Proceedings of the Seventeenth Annual Biochemical Engineering Symposium

Peter J. Reilly
Iowa State University, reilly@iastate.edu

Follow this and additional works at: http://lib.dr.iastate.edu/bce_proceedings

Part of the Biochemical and Biomolecular Engineering Commons

Recommended Citation
http://lib.dr.iastate.edu/bce_proceedings/19
This is the seventeenth of a series of symposia devoted to talks by students about their biochemical engineering research. The first, third, fifth, ninth, twelfth, and sixteenth were at Kansas State University, the second and fourth were at the University of Nebraska-Lincoln, the sixth was in Kansas City and was hosted by Iowa State University, the seventh, tenth, thirteenth, and seventeenth were at Iowa State University, the eighth and fourteenth were at the University of Missouri-Columbia, and the eleventh and fifteenth were at Colorado State University. Next year's symposium will be at the University of Colorado. Symposium proceedings are edited by faculty of the host institution. Because final publication usually takes place elsewhere, papers here are brief, and often cover work in progress.

Attending this meeting at Iowa State University were the following:

University of Colorado: Brian Batt, William Bentley, Max Bynum, Dhinakar Kompala, Charles Parnham, and Harold van Deinse.


University of Iowa: Allan Bream, In-Chul Kong, and Randall Yoshisato.

Iowa State University: Kathleen Clark, Charles Glatz, Diane Holm, Merri Johnson, Leah Patterson, Sabine Pestlin, Peter Reilly, and Michael Sierks.

Kansas State University: Larry Erickson, Rodney Fox, Ayush Gupta, Purboyo Guritno, Travis Jones, K. H. Lee, Pat McDonald, Rizwan Mithani, Greg Sinton, Lourdes Taladriz, Brad Wright, and K. C. Zen.

University of Missouri-Columbia: Rakesh Bajpai, Kim Joong, and Peter Sohn.

Washington University: Ales Prokop.

Inquiries about the research described in these papers should be directed to the biochemical engineering faculty at the various institutions: Robert Davis and Dhinakar Kompala at the University of Colorado, Bruce Dale, Naz Karim, James Linden, and Vincent Murphy at Colorado State University, Gregory Carmichael and Randall Yoshisato at the University of Iowa, Charles Glatz and Peter Reilly at Iowa State University, Larry Erickson and L. T. Fan at Kansas State University, and Rakesh Bajpai at the University of Missouri-Columbia.
<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Effect of Polymer Dosage Conditions on the Properties of Protein-Polyelectrolyte Precipitates, K. M. Clark and C. E. Glatz, Iowa State University</td>
<td>1</td>
</tr>
<tr>
<td>An Immobilized Enzyme Reactor/Separator for the Hydrolysis of Casein by Subtilisin Carlsberg, A. J. Bream, R. A. Yoshisato, and G. R. Carmichael, University of Iowa</td>
<td>11</td>
</tr>
<tr>
<td>Cell Density Measurements in Hollow Fiber Bioreactors, Thomas Blute, Colorado State University</td>
<td>23</td>
</tr>
<tr>
<td>The Hydrodynamics in an Air-Lift Reactor, Peter Sohn, George W. Preckshot, and Rakesh K. Bajpai, University of Missouri-Columbia</td>
<td>27</td>
</tr>
<tr>
<td>Local Liquid Velocity Measurements in a Split Cylinder Airlift Column, G. Travis Jones, Kansas State University</td>
<td>37</td>
</tr>
<tr>
<td>The Effect of 2,4-D Concentration on the Growth of \textit{Streptanthus tortuosis} Cells in Shake Flask and Air-Lift Fermenter Culture, I. C. Kong, R. D. Sjolund, and R. A. Yoshisato, University of Iowa</td>
<td>59</td>
</tr>
<tr>
<td>Protein Engineering of \textit{Aspergillus niger} Glucoamylase, Michael R. Sierks, Iowa State University</td>
<td>71</td>
</tr>
<tr>
<td>Structured Kinetic Modeling of Hybridoma Growth and Monoclonal Antibody Production in Suspension Cultures, Brian C. Batt and Dhinakar S. Kompala, University of Colorado</td>
<td>77</td>
</tr>
<tr>
<td>Modelling and Control of a \textit{Zymomonas mobilis} Fermentation, John F. Kramer, M. N. Karim, and J. Linden, Colorado State University</td>
<td>87</td>
</tr>
<tr>
<td>Modeling of \textit{Brettanomyces clausenii} Fermentation on Mixtures of Glucose and Cellobiose, Max T. Bynum and Dhinakar S. Kompala, University of Colorado, Karel Grohmann and Charles E. Wyman, Solar Energy Research Institute</td>
<td>97</td>
</tr>
<tr>
<td>Master Equation Modeling and Monte Carlo Simulation of Predator-Prey Interactions, R. O. Fox, Y. W. Huang, and L. T. Fan, Kansas State University</td>
<td>107</td>
</tr>
<tr>
<td>Kinetics and Equilibria of Condensation Reactions Between Two Different Monosaccharides Catalyzed by \textit{Aspergillus niger} Glucoamylase, Sabine Pestlin, Iowa State University</td>
<td>117</td>
</tr>
</tbody>
</table>
Biodegradation of Metalworking Fluids, S. M. Lee, Ayush Gupta, L. E. Erickson, and L. T. Fan, Kansas State University

Redox Potential, Toxicity and Oscillations in Solvent Fermentations, Kim Joong, Rakesh Bajpai, and Eugene L. Iannotti, University of Missouri-Columbia

Using Structured Kinetic Models for Analyzing Instability in Recombinant Bacterial Cultures, William E. Bentley and Dhinakar S. Kompala, University of Colorado
The Effect of Polymer Dosage Conditions on the Properties of Protein-Polyelectrolyte Precipitates

K. M. Clark and C. E. Glatz
Dept. of Chemical Engineering, Iowa State University, Ames, IA

Abstract
The properties of aggregates formed by the precipitation of egg white proteins by carboxymethyl cellulose (CMC) were examined. Both the final level and the number of incremental additions of the polyelectrolyte were varied. The protein and lysozyme recoveries, particle zeta potential and protein composition of the precipitate were found to vary with only the final CMC level. Particle size and size distributions were dependent on both the polymer dosage and manner of addition. Overdosed systems, or systems in which the CMC dosage exceeded that required for optimal total protein removal, produced the smallest particles with size increasing as the number of incremental polymer additions increased. Optimally dosed systems produced the largest particles.

Introduction
The recovery of a single protein fraction from a fermentation broth is a primary focus of many downstream processing operations. This task is complicated by the low concentration of the protein in the broth, and the difficulty in separating it from other biomolecules in solution. Furthermore, severe recovery conditions must be avoided since it is often necessary to retain the activity of the protein.

Precipitation is an attractive scheme for the recovery of proteins from dilute solution since it serves as both purification and concentration steps, reducing the volume of material that must be processed. Isoelectric precipitation [1], salting-out [2], and precipitation by the addition of non-ionic polymers [1] have been successfully employed in protein recovery processes, but the fractionation potential is limited.

Protein precipitation by polyelectrolytes offers several advantages. Low polyelectrolyte dosages are effective, and several authors [3-10] have reported high protein removal levels. Although the cost of the polyelectrolyte may be high, the feasibility of polyelectrolyte reclamation and recycling has been demonstrated [4, 8]. Fractional precipitation may be accomplished through careful selection of precipitant and precipitation conditions [3, 8, 11, 12]. Sternberg and Hersherberger [8] noted that up to 92.3% of enzyme activity may be retained following precipitation. However Gekko and Noguchi [13] have indicated that protein may be more susceptible to thermal denaturation following the addition of ionic polysaccharides.

Many factors affect protein removal and fractionation efficiencies by polyelectrolyte precipitation. Increased ionic strength leads to lower protein removal efficiencies [6, 7, 9]. The success of fractionation is highly dependent on the process pH [3, 4, 5, 8, 9]. The charge density and the molecular weight of the polyelectrolyte will affect protein recovery levels [5-8]. Several investigators [5-9] have indicated the importance of careful control of the polyelectrolyte dosage. Typically, protein removal increases with dosage to an optimum, then decreases with further polymer addition. In non-protein systems, the importance of the manner of polymer addition has also been noted. Walles [14] found that flocculation of clay suspensions by poly (sodium styrene sulfonate) improved when the number of polymer additions was increased.
The formation of a protein-polymer aggregate is the result of a two-stage process: formation of a solid phase or complex and aggregation of this complex into flocs. The solubility of the protein-polymer complex is expected to depend on three factors: excluded volume, salting out and protein/polymer interactions resulting from electrostatic, hydrophobic or hydrogen bonding [13]. Electrostatic forces are expected to be dominant in the present work. Floc formation may proceed through a number of mechanisms, including patching [15-18], bridging [19-21], or charge neutralization [22, 23].

In this work we have investigated the effect of polyelectrolyte dosage and addition on the formation of protein-polymer precipitates using egg white protein and carboxymethyl cellulose. Protein solubility and lysozyme activity were determined and several properties of the protein-polyelectrolyte aggregates were evaluated: zeta potential, protein composition and fractionation, particle size and size distribution. Through the particle size and particle size distribution behavior with polymer dosage and addition, the role of the polyelectrolyte in floc formation was studied. Characterization of the particles resulting from staged addition of CMC to egg white proteins allowed insight into the mechanism of aggregate formation.

Materials and Methods

Materials
The carboxymethyl cellulose used was a commercial sample from Hercules Inc. (Wilmington, DE) of average molecular weight 250,000 daltons and degree of substitution of 1.2. The dried egg white solids, type P-110, were obtained from Henningsen Foods, Inc. (White Plains, NY). Micrococcus lysodeikticus (ATCC 4698) was from Sigma Chemicals (St. Louis, MO). All other chemicals were reagent grade.

Egg White Preparation
Fifteen grams of egg white solids were suspended in 100 ml deionized water. The pH was adjusted to 4.5 with 5 M acetic acid. Upon solubilization the mixture was diluted 1:1 with pH 4.5, I = 0.02 M acetate buffer. The solution was centrifuged at 20,000 g for 45 minutes to remove insolubles. Immediately prior to precipitation, the egg white was filtered through a 0.45 μm capsule filter. Final solution conditions were pH 4.5, I = 0.07 M and 55 mg/ml protein. Earlier work had indicated that at pH 4.5 lysozyme removal levels are high, little ovalbumin, which comprises 54% of egg white protein is precipitated, and sufficient particles are produced for particle size distribution analysis. The selective precipitation of lysozyme (pI 10.7) [24] by CMC is expected since the primary basis for fractionation appears to be the pI or the net charge of the protein species.

Precipitation
The precipitation was carried out at room temperature using a 400 ml baffled batch reactor. Agitation was provided by a 45° pitched, 6-bladed turbine at 200 rpm. Total mixing time was 30 minutes, with a mean shear rate of 45 sec⁻¹. CMC was prepared as a 0.35% solution in pH 4.5, I = 0.07 M acetate buffer and injected into the reactor from a syringe over a period of 5-15 seconds. On the basis of earlier small-scale solubility studies, three dosage levels were investigated: optimum, corresponding to maximum total protein removal, underdosed, and overdosed. The CMC/total protein mass ratios were 0.04, 0.02 and 0.10, respectively, for these conditions. To examine the effect of polymer addition, CMC was added in increments to reach the final
dosage level. One, two and three-stage polymer additions were investigated.
For one-stage additions, the entire polymer aliquot required to reach each of
the three dosage levels was added at the start of the precipitation. Mixing
time was 30 minutes. Two, 2-stage additions were investigated. For the first
stage, sufficient CMC was added to reach the underdosed level, followed by 15
minutes of mixing. The second polymer aliquot was then added, to attain
either optimum or overdosed levels, again followed by 15 minutes of mixing.
The three-stage polymer addition was performed by adding three polymer
aliquots, to reach the underdosed, optimum and overdosed levels, successively.
Each polymer addition was followed by 10 minutes of mixing.

Protein and Lysozyme Assays

Total protein determinations were accomplished using a modified biuret
assay [25]. Initial protein concentration was that of the egg white solution.
Final protein concentrations were determined on the supernatant of a slurry
aliquot centrifuged at 50 000 g for 45 minutes. Lysozyme concentrations were
found by following the rate of lysis of a suspension of M. lysodeikticus cells
[26].

Small-Scale Solubility Studies

Initial, small-scale, solubility studies were conducted in order to
select dosage conditions for the 400 ml polymer addition and dosage studies.
Egg white solution, prepared as described earlier, and pH 4.5, I = 0.07 M
acetate buffer were pipetted into a centrifuge tube. CMC was added as a 0.35%
solution to dosage levels (CMC/total protein mass ratios) of 0 to 0.2. Mixing
was by inversion. Total solution volume was 25 ml. The slurries were
centrifuged at 50 000 g for 45 minutes. Supernatant protein and lysozyme
concentrations were determined by methods described earlier.

Particle Size Distributions

Particle size distributions were obtained using a Model TA II Coulter
Counter (Coulter Electronic, Hialeah, FL), equipped with a 70 µm aperture
tube and calibrated with 3.14 µm latex particles. A 500-fold dilution of the
precipitated slurry into pH 4.5, I = 0.07 M acetate buffer was performed to
prevent further aggregation and to insure that the concentration index was
under 5%. Three counts were determined for each diluted sample.

Zeta Potential Measurements

Zeta potentials were determined using a Zeta-Meter System 3.0 (Zeta-
Meter Inc., Long Island City, NY). A 5 ml slurry aliquot was diluted to 50 ml
with pH 4.5, I = 0.07 M acetate buffer. The zeta potential reported is the
average of 20-25 measurements. The applied voltage was 75 V DC for all
determinations except the underdosed trial for which 100 V DC was used.
Measurements were performed as rapidly as possible to minimize errors due to
thermal convection.

SDS-PAGE

SDS-PAGE was performed on a 10% gel following the procedure of Laemmli
[27]. Gels were stained and fixed overnight in a 5:2:12 isopropanol-acetic
acid-water 0.06% Coomassie blue solution. The gel was destained in a 10% acetic acid solution and photographed.
Complex Composition
The protein content of the precipitate was determined after collecting the precipitate by centrifuging at 5000 g for 20 minutes. The precipitate was washed in a pH 4.5, I = 0.07 M acetate buffer, recentrifuged and freeze-dried. A portion of the precipitate was then weighed and dissolved in 40 ml of deionized water adjusted to pH 12 with 2 M NaOH. The protein concentration was determined by biuret assay.

Results and Discussion

Solubility
The results of the small-scale batch solubility studies are shown in Figure 1. Although the maximum total protein removal is only 36%, lysozyme is nearly quantitatively precipitated, with an optimum removal level of 96%. The maximum precipitation level occurs at a CMC/total protein mass ratio near 0.04 for both lysozyme and total protein removal, and addition of polyelectrolyte beyond this level results in lower removals of both. This resolubilization appears to be characteristic of protein precipitation by highly ionized polyelectrolytes [5-9]. Hidalgo and Hansen [5] have explained this as the result of the redistribution of protein on available CMC and the subsequent breakdown, but not dissociation of the complex.

This figure also shows that the better mixing condition in the incremental addition studies improves the selectivity for lysozyme removal. The effect of dosage remains the same and the number of polymer additions had no effect on recovery. The method of contacting has been observed to be important for other protein [1, 28] and polyelectrolyte [29] precipitations.

After redissolving these precipitates, no loss of lysozyme activity was observed.

Zeta Potential
The zeta potentials of the aggregates are shown in Figure 2. As polymer dosage is increased, the zeta potential of the aggregate becomes more negative, indicating a higher proportion of anionic groups or CMC in the complex. At a constant final dosage level, incremental polymer addition had no effect on the zeta potential.

Complex Composition
The protein content of the precipitate is shown in Figure 2. CMC and salt are the other components. Protein content decreases with increasing CMC dosage and is unaffected by number of CMC additions. This is consistent with both the solubility and the zeta potential behavior with dosage. Although total protein removal levels were the same (23%) for both under- and overdosed trials, the protein content of the precipitate decreases from 95% for the underdosed to 71% for the overdosed conditions. This, along with the change in zeta potential from +10.4 mV to -22.6 mV indicates the incorporation of more polyelectrolyte in the complex as polymer dosage is increased.

It is likely that additional polymer is distributed throughout the solid, rather than merely on the surfaces of the primary particles. On the basis of a 400 ml suspension of 10 μm particles composed of a loose random packing [30] of primary particles 0.6 μm in diameter, and a specific polyelectrolyte adsorption of 1 x 10⁻⁴ g polyelectrolyte adsorbed/m² surface area [31], only 1.4 x 10⁻⁴ g of polyelectrolyte is required to saturate the available surface area in the floc. This is far less than the 0.09 to 0.35 g of CMC added.
SDS-PAGE

SDS-PAGE analysis of the egg white solution, supernatants and resolubilized precipitates is shown in Figure 3. Fractionation of egg white by CMC precipitation is evident and confirms the electrostatic basis for selectivity. Proteins with high pI's such as lysozyme and conalbumin, with pI's of 10.7 and 6.6 [24], respectively, are precipitated. The only major protein fraction not largely precipitated is ovalbumin with a pI of 4.6 [24], only slightly above the precipitation pH of 4.5.

Particle Size

The number concentration (# particles/volume solution) data obtained from the Coulter Counter analyses were converted to number density (# particles/volume • channel width). In order to characterize the particle size distribution, a characteristic diameter, the D₅₀, was chosen. The D₅₀ is the particle diameter at which 50% of the total particle volume is in larger particles.

A plot of D₅₀ as a function of polymer dosage and addition is shown in Figure 4. Several points should be noted. First, Duncan's multiple-range test indicates that all six treatments produced statistically different D₅₀'s, indicating a significant effect of both polymer dosage and manner of addition on particle size. The largest particles are obtained at an optimum dosage level, regardless of manner of polymer addition. This corresponds to the aggregates with the lowest zeta potential. The smallest particles result from overdosed conditions, again, regardless of number of polymer additions. At overdosed conditions, particle size clearly increases with incremental addition of polyelectrolyte.

A schematic of the proposed aggregation process is shown in Figure 5. Three levels of aggregation may occur in floc formation. Fractionation occurs during electrostatic association of protein and polyelectrolyte. These insoluble complexes aggregate to form primary particles. Finally, the aggregation of the primary particles into floes may result from a charge neutralization, patching or bridging mechanism.

The increase in particle size of overdosed aggregates with incremental addition of polymer at a constant final dosage level (Fig. 6), may be interpreted in terms of the bridging theory. Incremental addition results in the presence of preexisting particles on which polyelectrolyte increments after the first may adsorb. The surface adsorption would provide a chance for bridging to improve the otherwise small extent of floc formation at these conditions.

Conclusions

The effects of CMC dosage level and addition on the precipitation of egg white proteins at pH 4.5 and I = 0.07 M are summarized below:

1. At a CMC/protein dosage level of 0.04, removal levels of 28% and 96% for total protein and lysozyme, respectively, were obtained.
2. No lysozyme activity was lost as a result of the precipitation process.
3. At a given final dosage level, incremental polymer addition had no effect on lysozyme or total protein solubility, fractionation, aggregate zeta potential or the protein content of the precipitated complex.
4. Both the zeta potential and the protein content of the precipitate decreased with increased polyelectrolyte dosage.
5. Particle size was affected by both the polymer addition and dosage level:
  a. Largest particles, with a $D_{50}$ of 11.4 $\mu$m, were obtained by one-stage polyelectrolyte addition to an optimal dosage level, while one-stage polymer addition to an overdosed condition produced the smallest particles, with a $D_{50}$ of 3.34 $\mu$m.
  b. For overdosed treatments, the increase in particle size with incremental polyelectrolyte addition is attributed to the enhanced bridging opportunities available to newly added polyelectrolyte.
  c. One-stage addition to an optimum dosage level produced larger particles than two-stage addition to the same final dosage level.

Acknowledgements
This work was supported by the Engineering Research Institute of Iowa State University through National Science Foundation Grant Nos. CPE-8120568 and ECE-8514865. The CMC was a gift from Hercules Inc.

Literature Cited


Figure Legends

Figure 1. Total protein and lysozyme removals from egg white by CMC precipitation. Closed symbols indicate total protein removals; open symbols indicate lysozyme removal. □, △ indicate results of small scale solubility studies. ◆, ◆ indicate one stage polymer addition. ▲, ▲ indicate two stage polymer addition. ▼, ▼ indicate three stage polymer addition. Standard errors of treatment means: 0.74% and 0.48% for small scale total protein and lysozyme removals, respectively; 1.2% and 4.1% for total protein and lysozyme removals for staged polymer addition.

Figure 2. Zeta potential and protein content of protein-CMC precipitates as a function of polymer dosage.
□ One-stage polymer addition; △ Two-stage polymer addition; ◆ Three-stage polymer addition
Standard errors of treatment means: 0.18 mV for zeta potential, 5.1% for protein content.

Figure 3. SDS-PAGE analysis of egg white, supernatants and resolubilized precipitates of CMC-protein complexes formed at pH 4.5 and I=0.07 M. EWS: Egg white solution; SUP: Supernatant; PPT: Precipitate. −, 0 and + indicate underdosed, optimally dosed and overdosed treatments respectively. 1, 2 and 3 indicate the number of incremental additions of polymer.

Figure 4. Zeta potential and diameter of protein-CMC aggregates as a function of polymer dosage.
□ One-stage polymer addition; △ Two-stage polymer addition; ◆ Three-stage polymer addition
Standard errors of treatment means: 0.18 mV for zeta potential and 0.16 μm for $D_{50}$.

Figure 5. Schematic of a proposed aggregation process for oppositely charged protein and polyelectrolyte, resulting in the formation of flocs.
Figure 1.

Figure 2.

Figure 3.
Figure 4.

Figure 5.
AN IMMOBILIZED ENZYME REACTOR/SEPARATOR
FOR THE HYDROLYSIS OF CASEIN
BY SUBTILISIN CARLSBERG

A.J. BREAM, R.A. YOSHISATO, AND G.R. CARMICHAEL

DEPARTMENT OF CHEMICAL AND MATERIALS ENGINEERING,
THE UNIVERSITY OF IOWA
IOWA CITY, IOWA 52242

ABSTRACT

Subtilisin Carlsberg was immobilized by glutaraldehyde crosslinking within the spongy layer of a hollow fiber ultrafiltration membrane cartridge for continuous casein hydrolysis. The free enzyme kinetic constants, $K_m$, $V_{max}$, $k_1$ (second order enzyme deactivation constant), and $K_p$ (competitive product inhibition constant) were measured at pH 8.5 and 60°C. A model based on an integrated form of the Michaelis-Menten equation with second order enzyme deactivation and competitive product inhibition terms agreed well with experimental data obtained from the free enzyme, batch hydrolysis of casein. The stability and hydrolysis rate of the free enzyme is compared to that of the immobilized enzyme.

INTRODUCTION

Enzymatically hydrolyzed proteins can be used for nutritional enrichment of soups, fruit juices, and soft drinks (1,2). Also, protein hydrolysates can be used as a protein source for those that have problems digesting whole protein (3). Enzyme hydrolysis, unlike alkaline or acid hydrolysis, retains the full nutritional value of the hydrolysates (1). The products of hydrolysis, peptides, are soluble at low pH and temperature whereas whole protein is not.

Batch, free enzyme hydrolysis of proteins suffers from several drawbacks. Most notable is the inability to reuse the often expensive proteolytic enzyme. The hydrolysis of many proteins is slowed by product inhibition (1, 4-7). Enzymes, particularly proteases, are subject to deactivation especially at the higher temperatures favorable for hydrolysis. Proteolytic enzymes can be deactivated by heat denaturation or auto-degradation. Enzyme immobilization enables the reuse of enzymes and stabilizes the enzymes against auto and thermal degradation (8). Operation of an immobilized enzyme reactor in a plug flow reactor mode can lessen the effects of product inhibition (9).

Boudrant and Cheftel have observed that Subtilisin Carlsberg is very active towards casein and is inhibited by casein hydrolysates (1). In another study, enzyme deactivation was reported to be significant for free Subtilisin Carlsberg in solution at 60°C (4). This study used a hollow fiber ultrafiltration membrane cartridge for the immobilization of a proteolytic enzyme, Subtilisin Carlsberg. The enzyme was immobilized within the spongy layer of a hollow fiber by crosslinking the enzyme with glutaraldehyde. Glutaraldehyde crosslinking has been used successfully with Subtilisin Carlsberg (10-13). The substrate, casein, enters the shell side of a hollow fiber cartridge under pressure. Casein (monomer molecular weight of 25,000 daltons, but tetramers and larger aggregates can form (14)) is retained by the membrane (MWCO = 10,000) while it is hydrolyzed within the spongy layer. As casein is hydrolyzed, the inhibiting, small peptides pass through the membrane into the hollow fiber lumen (Figure 1).

The objectives of this study were: (1) Determine the optimum pH and temperature of the free enzyme hydrolysis. (2) Determine the enzyme deactivation rate, $K_m$, $V_{max}$, and...
product inhibition constants at the optimal conditions. (3) Determine the feasibility of the proposed immobilized enzyme reactor/separator. (4) Compare the stability and hydrolysis rate of the free enzyme, batch reactor to the continuous, immobilized enzyme system.

**PROPOSED FREE ENZYME HYDROLYSIS KINETICS MODEL**

**Mechanism for Protein Hydrolysis**

Enzymatic protein hydrolysis is a complex process, producing peptides of various sizes and amino acid composition. Smaller peptides can compete with larger peptides and unhydrolyzed protein for the enzyme active site resulting in competitive product inhibition. Using the initial rate constants obtained from the Eadie-Hofstee plot and integrating the Michaelis-Menten equation (Equation (1)) with \([S] = [S_0](1 - X)\), one can predict the theoretical time course of the hydrolysis with Equation (2).

\[
\begin{align*}
V & = \frac{d[P]}{dt} = \frac{-d[S]}{dt} = \frac{V_{\text{max}}[S]}{K_m + [S]} \\
\frac{[S_0]X - K_m\ln(1 - X)}{V_{\text{max}}} & = t
\end{align*}
\]

The substrate and product concentration are in terms of equivalents of peptide bonds and cleaved bonds per liter, respectively. Reaction velocities are expressed as equivalents of cleaved peptide bonds per liter per minute.

Assuming second order enzyme deactivation and competitive product inhibition, the Michaelis-Menten equation, Equation (1), can be modified.

\[
\begin{align*}
V & = \frac{d[P]}{dt} = \frac{-d[S]}{dt} = \frac{k_j[E_t][S]}{K_m(1 + [P]/K_p) + [S]} \\
\frac{[E_t]}{k_j t + 1/[E_0]} & = 1
\end{align*}
\]

\(K_m, V_{\text{max}}, \text{and } k_j\), the second order deactivation constant, are determined experimentally. Integrating Equation (3), with \(V_{\text{max}} = k_j[E_t] \cdot [P] = [S_0] - [S]\), and \(K_p\), the competitive product inhibition constant, yields the intermediate equation:

\[
\frac{[(1 - K_m/K_p)[-[S_0]X] + K_m(1 + [S_0]/K_p)\ln(1 - X)] - k_j/k_1 \ln(k_1[E_0] + 1)}{[S_0]X - K_m\ln(1 - X) - k_j/k_1 \ln(k_1[E_0] + 1)}
\]

Equation (5) can be manipulated to obtain relationships for \(K_p\) and \(t\), reaction time:

\[
K_p = \frac{K_m [S_0](X + \ln(1-X))}{[S_0]X - K_m\ln(1 - X) - k_j/k_1 \ln(k_1[E_0] + 1)}
\]
Using 4 hour batch hydrolysis data and Equation (6), \( KpJ \) can be calculated. Equation (7) can be used to predict the time required to achieve a given conversion, \( X \), for free enzyme batch hydrolysis.

