

Enzymatic Saccharification of Cellulose in Semi-and Continuously Agitated Systems

TARUN K. CHOSE' and JOHN A. KOSTICK

Microbiology Division, Food Laboratory, U. S. Army Natick Laboratories,
Natick, Mass. 01760

Enzymatic saccharification of cellulose pulp (Solka Floc) in semi- and continuously agitated systems has been studied using untreated enzymes obtained from submerged fermentation of Trichoderma viride (T. viride). Pretreatment of the cellulose substrate by heating, heating followed by grinding, and grinding followed by heating, leads to considerable difference in the rate of saccharification. It is possible to reactivate the residues from digestion to a high degree of susceptibility to enzymatic hydrolysis by heat treatment and milling. Saccharification of cellulose has been shown to be possible by milling the substrate in contact with T₀ cellulase. Semi-continuous hydrolysis of heated and milled Solka Floc (10% w/v) by enzyme (with daily replacement of 40% of the reaction volume with fresh enzyme substrate suspensions) maintained the sugar level in the effluent over a period of several days at 5.1-5.6%.

A large number of rumen and soil microorganisms representing extreme ranges of aerobic and anaerobic species are found to possess the capacity of degrading native cellulose into a wide range of products, including cellobiose, glucose, methane, alcohols, and organic acids. Yet, not a single commercial undertaking exists today to produce any of these chemicals through this microbial pathway. The desirability of having cellulose converted into glucose by microbes has nevertheless been emphasized because of the numerous problems involved in the chemical means of doing it. Although there is much uncertainty concerning the nature and mechanism of the action of enzymes, there is considerable

'NRC Senior Visiting Scientist on leave from Jadavpur University, Calcutta, India.

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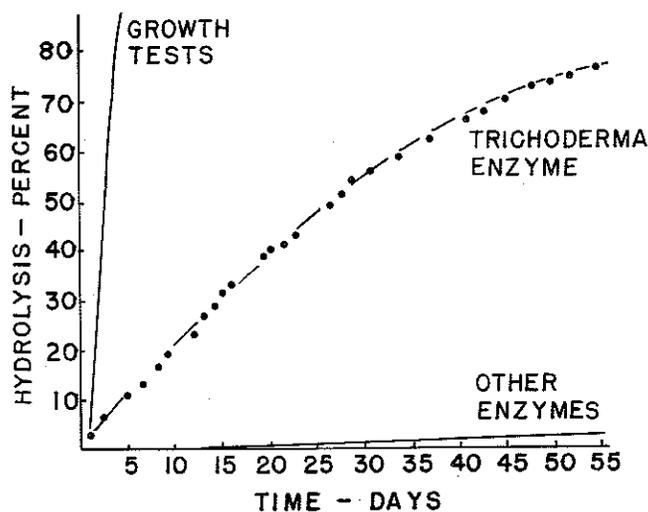
knowledge of what they do. Generally, the slow rates, the diverse end-products, and the problems of economic recovery of the products are some of the factors which stand in the way of biochemical utilization of cellulose. The success with which enzymatic conversion of starch into sugars has been attained (12, 18) motivates us to consider what might be similarly achieved in the case of cellulose.

Cellulolytic enzymes exist in nature, and many have been studied. Perhaps the best performing ones are not yet known to us, but the ones already produced from *Trichoderma viride* are good enough to saccharify cellulose in a manner comparable with that by which starch is converted into sugar. The success of such a program would lead to the creation of a tremendous new source of fermentable sugars from agricultural wastes. Besides discovering a more active source of cellulolytic enzyme, one of the most formidable goals to be accomplished lies in the modification of the native cellulose into a form more susceptible to hydrolysis. In addition to the complete conversion of cellulose into glucose, cellulase has been used to break down the cell walls of cereals, legumes, a large number of vegetables, and fruits, to make their edible contents more available to human digestive system. Other food uses of cellulase enzyme include clarification of citrus juices, removal of fiber from edible oil press cakes, higher yields in starch recovery from sweet potato, and cassava roots, extraction of proteins from leaves and grasses, tenderizing fruits and vegetables prior to cooking, extraction of essential oils and flavoring materials from roots, seeds, and barks containing large contents of crude fibers, etc. These applications are more fully described elsewhere (20).

A description of the difference between the mechanism of acid and enzymatic saccharification of cellulose has been reported (22). It has been shown that enzymatic hydrolysis of cellulose causes a slower loss in degree of polymerization (DP) than the acid hydrolysis. The most probable reason for such a difference is the relative sizes of the two catalysts (enzyme and mineral acid) and their ability to permeate the fine structure of cellulose. Large cellulase molecules (25,000-67,000 mol. wt.) are able to penetrate only into the larger intercrystalline space and on account of their high catalytic activity, all chain linkages (β -1,4 glucosidic) which can be contacted are readily hydrolyzed to form soluble sugars. On the other hand, the relatively small acid molecules are capable of diffusing into the smaller intercrystalline spaces and then attack glucosidic bonds which are otherwise not available to the enzymes. As a corollary to this, a finely ground cellulose would be expected to result in a higher conversion into glucose units by cellulase because of the reduction in crystallinity on one hand, and proportional increase of the surface on the other (3, 4, 7, 8).

A comparison (15) of activities of acid and enzyme catalysts on three cellulosic substrates at 50°C. shows that 100,000 times as much acid is required to bring about the same degree of hydrolysis. At the molecular level the difference is further increased because of the disparity in mol. wt. [(HCl—36, cellulase 63,000 (23)], so that approximately 10^8 HCl molecules are required to perform the work of a single enzyme under certain conditions. This is a point strongly in favor of enzyme hydrolysis of cellulose.

Microorganisms have no difficulty digesting cellulose (Figure 1). They accomplish it rapidly and effectively. Why is it then that we cannot utilize their systems to develop a practical conversion of cellulose to sugar? The answer is rather simple; we can—if we pour into this problem the effort it rightly deserves.



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Figure 1. Hydrolysis of cotton by organisms and by cell-free enzymes (17). Growth tests—Consumption of cellulose by active cellulolytic fungi. Trichoderma enzyme—(●—●—●—●— weight loss of cellulose). Other enzymes—Cell-free cellulases from organisms other than Trichoderma

Accessibility of Substrate and Rate of Saccharification. Let us re-examine the problem. Enzymes which catalyze the hydrolysis of simple soluble compounds may split a million (or more) bonds per minute per enzyme molecule. This is the ideal system towards which we must work.

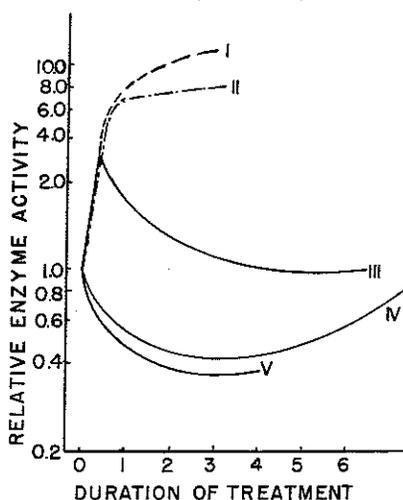
It involves molecules which in solution pose no problem of accessibility to enzyme. What happens when the substrate molecule gets much larger—*i.e.*, as one moves from the monomer to polymer? The interaction between large enzyme and large substrate molecules may be somewhat impeded—*i.e.*, it is likely to be more difficult for the enzyme to accommodate itself to the site at which reaction is to occur. But even here the rate of catalysis is high, in the neighborhood of 18,000 bonds per minute per enzyme molecule for α -amylase and 240,000 bonds for β -amylase. Viscous polymer and branched polymers present additional impedence to enzyme attack, the first through restricting enzyme movement and the second through blocking hydrolysis sites. Substituents on the substrate may also bind enzyme through electrostatic charges, further lessening the rate of hydrolysis.

The above factors, however, limit hydrolysis far less than conversion of the soluble polymer to the solid state, and most resistant of all substrates are those in which molecules are in a highly oriented or crystalline state. Into these it is difficult even for small water molecules to penetrate. What chance, then, for the large enzymes?

This resistant condition is the state of most cellulose. Our problem, thus, is to lessen the resistance by converting it into one of the more susceptible forms. Some years ago, cellulose highly susceptible to enzymes was produced (22) by swelling it in acid, and subsequently precipitating the same in water. Other methods (Figure 2) which increase accessibility are physical size reduction (3, 4, 7, 8) and alkali swelling (1). Gamma rays in low doses decrease susceptibility, but in high doses exercise the opposite effect (14). Boiling in dilute acid produces a more resistant residue. All these methods yield an insoluble cellulose. Chemical modification on the other hand results in soluble derivatives of cellulose. When these have only sufficient substituents to lend solubility (CMC, HEC, C-SO₄), they are much more rapidly hydrolyzed than are any of the insoluble cellulosic forms. Greater substitution (DS1) may produce a more soluble product, but it is then resistant to enzyme attack.

Both the swollen and the soluble celluloses can be rapidly saccharified, and the former completely converted into sugar in a relatively short time. The problem with them is that the maximum concentration obtainable is quite low. A 5% suspension of swollen cellulose is a solid mass. Even complete conversion can produce only 5% plus glucose in the hydrolysate. We need to reach higher levels.

