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Title: Characterization of Substrate - Velocity Relationships for the Cellulase Enzyme Complex from *Trichoderma viride*.

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The influence of substrate and enzyme concentration on the rate of saccharification of two defined, insoluble, cellulose substrates, Avicel and Solka-Floc, by the cellulase enzyme system of *Trichoderma viride* has been evaluated. Assays utilized enzyme concentrations ranging from 0.014 to 0.056 filter paper unit per mL and substrate concentrations up to 10% (w/v). Analysis by initial velocity methods found the maximum velocity of the enzyme to be nearly equivalent for the two substrates and the km for the two substrates to be of similar magnitude, i.e., 0.20% for Solka-Floc and 0.63% for Avicel (w/v). Studies utilizing relatively high substrate concentrations (greater than 15 times the Km) demonstrated that the enzyme exhibits very different apparent substrate inhibition properties for the two substrates. The rate of saccharification of Avicel at relatively high substrate concentrations was up to 35% lower than the maximum rate

which was obtained at a lower substrate concentration. The Avicel concentration corresponding to the maximum rate of saccharification was dependent on enzyme concentration. In contrast to the results with Avicel, the enzyme did not exhibit substrate inhibition with the Solka-Floc substrate. Potential differences in the degree of substrate inhibition with different substrates, as reported in this paper, is particularly relevant to the experimental design of comparative studies.

Characterization of Substrate - Velocity Relationships for the Cellulase Enzyme Complex from *Trichoderma viride*

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Review of Literature:

Cellulose in nature and its utilization

Cellulose, a high molecular weight polymer of glucose units linked in a β-1,4 configuration, is an orderly crystalline structure associated with hemicellulose and lignin in plant cell wall. The proportions of these three components of plant cell wall are varied with the sources, 35 to 50% for cellulose, 20 to 30% for hemicellulose, and 20 to 25% for lignin (30). Cellulose is not only a major component of cotton, wood and biomass material but also exists in a significant amount in domestic, agricultural, and industrial wastes.

Currently, two major sources of cellulosic residue are easily obtainable. One of these is municipal paper refuse of which 150-250 million dry tons are produced annually in the U.S. Another major potential source of cellulosic substrate is agricultural wastes, such as crop wastes, animal wastes (manures), and forestry residues. It has been estimated that between 600 and 700 million tons (dry) of agricultural refuse are produced annually in the United States, over half of which is left in the field following the harvest of agronomic crops (43). Therefore, if we assume that, on the average, plants contain 40% cellulose and if we apply this value to the estimated

terrestrial plant biomass in the world, 1.8×10^{12} metric tons, we can estimate the total world supply of cellulose to be 7.2×10^{11} metric tons (98). Thus, cellulose is the most abundant renewable resource in the world.

There is no question that cellulosic materials could be put to a number of uses, thereby mitigating the effects of anticipated growing shortages of food and raw materials. The enzymatic conversion of cellulose to glucose is of importance relative to several industrial processes, including single cell protein, industrial chemical and energy production (22,43,53,89). Reduction of glucose to glucitol has been practiced on a fairly large scale for decades (35). Glucitol partly replaces artificial sweeteners and sucrose, and is used as an intermediate in the synthesis of ascorbic acid. Glucose also can be oxidized under either acidic or alkaline conditions to mono- and dicarboxylic acids. Monocarboxylic acid has metal-complexing properties and is commonly used in the food industry (27). Current uses of cellulose are restricted mainly to high-purity products for the food and pharmaceutical industries (7,92).

Structure of Cellulose

Natural fibers of cotton and wood are the most important commercial sources of cellulose. The knowledge of their structure is required for the successful exploitation of cellulose as a renewable chemical and energy resource. The cellulose in cotton and wood are

very similar in molecular structure (20). The celluloses are linear polymers of D-anhydroglucopyranose units, linked by β-1,4-glucosidic bonds. The number of glucose units per molecule (degree of polymerization, DP) ranges from as few as 15 or less to as many as 10,000 to 14,000 (14). It is generally assumed that cellulose molecules are arranged in a parallel fashion, i.e., with adjacent molecules running in the same directions (20,30).

X-ray diffraction data showed that cellulolytic enzymes degraded the more accessible amorphous portions of regenerated cellulose but were unable to attack the less accessible crystalline region (67). Thus, any treatment which alters the proportion of crystalline material or the degree of perfection of the crystallites present may modify the susceptibility of cellulose to hydrolysis by enzymes, strong acids, or other reagents. Treatments that may increase susceptibility include: reprecipitation from solution, and mechanical disruption such as in a vibratory ball mill. King (45) suggested that the greater resistance of crystalline as compared to amorphous cellulose may be due not just to its physical inaccessibility to enzyme molecules but also to the conformation and steric rigidity of the anhydroglucose units within the crystalline regions. Experimental verification of these speculations is a great challenge to those studying the stereospecificity of enzyme reactions.

When the degree of polymerization (DP) of cellulose is reduced during acid hydrolysis, the broken ends of cellulose chains in the amorphous regions have a tendency to recrystallize and make the residue more resistant to enzymatic hydrolysis. When the DP of cellulose is reduced to such an extent (DP < 7) that the molecules are soluble and thus no longer maintain their structural relationships with one another, there will be a great increase in susceptibility to enzymatic hydrolysis (20).

Cellulase enzyme complexes

Cellulases are formed by many bacteria, fungi, higher plants, and some invertebrate animals (31,106). These enzymes perform at least three physiological functions. Firstly, they can be used as morphogenic agents which weaken cellulose-containing cell walls in preparation for growth. Plant cellulases and some fungal cellulases perform this function. Secondly, these enzymes can be used as invasive agents which can, for example, enable a microbial plant pathogen to penetrate the tissues of its host. Thirdly, these enzymes can be used as digestive agents which render plant tissues penetrable by other enzymes and render the cellulose itself usable as a carbon source. The cellulases of invertebrate animals and of many microorganisms perform this function.

Both the quantity and properties of the cellulases produced by microorganisms depend on the culture conditions. The complexity of the crude cellulosic carbon source usually leads to the production of a mixture of hydrolytic enzymes which may include amylases, chitinases, etc., in addition to the cellulases (21). Many microorganisms are able to produce a wide variety of polysaccharide degrading enzymes. Cellulases and xylanases are commercially significant types of these enzymes because they can potentially be employed to utilize two of the most abundant polysaccharides: cellulose (54) and xylan (44), respectively.

Papers continue to appear on the separation and characterization of the cellulase enzymes of various microoganisms (13,29,42). Moreover, many components have been separated and well characterized as individual enzymes with different modes of action. Most of the cellulolytic fungi appear to have similar cellulase systems, each containing one to several β-glucosidases, endo-β-glucanases and exo-β-glucanases which act synergistically to hydrolyze insoluble cellulose (59).

Due to the efficient degradation of cellulose and interest in utilizing cellulose as a source of chemicals and liquid fuels, cellulases from fungal origin have been widely studied (62,86,87,88,101). The advantages of *Trichoderma* as a source of cellulase are that (1) it

produces a complete cellulase with all the components required for total hydrolysis of crystalline cellulose and (2) it yields very high amounts of cellulase protein . These microorganisms produce a multi-component enzyme system, including the β-1,4-D-glucan glucanohydrolase (endoglucanase; EC 3.2.1.4), the β-1,4-D-glucan cellobiohydrolase (exoglucanase; EC 3.2.1.91) and the β-D-glucoside glucohydrolase (β-glucosidase; EC 3.2.1.21).

Sprey and Lambert (96) indicated that the fungi release a tightly bound complex of several hydrolases in order to attack a multicomponent substrate of plant cell wall polysaccharides. Similar results were obtained by Shikata and Nisizawa (85); they found a cellulase that showed xylanase and β-glucosidase activity. Therefore, it is important to identify each component of the cellulase mixture. Using isoelectric focusing, Biely (12) divided the enzyme mixture of cellulase from *Trichoderma ressei* into specific xylanase, specific glucanases and nonspecific glucanases. Similarly, Bledman (8) successfully isolated three different types of β-glucosidase which showed aryl-β-D-glucosidase, as well as cellobiase activity.

The stability of the cellulases of several organisms under defined conditions has been investigated (75). In general the β-glucosidases

and endo-β-glucanases are more resistant to heat, pH extremes, and chemical inhibitors than are the exo-β-glucanases.

Degradation of cellulosic materials

The feasibility of hydrolyzing wood and cellulosic materials by enzymes has been studied intensively in the last decade (16,35,91). Although when compared to acid hydrolysis, enzymatic degradation is slow, it proceeds in non-corrosive conditions and produces fewer harmful by-products (3).

Native celluloses are most appropriate for studying complete cellulase preparations, although some individual exo- and endo-glucanases also display activity (38,108). However, it is more common to use either swollen or regenerated cellulose as substrates because of their greater susceptibility to enzymatic degradation (103,104). A widely used procedure in the determination of total cellulolytic activity (60) is to incubate cellulases with a 2.5 % w/v suspension of filter paper for 1 hr at 50°C and determine the reducing power generated in solution via Nelson's method (94). However, it is important to recognize that hydrolytic activity does not necessarily generate soluble products. Wong (108) reported that endo-glucanases catalyzed the hydrolysis of 10% of the glycosidic linkages in fibrous cellulose without releasing glucose or cellodextrins into the solution.

The physical and chemical features of cellulosic materials which determine their susceptibility to enzymatic degradation include: the moisture content, the ratio of accessible to inaccessible surface area of the substrate, the location and size of amorphous regions of the substrate, the degree of crystallinity of the cellulose, the degree of polymerization of the cellulose, and the composition of substituent groups of the substrate.

Assay methods for cellulase activities

There are several assay methods for determining the cellulase activity (10). Selection of an appropriate assay for cellulase activity depends on which enzyme is measured. The activity of complete cellulase complex can be measured using crystalline cellulose such as cotton fiber, filter paper or Avicel. A common method for the activity of complete cellulase complex, to determine the reducing sugar is by using filter paper. Usually the activity of endoglucanase is measured by using carboxymethylcellulose (CMC) as a substrate. The activity of endoglucanase also can be determined by measuring the decrease in viscosity using hydroxyethylcellulose as a substrate. There is no specific substrate for cellobiohydrolase, an exoglucanase, however the activity of this enzyme which must be pure and can be measured using Avicel. However there is no satisfactory way of directly measuring exo-activity in the presence of endo-activity (28).