**EXPERIMENTAL MATERIALS AND METHODS**

**Materials**

Crystallized and lyophilized Subtilisin Carlsberg, purified casein powder, 25% glutaraldehyde solution, NaBH₄, N-trans-cinnamoylimidazole, leucine, and trinitrobenzenesulfonic acid were obtained from Sigma Chemical Co., (St. Louis, Mo.). The water used to prepare solutions was obtained from a Barnstead NANOpure II water deionization system. All other materials used were of reagent or HPLC-grade.

**Apparatus**

A schematic of the immobilized enzyme experimental apparatus is shown in Figure 2. The reactor was a polysulfone hollow fiber ultrafiltration cartridge, Model H1P10-20 from Amicon Corporation. A DH2 hollow fiber cartridge holder was also purchased from Amicon. The substrate solution was delivered to the reactor via a Lapp Microflo Metering pump, Model LS-10. The temperature of the reactor was maintained to \( \pm 0.1^\circ\text{C} \) by a Lauda RC20 circulating water bath. Polypropylene tubing and fittings were purchased from Cole-Parmer. Stainless steel pressure gauges, 0-30 psi., were obtained from Omega Engineering, Inc. Rotameters were supplied by Brooks Rotameter, tube size R-2-15-D, with glass floats.

**Analytical Methods**

The trinitrobenzenesulfonic acid (TNBS) assay (2) is a spectrophotometric method used to determine the concentration of "free" amino groups formed upon peptide bond cleavage during hydrolysis. One "free" amino group is produced per peptide bond cleaved. The TNBS forms a chromophore with the "free" amino group, with the absorbance at 340 nm linear with respect to the "free" amino group concentration. Concentration of the "free" amino groups is proportional to the degree of hydrolysis. An activity assay, using a low molecular weight substrate, N-trans-cinnamoylimidazole (15), was performed to determine the enzyme activity with time. The absorbance of N-trans-cinnamoylimidazole is significant at 305 nm whereas the products of hydrolysis are not, thus, the linear decrease of absorbance with time was monitored by a Perkin-Elmer Lambda 3A UV-VIS spectrophotometer connected to a Perkin-Elmer R100 chart recorder. The slope of the absorbance decrease was proportional to the amount of active enzyme present. The deactivation rate of the free enzyme at pH 8.5 and 60°C was determined by comparing the activity of the initial enzyme solution to the activity of the enzyme solution at a given time.

High performance size exclusion chromatography was used to analyze the amount of enzyme immobilized within the spongy layer. The initial enzyme solution, the water wash, and the enzyme solution after immobilization were analyzed. The HPLC was performed with a Beckman Model 110A pump connected to an injector with a 20 ul loop, a Beckman Spherogel-TSK 2000SW (7.5 mm x 300 mm) column, and a Beckman Model 164...
variable wavelength detector. The mobile phase, 0.1 M Na₂HPo₄/NaH₂PO₄ buffer, pH 6.8, containing 0.05 M NaCl and 0.02% NaN₃ (16), flowed through the column at 1.0 ml/min. The detector wavelength was set at 280 nm. The data from the UV detector was collected and analyzed with an IBM XT personal computer using Chromatochart-PC chromatography software from Interactive Microware, Inc (State College, Pa.).

**Experimental Procedures**

**Kinetics Experiments**

Preliminary experiments were carried out in order to determine the optimum conditions over the temperature range 40°C to 70°C and pH range 7.0 to 10.0. The optimal conditions were pH 8.5 at 60°C. To measure the various kinetic constants, experiments were conducted at the optimal conditions. The experiments were performed in a 25 ml glass, stirred, thermostated batch reactor. The samples were analyzed for the extent of hydrolysis using the TNBS assay. Kinetic constants could be estimated from the rate of hydrolysis data.

**Enzyme Deactivation Experiment**

The enzyme deactivation experiment was performed at pH 8.5 and 60°C. A 1.46 x 10⁻⁴ M Subtilisin Carlsberg solution was prepared by dissolving 0.1 gm enzyme in 25 ml of a buffered 10 gm/l casein solution, (0.5 M tris-HCl, pH 8.5). Aliquots were withdrawn from the stirred batch reactor at various times, quenched in an ice bath, and then analyzed by the activity assay.

**Immobilized Enzyme Experiments**

The apparatus used for the immobilized enzyme experiments is shown in Figure 2. The substrate solution was placed on a magnetic stirrer and pumped to the hollow fiber cartridge. The hollow fiber reactor operates in the "backflush" mode, meaning that the substrate enters on the cartridge shell side, reacts within the spongy region of the hollow fiber with the hydrolyzed products leaving through the tube outlet. The tube inlet and shell side outlet ports were closed.

Subtilisin Carlsberg was immobilized by crosslinking the enzyme with glutaraldehyde within the spongy layer of the hollow fibers. The enzyme solution in 0.1 M Na₂HPo₄/NaH₂PO₄ buffer, pH 7.0, was circulated through the membrane in the "backflush" mode for 60 minutes. To flush the loosely bound enzyme from the membrane, deionized water was circulated through the membrane. To crosslink the enzyme within the spongy layer a 0.1% glutaraldehyde solution in 0.1 M Na₂HPo₄/NaH₂PO₄ buffer, pH 7.0, was circulated for 30 minutes. A 0.05 M NaBH₄ solution was circulated for 15 minutes to reduce the excess glutaraldehyde (16). The original enzyme solution, the enzyme solution remaining after immobilization, and the water wash were analyzed by HPLC.

Experiments at flowrates of 1.6, 3.3, and 6.4 ml/min, pH 8.5 and 60°C, with a casein concentration of 10 gm/l were conducted. During reactor operation, 2.0 ml samples were taken periodically and diluted in 2.0 ml of 1.0% SDS. The diluted samples were analyzed by the TNBS assay for degree of hydrolysis.

**RESULTS AND DISCUSSION**

The results of the free enzyme kinetics experiments are shown in Table 1. The constants $K_m$ and $V_{max}$ were obtained by plotting the initial rate data on an Eadie-Hofstee plot, $V$ versus $V/[S]$. There are 8.2 milliequivalents of peptide bonds per gram of casein. Figure 3 compares a first order deactivation and a second order deactivation mechanism with the experimental data. The second order mechanism fits the data very well which
would suggest an enzyme-enzyme interaction. The free enzyme at 60°C deactivates very rapidly, with a half life of 25 minutes and only 17.2% residual activity after 2 hours. The second order deactivation constant is found in Table 1. In contrast, O'Meara and Munro (4) showed that the deactivation of Alcalase (Subtilisin Carlsberg) is first order in the presence of meat protein. The theoretical conversion based on initial rate constants, Equation (1), is compared to the results from the 4 hour batch experiment, $[S_0] = 50 \text{ gm/1}$, in Figure 4. The combined effects of product inhibition and enzyme deactivation can be seen as the gap widens with time between the theoretical and experimental conversion. To determine $K_{P1}$, the competitive product inhibition constant, Equation (6), the initial rate constants, and the results of the 4 hour batch experiment were used. The 4 hour batch data was fit to Equation (7) using one adjustable parameter, $K_{P1}$. Each experimental point generated a value for $K_{P1}$. These values of $K_{P1}$ were averaged and substituted into Equation (7). Figure 5 shows the excellent agreement between the 4 hour batch experiment and the model prediction.

Three immobilized enzyme runs were performed at pH 8.5, 60°C, $[S_0] = 10 \text{ gm/l}$, and flow rates of 1.6, 3.3, and 6.4 ml/min. Run 1 at a flowrate of 6.4 ml/min ended after 330 minutes due to a high pressure drop from shell side to lumen side of the hollow fibers. The maximum allowable transmembrane pressure drop is 10 psi. After 240 minutes, substrate was pumped rapidly through the shell side to sweep the surface of the hollow fibers. After 255 minutes, pressure decreased from 6.8 psi to 2.2 psi and the hydrolysis rate increased. This suggested that part of the decrease of hydrolysis rate was due to membrane clogging and blocking off of the active enzyme. Runs 2 and 3 continued for 12 hours at flowrates of 1.6 and 3.3 ml/s/min and final transmembrane pressure drops of 2.8 and 9.3 psi, respectively. Figures 6 and 7 show that the initial apparent hydrolysis rate of the 3 runs was proportional to flowrate. The hydrolysis rate of the free enzyme is compared to the immobilized enzyme apparent hydrolysis rate in Figure 6. For the free enzyme, values beyond 4 hours are based on the model prediction. The initial hydrolysis rate of the free enzyme is much higher than the immobilized rate. At the end of runs 2 and 3 the discrepancy between the free and immobilized rate narrows considerably. The residual activities of the free and immobilized enzyme are compared in Figure 7. The residual activity of the free enzyme declines rapidly and is only 1.7% after 12 hours. The residual activities of the immobilized enzyme trials at the end of each run are 58.1% (6.4 ml/min), 65.6% (3.3 ml/min), and 45.5% (1.6 ml/min).

CONCLUSIONS

- For the free enzyme, competitive product inhibition and enzyme deactivation is significant at pH 8.5 and 60°C.

- At 60°C, the free enzyme deactivation is a second order process, consistent with an enzyme-enzyme deactivation mechanism.

- The integrated Michaelis-Menten equation with enzyme deactivation and product inhibition terms predicts the 60°C batch reactor performance well.

- The proposed enzyme membrane reactor/separator has potential as a protein hydrolysis reactor but membrane clogging must be overcome. This might be done by recycling the substrate solution through the shell side of the reactor to produce enough shear to prevent protein build-up.

- The initial hydrolysis rate of the free enzyme is much greater than the immobilized enzyme but is comparable at time greater than 10 hours.
The immobilized enzyme is more stable than the free enzyme based on residual activity.

**NOMENCLATURE**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_0$</td>
<td>Total active enzyme concentration at time 0 (m/1)</td>
</tr>
<tr>
<td>$E_t$</td>
<td>Total active enzyme concentration at time t (m/1)</td>
</tr>
<tr>
<td>$k_j$</td>
<td>Turnover number (min$^{-1}$)</td>
</tr>
<tr>
<td>$k_i$</td>
<td>Second order enzyme deactivation constant (1/m min)</td>
</tr>
<tr>
<td>$K_p$</td>
<td>Inhibition constant, (meqv/l)</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant, (meqv/l)</td>
</tr>
<tr>
<td>$P$</td>
<td>Inhibitor (cleaved peptide bond) concentration (meqv/l)</td>
</tr>
<tr>
<td>$S$</td>
<td>Substrate concentration at time t, in terms of peptide bonds (meqv/l)</td>
</tr>
<tr>
<td>$S_0$</td>
<td>Substrate concentration at time 0 (meqv/l)</td>
</tr>
<tr>
<td>$t$</td>
<td>Reaction time (min), (hr)</td>
</tr>
<tr>
<td>$V$</td>
<td>Reaction velocity (meqv/l min)</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximal reaction velocity, $V_{max} = k_j[E_0]$ (meqv/l min)</td>
</tr>
<tr>
<td>$X$</td>
<td>Conversion, $X = (S_0 - S)/S_0$</td>
</tr>
</tbody>
</table>

**REFERENCES**


TABLE 1  Kinetic Constants of Subtilisin Carlsberg Catalyzed Hydrolysis of Casein at pH 8.5, 60°C

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis Constant, $K_m$</td>
<td>72.2 meqv/l</td>
</tr>
<tr>
<td>Maximal Velocity, $V_{max}$</td>
<td>2.26 meqv/l min</td>
</tr>
<tr>
<td>Turnover Number, $k_j$</td>
<td>2260 min$^{-1}$</td>
</tr>
<tr>
<td>Second Order Deactivation Constant, $k_I$</td>
<td>269.0 l/m min</td>
</tr>
<tr>
<td>Competitive Product Inhibition Constant, $K_{PI}$</td>
<td>$1.20 \pm 0.12 \times 10^{-4}$ meqv/l</td>
</tr>
</tbody>
</table>

FIGURE 1  Hollow Fiber Cross Section
FIGURE 2  Diagram of experimental apparatus

FIGURE 3  Residual Activity (%) of Subtilisin Carlsberg
Versus Time at 60°C in the Presence of 10 gm/l Casein, 0.5 M tris-HCl, pH 8.5, [E₀] = 1.46 x 10⁻⁴ M,
Experimental Data Points are Compared to a First and Second Order Deactivation Mechanism.
FIGURE 4  Experimental Conversion Compared to Theoretical (Initial Rate Constants Substituted into the Integrated Form of the Michaelis-Menten Equation, Equation (2)) Conversion Versus Time.

FIGURE 5  Experimental Conversion (Data Points) Compared to Model (Initial Rate Constants Substituted into the Integrated Form of the Michaelis-Menten Equation with Second Order Deactivation and Competitive Product Inhibition Terms, Equation (7)) Conversion Versus Time.
FIGURE 6  Comparison of Free and Immobilized Enzyme Hydrolysis Rates Versus Time. Hydrolysis Rate - (meqV/1 min per μM enzyme)

FIGURE 7  Comparison of Free and Immobilized Enzyme Residual Activity Versus Time.
Hollow-fiber devices have always drawn attention to the high mass transfer rates achieved in blood dialysis and gas separations. As the fibers have become smaller and smaller, the tremendous surface area available per unit volume has generated speculation as to the utility of hollow fiber units as a novel means of immobilizing mammalian cells, microorganisms, and enzymes. Indeed, these systems have been successfully used to produce monoclonal antibodies from mammalian cells with much greater efficiency than previous methods.

At Colorado State University, the Departments of Biochemistry and Chemical Engineering have been engaged in developing hollow-fiber perfusion systems for maintaining very high density mammalian cell cultures. These cultures, by virtue of their unprecedented density, enable the real-time study of cellular processes using Nuclear Magnetic Resonance spectroscopy. However, to obtain cell cultures with densities approaching that of animate tissue requires that the environment within the bioreactor be optimized. The final goal of this optimization is to achieve maximum cell density. This is a common goal with researchers who are interested in exploiting hollow fiber units for mammalian cell protein synthesis.

In order to optimize a given system variable, it is a prerequisite that the variable be a measurable quantity. Until this time, cell density in an HFBR has been unmeasureable. If the cells are grown in a suspension, a sample may of course be withdrawn and the cell population counted microscopically or with lengthy protein or DNA determinations. However, when the cells are immobilized within the fiber bundle, direct access is impossible. The system geometry and operational restrictions (such as maintaining the integrity of the fiber bundle) present difficulties in cell density determination.

The bioreactor developed by CSU has many features common to most hollow-fiber units (Figure 1). The growth medium which flows through the fiber lumena diffuses into the cell matrix in the extracapillary space. Waste products diffuse into the fibers from the cell matrix and are swept away. One new development is the use of an interfiber gel, either agar or collagen, to provide anchorage for the cells. Thus the cells are not limited to the fiber surface for attachment and growth.

These units are constructed in the following manner: First, the hollow fibers are laid down on two strips of double-stick tape. When a sufficient length of tape is covered, (about two feet of tape) the bundle is formed by rolling up the tape upon a plastic spindle. The interfiber spacing is therefore
determined by the fiber-guide slot spacing and the tape thickness. The bundle is inserted into an acrylic shell, secured at both ends, and potted centrifugally with polyurethane. In this manner, the fiber spacing is both uniform and easily established.(4)

Immobilization of the cells within the fiber bundle, as mentioned previously, causes the greatest difficulty in cell density measurement. Currently, two techniques have been used in the laboratory to determine the cell density. The first requires the bioreactor to be taken off-line, and destroyed.(5) The fibers are manually separated from the cells, which are then enumerated with a hemocytometer. This method is costly, inefficient and not amenable to continuous operation. The second method uses the (measured) oxygen uptake rate(6), or the glucose consumption rate(4) to infer the cell density, by means of a constant specific cellular uptake rate. It has been shown, for swine testicular cells, that the oxygen demand of a cell can drop as much as 93 percent due to a change in extracellular glucose concentration.(7) Since the metabolite concentrations are different at every point in the reactor (due to axial and radial gradients), the uptake rates of these metabolites can thus vary considerably from point to point. Inferential measurements, therefore, are useful as a first approximation, but are not acceptable for a rigorous optimization.

The cell-density measurement which has been developed in this work is based on a relationship between the electrical conductivity of a suspension of membrane-bound vesicles and the volume fraction enclosed by those membranes. The theoretical development, described by Hugo Fricke in 1925, leads to the following relationship:(8)

\[
\frac{K}{K_1} - 1 = \frac{K_2}{K_1} - 1
\]

\[
\frac{K}{K_1 + x} = \frac{K_2}{K_1 + x}
\]

where

- \(K\) = electrical conductivity of the suspension
- \(K_1\) = electrical conductivity of the suspending medium
- \(K_2\) = electrical conductivity of the suspended phase
- \(x\) = shape factor (equals 2 for spheres)

Since the vitality of a mammalian cell relies on the existence of an ion-impermeable membrane, the cells can be treated as non-conducting spheroids (although other shapes can be accommodated, the effect is slight). An early work by Stewart found good agreement between Equation 1 and measured values for suspensions of blood cells up to 90 percent volume fraction.(9) More recently, Lovitt et al. has shown the relation to hold for suspensions of yeast cells (S. cerevisiae).(10)

The main challenge to using this principle in the measurement of HFBR cell densities is to incorporate a conductivity probe of suitable sensitivity into the hollow fiber bundle. This has been accomplished as seen in Figure 2. Before
the fiber bundle is rolled up, two parallel wires are stretched down the length of the fiber plate and fastened to thin plastic strips which are placed among the fibers at regular intervals. When the bundle is rolled up, the wires form themselves into two parallel coils, facing each other in the space between the successive wraps of fiber. This allows the conductivity meter to sample the volume between the fibers, precisely where the cells proliferate.

Preliminary results indicate that this configuration of probe and bioreactor can be used to determine cell density in-situ and non-invasively. An HFBR with a conductivity probe has been constructed and cell slurries of different cell densities were injected into the extracapillary space. The resulting plot of the ratio of cell slurry conductivity to media conductivity shows a 20 percent decrease from 1.0 to 0.8 for cell densities ranging from 0 to \(10^8\) cells/ml. This indicates that monitoring cell density in a hollow-fiber bioreactor is possible using electrical conductivity measurements.
REFERENCES


9. G. N. Stewart, J. Physiology, 24, 356 (1899)

The Hydrodynamics of an Airlift Fermenter

Peter U. Sohn, George W. Preckshot, and Rakesh K. Bajpai
Department of Chemical Engineering
The University of Missouri-Columbia
Columbia, MO 65211

Introduction

An airlift reactor is a very promising gas-liquid contactor which has received much attention in the past few years. The disadvantages of using a conventional stirred tank reactor, such as high energy input, complexity in construction, and its difficulties in scaleup can be avoided by reactor concepts without moving parts, in which incoming gas for gas-liquid absorption drives the liquid for the mixing purposes (6).

Several investigators (1,2,5) have tried to develop hydrodynamic relationships among parameters such as superficial gas velocity ($J_2$), superficial liquid circulation velocity ($J_1$), and holdup of gas in the riser column ($\alpha$) in case of an airlift reactor with external loop. However, the correlations developed thus far do not seem to agree with data obtained by others, and also there are no correlations which distinguish different hydrodynamic characteristics of flow regimes.

In this work, an attempt was made to relate $J_1$, $J_2$, and $\alpha$ in an airlift reactor with external loop with the help of momentum balance proposed by Hsu and Dudukovic (2) and drift flux model (9).

Theoretical Development

1. Momentum balance

Hsu and Dudukovic (2) proposed the following momentum balance equation around an airlift reactor:

$$
\left[ \alpha \bar{\rho}_2 + [1 - \alpha] \bar{\rho}_1 \right] H_D - \left[ \alpha \bar{\rho}_2 + [1 - \alpha] \bar{\rho}_1 \right] H_U
$$

Hydrostatic head of downcomer column
Hydrostatic head of riser column

$$
= \left[ \frac{4 f_{TP} L}{D} \left[ \frac{\bar{\rho}_m J_2^2}{2 g_C} \right] + \left( 1 + k_1 \right) \frac{\rho_m J_1}{2 g_C} \frac{J_1}{1 - \alpha} \frac{J_1}{g_C} \right] U
$$

Friction loss
Losses due to exit and bends
Acceleration loss across the gas injection

\[ \text{losses due to exit and bends} \]

\[ \text{acceleration loss across the gas injection} \]
Friction loss, Entry loss, and loss by valves and bends

\[ \left[ \frac{4f_{TP}L}{D} \left( \frac{\bar{\rho}_mJ}{2g_C} \right) + \left[ k_2 + k_3 \right] \frac{\bar{\rho}_mJ^2}{2g_C} \right] \]

(Eq. 1)

where \( k_1 \) accounts for head losses due to bends or other fittings in the riser column, \( k_2 \) is for the entry loss of the downcomer, and \( k_3 \) is for the sum of losses due to valves and fittings in the downcomer.

In the case where \( \frac{D_U}{D_D} > 1 \), \( H_U = H_D \), and \( c_D = 0 \), the frictional losses in the riser section will be negligible compared to those in the downcomer, and the above momentum balance equation may be simplified as:

\[ \bar{a} = K \bar{J}^2 \]

(Eq. 2)

where \( K \) accounts for reactor geometry and fluid properties. It is essentially a measure of resistance to fluid flow per unit height of reactor, and can be theoretically predicted based upon column configuration using established relations for fluid flow in pipes (7).

2. Velocity-holdup relationship

Drift flux velocity, \( V_{2J} \), in a two phase flow system has been defined by Zuber and Findlay (9) as follows:

\[ V_{2J} = v_2 - J \]

(Eq. 3)

where \( J = J_1 + J_2 \) is the net velocity of the two phase flow system in the riser section, and \( v_2 \) is the instantaneous gas velocity. In the presence of velocity and holdup profiles at a given cross section, an average gas velocity may be defined as:

\[ \bar{v}_2 = \frac{\bar{a}v_2}{\bar{a}} = \frac{\bar{J}_2}{\bar{a}} \]

(Eq. 4)
which upon introduction of drift flux from equation 3 results in

\[
\frac{J_2}{a} = \frac{a J}{a} + \frac{a V_{2J}}{a} \quad \text{(Eq. 5)}
\]

Zuber and Findlay wrote this equation as:

\[
\frac{J_2}{a} = C_0 \bar{J} + \frac{a V_{2J}}{a} \quad \text{(Eq. 6)}
\]

where \( \bar{J} \) is the average net flow rate of the two phase system and

\[
C_0 = \frac{a J}{a} \quad \text{(Eq. 7)}
\]

The second term on the right hand side of equation 6 is correlated to the terminal rise velocity of gas and to the volume fraction of gas in the column as follows (8):

\[
\frac{a V_{2J}}{a} = V_\infty \left[ 1 - \bar{a} \right]^n \quad \text{(Eq. 8)}
\]

Substitution of equation 8 into equation 6 gives drift flux model as in equation 9.

\[
\frac{J_2}{a} = C_0 \bar{J} + V_\infty \left[ 1 - \bar{a} \right]^n \quad \text{(Eq. 9)}
\]

In equation 9, \( C_0 \) is a parameter to account for the nonuniformity of velocity and gas holdup profile in the riser column. Wallis(8) suggested that \( n=0 \) for large bubbles and \( n=2 \) for small bubbles.

**Experimental**

A schematic diagram of the experimental apparatus is shown in figure 1. The material of construction for the apparatus was Plexiglas. Dimensions of apparatus are given in table 1.
Spherical and flat-disk sintered spargers were used as the gas distribution system. In order to obtain different liquid circulation rates for the same gas velocity, different sizes of orifices were introduced in the middle of the downcomer section with the help of which frictional losses could be independently manipulated without changing column height. Sizes of the restriction orifices are given in table 2. Experiments were carried out in semi-continuous fashion, where liquid stays in the reactor, and the gas is continuously sparged. Properties of the different liquids used for the experiment are shown in table 3. Air in all the experiments was filtered to remove any entrained oil-droplets before sparging. Gas velocity was measured with the help of either an area flowmeter or a mass flowmeter. The liquid circulation velocity was measured by pressure drop in a venturi flowmeter which was located in the gas-free downcomer. A pressure transducer and digital voltmeter were used to monitor pressure drop across the venturi meter. A calibration curve was used to determine liquid circulation rates from the measured pressure drops. The gas holdup was measured by using manometers which were connected to different sections of the riser column.

Results

1. Momentum balance

The validity of momentum balance equation was checked with the experimental data obtained in this investigation. And it was found that the relation between the holdup and the superficial liquid velocity is well represented by equation 2. In figures 2 and 3 the typical data are shown for 17.1 %wt glycerine + distilled water solution and tap water solution respectively. The resistance factor \( K \) in equation 2 is dependent on physical properties and reactor geometry. In the present work, these were obtained as slopes of lines in figures 2 and 3.

2. Velocity-holdup relationships

The data obtained from this investigation were plotted according to drift flux model. Figure 4 represents a typical plot. And it was found that there exists an inevitable need to analyze the velocity-holdup relationship by considering different flow regimes, namely, homogeneous, transition, and churn turbulent.

In homogeneous flow regime, bubbles generated at the sparger move through the column undisturbed. The extents of coalescence and redispersion are minimal. Bubbles are almost of uniform size except small variations occurring due to the hydrostatic head of the liquid. As can be seen in figure 5, the velocity-holdup relationship can be described satisfactorily by the drift flux model for homogeneous flow regime, where the intercept is the terminal rise velocity and the slope is the value of \( C_0 \). Here \( n=2 \) (equation 9) was used in homogeneous flow since bubbles tend to be small and do not coalesce. The terminal
rise velocity can also be obtained as the reciprocal of the slope of the plot of holdup vs. \( J_2 \) curve (figure 6) in a bubble column experiment involving the same geometry as the riser section of an airlift reactor.

In churn turbulent flow regime, a characteristic bubble size is determined by bulk flow characteristics in which bubbles continuously undergo coalescence and redispersion. The bubble size distribution is relatively wide. The turbulence characteristics are isotropic and are influenced by the column diameter (3). The turbulence intensity is much higher than that in the homogeneous flow regime. The drift flux model can also represent the velocity-holdup relationship for this flow regime with \( n=0 \) (figure 7) since the bubble size tends to be large. Here the intercept is, of course, higher than that in the homogeneous flow regime since the terminal rise velocity is higher.