Our aim then is modified somewhat. We require not only a susceptible substrate, but one which has a high bulk density, one which can be used in suspensions of 30–50%. At first glance, this would seem to be an impossible requirement; for if swelling leads to increased susceptibility, it also results in decreased concentrations in a given volume of



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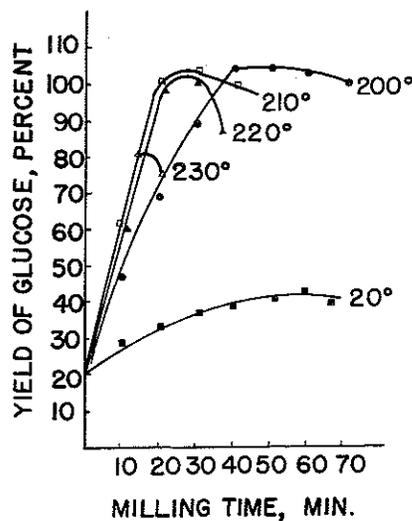
Figure 2. Effect of various treatments on the susceptibility of cotton cellulose to enzymatic hydrolysis (16)

- I—Effect of 85% H_3PO_4 , or 72% H_2SO_4 , abscissa in hours;
 II—Effect of ball milling (vibratory), abscissa in hours;
 III—Effect of 30% NaOH, abscissa in days exposure to alkali swollen material to air;
 IV—Effect of irradiation with cathode rays (van de Graaf), abscissa 0–60 megareps;
 V—Effect of weak organic acids, abscissa in days of refluxing (loss of weight = 5 to 6% in 3 days)

enzyme. The recent Russian discovery (8) gets us out of this dilemma. It has been shown (Figure 3) that milling of wood (fir) pulp cellulose at 200°C. for 40 min. in a specially constructed grinding unit increases the capacity of the cellulose to be hydrolyzed by acid at a faster rate. The glucose yield reaches 105%—*i.e.*, almost equal to theoretical. The comparative yields of glucose from cellulose milled under heated conditions at various temperatures show that a temperature range between 200°–220°C. appears to be most favorable for the kind of heat transfer and size reduction systems which the cellulose is exposed to. At 230°C. the cellulose increases in susceptibility to hydrolysis (~80%) up to 20 min. of milling and heating period and then falls off. Katz and Reese (4) have found that suspensions up to 50% w/v of ground and heat treated cellulose can be prepared; that this product is readily hydrolyzed by enzymes; and that concentrations of glucose of more than 30%

can be obtained. Our colleagues (Mary Rollins and Verne Tripp, U.S.D.A., SURDD, New Orleans, La.) have examined cellulose samples, heat treated and ground, which we sent them for examination. They report that the samples are probably slightly oxidized and their crystalline structure destroyed as evidenced by both infrared spectra and x-ray diffraction scans. The control samples (Solka Flocc, a sulfite process spruce pulp) has an x-ray crystallinity index of 83 which is comparable with that of cotton (85-90). Milled, and milled and heated samples show severe loss of crystallinity (to a cryst. index of about 41 (and reduced degrees of polymerization (to DP 200 or less). In the enzyme digested sample of heated and milled cellulose there appears to be more fibrillar structure visible and an increase in crystallinity implying that non-fibrillar material may have been digested away. Staining tests indicated that the milled cellulose and subsequent samples were more accessible to methylene blue and to iodine than was the control sample.

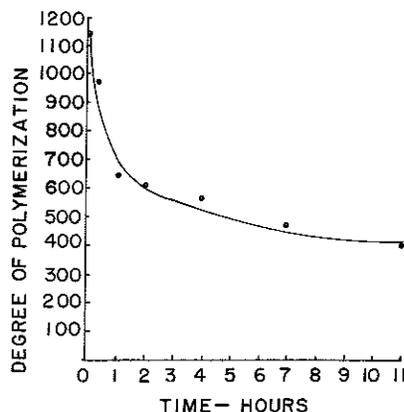
More than 60% reduction in the degree of polymerization of cellulose (about 1150 to 440) in the first eleven hours of contact with *Penicillium variable* cellulase (Figure 4) has been reported (2). Further depolymerization was very slow, implying that the amorphous portion of the cellulose has reacted rapidly, leaving a more resistant crystalline structure represented by a DP of about 440. These conclusions strongly



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Figure 3. Influence of temperature and duration of dry milling of fir tree cellulose on subsequent hydrolysis with diluted sulfuric acid (8)

support our experimental data (Figures 15, 16)—*i.e.*, the activated cellulose showing increased digestibility. The compact nature of our product with a high bulk density (15–30 lbs./cu. ft.) is caused by both oxidative heat treatment and size reduction. The Russian report (8) claims that the readily hydrolyzable cellulose obtained by dry milling of wood pulp at 200°–240°C. is soluble in cold water; but such a high degree of solubility could not be accomplished by either Katz and Reese (4) or Chose (3).



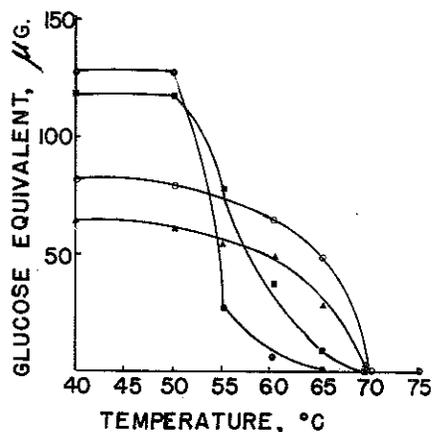
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Figure 4. Change of the degree of polymerization by enzyme action. *Penicillium variable cellulase*—wood pulp system (2)

In heated and milled cellulose, we have a material which is by far the best substrate yet found for cellulose saccharification, where the major goal is to obtain high glucose concentrations at a rapid rate.

Environmental Factors Affecting Rate. Temperature, pH, substrate concentration, and enzyme concentration are important factors in determining the rate of hydrolysis of any substrate. King (6) has summarized the advantage of several enzymes providing optimum activity at 50°C. (Figure 5). Li, Flcra, and King (9) have demonstrated that the effect of temperature on reaction velocity in the cellulose *T. viride* enzyme system depends on the nature of cellulosic substrate used (Figure 6). For amorphous cellulose, the Arrhenius plot is linear over the temperature range from 10°–57°C. (activation energy—5.1 kcal./mole). Because of the phase change from a sol at temperatures above 37°C. to a gel at lower temperature the carboxymethylcellulose profile represents two activation energy values (6.4 kcal./mole from 37°–60°C. and 16.7

kcal./mole below 37°C.). King (5) showed a fragility effect by the cellulase (most probably C₁ component) acting on hydrocellulose. This is reflected in a marked increase in the total number of crystalline hydrocellulose substrate particles (assumed spherical) during the initial substrate enzyme contact (Figure 7), implying that the enzyme action is not exclusively a surface erosion effect, but fragmentation of the larger initial particles into smaller ones. The surface area provided by the particles is a linear function of the surface destroyed by the cellulase.



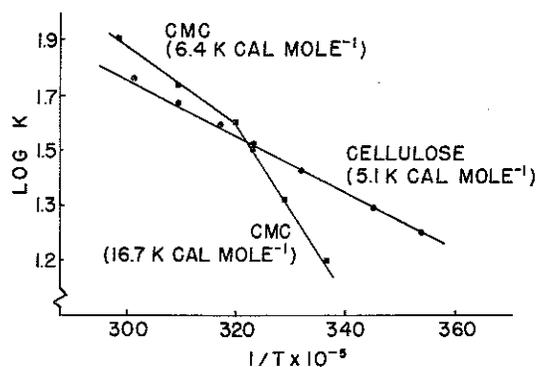
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Figure 5. Effect of heating on enzyme activity (6). Reaction mixtures contained substrate (2 mg.), 0.1M—acetate-buffer, pH—5.0 (2.0 ml.), and 0.1 ml. of an enzyme solution that had previously been held at the temperature shown for 10 min. Glucose equivalent was then estimated by the standard assay procedure described in the text

○—○—○— hemicellulase
 ▲—▲—▲— cellulase
 ●—●—●— amylase
 ■—■—■— laminarinase

The culture filtrate used in the starch and laminarin experiments was diluted 1:10

For *T. viride* cellulase substrate system a temperature of 50°C. and a pH of 4.5–5.5 are reported (3) to be optimal. Over this pH range, loss of enzyme activity may be caused by adsorption on adsorbents such as Fuller's earth or on substrates. Enzyme alone does not appear to lose activity at the temperature and pH of the reaction (50°C., pH 4.8–5.0) mixture. Rates of hydrolysis increase with enzyme and substrate con-



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Figure 6. Arrhenius plot showing effect of temperature on the rate of hydrolysis of amorphous cellulose and carboxymethylcellulose by the endoglucanase (9)

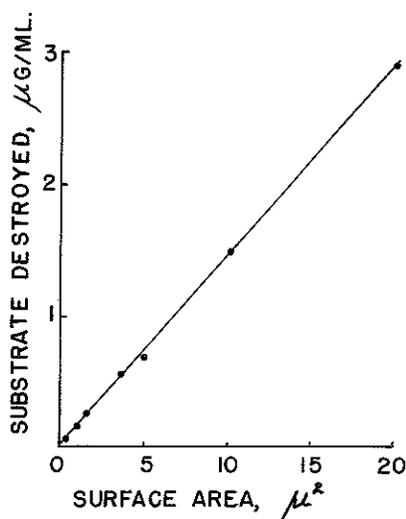
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Figure 7. Relationship of the rate of degradation of crystalline hydrocellulose particles to their surface area (5)

centrations (3, 21). No one has yet reported a decrease in rate as the cellulose concentrations become very high. However, a decreased rate has been observed with carboxymethylcellulose at concentrations over 1% but not with cellulose sulfate with the same *T. viride* enzyme (10). Similarly, Nisizawa *et al.* (13) have shown that the rate of hydroly-

sis of cellopentaose and cellotetraose by *Irpex lacteus* cellulase decreased markedly when a particular substrate concentration had been exceeded (Figure 8). Cellopentaose inhibited strongly at a concentration as low as 1 mg./ml. of reaction mixture. It required much more of the tetramer (7 mg./ml.) and considerably more of the trimer (500 mg./ml.) to reach inhibitory levels. These data show that cellulase can be inhibited at high concentrations by some of its substrates, but as indicated above, no such inhibition has been reported in the case of solid unmodified cellulose.

