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TEXTILE TECHNOLOGY

Enzymatic Hydrolysis of Cotton Fibers: Modeling Using an Empirical Equation

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ABSTRACT

In recent years, there has been a tremendous growth in the use of enzymes in wet-processing of textiles. Of the many enzymes suitable for textile applications, cellulase is one of the most important. Cellulase is used in biopolishing of cotton fibers to improve fabric smoothness and softness and in biofinishing denim garments to produce a worn look. To harness the full potential of cellulase and discover additional novel uses for this enzyme in cotton processing, it is imperative to understand the kinetics of the enzymatic action of cellulase on cotton cellulose. In this study, enzymatic hydrolysis of cellulose in cotton fibers by a cellulase mixture was monitored by measuring products of hydrolysis as a function of time in a test reaction vessel. Subsequently, an empirical equation was applied to the data to characterize the cotton cellulose-cellulase system. In spite of its simple form, the empirical equation provided a good fit to the experimental data. In addition, the empirical equation provided pertinent mechanistic information without resorting to the use of complex kinetic models.

Based on the fact that 42 billion pounds of this fiber is consumed annually in textile use, cotton is the international fiber of choice among the world's population (Etters, 2003); however, there are severe environmental costs associated with the popularity of cotton. Wet-processing of cotton during preparation prior to dyeing involves the use of harsh chemicals. Also, chemicals are used during finishing to impart desirable attributes such as softness, durable press, and dimensional stability. Fortunately, in the past decade, commercially viable alternative methods for preparing and finishing cotton fiber substrates based on the use of enzymes have emerged. Such methods will ensure the supremacy of cotton over other fibers for decades to come. Enzymes are biological catalysts usually derived from a fungal or a bacterial source and consist of complex, three dimensional proteins that are composed of polypeptide chains (Etters et al., 1999). Enzymes primarily function by promoting hydrolysis of specific substrates, a process by which water-insoluble material is converted to products that dissolve in water and can be washed away (Etters et al., 1999). Enzymatic hydrolysis has been successful in biofinishing and biopolishing of cotton. In biofinishing, dyed denim jeans are treated with solutions of cellulase under the proper conditions of temperature and pH. The surface of the treated fiber is hydrolyzed releasing dye in a random manner to produce the popular washed-down or worn appearance. Enzymatic biofinishing has largely replaced the use of environmentally harsh pumice stones soaked in sodium hypochlorite or potassium permanganate that were previously used to obtain the worn effect. Cellulase is also highly effective in removing loose fibers from fabric surfaces, a process known as biopolishing. Not only does biopolishing produce a smooth fabric surface, it also assists a dyed fabric in retaining its color depth during laundering. This latter effect is achieved by incorporating cellulase in detergent formulations. Any fibers brought to the fabric surface by abrasion during laundering are hydrolyzed, which avoids the light scattering phenomenon that reduces color depth (Etters et al., 1999).

The term cellulase refers to a group of enzymes that act synergistically to hydrolyze cellulose. Cellulases perform a specific catalytic activity on the 1, 4– β – glucosidic bonds of the cellulose molecule. The hydrolysis of this bond cleaves the molecule into smaller parts that may be further reduced. Commercial cellulases, which are usually produced by submerged fermentation of *Trichoderma reesei*, are multi-component enzyme systems typically containing one or more exo-cellulase activities known as exo-cellobiohydrolases, multiple endo-glucanases, and beta-gluocosidases. Exo-cellulases act on cellulose polymer chain ends and produce primarily cellobiose. Endo-cellulases act randomly along the

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cellulose polymer chains breaking very long polymers into shorter chains. Beta-glucosidases act on short, soluble oligosaccharides to produce primarily glucose (Karmakar, 1998; Kumar and Harnden, 1998). Synergism between the different components in the cellulase system has been documented, but detailed explanation of their mechanism and kinetics is not completely understood. The most widely proposed mechanism of hydrolysis of cellulose can be conveniently divided into the following stages (Lee and Fan, 1982): (a) transfer of enzyme molecules from the aqueous phase to the surface of cellulose molecules; (b) adsorption of the enzyme molecules onto the surface of cellulose resulting in the formation of an enzyme-substrate (E-S) complex; (c) transfer of molecules of water to the active sites of the E-S complex; (d) surface reaction between water and cellulose catalyzed by the E-S complex; and (e) transfer of the products of the reaction of cellobiose and glucose to the aqueous phase.