In between homogeneous and churn turbulent flow regimes, there exists a phase during which bubble coalesce but have not yet reached the maximum size. The data obtained for transition flow regime still seem to follow the drift flux model for the same gas flow rate. However, the terminal rise velocity changes as the representative bubble size changes and it is no more a constant. Moreover, as can be seen in figure 8, \( \bar{J}_2/\bar{a} \) is linearly dependent on \( \bar{J} \) with the origin as the intercept, which implies that terminal rise velocity increases linearly with the total gas and liquid velocity, \( \bar{J} \). Then, this part of data can be represented as:

\[
\frac{\bar{J}_2}{\bar{a}} = M \bar{J}
\]

(Eq. 10)

Experimental data show that each line in figure 8 represents a group of constant resistance factor, \( K \), and, therefore, \( K \) was included to correlate \( M \) in equation 10. The other variable used to correlate \( M \) was the coalescence characteristic of fluids in a reactor. This was characterized by the plateau holdup \( (\bar{a}_p) \) values of gas phase in a bubble column having the same geometry as the riser section of an airlift reactor (figure 6). These have been suggested (4) to be established by coalescence resulting in larger bubbles with higher terminal rise velocity. On the other hand of this spectrum, column diameter governs the size of bubbles and gas holdup increases sharply again. Multiple regressions were performed with the data obtained from this investigation, and the resulting equation is as follows.

\[
\frac{1/a_p - M}{M - C_0} = 12.9 \left[ \sqrt{\frac{1}{a_p}} - 1 \right] \sqrt{K g H}
\]

(Eq. 11)
The goodness of fit for equation 11 can be seen from figure 9 where abscissa is an experimentally obtained $M$ and ordinate is predicted $M$. The equation is accurate within $7\%$.

**Conclusion**

--- Momentum balance equation, equation 2, is valid for any type of flow regime, especially for a large ratio of riser to downcomer diameter.

--- Velocity-holdup relationship

The drift flux model with $n=2$ for homogeneous and with $n=0$ for heterogeneous flow regimes satisfactorily describes the velocity-holdup behavior. The terminal rise velocity for homogeneous flow regime can be obtained from holdup and gas velocity experiment for bubble column. For transition flow regime also, the drift flux model appears to be valid. But it cannot be used for correlation purposes because of the difficulty of determining the specific terminal rise velocity. However, equations 10 and 11 were found to describe the flow behavior fairly accurately.

**Further work needed**

--- The parameter $C_0$ in the drift flux model needs investigation.

--- The transition flow regime holdup for bubble column has to be investigated against liquid properties (viscosity, density, and surface tension) and geometrical factors (column diameter, height of the column, sparger type, ratio of sparger to column diameter).

**References**


Nomenclature

\[ C_0 \] : Parameter in Drift Flux Model to Account Nonuniformity of Holdup and Total Velocity, dimensionless.
\[ D \] : Diameter of a Column, cm.
\[ D \text{D} \] : Represents Downcomer Column as a Subscript, dimensionless.
\[ f \] : Friction Factor, dimensionless.
\[ g \] : Gravitational Constant, cm/sec^2.
\[ H \] : Height of the Airlift Column, cm.
\[ J \] : \( J_1 + J_2 \), cm/sec.
\[ J_1 \] : Superficial Liquid Circulation Velocity, cm/sec.
\[ J_2 \] : Superficial Gas Velocity, cm/sec.
\[ k_s \] : Resistance Factor in a Column, dimensionless.
\[ K \] : Resistance Factor in Equation 2, (cm/sec)^{-2}.
\[ L \] : Length of the Column, cm.
\[ M \] : Parameter in Equation 4, dimensionless.
\[ n \] : Parameter in Drift Flux Model, dimensionless.
\[ U \] : Represents Riser Column as a Subscript, dimensionless.
\[ V \] : Terminal Rise Velocity of a Bubble, cm/sec.
\[ V_2 \] : Drift velocity of gas, cm/sec.
\[ v_2 \] : Gas velocity, cm/sec.
\[ \rho \] : Density of Gas, g/cm^3
\[ \rho_l \] : Density of Liquid, g/cm^3
\[ \rho_m \] : Density of a Mixture, g/cm^3
\[ \sigma \] : Surface Tension, dyne/cm.
\[ \alpha \] : Holdup of Gas in a Column, dimensionless.
\[ \alpha_p \] : Holdup of Gas in Transition Flow Regime for Bubble Column Experiment, dimensionless.

Table 1 : Airlift reactor dimension

<table>
<thead>
<tr>
<th>Diameter</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DU</td>
<td>3 inches</td>
</tr>
<tr>
<td>DD</td>
<td>1.5 inches</td>
</tr>
<tr>
<td>H</td>
<td>67 inches</td>
</tr>
</tbody>
</table>

Table 2 : Size of Restriction Orifice Compared to Downcomer Diameter

<table>
<thead>
<tr>
<th>Orifice</th>
<th>Diameter (inch)</th>
<th>( D_0/D_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Orifice</td>
<td>3/2</td>
<td>1.000</td>
</tr>
<tr>
<td>Media</td>
<td>$\rho$ (g/cm$^3$)</td>
<td>$\mu$ (cp)</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Tap Water</td>
<td>0.998</td>
<td>0.886</td>
</tr>
<tr>
<td>2.6 %wt glycer.</td>
<td>1.007</td>
<td>0.959</td>
</tr>
<tr>
<td>5.4 %wt glycer.</td>
<td>1.013</td>
<td>0.994</td>
</tr>
<tr>
<td>12.0 %wt glycer.</td>
<td>1.028</td>
<td>1.220</td>
</tr>
<tr>
<td>17.1 %wt glycer.</td>
<td>1.042</td>
<td>1.586</td>
</tr>
<tr>
<td>47.9 %wt glycer.</td>
<td>1.121</td>
<td>4.671</td>
</tr>
<tr>
<td>56.5 %wt glycer.</td>
<td>1.158</td>
<td>9.189</td>
</tr>
<tr>
<td>77.6 %wt glycer.</td>
<td>1.200</td>
<td>27.541</td>
</tr>
</tbody>
</table>

* Surface tensions of Liquids are 68 - 73 dyne/cm.
* All glycerine mixture solutions were made of glycerine and distilled water.

---

**Figure 1.**

**Figure 2.**
**Figure 3.**

**Figure 4.**

**Figure 5.**

**Figure 6.**
Figure 7.

Predict. M vs. Exp. M.

$$\frac{\bar{G}_p - M}{M - C_0} = 12.9 \left[ \sqrt{\frac{1}{\bar{G}_p - 1}} \right] [K S H]^2$$

Figure 8.

Figure 9.
LOCAL LIQUID VELOCITY MEASUREMENTS IN A SPLIT CYLINDER Airlift Column

G. Travis Jones

Department of Chemical Engineering
Durland Hall
Kansas State University
Manhattan, Kansas 66506

Introduction

Airlift reactors are frequently used for animal cell cultivation. For example, Katringer and Scheirer cultivated cell lines BHK and NAMALWA and Birch et. al. cultivated twenty two different cell lines of mouse, rat and human origin using airlift fermenters [1,2]. However, hydrodynamic conditions in the fermenter may adversely affect the growth of such cells. In fact, large shear rates can disrupt membrane integrity of both animal and plant cells, cause physio-chemical changes or cause the loss of cell viability [3]. Nevertheless, an airlift fermenter provides gentler mixing and thus a lower average fluid shear rate when compared to the more traditional stirred tank reactor.

The airlift reactor has a number of other advantages when compared to the stirred tank reactor. Liquid circulation in a airlift reactor is promoted by sparging air into the column and providing a pathway for the degassed liquid at the top of column to return to the bottom; this avoids the moving seal that is present in the stirred tank reactor through which contamination can enter. The use of expanding gas by the airlift to promote liquid circulation results in a lower power consumption than a stirred tank and at the same time provides good oxygen transfer. However, one of the more important considerations for animal and plant cell culturing is that the airlift fermenter provides a low shear environment for mixing and circulation of the broth.

Many considerations exist for the study of the liquid phase hydrodynamics within an airlift reactor. Interaction of bubbles with liquid-phase turbulence partially determines the frequencies of bubble breakage and coalescence occurring within the column, which are required for population modeling. Coalescence-dispersion models can also contribute to a better understanding of mass transfer occurring between the liquid and gas phases. More importantly, operating conditions for which shear sensitive animal and plant cells are susceptible to damage by liquid-phase turbulence must be defined. The purpose of this research is the characterization of the hydrodynamics of the airlift with reference to the cultivation of animal and plant cells.
Theory

The classical study of turbulence based upon the Navier-Stokes equations has utilized the Reynolds decomposition [4] in which the local instantaneous velocity, $u_i'$, is decomposed into a mean flow component, $U_i$, and a velocity fluctuation $u_i'$ about the mean such that

$$u_i = U_i + u_i'$$  \hspace{1cm} (1)

where the subscript, i, refers to an Eulerian coordinate axis. The mean flow component is considered to be a time-averaged quantity and is defined by

$$U_i = \lim_{T \to \infty} \frac{1}{T} \int_{t_0}^{t_0+T} u_i \, dt$$  \hspace{1cm} (2)

The fluctuating component, $u_i'$, becomes zero for the statistically stationary process if it is time averaged. A more useful quantity is the standard deviation or root-mean-square velocity, $\overline{u_i}$, which is

$$\overline{u_i} = \sqrt{\lim_{T \to \infty} \frac{1}{T} \int_{t_0}^{t_0+T} u_i' u_i' \, dt}$$  \hspace{1cm} (3)

These parameters along with the turbulent energy dissipation rate, $\epsilon$, and the physical properties of the liquid phase (i.e. viscosity, density, surface tension, etc.) are important when defining liquid-phase hydrodynamics in relation to superficial gas velocities.

$\epsilon$ is one of the more important parameters to be measured when examining cell damage due to fluid shear within stirred tank reactors [5]. Specifically the turbulent dissipation rate is defined

$$\epsilon = 2\nu \overline{s_{ij}s_{ij}}$$

$$= \frac{\nu}{2} \left( \frac{\partial u_i}{\partial x_j} + \frac{\partial u_j}{\partial x_i} \right)^2$$  \hspace{1cm} (5)

where $\nu$ is the kinematic viscosity of the fluid, $s_{ij}$ is the fluctuating rate of strain and the overbar denotes a time-averaged quantity. The dissipation rate can be estimated using Taylor's [6] large-scale inviscid estimate.
\[ \epsilon = A \frac{u_1^3}{l} \]  

(4)

where \( A \) is a constant to be determined and \( l \) is the integral length scale that is characteristic of the system being studied.

Reported loss of cell viability due to fluid shear is related to the shear rate that is experienced by the cell under controlled conditions. The dissipation rate can be used to estimate the shear rate that would be experienced by the cell in turbulent flow; however, it is necessary to assume that the turbulence is isotropic. Therefore, the small scale structure of the flow is assumed to be independent of any orientation and \( \epsilon \) is related to the time average shear rate in the following manner [7]:

\[ \epsilon = 15 \nu \left( \frac{\partial u_1}{\partial x_1} \right)^2 \]

This allows order of magnitude estimates to be made concerning the time averaged shear rate that is encountered by a cell or a microcarrier in the reactor if the stipulated conditions are met. Thus, a possible estimate for the shear rate would be

\[ \frac{\partial u_1}{\partial x_1} \approx \sqrt{\frac{\epsilon}{15 \nu}} \]

(6)

The relative size of the turbulent eddy with respect to the entity is an important aspect of the determination of fluid-particle phenomena. A. N. Kolmogorov has shown by dimensional reasoning that the size of dissipative eddies, \( \eta \), is determined by the dissipation rate, \( \epsilon \), and the fluid's kinematic viscosity, \( \nu \), as

\[ \eta = \left( \frac{\nu^3}{\epsilon} \right)^{1/4} \]

(7)

For example, for water with \( \epsilon = 1000 \text{ cm}^2/\text{s}^3 \), the microscale eddies would be \( \eta \approx 56 \mu \text{m} \) and the time averaged shear rate \( 80 \text{ s}^{-1} \). For comparison, mammalian cells are typically \( 20 \pm 10 \mu \text{m} \) in diameter and microcarrier diameters are on the order of \( 180 \mu \text{m} \). Reported shear rates that cause loss of cell viability are: Pupal ovaries of the fall army-worm *Spodoptera frugiperda* = \( 1500 \text{ s}^{-1} \) [8]; Human embryonic kidney cells = \( 600 \text{ s}^{-1} \) [9]; Red blood cells = 10 to 100 \( \text{s}^{-1} \) [10]. Endothelial cells exposed to an average shear rate of 330 \( \text{s}^{-1} \) (pulsatile flow) lose viability [11], however at a steady shear rate of 500 \( \text{s}^{-1} \) this cell line remained stable [3].
Experimental Procedure

The airlift column used in these studies was an 151 cm tall and 15 cm diameter acrylic plastic cylinder with a vertical baffle along its axial length; it is shown in Figure 1. Four optically flat viewing ports along its length allow the use of back-scatter laser-Doppler velocimetry (LDV) to determine local liquid velocities. Air was sparged into the column through a sieve plate with 38 holes 1.6 μm in diameter. The measuring apparatus consisted of a TSI laser-Doppler velocimeter and a Nicolet 4094A digital recording oscilloscope. The light scattered by a particle passing through the measurement volume was reflected back along the axis of the laser and the detected signal is amplified by a photomultiplier mounted on top of the receiving optics assembly. Twenty data sets of 15,872 points were recorded to each diskette and the sample interval was either 1, 2 or 5 msec, depending upon the superficial gas velocity and the column region. Each data set was transferred to a Zenith 158 computer via RS-232 and analyzed using a program written to determine the Doppler burst frequencies present with in the data sets. Four systems were studied; Air-water; Air-1% NaCl by weight solution; Air-0.3% Carboxymethyl Cellulose (CMC) solution; Air-0.5% CMC solution.

Results and Discussion

Figure 2 displays a cumulative probability distribution for velocity in an air-water system at 25 C and 31 cm below the top of the dividing baffle. The results for three volumetric gas flow rates are shown for the downflow side of the column, 30, 60 and 90 standard cubic feet per hour (SCFH), and 30 SCFH for the upflow side. From this figure it is easily seen that increased volumetric gas flow rate causes the liquid velocity to increase. Further, it can be seen that there is a significant difference of liquid velocities on the upflow and downflow sides of the column for the air-water system at 30 SCFH.

Figure 3 shows the mean liquid velocities based on the ensemble average for the air-water system in relation to the relative height above the sparger. Four different superficial gas velocities were studied; 2.59 cm/s, 5.18 cm/s, 7.77 cm/s and 10.36 cm/s. The mean liquid velocities on the upflow side of the column agree with those reported by Lee et. al. [12]. Several trends can be observed for this system; as the superficial gas velocity increases the observed mean liquid velocity at the same relative height increases. The liquid velocity on the upflow side of the column appears to increase slightly as one approaches the top of the column. On the downflow side of the column the mean liquid velocity is seen to decrease as the bottom of the column is approached with all of the superficial gas velocities resulting in the approximately same velocity at the bottom. These results agree with general expectations. As bubbles rise from the sparger rapid changes in the bubble size distribution occur as bubble breakage and coalescence compete.
In Figure 3 the indicated values for the mean flows at the lower windows for the downflow region are believed to be in error based on measured circulation times. The LDV measurements were not made far enough into the column to be in the "core" of the flow as it came down to go under the baffle. This was due to the presence of back plates used in earlier experiments. Recent experiments have indicated that the mean velocity is significantly higher toward the center of the column. However, the flow in this region of the column is unlikely to adversely affect shear sensitive organisms.

Figures 4 and 5 display the mean liquid velocities in relation to the superficial gas velocities for various relative column heights for the upflow and downflow regions respectively for the air-0.3% CMC system. Figure 4 shows that as the superficial gas velocity increases mean liquid velocity on the upflow side increases gradually and approaches a plateau. Figure 5 shows the variation of mean liquid velocity with superficial gas velocity on the downflow side. In Figure 5 the mean liquid velocities are clearly segregated in relation to column height; the higher the relative column height the greater the mean liquid velocity for a given superficial gas velocity.

Tables 1 and 2 give estimated dissipation rates using Taylor's inviscid estimate [6] with the constant, A, assumed to be equal to 1 and the integral length scale assumed to be the Sauter mean bubble diameter. From Table 1 a general trend in the local dissipation rates for the upflow side of the air-water system is seen; as the superficial gas velocity increases the local dissipation rate increases at the same column height. Table 2 gives the estimated average dissipation rates for the upflow region of the column for the four systems investigated. The same general trend observed for Table 1 is reinforced by this data; the average dissipation rate increases as superficial gas velocity increases. The estimated average dissipation rates for the upflow side of the column for air-water and air-1% NaCl systems are in agreement with the results of Patel and Lee [13]. However, there are notable discrepancies for ε based on the CMC solution studies.

Conclusions

The mean liquid velocities on the upflow upside are in good agreement with values reported by Lee et. al. [12]. However, insufficient data were recorded to obtain accurate ensemble averages of the local mean velocities in all cases, but, more importantly many of the measured root mean squared velocity fluctuations can be considered only order of magnitude estimates. This resulted in poor estimates of the local dissipation rates. Additional work is needed to define the integral length scales on the downflow side of the column so that estimates of the dissipation rate can be made with reliability. Finally, additional work is needed to understand how hydrodynamic forces promote the loss of cell viability.
References


TABLE 1
Estimated local dissipation rates for the upflow section of the Air-Water system ($\epsilon = \nu^3/\dot{V}$).

<table>
<thead>
<tr>
<th>Relative Column Height</th>
<th>$\epsilon$ (cm$^2$/s$^3$)</th>
<th>Superficial Gas Velocity (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.59</td>
</tr>
<tr>
<td>0.073</td>
<td>2920</td>
<td>3790</td>
</tr>
<tr>
<td>0.238</td>
<td>2740</td>
<td>6470</td>
</tr>
<tr>
<td>0.629</td>
<td>1320</td>
<td>4320</td>
</tr>
<tr>
<td>0.808</td>
<td>4600</td>
<td>3610</td>
</tr>
</tbody>
</table>

$l =$ Sauter mean bubble dia. (cm)  
0.5 0.38 0.36 0.36

TABLE 2.
Estimated average dissipation rates for the upflow side of the airlift reactor.

<table>
<thead>
<tr>
<th>Superficial Gas Velocity (cm/s)</th>
<th>$\epsilon$ (cm$^2$/s$^3$)</th>
<th>System</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% NaCl</td>
<td>0.3% CMC</td>
</tr>
<tr>
<td>2.59</td>
<td>2900</td>
<td>2120</td>
</tr>
<tr>
<td>5.18</td>
<td>4550</td>
<td>3770</td>
</tr>
<tr>
<td>7.77</td>
<td>11000</td>
<td>5250</td>
</tr>
<tr>
<td>10.36</td>
<td>9250</td>
<td>12000</td>
</tr>
</tbody>
</table>
Figure 1. Acrylic plastic airlift column used in the experiments (all dimensions are in millimeters).
Figure 2. Comparison of probability distributions for velocity in airlift fermentor measured at a point 31 cm below the top of the dividing baffle. The superficial velocities at 30, 60, and 90 SCFH are 2.59, 5.18, and 7.77 cm/s, respectively. Data from air-water system at 25°C.

Figure 3. Mean liquid velocities for the Air-Water system at various superficial gas velocities(in cm/s): □, 2.59; †, 5.18; ◇, 7.77; ▽, 10.36.
Figure 4. Mean liquid velocities on the upflow side of the Air-0.3% CMC system at different relative column positions:
- □, 0.073; +, 0.238; ◊, 0.629; ▽, 0.808.

Figure 5. Mean liquid velocities on the downflow side of the Air-0.3% CMC system at different relative column positions:
- ■, 0.073; +, 0.238; ◊, 0.629; ▽, 0.808.
INTRODUCTION

Solid substrate fermentation (SSF) processes are slower than liquid fermentation because of the limitations of heat and mass transfer (Moo-Young et al., 1983). Fluidized bed reactor is expected to reduce these limitations and to enhance the fungal growth. The substrate covered with fungal biomass, then will be used as a starter culture (Corder et al., 1984).

OBJECTIVES

Both Liquid Fluidized Bed Reactor (LFBR) and Gas Fluidized Bed Reactor (GFBR) were investigated for the growth of Trichoderma reesei (Rut C-30). Fluidization characteristics such as bed expansions and pressure drops were observed. Liquid fluidized bed operation followed by stationary SSF seems to be more suitable for the fungal growth. Spore concentration of the inoculum was varied to know if it affects the fungal biomass production. Duration for fluidization in LFBR is an important parameter to obtain enough fungal biomass for the SSF following the fluidization. Fluidized beds were run with germinated spore inoculum to prepare a more rapid fermentation.

LITERATURE REVIEW

The technology of solid substrate fermentation (SSF) has been developed from the ancient times to obtain fermented foods. Solid substrate fermentations are often simpler and require less processing energy than the corresponding submerged fermentations. The low volume of water present in the media per mass of substrate can substantially reduce the space occupied by the fermenter. On the negative side, SSF processes slower than liquid fermentations due to the limitations of heat and mass transfer.
Fluidized bed reactor may become an alternative solution to serve better mass and heat transfer. Particles of substrate will provide a large surface area for biofilm formation and fungal growth. As the fungi cover the particles, the overall density of the biofilm coated particles decreases, which eventually will cause the carried over of the particles from the reactor. This can be prevented by controlling the bed height via intentional separation of the biomass coated particles (Shieh et al.).

Solid substrate fermentation following the fluidized bed reactor will maintain the growth of the fungal biomass in the exponential phase. Substrate particles covered with fungal biomass can be harvested from fluidized bed after a certain period, then the fermentation is continued in the stationary SSF. The risk of biomass detachment from the substrate can be eliminated in the SSF. After the maximum biomass level has been reached, spore formation can be induced by application of certain conditions appropriate for sporulation (Larroche et al., 1986). The substrate, biomass, and spores may be preserved to provide a good starter culture for further use (Tengerdy, 1985).

EXPERIMENTAL PROCEDURES

Microorganisms

The cellulolytic fungi of Trichoderma reesei (Rut C-30) from Rutgers University, New Jersey were grown on the solidified potato dextrose agar. The addition of furfural bran (5 g/l agar medium) was proved to enhance the sporulation. The spore suspension was obtained by washing the 10 day old cultures of agar in the roux bottle with the diluted Tween 80 (0.01 %) in sterilized water. The spore suspension was concentrated to 10^8 spores/ml by means of centrifuging, and stored at 4 °C before the use. Either germinated spores and ungerminated spores were used as an inoculum for the Liquid Fluidized Bed Reactor.

Substrates

Ground corn cob # 4060 from The Andersons (Maumee, OH) with particle size 0.25-0.42 mm (40-60 mesh) and density 500 kg/m^3 was used in the Gas Fluidized Bed Reactor. The chemical composition of corn cob is: lignin 7 %, cellulose 47 %, hemicellulose 37 %, and other materials. The ammonia (AFOREX) treated corn cob was used in the Liquid Fluidized Bed Reactor. In the AFOREX (Ammonia Freeze Explosion) pretreatment (Dale and Moreira, 1982), two grams of ammonia per gram of corn cob (30 % moisture) was mixed in the high pressure reactor at 250 psig, 27 °C for 30 minutes. The pressure was released suddenly into atmospheric pressure at the end of the pretreatment. Then the pretreated corn cob was exposed to the air for 24 h to release the ammonia from the particles. Furfural bran, the residue from corn cob after acid hydrolysis and extraction of furfural was obtained from Nitrochem. Works, Pet, Hungary in a granular form.
Furfural bran particles with 0.4-0.6 mm diameter and 442 kg/m³ bulk density were used for Gas Fluidized Bed Reactor.

Medium

The nutrient medium (Mandels and Weber, 1969) was prepared from: 11.5 g (NH₄)₂SO₄, 0.3 g MgSO₄, 0.79 g CaCl₂·2H₂O, 0.57 g Urea, 2.9 g peptone, 0.1 g lactose, 1.6 mg MnSO₄·H₂O, 1.4 mg ZnSO₄·7H₂O, 5.0 mg FeSO₄·7H₂O, and 2 mg CaCl₂ diluted in 1 liter of water. The pH was adjusted to 5.0 at 28 °C for the growth of Trichoderma reesei (Ghose et al., 1979).

Analytical Procedures

The protein content of the solid samples was estimated from the trichloroacetic acid (TCA) precipitable Kjeldahl nitrogen. Crude protein was determined by a micro-Kjeldahl apparatus and calculated as N x 6.25.

The cellulase enzyme used for substrate hydrolysis test were Celluclast (200-L) and Novozym obtained from Novo Enzyme Corporation. The enzymes were prepared in a 0.05 M Na-acetate buffer to provide 65 IU/g substrate. Ground corn cob and AFEX treated corn cob were hydrolyzed up to 24 h. Reducing sugar produced from the hydrolysis was measured using 3,5-dinitrosalicylic acid (DNSA) solution.

Gas Fluidized Bed Reactor (GFBR)

The glass reactor has the dimensions of 4 cm inside diameter and 150 mm length. A glass fiber filter with 1 micron average pore size was used as a distributor at the bottom of the reactor. The gas was bubbled through a water column to obtain a 100% relative humidity and recirculated using an air compressor. The fluidization velocity was 0.2 m/s and the moisture content of the substrate was 30%. The entrained light materials will be collected in a gas-solid separator (cyclon). To avoid vapor condensation, the whole apparatus, except the air compressor, was placed in a temperature controlled chamber at 28 °C. A typical arrangement of GFBR is shown in Figure 1.

Liquid Fluidized Bed Reactor (LFBR)

A jacketed glass column with the dimensions of 3.5 cm inside diameter and 1 m length was used to operate the liquid fluidization. A bed of 3 mm diameter glass balls were used as a distributor at the bottom of the reactor. The reactor was equipped with a constant temperature water bath at 28 °C. The nutrient medium was recirculated by a recirculation pump. The entrained materials will be collected in the liquid-solid separator and can be recycled back to the fluidized bed. Sterile air stream was bubbled through a sparger to provide oxygen for the fungal growth. The fluidization velocity was 0.2 cm/s and the aeration rate was 30 l/h. A typical arrangement of LFBR is shown in
Figure 2. The results from the LFBR will be compared to the ones obtained from a shaked culture run at 200 rpm with the temperature of 28 °C.

Stationary Solid Substrate Fermentation

Either GFBR or LFBR will only be operated for a short time and the fermentation will be continued in a stationary SSF. Dessicator equipped with trays inside was used to carry on the fermentation in SSF mode. For GFBR, the particles were unloaded from the reactor in a sterile condition and placed in petri dishes on the trays. For LFBR, the nutrient medium was drained and the solid particles were collected aseptically before transferring to petri dishes. The thickness of the substrate in the petri dishes was 1-2 cm. Sterile air was bubbled through the water at the bottom of the dessicator to provide the aeration rate of 0.048 l/h/g substrate (Abdullah et al., 1985). The dessicator was kept in a 28 °C constant temperature incubator.

RESULTS AND DISCUSSION

The main goal of the fluidization tests is to obtain fluidization characteristics with respect to the fluidizability and growth. In a low moisture content the bed can be fluidized well, but the moisture might not be sufficient for the growth. In a higher moisture content, although the growth may be better, the bed becomes unfluidizable. Bed expansion and pressure drop of GFBR for corn cob particles are shown in Figure 3 and Figure 4, while for furfural bran particles are shown in Figure 5 and Figure 6. Generally it can be formulated that the bed expansions increase with the decrease in moisture content. This parameter is significant because when the fungi start to grow, bed expansion will be much higher.