Product Inhibition. In most microbiological and biochemical systems accumulation of end products exercises an inhibitory effect on the rate of the forward reaction. Stimulation by end product is thermodynamically improbable. One of the major products of hydrolysis of cellulose is cellobiose. There is a stimulation by cellobiose of C_x activity of *Streptomyces* spp. filtrates only when the substrate is solubilized by the introduction of various substituents—*e.g.*, CMC, hydroxycellulose, cellulose acetate, etc. Stimulation is absent when unsubstituted cellulose is used (14). On the other hand, product "inhibition" is common. Cellobiose inhibits the hydrolysis of cellulose by filtrates of most of the 36 organisms tested. This action of cellobiose is believed to be that of an end product inhibiting an enzymatic hydrolysis in much the same manner that maltose inhibits hydrolysis of starch by α -amylase. The inhibitory effect of products varies with the organism from which the cellulase is derived. Thus, lactose is a very good inhibitor of the enzyme from

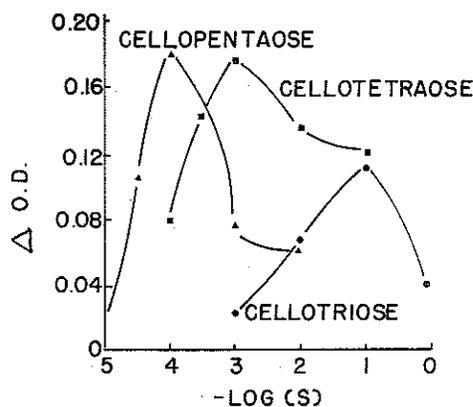


Figure 8. Activity—PS curves of cellulase II for cellotriase, cellotetraose and cellopentaose (13). Cellulase II: C_1 component obtained from a culture filtrate of *Irpex lacteus* by starch-zone electrophoresis. Conditions: Plastic tray $2 \times 5 \times 45$ cm., veronal buffer, pH 8.7, μ : 0.1, 24 hr., 5.6 V/cm., 2.0–3.0 mA./cm.²

Penicillium pusillum (Table I), but cellobiose is a good inhibitor of cellulases of many origins. Glucose inhibition is generally weak. For *T. viride* cellulases acting on heat treated cellulose, a concentration of 30% glucose gives only 40% inhibition (3, 4).

Product inhibition seems to be greatest for the larger soluble oligomers [cellopentaose (13)] and diminishes rapidly as the product molecular sizes become smaller. There would, therefore, seem to be a definite advantage in adding cellobiase to the cellulose hydrolysates for the conversion of cellobiose to glucose resulting in a decreased inhibition. In order further to cut down product inhibition and to keep the saccharification rate at its maximum, it would be of advantage to run the reaction in a steady state continuous system. A high bulk density of the substrate being only possible at a small particle size, a balance between the reaction time and glucose yield, may be drawn to reduce the inhibitory effects of glucose.

Table I. Inhibition of *Penicillium pusillum* Cellulase by Sugars (10)

| Sugar 1% | Inhibition of Hydrolysis, % | | | |
|-------------|-----------------------------|----------------------------------|---------------------------------|--------------------|
| | CMC Visc. | Swollen Cellulose Weight Loss | Ball Mill Cotton Weight Loss | Cotton Swelling |
| Cellobiose | 48 | 46 | 72 | 52 |
| Lactose | 88 ^a | 44 | 60 | 54 |
| Maltose | 5 | 4 | 1% stim. | NT |
| Glucose | 5 | NT | NT | 37 |

^a Lactose (0.1%) gave 52% inhibition; NT—Not tested.

Such a steady state continuous model has been favored for physiological studies in many microbiological systems primarily because environmental conditions can be kept constant, in sharp contrast with batch systems, where microorganisms are subject to continual change. The greatest advantage of a continuous biological system lies in the higher productivity than that for batch. Even a semi-continuous fermentation which eliminates a high incidence of microbial mutation appears advantageous. Engineering problems of aseptic control may also be avoided to some extent in a semi-continuous operation. Continuous culture is an excellent tool for studying mutation of microbial populations. Nevertheless, there are certain inherent practical problems associated with continuous fermentation. These are: (a) lack of homogeneity, especially in the case of thick and viscous media in large sizes, causing poor and fluctuating productivity at low dilution rates, (b) difficulty of maintaining a continuous fermentation under aseptic conditions over long period of time involving serious loads on the operations of sterilizing media and air,

and (c) inadequate stability relating to microbial strain and mechanical operation. Long continuous microbial fermentations are subjected to invariable mutation problems; this difficulty is by-passed by having semi-continuous operation in which the first vessel in series is occasionally re-seeded.

All the disadvantages cited are, however, applicable to microbial fermentations and none to enzymatic systems. As long as mechanical possibilities permit agitating and pumping thick slurries with high substrate concentrations (we have done this with 15% suspensions without difficulty) and, allowing fine free-flowing cellulosic substrates into the hydrolyzer at constant rate and under aseptic conditions, we do not visualize any major obstacle to run the system continuously. Maintaining it under sterile state is rather a less bothering problem particularly because of the possibility of having high glucose concentrations ($\sim 30\%$) in single or multi-vessel system.

Among the candidates important as substrates for single cell protein (SCP) cellulose is conspicuous. Unlike petroleum, a substrate about which much is said but so little is available in most of the overpopulated countries, cellulose is abundant all over the world. Unfortunately, few laboratories and but small efforts are engaged today in the big mission of economically rendering cellulose into its building blocks, glucose—a perennial source of carbohydrate for man and microbes. Those engaged in cellulase research today have had no success in selling the idea; but the successful production of sugar from cellulose at a cost less than 8¢ a pound will call attention to many who are interested in producing SCP or other food components from unconventional sources. We seem to be closer today to the accomplishment of saccharification of cellulose in a manner comparable to that achieved by glucoamylase in the conversion of starch to glucose.

The present study is aimed at investigating the increase in the susceptibility of cellulose towards cellulase in order to increase both rate and yield of sugar. It demonstrates the possibility of regenerating enzyme digested-resistant cellulose into highly susceptible form. It also describes a stable model reaction system for semi- or fully-continuous system for saccharification of cellulose into glucose using untreated culture filtrates of *Trichoderma viride*.

Materials and Methods

The Substrate and Its Modification. Solka Flocc—SW40A (Brown Co., Berlin, N. H., USA), a wood (spruce) pulp commonly used as a filter aid has been used as a basic cellulosic material for susceptibility tests and saccharification studies. The material was milled dry in a laboratory porcelain pot mill using glazed porcelain balls of 1 inch (2.65

kg.) and 1/2 inch (1.2 kg.) sizes over a period of time. Sweco 70 material used in several experiments was a dry milled product of Sweco Vibro Energy Mill received from Southwestern Engineering Company, Los Angeles, California. The dry milled products were screened down to 270 U.S. Standard Screens (opening 53μ). As heat treatment of the substrate was found to improve enzyme susceptibility further, the Solka Floc was heated to 200°C. for 25 min. either before or immediately after milling. To ascertain the extent of hydrolysis taking place during milling of cellulose in the presence of the cellulase, a few test runs were conducted with Solka Floc suspended in enzyme solution at room temperature (22°C.). Modification of Solka Floc was done by a combination of several kinds of treatments such as milling in pot mill, milling in Sweco Mill, heating in a laboratory forced draft air oven at 200°C. for 25 min., heating followed by milling, milling followed by heating, and milling in the presence of Tv cellulase. The following abbreviations are used in the text to designate various substrates:

- SF = Solka Floc SW40A without any treatment
- SF-M = Solka Floc pot milled
- SF-H = Solka Floc heated at 200°C. for 25 min.
- SF-MH = Solka Floc pot milled and heated as above
- SF-HM = Solka Floc heated and pot milled as above
- SF-MHD = Solka Floc pot milled, heated, and enzyme digested (~60%)
- SF-EM = Solka Floc pot milled in presence of cellulase enzyme
- SF-MHDWDr = Solka Floc pot milled, heated, enzyme digested, washed, and dried at 60°C.
- SF-MHDWDrH = Solka Floc pot milled, heated, enzyme digested, dried, and heated as above
- SF-MHDWDrM = Solka Floc pot milled, heated, enzyme digested, washed, dried, and milled as above
- SF-MHDWDrMH = Solka Floc pot milled, heated, enzyme digested, washed, dried, milled, and heated

Screen Analysis of the Substrate. 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 45 min. plus an additional 5 min. after the first weight 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-H, SF-HM, SF-MH, and Sweco 70 samples, all of which were subsequently tested. Summary of screen analyses reports is presented in Table II and Figure 9.

