates constant by feeding the reactor with a premixed enzyme–substrate suspension kept at 2°C in a reservoir. The effects of low temperatures on the rate of saccharification of SF-MH up to 48 hr were checked in a series of separate tests. These rates were fairly low at temperatures up to 4.4°C. (Fig. 9). Even at 10°C the difference in the rate is not appreciable, but beyond 10°C it rises considerably. Rate data on reducing sugar yields in respect to a 5-liter single stage stirred tank system with 5% substrate concentration are illustrated in Figure 10. Up to 40 hr the unit was run as a batch. Continuous feeding and discharge started from this point onwards and were maintained at a steady

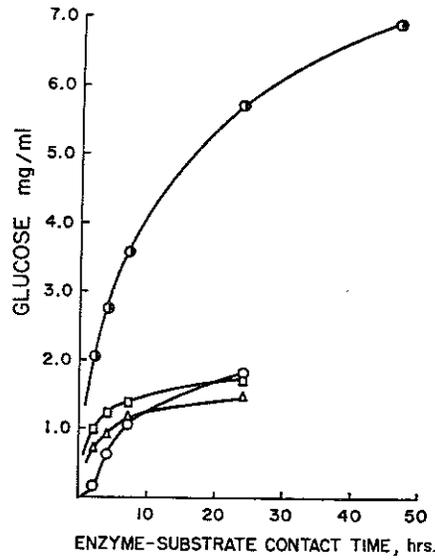


Fig. 9. Effect of lower temperature on saccharification of cellulose. (—○—) 0°C; (—△—) 4.4°C; (—□—) 10°C; (—●—) 20°C; cellulase = 1.0 FP activity; pH = 4.8; substrate concentration = 5% (SF-HM).

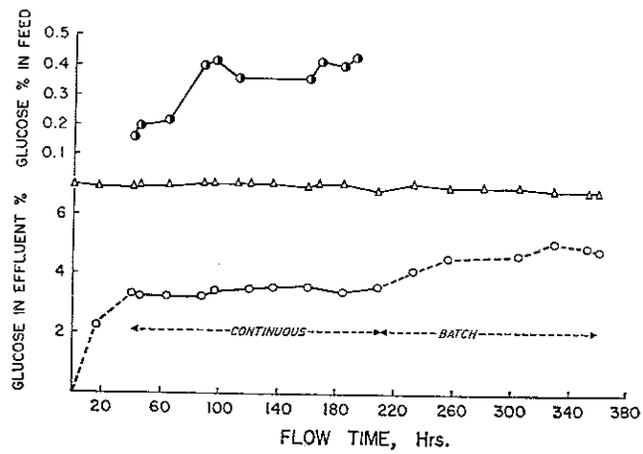


Fig. 10. Continuous saccharification of cellulose (SF-MH) with Tv culture filtrates at 50°C. (—○—) continuous steady state effluent glucose; (—○—) batch effluent glucose; (—●—) continuous feed glucose; (—△—) pH of effluent; cellulase = 1.2 FP activity; pH = 5.0; substrate concentration = 5%.

dilution rate of 0.025^{-1}-hr over 210 hr. The effluent and feed streams maintained reducing sugar levels between 3.3–3.6% and 0.16–0.43% respectively during the continuous phase. After the 210 hr of continuous operation, the feed and harvest streams were sealed off and the unit was run further as a batch system to see if any further conversion of the substrate would continue. In the next 119 hr of batch run, the sugar content of the effluent increased from 3.6 to 5.1%, and in the last 31 hr it fell to 4.8%. In terms of per cent conversion of substrate, the continuous phase maintained a level between 59.5% and 68.7%. This value then increased up to 92% in batch operation. This nearly 27% increase in the conversion of substrate into sugars is due to the residual activity of the cellulase still retained by the system. This corroborates very well with the batch data reported by Ghose and Kostick.²¹ The feed suspension (containing 1 mg/liter of merthiolate) was kept at 2°C in a jacketed, ice-cooled bath and was maintained in a homogeneous state under continuous agitation with a magnetic stirrer. Every 24 hr the feed reservoir was replenished with 3 liters of a freshly prepared suspension (5%) of substrate containing 3 mg of merthiolate. The ability of the system to run free of contamination is marked by the maintenance of a steady pH (4.8–5.0) during the entire period of 359.5 hr of both batch and continuous phases.

Four Stage: One of the most important aims of multistage continuous reaction system is to isolate the different phases of an overall reaction taking place consecutively in a closed system from one another. This provides an opportunity to study more closely the isolated reactions, their stoichiometry, orders and mechanisms and also to interpret the system as an engineering model.