It is most desirable that an accurate cellulase assay be developed, but this is difficult because cellulose is not a homogeneous substrate and cellulase is not a single enzyme.

Pretreatments of Substrate:

There are several studies which show that, after protracted enzymatic hydrolysis, the DP of the residual cellulose is not greatly reduced (77), and there is evidence that the crystallinity may actually be increased (59). It is quite clear that enzyme action occurs preferentially at amorphous regions, and microscopic examination confirms that degradation can be localized (15). Similar results have been obtained in the hydrolysis of Solka Floc (26) and wood pulp (17).

Many physical and chemical pretreatments for enhancing bioconversion of cellulose to glucose have been reported (5,61,64,73), but extensive and rapid conversion into glucose units by enzyme remains a problem because the strong crystalline structure of cellulose is resistant to enzyme attack. Judging by the characters of crystalline cellulose and cellulases, the most important key to the rapid and complete enzymatic hydrolysis of cellulose is the pretreatment of cellulose, which opens up the cellulose structure and eliminates the interaction between cellulose chains (47). Pretreated cellulose could be hydrolyzed by endo-glucanase and would not require exo-glucanase (83). Sasaki suggested that the crystalline structure of cotton cellulose powder was disrupted by acid dissolving treatment and thus was

changed to a noncrystalline form. Similar results in cardoxen-solubilized cellulose and DMSO-*p*-formaldehyde-solubilized cellulose were observed (79,80). The biological susceptibility of cotton cellulose depends on the degree of crystallinity of the cellulose structure. It is significant that the strongest correlation was observed between the crystallinity index by X-ray diffractogram and enzymatic hydrolysis (83).

Model of mechanism:

The study of enzymatic cellulose hydrolysis dates from four decades ago (74,76). Useful information has been obtained regarding biological sources, enzyme production and control, microbial deterioration of cellulosic substances, and the ecological roles of the cellulases (6,36,40,90). However, it has become evident that only by isolating, purifying, and chemically and physically characterizing the individual enzymes will scientists be able to understand the modes of action of cellulases.

The earlier proposal (24,38,109,111) of so-called C1(exo-enzyme) and Cx (endo-enzyme) factors in cellulase complexes to explain enzymatic degradation of insoluble cellulose led to extensive research on the isolation of cellulase components. Three main enzymes have now been identified: 1) β-1,4-glucan glucanohydrolase (EC 3.2.1.4) which hydrolyzes cellulose polymer randomly and produces glucose

and cellobiose as end-products, 2) β-1,4-glucan cellobiohydrolase (EC 3.2.1.91) which attacks the nonreducing end of a cellulose polymer chain to produce primarily cellobiose, and 3) cellobiase or β-glucosidase (EC 3.2.1.21) which acts primarily on cellobiose to produce glucose (69).

A combination of these three types of enzymes is necessary for the complete hydrolysis of crystalline cellulose. Endoglucanase and exoglucanase are known to act synergistically in cellulose hydrolysis (113), while β-glucosidase is required for the removal of cellobiose, which is a strong inhibitor of both endoglucanase and exoglucanase activity. The commonly accepted hydrolysis model (68) suggested that endoglucanase randomly hydrolyzes amorphous cellulose at its glucoside linkages into its fragments, while exoglucanase attacks the nonreducing end of crystalline and a fragment of amorphous cellulose, thereby releasing cellobiose, while cellobiose is hydrolyzed to glucose by β-glucosidase .

A model for the enzymatic hydrolysis of cellulosic materials must take into account the effects of the physical structure of the substrate, the nature of the cellulase complex, and the inhibitory effects of both substrates and products, including the presence of material in the substrate other than cellulose, e.g. hemicellulose and lignin (107). The major structural features that determine susceptibility to enzymatic

degradation are crystallinity, and accessibility, which is defined as the surface area accessible to enzymatic attack (26,83). Thus, pretreatment has a profound effect on hydrolysis. Okazaki and Moo-young (69) developed a model of cellulose degradation to describe the synergistic effect of cellulases, the dependency of hydrolysis rate on the degree of polymerization of the substrate, and the effects of the substrate inhibition.

Despite many kinetic studies on the hydrolysis of cellulose, little is known quantitatively about the individual and overall rate retarding effects or about the useful rate expression. This is probably due to the fact that the chemical and physical properties of cellulosic materials vary widely and to the fact that the enzyme consists of many components (68). Rabinovich (72) suggested that the weakly- and strongly-adsorbed enzymes attack different sites on the substrate and that this affects the hydrolysis rate of cellulose. However, the debate over the precise mechanism of cellulose hydrolysis continues.

Synergistic effects:

The specific activity of the purified components towards native and derivatized cellulose is often extremely low, whereas the activity of the recombined enzymes is much higher than the sum of the individual components. Thus the enzymes appear to act in a cooperative or synergistic manner (28,39). Reese (76) seems to have been the first to have suggested that some form of synergism was

involved in cellulose degradation. This suggestion arose from his observation that some microorganisms were able to attack native cellulose while others were able to degrade only cellulose derivatives such as carboxymethylcellulose (CMC). Reese suggested that the function of one enzyme in the complex was to break the intercrystalline hydrogen bonds, thus making the cellulose molecules accessible to attack by other enzymes.

The enzymes which play major roles in synergism are the endoglucanase and the exoglucanase (8,87,88,116,117). Eriksson (24) and Streamer (99) provide the most convincing evidence in support of the cooperative action of the exo- and endo-β−1,4-glucanases by demonstrating that de-waxed cotton and microcrystalline cellulose, when pretreated with the endo-β−1,4-glucanases, and , when subsequently treated with the exo-β-1,4-glucanase, released much more soluble degradation products than substrates which had not been pretreated. Synergism between the two exoglucanase of *T. reesei* has been reported (25).

A synergistic effect resulting from the combination of endo- and exocellulases and/or cellobiase on the hydrolysis of cellulose has been observed by many workers using purified enzyme systems obtained from various microbes (9,10,76,84,108,109,115). Okazaki's (69) model was an attempt at an explanation of this synergistic effect, which is

affected by several factors, principally the DP of the cellulose, the ratio of endo/exo, the concentrations of endo and exo, the Michaelis constants, product inhibitions, and substrate concentrations. However, this model assumed a soluble substrate and homogeneous reaction conditions. When insoluble crystalline cellulose is used, the reaction is heterogeneous. Additionally, another factor, the selective adsorption characteristics of the individual endo- and exo-glucanases, should be considered (9,10).

Wood and McCrae (113) stated that the formation of a complex of endoglucanase and exoglucanase on the surface of the cellulose chain is essential for synergism. Another possible explanation of synergism was given by Ryu and co-workers (81) who found that synergism was gradually introduced into the non-adsorbed enzyme fraction by competitive adsorption of one of the glucanases.

Beldman's data (10) showed that the degree of synergistic effect (DSE) for a specific combination of endo- and exoglucanase, is dependent upon at least two main factors: firstly, the ratio in which both enzymes are combined and, secondly, the nature of the substrate which is used. In agreement with the findings of Wood and McCrae (112), the maximal DSE occurred at a specific optimal ratio of adsorbed endoglucanases and exoglucanases. It is very difficult to predict the optimal composition of an enzyme mixture necessary to obtain maximal synergism (10).

Although Avicel is not a completely native cellulose as cotton fiber is, Avicel is commonly used as a model substrate to investigate the process of the hydrolysis of insoluble crystalline cellulose (81,99).

Adsorption of enzyme on substrate

The mechanism of cellulase adsorption and its effect on cellulosic material is still not completely understood, and remains perhaps the most difficult problem in the enzymatic hydrolysis of insoluble cellulosic materials (2). It is very important that it be understood because adsorption is a prerequisite for subsequent hydrolysis reaction. The multiplicity of cellulase components and of sources of cellulosic materials complicate any systematic investigation of the adsorption phenomena in the cellulose/cellulase system. This reaction process is quite different from the splitting reaction of the soluble substrate; it is dependent upon the physicochemical properties of the cellulose adsorbent used.

Some important observations (1,50,51,72,97,100) on celluase adsorption have been made. These include observations on the effects of various pretreatments of cellulose and sample preparations on the adsorption of the cellulase complex, and on the relationship between the specific surface area of the cellulose particle and the adsorbed amount of soluble protein (52). In agreement with Rabinovich's results (72) suggested that the affinity of the enzymes for the insoluble substrate is one of the factors which determine the effect

of the cellulolytic enzymes on crystalline cellulose. The effects of the adsorption characteristics of the cellulase complex on cellulosic material involve many intriguing and complex phenomena. A complete understanding of the adsorption phenomena of cellulase components may provide some clues to the true reaction mechanism and to the synergism of the cellulase complex.

From the adsorption affinity point of view , reversible adsorption and irreversible adsorption coexist and, with respect to the hydrolysis reaction, the productive and nonproductiove adsorptions are also involved in the adsorption process (48). Klyosov believed that the composition of cellulase components in cellulase complex affects the extent of adsorption on a cellulose adsorbent due to the different adsorption affinities of the cellulase components. Some investigators (48,51,52,71) reported on the effect of temperature on the adsorption of cellulase complex and on the adsorption characteristics of the cellulase complex which depend on the physicochemical properties of cellulose adsorbents. Their results , however, were inconclusive and somewhat conflicting. Since the adsorption phenomena of the cellulase enzyme reflect the behavior of cellulase components on the surface of cellulose, the synergistic actions of the cellulase components can be observed from the viewpoint of adsorption kinetics and the characteristics of cellulase components (114).

Substrate inhibition

Substrate concentration is one of the most important of those factors which determine the velocity of enzyme reactions. In most cases when initial velocity is plotted against substrate concentration a section of a rectangular hyperbola is obtained. It is not uncommon to find that, while the Michaelis equation is obeyed at lower substrate concentrations, the velocity falls off again at high concentrations. This effect may be due to several causes (23). Firstly, the enzymes have several active sites which can react with particular parts of the substrate molecules. In high substrate concentration, the velocity decreases due to increasing the chances of formation of ineffective complexes. Secondly, since all enzymes act in aqueous media, very high substrate concentration will imply a reduction in the concentration of water, which may lower the velocity especially if one of the reactants is water. Thirdly, enzymes require an essential activator, such as metal ion. The excess of substrate binds the activator and lowers the effective concentration of the enzyme activator, so that the velocity decreases. Finally, the presence of a contaminant in the substrate which act, as a mixed or as an uncompetitive inhibitor can result in the appearance of high substrate inhibition.