It is apparent from the steps in hydrolysis that the properties of the substrate, the multiple nature of the cellulase complex, and the mass transfer effects influence cellulose hydrolysis. A prerequisite for hydrolysis to occur is direct physical contact between the enzyme molecules and the surface of cellulose (Lee and Fan, 1982). Cellulase enzymes have a specific three-dimensional shape and their catalytic power depends on adsorption onto the surface of a substrate in lock-and-key fashion (Etters, 1998). A higher surface area enhances the accessibility of the enzyme molecules to the surface (Lee and Fan, 1982). Since cellulases are highly substrate specific in their action, any changes in the structure and accessibility of the substrate has a profound influence on the kinetics of the hydrolysis reaction. Yarn type and fabric construction influence the hydrolysis rate (Karmakar, 1998). Key parameters for the cellulose substrate are accessible surface area, crystallinity, and pore dimensions. Changes in any of these factors, such as structural changes brought about by pre-treatments, influence the hydrolysis reaction. It has been reported that mercerized and raised fabrics are more accessible to enzymatic attack, because they have a more accessible structure (Cavaco-Paulo and Almeida, 1996a). Crystallinity is another structural feature regarded as important. The cellulolytic enzyme acts to a greater degree on the more accessible amorphous regions, so as crystallinity increases, cellulose becomes resistant to further hydrolysis (Lee and Fan, 1982). Milling of cellulose can increase the hydrolysis rates by reducing crystallinity or increasing the surface area (Buschle-Diller et al., 1998). Also, exo-cellulases assist in degradation of cellulose by disrupting the local crytalline cellulose structure, which makes the region more prone to attack by endo-glucanases (Karmakar, 1998).

Mass transfer effects play a decisive role in the kinetics of reaction. For example, enzyme diffusion plays an important role in the heterogeneous system of soluble enzyme and insoluble substrate. Kinetics of the reaction is therefore dependent on the diffusion of enzyme to and into the solid phase of the substrate and the diffusion of the reaction products out of the solid phase into the aqueous phase. Since cotton possesses only limited amorphous areas, diffusion into the interior of fibers is restricted, and the catalytic action of cellulase is confined to the fiber surface (Heine and Hocker, 1995). Consistent with this theory, researchers have reported that the transfer of enzyme molecules to the surface is facilitated by agitation of the reaction mixture (Cavaco-Paulo and Almeida, 1996a), which helps enzyme adsorption and desorption, and aids in the removal of enzymatically loosened material from fiber surfaces (Buschle-Diller et al., 1998). For example, mechanical treatment in rotating drum washers and jets simultaneously with enzymatic hydrolysis is necessary in biopolishing to remove the fibers protruding from the surface (Cavaco-Paulo and Almeida, 1996b). Where a two-step process is adopted with prior mechanical treatment followed by hydrolysis, a decrease in the efficiency of hydrolysis is observed (Zeyer et al., 1994). A smaller change in weight loss was also observed when a washing process followed cellulase treatment (Cavaco-Paulo et al., 1997). These observations confirm the importance of simultaneous mechanical agitation in the enzymatic hydrolysis process. In biofinishing of denim, mechanical action opens the outermost layers of the cellulosic crystal, which results in more of the cellulose being accessible to cellulase and in greater enzymatic removal of indigo. Use of ultrasonic energy in conjunction with mechanical agitation has also been reported to enhance efficiency of enzymatic treatment through reduced processing time, less concentration of enzyme, and better uniformity of enzymatic treatment (Yachmenev at al., 1998).

