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A STUDY ON ENZYMATIC HYDROLYSIS OF CELLULOSE IN AN ATTRITION BIOREACTOR

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Abstract—In the biological conversion of cellulose, a physical or chemical pretreatment proceeds the hydrolysis by an enzyme. The hydrolysis rate however, is slowed down as the active sites in the pretreated substrates are reduced.

In this study, attempts were made to use an attrition bioreactor in which the pretreatment and the hydrolysis were carried out at the same time, where higher hydrolysis rates were achieved.

Glass beads of 0.3-cm-diameter and sand were used as the milling media in a batch reactor where pure cellulose, Solka Floc BW200 was hydrolyzed by cellulase secreted from a fungus mutant, *Trichoderma reesei*.

The higher rates observed are believed due to the synergistic effects of the size reduction and the conversion of the crystalline to the amorphous form of cellulose which was observed by comparing the X-ray diffractorgrams of the cellulose hydrolyzed in the reactors with and without milling medium.

A simple kinetic model was found satisfactory in depicting the hydrolysis mechanism, and the kinetic parameters were estimated.

Higher power consumption as compared to a regular stirred reactor was observed and a quantitative expression was derived for its estimation.

INTRODUCTION

There is a great potential for biomass conversion to ease our dependence on nonrenewable resources used as feed-stocks in chemical, fuel, and food production. Cellulosic biomass is particularly promising because of its abundance and regenerative capacity. Reports estimate that on the land area of the earth approximately 16 billion tons of carbon per year are fixed by photosynthesis, of which about one half appears initially in the form of cellulose [1]. Efforts have been focused on developing effective bioconversion processes for a wide variety of cellulosic substrates [2-5], which many consist of physical or chemical pretreatment steps followed by enzymatic hydrolysis.

Pretreatment is essential to open up the cellulose matrix allowing more rapid diffusion of the enzyme into the matrix pores. Physical pretreatments such as milling [2-6], irradiation [7-8], heating [2, 9, 10], and heating with other pretreatment [11-13], chemical pretreatments such as the use of swelling agents [2, 9, 11, 14-18], delignification [8, 19-21], dissolving and reprecipitation [22, 23], and steaming [24] have all been used in obtaining high yield for the subsequent enzymatic hydrolysis.

Milling has been recognized as one of the most effective methods [9, 25]. It reduces crystallinity and increases both contact surface and bulk density so that highly concentrated suspensions can be handled in the reactor [4, 12]. However, the cost makes pretreatment one of the most critical phases in terms of overall economic factors.

The prime objective of this study is to investigate a continuous generation of accessible sites and a sustained rapid hydrolysis rate in an "attrition bioreactor" in which the pretreatment and the hydrolysis are carried out at the same time.

ENZYMATIC HYDROLYSIS

The ability of cellulolytic microorganisms and that of cell free cellulolytic enzymes to degrade cellulose varies greatly with the nature of the substrate. Cowling and Brown [27] and Cowling [26, 28] have discussed comprehensively the influence of fiber structure on its susceptibility to enzymatic degradation. They have pointed out that the structural features of cellulosic materials determine their susceptibility to enzymatic degradation.

The amorphous component of cellulose is digested

more readily by enzymatic attack than the crystalline component, and any means that will increase the amorphous content will enhance the hydrolysis rate. The limitation of available sites for enzymatic attack stems from the fact that the average size of the capillaries in biomass is too small to allow the entry of large enzyme molecules, and enzymatic attack is confined, therefore, to the external surface.

Thus, pretreatment is an essential prerequisite to enhance the susceptibility of cellulose to enzyme action. An ideal pretreatment would accomplish reduction in crystallinity, concomitant with a reduction in lignin content and an increase in the surface area.

Many different pretreatments have been attempted and the literature on this subject is voluminous [21, 38].