The results of enzyme hydrolysis tests are shown in Figure 7. Two particle sizes of corn cob (40-60 mesh and 14-20 mesh) and one particle size of furfural bran were tested. Results indicated that AFEX treatment increased the susceptibility of the substrate more than twice in 24 h. The reducing sugar produced still increased even after 24 h for AFEX treated corn cob. It indicates that more surface area is still available for the enzymatic attack of cellulose.

The duration for the liquid fluidization in LFBR and the spore concentration of the inoculum affected the fungal biomass produced as shown in Figure 8 and Figure 9. Fungal biomass will increase as the spore concentration is increased and the duration is increased. The bed became unfluidizable after 24 h duration because slugging dominated the operation. After 24 h fluidization, the fermentation is continued in the stationary SSF.
Fungal biomass produced from some different mode of fermentations are shown in Figure 10. Fermentations in GFBR, LFBR, shaked culture and stationary SSF were compared. Highest fungal biomass was obtained from 24 h liquid fluidization followed by 4 day SSF.

CONCLUSIONS

Both GFBR and LFBR have been investigated to produce fungal biomass. LFBR gave the highest biomass production because of the better condition for the growth was achieved. Mass and heat transfer limitations can be eliminated in the very beginning of the growth phase that is a critical point for the fermentation. The growth in the GFBR was very limited because of the low water content in the substrate. Higher moisture can not be provided for the growth because the bed becomes unfluidizable at the higher moisture content.

REFERENCES


ACKNOWLEDGEMENT

This work was supported in part by NSF grant No. CBT-8502125.
Figure 1. Gas Fluidized Bed Reactor
Figure 2. Liquid Fluidized Bed Reactor

1. Reactor
2. Substrate particles
3. Glass balls
4. Water bath
5. Recirculation pump
6. Liquid-solid separator
7. Sparger
Figure 3. Bed expansions of corn cob 4060 in GFBR

Figure 4. Pressure drop of corn cob 4060 in GFBR
Figure 5. Bed expansions of furfural bran (0.4-0.6 mm) in GFBR

Figure 6. Pressure drop of furfural bran (0.4-0.6 mm) in GFBR
Figure 7. Cellulase hydrolysis of corn cob & furfural bran

Figure 8. Effect of spore concentration in LFBR
Figure 9. Effect of the duration for the fluidization in LFBR

Figure 10. Fungal biomass in different fermentation systems
THE EFFECT OF 2,4-D CONCENTRATION ON THE GROWTH OF STREPTANTHUS TORTUOSIS CELLS IN SHAKE FLASK AND AIR-LIFT FERMENTER CULTURE

I.C. Iona, R.D. Sjolund*, and R.A. Yoshisato

Department of Chemical & Materials Engineering
Department of Botany
University of Iowa
Iowa City, Iowa 52242

Abstract

Plant cell suspension culture is of potential use in the production of chemicals from plant based sources. The effect of plant hormones and culture conditions on the development of plant cells in culture is not well understood. The objective of this study was to determine the effect of 2,4-dichlorophenoxyacetic acid (2,4-D), a plant hormone, on the cell growth rate and aggregate size in shake flask and airlift fermentor culture. 2,4-D increases cell growth in moderate concentrations, but seems to inhibit growth at higher concentrations in both shake flask and airlift fermentor culture. Results indicate that the optimum 2,4-D level is about 0.4 ppm and is not dependent on the bioreactor configuration used. Cell growth rate was significantly increased in the airlift fermentor at all 2,4-D levels used, presumably due to the constant supply of 2,4-D. Mean cell aggregate size was smaller in the airlift fermentor than in the shake flask experiments. 2,4-D tended to decrease mean cell aggregate size.

I. Introduction

Plants are major sources of pharmaceuticals, flavorings, foods, and other fine chemicals (Staba, 1980; Fowler, 1983). The suspension culture of plant cells is receiving increasing attention as a way to minimize some of the problems associated with the production of chemicals from whole plants. Before the commercial potential of plant cell culture for the production of chemicals can be fully realized, it is necessary to develop methods for consistently producing large quantities of plant cells under controlled conditions. Small quantities of plant cells can be grown successfully under batch conditions on shaker flask tables; however, this method is unsuitable for growing large quantities and other bioreactor configurations are under investigation.
Unlike microbial cultures, plant cell grow in multi-cell aggregates and have complex nutritional requirements for growth and differentiation. The role of plant hormones and important nutrients in the growth and development of plant cells in culture is poorly understood, yet critical for the industrial use of plant cell culture technology.

The purpose of this study was to investigate the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on the specific growth rate and cell aggregation size of *Streptanthus tortuosus* cells in culture. 2,4-D is a synthetic auxin which affects plant growth. The experiments were carried out under batch conditions in 250 ml shake flasks and in a 14 liter air-lift fermenter with 2,4-D concentration controlled.

II. Materials and Methods

2.1 Cell Line

*Streptanthus tortuosus* is a small plant in the Brassicaceae family found in the Sierra Nevada mountains of California. A suspension culture of *S. tortuosus var. orbiculatus* cells was originally isolated in the early 1970's by Sjolund (1971). A stock suspension culture of *S. tortuosus* (cell line 880-3) was obtained from Professor R.D. Sjolund of the University of Iowa, Department of Botany.

2.2 Media Preparation

The suspension culture medium (referred to as Z-medium) used for this study was developed by Sjolund (1971). Z-medium is based on the Murashige and Skoog (1962) basal medium with yeast extract. Z-medium components and stock solutions are listed in Tables I - III. 2,4-D was added to the desired experimental level. Shake flask experiments were carried out at concentrations of 0, 0.4, 2.0, 5.0 and 10.0 ppm. Airlift fermentor experiments were performed at 0.4, 2.0 and 10.0 ppm.
2.3 Culture Condition and Analysis Method

The suspension culture experiments were carried out in 250 ml shake flask and a Chemap 1-liter airlift fermenter. The culture conditions for the experiments are listed in Table IV. Shake flask runs were 20 days long and airlift fermentor runs were 14 days. A 25 ml sample of cells was collected at two day intervals. Cells were filtered from the liquid medium and weighed to obtain the cell fresh weight. The recovered liquid fraction was analyzed for 2,4-D level. During airlift fermentor experiments, 2,4-D was added in order to maintain the 2,4-D level within 10% of the initial level.

The 2,4-D concentration was analyzed using the HPLC method of Connick and Simonaux (1982). A Beckman Model 330 Isocratic HPLC system with a Beckman Ultrasphere ODS (C₄) reversed phase column was used. The mobile phase was a 50% CH₃CN - 49% H₂O - 1% HOAc solution. Peak detection was performed at 280 nm with a Beckman 164 variable UV detector.

The mean aggregation size was determined from scanning electron micrographs of known magnification. For S.E.M samples, the tissue was dehydrated through an ethanol series, critical point dried in CO₂, affixed to aluminum stubs and coated with 50 nm of gold-palladium. The samples were then examined in a JEOL JSM-35C scanning electron microscope at an accelerating voltage of 15 KV.

III. RESULTS AND DISCUSSIONS

3.1 Growth in Culture

The plant cell density based on fresh weight as a function of time for each run is shown in Figure 1. The initial level of 2,4-D in the shake flask had a marked effect on the growth of plant cells. The growth of cells increased quite dramatically when the 2,4-D level was increased from zero to 0.4 ppm. Generally, the presence of 2,4-D
increased the growth of plant cells; however, at some level near 10 ppm, 2,4-D became inhibitory to cell growth. In addition, at 10 ppm, the lag phase increased from less than one day to greater than two days. Similar trends were observed in the airlift fermentor experiments. The airlift fermentor seemed capable of producing slightly higher cell densities than the shake flask culture; although, it should be mentioned that the airlift fermentor had a slightly higher inoculum density. The growth of plant cells in the airlift fermentor at 10 ppm was greatly inhibited and the lag phase nearly four days. In addition, at this level, the airlift fermentor had a lower cell density than the shake flask. Due to the larger inoculum volume required for the airlift fermentor, it was not possible to do investigate as many 2,4-D levels as in the batch shake flask experiments.

The specific growth rate and the total cell mass produced are given as a function of 2,4-D level in Table V. The highest specific growth rate for the shake flask culture was obtained at 0.4 ppm; although, the specific growth rate fell slowly as 2,4-D level increased to 2.0 ppm. The specific growth rate dropped only 7.1% indicating that cell growth is relatively insensitive to 2,4-D level between 0.4 and 2.0 ppm. Total cell mass produced was similarly related to 2,4-D level. The airlift fermentor data indicates that the specific growth rate is increased dramatically when the 2,4-D level is controlled over the course of the experiment. The specific growth was 28% higher in the airlift fermentor than the shake flask at the 0.4 ppm level. At 10 ppm, the airlift fermentor had a specific growth rate only 8% greater than the shake flask culture. The relationship between 2,4-D level and specific growth is shown graphically in Figure 2. Growth rate increases dramatically at 0.4 ppm and falls proportionally as 2,4-D level as is increased further. This may be explained based on studies conducted by Bayliss (1977). Bayliss' results showed that the slow growth rate at a high 2,4-D concentration
was due to the long mitotic duration time. The higher levels of 2,4-D in our experiments may be suppressing cell mitosis.

3.2 Comparison of Aggregation Size

Cell aggregate morphology was examined using scanning electron microscopy. Generally, suspension plant cultures grow as aggregates as shown in Figure 3a. These aggregates are composed of discrete cells as shown in Figure 3b. As shown in Table V and Figure 4, the mean aggregation size is affected by 2,4-D concentration and culture system. In Figure 4 the darkly shaded bars represent the fermentor culture data. A high 2,4-D concentration and greater mixing level tend to lower cell aggregate size. In addition, plant cells grown in the airlift fermentor were of more uniform aggregate size even when grown under different 2,4-D levels. Possible reasons are that high 2,4-D concentration reduces growth and thus, leads to smaller cells and that increased shear from improved mixing causes large aggregates to break into smaller aggregates. In all cases, plant cells were generally healthy and growing despite variations in cell aggregate size.

IV. CONCLUSIONS

Based on our results we can conclude that the 2,4-D level and culture conditions affect the growth rate, mass production and cell aggregate size of plant cells grown in culture. The specific growth rate is affected by changes in low levels of 2,4-D. A level around 0.4 ppm appears to be optimum regardless of culture condition. Growth rate decreases proportionally with 2,4-D levels higher than 0.4 ppm. Mass production was also greatest at 2,4-levels of 0.4 ppm and dropped as 2,4-D level was increased. In almost all cases, the airlift fermentor culture was capable of operating at higher cell densities. Aggregation size appears to be a function of 2,4-D level and culture condition. High 2,4-D levels tend to reduce cell aggregate size, possibly by suppressing cell mitosis.
Plant cells grown under the higher mixing conditions of the airlift fermentor were smaller than cells grown in shake flask culture. In the airlift fermentor, aggregate size was more uniform and seemed less sensitive to 2,4-D level than in the shake flask culture. The increased shear in the airlift fermentor may serve to limit the maximum size of plant cell aggregates.

V. REFERENCES


Table I. Z-medium Component (based on 1 liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose</td>
<td>20 g</td>
</tr>
<tr>
<td>arginine</td>
<td>60 mg</td>
</tr>
<tr>
<td>yeast extract</td>
<td>10 mg</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>vitamin stock</td>
<td>5 ml</td>
</tr>
<tr>
<td>white stock</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Table II. White Stock Components (based on 1 liter)

<table>
<thead>
<tr>
<th>Component</th>
<th>quantity</th>
<th>Component</th>
<th>quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$·H$_2$O</td>
<td>1.75 g</td>
<td>Ca(NO$_3$)$_2$·4H$_2$O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>2.0 g</td>
<td>KNO$_3$</td>
<td>800 mg</td>
</tr>
<tr>
<td>KCl</td>
<td>650 mg</td>
<td>NaH$_2$PO$_4$·H$_2$O</td>
<td>160 mg</td>
</tr>
<tr>
<td>KI</td>
<td>7.5 mg</td>
<td>MnSO$_4$·7H$_2$O</td>
<td>45 mg</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>15 mg</td>
<td>ZnSO$_4$·7H$_2$O</td>
<td>15 mg</td>
</tr>
</tbody>
</table>
### Table III. Vitamin Stock components (volume 1 L)

<table>
<thead>
<tr>
<th>Component</th>
<th>quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxin : HCl</td>
<td>4.0 mL</td>
</tr>
<tr>
<td>d - Biotin</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Thiamin . HCl</td>
<td>0.6 mg</td>
</tr>
<tr>
<td>Nicotinic Acid ( Niacin )</td>
<td>2.8 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>4.0 mg</td>
</tr>
</tbody>
</table>

### Table IV. Culture Conditions for Fermenter and Shake flask

<table>
<thead>
<tr>
<th>System</th>
<th>Fermenter</th>
<th>Shake flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>capacity</td>
<td>14 L</td>
<td>250 ml</td>
</tr>
<tr>
<td>working vol</td>
<td>11 L</td>
<td>110 ml</td>
</tr>
<tr>
<td>inoculum density</td>
<td>0.5-0.6 g/dl</td>
<td>0.4-0.5 gm/dl</td>
</tr>
<tr>
<td>mixing</td>
<td>air sparge from bottom - 3 holes</td>
<td>rotating</td>
</tr>
<tr>
<td>mixing rate</td>
<td>1 L air/min</td>
<td>150 rpm</td>
</tr>
<tr>
<td>temperature</td>
<td>27°C</td>
<td>26°C</td>
</tr>
<tr>
<td>lighting</td>
<td>dark</td>
<td>dark</td>
</tr>
<tr>
<td>run time</td>
<td>14 day</td>
<td>20 day</td>
</tr>
</tbody>
</table>
Table V. Comparison Between Different Culture Systems

<table>
<thead>
<tr>
<th>2,4-D Conc. (ppm)</th>
<th>Specific Growth Rate (day⁻¹)</th>
<th>Mass Production (g)</th>
<th>Aggregation Size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF</td>
<td>AF</td>
<td>SF</td>
</tr>
<tr>
<td>0.4</td>
<td>0.215</td>
<td>0.276</td>
<td>4.20(20)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.199</td>
<td>0.238</td>
<td>3.90(20)</td>
</tr>
<tr>
<td>5.0</td>
<td>0.130</td>
<td>--</td>
<td>2.95(20)</td>
</tr>
<tr>
<td>10.0</td>
<td>0.099</td>
<td>0.107</td>
<td>1.54(20)</td>
</tr>
</tbody>
</table>

SF - Shake flask culture  
AF - Airlift fermentor culture  
+ ( ) - Run time (days)
Figure 1. Cell Fresh Weight as a Function of Time in Shake and Fermenter Culture.

Figure 2. Comparison of Specific Growth Rate in Two Culture Systems.
Figure 3. Scanning Electron Micrographs of *Streptanthus tortuous* Suspension Culture.

![Image of Scanning Electron Micrographs](image)

Figure 4. Comparison of Aggregation Size.

![Bar chart showing aggregation size comparison](chart)
INTRODUCTION

Glucoamylase is an exo-hydrolase that releases D-glucose from the nonreducing end of starch. Its primary activity is to hydrolyze $\alpha$-(1,4)-D-glucosidic bonds in maltooligosaccharides, although all other $\alpha$-linked bonds can be hydrolyzed at much lower rates. Glucoamylase is used industrially in conjunction with $\alpha$-amylase and glucose isomerase to produce high-fructose and high-glucose corn syrups. The $\alpha$-amylase is used to hydrolyze starch to fragments that are more readily attacked by glucoamylase. The released D-glucose is then converted to D-fructose by glucose isomerase.

While glucoamylase is already used commercially, it has a number of drawbacks. It is currently used at 60°C and pH 4.5, while $\alpha$-amylase and glucose isomerase are used at much higher temperatures and pH's. If the thermostability and pH tolerance of glucoamylase could be increased, it could be used in the same reactor with either of the other two enzymes, which would decrease overall costs. Glucoamylase can hydrolyze both $\alpha$-(1,4) and $\alpha$-(1,6) bonds found in starch, which is advantageous, but it also catalyzes the reformation of these $\alpha$-linkages from D-glucosyl units, which reduces the overall yield of D-glucose to 95%. If the specificity of glucoamylase could be limited to $\alpha$-(1,4) linkages, the reformation of other $\alpha$-linkages could be eliminated and the overall yield would be increased.

Since glucoamylase is a commercial enzyme, it has been extensively characterized. Kinetic studies of a number of different glucoamylases from various fungal sources have all provided the same description of the active site as consisting of six to seven subsites for the binding of D-glucosyl residues (1-8), with the catalytic site located between subsites 1 and 2. The affinities of each particular subsite are also consistent, with subsite 2 having the largest affinity, and subsites 4-6 or 4-7 having very small affinities.

Chemical modifications of the various glucoamylase isozymes to determine essential chemical groups in the active center have also led to similar results. A tryptophan residue believed to be essential for activity and located in subsite 1 has been detected in a number of the glucoamylases (1,9-14). A second tryptophan in subsite 4 has also been identified as essential for activity (15,16). Chemical modification of carboxyl groups has shown that there are one or more carboxyl groups essential for activity located in subsites 2 or 3 (15,17-21). Therefore, there appears to be a very high degree of homology in the composition of the active site of glucoamylases from different sources.
The entire amino acid sequences of glucoamylase from both A. niger and A. awamori have been determined and are identical (22,23). Glucoamylase is a glycoprotein that has a large number of short carbohydrate chains linked to serine and threonine residues located primarily in a 70-amino-acid sequence of the protein (24-28). The presence of the carbohydrates has been shown to increase the stability of the enzyme (29). There are two forms of glucoamylase from Aspergillus, designated GA I and GA II (26,30-31), having molecular weights of 82,500 (22) and approximately 70,000, respectively. GA II is missing the last 104 amino acids from the carboxyl terminal and does not maintain the ability to bind insoluble starch (32). Therefore, GA I can be hypothesized to consist of three main parts, the functional or catalytic area from residues 1-440, the glycosylated region, which adds to stability, from residues 441-514, and the carboxyl tail region, which is involved in adsorption to raw starch, from residues 514-616.

The amino acid sequences of glucoamylase from Saccharomyces diastaticus and Rhizopus oryzae have also been determined (33,34). Sequence homology studies between the three glucoamylase isozymes shows that there is a 40% homology overall, and a very high degree of homology in parts of the catalytic region (35) (see Figure 1). There are five conserved tryptophan residues among all three enzymes, corresponding to Trp 6, 52, 120, 170, and 178 in the A. awamori enzyme. There are also 11 conserved carboxylic acid residues, corresponding to Glu 106, 179, 180, and 308 and Asp 47, 55, 112, 126, 162, 176, and 309. Of these conserved residues, all four Trp residues, all four Glu residues, and Asp 47, 55, 126, 176, and 309 are in highly conserved areas. These residues are the ones most likely to correspond to the residues located in the active site by chemical modification studies. The Trp 120 residue has been identified as being essential for activity and located in subsite 4 (16). Identification of the other tryptophan residue and the carboxylic acid residues has not been accomplished, although preliminary results from chemical modification studies indicate Asp 126 and 176 and Glu 179 and 180, as well as Trp 212, 417, and 437, are in the active site (36). The carboxylic acid residues are all in highly conserved areas, which would support their being in the active site; however, none of the three tryptophans is in a conserved sequence, so the tryptophan residue in subsite 1 may well be found in the conserved areas.

To further understand the effect of specific amino acid residues on catalytic properties of an enzyme, individual residues can be studied by utilizing the technique of site-directed mutagenesis (36). This allows a particular amino acid to be replaced with any other amino acid without disturbing the rest of the protein. It is such work that is reported here.

MATERIALS AND METHODS

The glucoamylase gene of A. niger is encoded on a 2.1-kb HindIII fragment that had been inserted into the yeast expression vector pAC1 (23). The HindIII fragment contains the structural and signal sequences for glucoamylase. The pAC1 vector contains the promoter and terminator for the ENO-1 gene of Saccharomyces cerevisiae, and also separate replication origins and selectable markers for Saccharomyces and E. coli. The expression vector containing the glucoamylase gene is designated as pGAC9 and was a generous gift from Cetus Corp. The HindIII fragment was subcloned into the M13mp19
vector in preparation for oligonucleotide-directed mutagenesis using the method of Norris et al. (37). Mutagenic oligonucleotides were constructed at the Iowa State University Nucleic Acid Center. Mutations were verified by DNA sequencing using the Sanger dideoxy method (38). Following sequencing, the mutated genes were inserted into the expression vector and transformed into yeast, which secreted the enzyme during growth for subsequent biochemical characterization.

RESULTS AND DISCUSSION

Two sets of mutations have been constructed that create unique restriction endonuclease sites around two of the four areas of high sequence homology. By utilizing the redundancy of the genetic code, it is often possible to change the DNA sequence of a gene to create a restriction endonuclease site without altering the amino acid sequence. If this is the only occurrence of this specific sequence in the plasmid, then unique restriction sites can be created without altering the amino acid sequence. Such unique sites were constructed around the two homologous areas Asp 112-Arg 122 and Tyr 156-Arg 194. The latter region was constructed with a third unique site at Asn 181. Any of the homologous areas can be excised with the appropriate restriction endonucleases. After excision, the released wild-type fragment is removed and synthetic oligonucleotides containing the desired mutations can then be ligated into the gene.

Preliminary mutations in the Asp 112-Arg 122 cassette have focused only on the essential Trp 120 residue in subsite 4. The tryptophan has been replaced by leucine, tyrosine, histidine, and phenylalanine to study the function of the various side groups in activity. Mutations in the Tyr 165-Arg 194 cassette have been made to locate the carboxylic acid residues involved in catalysis and also to locate the tryptophan in subsite 1. The three conserved carboxylic acid residues, Asp 176, Glu 179 and Glu 180, have been individually changed to their amide equivalents. A fourth mutation changing both Glu 179 and Glu 180 to glutamines has also been constructed. The two tryptophans at 170 and 178 will also be changed individually to histidine to locate the tryptophan in subsite 1.

None of the mutated enzymes has yet been purified for biochemical characterization. However, preliminary results indicate that the first two substitutions at Trp 120, leucine and tyrosine, both substantially decrease but do not eliminate activity of glucoamylase on starch and maltose. Purification of the mutated enzymes is currently underway. Following purification, the effect of the mutated amino acid on the affinities of the subsite model and on other kinetic parameters will be determined.

ACKNOWLEDGMENT

This project is being supported by the Iowa State University Biotechnology Council. The author thanks Cetus Corporation for the gift of the glucoamylase-containing expression vector.
REFERENCES


Structured Kinetic Modeling of Hybridoma Growth and Monoclonal Antibody Production in Suspension Cultures

Brian C. Batt and Dhinakar S. Kompala
Department of Chemical Engineering
University of Colorado
Boulder, Colorado 80309-0424
June 23, 1987

INTRODUCTION

Monoclonal antibodies (MAbs) are an important biological product that are gaining widespread usage in many technical and medical fields. Because of their binding specificity, MAbs are being applied as research tools in biology and chemistry, in chemical assays, biological separation processes, and in the diagnosis and treatment of some diseases [1].

Currently, MAbs are produced by the culturing of hybridomas. A hybridoma is a cell resulting from the fusion of two mammalian lymphoid cells. One is an antibody-producing cell with a finite lifetime. The other is an "immortal", cancerous lymphocyte (a myeloma) incapable of producing antibody. The resulting hybridoma combines the principal characteristics of the two parent cells: it produces a single, specific type of antibody and can live indefinitely in culture.

The increasing demand for MAbs requires the scale-up of existing production methods and, possibly, the development of new ones. This also requires a better understanding of how physiological and environmental conditions affect hybridoma growth. Mathematical models are useful in predicting the behavior of such cultures, based on environmental conditions, and in optimizing their performance.

This paper describes a structured, kinetic model we have devised to predict hybridoma cell growth and the production of MAb and other extracellular products in a batch culture. The model is in a preliminary stage, but its promise is shown by a comparison of simulations with experimental batch results reported in the literature.

MAMMALIAN CELL METABOLISM

The model equations are based on the metabolic processes in a mammalian cell. Therefore, to understand the rationale for the model formulation, it is important to discuss cell metabolism. A schematic representation is shown in Figure 1.

Unlike bacteria and yeast, mammalian cells require two substrates. Glucose and glutamine are the primary carbon and energy sources in the culture medium. Glutamine also serves as the nitrogen source. Both substrates can enter the TCA cycle and form the major intracellular building blocks: amino acids, nucleotides, protein, and lipids. The amount of
each nutrient consumed in each pathway depends on the metabolic state of the cells, that is, glucose and glutamine are used in different proportions in each synthetic pathway [2]. Glutamine can provide between 30% and 65% of the energy required for cell growth [2] [3].

In addition to the intracellular products, hybridomas also secrete compounds. The principal ones considered in the model are antibody (or immunoglobulin) and the metabolic waste products, lactate and ammonia, which are toxins and can kill the cells when in a sufficiently high concentration [4] [5]. Lactate is derived from both substrates while ammonia is produced only from glutamine metabolism.

EXISTING MODELS

Mathematical models proposed by Bree and Dhurjati [6] and Miller, et al. [7], determine the specific growth rate of cultured hybridomas using a Monod relationship. The equation of Bree and Dhurjati has the form:

\[
\mu = \mu_{\text{max}} \left( \frac{\text{Glu}}{K_{\text{Glu}} + \text{Glu}} \right) \left( \frac{K_{\text{AI}}}{K_{\text{AI}} + A} \right) \left( \frac{K_{\text{LI}}}{K_{\text{LI}} + L} \right)
\]

where \(\mu_{\text{max}}\) is the maximum specific growth rate, \(\text{Glu}\) is the glutamine concentration, and \(A\) and \(L\) are ammonia and lactate concentrations, respectively. The equation presented by Miller, et al., is similar except that it contains an additional term, \(\frac{C_{c}}{C_{c} + K_{c}}\), to account for the contribution of glucose to cell growth (\(C_{c}\) is the glucose concentration). These represent simple, lumped models which take little account of hybridoma metabolism other than to acknowledge that glucose and glutamine are the primary cell nutrients and ammonia and lactate inhibit cell growth.

The model of Bree and Dhurjati [6] simulates hybridoma growth in a batch culture and, in addition to cell concentration, calculates only concentration changes in the two substrates, ammonia, lactate, and antibody. Concentration changes are determined using constant yield coefficients which precludes the simulation of culture dynamics. Indeed, the observed lag in cell concentration of an experimental culture was not predicted [7]. By structuring the cell mass in our kinetic model, we intend to enable it to predict dynamic changes in a hybridoma culture.

MODEL DEVELOPMENT

Based on the cell metabolism described previously (Figure 1), the model is structured by dividing the total cell mass into four constituent pools: amino acids (including their TCA cycle precursors), \(A\); nucleotides (including DNA and RNA), \(N\); protein, \(P\); and lipids, \(F\). The pools are treated as fractions of the total cell mass so that the sum of the fractions is unity.