The products of reaction, namely cellobiose and glucose, profoundly influence the kinetics of enzymatic hydrolysis of cellulose (Howell and Stuck, 1975; Lee and Fan, 1983; Ghose and Das, 1971).

Several researchers have reported that the accumulation of sugars inhibits the hydrolytic action of the cellulolytic enzyme. Ghose and Das (1971) was among the first to report that cellulase was competitively inhibited by cellobiose. Huang (1978) studied the enzymatic hydrolysis of insoluble amorphous cellulose by *T. reesei* and modeled the reaction by taking into account the fast adsorption of enzyme followed by slow reaction and subsequent product inhibition. Howell and Stuck (1975) developed a product inhibition on the assumption that non-competitive inhibition by cellobiose dominates the reaction kinetics for the *T. reesei* system. Applicability of the model was supported by agreement of predicted and experimental reaction progress when cellobiose was added to the reaction mixture or when product was continuously removed from the membrane reactor. A related work argued that the products of hydrolysis decreased the hydrolysis rate by facilitating a loss of enzymatic activity (Howell and Mangat, 1978). Other researchers have also observed a drastic decline in hydrolysis rate during the initial hours of hydrolysis (Lee and Fan, 1983). Two factors were identified as responsible for causing the reduction in the hydrolysis rate. The first factor was a structural transformation of cellulose into a less digestible form as evidenced by changes in the crystallinity index and surface area. The second factor was product inhibition. Effects of crystallinity of cellulose, thermal deactivation, and cellobiose and glucose inhibition on the hydrolysis rate of pure cellulose have been quantitatively investigated and an empirical rate expression has been developed (Ohmine et al., 1983). An excellent literature summary of cellulase inhibition classified according to enzyme, substrate, inhibitor, and nature of inhibition has been published (Holtzapple et al., 1990). Factors affecting product inhibition include the nature of substrate, substrate concentration, enzyme concentration, and composition of the cellulase multi-component system. Among these factors, the most important one is the enzyme/substrate ratio. Different product inhibition patterns may be observed depending on the enzyme concentration and variation in the range in substrate concentration (Gusakov and Sinitsyn, 1992).

It is apparent from the preceding discussion that a lot of effort has been expended on developing kinetic models to explain the complex phenomenon of enzymatic hydrolysis, but these studies have been conducted using variations of the Michaelis-Menten initial velocity kinetics. The Michaelis-Menten equation developed for enzymatic reactions in *solution* may not be valid since cellulose is an *insoluble substrate*. Also, due to the complexity of the hydrolysis process most models have tended to focus on one specific aspect of the hydrolysis process to the exclusion of other simultaneously occurring phenomenon. To concurrently take into account the various changes taking place during hydrolysis, an alternative approach is the application of empirical equations to model experimental data. An empirical rate expression was developed for several kinds of non-textile cellulosic substrates (Ohmine et al., 1983; Ooshima et al., 1982; Kurakake et al., 1995). Unfortunately, those equations do not hold very well for cotton fiber substrates. This manuscript reports the applicability of an empirical equation, which has been previously used successfully in dyeing kinetics, for the hydrolysis of raw cotton fibers. The rationale for using this equation for modeling enzymatic hydrolysis is the remarkable similarity in the stages of hydrolysis and steps in cotton dyeing. It was hoped the application of this empirical equation would reveal useful mechanistic information about the cotton fibers cellulose-cellulase system that were not achievable with other models.

MATERIALS AND METHODS

Raw cotton fibers (West Texas, Grade 52) were used as the substrate and were used to eliminate influence of any chemical pre-treatment on enzymatic hydrolysis. The cellulase used was Cellusoft L from Novozymes NA (Franklinton, NC). The strength of the enzyme was 750 EGU/g.