The physical treatments include ball milling to small mesh sizes, and two-roll milling[9, 21], and are effective in producing a more reactive cellulose. Chemical treatment with strong acids or bases such as sulfuric acid or sodium hydroxide or with other cellulose swelling/dissolving agents also effectively increase the hydrolysis of cellulose. The chemical agents are generally quite corrosive, costly, and must be recovered for reuse. Furthermore, they are often toxic or inhibitory to biological systems so that their removal from the treated cellulosic material must be complete. A further problem with the chemical treatments is that the recovery of the chemical agent generally requires a wash stream which itself must be purified. All these factors combine to increase the expense and difficulty of the chemical treatment methods. In addition to the treatments mentioned above, delignification by organic solutions, electron irradiation, and freezing have also been used to increase cellulose reactivity [9, 21].

Recently efforts have been made to investigate the performance of a new type of reactor, a "bioattritor" in which the physical pretreatment and the enzymatic conversion proceeds at the same time.

Detailed analyses on the performance of this reactor have been carried out by the investigators, Ryu and Lee, and Kelsey and Shafizadeh [49, 50]. In their works, higher hydrolysis rates were obtained while the power requirement was substantially increased.

In this work, investigations were made to elucidate the mechanism of the enhanced hydrolysis rates of cellulose in an attrition bioreactor, and the hydrolysis kinetics as well.

KINETIC MODEL

A simplified mechanism for the enzymatic hydrolysis of cellulose consists of the adsorption of enzyme E on cellulose S to form complex X, which is degraded to soluble product P. The product than combines with the enzyme to form complex X, which inhibits the enzyme action;

	Equilibrium Constant
$E+S \rightleftharpoons X_1$	K_1
$X_1 \rightarrow E+P$	
$E+P \rightleftharpoons X_2$	K_2

Assuming the second reaction is the rate-determining step, and the steady state is attained for X_1 and X_2, the rate equation is;

$$V=\frac{dP}{dt}=\frac{V'_m(S)}{K'_m-(S)} \tag{1}$$

where,

$$V'_m=\frac{k(E)_o K_1}{K_1-K_2} \tag{2}$$

$$K'_m=\frac{1+K_2(S)_o}{K_1-K_2} \tag{3}$$

Integration of equation (2) yields,

$$K'_m \ln\frac{(S)_o}{(S)}+[(S)_o-(S)]=V'_m t \tag{4}$$

or

$$\frac{\ln(S)_o/(S)}{(S)_o-(S)}=\frac{V'_m}{K'_m}\left[\frac{t}{(S)_o-(S)}\right]-\frac{1}{K'_m} \tag{5}$$

MATERTIALS AND METHODS

1. Meterials

1-1. Microbial Strain

The cellulase-enhanced mutant derived from *Trichoderma reesei (viride)* was used in this study. The fungus *Trichoderma reesei* QM 9414 was maintained on potato dextrose agar slants at 25 C.

1-2. Enzyme Production

Shake cultures for cellulase production were inoculated with a spore suspension and grown fore 120-200 hrs at 27 C on reciprocating shakers. The pH was adjusted to 5.0 with 1 N KOH. The cellulose source was Solka Floc BW 200 (Brown Co., Berlin, N. H.), a purified spruce wood cellulose. Proteose peptone (Difco) and Tween 80 (Atlas Chemical Industries, polyoxy ethylene sorbitan mono-oleate) were then added. The cultures were harvested by filtration through glass wool and the culture filtrates were used directly for the hydrolysis experiment.

1-3. Cellulose Substrate

The pure cellulosic material, Solka Floc BW 200 (Brown Co., Berlin, N. H.) was used as the main substrate in this study. Solka Floc BW 200 is a ball-milled sulfite pulp 200 mesh (75 μ m). Dry Solka Floc is at

Table 1. Physical characteristics of Solka Floc BW200.

Characteristics	Dimension
Color	White
Average fiber length	30-35 um
Screen analysis (Ro-Tap, Tyler)	
% on 35 mesh	0-0.5%
% through 100 mesh	95-99%
% through 200 mesh	80-90%
Bulk	1.8-2.2 ml/g
Apparent density	0.29-0.56 g/ml
Ash content	0.3-0.4%

least 99.5% cellulose and is virtually lignin free. The particle size and various physical structural parameters are listed in Table 1.