Enzyme and Its Assay. Culture filtrates from 10 liter batch or semi-continuous submerged fermentations of *Trichoderma viride* QM 6a were

used in the saccharification studies after adjustment of pH ($\sim 3 \rightarrow 5.0$). Most of these enzyme solutions were prepared by Mary Mandels (11). Following harvests, the culture fluids were filtered off on glass wool and made free of the suspended mycelium, pH adjusted to 4.8–5.0, and stored at 2°C. The enzyme strengths expressed in terms of FP activity (11) of the batch and semi-continuous culture filtrates used in the studies varied generally between 0.75 to 1.26 and 0.9 to 1.86. For continuous saccharification experiments requiring large volumes of enzymes with constant activity, several harvests were mixed together to bring the enzyme strength to about a FP activity of 1.0. The filtrates were sparkling clear with a light yellow-green coloration and an agreeable smell. Assays of enzyme activity of all samples received from fermenters were performed immediately after each harvest followed by filtration. For enzyme assay (FP activity) procedure, see Reference 11.

Table II. Screen Analysis of Solka Floc and Heated Solka Floc

| Materials | Particle Size Distribution, % | | |
|--|-------------------------------|-------------|------------|
| | > 149 μ | < 149 μ | < 53 μ |
| 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 24 hr.) | 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 | 1.1 | 99.3 | 93.8 |

Reducing Sugars. Estimation of total reducing sugars (RS) as glucose has been based on the DNS method (18) used in the cellulase assay. Actual procedure has been described in another communication (3).

Experimental

The factors affecting stability and activity of cellulase have been studied by several investigators. The present experiments include some of these factors particularly as they relate to a system being evolved for the production of high glucose concentrations.

Enzyme Studies. (1) EFFECT OF TEMPERATURE AND PH ON THE INACTIVATION OF CELLULASE. Details of experiments and results on the effects of temperature on the inactivation and adsorption of *Tv* cellulase, effect of pH on adsorption, activity, and inactivation of *Tv* cellulase, effect of enzyme concentration on saccharification of cellulose, and glucose inhibition of saccharification of cellulose have been reported in a separate communication (3). These data show that small enzyme losses (up to 10%) are difficult to detect. *Tv* cellulase alone does not appear to lose

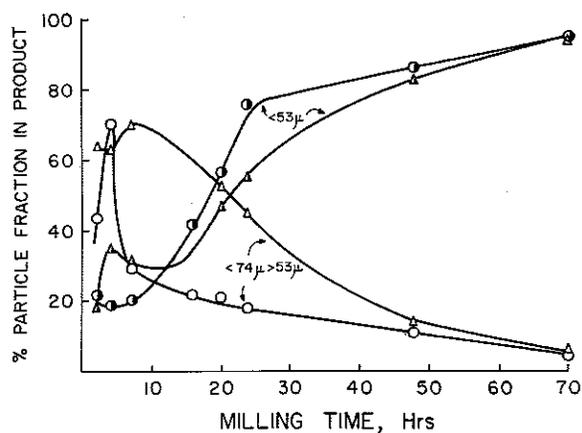


Figure 9. Effect of pre- and postheating of Solka Floc on particle size distribution owing to milling. Heating at 200°C. for 25 min. in forced draft air oven

Preheated - ○ - ○ - ○ - <math>< 74\mu > 53\mu</math>
 - ● - ● - ● - <math>< 53\mu</math>
 Postheated - △ - △ - △ - <math>< 74\mu > 53\mu</math>
 - ▲ - ▲ - ▲ - <math>< 53\mu</math>

any activity at 50°C. for one hour at pH 4.5. At 60°C. the inactivation amounts to 25% in one hour. In the presence of substrate (SF) at 50°C., the enzyme loss remains almost constant at about 13% up to one hour of contact. The cellulase alone remains most stable between pH 4.0 and 5.0 (50°C.; 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 the presence of substrate, however, the range for maximum cellulase stability is slightly broader—e.g., 3.5 to 5.5. The best range for enzyme activity (pH—4.8–5.0) falls within the range of maximum stability. It has further been demonstrated (3) that the highly heterogeneous nature of the suspension of the substrate enzyme system increases its initial rate of hydrolysis owing to agitation bringing the system close to homogeneous, even at a lower enzyme concentration. Secondly, at the same substrate level, increased concentrations of enzymes increases the rate of hydrolysis considerably. Inhibitory effect of product (glucose) accumulation in the system has also been shown.

(2) LOSS OF CELLULASE (C_x) ACTIVITY IN THE PRESENCE OF HIGHLY SUSCEPTIBLE SUBSTRATE AND ABSORBENT. Temperature and pH affect the inactivation and adsorption losses of *T. viride* cellulase in contact with Solka Floc and milled Solka Floc (3). Since several saccharification studies were conducted with highly reactive cellulose (Sweco 70 heated), a knowledge of the difference, if any, between adsorption and other losses of *T. viride* cellulase in contact with such substrates (at 50°C., pH—4.8) was necessary. Accordingly, *T. viride* cellulase was incubated with Sweco

70 heated substrate and with Fuller's earth as adsorbent (Figure 10). The enzyme alone suffers practically no loss of activity. In contact with Fuller's earth, a progressive loss of activity took place. In the first four hours, because of adsorption alone on Fuller's earth, 44% of the activity was lost and more than 60% was lost in 22 hours (both at 50°C.). At 60° the loss increased from 13% on immediate contact to 42% in one hour (3). In contact with substrate (Sweco 70 heated) a loss of 40% activity takes place in the first hour at 50°C. No additional loss is observed in the next 21 hours.

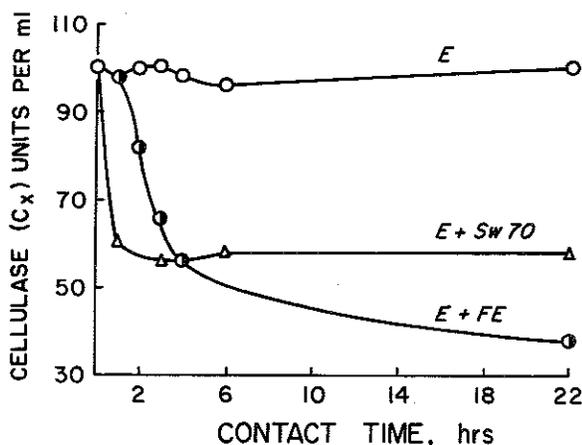


Figure 10. Loss of cellulase activity in contact with highly susceptible substrate (Sweco 70—93.8% < 53 μ)

- Cellulase alone (E)
- △—△—△—△—△— Cellulase plus Sweco 70 (E + Sw 70)
- Cellulase plus Fuller's earth (E + FE)

Cellulase activity—100 C_x units/ml.; substrate and adsorbent concentration—50 mg./ml. in tubes; temperature—50°C.; pH—4.8

(3) EFFECT OF pH LEVELS ON RATE OF SACCHARIFICATION. Studies on the rate of saccharification and cellulase inactivation have shown (3) that a pH range of 4.5–5.5 appears most effective for enzymatic hydrolysis of native cellulose. Since most of the experimental studies are conducted with modified cellulose (SF-HM, SF-MH) and under agitated systems, a verification of these facts was necessary. A 500 ml. glass stirred tank reactor was used for the study at 40°C. and 50°C. with SF-HM material and *T. viride* cellulase providing 1.26 FP activity. Each saccharification test was conducted for a period of 40 hr. at pH values of 3.0 to 6.5. The reducing sugar produced during this period is plotted against the pH values (Figure 11). As in all other experiments, each reaction mixture was provided with 1 mg./liter of Merthiolate to eliminate the possibility of contamination. It is clear from the two profiles representing two different temperatures of digestion that for both 40°C. and 50°C. a pH range between 4.5 and 5.5 appears most effective for high rates of con-

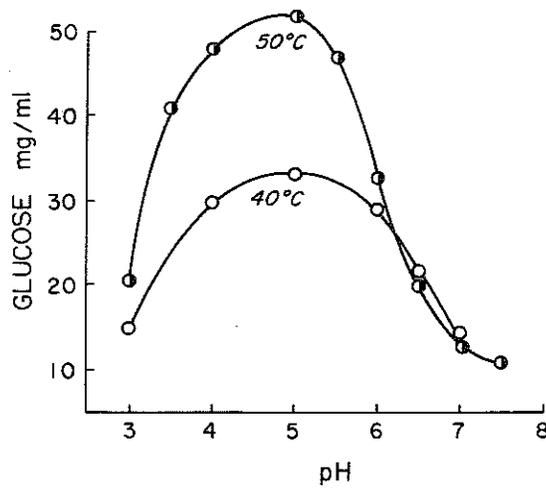


Figure 11. Effect of pH on the saccharification of modified cellulose (SF-HM, 76% < 53 μ)

—○—○—○—○— 40°C.
—●—●—●—●— 50°C.