The model consists of differential equations for each of the cell constituents as well as for the production of extracellular products: antibody, \(I\); ammonia, \(M\); and lactate, \(L\) (Table 1). Note that the extracellular products are assumed to be entirely secreted and, thus, not part of the cell mass. Therefore, their concentrations are expressed in g/L rather than as mass fractions. Each differential equation is composed of a synthesis rate term and depletion or turnover terms, where necessary. The \(k_{i}\) parameters are maximum rate constants; \(K_{ij}\) parameters are similar to Monod saturation constants; and \(a_{ij}\) parameters are stoichiometric coefficients needed to maintain the material balance between constituents.
In order to keep the model tractable, the metabolic complexity is simplified by lumping so that a minimal number of constants can be used to describe the metabolic processes. Consequently, the synthesis rate of a component varies with the concentrations of its primary precursors.

A unique feature of the model is that the synthesis terms for amino acids, lipids, and lactate are the sum of two rates. This accounts for the fact that these components are derived from both glucose and glutamine through essentially the same metabolic pathways, but that each substrate contributes a different amount to their formation.

The complete model also includes equations for the utilization of the substrates glucose, G, and glutamine, Q (Table 2). The subscript 'sG' refers to the contribution of glucose to the synthesis of a constituent, whereas the subscript 'sQ' accounts for the contribution of glutamine. Again, to maintain simplicity, only extracellular substrate is considered; substrate transport into the cells is not included.

Total cell mass is divided into two portions: viable, \( X_v \), and nonviable or dead, \( X_d \) (Table 2). The death rate, \( k_d \), is assumed to be a function of the concentrations of the cell toxins lactate and ammonia. These two portions are used to calculate the viable cell fraction in a culture.

The principal feature of the model formulation is that the instantaneous specific growth rate, \( \mu \), of the hybridoma cells can be calculated directly from the equations for the intracellular components. By definition, the sum of the intracellular mass fractions is always unity. Therefore, the time derivative of this sum is always zero. Using these results in the summation of equations 1-4 in Table 1 gives an independent expression for \( \mu \) (Table 2). This derivation for the instantaneous specific growth rate was first presented in general by Fredrickson [8].

The model framework presented here is similar to that devised by Bentley and Kompala [9] for recombinant E. coli. The differences reflect differences in mammalian and bacterial cell metabolism as well as the necessity of a second substrate and accounting for the secretion of cellular products.

A fourth-order Runge-Kutta algorithm was used to solve the model equations.

RESULTS AND DISCUSSION

Model simulations were performed and compared with experimental results for batch growth of hybridomas reported by Bree and Dhurjati [6]. These were selected because they were the most comprehensive results found in the literature.

Figure 2 is a plot of substrate utilization versus time. The model simulation agrees quite well with the experimental glutamine data. The agreement is not as good for glucose where the measured glucose concentration falls more rapidly than predicted. However, the model does predict a decrease in glucose utilization and approximately the same glucose concentration when glutamine is essentially exhausted.

The change in viable cell fraction with time is shown in Figure 3. The model simulation and experimental data are in close agreement until the glutamine concentration is negligible. With one of the nutrients absent, a rapid increase in cell death is expected. The model predicts this to occur sooner than the data show and the decrease in viability is not as rapid. Yet, the model demonstrates the same trend exhibited by the data.

The changes in lactate and ammonia concentrations in the culture medium as a function of time are shown in Figure 4. There is very little discrepancy between the model simulation and the measured data for ammonia. This is not the case for lactate. The experimental
lactate concentration increased rapidly shortly after the culture was started while the model predicts a much slower increase. Differences in the rate of glucose metabolism could explain the disparity. Figure 1 shows the model predicting a lower utilization rate than found experimentally. Since lactate is derived primarily from glucose, the observed and predicted changes in lactate concentration are consistent with the results for glucose.

The experimental lactate concentration reaches a plateau and begins to decrease at the time when glutamine is exhausted. The simulation, on the other hand, shows a continued increase in lactate concentration at this point, though at a reduced rate. Bree and Dhurjati [6] explain the decrease by asserting that lactate was being utilized by the cells. It is not clear that hybridomas can utilize lactate. Lactate is known to inhibit mammalian cell growth without being metabolized [5] although it has been observed that lactate concentrations up to 2.5 g/L can stimulate hybridoma growth [4]. The metabolism incorporated into our model does not include a mechanism for lactate utilization, which would explain the observed differences.

Figure 5 is a plot of Ig production in a batch culture. The model simulation agrees favorably with the experimental data, but predicts a more rapid initial increase in concentration and a more drastic decrease in production rate once the cells reach stationary phase. The initial increase could be reduced by lowering the value of the maximum synthesis rate constant for Ig production in equation 7.

CONCLUSIONS AND FUTURE STUDIES

Even though in a preliminary stage, the utility of our structured, kinetic model for predicting hybridoma growth and MAb production has been demonstrated by the generally favorable agreement between model simulations and experimental results from a batch culture.

By employing the same structural and metabolic framework, we intend to develop model equations for a continuous culture of hybridomas, including pulse and step changes in substrate concentrations. In addition, we want to develop a model for other culture schemes, such as fed batch. The purpose is to demonstrate the utility in using a lumped, metabolic model to predict dynamic changes in a cell culture.

References

REFERENCES


Figure 1. Schematic drawing of mammalian cell metabolism.
Table 1. Dynamic Equations for Pool Constituents and Extracellular Products

**Pool Constituents**

\[
\frac{dA}{dt} = k_{rAG} \left( \frac{G}{K_{MAG} + G} \right) \left( \frac{Q}{K_{AQ} + Q} \right) \left( \frac{K_{AA}}{K_{AA} + A} \right) + k_{rAQ} \left( \frac{Q}{K_{MAQ} + Q} \right) \left( \frac{K_{AA}}{K_{AA} + A} \right) \\
- a_{AP} \left[ \frac{dP}{dt} \right] - a_{AN} \left[ \frac{dN}{dt} \right] - a_{AF} \left[ \frac{dF}{dt} \right] - a_{AI} \left[ \frac{dI}{dt} \right] - \mu A
\]  

\( \tag{1} \)

\[
\frac{dN}{dt} = k_N \left( \frac{G}{K_{NG} + G} \right) \left( \frac{Q}{K_{NQ} + Q} \right) \left( \frac{A}{K_{NA} + A} \right) \left( \frac{N}{K_{NN} + N} \right) \left( \frac{K_{NI}}{K_{NI} + N} \right) \\
- \mu N
\]  

\( \tag{2} \)

\[
\frac{dP}{dt} = k_P \left( \frac{A}{K_{PA} + A} \right) N - k_{DP} P - \mu P
\]  

\( \tag{3} \)

\[
\frac{dF}{dt} = k_{rFG} \left( \frac{G}{K_{MFG} + G} \right) \left( \frac{A}{K_{FA} + A} \right) \left( \frac{K_{FI}}{K_{FI} + F} \right) \\
+ k_{rFQ} \left( \frac{Q}{K_{MFQ} + Q} \right) \left( \frac{A}{K_{FA} + A} \right) \left( \frac{K_{FI}}{K_{FI} + F} \right) - \mu F
\]  

\( \tag{4} \)

**Extracellular Products**

\[
\frac{dM}{dt} = k_M \left( \frac{Q}{K_{MQ} + Q} \right) \left( \frac{A}{K_{MA} + A} \right) X_v
\]  

\( \tag{5} \)

\[
\frac{dL}{dt} = k_{rLG} \left( \frac{G}{K_{MLG} + G} \right) + k_{rLQ} \left( \frac{Q}{K_{MLQ} + Q} \right) X_v
\]  

\( \tag{6} \)

\[
\frac{dIg}{dt} = k_I \left( \frac{A}{K_{AI} + A} \right) N X_v
\]  

\( \tag{7} \)
Table 2. Dynamic Equations for Substrate Utilization, Cell Mass, and Specific Growth Rate Determination

Substrate Utilization

\[
\begin{align*}
\frac{dG}{dt} &= -a_{GN}X_v \frac{dN}{dt} - a_{GA}X_v \frac{dA}{dt} - a_{GF}X_v \frac{dF}{dt} - a_{GL}X_v \frac{dL}{dt} \quad (1)
\end{align*}
\]

\[
\begin{align*}
\frac{dQ}{dt} &= -a_{QN}X_v \frac{dN}{dt} - a_{QA}X_v \frac{dA}{dt} - a_{QF}X_v \frac{dF}{dt} - a_{QL}X_v \frac{dL}{dt} \quad (2)
\end{align*}
\]

Cell Mass

\[
\begin{align*}
\frac{dX_v}{dt} &= [\mu - k_D]X_v \quad (3)
\end{align*}
\]

\[
\begin{align*}
\frac{dX_d}{dt} &= k_DX_v \quad (4)
\end{align*}
\]

where

\[
\begin{align*}
k_D &= k_{D} \left[ \frac{M}{K_{DM} + M} \right] \left[ \frac{L}{K_{DL} + L} \right] \quad (5)
\end{align*}
\]

Specific Growth Rate Determination

\[
\begin{align*}
\mu &= \left[ \frac{dA}{dt} \right] + [1 - a_{AN}] \left[ \frac{dN}{dt} \right] + [1 - a_{AP}] \left[ \frac{dP}{dt} \right] + [1 - a_{AF}] \left[ \frac{dF}{dt} \right] - a_{AI} \left[ \frac{dI_g}{dt} \right] \quad (6)
\end{align*}
\]
SUBSTRATE UTILIZATION

Figure 2.

HYBRIDOMA VIABILITY IN BATCH

Figure 3.
LACTATE AND AMMONIA PRODUCTION

Figure 4.

MONOCLONAL ANTIBODY PRODUCTION

Figure 5.
INTRODUCTION

A continuous culture of Zymomonas mobilis was controlled at predetermined optimal pH and temperature for specific ethanol productivity. The dynamic response of the reactor to step changes in dilution rate and input glucose concentration was studied. Structured and unstructured theoretical models are developed to predict the dynamic responses. A single input single output internal model control system was designed, simulated and implemented to control % CO2 in the exit gas with dilution rate as the input. This control design uses an empirical transfer function as the system model.

METHODS AND MATERIALS

Zymomonas mobilis strain ATCC 10988 was used throughout the study. The complex media composition was 1 g/l (NH4)2SO4, 1 g/l KH2PO4, 0.5 g/l MgSO4*7H2O, 3 g/l yeast extract, and 10 weight percent glucose.

The Chemap fermentation system is shown in Figure 1. The working volume was 3 l. Measurements of pH, Redox potential, turbidity, and CO2 percent in the exit gas. Also measured and controlled were agitation speed, temperature, and reactor weight. Nitrogen was bubbled through the reactor at 0.2vvm to maintain anaerobic conditions and to facilitate the CO2 measurement.

Off-line samples were taken to determine ethanol and glucose concentrations by HPLC. A 25 ml sample was also taken for dry biomass weight to be determined.

The fermenter, pumps and instruments were all connected to a data acquisition unit and micro-computer with measurements displayed in real time on the CRT screen. A menu driven data acquisition and control program was designed and used throughout the study.

THEORETICAL MODELS

A deterministic bioreactor model can be classified as either structured or unstructured. A structured model considers all cells to have average properties but considers the cells to have some variable internal structure. An unstructured model considers all cells to be the same at all times i.e. a chemical species.

The unstructured model used was an endogenous maintenance model because Z. mobilis is known to have a very high value for its cell maintenance coefficient. The microbial growth kinetics used were simple Monod kinetics. The model response was improved by the inclusion of a variable yield coefficient of biomass from substrate. The cell and substrate kinetics are given below:

1) \[ \dot{x} = \frac{U_m S X}{K_s + S} - U_e X \]
2) \[ \dot{S} = - \left( \frac{1}{Y_{sx}} \right) \dot{x} \]
3) \( Y_{sx} = a D + b \)

Where,
- \( R_x \) = Cell growth rate (g/l hr)
- \( R_s \) = Substrate consumption rate (g/l hr)
- \( U_{rn} \) = Maximum specific growth rate (hr)
- \( K_s \) = Monod constant (g/l)
- \( Y_{sx} \) = Yield of biomass from substrate
- \( U_e \) = Endogenous cell degradation rate
- \( S, X \) = Concentrations (g/l)
- \( a, b \) = constants

The mass balance equations for a CSTR were derived using the above kinetics. The non-linear ordinary differential equations are:

4) \[
\frac{dX}{dt} = \frac{U_m S X}{(K_s + S)} - U_e X - D X
\]

5) \[
\frac{dS}{dt} = -\frac{U_m S X}{(aD + b)(K_s + S)} + U_e X + (S_i - S) D
\]

The structured model used was a modified two-compartment model of Roels and Harder. The biomass is assumed to be either in a K or a G compartment. The physical interpretation of these compartments Roels explains as K compartment containing RNA, carbohydrates, and monomers of macromolecules. The G compartment is interpreted to contain DNA, protein, and lipids synthesized from K. Also included in the model are yield terms for the first order reactions between compartments. The rate equations are given below:

6) \[
R_s = \frac{U_m S X}{(K_s + S)}
\]

7) \( R_x = Y_{sk} R_s \)

8) \( R_k = k_k G K X \)

9) \( R_g = k_g G X \)

where;
- \( Y_{sk} \) = Yield of K from S
- \( R_i \) = rate of production of i
- \( K \) = fraction of biomass in K
- \( G \) = fraction of biomass in G
- \( k_k \) & \( k_g \) = first order rate constants (hr\(^{-1}\))

Using the above kinetics to derive the CSTR mass balances gives the following:

10) \( K + G = 1.0 \)

11) \[
\frac{dX}{dt} = \frac{U_m S X}{(K_s + S)} + K (1 - K) (Y_{kg} - 1) k_k X - D X
\]

12) \[
\frac{dS}{dt} = (S_i - S) D - \frac{1}{Y_{sk}} \frac{U_m S X}{(K_s + S)}
\]
CONTROL SCHEME
The control scheme used was internal model control using dilution rate to control % CO2 in the exit stream. This control design is based on a transfer function model for the plant. The components in the closed-loop are shown in Figure 1, where;

\[ G_m \text{ is the plant model} \]
\[ G_p \text{ is the actual plant} \]
\[ G_m \text{ is the controller} \]
\[ F \text{ is a first order filter} \]
\[ Y_{sp} \text{ is the setpoint} \]
\[ Y \text{ is the measurement} \]
\[ u \text{ is the input.} \]

The controller is designed as the invertible part of the model and the system is tuned with a single constant of the filter. This filter moderates the control input, u, such that even with mismatch between the model and plant the system is stable. The advantage over common PID controllers which are designed assuming no plant/model mismatch.

RESULTS
The experimental response of the system to step changes in dilution rate, input variable, were studied. The responses due to a step decrease from .205 hr\(^{-1}\) to .155 hr\(^{-1}\) are shown in Figures 2 thru 4. The responses of each state are as expected from the steady-state data. The glucose concentration is expected to decrease at the lower dilution rate, the ethanol is expected to increase slightly, and the biomass is expected to decrease due to the large maintenance requirements. The interesting aspect of the response is the damped oscillations as the new steady-state is approached. Another interesting result is the inverse response of the CO2 measurement. This seems to correlate with the turbidity or biomass measurement. The solubility of CO2 in the changing reactor composition was studied and no change was observed. To estimate the ethanol concentration the steady-state relation, Eq. 13, was valid even under reactor dynamics shown in Figure 5.

\[ P_{est} = Y_{ps} (S_i - S) \]

The model which predicted the correct movement in the states and a single overshoot was the maintenance model with variable yield. These results are shown in Figures 6 thru 8. The model parameters were not adjusted to fit the experimental response. They represent an a priori estimation of the system.

To design the control system a second order linear differential equation was empirically fit to match the CO2 response. The inverse response of the system was approximated with a deadtime of 2 hours. The controller is designed as the invertible part of the model. The system was simulated to tune the first order filter to give a stable and reasonably fast response with plant/model mismatches in gain and deadtime. Figure 9 shows the closed-loop performance of the CO2 measurement with a step change up to 38.5%. Even though the response is oscillatory the match to the model response, Figure 10, shows only a time shift difference between the model and actual plant. To correct this a longer model dead time was used and the system responded better with the step change, Figure 11, and a disturbance in initial glucose concentration, Figure 12. The reason for the deadtime mismatch is that the deadtime or inverse response is a strong function of the input , D. Thus the controller inputs the maximum D to increase the CO2 causing a longer inverse response than the smaller open-loop step change in D.
CONCLUSIONS

The dynamic response of the continuous fermentation showed damped oscillations between steady-states disturbed by step changes in dilution rate. To theoretically predict this behavior a unstructured maintenance model with variable yield was used as well as a two-compartment structured model. It was thought internal structure must be considered to predict the oscillations. However the model which to date describes the system was the maintenance model with variable yield. The ethanol could also be estimated given a substrate measurement during transients by using a steady-state relation.

Internal model control was designed with a input output model to control %CO2 with dilution rate. Implementation of the control scheme showed good agreement under closed-loop between the model and plant. The only discrepancy seemed to be a lag time which was corrected. The closed-loop responded reasonably well to setpoint changes and disturbances. The physical reason for the plant/model mismatch is that the system deadtime or inverse response is a strong function of the input dilution rate.

REFERENCES


Figure 1 Internal Model Control

Figure 2 Ethanol (Dilution Rate Down)
Figure 3 Glucose (Dilution Rate Down)

Figure 4 % CO2 (Dilution Rate Down)
Figure 5 Ethanol Estimation with $S$

Figure 6 Ethanol Dilution Rate Response
Figure 7  Glucose Dilution Rate Response

Figure 8  Cell Dilution Rate Response
Figure 9 Closed-loop Setpoint Change

% CO2
Measured

Figure 10 Model Response in Closed-loop

% CO2
Model
Figure 11 Closed-loop Setpoint Change

Figure 12 Closed-loop Disturbance
Modeling of *Brettanomyces clausenii* Growth on Mixtures of Glucose and Cellobiose

Max T. Bynum and Dhinakar S. Kompala
Department of Chemical Engineering
University of Colorado
Boulder, Colorado 80309-0424

Karel Grohmann and Charles E. Wyman
Solar Energy Research Institute
Golden, Colorado 80401

June 30, 1987

**Abstract**

The sequential utilization of glucose and cellobiose during anaerobic fermentations of *Brettanomyces clausenii* was investigated to develop a mathematical model for a simultaneous saccharification and fermentation process. Fermentation of cellobiose was found to be mediated by the presence of extracellular β-glucosidase, which is repressed and inhibited by glucose. A kinetic model containing a number of parameters, which can be determined experimentally, was developed for simulating the dynamics of yeast fermentation of mixtures of glucose and cellobiose. An alternative cybernetic model, which is based on the viewpoint that microbial behavior is the outcome of an instantaneous optimization strategy to maximize their growth rate, is also developed using a much smaller number of parameters. Both models were shown to be similarly capable of simulating the yeast growth dynamics in batch cultures, as well as perturbed cultures with intermittent additions of glucose.

**Introduction**

Simultaneous cellulose hydrolysis and carbohydrate fermentation to ethanol is an area of active research for the conversion of renewable resources, such as wood from short rotation forests, into liquid fuels such as ethanol. Simultaneous saccharification and fermentation (SSF) is considered advantageous relative to a two-step process of saccharification and fermentation since the fermentation of glucose and cellobiose prevents the accumulation of these sugars, thereby reducing the end-product inhibition of the cellulolytic enzymes. Operation, evaluation and improvement of this combined process require a clear understanding of the kinetics of both of the two process steps.

The kinetics of cellulose hydrolysis has been investigated by several researchers. \[1\] Cellulose hydrolysis by the commonly used cellulase is found to be a two-step process with the exo- and endo-glucanases catalyzing the production of cellobiose and

\[\text{To whom correspondence should be addressed}\]
β-glucosidase catalyzing the hydrolysis of cellobiose to glucose. The enzymatic reaction rates are variously modeled as modified Michaelis-Menten expressions, with the modifications representing competitive or non-competitive inhibitions by cellobiose and glucose. For any given cellulase system, the type of rate expressions to be used can be determined through initial rate experiments.

The kinetics of yeast fermentation on a mixture of glucose, cellobiose and higher cellodextrins are not as easily determined. To alleviate the end-product inhibition of the glucanases by cellobiose, an organism capable of growing on both glucose and cellobiose, e.g., *Brettanomyces clausenii*, is preferred for the SSF process [2]. Simulation of microbial growth and fermentation dynamics on multiple substrates using unstructured models, such as modified Monod terms or additive Monod terms etc., fails to predict the growth dynamics. This is mainly a result of the complexity of the microbial regulatory processes such as the repression/induction and inhibition/activation of some catabolic enzymes by the catabolites of faster growth supporting substrates.

In this paper, we develop structured models for growth dynamics of the yeast *Brettanomyces clausenii* on mixtures of glucose and cellobiose by incorporating the repression and inhibition of catabolic enzymes into the model equations. Two different approaches are followed for the development of these models: one is the more common kinetic modeling approach which requires the use of a large number of parameters, and the other is a new development in growth modeling known as the cybernetic modeling approach [3,4]. The cybernetic approach assumes the microorganism to be an optimal strategist, i.e., through the regulatory mechanisms of repression/induction and inhibition/activation, the microbial cell maintains some optimal condition which is discussed later. For more background on the cybernetic perspective, the reader is referred to the original literature [3,5]. Both kinetic and cybernetic model simulations are compared with the experimental results.

**Materials and Methods**

**Organism**

Parent yeast *Brettanomyces clausenii* (ATCC 10562), which was obtained from NRRL, gave rise to a faster growing, morphologically altered yeast after extended serial SSFs. This faster growing strain was the fermenter in all experiments.

**Medium**

Yeast Nitrogen Base (YNB) w/o amino acids, a completely defined carbon free medium containing vitamins and a nitrogen source, was obtained from Difco Laboratories and is described elsewhere [6]. Glucose and cellobiose were added to produce concentrated media which were then filter sterilized with disposable Nalgene 0.2μm filters.

**Inoculum Preparation**

The organism was stored in Nunc cryo tubes at -70°C in a 12% glycerol solution. Inocula were grown in 250-mL shake flasks at 30°C. Inocula media consisted of YNB w/o amino acids with glucose or cellobiose added according to the carbon source used in the fermentor to be inoculated.
Fermentor Description
Experiments were carried out in 5-L Braun Biostat V fermentors with initial culture volumes of 4 L. Temperature was controlled at 30°C and pH was controlled at 5.0. The medium was sparged with nitrogen for 30 minutes immediately following inoculation to remove oxygen.

Sample Analysis
Cell Concentration
Cell dry weight was estimated from absorbance measured at 590 nm. Absorbance varied linearly with cell dry weight up to approximately 0.17. Denser samples were diluted with measured quantities of sterile media to obtain absorbances below 0.17.

Glucose and Cellobiose Concentrations
Glucose concentrations were determined with a Yellow Springs Instrument Co. glucose analyzer. Cellobiose concentrations were determined by first hydrolyzing the cellobiose through addition of β-glucosidase (Sigma). Samples were incubated for \( \geq 3 \) hrs at 30°C. A second glucose assay was done for each sample. The result from the first glucose assay was subtracted from the second and the difference was multiplied by the stoichiometric coefficient to give the cellobiose concentration.

β-glucosidase Activity
β-glucosidase activity was determined via a colorimetric method. Sample aliquots were diluted in a citrate buffer of pH 5.2 and were incubated at 50°C with p-Nitrophenyl β-D-Glucopyranoside (Sigma) for 30 minutes. Incubation was quenched with 2.0 molar sodium carbonate and absorbance at 410 nm was determined.

Results
Figure 1 shows the sugar concentrations and the logarithm of optical density. The growth curve shows an initial lag phase, an exponential growth phase, during which only glucose is consumed, and an immediate second exponential growth phase with a slower growth rate, during which most of the cellobiose is consumed. The slower growth rate during the second growth phase is as expected and in complete agreement with the recently tabulated general characteristics of microbial growth on multiple substrates [7].

A surprising feature of the growth data is in the glucose concentration profile. After the sugar gets completely utilized during the first growth phase, the glucose concentration in the extracellular medium rises sharply during the initial growth on the remaining cellobiose and remains non-zero until cellobiose is almost completely utilized. Also, β-glucosidase assays showed a rapid increase in extracellular activity coincident with the beginning of cellobiose hydrolysis. These results form the basis for an assumption, in our subsequent modeling efforts, that cellobiose utilization by \( B. \ clauseni \) is solely through its extracellular hydrolysis to glucose and subsequent utilization of glucose thus formed.

Significant β-glucosidase activity was not found in the cell-free medium during the first growth phase when only glucose was consumed. This observation suggests
a repression of this enzyme synthesis by glucose when glucose is present in sufficient quantities. Near completion of the first growth phase, \( \beta \)-glucosidase synthesis appears to be derepressed due to the lower glucose concentration and induced by cellobiose, resulting in an immediate second growth phase without any intermediate diauxic lag. \( \beta \)-glucosidase activity was not detected after glucose depletion in the absence of cellobiose.

During growth on cellobiose only, i.e., when no glucose is added to the fermentor, the extracellular glucose concentration increases to a significant level during cellobiose hydrolysis: see figure 2. The glucose concentration is significant because it is of the order of the Monod constant for glucose growth and therefore can greatly effect the growth rate. This result further suggests extracellular cellobiose hydrolysis.

**Kinetic Modeling**

The growth of yeast is assumed to depend on the extracellular glucose concentration in a typical Monod form. As all cellobiose is hydrolyzed extracellularly, the Monod-type dependence on glucose alone will suffice for modeling of growth on cellobiose, provided the enzymatic hydrolysis kinetics are also included in the model equations. The original Monod term is unsatisfactory for simulating the growth dynamics in batch and continuous cultures; such as the initial lag phase or response to perturbations. A simple modification has been used [8] to improve this aspect of batch dynamic simulation by incorporating the intracellular level of a hypothetical key (or rate-determining) enzyme for the catabolism of glucose. Thus,

\[
\frac{dC}{dt} = \mu e_1 C \left[ \frac{s_1}{K_1 + s_1} \right]
\]

where \( C \) is the cell concentration (g dw/L), \( s_1 \) is glucose concentration (g/L), \( e_1 \) is the intracellular key enzyme mass fraction (g enzyme/g cell mass), \( K_1 \) is the saturation constant and the product, \( \mu e_1 \), replaces the maximum specific growth rate, \( \mu_{1,max} \), which is in the unmodified Monod equation. The rate equation of the intracellular key enzyme fraction can be written as:

\[
\frac{de_1}{dt} = \alpha_1 \left[ \frac{s_1}{K_{e_1} + s_1} \right] - e_1 \left( \frac{1}{C} \frac{dC}{dt} \right) - e_1 \beta_1
\]

where the first term on the right hand side corresponds to its synthesis. Induction occurs through the presence of the sugar \( s_1 \). The second term corresponds to a dilution of the intracellular mass due to cell growth [9], and the third term represents first order degradation of the enzyme. From this equation, the maximum value for \( e_1 \) during balanced growth phase on \( s_1 \), i.e., when \( de_1/dt = 0 \), in batch cultures can be obtained as:

\[
e_{1,max} = \frac{\alpha_1}{\mu_{1,max} + \beta_1}
\]

The maximum specific growth rate, \( \mu_{1,max} \), which is an experimentally determinable parameter, is the same as \( \mu e_{1,max} \) in this structured model. Thus,

\[
\mu = \frac{\mu_{1,max} (\mu_{1,max} + \beta_1)}{\alpha_1}
\]
If, at the beginning of a batch culture, the intracellular key enzyme fraction is smaller than its maximum balanced growth phase level (possibly due to its degradation during post-exponential or stationary phases of the inoculum culture), then the instantaneous specific growth rate will be proportionally smaller than $\mu_{1,\text{max}}$. This situation represents an initial lag phase. The synthesis of $e_1$ begins immediately provided $s_1$ is present and the instantaneous specific growth rate reaches its maximum.