2. Methods

2-1. Enzyme and Sugar Analysis

Standard procedures were employed for the analysis of the soluble protein, the filter paper activity and the reducing sugars [41, 43].

2-2. Crystallinity of Cellulose Substrate

The crystallinity was measured by the powder method of x-ray diffraction using a Norelco diffractometer. The specimen was mounted horizontally while the Geiger counter moved in a vertical arc. A Cuk_{α} target with a nickel filter was used. The substrate sample was prepared by a solvent-drying method and stored in a desiccator. Care was exercised in handling the samples to minimize exposure to the atmosphere because adsorption of moisture from the air tends to increase the crystallinity index.

The specimens were prepared by the method of Mc-Creery [55]. The diffraction intensity was measured between the Bragg [20] 10° and 30°. The following crystallinity index (CrI) proposed by Segal et al. [56] was employed:

$$CrI = \frac{(I_{002} - I_{am})}{I_{002}} \times 100$$

where I_{002} is the intensity of the 002 peak (at about $2\theta = 22°$), and I_{am} is the intensity at $2\theta = 18°$. The I_{002} peak corresponds to the amorphous fraction. The intensities were measured above an approximate baseline representing background intensity (Fig. 1).

2-3. Solvent Drying

A solvent-drying method was used to dry the cellulose samples. It was adopted to assure or preserve the cellulose structural parameters of the water-swollen state to the maximum extent possible.

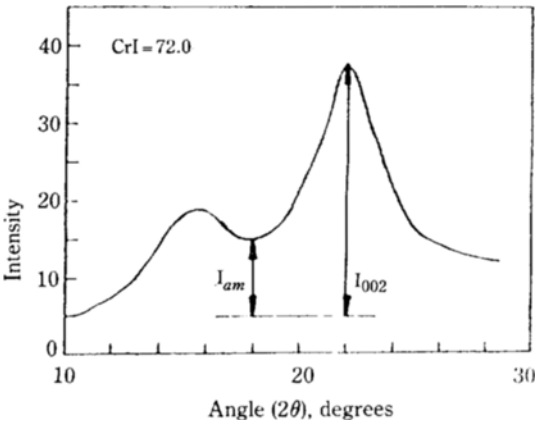

Fig. 1. X-ray diffraction pattern of Solka Floc.

The water-swollen cellulose was centrifuged, and the excess water was decanted. The water retained in the cellulose was replaced first by a polar solvent, methanol, using a Soxhlet extraction apparatus. Three extraction were performed to ensure the removal of all the water. Methanol was then replaced by a nonpolar solvent, benzene, using the same apparatus and procedure. The benzene was removed from the cellulose by air-drying at 80 C for 24 hrs. The solvent-dried samples were stored in a desiccator.

2-4. Attrition Bioreactor

The schematic diagram of the simple attrition bioreactor for the hydrolysis tests is shown in Fig. 2.

It is composed of a reaction vessel, milling media, and a stirrer. The reaction vessel of maximum capacity

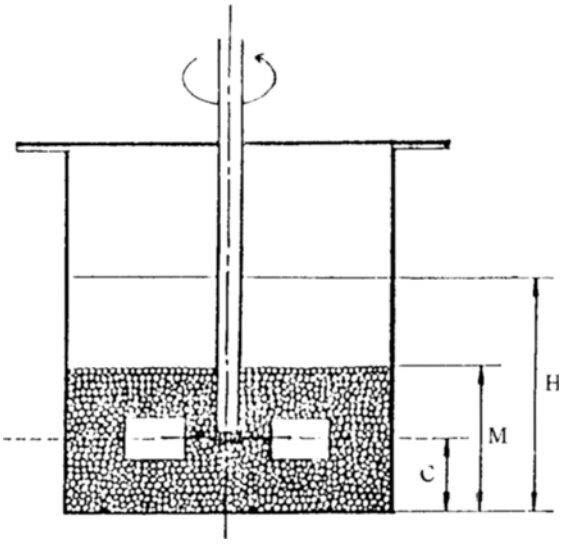

Fig. 2. Schematic diagram of attrition bioreactor.