Cellulase activity—1.26 FP units; substrate concentration 10%; total reaction time—40 hr.; 0.5 liter glass stirred tank reactor

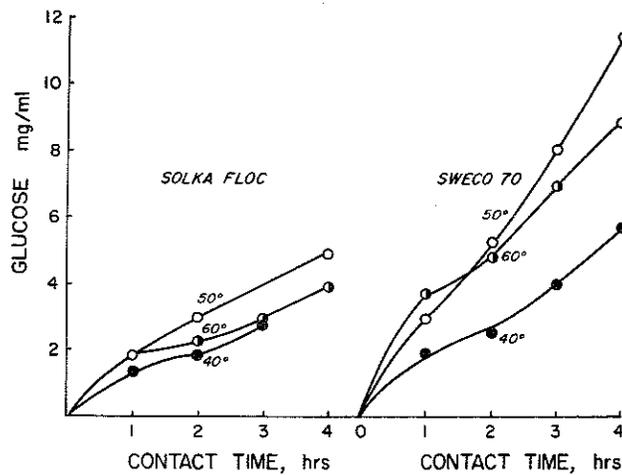


Figure 12. Effect of temperature on saccharification of native and modified cellulose

○—○—○—○— 50°C.
●—●—●—●— 60°C.
●—●—●—●— 40°C.

Cellulase activity—36 C_x units/ml.; substrate concentration 50 mg./ml. in tubes; pH—4.8

version. The difference of more than 18 mg./ml. glucose production between 40°C. and 50°C. (at pH 5.0) indicates superiority of higher temperature.

(4) EFFECT OF TEMPERATURE ON SACCHARIFICATION OF NATIVE AND MODIFIED CELLULOSE. Tests were conducted to determine the optimum temperature for enzymatic saccharification of modified cellulose under the conditions employed in the present studies. Two series of tests, one with SF, and the other with Sweco 70 were made at 40°, 50°, and 60°C. in tubes containing 10 ml. of 10% substrate suspension in *T. viride* cellulase (activity = 36 C_x units/ml.) over a period of 4 hr. The enzyme acts at an initially higher rate of hydrolysis at 60°C. than at 50°C. or 40°C. in the first hour. The steady rate of reaction at 50°C., however, remains high after the first hour while the rate at 60°C. declines, keeping an increasing margin of reducing sugar yield between itself and those at 40° or 60°C. This trend is also applicable to Solka Floc. Under agitated conditions the rates at 50°C. give higher values than those illustrated here (Figure 12).

Substrate Studies. (1) EFFECT OF HEAT TREATMENT OF THE NATIVE CELLULOSE (NOT MILLED). In order to ascertain the effect of heat treatment of Solka Floc under an oxidizing atmosphere without any reduction in particle size of the substrate, a series of hydrolysis tests was conducted in test tubes using SF heated to various temperatures. Fifty grams of

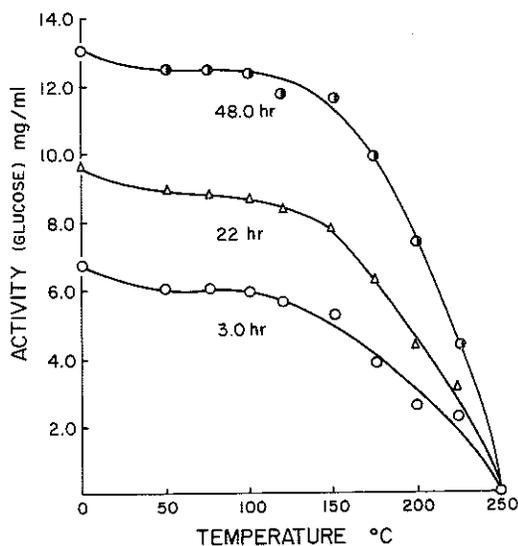


Figure 13. Susceptibility of Solka Floc (not milled) preheated at various temperatures. Hydrolysis conditions: Substrate—50 mg./ml.; Cellulase—0.75 FP activity; pH—4.8; Temperature—50°C.

○—○—○—○— 3 hr. hydrolysis
 △—△—△—△— 22 hr. hydrolysis
 ●—●—●—●— 48 hr. hydrolysis

samples of SW40A Solka Floc were evenly spread in 1/4-inch layers on enamelled trays and exposed to various temperatures in a forced draft air oven for 25 min., and then quickly cooled and kept in bottles. These heat treated samples were tested at 50°C. for their relative susceptibilities to *Trichoderma* cellulase (Figure 13). The results indicate that heating alone exercises an inhibitory effect on the hydrolysis by cellulase. There is a progressively increasing loss of susceptibility as the temperature of heating is increased and the enzyme action on cellulose heated to temperatures above 225°C. ceases. This unusual inhibitory effect may result from drying out the fibers in such a way that they do not again readily take up water. It is an effect quite the reverse of that obtained by similarly heating finely ground Solka Floc (Table III) where a considerable increase in saccharification has been observed.

Table III. Effect of Heat Treatment of Finely Ground Substrate (Sweco 70)

| Substrate 100 mg./ml. | Reducing Sugar Produced, mg./ml. | |
|----------------------------------|----------------------------------|------------|
| | In 2.75 hr. | In 7.0 hr. |
| Sweco 70 | 22.5 | 43.0 |
| Sweco 70 heated to 200°C. for | | |
| 25 min. | 38.0 | 50.0 |
| 40 min. | 27.0 | 51.0 |
| 60 min. | 29.0 | 50.0 |
| 120 min. | 21.5 | 42.0 |

Cellulase activity = 111 C_x units/ml.; temperature—50°C.; pH—4.8.

In view of the differences in the digestibility between the heated cellulose, and the milled and heated celluloses, as observed in the previous experiments (Table III) towards *T. viride* cellulase, it was necessary to study the extent of the effect of heat treatment of cellulose under an oxidizing atmosphere with particle size reduced to a low level. This was accomplished by milling Solka Floc for 2 to 48 hr. in laboratory porcelain pot mill. The milled products were screened down to < 53 μ size. This procedure was followed with both SF and SF-H materials to give SF-M and SF-MH. The results are presented in Figure 14. A comparison of the sugar values between the two substrates, SF-HM and SF-MH, suggests that the preheating provides a better substrate for cellulase than postheating. This may be owing to the presence of a larger proportion of smaller particles in the SF-HM material, particularly between 10 and 48 hr. of milling as evidenced from Figure 9. Variations in reducing sugar values in a few samples might have been owing either to difference in particle size of randomly weighed samples, or to non-uniform substrate enzyme contact, as the contents were not agitated, or to inadequate heat exposure of some of the particles, or a combination of all these effects. However, these samples represent less than 8% of all the samples tested.

(2) REACTIVATION OF RESIDUE (OF ENZYME SACCHARIFICATION) BY HEAT TREATMENT AND MILLING. Oxidative heat treatment of finely pulverized cellulose has been found to impart increased susceptibility to *T.*

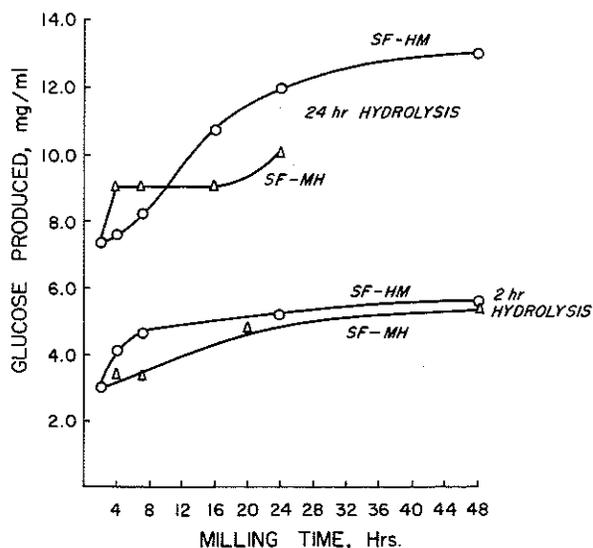


Figure 14. Effect of order of heating and milling of Solka Floc on its enzymatic saccharification

○—○—○—○— SF-HM ($< 53\mu$)
 △—△—△—△— SF-MH ($< 53\mu$)

Conditions of saccharification:
 2 hr. at 50°C. at pH—4.8
 24 hr. at 50°C. at pH—4.8
 Cellulase—0.92 FP activity

Table IV. Effect of Various Treatments of Substrates on the Production of Reducing Sugars

| Substrate (50 mg./ml.) | Nature of Treatment | Reducing Sugar Produced, mg./ml. | | |
|------------------------------|---|----------------------------------|---------|----------|
| | | 2.5 hr. | 4.5 hr. | 23.0 hr. |
| Solka Floc | None | 6.6 | 8.5 | 17.0 |
| Solka Floc | Milled for 48 hr. (82% $< 149\mu$) | 8.7 | 12.7 | 22.2 |
| Solka Floc | Milled and heated ^a in air (82% $< 149\mu$) | 16.0 | 21.0 | 33 |
| Sweco 70 (94% $< 53\mu$) | Milled and heated ^a in N ₂ (O ₂ free) | 7.8 | 11.0 | 25.5 |
| Sweco 70 (94% $< 53\mu$) | None | 11.2 | 13.0 | 31.0 |
| Sweco 70 (94% $< 53\mu$) | Heated ^a in air | 17.1 | 20.0 | 34 |
| | Heated ^a in N ₂ (O ₂ free) | 10.2 | 13.2 | 28.5 |

^a At 200°C. for 25 min.; cellulase—60 C_x units/ml.; temperature 50°C., pH—4.8.

viride cellulase (Table IV). It remains to be seen if similar treatments of the digested cellulose would produce any noticeable increase in its sus-

ceptibility. In other words, can the residues of the digested cellulose samples, which are not longer accessible to cellulase be modified by heat and milling in such a way that they become susceptible? Residues and several substrates were simultaneously tested with regard to their rates of saccharification at 50°C. at 10% substrate level using *T. viride* filtrates with 1:5 FP activity at pH 4.8. The substrates and enzyme digested residues tested were $S_1 = SF$; $S_2 = SF-M (< 53\mu)$; $S_3 = SF-HM (200^\circ C. \text{ for } 25 \text{ min., } < 53\mu)$; $S_4 = SF-MH (< 53\mu, 200^\circ C. \text{ for } 25 \text{ min.})$; $S_5 = SF-MHDWDr (\text{ digested up to } \sim 60\%, \text{ dried at } 60^\circ C.)$; $S_6 = SF-MHDWDrHM (\text{ heating and milling as others})$.

