ADCHEM 2006

International Symposium on Advanced Control of Chemical Processes
Gramado, Brazil – April 2-5, 2006

Control of High-Solids Saccharification using a Model-Based Methodology for Fed-Batch Operation

David B. Hodge, M. Nazmul Karim, Daniel J. Schell, James D. McMillan

Abstract—This work utilizes both insights obtained from experimental work and kinetic modeling to develop an optimization strategy for high-solids cellulose saccharification, which presents a problem for the operation of stirred tank reactors (STRs) at insoluble solids levels greater than ~15%. In this work, a previously developed model for enyzmatic hydrolysis of cellulose was modified to consider the effects of feeding a stream of pretreated corn stover (PCS) solids and cellulase for a fed-batch process. By solving the set of model differential equations while controlling for a feed stream, it was possible to develop an open-loop control feeding profile that controls the insoluble solids at a constant or manageable level throughout the course of the reaction. This prevents any difficulties that would otherwise be encountered in mixing high-solids slurries. Experimental application of two fed-batch feeding policies in bench-scale STRs resulted in similar cellulose conversion profiles to batch shake flasks, with final cellulose conversions reaching ~80% for fed-batch STRs fed at the equivalent of 25% initial insoluble solids.

I. Introduction

A. Models for Enzymatic Hydrolysis

Process modeling is ultimately motivated by one of two objectives. The first objective is to demonstrate an understanding of a physical process by proposing a model structure that suitably fits the experimental data. Another goal is to develop an application-based model for process design, simulation, or control. An application-based model could be either mechanistic or empirical. Many kinetic models of both types have been developed for the enzymatic hydrolysis of cellulose or cellulosic biomass to glucose and cellobiose [1]-[7].

B. Reactor Considerations for High-Solids

Biomass conversion technologies for high solids operation are ultimately motivated by favorable economics. Positive economic advantages associated with a high-solids saccharification process over a conventional low solids process include: lower capital costs due to the reduced volume; lower operating costs due to less energy required for heating and cooling; lower downstream processing costs due to higher product concentrations; reduced disposal and treatment costs due to lower water usage [8].

High-solids slurries of pretreated lignocellulosic biomass are challenging to manage due to the increasingly high viscosities and non-Newtonian rheological properties [9]. This rheology results in increased problems with mixing (mass transfer) and temperature control (heat transfer). A variety of reactors have been used to deal with these problems during high-solids enzymatic hydrolysis. Examples include fermentation shake flasks [10]-[12], the paddle-impeller reactor of Tengborg et al. [13], a horizontally revolving reactor developed from a laboratory ball mill [8]. Currently, all of these laboratory-scale reactors require either a large reservoir of temperature controlled air (incubator) or water (water bath) to circumvent the problem of accurate temperature measurement and feedback control. Additionally, high power requirements for agitation may also be a limiting factor in many types of high-solids reactor.

Fed-batch processes combine the benefits of both batch and continuous processes to improve reaction conditions. A fed-batch methodology is commonly applied in fermentation for the purposes of minimizing effects of inhibitors, maximization of growth associated products, and induction of desired products with a feed stream [14]. Several approaches have been applied in the past for the fed-batch enzymatic saccharification of cellulose, although all of these were based on ad hoc approaches to feeding rather than a rigorous design methodology. These process-derived motivations fall into three general categories, the first of which is enzyme recycle. Pristavka et al. [15] used an approach similar to fed-batch for high-solids saccharification of steam-exploded willow. For this, a high-solids slurry was saccharified, the liquid phase removed, and more solids added to the solids fraction such that a large portion of enzyme adsorbed to the solids could be reused. Total sugar concentrations averaged 120 g/L including glucose and hemicellulosic sugars from pretreatment. Another enzyme recycle approach is to add only pretreated solids but no enzymes. As the cellulose is hydrolyzed, enzymes should be released to back into liquid such that

Manuscript submitted October 5, 2005. This work was supported in part by the U.S. Department of Energy Office of the Biomass Programs.

D.B. Hodge is with the National Bioenergy Center at the National Renewable Energy Laboratory (NREL), Golden, CO, 80401 (email: david_hodge@nrel.gov).