of 5 liters is consisted of a 20 cm high, 18 cm inside diameter, and 0.8 cm thick glass vessel without baffles. The stirrer system installed in the vessel is consisted of a 12.85 cm diameter flat blade turbine impeller with 6-blade disk. It was equipped with a 1/2-hp drive motor and gear box for continuously variable output speeds from 0 to 1800 rpm. Sand (35/100 Tyler mesh) and glass beads (0.3 cm diameter) served as the milling media.

The dimensions of the reactor are given in Table 2.

2-5. Hydrolysis Tests

Hydrolysis tests were carried out in an attrition bioreactor containing 100 g of cellulose substrate, 100 m*l* of 1.0 M citrate buffer pH 4.8, 10 m*l* of 1% sodium aside to inhibit microbial growth, 1 *l* of culture filtrate, plus water to bring a 5% substrate solution. The temperature was maintained at 50 C in a water bath.

Samples were withdrawn, centriguged to remove solids, and the supernatant was refregerated for the analysis.

2-6. Power Consumption Measurement

The power consumption of the attrition bioreactor measured with a thrust bearing dynamometer system [47, 48] is given;

$$P = \frac{\Pi\ N\ d\ F}{30}$$

where P is the power consumption (W), N is the agitation speed (rpm), F is the force required to prevent the torque table from rotation (N), and d is the distance from the axis to the point of the force measurement (m).

RESULTS AND DISCUSSIONS

1. Hydrolysis

Solka Floc was hydrolyzed in the attrition bioreactor with glass beads (0.3 cm diameter) of sand(35/100 Tyler mesh) as its milling media. Agitation speed was 250 rpm. Shown in Fig. 3 are the changes in the concentration of reducing sugar with time. The hydrolysis time period of 8 hrs was chosen for economic process not only because it represents the hydrolysis before substantial product inhibition becomes important but also because a practical commercial process must deal with short reaction time.

Table 2. Dimensions of attrition bioreactor.

Vessel capacity	5*l*
Internal diameter of vessel, T	18.0 cm
Height of vessel	20.0 cm
Working volume	2*l*
Height of solution, H	12.8 cm
Height of milling media, M	7.9 cm
Height of impeller off bottom, C	3.9 cm
Impeller type:	Flat Blade Turbine
Impeller diameter, D	12.9 cm
Number of blades on impeller, B	6
Width of impeller blades, W	3.3 cm
Length of impeller blades, L	2.7 cm

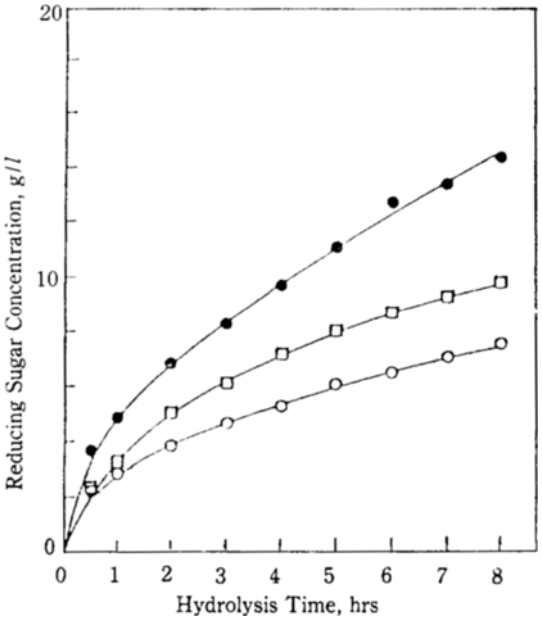

Fig. 3. Hydrolysis of Solka Floc: in a regular stirred reactor(○) without pretreatment; in an attrition bioreactor with sand(□), 0.3-cm-diameter glass beads(●), all with an agitation speed of 250 rpm.