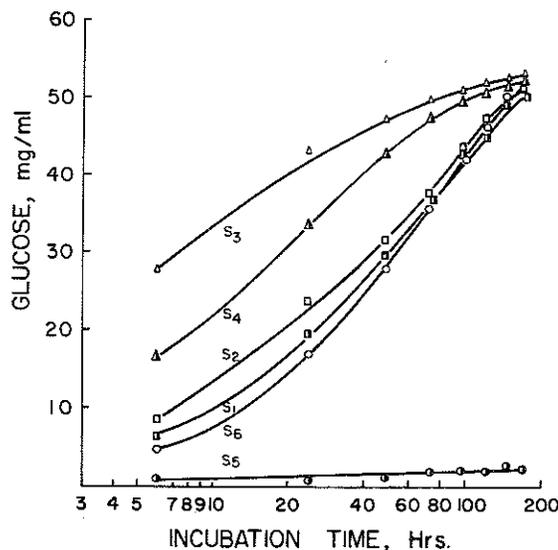


Figure 15. Effect of heat treatment and milling of cellulose on saccharification

- S_1 ■-■-■-■- SF (11.6% $< 53\mu$)
 S_2 □-□-□-□- SF-M
 S_3 △-△-△-△- SF-HM (200°C. for 25 min., 76% $< 53\mu$)
 S_4 ▲-▲-▲-▲- SF-MH (76% $< 53\mu$, 200°C. for 25 min.)
 S_5 ●-●-●-●- SF-MHDWDr (Dried at 60°C.)
 S_6 ○-○-○-○- SF-MHDWDrHM (200°C. for 25 min. $< 53\mu$)

Cellulase—1.80 FP activity; pH—4.8; substrate concentration 10%; temperature—50°C.

The results of tests carried out for 168 hr. (Figure 15) show conclusively that the residue of digested cellulose (S_5) which has been rendered extremely resistant to cellulase action can be reactivated into a highly reactive form (S_6) almost comparable to that of the initial Solka Floc (S_1). This has been possible by subjecting the fully digested residue to the same cycle of heating and milling as done to SF to obtain SF-HM (S_3). The results further show the beneficial effects of preheating cellu-

lose prior to milling in order to have maximum susceptibility of the substrate (S_3) to *T. viride* cellulase, compared with SF-MH (S_4). However, the ultimate percentage conversion of cellulose in its various modified forms after 168 hr. of saccharification appears to become more or less equal in all cases.

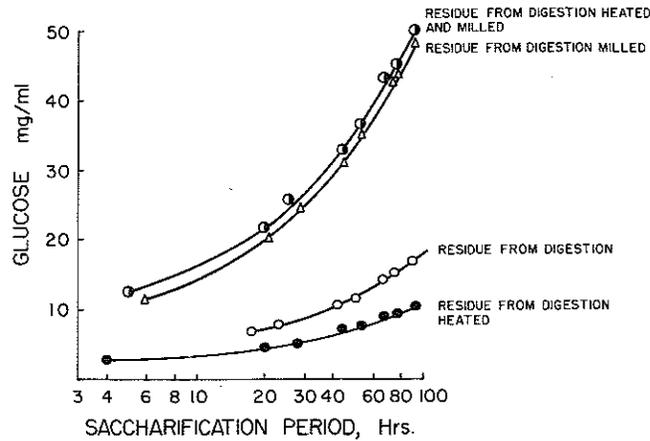


Figure 16. Reactivation of enzyme digested cellulose by heating and milling

- - ○ - ○ - ○ - SF-MHDWDr ($< 53\mu$)—Residue from digestion
- - ● - ● - ● - SF-MHDWDrH ($< 53\mu$)—Residue heated for 25 min. at 200°C.
- △ - △ - △ - △ - SF-MHDWDrM at 200°C. ($< 53\mu$)
- ⊖ - ⊖ - ⊖ - ⊖ - SF-MHDWDrHM at 200°C. milled ($< 53\mu$) heated as above

Substrate—10%; Cellulase—1.26 FP activity; pH—4.8–5.2; Temperature—50°C.; Reaction in 0.5 liter glass stirred tank reactor

Subsequently, four sample residues (SF-MHDWDr, SF-MHDWDrH, SF-MHDWDrM, and SF-MHDWDrHM) all comprising 100% $< 53\mu$ particles were separately hydrolyzed in 0.5 liter agitated glass reactors at 50°C. using 10% suspensions of the substrates in Tv cellulase (1.26 FP activity) at pH 4.8–5.2. The saccharification continued for nearly 90 hr. (Figure 16) indicating that a reactivation of the so-called resistant (digested) cellulosic substrate is possible by a combined treatment or milling and heating. The extent of increase in susceptibility brought about in the digested cellulose is comparable with that possible by similar treatments of SF. Much, however, depends on the fineness of the substrate particles. Susceptibility increases with increasing fineness of grind. It is thus possible to re-use the enzyme digested cellulose for saccharification in a closed cycle operation. The lack of difference between the residue heated and milled, and residue heated may be caused by the presence in the former material of a higher fraction of oxidized residue, which is seemingly inhibitory to enzyme action. As explained

before, heating of SF helps particle size reduction in the subsequent milling; but apparently such an assistance is not needed by already finely ground particles of cellulose.

(3) SEMI-CONTINUOUS SACCHARIFICATION OF SF-HM AND SF-MH. Following the initial studies on the enzymatic hydrolysis under changed environments of pH, temperature, cellulase activity, and other factors reported earlier, a few semi-continuous saccharification experiments were conducted. Comparative information on the differences in the rates between SF-HM and SF-MH was also necessary. For these two systems, the reactions were carried out in 0.5 liter agitated glass reactors of the same dimensions, at 50°C. using 10% substrate levels in each case and the same Tv cellulase (FP activity = 1.8). After an initial 40 hr. of batch hydrolysis, 200 ml. (or 40%) of the reaction mixture was removed and replaced by 200 ml. of fresh enzyme plus an amount of substrate equal to the fraction of cellulose converted into glucose at that time, plus the amount present in the 200 ml. of effluent removed. Both the original reaction volumes (0.5 liter) and the subsequent feeds were provided with 1 mg. of Merthiolate per liter. The cycles of discharge and feeds were repeated and the reactions continued over several days (Figures 17 and 18).

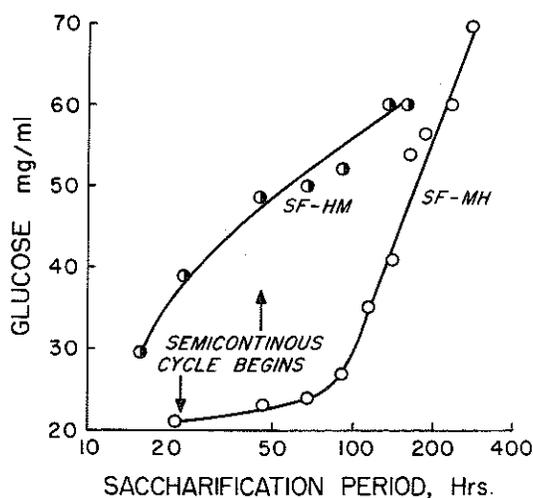


Figure 17. Semi-continuous saccharification of modified cellulose

- 10% suspension of a 54.9% < 53 μ size SF-MH substrate
- 10% suspension of a 76% < 53 μ size SF-HM substrate

Reactor conditions: 0.5 liter glass stirred tank; Cellulase—1.8 FP activity; pH—4.8; Temperature—50°C.; Substrate particle size SF-HM 76% < 53 μ ; SF-MH 54.9% < 53 μ . Semi-continuous harvest and feeding started for SF-HM at 45th hr. and for SF-MH at 22.5th hr.