Substrate purification. The fibers were cleaned by hand to remove impurities, such as seed fragments. Fibers were then boiled in water at 100°C for 30 min. After removal from the boiling water, the fibers were rinsed with deionized water and air-dried. Fibers were conditioned at 65% R.H. and 21°C for 24 h prior to enzymatic treatment.

Hydrolysis. Fibers were hydrolyzed in a reaction vessel equipped with a variable flow-rate pump for controlled circulation of the processing liquor. Hydrolysis was conducted at two flow rates, 0.12 L/min, and 0.84 L/min, to provide different rates of agitation. At each flow rate, the hydrolysis reaction was performed two times and the data reported are the averages of the two runs. The number of turnovers of liquid within the reaction vessel per minute was 0.6 and 4.2 for the 0.12 L/min and 0.84 L/min flow

rates, respectively. To ensure sufficient hydration and swelling prior to hydrolysis, 3.0 grams of fiber were introduced in the reaction chamber and treated for 12 hr in a buffer of pH 4.8 (acetate, 0.1M) at 50° C. At time zero, 2 g/L of enzyme preheated to 50°C was added. Total volume of the reaction mixture was 1 liter. Samples were withdrawn at intervals of 0.17, 0.33, 0.5, 1, 2, 4, 8, 12, and 24 h with a large hypodermic syringe and immediately filtered through a 0.45-μm filter to remove insoluble substrate. The filtrates were analyzed for concentration of soluble reducing sugars by the dinitrosalicylic (DNS) acid method (Miller, 1959). The DNS method is based on the principle that 3,5–dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicyclic acid and an equivalence is established between the amino-nitrosalicylic acid produced and the sugar present. Accordingly, 3 ml of DNS solution was placed in a test-tube and mixed with 3 ml of filtrate. The mixture was heated in a boiling water bath for 5 min. The test tubes were then cooled under running tap water and brought to ambient temperature. The characteristic color obtained was measured at 575 nm and the concentration of reducing sugars was determined by using a calibration curve obtained for a range of glucose concentrations.

RESULTS AND DISCUSSION

The concentration of soluble reducing sugars as a function of time at the two flow rates is presented in Table 1. The data was plotted as a figure (Fig. 1). The figure was plotted on square root of time scale because a compressed time scale provides a clearer picture of changes in product concentration at the beginning of hydrolysis. Salient features of the figure are the intercepts of the product formation curves on the time axis for both the flow rates studied, and increased sugar production increased with an increase in the flow rate. The intercepts on the time axis are characteristic of systems in which a resistance at the substrate surface exists (Etters, 1981). This resistance may be due to the presence of a diffusional boundary layer and suggest a reaction limited by diffusion. A diffusional boundary layer that exists at the surface of fibers offers resistance to the sorption of enzymes, as well as to the movement of products of reaction away from the fiber surface. There was a 12.98% increase in product formation at 24 h when the flow rate was increased from 0.12 L/min to 0.84 L/min. The results seem to suggest that at higher flow rates the efficiency of hydrolysis increased, presumably by decreasing the thickness of the diffusional boundary layer, which facilitated sorption of enzyme and also decreased probable product inhibition by more efficient removal of products of the reaction (Sarkar and Etters, 2001).

To model the experimental data, the following empirical equation was used (Etters, 1980).

$$\frac{P_t}{S_0} = [1 - \exp\{-k(t)^x\}]^{1/y} \quad (1)$$

where; P_t = product concentration at time t (mg/ml); S_0 = initial substrate concentration (mg/ml); P_t/S_0 = fractional conversion. Parameters x, k and y have

Table 1. Experimental and calculated product concentrations as a function of time at different flow rates