The rate equation for $s_1$ can be written as

$$\frac{ds_1}{dt} = -\mu e_1 C \frac{s_1}{K_1 + s_1} + \nu k_2 e_2 \left[ \frac{s_2}{K_2 + s_2} \right] \left[ \frac{K_3}{K_3 + s_1} \right]$$

(5)

where the first term corresponds to the consumption of $s_1$ for cell growth, related to the cell growth rate in Eq. 1 by the yield coefficient $Y_1$. The second term corresponds to the production of $\nu$ g glucose from 1g cellobiose. Cellobiose hydrolysis kinetics can be written as:

$$\frac{ds_2}{dt} = -\left[ \frac{k_2 e_2 s_2}{K_2 + s_2} \right] \left[ \frac{K_3}{K_3 + s_1} \right]$$

(6)

where $s_2$ is the cellobiose concentration, $e_2$ is the extracellular enzyme concentration, $K_2$ is the Michaelis-Menten constant, $k_2$ is the maximum enzymatic reaction rate and $K_3$ is the dissociation constant for the non-competitive inhibition of this enzyme by glucose. The synthesis rate of $\beta$-glucosidase is assumed to be the rate-determining step relative to the secretion rate of this enzyme into the extracellular medium. Hence, the rate equation for $e_2$ can be represented simply as:

$$\frac{dc_2}{dt} = \alpha_2 C \left[ \frac{s_2}{K_{e_2} + s_2} \right] \left[ \frac{K_4}{K_4 + s_1} \right] - \beta_2 e_2$$

(7)

where the first term in brackets accounts for the induction of enzyme synthesis by cellobiose and the second term in brackets represents the repression of enzyme synthesis by glucose. The last term accounts for any degradation by extracellular proteases or thermal denaturation.

Equations 1, 2, 5, 6 and 7 comprise the kinetic model for the dynamic growth behavior of the yeast B. Clausenii on mixtures of glucose and cellobiose. The constants $\mu_1$, $K_1$ and $Y_1$ are directly from the standard Monod model for microbial growth and can be estimated readily through initial growth rate experiments. Similarly, the constants $k_2$ and $K_2$ relate to the Michaelis-Menten kinetics of cellobiose hydrolysis and are also estimated readily through initial reaction rate experiments. The constants $\alpha_1$ and $\beta_1$ correspond to the intracellular key enzyme synthesis and degradation rates. As this key enzyme is hypothetical and not assayed, the estimation of these two constants is based on the synthesis and degradation rates of an average enzyme in the yeast cells. These two constants are also related to the maximum intracellular mass fraction of an average enzyme in yeast cells, which is estimated from the literature. The constants $\alpha_2$ and $\beta_2$ relate to the synthesis and degradation rates of the extracellular $\beta$-glucosidase. As this enzyme is assayed, its maximum synthesis rate $\alpha_2$ and its degradation rate $\beta_2$ are readily determined. The enzyme synthesis saturation constants $K_{e_1}$ and $K_{e_2}$ are more difficult to determine but can be estimated through curve fitting the initial lag during the growth on single substrates at several different initial concentrations. The constants $K_3$ and $K_4$ are the most difficult to determine and they are estimated through curve fitting the sugar concentration profile in the perturbed batch cultures (for $K_3$) and in
the simple batch cultures (for $K_A$) using various combinations of glucose and cellobiose concentrations.

The kinetic model explicitly includes terms for the repression/induction and activation/inhibition of key enzymes for substrate catabolism. Even this simple representation of complex internal regulatory mechanisms introduces parameters which are difficult to measure.

The complexity of the biochemical pathways constituting the regulatory mechanisms in the microbial cell present obvious difficulties when utilizing the kinetic modeling approach with any degree of thoroughness. The inclusion of terms representing phenomena such as catabolite repression is necessary if the kinetic model is to exhibit even a qualitative accuracy. However, the quantity and quality of the kinetic parameters rapidly increase in number and obscurity when additional pathways are represented in a kinetic model.

While striving for quantitative accuracy, an important goal of useful modeling techniques, kinetic modeling of biological systems quickly strays from the oversimplified Monod and Michaelis-Menten expressions. The major difficulty arising from including all known pathways associated with a biochemical event is that numerous chemical species must be included along with appropriate kinetic parameters. The concentrations of these species and parameters associated with their reactions are difficult to measure. Modeling assumptions which limit the number of experimentally determined parameters while not sacrificing qualitative or quantitative accuracy are valuable tools to be used, if possible, when model simplicity is desirable. The cybernetic approach to modeling biological systems utilizes such a tool.

**Cybernetic Modeling**

The cybernetic modeling approach views the microbial cell as an optimal strategist allocating a limited resource in order to maintain some optimal condition. The form of the resource need not be specified explicitly since the instantaneous strategy of optimization requires that the fractional allocation of this hypothetical resource must equal the fractional return from that allocation [3]. Therefore, only the form of the return, i.e., the quantity the cell is assumed to be maximizing needs to be specified. The resource has been hypothesized to be amino acids required for translation of key catabolic enzymes [5] and total transcription time allocated for transcribing genes coding for these enzymes [3]. Explanations of what this hypothetical resource physically represents are interesting, but the simplicity inherent in the cybernetic approach and the matching law result preclude the practical value of such explanations since the cybernetic model does not represent the mechanisms of resource allocation. The actual specification made for what the return will be in the model must be chosen based on experimental observations, and is not an unalterable feature of the cybernetic framework. The specific growth rate is the simplest choice for the return since researchers have repeatedly observed that the substrate supporting the faster growth is utilized preferentially to substrates supporting slower growth rates [7]. In addition, specific growth rate is easy to estimate through optical density measurements and can be accurately represented by a typical Monod form as was shown for the kinetic model;

\[
\frac{dC}{dt} = \mu e_1 v_1 C \left[ \frac{s_1}{K_1 + s_1} \right]
\]  
(8)

\[
\text{ddC} = p.e_1 \nu_1 C \left[ \frac{s_1}{K_1 + s_1} \right]
\]
where the cybernetic variable, $v_1$, represents inhibition/activation of the key enzyme for glucose catabolism, $e_1$, such that $0 \leq v_1 \leq 1$. For example, if $v_1 = 0$, $e_1$ is totally inhibited and if $v_1 = 1$, $e_1$ is at maximum activity. The rate equation for $e_1$ is written as:

$$\frac{de_1}{dt} = \alpha_1 u_1 - e_1 \left( \frac{1}{C} \frac{dC}{dt} \right) - e_1 \beta_1$$

(9)

where $u_1$ controls the induction/repression of $e_1$ synthesis such that $0 \leq u_1 \leq 1$. More specifically, $u_1$ represents the fractional allocation of some limited resource needed for the synthesis of $e_1$ and $u_1$ is defined in terms of a fractional return. The Monod type induction term containing $K_e$ in equation 2 has been replaced by $u_1$ in the cybernetic model. The rate equation for glucose is analogous to the kinetic version with the cybernetic variables $v_1$ and $v_2$ incorporated for implicit activation/inhibition of $e_1$ and $e_2$ respectively.

$$\frac{ds_1}{dt} = -\frac{\mu e_1 v_1 C}{Y} \left( \frac{s_1}{K_1 + s_1} \right) + \nu k_2 e_2 v_2 \left( \frac{s_2}{K_2 + s_2} \right),$$

(10)

The non-competitive inhibition term of equation 5 containing $K_3$ has been replaced by $v_2$. As expected, the induction and repression terms containing the constants $K_{e_2}$ and $K_4$ are replaced by the cybernetic variable, $u_2$, for the rate equation of $e_2$.

$$\frac{de_2}{dt} = \alpha_2 u_2 C - e_2 \beta_2$$

(11)

Previously, the return has been specified as a rate of growth [3]. In the present system, the return is specified as the rate of substrate utilization. The rate of substrate utilization and growth rate are simply related by the cellular yield. Therefore, the rate of growth and rate of substrate utilization are essentially the same when choosing the quantity the cell is assumed to be maximizing. However, if growth rate is chosen, each substrate must have a corresponding maximum specific growth rate. For the glucose-cellobiose system where cellobiose is hydrolyzed extracellularly, the specific growth rate depends only on the glucose concentration. Hence, the rate of substrate utilization was chosen as the form of the return in order to differentiate glucose growth from cellobiose growth. The rate of glucose utilization is defined as:

$$r_1 = \frac{\mu e_1 v_1 C}{Y} \left( \frac{s_1}{K_1 + s_1} \right)$$

(12)

and the rate of cellobiose utilization is defined as:

$$r_2 = k_2 e_2 v_2 \left( \frac{s_2}{K_2 + s_2} \right)$$

(13)

Thus, the fractional returns can be written as:

$$u_1 = \frac{r_1}{r_1 + r_2}$$

(14)

$$u_2 = \frac{r_2}{r_1 + r_2}$$

(15)

The cybernetic variables, $v_i$, which control enzyme activation/inhibition are written as: [8]

$$v_1 = \frac{r_1}{\max_i(r_1, r_2)}$$

(16)

$$v_2 = \frac{r_2}{\max_i(r_1, r_2)}$$

(17)
Simulation Results

Figure 3 shows simulation results from both the kinetic and cybernetic models. Several characteristics of the experimental data are represented. The first is the sequential utilization of first glucose then cellobiose. This results from the explicit glucose inhibition of β-glucosidase in the kinetic model and the implicit inhibition in the cybernetic model. The second feature is the change in the rate of exponential growth at the point of glucose depletion. Since the growth rate depends on only the glucose concentration in both models, eqns 1 and 8, the lower glucose concentration during cellobiose hydrolysis accounts for the lower growth rate. Thirdly, the glucose concentration increases after its initial depletion. Although the modeling assumption of extracellular cellobiose hydrolysis assumes all hydrolysis occurs outside of the cell, the magnitude of the increase for the simulations is lower than from the experimental data. The reason for this discrepancy in the magnitude of glucose accumulation is not clear. Incomplete parameter evaluation could be the major reason. Although it is possible that due to model simplifications, neither the kinetic nor the cybernetic model can accurately represent this aspect of the system in their present form.

Figure 4 shows simulation results from both models for perturbed batch simulations where glucose is suddenly added during exponential growth in the presence of only cellobiose. The experimental features represented are an immediate increase in the rate of exponential growth upon glucose addition, and a decrease in the rate of cellobiose hydrolysis due to inhibition of β-glucosidase by the increased glucose concentration.

The final set of simulations in Figure 5 is for growth on cellobiose only. Both the cybernetic and kinetic simulations show the increase in the glucose concentration presumably due to extracellular cellobiose hydrolysis. These simulations agree fairly well with the experimental data shown in figure 2.

Conclusions

Both the kinetic and cybernetic models contain gross oversimplifications of microbial regulatory mechanisms. Such simplifications are necessary for practical modeling purposes due to the immense complexity of the actual biochemical pathways constituting the regulatory mechanisms.

The distinguishing feature between the kinetic and cybernetic models is that the cybernetic model assigns an invariant strategy to the cell which is assumed to be sought by the cell's regulatory mechanisms at all times. This teleological approach is the basis of all modeling techniques categorized as cybernetic modeling and results in fewer parameters which must be determined experimentally.

In view of the oversimplifications made in both models presented here, there is a danger of creating parameters which become vague in their physical meaning or may not correspond to any phenomenon occurring within the microbial cell. Even in view of such cautions, simulations from both models have indicated a good agreement with the experimental data. Although this agreement is still at a qualitative level, there is little reason to suspect that further parameter evaluations can not render both models qualitatively and quantitatively more accurate.
References


Figure 1: Batch Growth on Glucose and Celllobiose

Figure 2: Batch Experiment with only Celllobiose added
Figure 3: Kinetic and Cybernetic Simulations of Batch Growth Experiment

Figure 4: Kinetic and Cybernetic Simulations for Perturbed Batch Experiment

Figure 5: Kinetic and Cybernetic Simulations with only Cellobiose Added
MASTER EQUATION MODELING AND MONTE CARLO SIMULATION OF PREDATOR-PREY INTERACTIONS

R. O. Fox, Y. W. Huang, and L. T. Fan

Department of Chemical Engineering
Durland Hall
Kansas State University
Manhattan, Kansas 66506

July 1, 1987

INTRODUCTION

The stochastic behavior of predator-prey interactions in a well-mixed chemostat has been studied using numerical (Monte-Carlo) simulations of the controlling birth-death master equation at two different values for a control parameter (holding time). Numerical solutions of the corresponding deterministic equations indicate that certain parameter values will result in trajectories through regions of low prey and/or predator populations. In such regions the stochastic nature of the process should be of great importance since fluctuations in population sizes can lead to extinction of one or both species. By studying the simulation results under two such parameter values, it has been concluded that fluctuations do indeed greatly affect the dynamic behavior of the process, leading to extinction in cases where the deterministic equations indicate stable populations. Generation of a fairly large number (50) of sample paths led to an estimate of the probability of extinction and to observations concerning the relative width of the probability distribution as the populations evolved.

Stochastic Model of Predation

A deterministic model for predation has been reviewed elsewhere by Stephanopoulos and Frederickson (1981). In this section a stochastic model which reduces to the deterministic model for the mean values of prey and predator population will be developed. The stochastic model will be based on the master equation as presented in the monographs of van Kampen (1981) and Gardiner (1983). The master equation is a general stochastic population balance applicable to Markovian systems undergoing linear or nonlinear interactions. In the case of nonlinear interactions, the master equation formulation leads to a straightforward Monte Carlo simulation procedure that can be successfully implemented on a digital computer.

Let $B(t)$ be the random variable denoting the number of prey cells at time $t$ in the chemostat, and $N(t)$, the number of predator cells at time $t$ in the chemostat. Changes in the values of $B(t)$ and $N(t)$ will be modeled as a birth-death master equation governing the following joint conditional probability

$$
Pr\{B(t)=j, N(t)=m \mid B(0)=i, N(0)=n\}
$$
Let this conditional probability be denoted by $P_{j,m}(t)$. If we assume that in the prey-predator problem a change in either $B(t)$ or $N(t)$ will occur independently of a change in the other, i.e., $B(t)$ can change from $j$ to $j+1$ without a change in $N(t)$. The master equation for this birth-death process can be written as (Gardiner, 1983):

$$
\frac{d}{dt} P_{j,m}(t)
= \lambda_{j-1} P_{j-1,m}(t) + \mu_{j+1} P_{j+1,m}(t) - (\lambda_j + \mu_j + \eta_m + \gamma_m)P_{j,m}(t)
+ \eta_{m-1} P_{j,m-1}(t) + \gamma_{m+1} P_{j,m+1}(t)
$$

(1)

with

$$
P_{j,m}(0) = \delta_{j,1} \delta_{m,n}
$$

Following Stephanopoulos and Fredrickson (1981), the specific growth rate of prey cells, $\lambda_j$, will be assumed to be dependent upon the amount of nutrient or feed, $s$, present in the chemostat. This relationship is non-linear; $\lambda_j$ attains its maximum value, $\lambda_{j,\max}$, as $s$ increases. The Monod model is often employed to represent the nonlinearity (Drake et al., 1968, and Bungays, 1968); it is expressed as

$$
\lambda_j = \left[ \frac{\lambda_{\max}s}{K + s} \right] j
$$

(2)

The two mechanisms by which prey cells are lost from the system are: (1), exiting with the outlet stream, and (2), consumption by predator cells. The first of these is analogous to emigration and is modeled as

$$
D_j
$$

where $D$ is the dilution rate. The second depends on the number of prey cells consumed per predator cell produced multiplied by the rate at which predator cells multiply. If the specific growth rate of predator cells, $\eta_{m}$, is again modeled by the Monod model, this can be written as

$$
\eta_m = \left[ \frac{\eta_{\max,j}}{VL + j} \right] m
$$

(3)

the specific death rate of prey cells becomes

$$
\mu_j = \alpha \left[ \frac{\eta_{\max,j}}{VL + j} \right] m + D_j
$$

(4)

Finally, the specific death rate of predator cells, $\gamma_{m}$, corresponding to the rate at which predator cells leave the system, is assumed to be
Substitution of \( \lambda_j, \eta_m, \mu_j, \gamma_m \) into Eq. (1) yields the following master equation:

\[
\frac{d}{dt} P_{j,m}(t) = \left[ \frac{\lambda_{\text{max}}^s}{K + s} \right] (j-1)P_{j-1,m}(t) + \left\{ \alpha \left[ \frac{\eta_{\text{max}}^j}{VL + j + 1} \right] m + D(j+1) \right\} P_{j+1,m}(t)
\]

\[
+ \left[ \frac{\eta_{\text{max}}^j}{VL + j} \right] (m-1)P_{j,m-1}(t) + D(m+1)P_{j,m+1}(t)
\]

\[
- \left[ \frac{\lambda_{\text{max}}^s}{K + s} \right] j + \alpha \left[ \frac{\eta_{\text{max}}^j}{VL + j} \right] m + D(j) + \left[ \frac{\eta_{\text{max}}^j}{VL + j} \right] m + Dm \right\} P_{j,m}(t)
\]

The deterministic variable \( s \) is also a function of time and obeys the ordinary differential equation

\[
\frac{d}{dt} s(t) = Ds_f - Ds - \frac{1}{Y} \left[ \frac{\lambda_{\text{max}}^s}{K + s} \right] J
\]

The deterministic equations governing the mean values of the number of prey and predator cells are

\[
\frac{d}{dt} b = \left[ \frac{\lambda_{\text{max}}^s}{K + s} \right] b - Db - \alpha \left[ \frac{\eta_{\text{max}}^b}{L + b} \right] g
\]

\[
\frac{d}{dt} g = \left[ \frac{\eta_{\text{max}}^b}{L + b} \right] g - Dg
\]

These are the equations used in a previous deterministic study of this system (Stephanopoulos and Frederickson, 1981).

The numerical integration of the equations for \( b, g, \) and \( s \) reveal that three possible steady-states exist (Stephanopoulos and Frederickson, 1981):

1) extinction of both predator and prey cells

2) extinction of predator cells only

3) coexistence of predator and prey cells

Region III is further subdivided into two regions, one where the populations reach constant values, and the other where oscillations are sustained (limit cycle). In the limit-cycle region the population of prey cells varies from \( 10^7 \) cells to a minimum near zero. If the parameters are chosen correctly,
the minimum can be pushed closer and closer to zero, but will always cycle back to \(10^7\) cells. Since the population growth is stochastic and the number of cells is an integer value, when the prey population is near zero there is a probability that the population will become extinct, thus ending the limit cycle. The predator population will also become extinct and the new steady-state will be extinction of both prey and predator. Therefore, to determine the frequency of extinction, the random variables \(B(t)\) and \(N(t)\) can be studied for operating parameters where the limit cycle approaches low prey population values. The complexity of the master equation for this process prohibits its solution for an analytical expression. Consequently, we shall resort to numerical simulation using a random number generator to construct sample paths. The desired extinction probabilities can be estimated from the resultant sample paths.

Results of Simulation

The parameters used in the simulation are as follows:

\[
\begin{align*}
\lambda_{\text{max}} &= 1.3043 \text{ hr}^{-1} \\
K &= 0.002 \text{ mg/ml} \\
\eta_{\text{max}} &= 0.20261 \text{ hr}^{-1} \\
\text{VL} &= 10^6 \text{ cells} \\
Y &= 10^8 \text{ cells/mg} \\
\alpha &= 10^4 \text{ cells of prey/predator cell} \\
\text{sf} &= 0.10 \text{ mg/ml}
\end{align*}
\]

These parameters roughly correspond to those used by Stephanopoulos and Fredrickson (1981) in their Figure 2. There it is noted that similar values can be used to model the feeding of *Tetrahymena pyriformis* on *Azotobacter vihelandii* as done by Jost (1972).

The parameter \(D\) is a free parameter whose value determines the steady-state region (I,II,III) in which the system operates. In this study the two values chosen for \(D\) are 0.0435 \text{ hr}^{-1} and 0.926 \text{ hr}^{-1}. The former corresponds to the limit cycle region, region III, while the latter corresponds to region II (Stephanopoulos and Fredrickson, 1981).

**Region II:** \(D = 0.926 \text{ hr}^{-1}\)

Fifty sample paths have been generated beginning with \(N(0)\) and \(B(0)\) equal to 100 cells; the results are shown Figure 1, where several sample
paths are illustrated. All other paths lie among these sample paths; concentrated near the center of the region. Each sample path is constructed by connecting the maximum and minimum numbers of prey cells at a given number of predator cells. The solid line represents the solution to Eqs. (7), (8), and (9) for the deterministic representation of this system. The number of predator cells converges rapidly to zero while the number of prey cells continues to grow until the growth is constrained by lack of feed. The simulation indicates that the lowest value of the prey population when the predator population first becomes extinct is 124, while the highest is 513; the difference between them is 389. To obtain an estimate of the spread of the probability distribution, we can examine the distance of the dashed lines from the solid line along lines normal to the solid line. At the maximum (prey = 108, predator = 33) the distance above and below averages to approximately 45. However, to find a more accurate estimate of the width of the distribution, it would be necessary to plot all sample paths (and to generate many more) in order to determine the relative probability of finding sample paths with a distance from the mean. It would also be necessary to find the mean value since it does not necessarily correspond to the deterministic value.

Despite the limited number of sample paths generated, it is interesting to note that within a short distance from the starting point the number of prey cells varies widely from the starting value of 100 even though the deterministic solution drops quickly to low predator values before the prey cells begin to increase. This results from the fact that although the net change in the number of prey cells in this region is approximately zero, the birth and death rates are rather large and equal in magnitude; this results in large variances in the population sizes. Note that in the simulation, the initial variance is equal to zero since all paths begin at the same point. It would also be interesting to begin the simulation with a larger population, size to allow the variance to reach its steady-state value before the predator cells become extinct. Such simulations would most likely lead to larger variances, perhaps resulting in prey populations near extinction even though the deterministic value is near 100.

Region III: \[ D = 0.0435 \text{ hr}^{-1} \]

In this simulation, fifty sample paths have again been generated beginning with \( N(0) \) and \( B(0) \) equal to 1000. From this starting point, the deterministic solution indicates that the prey population will drop very rapidly, with the predator population following slowly, passing a low point of about 16 prey cells and 610 predator cells before it increases rapidly again. By beginning the simulation at 1000 in each population, the initial conditions will have little effect on the variances in the region of low prey populations; this requires that more than 25,000 random numbers be generated for each sample path. Figure 2 shows a few representative sample paths plus extrema for the region between 800 and 400 predator cells. After this region the number of prey cells increases rapidly, and no extinction of the prey population is found even though one sample path crosses the 400 axis at a value of six prey cells before growing beyond the range of extinction.
In Figure 2, the solid line indicates the deterministic value of the controlling equations found by numerical integration using the same initial values. The dashed lines indicate the approximate width of the probability distribution as determined from the fifty sample paths generated. The axis where the prey population is equal to zero acts as an absorbing boundary. When a sample path touches this axis, the prey population becomes extinct, and the sample path will then continue, without increase, down the axis until the predator population is also extinct.

For simulations of long duration, the populations would pass along the solid line, from the left to the right, in Figure 2, arbitrarily many times. Nevertheless, each time a probability exists that both populations may become extinct. In this simulation fifty such simulated passes. For large values of the prey population \((10^7)\) the random process will show very little relative variance and thus pass near the starting point of the simulation on each cycle. By determining the number of passes resulting in extinction out of the total fifty passes, we can estimate the probability of extinction for these operating parameters. Here, 21 sample paths have resulted in extinction. Thus, we can estimate the probability of extinction during any given cycle to be 0.42. To obtain a more accurate estimate, additional sample paths would be necessary. Of interest is the fact that the extrema in the predator population at which extinction has occurred are 695 for the high and 524 for the low. At these points the deterministic value for the prey population is approximately 21. From this result we can roughly conclude that the probability of extinction will be of increasing importance for operating parameters yielding limit cycles whereby the prey population falls somewhere below 25. This, however, is a rough estimate since the length of the interval will also be important (here 175). The longer the interval, the more likely the process will become extinct. This estimate will also be dependent on the parameter values chosen since the variance may be larger or smaller with different choices.

To find the probability of extinction after a given number of cycles, \(M\), it suffices to note that each cycle will be essentially independent; the desired probability for the present simulation is thus equal to \(1 - 0.42^M\). As \(M\) increases the probability of extinction goes to one; this is true as long as the probability of extinction on given cycle is greater than zero. Note, however, that if the latter is nonzero but small, the number of cycles which the system may exhibit before extinction can be very large. Experimental observation of extinction in such cases would require long operating periods which may not be realizable. This fact may be the reason why extinction has not been reported in the literature (Stephanopoulos and Fredrickson, 1981).

Using the dashed lines in Figure 2, we can make some observations about the variance of the probability distribution as the process travels around the limit cycle. First, it seems apparent that the distribution is spread out farther above the deterministic value than below. This would indicate a non-Gaussian distribution, with a higher probability of finding a sample path a given distance inside the limit cycle than at the same distance outside. Since most linear processes exhibit a Gaussian distribution, the skewness of this distribution may be a result of the nonlinearities in the
birth and death rates; however, these observations must be seen as approximate since a relatively small number of paths was generated.

It may be safer to make some conclusions about the variance at different points along the predator axis; the most obvious such observation is that the variance is minimum at approximately the same point as where the deterministic value for the prey reaches a minimum (predator = 610). Before this point, the variance decreases slowly, after this point, the variance increases rapidly as the prey population quickly increases. For the sample paths generated, the maximum size of the prey population for a predator population of 800 is 107, and the maximum size for a predator cell of 400 is 833 (not the same path). The deterministic values at these points are 44 and 142, respectively. The difference in the maximum and the deterministic value at the lower end point is thus almost 700. A more accurate assessment of the width can be made by measuring the normal distance of the dashed line from the solid line. At its maximum, this distance reaches approximately 120, representing a wide variation in the sample paths. This again may be a result of the nonlinear dependence of the growth rates of the populations.

ACKNOWLEDGEMENTS

This material is based upon work supported under a National Science Foundation Graduate Fellowship awarded to the first author.