M.N. Karim is with the Department of Chemical Engineering, Texas Tech University, Lubbuck, TX USA (e-mail: naz.karim@coe.ttu.edu).

D.J. Schell is with the National Bioenergy Center at the National Renewable Energy Laboratory (NREL), Golden, CO, 80401 (email: dan_schell@nrel.gov).

J.D. McMillan is with the National Bioenergy Center at the National Renewable Energy Laboratory (NREL), Golden, CO, 80401 (email: jim_mcmillan@nrel.gov).

more cellulosic biomass could be added to reactor as these enzymes are released.

The second category uses fed-batch SSF [16]-[17] to mitigate the inhibitory effects of compounds in hydrolyzate liquors on the fermentative microorganism. Effective insoluble solids were also increased from 3% to 5% or to 8.4% for the two studies, respectively.

The third motivation for fed-batch saccharification is to utilize feeding to increase the equivalent insoluble solids level during saccharification to overcome rheological limitations in the reactor as in the work of Ballesteros *et al.* (2002) for SSF by *Kluyveromyces marxianus*. In this work, the insoluble solids were increased from 10% to 20%, which the reactor would have been incapable of handling initially, as well as reducing the effects of inhibitors in the hydrolyzate. Due to problems encountered in mixing wet oxidation pretreated corn stover at insoluble solids greater than 12%, Varga *et al.* [10] used a fed-batch hybrid hydrolysis and fermentation (HHF) approach to achieve 17% insoluble solids with high ethanol yields. Mohagheghi *et al*. [8] compared batch to fed-batch SSF and determined that the feeding of various combinations of pretreated biomass, media nutrients, and fresh inocula did not improve either ethanol yield or productivity in high-solids SSF for the ball-mill reactor system used.

C. Mathematical Approaches to Fed-Batch

In order to develop only a feeding policy without requiring online measurements of insoluble solids, an open-loop approach can be used. This doesn't rely on feed-back from process measurements, but rather is based only on the model predictions. Since open-loop control schemes rely solely on process models or speculation on the physical parameters governing the process, these control methods are only as good as the model.

Using a system of model differential equations, an exact analytical solution for a feeding profile can be obtained by developing and solving an optimal control problem. Optimal control fed-batch operation has been applied to cell cultures in bioreactors for a variety of objectives such as to maximize the production of cell mass, penicillin, amino acids, enzymes, and bioengineered products such as penicillin, ethanol, and recombinant proteins [14], [18], [19]. There are two general classes of methods to determine a numerical solution to this problem [20]. The first of these applies Pontryagin's maximum principle for determining the necessary conditions for the optimizer, which takes the form of a multi-point boundary problem for state and adjoint equations. The points at which the solution enters the boundary of the set of feasible conditions must be known *a priori* resulting in either singular or nonsingular control schemes. The second class of methods involves the discretization of the optimal control problem into a nonlinear programming (NLP) problem [20], which is solvable by optimization methods. For this method, as the number of time steps is increased, the calculated profile approaches the continuous optimal profile [18].

A general form for the continuous-time nonlinear system that is approximated by the model system in the optimal control problem can be described by the following system of equations as:

$$\dot{x} \equiv \frac{dx}{dt} = f(x(t),u(t)) \tag{1}$$

where $x(t)$ is the vector of state variables and $u(t)$ contains the controlled variables. A continuous form for the objective function for this algorithm seeks to minimize the difference between the desired process trajectory and the predicted process trajectory giving the minimization algorithm as:

$$\min_{u(t)} \Phi(x) = \int_0^{t_f} [r(t) - x_i(t)]^2 \, dt \tag{2}$$

subject to:

$$\begin{cases} \dot{x} = f(x(t),u(t),t) \\ t \in [0,t_f], x(0) = x_0 \end{cases}$$

where $r(t)$ is the desired output trajectory of the controlled variable and $x_i(t)$ is the controlled variable. The optimal path for $u(t)$ can be determined by reformulating the problem in terms of a Hamiltonian equation and developing a set of adjoint equations in order to solve the optimization problem under the conditions of the Pontryagin maximum principle [21]. Another approach is to discetize this continuous form of the objective function by subdividing the time region into K equal intervals as:

$$\mathbf{\Phi} = [\phi_1,\ldots,\phi_{k-1},\phi_k,\ldots,\phi_K] \tag{3}$$

and the discretized form of the minimization algorithm generated becomes a series of optimization tasks as:

$$\min_{u(k)} \phi_k = [r(k) - x_i(t_k)]^2 \tag{4}$$

subject to:

$$\begin{cases} \dot{x} = f(x(t),u(k),t) \\ t \in [t_{k-1},t_k], x(t_{k-1}) = x_{k-1} \end{cases}$$

Using this approach, only K values for the control variable $u(k)$ are required, and these are fixed over each time interval, resulting in a straightforward optimization problem.