Comparison of results in the attrition bioreactor with those in the control system employing the same reactor without milling media (regular stirred reactor) has shown; (1) In the regular stirred reactor without milling media (the control), reducing sugar concentration of 7.4 g/*l* was produced in 8 hrs. While reducing sugar concentration of 14.2 g/*l* was obtained for the same reaction time in the attrition bioreactor, when glass beads of 0.3 cm diameter were used as milling medium. (2) Sand was not as effective as the glass beads as a milling medium but still showed significant improvement over the control; conversion could be improved by 92% with the glass beads (0.3 cm diameter) medium and by 30% with the sand medium in the attrition bioreactor over the control when the reaction time was 8 hrs.

2. Changes in Crystallinity of Cellulose

The changes in crystallinity indices of the cellulose in two type of reactors are shown in Fig. 4a, 4b.

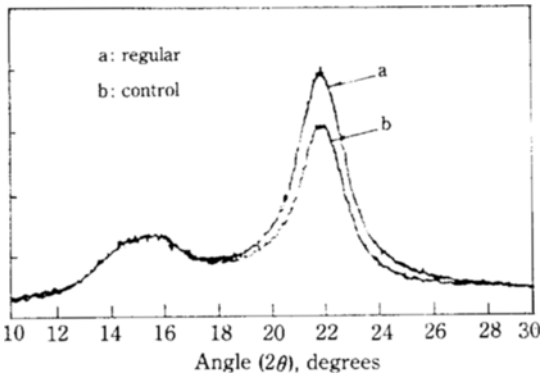

Fig. 4a. X-ray diffractograms of Solka Floc in a regular stirred reactor.

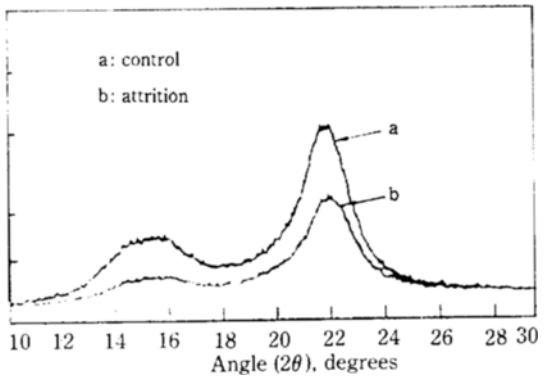

Fig. 4b. X-ray diffractograms of Solka Floc in an attrition bioreactor.

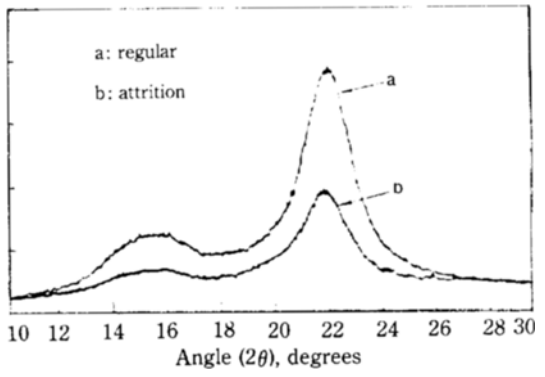

Fig. 4c. X-ray diffractograms of Solka Floc.

The index in a regular stirred reactor increased from 72.0 to 75.3 in 8 hrs indicating that the amorphous prtion was readily hydrolyzed during earlier reaction period. Whereas the decrease in an attrition bioreactor from 72.0 to 71.1 indicates the augmentation of amorphous fraction by the shearing and compressive force of the milling action.

Table 3. Parameters of kinetic model for each reactor.