INTRODUCTION

Characterization of Substrate-Velocity Relationships for the Cellulase Enzyme complex from *Trichoderma viride*

The enzymatic saccharification is catalyzed by a complex enzyme system which typically includes at least three distinct classes of enzyme; endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The relative activity of the component enzymes in a given cellulase enzyme preparation is dependent on the source of the enzyme (19).

The principal methods used to characterize the saccharification properties of these enzyme systems are based on kinetic analyses. Kinetic studies are utilized to compare the relative cellulolytic capacity of enzyme systems from different sources (49,82) as well as the relative enzymatic susceptibility of different cellulosic substrates (33,70). Generally, these comparative studies include a measure of the time course of saccharification over some initial reaction period, as well as, a measure of the total extent of hydrolysis after a fixed, relatively long reaction period. Kinetic studies are also used to evaluate potential mechanisms of the component cellulase enzymes (8,9) and to develop kinetic models which may be used to predict the rate of saccharification of a particular substrate (37,40,63). The latter studies have resulted in mechanistically distinct models, each of which are

capable of simulating a portion of the time course of saccharification for a given substrate under defined conditions . Although many of these kinetic studies have been reported, the kinetic mechanism of the cellulase enzyme complex is not well understood.

The rate of degradation of a cellulosic substrate is affected by several parameters, including the source of the cellulase enzymes (19), the physicochemical properties of the substrate (105) and the extent of product (57) and substrate (51) inhibition. Of these parameters, substrate inhibition has received the least amount of experimental scrutiny. Substrate inhibition is a fundamental kinetic property which reflects a deviation in the expected saturation kinetics of enzyme catalyzed reactions. Apparent substrate inhibition is not uncommon when enzymes are acting at relatively high substrate concentrations and the property may be the effect of several causes (23). Along with its inherent mechanistic information, substrate inhibition is of importance relative to several applied aspects of cellulase/cellulose research. When evaluating the relative maximum cellulolytic capacity of a microbial enzyme system, it is essential to consider that particular enzyme's potentially unique substrate inhibition properties. Similarly, substrate inhibition is of relevance to the design of experiments analyzing native and modified cellulosic substrates to identify potential pretreatments that are capable of increasing the reactivity of cellulosic materials.

The cellulase enzyme complex from *Trichoderma viride and Trichoderma reesei* have been the focus of considerable research due to their commercial potential. The heightened interest in these enzymes is largely because they are complete, extracellular enzyme systems capable of hydrolyzing crystalline cellulose and because they may be obtained in relatively high yield. The complete nature of these enzyme systems, coupled with the historical use in cellulase research, have made them among the primary enzymes against which newly discovered enzyme complexes are compared (11,55). Their complete nature has also made these enzymes a primary focus of studies attempting to model the hydrolysis of crystalline cellulose (63,68). Despite the large number of comparative and mechanistic studies done with *Trichoderma* enzymes, there are relatively few papers which consider their apparent substrate inhibition properties. Okazaki and Moo-Young (69) have presented a generalized mechanistic model for the enzymatic hydrolysis of cellulose which, based on concurrent random and endwise attack of the substrate, predicts substrate inhibition. These authors also state that apparent substrate inhibition has been observed in their unpublished studies. Lee and Fan (51) have presented initial velocity data which reflect apparent substrate inhibition of the *T. reesei* enzyme. The authors attribute the inhibition to hydrodynamic factors and therefore focus their initial velocity study on reaction conditions which do not exhibit substrate inhibition.

Apparent substrate inhibition of *Trichoderma* and *Aspergillus* cellulase complexes by a relatively complex cellulosic substrate, leached beet cosette, has also been reported (18). The current study extends these previous observations by characterizing the apparent substrate inhibition properties of the *T. viride* enzyme system with respect to two substrates commonly used in cellulase/cellulose research.

In the present paper, the substrate inhibition properties of the cellulase enzyme system from *T. viride* are characterized through analysis of substrate-velocity profiles for two substrates, Avicel and Solka-Floc, over a range of enzyme and substrate conditions. The results presented demonstrate that the substrate inhibition properties of this enzyme are indeed complex and dependent on the cellulosic substrate utilized. The importance of these results relative to appropriate experimental designs in cellulase/cellulose research is discussed.

MATERIALS AND METHODS

Materials

Substrates were obtained commercially; Avicel PH101 (FMC Corp., Philadelphia, PA) and Solka-Floc BW 200 NF (James River Co., Berlin, NH). The following potentially variable chemicals and reagents were obtained from the designated supplier: BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL); bovine serum albumin (Sigma Chemical Co., ST. Louis, MO), formic acid (95-97%) and soluble starch (Aldrich Chemical Co., Milwaukee, WI); and cupriethylenediamine hydroxide solution (Synmet Inc., Baton Rouge, LA).

Enzyme preparation

A commercially available (Calbiochem Corp., San Diego, CA) cellulase enzyme system from *Trichoderma viride*, "Cellulysin", was used without modification. Filter paper activity of the enzyme preparation, determined by the method of Mandels *et al.* (56) as modified by Ghose (34) , was 1.75 filter paper units (FPU) per mg enzyme preparation (see Appendix). The protein content, determined utilizing the modified biuret method of Smith *et al.* (93) , was 0.53 mg bovine serum albumin equivalents per mg enzyme preparation (see Appendix).

Substrate characterization

The percent cellulose content of the two substrates was determined by extraction of the non-cellulosic polysaccharides with acetic/nitric acid (see Appendix) as described by Updergraff (102) followed by quantitation of residual cellulose as described by Southgate (95) utilizing the anthrone reagent with glucose as the calibration standard.

The crystallinity index of the substrates was determined by X-ray diffraction methods (see Appendix) as described by Hsu and Penner (41). The degree of polymerization of substrates was determined by multiplying their experimentally determined intrinsic viscosity (see Appendix) in 0.5 M cupriethylenediamine solution by 190 (4). The small molecule accessibility of the substrates was determined by the Eberstadt formylation method as described by Nickerson (32,66). Briefly, cellulose substrates are formylated in 90% formic acid for varying lengths of time at 25°C to establish a time course of formylation. The time course is then extrapolated back to zero time in order to estimate the accessibility of the native substrate prior to any modification resulting from formylation. Percent accessibility of the substrates was then determined by quantitative comparison of the extent of formylation of the analyzed substrate with the extent of formylation of an equivalent weight of soluble starch (see Appendix). The water retention volume (WRV), defined as the weight of water

retained per unit weight of substrate following centrifugation at 1,300 g for 5 min, was determined as described by Lee and Fan (51) utilizing reaction mixture buffer as the aqueous phase (see Appendix).

Enzymatic saccharification assays

The standard assay was performed in 50 mM sodium acetate buffer, pH 5.0, at 40°C. Cellulose substrate was added to the reaction flask (125 mL Erlynmeyer) containing buffer and equilibrated to 40°C. The reaction was then initiated by the addition of buffered enzyme solution. The final volume for each reaction mixture was 62 mL. Reaction mixtures were agitated at 140 rpm in a constant temperature, orbital shaking water bath (Lab-Line Model 3540, Melrose Park, IL). Substrate concentrations, expressed as % cellulose (w/v), and enzyme concentrations, expressed as FPU per mL, were varied as described in the Results section. In experiments evaluating bulk mass transfer, the standard assay conditions were utilized except the amount of agitation was varied as described in the Results section.

Saccharification reactions were terminated at appropriate times, 2 h or 6 h, by filtering the reaction mixture through a 0.22 μm membrane filter (Millipore Corp., Bedford, MA) followed by immediate assay for reducing sugars and total sugars equivalents. Reducing sugars were measured (see Appendix) by the cupric ion reduction method of Nelson (65,94) and total sugars (see Appendix) by the

anthrone method of Roe (78) as described by Southgate (95). Glucose was used as the calibrating standard for both sugar assays. Kinetic constants were determined from double-reciprocal plots of experimental data (23).

RESULTS

Compositional and structural parameters of cellulose substrates

The compositional data for the two substrates indicates their similarity in that both substrates contain greater than 90% cellulose (Table 1). However, it is informative with these substrates to compare the relative amount of non-cellulosic material. In this regard, the Solka-Floc substrate contains approximately 20-fold more non-cellulosic material than the Avicel substrate. Selected structural parameters of the two substrates are also given in Table 1. The crystallinity index (CrI) is an empirical estimate of the ratio of crystalline to amorphous structure in the cellulose sample. Avicel has a slightly higher CrI than Solka-Floc, indicating that a greater percentage of the cellulose molecules in the Avicel substrate are involved in highly ordered, crystalline regions of low reactivity. Both cellulose substrates, however, show much more crystalline character than commonly used "amorphous" substrates such as ball-milled cellulose (41). The small molecule accessibility of the celluloses, estimated by measuring the extent of formylation of potentially reactive hydroxyl groups, suggests that the majority of hydroxyl groups in both substrates are unavailable for reaction with solvent molecules. These results, which are consistent with the CrI values, indicate that the accessible surface area of Avicel is somewhat less than that of Solka-Floc. The degree of polymerization of the substrates reflects an apparent 3.2-fold greater molecular weight for the cellulose

molecules in Solka-Floc compared to those in Avicel. The water retention volume of a fibrous substrate is an estimate of the amount of water which may be entrapped within the substrate. The results indicate that Solka-Floc absorbs roughly 40% more water per unit weight than Avicel under the defined reaction conditions.

Analysis of Kinetic Constants

Initial velocity methods were used to determine the Km and the maximum velocity of the enzyme with respect to both of the substrates (Table 2). The Km for the two substrates was of similar magnitude, 0.20% for Solka-Floc and 0.63% for Avicel. Consistent with the assumptions of Michaelis-Menten kinetics, the Km was not affected by changes in the enzyme concentration. The maximum velocity obtained for the two substrates was nearly identical when equivalent enzyme concentrations were compared. As predicted by Michaelis-Menten kinetics, the maximum velocity was proportional to the enzyme concentration in the reaction mixture. The amount of sugar solubilized in each of the reaction mixtures corresponded to less than 2.5% of the initial substrate present.