II. MATERIALS AND METHODS

A. Shake Flask CSS

Corn stover was pretreated with dilute sulfuric acid in NREL's Sunds vertical pulp digester as the batch designated PS030312CS. The solids are composed of 53.2% cellulose by dry mass, and are washed with >10 times the mass of DI water as solids and centrifuged at 15800 x g. Washed solids are used to minimize any inhibition effects from background sugars entrained in the solids. The final glucose concentration in the wash water is verified to be less than 0.1 g/L.

The cellulase / β-glucosidase enzyme mixture used for this study was Spezyme CP (Genencor International, Lot # 301-00348-257). Total protein was assayed at 106.6 mg/mL (Bio-Rad assay) and the activity was determined to be 0.27 FPU/mg of protein using the NREL protocol LAP-006. For the modeling work, cellulase : β-glucosidase is assumed to

be 0.975:0.025. 1 M citrate buffer is prepared by adjusting the pH to 4.8 with NaOH, and is used as 5% of the total mass to yield an effective molality of 0.05 mol/kg. Enzyme, DI water, and citrate buffer are filter sterilized before use. PCS is added to the shake flasks and weighed before autoclaving. After autoclaving the shake flasks are weighed again so that any water lost could be made up. Water loss to evaporation in addition to reactor mass changes associated with feeding and sampling are tracked by weighing each flask before and after sampling and feeding.

B. Bench-Scale STR Fed-Batch Saccharification

Bench-scale fed-batch saccharification is performed using the same PCS substrates and enzymes as the shake flask reactors. Washed PCS is air-dried at ambient conditions to ~45% insoluble solids and the insoluble solids content is determined in triplicate using the 105ºC oven. New Brunswick Bioflo 3000 (7 L vessel size) reactors are autoclaved empty and allowed to cool in a sterile biological hood. The appropriate amounts of solids are measured and autoclaved and the amount of water lost is determined. Solids and other sterile components are next added to the reactors and allowed to mix and reach steady-state temperature before enzyme is added. Feeding policies are determined based on the solution to the optimal control problem as outlined in the **RESULTS AND DISCUSSION.** Feeding is performed by adding both autoclaved solids and filter-sterilized enzyme at discrete times. The solids and enzyme feeding policy is determined from the model kinetic equations in MATLAB to control the reactors using two feeding policies (F1 and F2). For F1, the actual insoluble solids level is increased from 12% to 15% as the reaction proceeds proportionally to the sugar produced. For F2 the insoluble solids are maintained at 15% insoluble solids. For both cases the feeding is continued until the reactors reach the equivalent of 25% initial insoluble solids. HPLC measurements for the reactor are taken both prior to and after feeding. Temperature is maintained at 45ºC by water circulating to a heating jacket from a temperature controlled water bath (maximum T = 52ºC). Enzyme loading is 40 mg protein / g cellulose (10.7 FPU / g cellulose), and enzyme is fed proportionally to the amount of PCS solids added. This can be considered as equivalent to the same amount of enzyme required for a given amount of PCS as would be used in an equivalent batch reaction. New Brunswick reactors are insulated and equipped with two marine impellers (d/D = 0.68) maintained at 400 rpm. Cellulose conversion for fed-batch reactors is determined based on the percentage of the final cumulative cellulose converted to glucose and cellobiose rather than only the initial or currently fed cellulose.