A four-vessel, open-circuit, forward-feeding, continuous saccharification unit, using stirred tank glass reactors, was set up in the sequence of volume, reactor 1 (0.5 liter), reactor 2 (1 liter), reactor 3 (1.5 liter), and reactor 4 (2.0 liter) representing a total retention period of 40 hr distributed in the four stages according to the individual volumes. Enzyme-substrate suspension (10% w/v) was kept under agitated condition in a reservoir ice-cooled at 2°C. The enzyme filtrate used contained 1.00 FP activity and the substrate used was SF-HM. Reaction temperature was maintained constant at 50°C. Pumping rates at all stages were maintained by independent

peristaltic pumps through rubber tubings (1/8 in. ID, 3/16 in. OD.) feeding in and discharging out of each reactor at the rate of 125 ± 5 ml/hr of the homogenous suspension. Every 24 hr 3 liters of a fresh enzyme-substrate suspension containing 1 mg/l of merthiolate were added into the reservoir. Figure 11 shows a diagram of the continuous setup. It was possible to maintain the continuous system for a period of 96 hr. The reducing sugar concentrations in the various vessels were checked frequently and were found to contain 0.74–5.5 mg/ml in the feeding reservoir, 21–25 mg/ml in reactor 1, 27.5–31 mg/ml in reactor 2, 38–42 mg/ml in reactor 3, and 48.6–55.0 mg/ml in reactor 4. While this experiment established the possibility of maintaining a steady-state continuous biochemical reaction involving a solid substrate, continuation of the system beyond 4 days had to be stopped because of the mechanical failure of the pumping system. The rate curve of saccharification are illustrated in Figure 12.

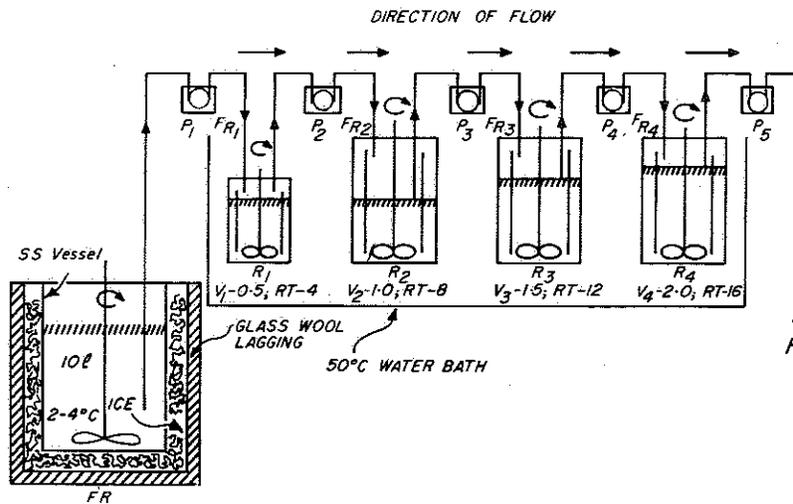


Fig. 11. Continuous, forward-feed, steady-state, four-vessel cellulose saccharification system. P_1, P_2, P_3, P_4, P_5 ; feed and harvest pumps working continuously between feed reservoir and fourth reactor; R_1, R_2, R_3, R_4 : forward feed agitated reactors in series; FR_1, FR_2, FR_3, FR_4 : feeds entering R_1, R_2, R_3 and R_4 ; H : harvest from R_4 ; FR : feed reservoir; RT : retention time, hr; V_1, V_2, V_3, V_4 : volumes of reactors, liters; $//////$ fluid level in reactors.

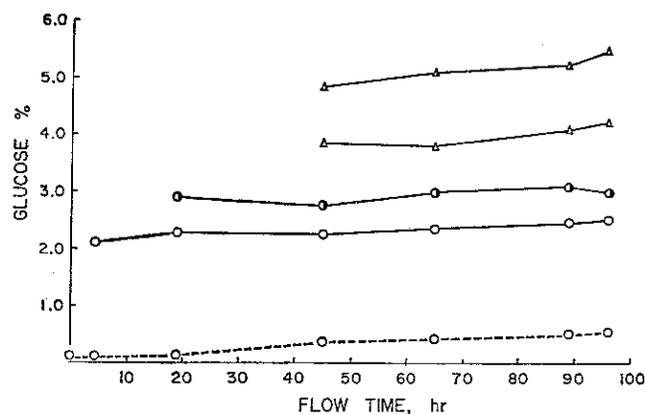


Fig. 12. Continuous steady state saccharification of modified cellulose (SF-HM) in forward-feed, four-vessel agitated system at 50°C. (Δ) Reactor 4, vol. 2.0 liter glucose level; (\triangle) Reactor 3, vol. 1.5-liter glucose level; (\bullet) Reactor 2, vol. 1.0-liter glucose level; (\circ) reactor 1, vol. 0.5-liter glucose level; cellulase = 1.0 FP activity; pH = 4.7-5.0; substrate concentration = 10%; feed stream temperature = 2-4°C.