Effect of substrate concentration on the rate of saccharification

The effect of substrate concentration on the quantity of solubilized reaction products released from Avicel and Solka-Floc substrates, after 2 h and 6 h reaction time periods is depicted in Figure 1.

Utilizing Solka-Floc as the substrate, the enzyme system appears to obey classical saturation kinetics over the concentration range studied. In contrast, when Avicel is the substrate the enzyme system deviates substantially from this classical behavior. The marked decrease in the apparent reaction rate at relatively high Avicel concentrations results in an optimum substrate concentration, above which substrate inhibition is observed. The maximum amount of substrate inhibition observed corresponded to an approximate 35% decrease in the rate of saccharification relative to that observed at the optimum substrate concentration. The profile of the substrate-velocity curves for the respective substrates are similar whether the extent of the reaction is measured by total sugar solubilized or by solubilized reducing sugar (Figure 1). The greater quantity of total sugar versus reducing sugar produced indicates the measured reaction products contain some cellobiose and/or other cellooligosaccharides . Comparison of the substrate-velocity curves determined at 2 h and 6 h reaction times indicates that the observed substrate inhibition properties are expressed consistently throughout this time period.

The relevance of bulk mass transfer to the observed reaction rates was estimated by measuring the rate of saccharification of Avicel at varying degrees of reaction mixture agitation (Figure 2). It was visually obvious that substrate was setting out of suspension in

reaction mixtures receiving the lowest amount of agitation, 100 rpm, and the corresponding rate of saccharification in those reaction mixture was consistently lower than that in reaction mixtures which were more aggressively agitated. Substrate in reaction mixtures agitated at 140 or 180 rpm remained in suspension and the corresponding rates of saccharification for these reaction mixtures were similar, suggesting that under the standard assay conditions used in this study there was a relatively small bulk mass transfer influence on the rate of cellulose saccharification.

Effect of enzyme concentration on the Substrate-velocity profiles

The substrate-velocity profiles for the Solka-Floc and Avicel substrates at varying enzyme concentrations are presented in Figure 3. The cellulase system with Solka-Floc as the substrate behaved as expected with a given substrate concentration providing nearly equivalent V/Vmax ratios at the different enzyme concentrations. However, the relationship between enzyme concentration, substrate concentration and the rate of saccharification is more complex with the Avicel substrate. The rate versus substrate curves at the two lower enzyme concentrations, 0.014 and 0.028 FPU per mL, both demonstrate significant substrate inhibition while the corresponding curve at 0.056 FPU per mL does not . The curves presented in Figure 3 show that the Avicel concentration required for the maximum rate of saccharification is dependent on the enzyme concentration of the

reaction mixture. When the enzyme concentration is doubled, from 0.014 FPU to 0.028 FPU per mL, the Avicel concentration required for maximum activity also approximately doubled, from 2% to 4%. Similarly, the reaction mixture containing 0.056 FPU per mL appears to have reached its maximum rate of saccharification at an Avicel concentration of approximately 8%.

DISCUSSION

The cellulase enzyme system of *T. viride* appears to obey Michaelis-Menten kinetics at relatively low substrate concentrations when utilizing either the Solka-Floc or Avicel substrate. However, at relatively high substrate concentrations the rate of saccharification of Avicel decreases, indicative of substrate inhibition. Substrate inhibition of the enzyme is not observed with the Solka-Floc substrate under equivalent conditions. The apparent substrate inhibition of the enzyme complex by the Avicel substrate demonstrates that any mechanistic interpretation of the kinetic constants, Km and Vmax, is indeed complex and that these parameters are best used for only comparative purposes. The term "substrate inhibition". as used in this paper, refers to any apparent decrease in the rate of the reaction which accompanies an increase in substrate concentration. The cause of the substrate inhibition characterized in this paper is not clear. Previous observations of substrate inhibition have been rationalized by mechanisms involving a decrease in the extent of synergism between component enzymes (69, B. H. Van Dyke, Jr., Ph.D. thesis, Massachusetts Institute of technology, Cambridge, 1972) or a decrease in the movable aqueous phase of the reaction mixture (51). Further studies are required to conclusively determine the actual mechanism(s) which governs this behavior.

Knowledge of the substrate inhibition properties of a cellulase enzyme complex is of most obvious application to the design of reactors for the saccharification of cellulosic substrates. When considering reactor conditions it is clear that classical saturation kinetics should not be assumed and that a potential optimum substrate to enzyme ratio must be considered. The differences in the substrate inhibition properties of the enzyme with the two cellulose substrates used in this study strongly suggests that each cellulosic material must be analyzed independently to ascertain its optimum concentration. The present study compared relatively refined cellulose substrates derived from wood pulp. It is not known how the inhibitory properties of these substrates compare with those of cellulosic substrates derived from other sources by other methods. Our results considered in conjunction with previously observed substrate inhibition by leashed beet cosette (18) and ball-milled Solka-Floc (B. H. Van Dyke, Jr., Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1972) suggest that the extent of substrate inhibition is dependent on the structural properties of the substrate and that it is not a unique property of Avicel.

Substrate inhibition as described in this paper is of importance relative to the appropriate experimental design for comparative studies which analyze the rate of digestion of different cellulose type substrates. Cellulose substrates with a range of structural properties

are often utilized to analyze the degree to which specific physical properties of cellulose are of relevance to their susceptibility to cellulolytic enzymes (105). In related studies, cellulose substrates are compared to identify appropriate pretreatments to increase the enzymatic susceptibility of lignocellulosic materials (33). Comparative studies of this nature often utilize a single set of reaction conditions, including a single substrate and enzyme concentration, to determine which of the cellulose substrates is most susceptible to saccharification. The present data suggest that the conditions chosen for the assay may significantly affect the results of such a study. This point is graphically illustrated in Figure 4 which depicts the rate of saccharification of the two celluloses used in this study, Solka-Floc and Avicel, as determined by total sugars produced after a 6 h reaction time. The figure illustrates that the substrate with which the enzyme shows the most activity is a function of the substrate concentration utilized in the comparative assays. Assays utilizing 2% substrate indicate that Avicel is degraded at a rate 50% greater than that of Solka-Floc. However, assays utilizing 8% substrate indicate that Solka-Floc is degraded at a rate nearly 60% greater than Avicel.

Substrate inhibition properties may similarly affect the conclusions drawn from studies designed to compare the relative activity of different cellulase enzyme preparations. This type of study is commonly done to evaluate the industrial potential of novel

cellulase enzyme systems (11,55). The Avicel substrate is often used in this type of study due to its microcrystalline character. Figure 5 illustrates the influence that substrate concentration may have on the results from this type of study. In the example provided, the enzyme concentration was increased four-fold from 0.014 to 0.056 FPU per mL and the rate of saccharification was determined at two substrate concentrations. At the lower substrate concentration, 2%, there was nearly a linear relationship between enzyme concentration and rate of saccharification. However, at the higher substrate concentration, 8%, the relationship was nonlinear , so that the actual four-fold increase in enzyme concentration was measured to be nearly nine fold. The presented data demonstrate how the conclusions drawn from comparative studies measuring the relative avicelase activity of two enzyme preparations may unintentionally be biased if substrate-velocity interrelationships are not considered.

SUMMARY AND CONCLUSION

1. Kinetic parameters were calculated from initial reaction velocities, and were consistent with the assumptions of Michaelis-Menten kinetics. Km was not affected by changes in the enzyme concentration and the maximum velocity was proportional to the enzyme concentration in the reaction mixture. The Km for the two substrates was of similar magnitude, 0.20% for Solka-Floc and 0.63% for Avicel. The maximum velocity of the enzyme was nearly equivalent for the two substrates.
2. Apparent Substrate inhibition was observed with Avicel, but not in Solka-Floc. Utilizing Avicel as the substrate, the enzyme did not obey classical saturation kinetics over the concentration studied. The rate of saccharification of Avicel at 10% (w/v) showed an approximate 35% decrease relative to the rate obtained at the optimum substrate concentration. Substrate inhibition properties of this enzyme system were similar over 2h and 6h reaction period.
3. The Avicel concentration required for the maximum rate of saccharification was dependent on the enzyme concentration of the reaction mixture (2% for 0.014 FPU/mL, and 4% for 0.028FPU/mL).
4. The causes of the shift in the optimum substrate concentration with changes in enzyme concentration and lack of substrate inhibition with Solka-Floc was unclear.

5. This study revealed that conclusions based upon a single set of reaction conditions may be biased; different conclusions will be reached depending on the substrate concentration used.

FUTURE STUDIES

Purification of the cellulase enzyme complexes is a most important and necessary for determining the mechanism of substrate inhibition. It also provides information necessary for the construction of a reasonable model that could be used to explain how complex cellulase enzymes act on distinct celluloses. Identification of individual components that play an important role in hydrolysis reaction might be accomplished by experimentation with different relative compositions of endo/exo enzyme in the reaction system. Individual cellulase components will be applied to the same reaction system as described in the current study. Consequently, purification of cellulase enzyme complexes from *Trichoderma viride,* in order to obtain pure individual cellulase components is an important future task.

Comparison of activity curves associated with individual components with those of the complex cellulase enzyme may aid development of a method for measuring the maximum exo-enzyme activity in the presence of endo-enzyme. The present study revealed that a simple assay for measuring the maximum exo-enzyme activity could be standarized using a specific substrate (Avicel PH 101). However, more experiments are required to develop an accurate assaythat could be applied to most cellulase enzyme complexes.

Table 1. Properties of cellulose substrates.[a]

Substrate	% Cellulose	CrI[b]	Small molecule accessibility[c]	DP[d]	WRV[e]
Avicel	99.6 (0.5)	80	16.5	219 (3)	2.17 (0.05)
Solka-Floc	91.8 (2.0)	73	26.0	703 (20)	3.06 (0.06)

[a] Values reported are means with standard error of the mean in parenthesis.

[b] Crystallinity Index; obtained by X-ray diffraction methods.

[c] Values reflect the percentage of substrate hydroxyl groups esterified in 90% formic acid.

[d] Degree of Polymerization; obtained from intrinsic viscosity measurements.

[e] Water Retention Volume; grams of water retained per gram cellulose after centrifugation.