C. Sampling and Data Analysis

For the batch mode reactors, samples are taken approximately every 24 hours. Samples are removed with 5 mL pipettes (Falcon, Inc.) that had the tips broken off in order to accommodate the high level of insoluble solids. These are transferred to 2.5 mL microcentrifuge tubes and the pH is measured. The samples are next centrifuged at 10000 rpm for 5 minutes. The supernatant is decanted and diluted 1:5 and syringe filtered into HPLC vials for subsequent HPLC analysis. HPLC analysis is performed on all saccharification samples to determine the glucose, xylose, and cellobiose concentrations. An Agilent 1100 series HPLC equipped with a differential refractive index detector is used. This is equipped with a Bio-Rad HPX-87H organic acid column operating at 55^{o}C with a 0.01 N H_2SO_4 mobile phase at a flow rate of 0.6 mL/min. Component concentrations obtained from HPLC measurement are converted to g/kg concentrations based on the estimated insoluble solids level in the reactor. For fed-batch and CSS, cellulose conversions are based on cellulose converted to glucose and cellobiose, while for SSF conversions are based on cellulose converted to glucose, cellobiose, and ethanol.

III. RESULTS AND DISCUSSION

A. Development of Optimal Control Problem

The mechanistic model of Kadam *et al.* (2004) was used in this study. This model is based on the enzymatic hydrolysis of dilute acid pretreated corn stover (PCS) and considers the adsorption of β-glucosidase and that of CBH/EG enzymes separately while including an inhibition term for xylose. The kinetic model was implemented in MATLAB, and was modified to consider changes in volume for fed-batch operation, as well as introducing several new state variables. New state variables that need to be added include insoluble solids, enzyme concentration, and total reactor working mass. In addition to these new variables, all of the other differential equations needed modification to account for the rate changes associated with feeding and the change in reactor working mass for fed-batch operation. A new variable that needs to be introduced for systems where reactor working mass and/or concentrations is changing due to a feed stream is the dilution rate (D). This term can be defined as the feed flow rate / reactor volume, and can be considered the inverse of the reactor residence time.

All of the expressions for rate terms used in this model and the parameters within the rate terms can be in found in Kadam *et al.* [1]. It is important to note that enzyme inactivation is not considered in this model, and should be more significant for fed-batch operation when there is a large disparity between the ages of the enzymes and fractional utilization of substrates in the reactor.

B. Fed-Batch Saccharification Model Simulation

One barrier to developing a high-solids saccharification process in stirred tank reactors (STRs) is rheological limitations manifested as problems in mass and heat transfer. In our previous unpublished work, we demonstrated that the saccharification reaction is scalable from the shake flask to the bench scale STRs at insoluble solids levels of 15% or less using PCS solids. Above this level, STRs encounter problems with mixing and temperature control, which is primarily correlated to the amount of free water in the slurry. Based on this finding, the fed-batch approach control objective is to optimize reactor conditions (insoluble solids levels) to facilitate sufficient

mixing and temperature control. A large number of variables exist that can be altered to achieve this objective., although during the process of feeding, the feed rate is the only variable that is free to be changed.

Using the kinetic model, a feeding policy can be developed based on controlling the insoluble solids below a defined critical value during the saccharification reaction. This is possible by feeding a stream of PCS solids and cellulase at a rate that approximately matches the rate at which cellulose in the reactor is depolymerized and solubilized. Using the model equations, the rate of change for insoluble solids can be determined based on a set of initial operating conditions and a feed rate.

To determine a solution for the feeding policy, the set of equations must be integrated over time with the value for feed rate free in order to satisfy the control objectives of insoluble solids. For this, the insoluble solids rate equation can be either set to 0 as:

$$\frac{dS_I}{dt} = D \cdot \left(S_{IF} - S_I\right) - r_1 - r_2 = 0 \qquad (5)$$

or set the insoluble solids level (S_I) to a specified trajectory $(S_{I,SP}(t))$ as a function of the sugar level:

$$S_{I,SP}(t) = f(G, CB, S_I) \qquad (6)$$

since sugar level can be correlated to the substrate conversion and consequently to slurry rheology. To achieve the control objectives of both (5) and (6), the objective function of the optimization problem of (4) becomes:

$$\min_{F(k)} \phi_k = \left[S_I(k \mid k-1) - S_{I,SP}(k)\right]^2 \qquad (7)$$

while the constraints become the system of model equations.