Type	V'_m	K'_m	V'_m/K'_m
Regular stirred reactor	-0.067	-50	0.00134
Attrition bioreactor			
sand	-0.150	-51	0.00294
glass beads	-0.336	-51	0.00659

The contrast in the change of X-ray diffractograms in two type reactors, i.e., the increase in the regular stirred reactor and the decrease (considered to be substantial due to the fact the amorphous portion was readily hydrolyzed while being generated) in the attrition bioreactor, is quite noticable (Fig. 4c).

The simultaneous milling and hydrolysis in an attrition bioreactor, thus enhanced the rate of hydrolysis by continuously generating the accessible and readily reactive sites.

3. Kinetic and Parameter Estimation

The $\frac{\ln (S)_o/(S)}{(S)_o-(S)}$ vs. $\frac{t}{(S)_o-(S)}$ plots yielded straight lines (Fig. 5) indicating that the simplified model could serve as the hydrolysis kinetics.

In order to compare the performances of a attrition bioreactor and a regular stirred reactor, the kinetic parematers, V_m' and K_m' in the rate equations derived from proposed model, were obtained employing the plots (Table 4).

4. Power Consumption in an Attrition Bioreactor

The power consumption for an attrition bioreactor

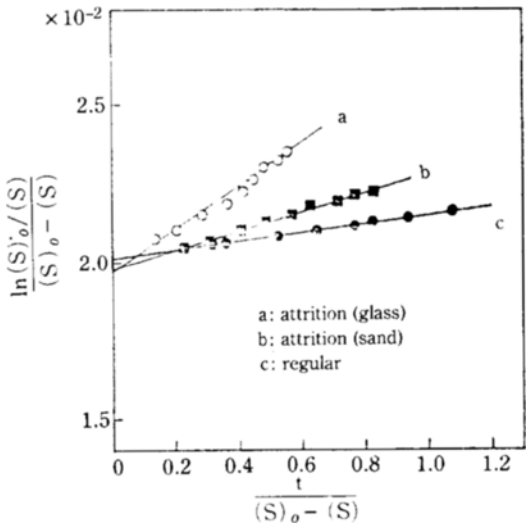

Fig. 5. Kinetic parameters calculation.

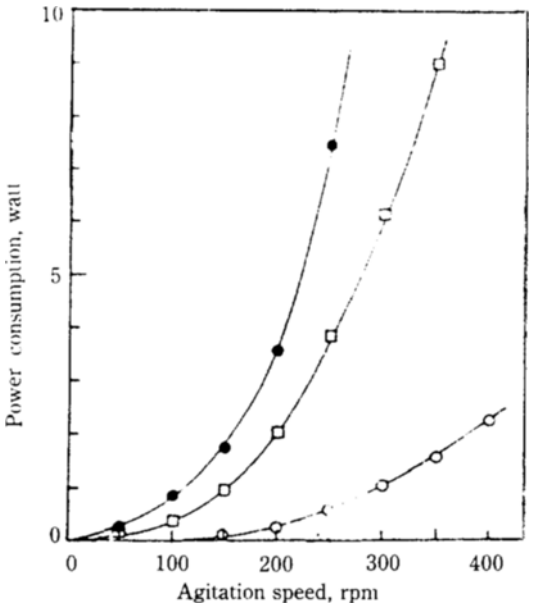

Fig. 6a. Power consumption versus agitation speed of attrition bioreactor.
(●) 0.3-cm-diameter glass beads: (□) sand, (○) without milling media.

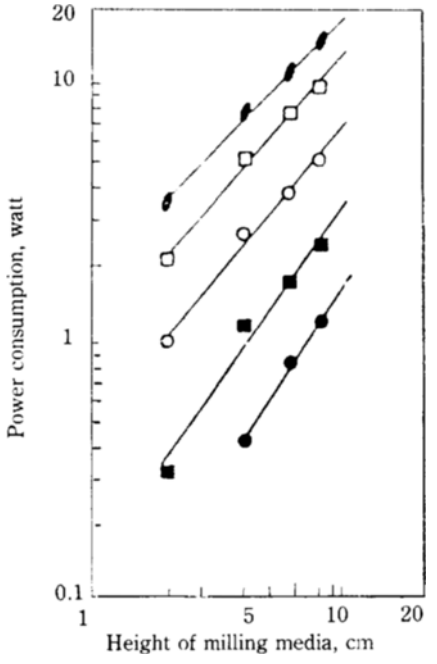

Fig. 6b. Power consumption versus the height of milling medium (0.3-cm-diameter glass beads) in an attrition bioreactor.
Agitation speed: (●) 100 rpm, (■)150 rpm, 200 rpm (○), 250 rpm (□), 300 rpm (◐).