Table 2. Kinetic constants for *Trichoderma viride* cellulase .[a,b]

enzyme conc.	Avicel		Solka-Floc	
	Km	Vmax	Km	Vmax
(FPU/mL)[c]	(%,w/v)	(µmol RSE/h)[d]	(%,w/v)	(µmol RSE/h)[d]
0.014	0.63 (0.02)	0.11 (0.01)	0.20 (0.01)	0.08 (0.02)
0.028	0.63 (0.01)	0.21 (0.02)	0.19 (0.01)	0.16 (0.02)
0.056	0.62 (0.01)	0.40 (0.01)	0.20 (0.01)	0.33 (0.01)

[a] Experimental conditions were 50 mM Na acetate buffer, pH 5.0, 40°C, 2 h reaction time and variable substrate concentration ranging from 0.2-2% (w/v).

[b] Values reported are means with standard error of the mean in parenthesis.

[c] Filter paper units per mL.

[d] µ moles of reducing sugar equivalents solubilized per h.

FIG. 1 Effect of substrate concentration on rate of saccharification. Experiments were performed in 50 mM Na acetate, pH 5.0, 40°C, at 140 rpm agitation and an enzyme concentration of 0.014 FPU per mL. The substrate was either Avicel (A) or Solka-Floc (B). Total sugar equivalents solubilized and reducing sugar equivalents solubilized were measured at 2 h (○) and 6 h (■) reaction times.

FIG. 1

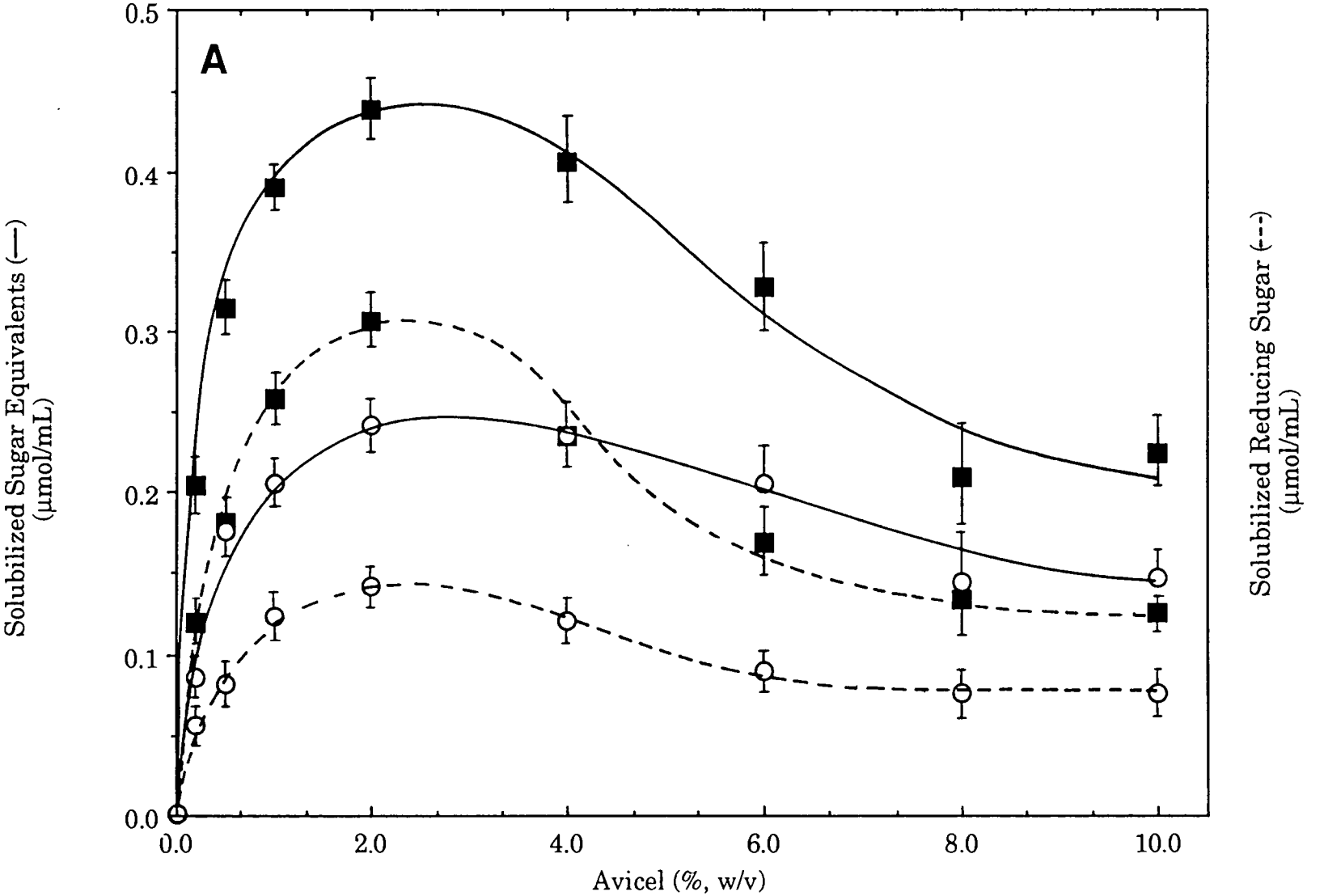

A
0.5
0.4
0.3
0.2
0.1
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Avicel (%, w/v)
Solubilized Sugar Equivalents (—)
(μmol/mL)
Solubilized Reducing Sugar (---)
(μmol/mL)

FIG. 1 Continued

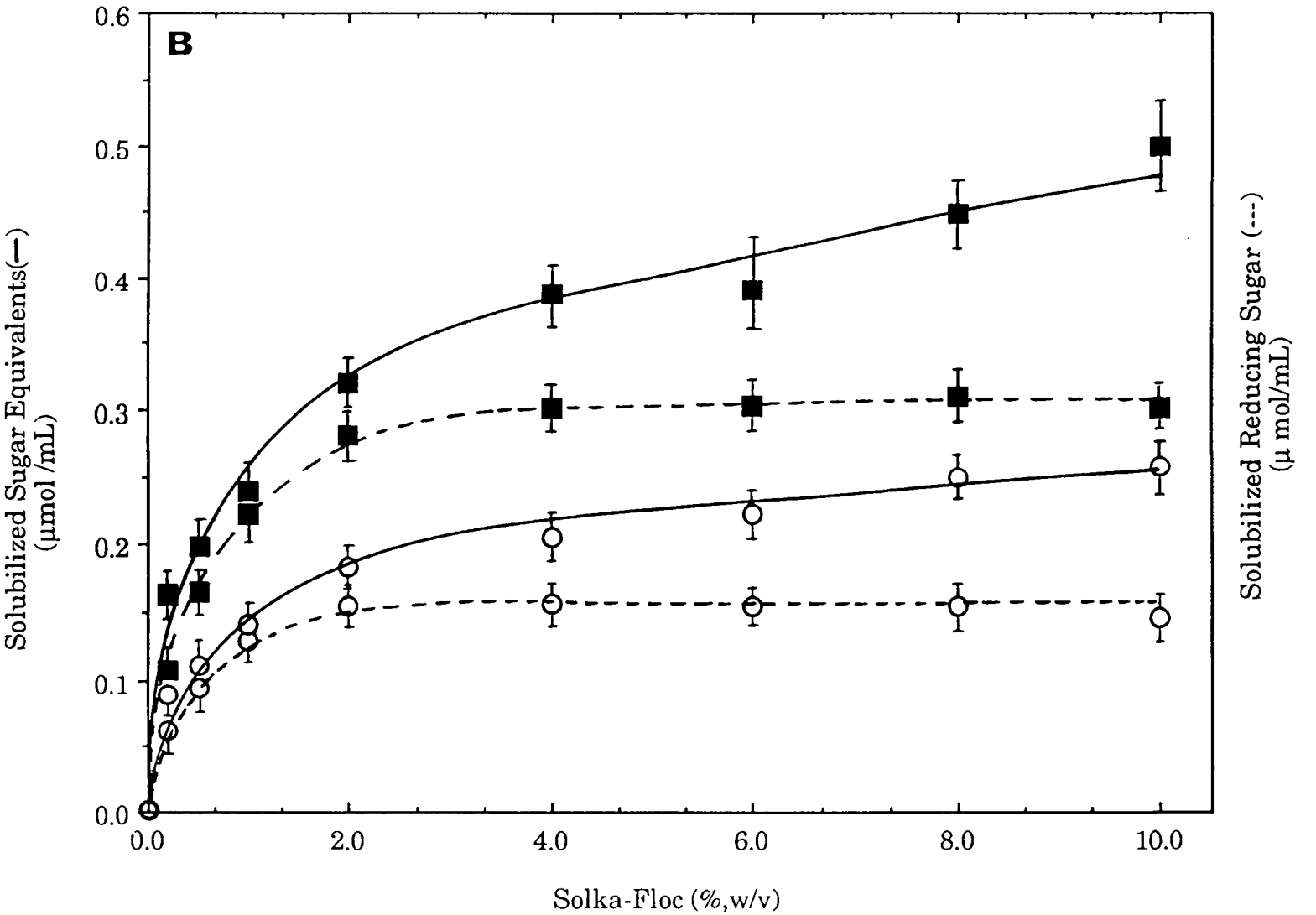


FIG. 2 Effect of degree of reaction mixture agitation on rate of saccharification. Experimental conditions were as in FIG. 1 with 0.028 FPU enzyme per mL. Solubilized reducing sugar equivalents were determined after a 2 h reaction period. Reaction mixtures were agitated at either 100 (▲), 140 (●), or 180 (□) rpm.