NOTATION

\[
\begin{align*}
  b & = \text{mean number of prey cells per unit volume} \\
  B(t) & = \text{number of prey cells at time } t \\
  D & = \text{dilution rate} \\
  g & = \text{mean number of predator cells per unit volume} \\
  j & = \text{value of random variable } B(t) \\
  K & = \text{saturation constant} \\
  L & = \text{growth parameter} \\
  m & = \text{value of random variable } N(t) \\
  N(t) & = \text{number of predator cells at time } t \\
  P_{j,m}(t) & = \text{joint probability for } B(t) \text{ and } N(t) \text{ as discrete variables} \\
  s & = \text{feed concentration} \\
  s_f & = \text{inlet feed concentration} \\
  V & = \text{volume of chemostat} \\
  Y & = \text{number of prey cells produced per unit mass of feed consumed}
\end{align*}
\]

Greek Letters

\[
\begin{align*}
  \alpha & = \text{number of prey cells consumed per predator cell produced} \\
  \gamma_m & = \text{specific death rate of predator cells} \\
  \eta_m & = \text{specific birth rate of predator cells} \\
  \lambda_j & = \text{specific birth rate of prey cells} \\
  \mu_j & = \text{specific death rate of prey cells} \\
  \lambda_{\text{max}} & = \text{specific maximum growth rate of an individual prey cell}
\end{align*}
\]
\[ \eta_{\text{max}} = \text{specific maximum growth rate of an individual predator cell} \]

REFERENCES


Figure 1. Simulation results for $D=0.926 \text{ hr}^{-1}$.
Figure 2. Simulation results for $D=0.0433 \text{ hr}^{-1}$. 
INTRODUCTION

Glucoamylase is one of the most useful of industrial enzymes, catalyzing the production of D-glucose from maltooligosaccharides formed from starch by \( \alpha \)-amylase-catalyzed hydrolysis. However, at the high solids contents used in industrial processes the yield of D-glucose is not complete, as a mixture of oligosaccharides is formed. There has been a longstanding controversy over the cause of the reactions that produce these products. One possibility is that they are catalyzed by transglycosylases that contaminate the Aspergillus niger glucoamylase preparations used industrially. Another possibility is that the glucoamylase itself can catalyze condensation reactions starting from D-glucose. A further controversy has been over the identity of the oligosaccharides that are formed. While virtually all previous research has suggested that the main products are isomaltose, isomaltotriose, and maltose, there has been wide disagreement over other products, caused partly by the different analytical methods employed to identify them.

To answer these questions, a long investigation has been conducted in our laboratory, using extremely sensitive gas chromatographic (GC) methods that we developed to analyze disaccharides (1) and trisaccharides (2). This work, the subject of most of two doctoral dissertations (3,4), has led to the following conclusions: 1) Glucoamylase, when purified free of transglycosylases and other amylases, is capable of catalyzing condensation reactions using D-glucose as a starting material; 2) The chief products of these condensation reactions, in descending order, are isomaltose, isomaltotriose, kojibiose, nigerose, maltose, \( \alpha, \beta \)-trehalose, and panose. In support of these conclusions the glucoamylase active center was subsite-mapped with malto- and isomaltooligosaccharides (4), kinetic data were gathered for both hydrolysis (4) and condensation (3) reactions, and equilibrium data were obtained for all products obtained from D-glucose (3). A model incorporating all reactions was formulated, and subsite affinities and hydrolysis and equilibrium data were fed into it to generate predicted condensation data (3). These predicted data closely matched experimental condensation data (3).

The work to be reported in this paper is a direct continuation of that just described. It is an investigation of the ability of Aspergillus niger glucoamylase freed of transglycosylases and other amylases to catalyze the formation of condensation products from aqueous mixtures of D-glucose and a second monosaccharide. Six other monosaccharides, D-arabinose, D-fructose, D-galactose, myo-inositol, D-mannose, and D-xylose, were used. First, inhibition constants were obtained for D-arabinose and myo-inositol in the
presence of maltose to supplement already available data for D-galactose, D-mannose, and D-xylose (5). Then kinetic and equilibrium data were obtained for the main products of each of the six reacting systems, and a start was made toward the identification of the products from each. When all major products are identified, the specificity of the first two glucoamylase subsites for the seven different monosaccharides tested in this work should be clear, as should the ability of glucoamylase to form different bonds between D-glucose and each of the other six monosaccharides.

MATERIALS AND METHODS

Glucoamylase Source and Purification

Purified glucoamylase was obtained from a Diazyme preparation donated by Dr. J. Shetty (Miles Laboratories, Elkhart, IN). The raw material was dissolved in 0.025M sodium citrate-sodium phosphate buffer, pH 7.9, filtered, and passed through a 850-mm x 26-mm i.d. DEAE-Sephadex column, using 1 mL/min of a decreasing linear pH gradient made of equal amounts of the same buffer, except at 0.05M, at pH 7.9 and pH 3.2 as eluent. Four major protein peaks, measured at 280 nm, were eluted from the column, with the middle two being glucoamylase II and glucoamylase I (6,7). The latter, by far the largest peak, was collected, desalted, and concentrated with an Amicon 10 PM10 membrane. It was tested for homogeneity by ultrathin-layer isoelectric focusing (8,9) with a pH 3-10 ampholyte and by ultrathin-layer sodium dodecyl sulfate polyacrylamide gel electrophoresis (10). Visualization of the protein in both cases was by silver staining (11). The first procedure yielded one band with a pI of 3.5, while the second gave one major band and three lighter bands, evidently caused by sample preparation (2), at higher molecular weights. No band corresponding to transglycosylase or to any amylase other than glucoamylase was observed.

Other Materials

D-arabinose was obtained from Pfanstiehl (Waukegan, IL), D-fructose from Fisher (Fair Lawn, NJ), D-galactose, D-glucose, myo-inositol, D-mannose, and D-xylose from Sigma (St. Louis, MO), and maltose from Baker (Phillipsburg, NJ).

Reaction Studies

Reactions were conducted in agitated 5-mL Pierce (Rockford, IL) Reacti-Vials at 35°C (inhibition studies) or 45°C (condensation studies).

For determination of inhibition constants, glucoamylase I was added to varying concentrations of maltose and 0.2M of D-arabinose or myo-inositol dissolved in 0.05M acetate buffer, pH 4.5, to obtain total volumes of 4 mL. Seven 0.4-mL samples were taken from each vial in 4-min intervals and the reaction was stopped by pipetting them into 0.1-mL aliquots of 5M TRIS, pH 7.1 (12). Glucose was determined by the addition of 1 mL glucose oxidase solution to each sample, followed by incubation at 37°C for 1 h, addition of 2 mL of 7M HCl, and measurement of absorbance at 525 nm (13). Kinetic parameters were calculated by a nonlinear regression routine, SAS NLIN (SAS Institute, Cary, NC).
For determination of condensation kinetics and equilibria, glucoamylase I was added to 4 mL of a solution of 15% (w/v) glucose and 45% (w/v) of the other monosaccharide (20% for myo-inositol) dissolved in the same buffer. Samples of 0.03 mL were taken at various time intervals, pipetted into 0.006 mL of a 0.2M NaOH solution to stop the reaction, freeze-dried, and trimethylsilylated in pyridine (1). Sucrose was added as an internal standard. Derivatized samples were analyzed with a Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph coupled to a Hewlett-Packard 3492A integrator, using a 30-m x 0.26-mm i.d. fused silica capillary column (J & W Scientific, Rancho Cordova, CA) coated with a 0.1-μm film of DB-5 liquid phase. Helium at 0.24 m/s was used as the carrier gas. Nitrogen, the makeup gas, flowed at 30 mL/min, while air and hydrogen flowed to the flame ionization detector at 300 mL/min and 30 mL/min, respectively. Column temperature was 217°C for 31 min, then increased at 10°C/min to 250°C, where it remained for 10 min. Flame ionization detector and injector temperatures were 280°C and 270°C, respectively. Initial disaccharide formation rates were calculated by fitting the experimental data to the equation \( c = \frac{at}{1 + bt} \), where \( c \) is disaccharide concentration and \( t \) is elapsed time, and evaluating the first derivative at \( t = 0 \).

**Product Separation**

The remaining product solution from the condensation reactions between D-glucose and D-arabinose and between D-glucose and D-mannose was subjected to descending paper chromatography (14) by applying 0.1-mL samples to 550-mm long, 300-mm wide Whatman 3MM paper sheets and eluting with 80% (v/v) n-propanol:20% (v/v) water for 18 h at 70°C. Fractions containing disaccharides were cut out, eluted with 10 mL water, freeze-dried, dissolved in 1 mL water, and filtered. The disaccharides were separated from each other by high-performance liquid chromatography (HPLC) with a Dionex (Sunnyvale, CA) special-order Ion Pac preparative column (ref. 264-86-941), using Johnson's method (15). The HPLC system consisted of an Erma (Tokyo, Japan) ERC-3510 solvent degasser, an ISCO (Instrumentation Specialties, Lincoln, NE) Model 2360 gradient programmer, and an Erma ERC-7510 refractive index detector. The eluent, a 60mM Ba(OH)_2 solution, was degassed by aspiration and submersion in an ultrasonic bath and filtered before use to remove BaCO_3 that formed upon exposure to air. Eluent flow rate was 2 mL/min, while sample size was 0.02 mL. Fractions were collected, neutralized with 0.1M H_2SO_4, and centrifuged, with the supernatant being freeze-dried.

In an attempt to identify the components of the fractions, which were not necessarily pure, the latter were subjected to GC in four states: as is, after hydrolysis, after reduction, and after reduction and hydrolysis. Hydrolysis was carried out by dissolving the disaccharides in 1% (v/v) aqueous trifluoroacetic acid and holding them under nitrogen for 90 min at 130°C. Reduction occurred by dissolving each fraction in 1 mL water, adding a 15% molar excess of a 1% (w/v) aqueous NaBH_4 solution, and holding the mixture in boiling water for 10 min. After cooling the reduced fraction, 1 mL of a 10:1 (v/v) CH_3OH:HCl solution was added to it to form a volatile borate-methanol complex (16) and the solution was evaporated to dryness at 40°C. Addition of CH_3OH and evaporation was repeated six times, and was followed by freeze-drying. GC of the hydrolyzed and reduced samples was conducted as previously described, except that the initial temperature was 160°C instead of 217°C so that monosaccharides and alditols could be
determined.

In a further attempt to identify condensation products, samples after HPLC separation were sent to Dr. Morey Slodki of the USDA Northern Regional Research Center, Peoria, IL, for permethylation analysis.

The freeze-dried samples were dissolved in water and reduced with sodium borodeuteride (17). The latter was decomposed with cation exchange resin, and residual boric acid was removed by dissolution in methanol and evaporation in vacuo. The dried product was dissolved in dimethyl sulfoxide under N2, and Hakomori's reagent (18) and iodomethane were added. The reaction mixture was partitioned between chloroform and water, with the chloroform layer dried by molecular sieve and then vacuum evaporation. The residue was hydrolyzed with trifluoroacetic acid, and the excess of the latter was removed by evaporation and passage through a column packed with anion exchange resin. Combined water and methanol column washes were evaporated to dryness, and the residue was derivatized with acetic anhydride in hydroxylamine/pyridine (19) to give, for each dihexosyl disaccharide initially in the sample, a tetra-0-methyl-per-0-acetylaldononitrile and a 1-d1-penta-0-methylalditol monoacetate. Derivatized samples were subjected to GC on methyl silicone fused-silica capillary columns. Peaks were identified by comparison with the retention times of authentic samples and by mass spectroscopy.

RESULTS AND DISCUSSION

Inhibition of Hydrolysis Reactions by D-Arabinose and myo-Inositol

When varying concentrations of maltose between 0.25mM and 4mM were incubated with glucoamylase I at 35°C and pH 4.5, a Michaelis constant (Km) of 1.14mM ± 0.08mM, the second value being the standard deviation, was obtained. When maltose at these concentrations was individually incubated under the same conditions with glucoamylase I and 0.2M of D-arabinose or myo-inositol, inhibition by the latter two was competitive. Values of Km for maltose were 1.11mM ± 0.07mM with D-arabinose inhibition and 1.17mM ± 0.07mM with myo-inositol inhibition, not significantly different from the value determined without inhibition or from the value of 1.23mM ± 0.19mM determined earlier by Meagher (4). Inhibition by D-arabinose and myo-inositol gave Ki values of 0.112M ± 0.007M and 0.428M ± 0.050M, respectively. These may be compared with the Ki values of 0.22M, 0.16M, and 0.23M found for D-galactose, D-mannose, and D-xylose inhibition, respectively, by Hiromi et al. (5), using p-nitrophenyl-α-D-glucoside as substrate.

Kinetics and Equilibria of Condensation Reactions

When each of the other six monosaccharides was incubated alone with glucoamylase I, no condensation products were formed. However, each of the other six formed condensation products when incubated with D-glucose and glucoamylase I. The only diglucosyl disaccharide detected was isomaltose. When D-glucose at concentrations of 25% and above was incubated alone with glucoamylase, maltose was the fastest formed product (3), but it was not detected in this work when only 15% D-glucose was initially present. This was expected, as equilibrium concentrations of maltose are much lower than
those of isomaltose at all initial D-glucose concentrations, and as the latter decreased, maltose would become undetectable.

In all, incubations of D-glucose with D-arabinose yielded six quantifiable GC peaks. With D-fructose five were formed, with D-galactose twelve, with myo-inositol nine, with D-mannose ten, and with D-xylose seven. Since in nearly all cases two peaks represent the anomic forms of a single disaccharide, the total number of condensation products is half (or slightly above half) of the total number of peaks. In all six monosaccharides the fastest forming two peaks by far were those of α- and β-isomaltose, signifying that glucoamylase is highly specific for D-glucosyl residues in the two subsites about the catalytic site. The ratio of the initial rates of formation of α- and β-isomaltose in all cases was in agreement with their equilibrium concentrations, which indicated that the two anomers reached mutarotation equilibrium as they were being formed. In addition, the variation of initial rates of isomaltose formation among the six incubations could be roughly correlated with the inhibition constant of the second monosaccharide.

While isomaltose is produced much faster than any other condensation product, it is not always overwhelmingly favored at equilibrium. With incubations containing D-arabinose, three other peaks reached at least 25% the equilibrium concentration of β-isomaltose, the predominant anomer. With D-fructose, all three other peaks reached this threshold, with D-galactose five, with myo-inositol only one, with D-mannose seven (including two that were much larger than the isomaltose peaks), and with D-xylose two. It is to be expected that some of these large peaks represent disaccharide anomers that are linked through a hydroxyl group attached to a primary carbon atom, as this is thermodynamically favored.

Identification of Products

Paper chromatography of the mixtures that resulted from reactions between D-glucose and D-arabinose and between D-glucose and D-mannose yielded three carbohydrate bands for the first system and four for the second. In both cases the fastest moving band contained monosaccharides, the second contained condensation products, and the others had materials that were not identified as condensation products by GC.

Separation by preparative anion exchange HPLC of the collected paper chromatographic bands yielded five peaks for the D-glucose/D-arabinose system, of which the last four had condensation products. One of the HPLC peaks (A2) had a single peak by GC, two (A3 and A5) had two peaks, and the fourth (A4) had four, of which two were the isomaltose anomers. Six peaks, four that could be collected, were observed upon HPLC of the D-glucose/D-mannose system. The middle two of the four collected had condensation products. One of these peaks (M2) had two peaks by GC, while the other (M3) had four, two being the isomaltose anomers.

Portions of each of the six collected peaks were separately or sequentially reduced and hydrolyzed. Peak A2 could not be reduced; when hydrolyzed
it gave approximately equal amounts of D-arabinose and D-glucose by GC. Reduc-
tion followed by hydrolysis yielded D-glucose and D-arabinose in a 1.4:1 ratio, but neither D-arabitol nor D-sorbitol, suggesting that the major prod-
uct in A2 was a nonreducing D-glucosyl-D-arabinoside.

Peak A3 yielded one GC peak after reduction, while hydrolysis gave
D-glucose and D-arabinose in a 0.7:1 ratio. Reduction and hydrolysis pro-
duced D-glucose and D-arabitol in a 4:1 ratio. Despite the product distri-
bution favoring the aldose over the alditol, a common occurrence, the
predominant disaccharide in A3 appears to be a D-glucosyl-D-arabinoside.

Peak A4 upon reduction gave two GC peaks. Reduction yielded D-glucose
and D-arabinose at a 20:1 ratio, caused by the high amount of isomaltose
initially present. Reduction and hydrolysis led to D-arabitol, D-glucose,
and D-sorbitol, but no D-arabinose, with the middle two being the largest and
having a ratio of approximately 1:1 as expected. Therefore the material
present in A4 in higher quantity was isomaltose, with the other being a
D-glucosyl-D-arabinoside.

The reduction of Peak A5 resulted in one peak, while hydrolysis yielded
very low concentrations of D-glucose and possibly D-arabinose. Reduction
followed by hydrolysis led to D-glucose and D-arabitol, but neither
D-sorbitol nor D-arabinose, suggesting that the disaccharide in A5 was a
D-glucosyl-D-arabinoside.

Peak M2 gave one GC peak when reduced. When it was hydrolyzed, D-glucose
and D-mannose in a 1.2:1 ratio appeared. Reduction and hydrolysis
yielded D-glucose and D-mannitol at an 8:1 ratio, but neither D-sorbitol nor
D-mannose. This indicates that the predominant product is a D-glucosyl-D-
mannose.

Reduction of Peak M3 gave three GC peaks, one for isomaltitol, one for a
nonreducible material whose equilibrium concentration was 60% that of isoma-
tose, and one derived from a peak representing a single anomer with an equil-
librium concentration 1.4 times that of isomaltose. M3 after hydrolysis
yielded D-glucose and D-mannose in a 0.9:1 ratio. Reduction and hydrolysis
led to a large amount of D-glucose and smaller amounts of D-mannose, D-manni-
tol, and D-sorbitol, leaving the composition of the four main components of
M3 unclear.

Peaks A2, A3, A4, M2, and M3 were subjected to permethylation analysis
at the Northern Regional Research Center by the procedure already described.
Peak A2 appeared to contain 5-O-D-glucopyranosyl-D-arabinofuranose, which
should be reducing, contrary to the evidence provided by reduction and
hydrolysis experiments. Peak A3 was composed largely of 2-O-D-glucopyranos-
yl-D-arabinose. No good results came from the permethylation of Peak A4.
Peak M2 contained a mixture of products requiring further interpretation.
Peak M3 was composed largely of 4-O-D glucopyranosyl-D-mannose, with a
smaller amount of 3-O-mannopyranosyl-D-glucose. It appears that either a
glucopyranosyl or a mannopyranosyl residue was linked through its anomeric
hydroxyl group to the 3-hydroxyl of a pentosyl residue. Surprisingly, there
is no evidence of isomaltose, which GC showed to be present. It is obvious
that the composition of this peak is yet unclear, and that more work is
necessary on it.
FUTURE WORK

Not only must Peaks A2, A4, M2, and M3 be completely identified, but so must the various peaks from the condensation of D-glucose with D-fructose, D-galactose, myo-inositol, and D-xylose. The mixtures produced by these reactions are at present being separated. Further work not covered in this paper dealt with the condensation of D-glucose with D-lyxose and D-ribose (20), and the products of these reactions are also in the process of separation.

ACKNOWLEDGEMENTS

The author thanks Dr. M. E. Slodki for his work in identifying the reaction products and Dr. J. Shetty for the gift of glucoamylase. This work was supported by the Engineering Research Institute, Iowa State University.

REFERENCES

10. Application Note 320, LKB-Producter AB, Bromma, Sweden, 1981.


Metalworking fluids are used extensively in the metalworking industries to primarily perform two functions viz. the removal of heat from the metal surface and to minimize the friction at the metal site. Semi-synthetic metalworking fluids are chemical emulsions containing some oils, whereas the synthetic metalworking fluids contain no oil and are true solutions of complex organics in water. These metalworking fluids have successfully replaced the conventional oil in water emulsions. The addition of various chemical agents to the semi-synthetic and synthetic metalworking fluids renders them superior in terms of rust protection, stabilization, increased tool life, reduction of surface tension, and extreme pressure lubrication.

From the perspective of high initial costs of these fluids, they are required to serve for extended periods in the industry. It has been observed that bacterial growth is responsible for the deterioration of fluid quality over a period of time. An effort, therefore, has been made to use glycols, blending agents, humectants and germicides for the control of bacterial growth. Nevertheless, increased bio-resistance presents a considerable hardship in terms of disposal of the waste emulsions. The existing wastewater treatment processes are incompatible to the synthetic metalworking fluids because they contain little or no oil, but only water soluble organic compounds. Thus, their removal from the wastewater stream cannot be accomplished by the processes for removing oils. This leads to increased BOD and COD levels in the effluent. It therefore becomes imperative to either remove the non-biodegradable component of the used emulsion prior to disposal or to explore the feasibility of successfully performing biodegradation of the waste emulsion.
PURPOSE

An experimental study was undertaken on the laboratory scale to study the biodegradation of thirteen different metalworking fluids obtained from five different sources (Table 1). The primary aim of the study was to determine the biodegradability of these fluids, in general, and the most favorable conditions for biodegradation. The present biodegradation studies also included the adaptation of mixed cultures.

BATCH EXPERIMENTS

Experiments were carried out in 500 ml Erlenmeyer flasks with heterogeneous microbial populations of sewage origin and with metalworking fluids, each containing a synthetic medium as the inorganic nutrient source. The system parameters measured include biomass concentration in terms of the dry weight, chemical oxygen demand (COD), biological oxygen demand (BOD), pH, dissolved oxygen and temperature. The experiment was continued for a period of over 40 days with the shake flasks maintained at 175 rpm (Table 2).

The dilution effect was studied by selecting Sample no. 4 as the growth limiting substrate. This sample was fed to the shake flasks at concentrations of 1%, 0.8%, 0.5%, and 0.2%. Biodegradation was carried out for 55 days (Table 3).

The effect of adaptation was investigated by harvesting a portion of the mixed culture at the end of the 55th day from the flask used in the study of the dilution effect. This portion of mixed culture was employed for the degradation study, of Sample no. 4.

Sample no. 2 was selected to study the effect of pH on biodegradation. The experiment was carried out in four, one liter fermentors (LH 500 series). The pH was maintained with 0.1 N sulfuric acid and 0.1 N sodium hydroxide to the culture vessel using peristaltic pumps. The stirrer speed was maintained at 700 rpm, the air flow rate at 1.0 vvm and the temperature at 24°C. The pH was controlled at 6.0, 7.5, 8.0 and 9.0 in the four fermentors.
CONCLUSIONS

From the above mentioned experiments on biodegradation of metalworking fluids the following conclusions were arrived at.

1. The results of the biodegradation studies show that more than half of the samples of metalworking fluids selected (8 of 13) are readily biodegradable with mixed populations of sewage origin. COD removal efficiencies of these metalworking fluids ranged from 59% to 90% in the experiments conducted.

2. In the experiments conducted samples 8, 9, 10, 12 and 13 are not easily biodegradable. COD reduction of these samples ranged from 15% to 40%.

3. Occurrence of multiple lag phases is fairly common in the biodegradation of metalworking fluids - containing multiple carbon source - with mixed microbial populations.

4. The studies involving adaptation have revealed that the extent of initial lag for adapted microorganisms of sewage origin in batch culture is smaller than for unadapted mixed populations of sewage origin. However, it has been observed that the adapted microorganisms have the same ability to degrade the synthetic metalworking fluid as the unadapted microorganisms. This suggests that microbial strains with degradative capabilities would be generated in the laboratory with continuous culture techniques(Fig. 1). This conclusion is further strengthened by observing similar kind of behavior in the preliminary studies conducted, involving the biodegradation of samples 3 and 11.

5. The study of dilution effect suggests that the concentration of substrate should be kept below 1% (v/v), i.e. between 3000 and 6000 mg/lit. of COD(Fig. 2).

6. The studies of the effect of pH indicates that it should be adjusted between 7.5 and 8.0 with any inorganic acid or base for optimum biodegradation operation(Fig. 3).

ACKNOWLEDGEMENTS

This work was made possible through the financial support of Advanced Engineering Staff, General Motors Corporation, Warren, Michigan. Thanks are also due to Dr. P. N. Mishra of General Motors Corporation for his assistance.
REFERENCES


Table 1. Initial substrate concentration of samples.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Manufacturer's Name</th>
<th>Product Name</th>
<th>Color</th>
<th>COD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cincinnati Milacron**a</td>
<td>CM A</td>
<td>colorless</td>
<td>8940</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>CM B</td>
<td>colorless</td>
<td>5990</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>CM C</td>
<td>white</td>
<td>29360</td>
</tr>
<tr>
<td>4</td>
<td>2V Industries**b Mach-3 Synthetic</td>
<td></td>
<td>colorless</td>
<td>8900</td>
</tr>
<tr>
<td>5</td>
<td>E.F. Houghton**c</td>
<td>Product A</td>
<td>blue</td>
<td>13450</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>Product B</td>
<td>green</td>
<td>11580</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>Product C</td>
<td>yellow</td>
<td>7130</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>Product D</td>
<td>yellow</td>
<td>15150</td>
</tr>
<tr>
<td>9</td>
<td>Int.Refining&amp;Mfg.**d</td>
<td>IRMCO 141</td>
<td>colorless</td>
<td>9150</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>IRMCO 156</td>
<td>colorless</td>
<td>8670</td>
</tr>
<tr>
<td>11</td>
<td>Van Straaten Chem.**e</td>
<td>826</td>
<td>green</td>
<td>9920</td>
</tr>
<tr>
<td>12</td>
<td>&quot;</td>
<td>902</td>
<td>blue</td>
<td>8210</td>
</tr>
<tr>
<td>13</td>
<td>&quot;</td>
<td>930</td>
<td>blue</td>
<td>9150</td>
</tr>
</tbody>
</table>

Note:
- After 1% (v/v) dilution and pH adjustment
- Address of the manufacturers
  a. 4701 Marburg Ave., Cincinnati, OH 45209
  b. 48553 West Road, Wixon, MI 48096
  c. Unknown
  d. 2117 Greenleaf St., Evanston, IL 60202
  e. 630 W. Washington Blvd., Chicago, IL 60606
## Table 2. BOD and COD values of the samples.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>BOD$_5$ (mg/L)</th>
<th>BOD$_{20}$ (mg/L)</th>
<th>Initial COD (mg/L)</th>
<th>Final COD (mg/L)</th>
<th>Sampling Time (days)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>660</td>
<td>3160</td>
<td>940</td>
<td>2770</td>
<td>40</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>1020</td>
<td>2030</td>
<td>5930</td>
<td>1770</td>
<td>40</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>6840</td>
<td>12300</td>
<td>29360</td>
<td>3030</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>590</td>
<td>2160</td>
<td>8900</td>
<td>3170</td>
<td>55</td>
<td>64</td>
</tr>
<tr>
<td>5</td>
<td>660</td>
<td>3480</td>
<td>13450</td>
<td>3030</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
<td>6</td>
<td>1200</td>
<td>3450</td>
<td>11580</td>
<td>4790</td>
<td>55</td>
<td>59</td>
</tr>
<tr>
<td>7</td>
<td>420</td>
<td>1920</td>
<td>7130</td>
<td>1770</td>
<td>55</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>840</td>
<td>2820</td>
<td>15150</td>
<td>11600</td>
<td>40</td>
<td>23</td>
</tr>
<tr>
<td>9</td>
<td>660</td>
<td>2030</td>
<td>9150</td>
<td>5450</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>840</td>
<td>8670</td>
<td>6270</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td>11</td>
<td>1500</td>
<td>3310</td>
<td>9920</td>
<td>2130</td>
<td>40</td>
<td>79</td>
</tr>
<tr>
<td>12</td>
<td>720</td>
<td>1080</td>
<td>8210</td>
<td>6600</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>13</td>
<td>1140</td>
<td>1620</td>
<td>9150</td>
<td>7790</td>
<td>40</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 3. Experimental data for dilution effect.