The difference of nearly 21% of fine ($< 53\mu$) particles (Table II, Figure 9) between the two substrates SF-HM and SF-MH makes a contribution to increased susceptibility of the SF-HM substrate over the other. Heating most likely renders the cellulose fragile and subsequent milling results in a disappearance of a large portion of its fibrillar structure, which is replaced by fine and more amorphous materials. This steady rate of hydrolysis after the first 40 hr., in the case of SF-HM,

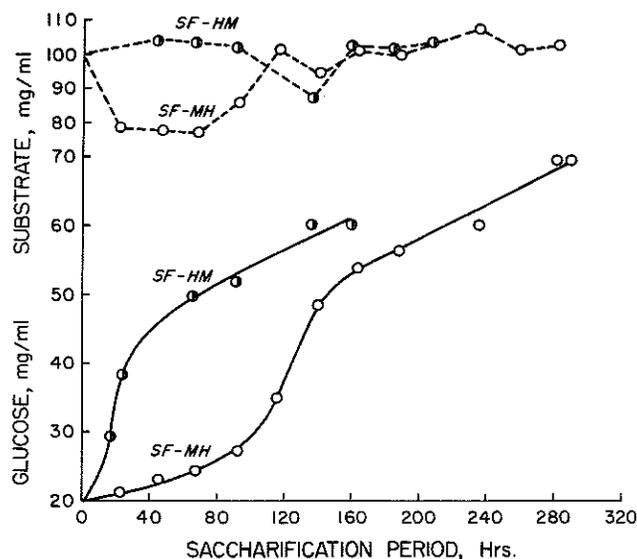


Figure 18. Relationship between substrate concentration and saccharification rate of modified cellulose (SF-HM, SF-MH) in semi-continuous agitated reactors

---●---●---●---● SF-HM Glucose level
 ---○---○---○---○ SF-MH Glucose level
 ---●---●---●---● SF-HM Substrate level
 ---○---○---○---○ SF-MH Substrate level

Reactor conditions: 0.5 liter glass stirred tank reactor; Substrate concentration 77–108 mg./ml. (av.—100 mg./ml.); Cellulase—1.8 FP activity; pH—4.8; Temperature—50°C.; Substrate particle size—SF-HM—76% $< 53\mu$; SF-MH—54.9% $< 53\mu$; Semi-continuous feeding and harvest started at 45th hr. for SF-HM, and at 22.5th hr. for SF-MH

progresses steadily. The other substrate (SF-MH) containing a larger portion of large particles ($> 149\mu$) and smaller portion of fines keeps down the initial rate up to about 90 hr. and then progresses at a fast rate, almost equal to the initial rate exhibited by SF-HM. The two rates become equal after 160 hr. of hydrolysis. It was evident from these experiments that the hydrolysate in batch and semi-continuous saccharification of cellulose, a level of about 5% sugars is reached around 40 hr. in the SF-HM and Sweco 70, whose average particle size consisted of between 85–94% $< 53\mu$. Generally higher percent conversions were

obtained in Sweco 70 which contained more than 93% of 53μ or smaller particles as against little over 40% for SF-MH and around 85% for SF-HM, both milled for 48 hr. under the same conditions.

(4) CONTINUOUS SACCHARIFICATION OF MODIFIED SUBSTRATE IN SINGLE STAGE. Because of limited supply of Sweco 70, most of the continuous studies were based on SF-MH and SF-HM. One of the major difficulties encountered in feeding the solid substrate into the reactor continuously was the small reactor volume and necessarily low feed rates. Constant feeding has therefore been made in the form of an enzyme substrate suspension kept under continuous agitation at 1° - 2°C . in an ice-cooled jacketed bath (Figure 19). Details of the method are described in another communication (3). Rate data on reducing sugar yields in a 4 liter single stage stirred reactor using 10% substrate (SF-HM) and *T. viride* cellulase are illustrated in Figure 20. The unit was run

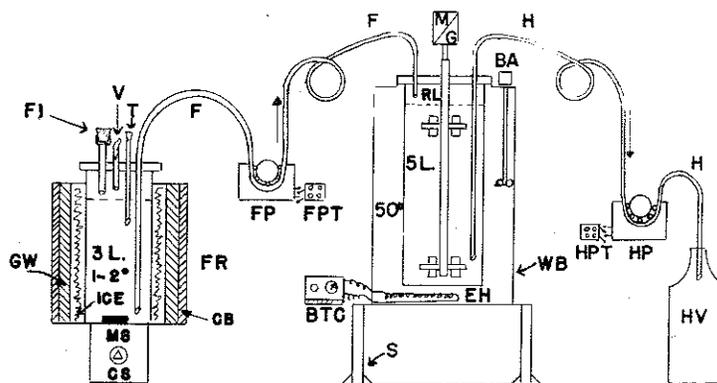


Figure 19. Continuous forward feed steady state single vessel saccharification system

Legend..

- GW = Glass wool lagging
- CB = Cardboard lagging
- FR = Feed reservoir
- FI = Feed (Enzyme + Solid Substrate) Inlet
- V = Vent
- T = Thermometer
- MS = Magnetic stirrer
- CS = Magnetic stirrer control switch
- F = Feed line
- H = Harvest line
- M/G = Speed geared motor
- BA = Bath agitator
- RL = Reactor level
- EH = Electrical heating element
- FP = Feed pump
- FPT = Feed pump timer
- HP = Harvest pump
- HPT = Harvest pump timer
- WB = Water bath
- S = Support
- HV = Harvest vessel
- BTC = Bath thermostat control

as a batch up to the first 40 hr. Continuous feeding of enzyme substrate slurry from the ice-cooled reservoir, and discharge of the effluent from the reactor, both at the rate of 100 ml. per hour, were started from this point onward and were maintained at this steady dilution rate of 0.025^{-1} hr. over a period of 170 hr. The effluent maintained a reducing sugar level between 5.1–5.6 mg./ml. during the continuous phase. Because certain mechanical troubles developed in the agitation system of the reactor, the experiment had to be discontinued beyond this period.

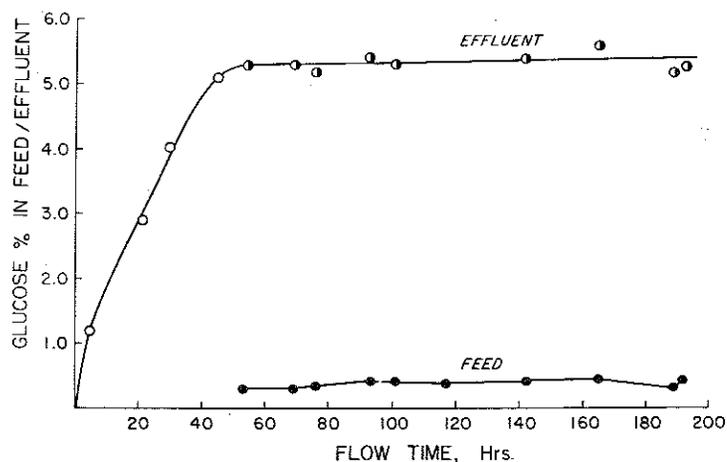


Figure 20. Continuous steady state saccharification of modified cellulose (SF-HM)

- Batch rate profile
- ◐—◐—◐—◐— Continuous phase
- Reducing sugar in feed

Enzyme—1.0 FP activity; Substrate—SF-HM (76% 53μ—v0%; pH—4.05–5.2; Saccharification temperature—50°C.; Dilution rate in continuous phase— 0.025^{-1} hr.; Reactor conditions: 4.0 liter glass stirred tank reactor; Feed temperature—1°–2°C.

(5) HYDROLYSIS DURING MILLING OF CELLULOSE IN THE PRESENCE OF ENZYMES. Since the size reduction operation takes the major portion of the total time needed in converting cellulose into reducing sugar, it seemed desirable to see if hydrolysis could be done simultaneously with the size reduction operation. No published information is available on such an approach. Cellulose was subjected to milling at room temperature (22°C.) in presence of cellulase in a porcelain pot mill containing porcelain balls, both previously steam sterilized. The substrate (SF-H) was kept at 10% consistency in 1.0 liter of *T. viride* cellulase. After 48 hr. of milling, 0.5 liter of the milled enzyme substrate slurry was transferred into a 0.5-liter stirred tank reactor, and further hydrolysis continued in both of the systems (mill and agitated reactor) at 22°C. and 50°C., respectively. The progress of saccharification is shown in Figure 21. The enzyme still remains active after 48 hr. of abrasive milling in contact with substrate. While this is in no way a conclusive study of the new

system, it illustrates the possibility of further experimentation on milling of substrate in contact with enzymes at 50°C. Although there is likely to be more loss or inactivation of enzyme in such a system, the gain in time and milling cost is worth looking into.