FIG. 2

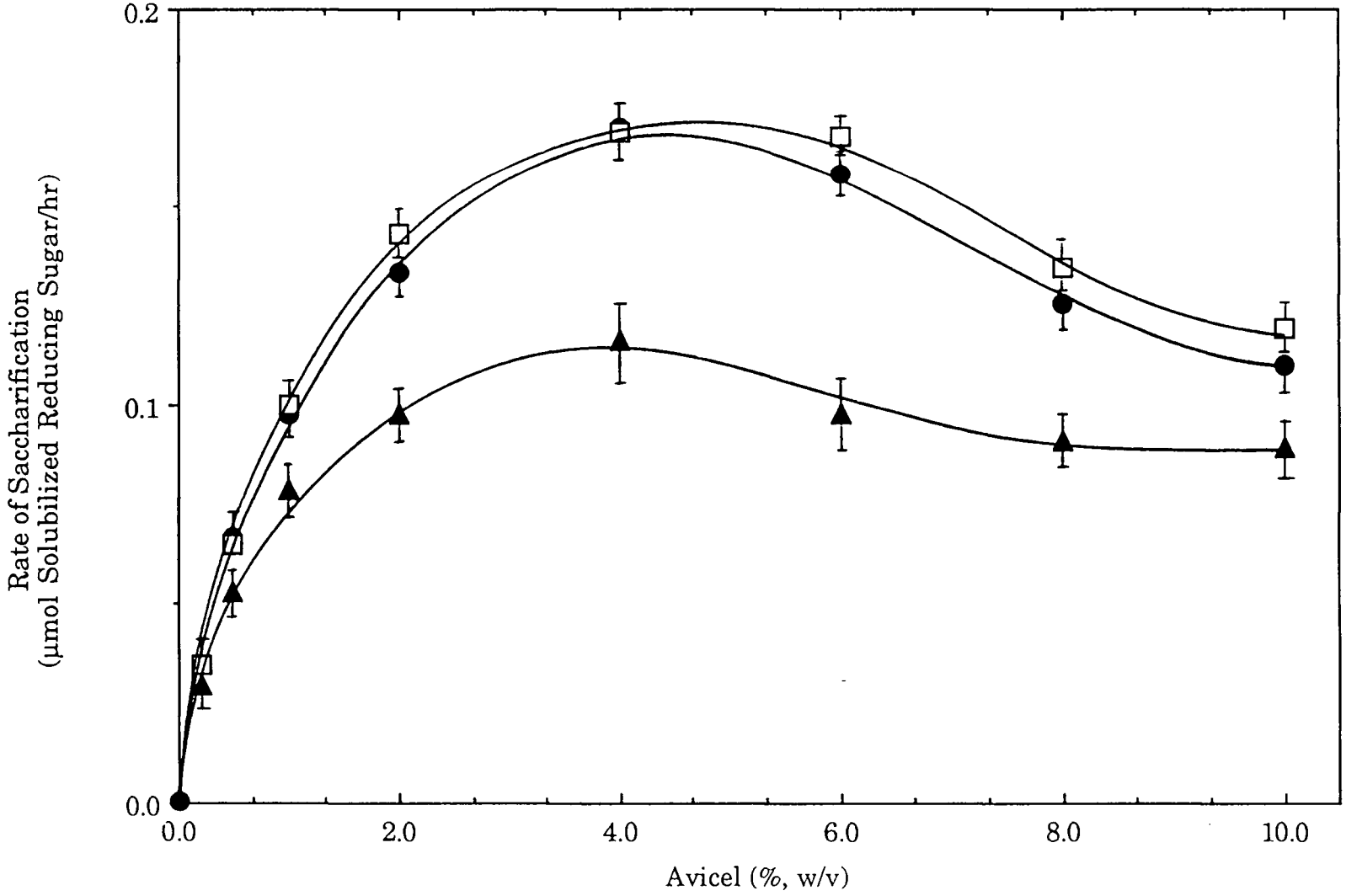

0.2
0.1
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Rate of Saccharification
(µmol Solubilized Reducing Sugar/hr)
Avicel (%, w/v)

FIG. 3 Effect of enzyme concentration on rate of saccharification. Experimental conditions were as in FIG. 1 utilizing either the Avicel (A) or Solka-Floc (B) substrate. Solubilized reducing sugar equivalents were determined after a 6 h reaction period. Reaction mixtures contained either 0.014 FPU (●), 0.028 FPU (□), or 0.056 FPU (▲) per mL. Arrows indicate apparent optimum Avicel concentration.

FIG. 3

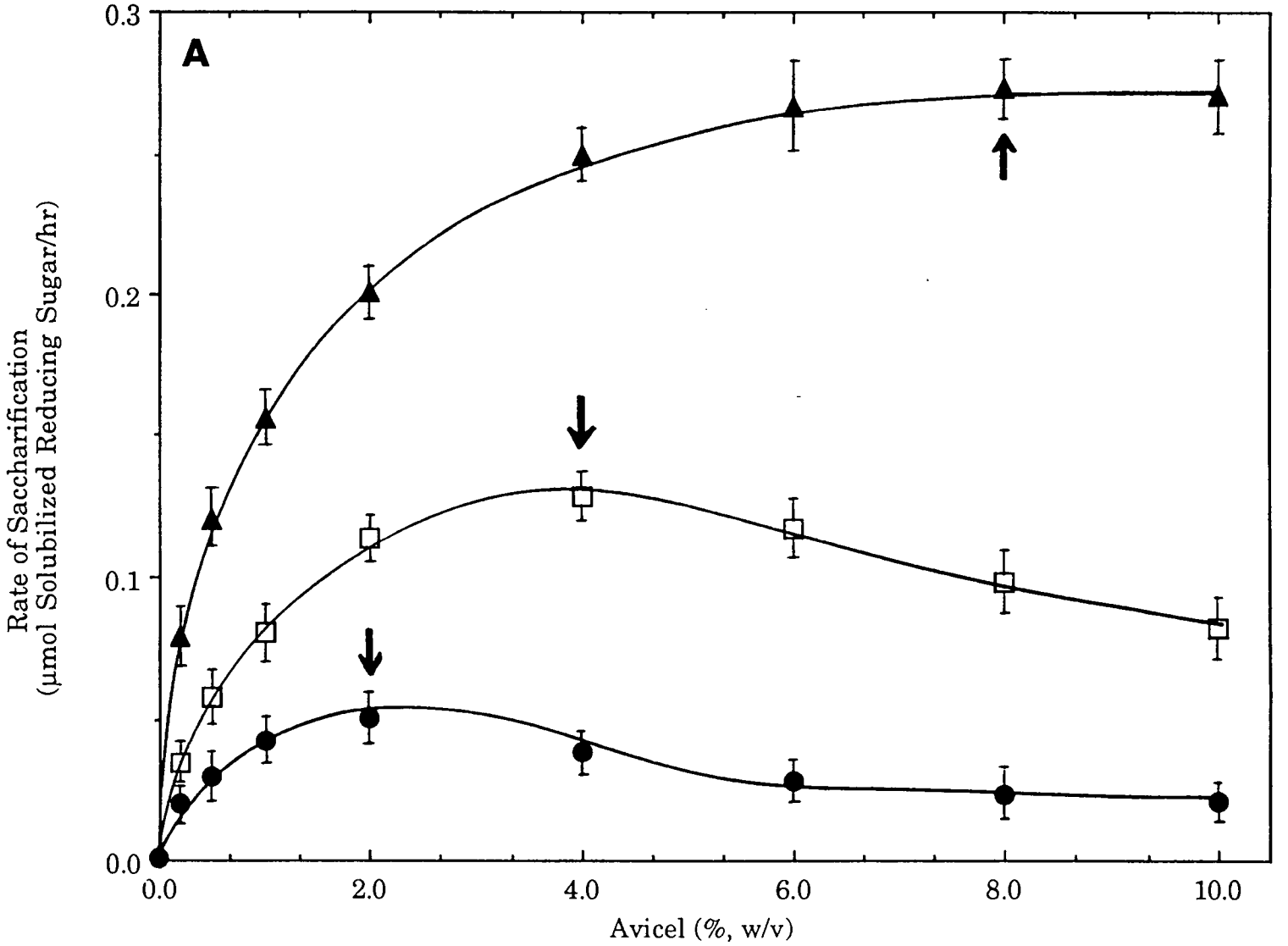

A
0.3
0.2
0.1
0.0
0.0
2.0
4.0
6.0
8.0
10.0
Rate of Saccharification
(μmol Solubilized Reducing Sugar/hr)
Avicel (%, w/v)