Using this optimal control algorithm, fed-batch feeding policies can be developed, and a related set of feeding curves over various process objectives and initial conditions can be generated to determine the theoretical physical limitations and potential for using this type of fed-batch approach. Ultimately, physical limitations exist that limit the equivalent insoluble solids level to which a reactor under given initial conditions can be fed. The two important variables for this are reactor volume (V, or in dimensionless form as V/V_0) and insoluble solids level in the feed (x_{sf}). As the reactor is fed, the level of equivalent initial insoluble solids increases. The equivalent initial insoluble solids is defined as the level of initial insoluble solids that would be present if all of the solids were added initially and the reactor was operated in batch mode.

Simulations were performed using an enzyme loading of 40 mg protein / g cellulose (10.7 FPU / g cellulose for Spezyme CP). These simulation results in Fig. 1 show that higher solids levels in the feed result in both smaller reactor volumes and shorter residence times to achieve the same feeding objectives. The reason for this is that feeding a lower solids stream adds more water and is effectively diluting the product in the reactor.

Another option for a feeding policy is to use a non-constant insoluble solids level for the control trajectory. This could be based on our previous findings that cellulose removal affects the mixing behavior in STRs by allowing higher levels of glucan-depleted solids to be mixed effectively relative to the same level of glucan-rich solids. For this scheme, insoluble solids are initially maintained at 12% and are allowed to increase to about 15% during feeding as the equivalent cumulative solids approach 25%. The rate of increase in the insoluble solids set point ($S_{I,SP}$) is proportional to the amount of glucose produced. Estimating the final sugar concentration when the feeding was complete resulted in the development of the following relation:

$$S_{I,SP} = S_{I0} + (G + CB) / 4800 \qquad (8)$$

This approach (F1) was one of the two feeding policies selected for experimental validation of the modeling results. The other (F2) was based on controlling the insoluble solids at 15%, which was shown in simulation to have relatively rapid performance for 45% insoluble solids in the feed. Fig. 2 shows the two feeding policies (F1 and F2) chosen for experimental testing in the bench-scale STRs (New Brunswick Bioflo 3000). As seen in Fig. 2, by maintaining the insoluble solids level at 15% as in F2, solids can be fed up to 25% equivalent within a reasonable timeframe.

C. Experimental Validation of Theoretical Results

The two feeding policies chosen in Fig. 2 were performed experimentally in the bench-scale STRs. Batch saccharification shake flasks are also performed as controls in triplicate at 25% initial insoluble solids under similar conditions to determine maximum saccharification potential. The shake flask data is meant to be applied as a "best case" condition for these reaction conditions since these can be assumed to have demonstrated uniform temperature throughout the saccharification due to the reaction being performed in an air-temperature controlled shaking incubator. However, concentration gradients and rate limitations due to problems with diffusion of sugars and enzymes cannot be ruled out in shake flasks.

Fig. 3 shows that the glucose and cellobiose time profiles for both F1 and F2 meet the profiles for the batch shake flask reactors at approximately the end of the feeding, indicating that this fed-batch feeding policy will not require significantly higher residence times than a batch reaction. The "sawtooth" pattern for the fed-batch reactors is due to the change in volume and subsequent dilution of reactor components after addition of the feed. The reactor using feeding policy F1 is slower due to the slower feeding rate, while F2 shows more rapid rates as was predicted in simulation. From this work it was concluded that the fed-batch approach could allow STRs to saccharify the equivalent of 25% insoluble solids without significantly different residence times than shake flask reactors operating in batch mode at 25% solids. Interestingly, the much faster rate in batch predicted by the kinetic model was found not be consistent with experimental data, which show approximately equivalent rates for both batch and fed-batch reactors.