Time (h)	Flow rate (0.12 L/min)		(0.84 L/min)	
	P_{exp}[z] (mg/ml)	P_{calc} (mg/ml)	P_{exp}[z] (mg/ml)	P_{calc} (mg/ml)
0.17	0.029 (0.008)	0.020	0.042 (0.000)	0.036
0.33	0.033 (0.007)	0.032	0.054 (0.008)	0.053
0.50	0.034 (0.008)	0.041	0.057 (0.008)	0.068
1.0	0.050 (0.003)	0.065	0.098 (0.005)	0.103
2.0	0.083 (0.008)	0.102	0.139 (0.007)	0.156
4.0	0.152 (0.006)	0.160	0.217 (0.011)	0.236
8.0	0.286 (0.005)	0.249	0.404 (0.005)	0.352
12.0	0.368 (0.017)	0.322	0.523 (0.011)	0.443
24.0	0.539 (0.010)	0.492	0.609 (0.016)	0.649

[z] Values in parenthesis are standard deviations.

specific values for each flow rate studied and were determined statistically using SAS (SAS Institute Inc.; Cary, NC). At a flow rate of 0.12 L/min, the values of x, k, and y were 0.66, 0.022, and 1, respectively. At 0.84 L/min, the values were 0.61, 0.035, and 1 for x, k, and y, respectively. Using these values, the expected product concentrations were calculated. Results are tabulated in Table 1 and plotted in Figure 1, which show the empirical equation calculates experimentally obtained results with good accuracy, especially at the beginning of hydrolysis. From a textile processing perspective, the ability to accurately model the beginning of hydrolysis is more significant because most enzymatic treatments of cotton fiber are less than 1 hr in duration. Other mechanistically useful information is also acquired. For example, the parameter k is a measure of overall rate of the reaction. An increase in the flow rate resulted in an increase in the rate of the reaction from 0.022 at a flow rate of 0.12 L/min to 0.035 at a flow rate of 0.84 L/min. From a fiber processing perspective, the result has important implications, because it implies that effective hydrolysis of cotton by cellulase is dependent on effective agitation of the reaction mixture. Therefore, cellulase treatment of cotton should be done in jets, rotating drum washers, and becks, which are all batch processes with high levels of agitation. Parameter x is also important, because it depends on the sterical structure of the system ranging from 1 for very thin films to 0.5-0.6 for high resistance structures with intermediate values describing varying degrees of structural resistance of the system (Chrastil and Wilson, 1982). For the cotton fiber cellulose-cellulase system in this study, the values of x suggest that cotton fibers were resistant to the cellulase treatment, because of the raw condition of the fibers. Practically, this means that cellulase hydrolysis will be more effective when fibers are pretreated prior to finishing with cellulase.

The robustness of Equation 1 is further demonstrated by applying the equation to data in other studies (Howell and Stuck, 1975; Ghose and Das, 1971). Ghose and Das (1971) followed the enzymatic saccharification of fine cellulose in concentrated cellulose from *T. viride* with three initial cellulose concentrations. The reason for selecting this particular data set was to determine the applicability of the empirical equation to different changes in reaction parameters. In our experiment, the substrate concentration was held constant and the flow rate was varied as compared with the variation in substrate

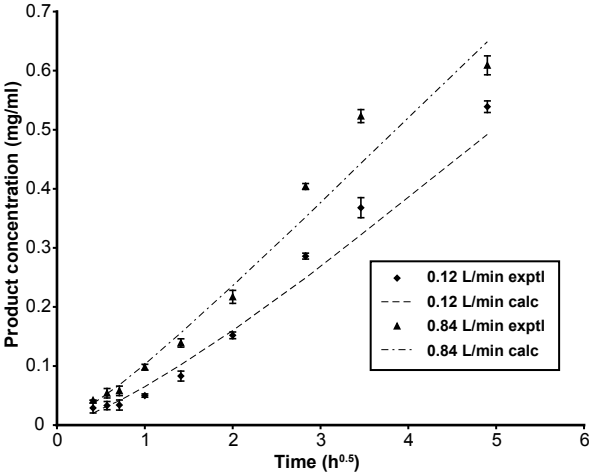

Figure 1. Calculated versus experimental concentration of product as a function of time at different flow rates