FIG. 3 Continued

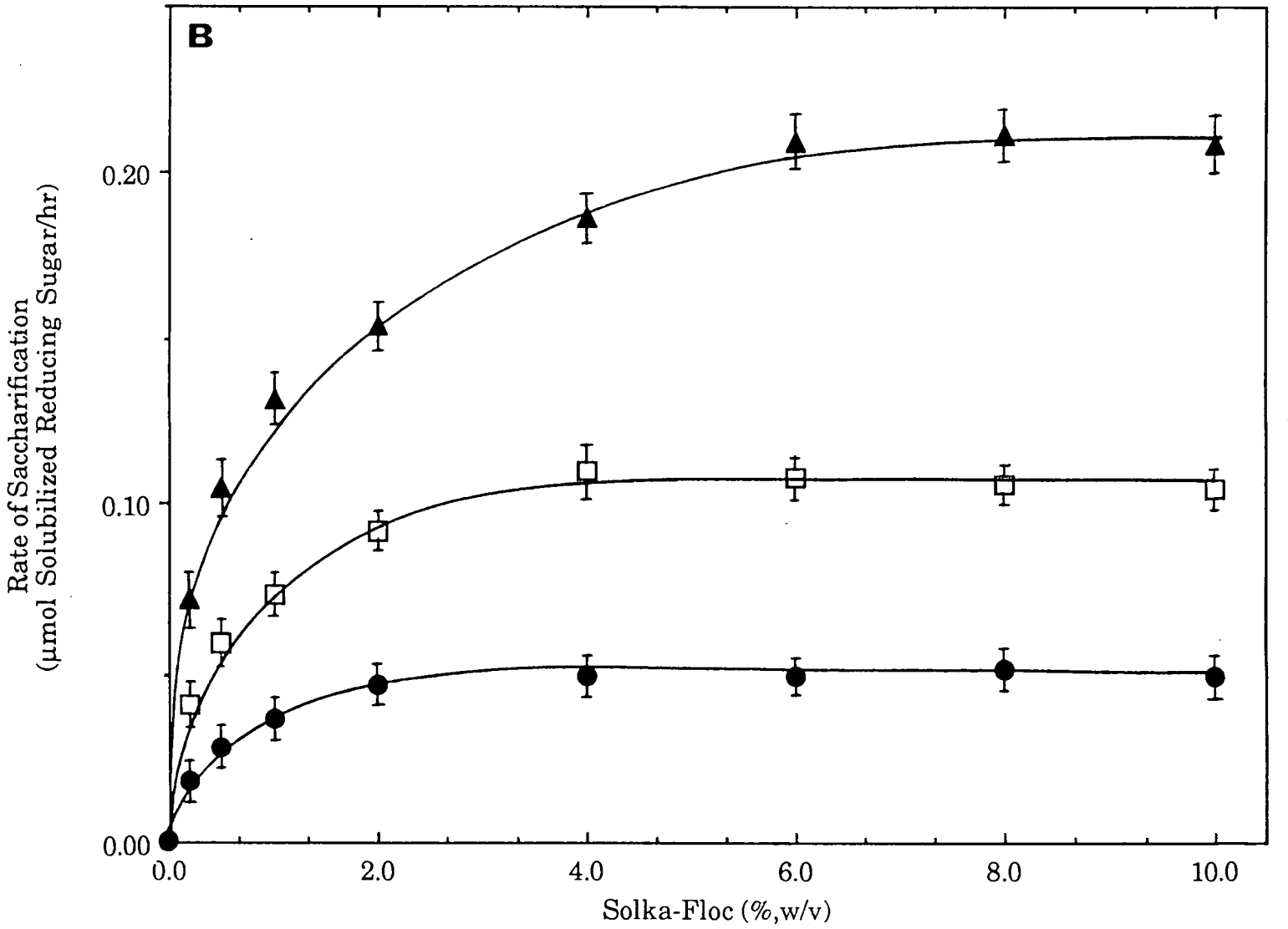


FIG. 4 Comparison of the substrate-velocity profiles for saccharification of Avicel and Solka-Floc. The plotted values are taken from data presented in FIG. 1A and 1B representing total sugar solubilized after a 6 h reaction preiod.

FIG. 4

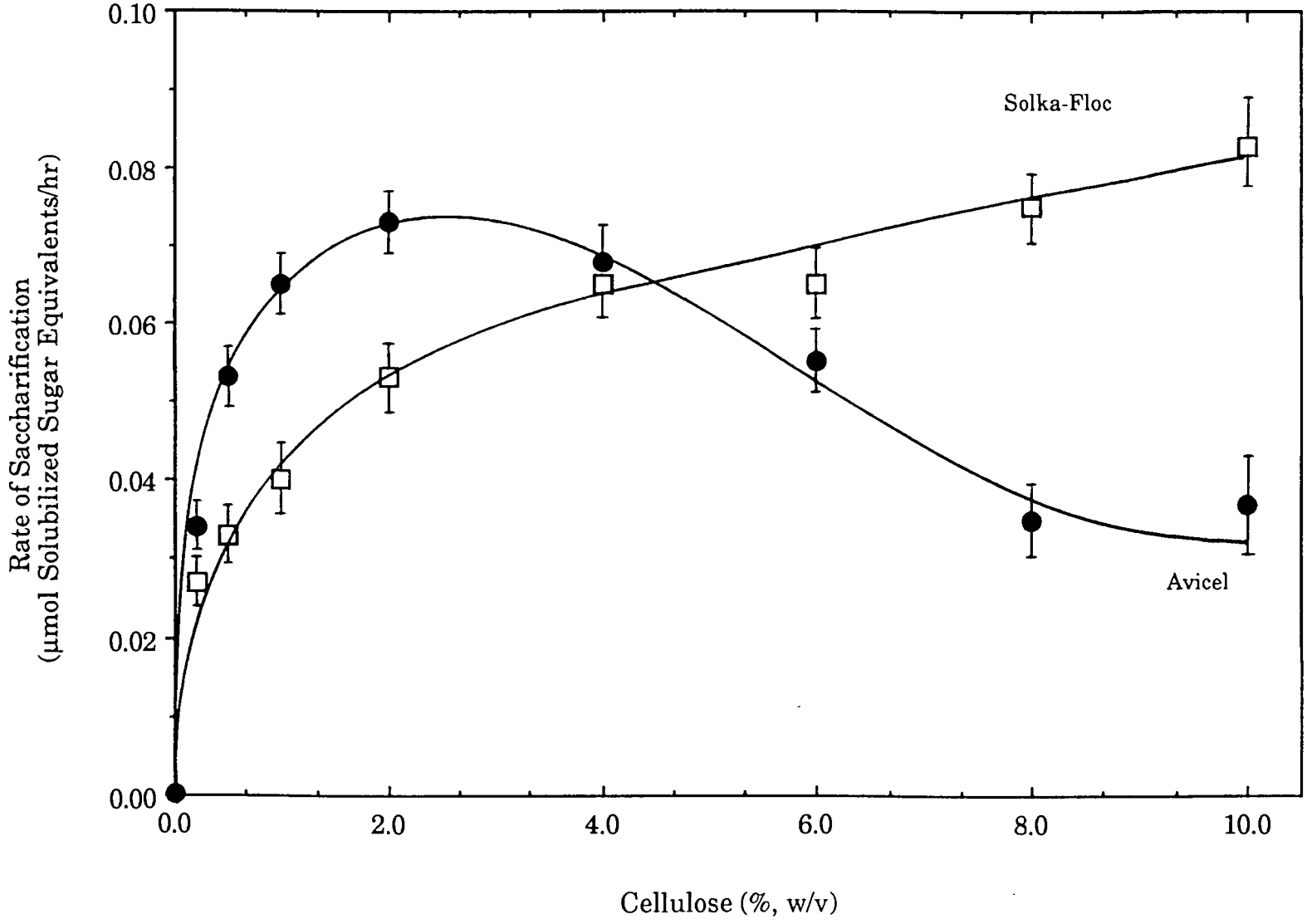
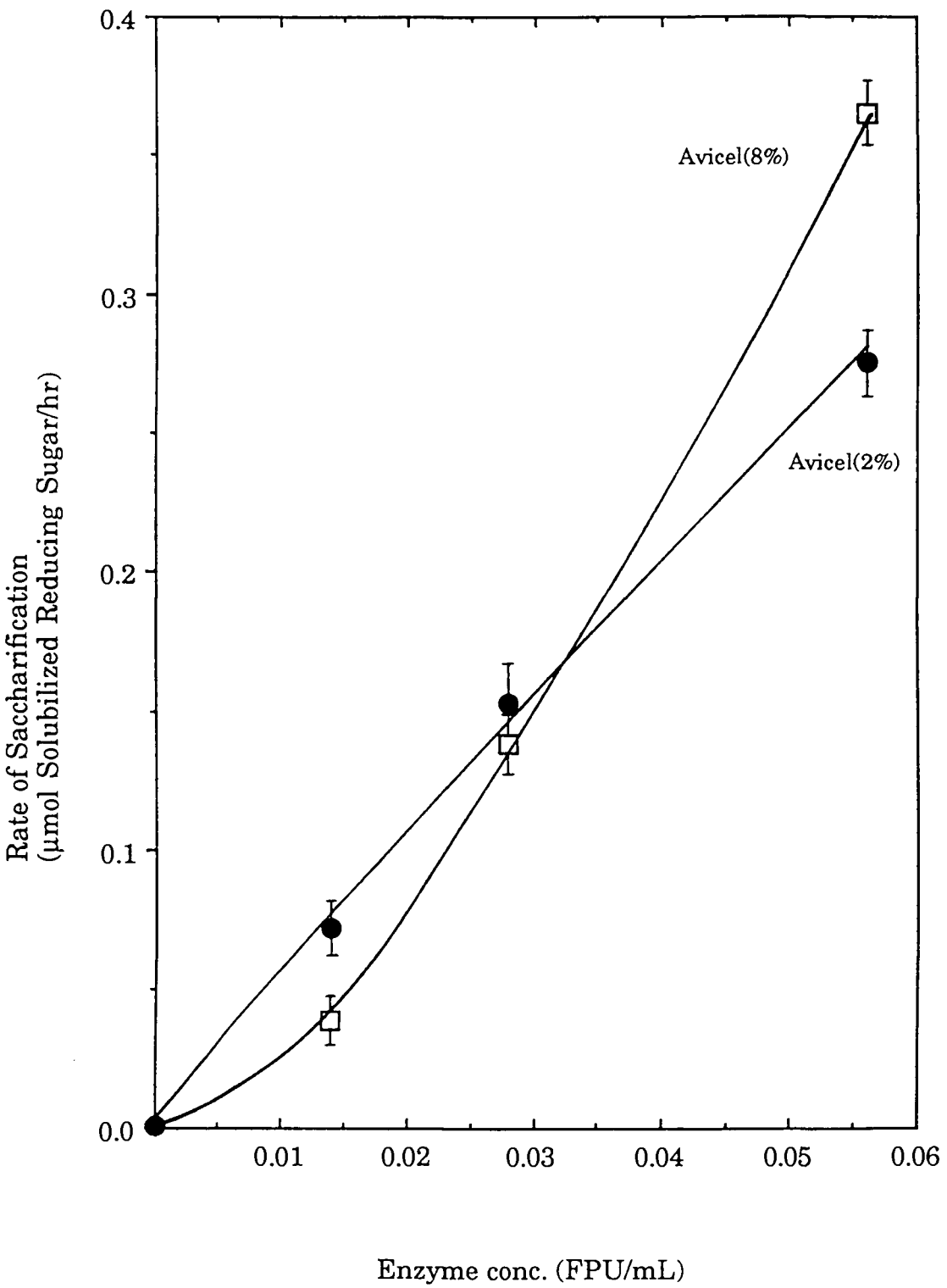

0.10
0.08
0.06
0.04
0.02
0.00
0.0
2.0
4.0
6.0
8.0
10.0
Solka-Floc
Avicel
Rate of Saccharification
(μmol Solubilized Sugar Equivalents/hr)
Cellulose (%, w/v)

FIG. 5 Effect of substrate concentration on the linearity of the enzyme-velocity profile for saccharification of Avicel. The plotted values are taken from data presented in FIG. 3A representing substrate concentrations of 2% and 8%.