<table>
<thead>
<tr>
<th>Sampling Time (hour)</th>
<th>Biomass Concentration (O.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8900* (mg/L)</td>
</tr>
<tr>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>18</td>
<td>0.005</td>
</tr>
<tr>
<td>24</td>
<td>0.015</td>
</tr>
<tr>
<td>27</td>
<td>0.020</td>
</tr>
<tr>
<td>29</td>
<td>0.040</td>
</tr>
<tr>
<td>36</td>
<td>0.150</td>
</tr>
<tr>
<td>39</td>
<td>0.200</td>
</tr>
<tr>
<td>42</td>
<td>0.260</td>
</tr>
<tr>
<td>45</td>
<td>0.310</td>
</tr>
<tr>
<td>49</td>
<td>0.365</td>
</tr>
<tr>
<td>51</td>
<td>0.460</td>
</tr>
<tr>
<td>60</td>
<td>0.480</td>
</tr>
<tr>
<td>63</td>
<td>0.500</td>
</tr>
<tr>
<td>69</td>
<td>0.500</td>
</tr>
<tr>
<td>72</td>
<td>0.430</td>
</tr>
</tbody>
</table>

Note:

* Initial substrate concentration.
Fig. 1. Growth and substrate removal for mixed population in raw sewage (solid lines) and developed culture (dotted lines) growing on sample no. 4.
Fig. 2. Effect of dilution on the specific growth rate of mixed populations in batch culture with sample no. 4 as the carbon source.
Fig. 3. Effect of pH on the specific growth rate of mixed populations in batch culture with sample no. 2 as the carbon source.
Redox Potential, Toxicity and Oscillations in Solvent Fermentations

by

Joong Kim, Rakesh Bajpai, and Eugene L. Iannotti*

Chemical Engineering Department and
* Agricultural Engineering Department
University of Missouri-Columbia

In batch and continuous cultivations of Clostridium acetobutylicum ATCC 824 on glucose and lactose, a strong relationship was observed between redox potential of broth and the cellular metabolism. The specific productivity of butanol as well as of butyric acid were maximum at the redox potential of -250 mV. For butyric acid, it decreased rapidly as the redox potential decreased further. For butanol, it achieved a lower but stable value. This was true for steady as well as dynamic operation. The continuous cultivations involving lactose as a carbon source, exhibited sustained oscillations at lower dilution rate, which appeared to be related to butanol toxicity. No oscillations were observed at higher dilution rates (low butanol concentrations) and when glucose was used as carbon source, suggesting a butanol mediated inhibition of lactose transport/hydrolysis.
Introduction

Distinct acid and solvent production phases are observed in acetone-butanol fermentation with Clostridium strains which have been recently studied [1-4]. Different proportions of acids and solvents can be obtained depending upon the dilution rate in continuous culture. During the first acidogenic phase in typical batch fermentations, cellular growth is predominant and still occurs during the initial phase of solvent excretion. However it is strongly inhibited at high butanol concentrations, often associated with significant lysis of cells. This shift in cellular metabolism has been a subject of intensive study [3,5,6]. With several strains of C. acetobutylicum, an acidic pH which is essential regulatory parameter below 5.0 was found optimal for acetone-butanol production. At higher pH values, only acids are generally excreted. Optimum butanol yield has been found at a controlled pH of 5.0 [7].

Pyruvate metabolism, specifically the reactions associated with the formation of acetyl-CoA, appears to be a key to the diversion of carbon skeletons to acids or to solvents [8-10] (Figure 1). These reactions are predominantly concerned with the transfer of electrons between the reduced and oxidized forms of nucleotides and ferredoxin, and their ultimate release as hydrogen. These reduced compounds as well as hydrogen influence the oxidation-reduction potential of the broth[11,12].

The optimum growth of strictly anaerobic bacteria often requires a low redox potential in the medium. Exclusion of oxygen from the culture medium and gas phase is normally necessary since oxygen helps determine redox potential and it also results in formation of toxic free radicals. To provide a better understanding of bacterial culture behavior, redox potential has been used as an important measurement.

This study deals with the relationships between redox potential of culture broth and growth and product formation phenomena observed in continuous acetone-butanol fermentations.
MATERIALS AND METHODS

Microorganism

Clostridium acetobutylicum ATCC 824 was used in this study. Spores were maintained on a corn meal medium under anaerobic conditions at 4°C [13].

Medium

A soluble complex medium was used for inoculum preparation and for experiments. The medium contained following components per liter of distilled water: lactose, 60.0 g; KH$_2$PO$_4$, 0.75 g; K$_2$HPO$_4$, 0.75 g; MgSO$_4$, 0.02 g; MnSO$_4$·H$_2$O, 0.01 g; FeSO$_4$·7H$_2$O, 0.01 g; NaCl, 1.0 g; Cystein, 0.5 g; Yeast Extract, 5.0 g; Asparagine, 2.0 g; (NH$_4$)$_2$SO$_4$, 2.0 g. The pH of the soluble medium was adjusted to 6.5 with cystein HCl and carbonate prior to autoclaving (15 Min. at 121°C & 20 psig) [14]. The corn meal medium contained following components per liter of distilled water: corn meal, 100 g; agar, 1.5 g; resazurin, 0.1 ml.

Fermentor Assembly

The experiments were conducted a 2L-Virtis Omni-Culture fermentor with a 1L working volume equipped with an overflow tube for chemostat operation. The agitation speed and the temperature were maintained at 200 rpm and at 37°C, respectively. pH was controlled at 5.0 by automatic addition of 5N-NaOH. The pH and redox potential of the culture medium were measured with the help of autoclavable pH (Ingold, Model 5003-k9) and redox (Ingold, Model Pt 4865-35-k9) probes. The redox electrode was calibrated using Ingold's standard redox buffer (Cat. No. 18503) at 25°C and pH 7.0. Foaming was controlled manually by adding a few drops of 1:10 diluted SAG 5693 silicone antifoam agent when foaming became particularly severe. Head
space of the fermentor was continuously flushed with oxygen-free carbon dioxide gas in order to ensure anaerobic environment.

Continuous fermentations were initiated from actively growing batch cultures by starting continuous flow of feed solution maintained anaerobically. Oxygen-free CO₂ gas was sterilized by passing it through a sterile cotton-wool filter, and then introduced into the fermentor. The dilution rate was first at a low value and then increased stepwise from 0.02 to 0.05 hr⁻¹. At each dilution rate, a steady state was considered to be reached when the concentrations of biomass, substrate, and products remained approximately constant after about 4-5 residence times. Feed medium was changed every five days with freshly prepared solutions. Working volume of the fermentor was kept equal to 1 liter by an overflow device. Samples were taken from the effluent twice a day. Absence of contamination was regularly controlled by microscopic observation and by strip and pour plating.

Sample Analysis

Sample were analyzed for the concentrations of cells, sugar, and products in the culture broth. Cell concentration was estimated by measuring the absorbance at 630 nm using a spectrophotometer (Bausch & Lomb Spectronic 2000) as well as by measuring dry cell weight after centrifugation at 10,000 rpm for 10 minutes and several washes. The supernatant was stored frozen for subsequent analyses.

Concentrations of solvents (ethanol, acetone, and butanola) and acids (acetic acid and butyric acid) were determined by injecting supernatant liquid into a Varian Model 1520 gas chromatograph equipped with a flame ionization detector. Separation took place in a glass column, 6ft long by 2 mm in diameter, and packed with coated Chromosorb W-AW, 80/100 mesh. The flow rates of helium, hydrogen and air were 30 ml/min, 40 ml/min and 200 ml/min, respectively. The column temperature was programmed to increased from 70°C to 140°C at a rate of 30°C/min. The temperature of the injector and detector were 250°C and 220°C, respectively. The analysis of chromatographic data were carried out by a Perkin-Elmer LCI-100 Laboratory Computing Integrator.

Residual lactose concentration was measured by Nelson-Somogyi reducing sugar test [15]. Glucose and galactose concentrations were assayed by YSI Model 23A Glucose Analyzer and HPLC (Perkin-Elmer Series 4 Liquid Chromatograph), respectively.
RESULT

The steady state conditions in continuous culture with *C. acetobutylicum* were investigated at different dilution rates ranging from 0.02 to 0.05 hr⁻¹. The concentrations of solvents and redox potential showed cyclic behavior in all the measured culture parameters in Figure 2. Similar oscillations were obtained with the concentrations of biomass and acids (data not shown). Higher concentrations of the solvents were obtained at the lower dilutions rate. However, redox potential varied inversely with cell and solvent concentrations. High dilution rates resulted into greatly reduced cyclic behavior. Several repeated runs at low dilution rates gave the same cyclicity.

In Figure 3, the redox potentials at different dilution rates are shown. Under steady state conditions, the lower the dilution rate, the lower were the redox potentials in broth. The bars represent the range of redox potentials at each dilution rate. The operation at D=0.03 hr⁻¹ was unsteady state which came from a malfunction in pH control. The results are included in this analysis as this state corresponded to unusually high redox potential in broth.

The concentrations of various fermentation products as well as the amount of lactose consumed as a function of broth redox potentials for the different dilution rates are shown in Figures 4 and 5. Production of cell mass, solvents, and lactose consumption increased at lower redox potential while that of butyric acid decreased. The concentration of acetic acid was not dependent upon broth redox potential. The metabolism appears to be strongly inhibited above -200 mV.

To obtain the optimal redox potential range in order to improve the productivity of butanol, the specific rates and redox potential values were plotted in Figure 7. The maximum specific productivity of butanol and butyric acid were obtained at about -250 mV. However, at the Eₖ less than -250 mV the specific productivity of the butyric acid was found to decrease quite rapidly, whereas the specific productivity of the butanol did not decrease as much as that of butyric acid.
DISCUSSION

The possibility of oscillations in steady states of continuous operations is expected when toxic products exist in the system. The toxicity of acids and solvents to cellular metabolic phenomena is well documented [16]. Yet, no oscillatory steady states have been reported using a glucose medium in the considerable amount of published data. We also found no such oscillations with few our experiments (data not shown). However, an oscillating steady state was observed with lactose as a carbon source (Figure 2). The level of solvents obtained in these two systems (glucose and lactose) were similar. A careful analysis revealed that a butanol level of 7 g/liter was associated with a minimum in redox potential. At this point, microscopic observation also showed changes in cell morphology and cell density started to decrease. The solvent peak at 12 g/liter and reduced to 6.5 g/liter before an increase in cellular activity associated with decrease in redox potential is seen. Only negligible amounts of monosaccharides, primarily glucose were detected at the peak.

Butanol has been reported to be the most toxic product in this fermentation system [17]; 7-13 g/liter of butanol reduces growth rates of C. acetobutylicum cells by 50%. Since no similar oscillations have been found in continuous cultivations on glucose, one may postulate that butanol is more inhibitory to parts of lactose metabolism that are different from that of glucose. Solvent inhibition of sugar transport system has been reported in Saccharomyces cerevisiae cells [18] and the inhibitory effect of butanol is dependent upon the nature of a carbon source [19].

Interactions between oxidized/reduced forms of ferredoxin and nicotinamide-adenine dinucleotide, and those between reduced ferredoxins and an iron containing hydrogenase play an important role in determining the flow of carbon skeletons to the acids or to the solvents [20-22]. Ferredoxin can both accept and donate electrons at a very low potential. Under suitable conditions, reduced ferredoxin can donate electrons with the help of hydrogenase to form hydrogen. Maximum hydrogen productivity would be expected to occur during the acid phase of the fermentation. During the solvent phase of fermentation, reducing equivalents can be disposed of by forming butanol and ethanol as well as hydrogen. Butyric acid produced during the acid phase is read sorbed and reduced to butanol (Figure 1). Reduction of hydrogenase activity [9,23,24] or increase of hydrogen pressure [4,25-27] have been reported to improve solvent production. This effect be related to H+/hydrogen redox
potential in the cell [28]. The experimental data presented here show correlation between the redox potential of broth and the metabolic state of the cells (Figures 4-6). This relationship can be seen in the light of the primary oxidation reduction reaction. Thus this study support the postulation that changes in intracellular redox potential should be linked with the changes in electron flow in the cell.

From the observation of decreasing specific butanol productivity (Figure 7), it would appear that too high reduction-oxidation levels in the system reduce the overall flux of carbon through the glycolytic cycle. This is supported by the observations of a maximum in the specific lactose uptake rate from Figure 5.

**CONCLUSION**

Sustained oscillations were obtained under conditions favoring solvent productions. The specific productivities of butanol and butyric acids were maximum at -250 mV and were found to be well correlated with the broth redox potential.

**LIST OF FIGURES**

Figure 1. Schematic biochemical pathways in _Clostridium acetobutylicum_.

Figure 2. Concentration of solvents and redox potential in broth.

Figure 3. Broth redox potential as a function of dilution rate.

Figure 4. Concentrations of products as function of redox potential.

Figure 5. Change in cell concentration and lactose consumption as function of redox potential.

Figure 6. Variations in yield of products (based upon lactose consumed) with redox potential.

Figure 7. Specific rates of formation of butanol and butyric acids as functions of redox potential.
Figure 4

Figure 5

Figure 6

Figure 7
Figure 1

![Diagram of metabolic pathways involving glucose, glycerol, pyruvate, and fatty acid metabolism]

Figure 2

![Graphs showing solvent, pH, and Eh changes over time]

Figure 3

![Graph showing Eh vs. dilution rate]
REFERENCE

Using Structured Kinetic Model for Analyzing Instability in Recombinant Bacterial Cultures

William E. Bentley and Dhinakar S. Kompala

Department of Chemical Engineering
University of Colorado
Boulder, Colorado 80309-0424

July 2, 1987

Abstract

The instantaneous specific growth rate of a recombinant bacterial culture is directly calculated using a simple structured kinetic modeling approach. Foreign plasmid replication and foreign protein expression represent metabolic burdens to the host cell. The individual effects of these plasmid-mediated activities on the growth rate of plasmid-bearing cells are estimated separately. Instability in continuous cultures of recombinant bacteria results from the emergence of plasmid-free host cells due to unequal partitioning of plasmids upon cell division and a growth rate differential between the plasmid-free and plasmid-bearing populations in the resulting mixed culture. Plasmid segregation kinetics are included to enhance quantitative accuracy of continuous culture simulations. The dynamic model predictions of culture stability show remarkable agreement with experimental trends in extended batch and continuous cultures.

INTRODUCTION

Structural and segregational instabilities have been identified which can result in the dramatic loss of a recombinant strain from continuous cultures [1] [2]. In addition, the recombinant strain can be eliminated from continuous cultures due to a growth rate differential between the recombinant (plasmid-bearing) strain and the plasmid-free host. Several experimental studies have established

*To whom correspondence should be addressed
that as plasmid copy number and foreign protein overexpression increase, the growth rate of the recombinant cell decreases [3] - [7]. It is generally recognized that the replication of high copy number plasmids and the overproduction of plasmid-encoded proteins represent an additional "metabolic burden" on the normal chromosome-directed metabolism of the bacterial cell, thereby reducing the cell's growth rate.

Segregational instability is described as the generation of a small fraction of plasmid-free cells upon cell division of the recombinant strain, due to unequal plasmid partitioning between the daughter cells. Coupling these two effects (plasmid segregation and growth rate differential) results in the loss of plasmid-containing cells and their replacement by plasmid-free cells in continuous fermentations.

In the present work, we have extended the simple structured kinetic model, capable of predicting the growth rate of the bacterial cell mass as a dynamic function of the additional metabolic burdens [8], to include the kinetics of segregational instability. We associate "segregational" and "structural" instabilities with "plasmid" instability since they refer to actions directly involving the plasmid. Incorporating the growth rate differential in our mathematical characterization necessitates the use of "culture" instability when referring to the presence or absence of the desired recombinant strain in continuous or extended batch cultures. We demonstrate the model by predicting experimentally observed "culture instability" phenomena.

MODEL OVERVIEW

The structured kinetic model which directly calculates the instantaneous specific growth rate was previously discussed in detail [8], and is briefly reviewed here. A key feature of the modeling framework is the uncommon representation of the state of a microorganism in terms of the fractional mass levels of its intracellular constituents.

Our lumped metabolic model of recombinant cells includes eight major intracellular constituent pools. These pools provide our model with sufficient metabolic detail without becoming numerically burdensome. Included are: protein, \( P \); foreign protein, \( P_f \); chromosomal DNA, \( G \); plasmid DNA, \( G_f \); ribosomes, \( R \); lipids, \( L \); nucleotides, \( N \); and amino acids, \( A \). The level of each of the internal constituent pools is expressed in our model equations as mass fraction or gram constituent per gram dry cell mass. Table I lists the specific net synthesis rates for each constituent pool, whereas
Table II contains equations describing the overall dynamic response for each constituent pool.

The summation of all eight equations in Table II yields:

$$\sum_{i=1}^{8} \frac{dC_i}{dt} = \sum_{i=1}^{8} \sum_{j=1}^{8} r_{ij} - \mu \left[ \sum_{i=1}^{8} C_i \right]$$

(1)

where $C_i$ denotes each constituent pool: $A, N, P, P_f, G, G_f, L,$ and $R,$ and $r_{ij}$ corresponds to the synthesis and depletion terms shown in Table II. Since the entire cell mass is divided into lumped constituent pools, the sum of all their fraction mass levels adds to unity at all times. Furthermore, the time derivative of this sum is zero at all times. By noting these results it can be readily shown that:

$$\mu = \sum_{i=1}^{8} \sum_{j=1}^{8} r_{ij}$$

(2)

This simple equation for calculating the instantaneous specific growth rate was first derived in general form by Fredrickson [9]. The instantaneous specific growth rate for a recombinant bacterial culture as represented by the equations listed in Tables I and II is then:

$$\mu = \left[ \frac{dA}{dt} \right] + (1-\epsilon_1) \left[ \frac{dN}{dt} \right] + (1-\epsilon_2) \left[ \frac{dL}{dt} \right] + (1-\gamma_1) \left[ \frac{dP}{dt} \right] + \left[ \frac{dP_f}{dt} \right] + (1-\gamma_2) \left[ \frac{dC}{dt} \right] + \left[ \frac{dG_f}{dt} \right] + \left[ \frac{dR}{dt} \right]$$

(3)

The concept of fractional mass units was employed for a corrected form of Williams' two compartment model [10] by Bailey and Ollis [11]. To our knowledge, this was the only attempt at using this powerful approach for the structured modeling of biological systems.

**PLASMID SEGREGATION**

One major contributing factor to culture instability in continuous systems is the growth rate differential between the competing populations. The existing model successfully calculates this dynamic growth rate differential in both batch and continuous cultures.

A second contributing factor is the unequal partitioning of plasmids from the mother to daughter cells. A comprehensive description of culture instability must include these plasmid segregation kinetics. The magnitude of this instability depends upon the plasmid copy number at the time of cell division and presence or absence of a partitioning function. Most plasmids control their own replication genetically through negative control. Inhibitors include proteins, RNA, or series of direct repeats [12]. When the inhibitor (e.g. RNA I in ColEI [13]) reaches an appropriate concentration, replication terminates, leaving the cell with a defined copy number.
If no mechanism exists for active segregation of plasmid molecules to the daughter cells during division then the probability $\delta$ that either daughter cell will fail to inherit a plasmid is given by the binomial distribution

$$\delta = 2^n C_0 \left( \frac{1}{2} \right)^n \left( \frac{1}{2} \right)^6$$

where $n$ is the copy number per cell at division. If the mechanism which controls plasmid replication is able to correct anomalies in plasmid copy number produced by this random segregation, then all cells will contain equivalent numbers of plasmids at the next division, irrespective of the precise number of plasmids that each daughter cell inherited. Therefore the probability of producing a plasmid-free cell at the next division will remain unchanged, and plasmid-free cells will be produced at a constant frequency [14].

Since the intracellular constituents are expressed in units of mass fraction in our model, the plasmid copy number is expressed as copies per chromosome equivalent which provides the relative significance of plasmid activity to the cell. Furthermore, this quantity is readily determined experimentally. In order to incorporate plasmid segregation using the above probabilistic approach, we must evaluate plasmid content on a per cell basis. This can be accomplished using the Cooper-Helmstetter model which provides an estimate for the number of genome equivalents per cell in steadily dividing cell cultures [15].

$$G = \frac{T}{Cln2} \left[ 2^{\frac{2G}{D}} - 2^B \right]$$

This equation is based on the cell-number-derived growth rate, $\omega = ln2/T$, and the C and D periods (min.) of the E. coli growth cycle.

By combining the Cooper-Helmstetter result with our modeling framework, the average cell size, $m$, is calculated as

$$m = \frac{G MW_g}{G N_{av}}$$

where $MW_g$ is the molecular weight of the genome, $G$ is the genome content per cell mass, and $N_{av}$ is Avogadro's Number. The plasmid copy number per average cell then becomes

$$< N_p > = \frac{m G_p N_{av}}{MW_p}$$

where $G_p$ is the plasmid content as gram plasmid per gram dry cell mass, and $MW_p$ is the plasmid molecular weight.
In order to calculate the number of plasmids in a single cell that is about to divide, we make the approximation that the plasmid copy number increases linearly over the age of a single cell. In this way, the copy number at cell division, \( N_{p(a=1)} \), is

\[
N_{p(a=1)} = [1 - <a>] \frac{dN_p}{da} + <N_p>
\]

where \(<a>\) is the average cell age and \(\frac{dN_p}{da}\) is the rate of plasmid replication as calculated in the model after the transformation \(\frac{dN_p}{dt} = \frac{dN_p}{da}\) where \(\frac{dt}{da} = 1\) is made. The average age in a steadily growing culture can be found by integrating

\[
<a> = \int_0^1 a W(a) da
\]

where \(W(a)\) is the cell age distribution as shown by Seo and Bailey [16] as

\[
W(a) = \left( \frac{2 - \delta}{1 - \delta} \right) we^{-\omega a}
\]

### Reactor Dynamics

The equations listed in Table II describe the kinetics of all intracellular constituents of the recombinant bacteria and consequently determine the instantaneous specific growth rate. This set of kinetic equations was incorporated into continuous flow stirred tank reactor (CFSTR) material balances. CFSTR dynamics are described by adding the following equations to those in Table II:

\[
\frac{dS}{dt} = -\frac{1}{Y_s} \mu^+ X^+ - \frac{1}{Y_s} \mu^- X^- + D(S_f - S)
\]

\[
\frac{dX^+}{dt} = \mu^+ X^+ - \gamma \mu^+ X^+ - DX^+
\]

\[
\frac{dX^-}{dt} = \mu^- X^- + \gamma \mu^+ X^+ - DX^-
\]

The cell mass, \(X\), is in units of grams dry weight/liter and the yield coefficient, \(Y_s\), is in units of gram cell mass/gram substrate. The calculated growth rate, \(\mu\), is of dimension hr\(^{-1}\). The plasmid-bearing and plasmid-free populations are represented by a superscript plus and minus respectively. In these equations, the dilution rate, \(D\), and substrate feed concentration, \(S_f\), are in units hr\(^{-1}\) and gm/liter respectively. Koizumi and Aiba [17] have shown that the segregation coefficient, \(\gamma\), can be calculated as a function of \(\delta\), the probability of producing a plasmid free cell upon division.

\[
\gamma = 1 - \frac{\ln(2 - \delta)}{\ln 2}
\]
In order to maintain simplicity, we have assumed that the cell growth rates based on mass and cell number were identical \((\mu = \omega)\). Alternatively, the mean cell size changes as slowly as the growth rate so that in continuous cultures, the process can be described as a series of steady state increments. This is reasonable for the description of plasmid instability in slowly evolving chemostat cultures.

**RESULTS AND DISCUSSION**

**Steady State Determination of Replication Constants**

Before predicting behavior in chemostat cultures, the plasmid associated replication constants were determined. The model simulations of the variation in steady state plasmid copy number as a function of growth rate are shown in figure 1. We have taken the data from Seo and Bailey [18] and Siegel and Ryu [19] and expressed their results as number of plasmids per genome equivalent. Like Seo and Bailey, we assumed that chromosomal content varied with growth rate according to the Cooper-Helmstetter model [15]. The values of \(\mu_s\) and \(K_{G,N}\) which best represent the data are \(1.8 \times 10^{-3}\) \(g\) plasmid \(g\) cell mass h\(^{-1}\) and \(10^{-6}\) \(g\) \(G_i\) \(g\) cell mass for Seo and Bailey’s pDM247 (5.85 MD) and \(1.07 \times 10^{-3}\) \(g\) \(G_i\) \(g\) cell mass and \(7.5 \times 10^{-4}\) \(g\) for Siegel and Ryu’s pPLc23 trpAl (6.5 kb). It is apparent that the model accurately describes the plasmid content in these systems.

**Mixed Populations: Stability of Chemostat Cultures**

We have predicted the culture stability of chemostats containing mixed cultures of two competing populations: the plasmid-free host and the plasmid-bearing recombinant strain (figures 2 & 3). In these simulations, we assumed that each population could be represented by an “average” cell. Simulations were performed by coupling two chemostat models, one simulating the plasmid-free host population \((\mu_4 = \mu_5 = 0)\) and the other simulating the plasmid-bearing recombinant population. The initial population mixture was 99% recombinant cells and 1% plasmid-free cells.

When describing the results from Siegel and Ryu (figure 2 [19]), the plasmid replication constants were selected as described above, the additional constants which describe foreign protein dynamics were selected so that the growth rate differential gave the appropriate response for a single dilution rate. Comprehensive foreign protein data were not given, precluding independent constant determination. The dilution rates were then varied while plasmid and foreign protein con-
stants remained fixed. Model simulations show remarkably close agreement with the experimentally determined results.

In both cases (Siegel and Rvu (figure 2 [19]) and Bron and Luxen (figure 3 [20])), the plasmid copy number at cell division was sufficiently high to preclude plasmid segregation as a mechanism for the washout of the recombinant strain \( \gamma = 0 \). Hence, the population composition was strictly dependent upon the growth rate differential between the plasmid-free and recombinant strains. Also, in figure 2, it is apparent that our simulations predict higher chemostat stability at higher dilution rates which is in good quantitative agreement with these experimental results and in good qualitative agreement with other experimental studies which found similar phenomena [21] [4] [22].

Segregational instability is significant, as described here, when average number of plasmids per cell is less than approximately ten (figure 4). The calculated plasmid copy number at cell division can be nearly twice the average, which tends to stabilize the recombinant population. In figure 4, we have illustrated the expected increasing instability as the plasmid copy number approached four. In general, low copy number plasmids \( < 4 \) contain a \( \text{par} \) locus sufficient for stabilizing partitioning dysfunction [12].

A third mechanism for cultural instability, which has not been considered here, results from a continual selective pressure for subpopulations with decreasing plasmid copy numbers. In plasmids with negative, autonomously controlled replication however, there will always be a defined plasmid copy number at cell division thus obviating this mechanism from consideration.

The authors wish to acknowledge the National Science Foundation (grant No. ECE-8611305) and the Amoco Foundation Engineering Faculty Grant for the support of this work.

References


Effects of Plasmid Segregation in Chemostat Cultures with Constant Growth Rate Differential

Chemostat Instability
dependent on growth rate differential

Plasmid Copy Number: >30

Chemostat Instability
also dependent on growth rate differential

figure 1.

figure 2.

figure 3.

figure 4.