concentration while other parameters were constant in the experiment by Ghose and Das (1971). Their experimental data is summarized in Table 2 with calculated values obtained by using Equation 1. Again, there is good agreement between the experimental and calculated values (Fig. 2). An interesting observation was the decrease in the rate coefficient, k, from 0.51 at a substrate concentration of 10 mg/ml to 0.33 at a substrate concentration of 50 mg/ml, which suggests substrate inhibition at higher concentrations of cellulose. This conclusion is consistent with findings reported in the literature in which cellulose is inhibited by concentrations in excess of 15 mg/ml (Ghose and Das, 1971). Further proof is provided by the values of the parameter x. At higher substrate concentrations, the value of x is lower that indicate increased resistance to hydrolysis.

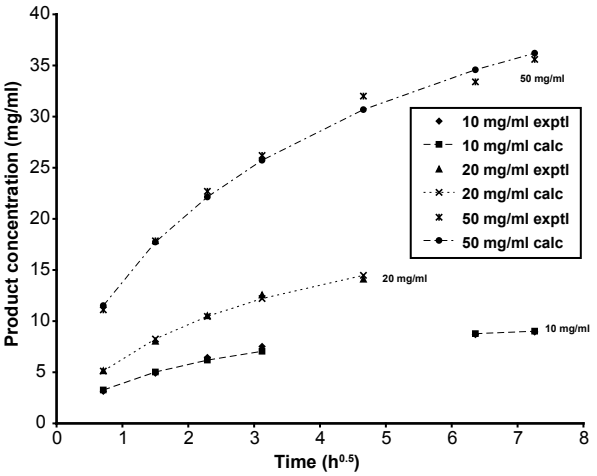

Figure 2. Calculated versus experimental concentration of product as a function of time at different substrate concentrations

Table 2. Experimental and calculated product concentrations as a function of time at different substrate concentrations (data from Ghose and Das, 1971)

Time (h)	Initial substrate conc. (10 mg/ml)		Initial substrate conc. (20 mg/ml)		Initial substrate conc. (50 mg/ml)	
	P_{exp} (mg/ml)	P_{calc}[x] (mg/ml)	P_{exp} (mg/ml)	P_{calc}[y] (mg/ml)	P_{exp} (mg/ml)	P_{calc}[z] (mg/ml)
0.5	3.14	3.26	5.14	5.12	11.10	11.52
2.25	4.91	5.03	8.04	8.25	17.85	17.73
5.25	6.45	6.19	10.52	10.46	22.70	22.14
9.75	7.52	7.05	12.56	12.21	26.20	25.73
21.75	-	-	14.10	14.48	32.00	30.68
40.50	8.69	8.77	-	-	33.40	34.58
52.75	8.95	9.02	-	-	35.60	36.20

[x] Parameters x, k, and y in Equation 1 were 0.38, 0.51, and 1, respectively

[y] Parameters x, k, and y in Equation 1 were 0.39, 0.39, and 1, respectively

[z] Parameters x, k, and y in Equation 1 were 0.34, 0.33, and 1, respectively

CONCLUSIONS

The empirical equation adequately described the hydrolysis of cotton fibers, and the hydrolysis of non-textile cellulosic substrates. In addition, useful mechanistic information providing insight into the structural nature of the cellulose-cellulase system was obtained. Such information included the rate of the reaction, the sterical and diffusional features of the system, and probable inhibitory effects of excess substrate and products of reaction on enzymatic hydrolysis. Theoretical analysis of enzymatic reactions using such simple empirical equations will hopefully pave the way for efficient kinetic studies. As more enzymes are introduced in the wet-processing of cotton, there is a need for quick but reliable data to judge the effectiveness of the enzyme-substrate system. Modeling using empirical equations fills the requirement admirably.

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