FIG. 5


0.4
0.3
0.2
0.1
0.0
0.01
0.02
0.03
0.04
0.05
0.06
Avicel(8%)
Avicel(2%)
Rate of Saccharification
(μmol Solubilized Reducing Sugar/hr)
Enzyme conc. (FPU/mL)

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APPENDIX

APPENDIX A-- Detailed Assay and Characterized Methods

Filter Paper Activity Assay

The assay method developed by Mandels et. al. and modified by T. K. Ghose was chosen for the determination of cellulase activities. Half mL of enzyme solution (0.5 mg/mL) was added to a test tube which contained 1 mL of 50 mM Na-acetate buffer, pH 4.8. Three different enzyme dilutions, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, were prepared for each investigation. One filter paper strip (10 mm x 60mm) was added, mixed and incubated at 50°C for an hour. Three mL DNS reagent (dinitrosalicylic anid) was added , mixed and boiled for exactly 5.0 min in a vigorously boiling water bath. After boiling, the sample was transfered to a cold bath, and 20 mL of distilled water was added. The sample was mixed by complete inversions of the tubes and the absorbance was measured at 540 nm.

Protein Content Assay

a. BCA reagent:

Reagent A: 1000 mL of base reagent which contains; sodium carbonate, sodium bicarbonate, bicinchoninic acid (BCA) detection reagent and sodium tartarate in 0.1 N NaOH.

Reagent B: 25 mL of 4% Copper Sulfate solution.

BCA Protein Assay Working reagent: mixed 50 parts of Reagent A with 1 part of Reagent B.

b. Procedures:

This assay was developed by Pierce Chemical Company and is based on the principles of Lowry method. A set of appropriate protein standards was prepared by diluting a stock solution of BSA (bovine serum albumin), in the same diluent as the unknown samples. One-tenth ml of each standard or unknown protein sample was pipeted into the labelled test tube. Two ml BCA (Bicinchoninic acid) working reagent (freshly prepared) was added to each tube, mixed well,and incubated all tubes at 37°C for 30 min. After incubation, all tubes were cooled to room temperature,and the absorbance was measured at 562 nm. A standard curve was prepared by plotting the absorbance at 562 nm vs. protein concentration. Using this standard curve, determined the protein concentration for each unknown protein sample.

Determination of the Cellulose Content of Substrates

One mL of acetic/nitric acid (89:11, v/v) was added to a test tube which contained 50 mg of cellulose substrates (Avicel or Solka-Floc) and mixed well. Then, 2 mL of acetic/nitric acid was added and mixed again. After heating at 100°C for 30 min, the substrate was cooled down and centrifuged, the supernatant was then removed. The precipitate was washed twice with 3 mL acetic/nitric acid and 4 times with 3 mL acetone. The cellulose sample was dried at room temperature for several hours, and the dried samples were added

with 1 mL 12 M sulfuric acid. The acid concentration of reaction mixture was diluted to 1 M by adding 11 mL distilled water after incubation in a water bath at 35°C for 1 hour. Samples were placed in a boiling-water bath for 2 hours. The samples were cooled down and determined for the total sugar content by the anthrone method. Glucose was employed as the calibrating standard.

Crystallinity Index (X-ray)

The X-ray data were obtained with an automated Philips X-ray diffractometer using nikel-filtered CuKα radiation in which the intensity of diffraction was measured between Bragg angles (2 θ) of 10° and 30°. The crystallinity index was then calculated using the following empirical relationship:

$$CrI\ (\%) = [\ 1 - I\ (am) / I\ (002)\ ] \times 100$$

where I (002) denotes the maximum intensity of the (002) lattice diffraction at $2\theta = 22.5^{\circ}$ and I (am) is the intensity of diffraciton at $2\theta = 18^{\circ}$.

Degree of Polymerization

The degree of polymerization of cellulose was determined by measuring the intrinsic viscosity (η) of cellulose . The method for determining the viscosity of cellulose solutions involved the use of 0.5M cupriethylenediamine (CED) as a solvent and a

Cannon-Fenske capillary viscometer. The precise value of viscosity was obtained by finding the relative viscosity (η_{rel}) of three or more sample concentrations (c), plotting [(η_{rel} - 1)/c] against c and extrapolating the straight line through the points to c=0. The intercept gave the intrisic viscosity. In this study, the sample concentrations were 0.05, 0.1, 0.15, and 0.2% (w/v %). A calculated amount of air-dried cellulose was weighed out and transfered to a 125 mL flask and to which 30 mL 0.167M CED was added (This was made by 1M CED and distilled water in 1 to 5 ratio under N_2 gas) by buret. Flasks were placed in shaker bath at 150 rpm and 25°C to disperse the sample for about an hour. Using buret to add 20 mL 1M CED, the final CED concentration was reduced to 0.5M. Depending on the type of samples, the flasks were kept shaking until the substrates were completely dissolved. Ten mL of the solution was transfered by means of a pipet to a viscometer previously placed in the constant temperature bath at 25.0 $\pm$0.1°C and allowed at least 5 minutes for the solution to reach the temperature. The solution was drawn up into the upper bulb and measured the time (t) required for the meniscus to pass between the two marks. The results was repeated twice and averaged , which did not differ by more than 0.3%. In the same way, the outflow time (t_o) was measured with the solvent as control. The relative viscosity was calculated by the ratio of outflow times (t /t_o).

Initial Accessibility

Preparation of formylated cellulose

One hundred mL of 90% formic acid was added to a 125 mL Ermyler flask which contained 1.2 g cellulose (Avicel or Solka-Floc), and was incubated in a shaker bath at 40°C and 140 rpm. Periodically, the flask was taken from the shaker bath, and 125 mL of methanol/n-propanol (3/1, v/v) was added to the flask to terminate the reaction and precipitate any solubilized carbohydrate. Suspension was collected onto Whatman 1 filter paper via vacuum filtration and washed five times with 100 mL methanol. Formylated cellulose was air-dried overnight, and the resultant product was analyzed by the "Eberstadt titration".

Eberstadt titration

Accurately weighed 0.5 g samples were placed in a 125 mL Ermyler flask, and 20 mL 75% ethanol was added to each flask. The flasks were then heated for 30 min at 50°C in a shaker bath at 100 rpm. This was followed by the addition of 40 mL 0.25 N sodium hydroxide solution into the flasks and which were then heated at 50°C and 100 rpm in shaker bath for 15 min. The flasks were stoppered tightly and allowed to stand at room temperature for 24 hours by shaker bath at 100 rpm. At the end of the time, the excess alkali was back-titrated with standard 0.25 N HCl indicated by phenolphthalein. Blanks containing alcohol and alkali were run with each set of samples. The standard soluble starch was prepared

in the following way. Approximately 1.8 g of dry starch was placed in a 125 mL Ermyler flask with 50 mL 90% formic acid. The flask was stoppered and rotated until dispersion was completed, and incubated at 40°C by shaker bath at 100 rpm. At the end of selected hours, the reaction was quenched by the addition of 200 mL of chilled methanol to each flask. The precipitates were filtered off, washed several times with methanol, dried in the hood overnight, and subsequently saponified with 0.25 N HCl.

Determination of Water Retention Volume (WRV)

The weight of the centrifuge tube was weighed and recorded . Following that, 2 g (W_1) of cellulose was immersed in the centrifuge tube with excess water (20 mL) or buffer (50 mM acetate buffer, pH 5.0, 20 mL) and stirred with a glass rod every 30 min at room temperature for 4 hours. Terminated the incubation by centrifuging at 1,300g for 10 min with Beckmen Model TJ-6 Centrifuge, discarded the supernant, and recorded the weight of percipitate (W_2). The WRV was calculated using the following formula. $WRV(g/g) = (W_2 - W_{1)} / W_1$

Determination of Reducing Sugar by Nelson's Method

A. Reagents:

Glucose (dextrose) (0.5 mM) -- carefully weighed out 0.09 g of

glucose which was dissolved in distilled water to a final volume of 1,000 ml (volumetric flask).

Nelson's reagent A-- dissolved 12.5 g of anhydrous Na_2CO_3, 12.5 g of potassium sodium tartrate, 10.0 g $NaHCO_3$, and 100 g of anhydrous Na_2SO_4 in a final volume of 500 ml of distilled water. Left the solution at room temperature and if a sediment formed removed it by filtration.

Nelson's reagent B-- dissolved 15.0 g of $CuSO_4 \cdot 5H_2O$ in 90 ml of distilled water which contained 2 drops of concentrated H_2SO_4. Made up to a final volume of 100 ml with distilled water.

Arsenomolybdate reagent -- dissolved 25.0 g of ammonium molybdate in 450 ml of distilled water. To this solution was added 21.0 ml of concentrated sulfuric acid (did this slowly and carefully with stirring). Dissolve 3.0 g of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) in 25.0 ml of distilled water and add this solution to the acid molybdate solution with stirring. Incubate the resulting solution at 37°C for 24 hours. Store the arsenomolybdate reagent in a brown bottle.

B. Protocol of Nelson's method:

One mL sample solution was withdrawn and filtered through 0.22 μm membrane at 0, 2, and 6hr. reaction times. One mL Nelson's

reagent, which was freshly prepared each time by mixing 25 mL Nelson's A and 1 mL Nelson's B was immediately added to the filtrate. The mixture was placed in boiling water for exactly 20 minutes, then cooled to room temperature. 1 mL arsenomolybdate reagent was added, mixed well, and allowed to stand for a few minutes. The final volume was made up by 10 mL of distilled water. The solution was mixed well and detected OD 510nm by spectrophotometer. All the data points were collected three times. The standard curve was established by a series of seven tubes each containing concentrations of glucose ranging from 0 to 0.5 mM.

Determination of Total Sugar by Anthrone Method

A. reagents

The anthrone reagent was made up of 10 g thiourea, 0.5 g anthrone, and 1 L 66% sulfuric acid. The 66% (v/v) sulfuric acid was prepared with conc. sulfuric acid and distilled water in a 66 to 34 ratio. When the solution cooled down to 80 ~ 90°C, thiourea and anthrone were added. Until the solute dissolved completely, the solution was stored at 0 ~ 4°C.

B. protocols of the anthrone method.

The sample solutions were prepared as those in the Nelson's method had been. At the same reaction times, 0, 2, and 6 hr, withdrew 1 mL sample solution into a glass tube already filled with 10 mL anthrone reagent. The tubes were stopped tightly and

swirled to mix the content for 10 seconds. They were then placed in a water bath to maintain the temperature equilibrium at room temperature. Followed by heating in a boiling-water bath for 15 minutes, the sample tubes were cooled to room temperature and left in the dark for 25 minutes. The absorbance at 620 nm of each sample was immediately measured. The data were complied three times and glucose was used as a calibration standard range from 0.0 to 1.0 mM.