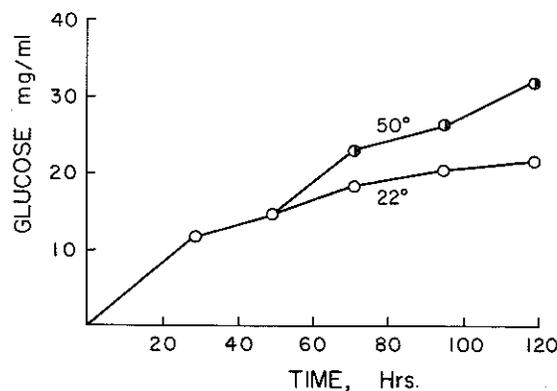


Figure 21. Effect of milling cellulose in contact with cellulase on saccharification

- — ○ — ○ — Solka Floc milled in the presence of *T_v* cellulase at 22°C.—10% suspension
 ● — ● — ● — Solka Floc milled in contact with *T_v* cellulase subjected to further hydrolysis at 50°C. in agitated reactor—10% suspension

Enzyme—0.9 FP activity; pH—5.0; Mill size—1.0 liter in porcelain pot mill (2.5 gal.) previously steam sterilized

Discussion

Experimental results show that heating of cellulose exercises an "inhibitory" effect on the action of cellulase on the substrate and there is a progressive increase of inhibition of the rate of hydrolysis with increasing temperature (Figure 13). But a considerable increase in susceptibility of the substrate to the cellulase is possible by a two-step process of size reduction and oxidative heat treatment (Table III, Figures 14 and 15). Reduction in particle size and consequent exposure of hidden reactive points of the cellulose through their opening up plays a big role in the increase in susceptibility of the substrate. The combined effects of heat treatment and particle size reduction of cellulose make a distinct difference as a reactive substrate, either from size reduction alone, or from size reduction combined with heat exposure (Figure 15). Improvement in the quality of the substrate through preheating in air at 200°C. for 25 min. followed by milling is, in part, owing to the presence of high

percentage of fines ($< 53\mu$ size) in the product. A highly resistant cellulose left as residue of enzyme digestion can be regenerated into a susceptible substrate by repeating the combined operation of heating and milling (Figure 16). It has been shown, however, that heating alone of the digested cellulose does not improve its quality. These results suggest that as far as cellulase action on cellulose is concerned, availability of active areas of the substrate is of primary importance. Heating assists the process of particle size reduction, but does not, in itself, make any contribution to increase susceptibility. The period of complete saccharification is only prolonged in the presence of increasing concentration of substrate molecules whose hidden reactive areas do not readily become available for further hydrolysis. Milling, or heating plus milling, make these areas available to the enzyme, and this so-called resistant substrate again becomes susceptible (3). As mentioned earlier, the infrared spectra and x-ray diffraction analyses of the treated cellulose (SF-M, SF-HM, SF-MH, SF-HMD) samples show that they are probably slightly oxidized and their crystalline structure destroyed. The control sample (SF) resembles that of cotton in terms of x-ray crystallinity index (83 against 85-90). Milled samples (SF-M, SF-MH, SF-HM) show severe loss of crystallinity and reduced degrees of polymerization (DP 200 or less). In the enzyme hydrolyzed samples of heated and milled cellulose, there appears to be more fibrillar structure visible implying that the amorphous nature so characteristic of the finely ground cellulose may have been hydrolyzed away. These conclusions strongly support our experimental data (Figures 15 and 16) which illustrate that a considerable portion of the fibrillar structure of the hydrolyzed cellulose residue can be reactivated by a heating and milling process. *T. viride* cellulase does not seem to suffer any detectable loss of activity (C_x) at pH 4.8 and 50°C. in 22 hr. (Figure 10). The loss of activity in contact with highly a reactive substrate is about 40% in the first half hour of contact with substrate and it remains almost at that level over the next several hours.

The results of stirred tank saccharification of modified cellulose (SF-HM) at various pH (Figure 11) confirms the optimum pH to be 4.5-5.5 as shown in the case of untreated Solka Flocc substrate studied in unagitated tube experiments (3). It has been demonstrated (Figure 21) that saccharification of cellulose is possible while the latter is milled in contact with *T. viride* cellulase. Although no published information is available describing a solid substrate reacting with an enzyme solution under high impact and shear conditions, the preliminary data reported here illustrate the possibility that such an approach may be advantageous.

The saccharification of cellulose in crystalline (Solka Flocc) or amorphous (finely pulverized Sweco 70) forms by *T. viride* cellulase has a maximal rate at 50°C. as compared with either 40°C. or 60°C. Up to the

first hour the rate at 60°C. appears faster than at 50°C. and 40°C., but thereafter the 50°C. system progresses at a much higher rate than at 40° or 60°C. The inactivation of C_x at 60°C. appears to be the main reason for this.

The results of the semi-continuous saccharification (Figures 17 and 18) of the two substrates (SF-HM and SF-MH) can be summarized:

The SF-HM cellulase system in a 10% suspension reaches a maximum rate of saccharification at about 40 hr. The SF-MH cellulase system at the same consistency maintains a sluggish hydrolytic rate up to about 90 hr., then starts to rise and becomes almost equal to the rate exhibited by the SF-HM system beyond 100 hr. A comparison of the SF-HM and SF-MH (Figure 1) systems shows a distinct difference between the capacities of the two substrates to hydrolyze under the same conditions of enzyme concentration, temperature, and pH. Overfeeding or feeding prior to the time maximum rate is reached causes a falling of rate or temporary accumulation of substrate. Beyond 90 hr. of hydrolysis with agitation, rates of saccharification of both the substrates become equal. The difference in the susceptibility of the two substrates is attributed to the major difference in the particle size distribution in the two substrates (Figure 9, Table II)—*e.g.*, SF-HM material containing 76% of $< 53\mu$ particles and SF-MH material containing about 55% of $< 53\mu$ particles.

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Discussion

E. T. Reese: "The highest concentrations of glucose that we can get in a digest must compare favorably with the 30% or more that one gets in a glucamylase digest. One of our problems then is to get a lot of cellulose into the initial suspension. If we swell the cellulose, we can not get very much in, maybe a 5 to 10% suspension, and that is a mush. But the milling plus heating process that Dr. Ghose is using allows one to get suspensions of up to 50% cellulose. We need these high concentrations in order to reach 30% glucose concentrations in the digest."

J. Saeman: "If you take your digested material and remove all substrate and product, do you find the same cellulase activity that you had initially? In other words, is there a loss in cellulase activity as the digestion proceeds?"

T. K. Ghose: "It has been shown in one of the slides that in contact with Sweco 70 (cellulose) substrate we did find that 40% of the enzyme activity disappeared. We would designate this as adsorbed loss rather than inactivation. At 50°C. the loss in enzyme activity in the *absence* of substrate over four hours is practically nil."

J. Saeman: "Did you try treating cellulose with sodium hydroxide?"

T. K. Ghose: "We did try. In one case it was useful, in another case it was detrimental."

K. Selby: "Did you use any antiseptic in the system while you were doing the continuous digestion?"

T. K. Ghose: "Yes. We used 1 mg. of Merthiolate per liter of enzyme substrate suspension."

L. Baribo: "Dr. Reese, you made a comment that you could swell. How did you swell the cellulose?"

E. T. Reese: "Yes, we have used swollen cellulose in some of our earlier work. We were comparing various methods of swelling with the rate of subsequent hydrolysis. We used alkali swelling followed by washing out the alkali, but never drying the sample. Under these conditions there was a two-fold increase in susceptibility."

L. Baribo: "Well, then you might do what they are doing in the starch industry, that is to decrease the molecular weight of the cellulose so that you can get a higher concentration in your substrate media. They could not run a 30% gelatinized starch either, unless they degraded it to something like 10-12 DE. But if you do swell, then you can not get a high concentration in suspension."

T. K. Ghose: "With swelling you decrease the bulk density of the substrate in the reactor. In other words, if you have a solid fluffy material like Solka Floc you do not expect to charge the reactor in high concentrations. On the other hand when the particle size is reduced, not only does the total surface increase, the reactor capacity is also increased due to increased bulk density of the substrate."

E. T. Reese: "We have no way of hydrolyzing the cellulose readily to a soluble oligosaccharide fraction. If we use anything like the strong sulfuric acid treatments, then we may as well be doing acid hydrolysis again."

J. M. Leatherwood: "Did you try percolating the enzyme through a column of cellulosic substrate?"

T. K. Ghose: "Yes, in fact, our first experiment was done in a column containing Solka Floc and fresh enzyme and hydrolysate respectively, pumping from the top to the bottom. After a period of operation the material swelled so much that it was impossible to operate at a steady rate, and ultimately the whole thing plugged up."

J. M. Leatherwood: "Have you tried to measure adsorption loss in other susceptible substrates used by you?"

T. K. Ghose: "Do you mean in place of Sweco 70, whether we attempted to use Sweco 70-heated in the activity loss studies? No, we did not. We expect essentially the same trend of activity loss as in the case of Sweco 70 unheated."

J. M. Leatherwood: "What about the adsorption of enzymes on the digested cellulose?"

T. K. Ghose: "No, we did not do that."

M. Mandels: "In previous long term digestions of cotton, the cellulase was recovered approximately 100% after the cellulose had been completely hydrolyzed."

L. Underkofler: "May I say something further about the starch situation. It is correct that the amylases do not attack undamaged starch granules. However, if you ball-mill starch then the raw starch can be attacked by the enzymes. But the best way is to gelatinize the starch. Then, of course, it becomes susceptible. The thinning of the starch suspension is just a matter of reducing the viscosity for easier handling. In the laboratory one can make a 30% starch suspension, bring the enzyme in, and convert it to glucose. But in the plant you carry out a prehydrolysis step to reduce the load on the stirrers, and to get the enzyme mixed in. Glucoamylase does act very rapidly on the large starch molecules. If you can get the enzyme mixed in, then you get practically the same rate of hydrolysis whether the starch is thin or viscous."