The implications of this work are that fed-batch saccharification enables high solids levels to be hydrolyzed at high conversions without the drawbacks associated with mixing, temperature, and pH control that would limit level

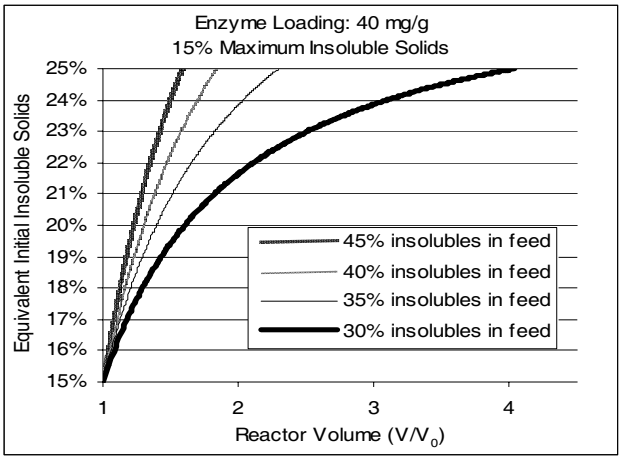

Figure 1: Effect of insoluble solids in the feed on equivalent initial insoluble solids; Reactors are fed to maintain insoluble solids at 15%.

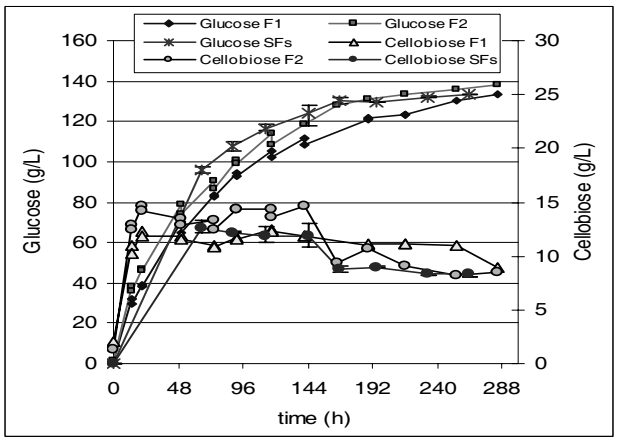

Figure 3: Glucose and cellobiose for the bench-scale STRs using the fed-batch feeding policies F1 and F2 and compared to batch CSS in shake flasks.

of solids used in stirred tank reactors. Final glucose concentrations >130 g/L and estimated cellulose conversions of 80% are achieved in both reactors, which is very promising economically. These high sugar levels are significant in that previous high-solids enzymatic saccharification work [8], [10] assumes that sugar inhibition will be a severely limiting factor, necessitating an SSF approach. Not only does this work show that current enzyme preparations are capable of robust activity at these high glucose levels, but that fed-batch can be used to perform these reactions at reasonable insoluble solids levels in STRs.

IV. CONCLUSIONS

The results of this work were significant in that it was demonstrated that through fed-batch, a reactor capable of handling approximately 15% insoluble solids, was able to achieve high cellulose conversions at 25% equivalent initial insoluble solids. It was also demonstrated that a kinetic model could be applied in an offline optimal control scheme

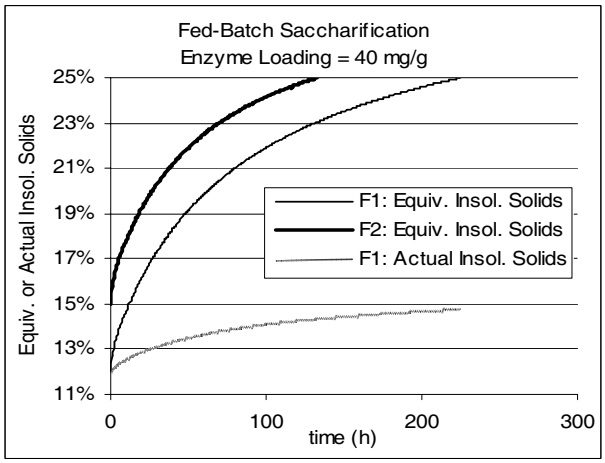

Figure 2: Equivalent total solids based on calculated feeding profiles for fed-batch operation at an enzyme loading of 40 mg/g and 45% insoluble solids in the feed stream.

to determine a fed-batch feeding policy capable of facilitating mixing and temperature control within the reactor, while achieving these high equivalent insoluble solids levels. When compared to batch saccharification results obtained in shake flask reactors, the overall rates in fed-batch reactors were slower due to the shorter cumulative residence times, although the final conversion results were similar. This is significant since slurry handling by process equipment (pumping, mixing, temperature, and pH control) is greatly simplified by operating at a lower solids level while gaining the economic advantages of a high-solids process.

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