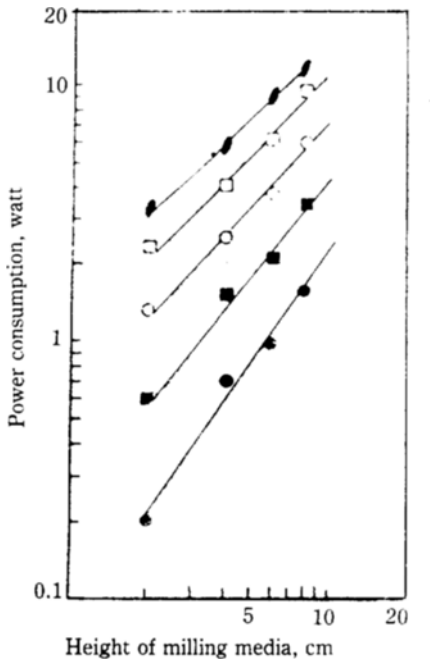

Fig. 6c. Power consumption versus the height of milling medium (Sand) in an attrition bioreactor.
Agitation speed: (●) 150 rpm, (■) 200 rpm, (○) 250 rpm, (□) 300 rpm, (◐) 350 rpm.

as compared to that in for a regular reactor has been substantially increase (Fig. 6a, 6b, 6c) as previously observed [49].

The power consumption P as a function of the height of the milling medium M could be expressed as;

$$P \propto M^n$$

where n has a value between 0.98 and 1.52 when glass beads of 0.3 cm diameter were used as a milling medium, and between 1.00 and 1.50 when sand was used as a milling medium. As the agitation speed was increased, however, n decreased.

CONCLUSIONS

1. The simultaneous milling and hydrolysis of cellulose in an attrition bioreactor was found effective, as compared to the hydrolysis of pretreated cellulose in a regular stirred reactor. The higher rates are believed to be attributed to:
   - the synergistic effects of the size reduction and the conversion of the crystalline to amorphous form of cellulose while the reaction proceeds in an attrition bioreactor, and
   - the enhancement of the mass transfer of the enzyme and the product in the reactor.

2. A simple kinetic model was found well representing the hydrolysis mechanism. The kinetic parameters in this model were estimated for both reactors, and those for the attrition bioreactor were found favorable as compared to those for regular stirred reactor.
3. The higher power consumption observed, in an attrition bioreactor, could possibly be compensated by the increased extent of hydrolysis and the substantial reduction of operating time.

NOMENCLATURE

CrI	:	Crystallinity index [dimensionless]
d	:	the distance from the axis to the point of the force measurement [m]
(E)	:	enzyme concentration [g/*l*]
$(E)_o$	:	total enzyme concentration [g/*l*]
F	:	the force required to prevent the rotation of a torque table [N]
I_{am}	:	intensity at 2θ=18° [-]
I_{002}	:	intensity at 2θ=22° [-]
k	:	rate constant [hr]
K_1, K_2	:	equilibrium constant
K_m'	:	kinetic parameter [g/*l*]
M	:	height of milling media [cm]
N	:	agitation speed [rpm]
P	:	Power consumption [W]
(P)	:	product (reducing sugar) concentration [g/*l*]
(S)	:	cellulose or substrate concentration [g/*l*]
$(S)_o$	:	initial cellulose concentration [g/*l*]
t	:	reaction time [hr]
v	:	reaction rate [g/*l*-hr]
V_m'	:	kinetic parameter [g/*l*-hr]

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