DISCUSSION

Experimental results show that considerable improvement in the susceptibility of cellulose (Solka Floc) to hydrolysis by Tv cellulase is possible by a two-step process of size reduction and oxidative heat treatment. Optimum effects of heat treatment is obtained at 200°C at 25 min. This effect is likely to increase further by bringing discrete particles in intimate contact with an oxidizing atmosphere in a fluidizing column whereby the contact time would also be reduced considerably. A difference in the susceptibility of modified cellulose is also noticed when heat treatment precedes milling operation. Preheated and postmilled material is more rapidly hydrolyzed than the premilled and postheated material. This is most likely due to the fact that preheating enables the polymer to reduce its particle size during subsequent milling much more smoothly than when the sequence is reversed. The most favorable way of improving susceptibility of the material is to carry out milling and heating operations simultaneously. A 71% increase in susceptibility in the case of finely pulverized Solka Floc (Sweco 70) heated to 200°C for 25 min over unheated Sweco 70 during 2.75 hr has been possible using highly active cellulase. Heat effects are principally oxidizing in nature as thermal

exposure in N_2 atmosphere exercises a retarding effect on the reducing sugar yield. Heating (Solka Floe) alone does not improve susceptibility. It must be followed or preceded by milling.

Loss of C_x activity in Tv cellulase in presence of substrate increases progressively with time and temperature. Between 0 and 60 min the loss increases from 19 to 67% in the presence of a highly active surface like Fuller's earth, but in contact with substrate, this loss is between 3 and 44%, both at 60°C. Between 40 and 50°C, the enzyme remains stable up to the first hour, but then begins to lose activity. The loss of activity of enzyme alone remains below 10%; while in contact with substrate the loss increases to 20% under the same conditions. For best enzyme activity and reducing sugar yield, a pH range of 4.8–5.2 appears to be optimum. From a study of the nature of profiles illustrated in Figures 6 and 8, all of which represent rates of enzymatic saccharification of cellulose (native and modified), there is a continuous change in the rates of hydrolysis over the entire period of contact between substrate and enzyme. The two separate tests comparing the effects of enzyme (C_x) concentrations on the rate of saccharification of Sweco 70 illustrate similar trends (Fig. 5). It is interesting to observe that each profile representing a defined activity of cellulase illustrates a distinctive rate of hydrolysis increasing with increasing concentration of enzymes. We may theorize that the slow action of C_1 on the cellulose in opening it up into a reactive form is very evident in the case of untreated Solks Floe (Fig. 8). The rates of C_x action on the reactive form are entirely dependent on the availability of the substrate. This phase of the cellulose hydrolysis is therefore accelerated by size reduction and heat treatment. A combination of these two steps brings about a sharp rise in the initial fast rates of saccharification within the first few hours of enzyme–substrate contact at the right pH. This process also continues until the substrates remaining become resistant enough to cause the rates to level off. Figure 6, representing several hydrolysis rates, shows that the initial fast rates are much higher than the subsequent rate steps and in most cases of enzyme–substrate contact the reactions come to a significantly low level, probably because of the buildup of more resistant crystalline substrate surface as suggested before. Ghose and Kostick²¹ showed that a considerable regain of the susceptibility of the resistant portion of the substrate is possible by heat treating the residues of enzyme digestion. Gradual buildup of glucose in the

reactor as the ultimate product of hydrolysis (Fig. 7) is also responsible for the decreasing rate of cellulose disappearance from the system. The initial fast rate of hydrolysis may be presented in the form of a simple straight empirical equation involving log-log function:

$$s = ct^n$$

where s is completion of hydrolysis in %, t is enzyme substrate contact time, min, c is rate constant, % hydrolyzed/unit time, and n is the slope of the profile.

Because of the existence of several factors like heterogeneity of substrate, interrelation between C_1 and C_x enzymes and the buildup of resistant cellulose during the course of hydrolysis, it is highly doubtful if the kinetic pattern as suggested for the initial phase of reaction is applicable to other stages of hydrolysis. Walseth²² suggested a similar rate equation for enzymatic hydrolysis of cellulose representing the initial fast rate, although the deviation from a straight line behavior of his data plotted beyond 12 hr of hydrolysis does not seem to justify only one rate process over the entire period of enzyme substrate contact.

In batch saccharification experiments at 10% substrate level without agitation, a conversion of more than 45% of modified cellulose (Sweco 70 heated) into glucose (5.0%) has been accomplished in 7 hr (Table I). Under agitated conditions, Sweco 70 (94% < 53 μ) yielded about 8% glucose in the hydrolysate in 40 hr at 10% substrate level (Fig. 8). In the case of SF-HM (76% < 53 μ) generally 50–65% conversions have been possible in batch experiments (Fig. 6). Under steady-state, continuously agitated systems employing SF-MH (55% < 53 μ) at 5% level, a maximum of 69% conversion (3.7% RS in the hydrolysate) has been achieved (Fig. 10). This conversion could be increased subsequently to 92% in batch operation of the system over a period of 143 hr resulting 5.1% glucose in the hydrolysate.

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