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Proceedings of the Nineteenth Annual Biochemical Engineering Symposium

Rakesh K. Bajpai
University of Missouri–Columbia

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Proceedings of the 19th Annual Biochemical Engineering Symposium

April 22, 1989

Rakesh K. Bajpai
Editor

Department of Chemical Engineering
College of Engineering
University of Missouri-Columbia
Columbia, Missouri
Proceedings
of the
Nineteenth Annual
Biochemical Engineering Symposium

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The nineteenth symposium was held at the University of Missouri - Columbia on April 22, 1989. A total of eighteen papers were scheduled for presentation, of which nine were in poster session. Finally, fifteen papers were presented and sixteen were submitted for this proceedings. It was attended by 53 participants from five institutions. A sixth group (from Colorado State University) was kept from attending the symposium due to mechanical problems on the road and we missed them. Since they worked hard at their presentations, I requested CSU-group to submit their papers for the proceedings and I am happy that they did.

The stated objectives of the symposium remain providing (i) a forum for informal discussion of biochemical engineering research being conducted at the various participating institutions, and (ii) an experience for students to present and publish their work. As a result, the papers were presented by the students and covered often the work still in progress. I was glad to note the quality of presentations. The papers submitted in this proceedings are brief and it is expected that these will eventually be refined and published elsewhere.

A list of participants is included at the end of this publication. The participating faculty members have been identified and enquiries concerning the research described in the papers should be directed to appropriate faculty members.

I would like to gratefully acknowledge the Department of Chemical Engineering (UMC), Office of the Dean, College of Engineering (UMC), Chapter No. 145 of the Honor Society of Phi Kappa Phi (UMC), and UMC Engineering Alumni Organization, for their financial support of the Symposium. This support has been used primarily for the publication of this proceedings, and also for the expenses incurred in the organization of the symposium.

The next symposium will be held at Kansas State University, Manhattan, Kansas.
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MATHEMATICAL MODELING OF A FLOUR MILLING SYSTEM

Kiyoshi Takahashi, Yiming Chen,
J. Hosokoschi* and L. T. Fan

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

June 28, 1989

ABSTRACT

A mathematical model has been constructed to simulate the performance of a flour milling system consisting of multiple processing units such as roll machines, sifters and purifiers; the system is continuously fed with wheat. For convenience, however, the resultant model visualizes that particle streams of two different types pass through a processing unit. One type is composed of only particles of pure endosperm, and the other type impure particles; each impure particle contains both endosperm and bran. Passage through the processing unit effects changes in the relative amounts and size distributions of these two types of streams. In the model, the extent of such changes is determined by the transition matrixes of the processing unit; the size distributions of particles in the products from the system are determined by combining the output streams from intermediate processing units. The present model will be useful for developing a systematic approach for the design of a flour milling system to replace the current ad hoc approach.

INTRODUCTION

The main purpose of flour milling is to separate bran from endosperm by grinding. The resultant particle stream from a roll machine is separated into several streams in an ensuing sifter. The smaller particles in the stream are relatively richer in endosperm than the larger particles in the stream; the finest particle stream, which is the richest in endosperm, becomes the product, and the remaining streams are conveyed to other processing units for further processing. Consequently, to model a flour milling system, it is necessary to consider bran contents and size distributions of particle streams flowing through the system.

A flour milling system consists of a complex network where intricate interconnections exist among various processing units. For example, while a sifter receives a single input from a roll machine or multiple inputs from other sifters, each of its outputs may become part of the final products or it may be conveyed to another roll machine, sifter or purifier for further processing. The common practice of design and modification for such a complicated system has been through experience or based on a trial-and-error procedure. The objective of this work is to develop a mechanistic mathematical model and propose a systematic approach to replace the current ad hoc approach for the design and modification of a flour milling system (Hosokoschi, 1988).

* Affiliated with the Technical Research Center, Nisshin Flour Milling, Saitama 354, Japan.
MODELING OF PARTICLE STREAMS

To model particle streams flowing through a flour milling system, the following simplifying assumptions are made.

1. Wheat comprises only two major components, endosperm and bran.

2. Two types of particle streams flow through each processing unit. One is composed of pure endosperm particles (E-particles), and the other impure or mixed particles (M-particles); each impure particle contains both endosperm and bran.

For convenience, particle sizes are represented by the size index. For example, the size index of the particles whose sizes are less than 50 $\mu$m is 1, and that between 50 and 100 $\mu$m is 2 (see Figure 1).

A particle stream is characterized by three matrixes, $E$, $M$ and $B$; they are expressed, respectively, as

\[
E = [e_1 \ e_2 \ ... \ e_i \ ... \ e_n] \\
M = [m_1 \ m_2 \ ... \ m_i \ ... \ m_n] \\
B = [b_1 \ b_2 \ ... \ b_i \ ... \ b_n]
\]

In these expressions, $E$ represents the size distribution of E-particles in the particle stream, whose element $e_i$ represents the amount of the E-particles with a size index of $i$; $M$ represents the size distribution of M-particles, whose element $m_i$ corresponds to the amount of the M-particles with a size index of $i$; and $B$ represents bran fractions of M-particles, whose element $b_i$ represents the bran fraction of the M-particles with a size index of $i$. The physical significance of matrixes $E$, $M$ and $B$ is illustrated in Figures 2, 3 and 4, respectively.

MODELING OF PROCESSING UNITS

The flour milling system being modeled comprises roll machines, sifters and purifiers (see, e.g., Naumov, 1967).

Roll Machine

Figure 5 illustrates the mode of grinding a particle stream. When E-particles are ground, they remain as E-particles but their sizes are reduced. When M-particles are ground, some of the resultant particles become E-particles, and the rest remain as M-particles in the output of the roll machine. The two E-particle streams, one produced from the E-particles and the other from the M-particles, are combined to form the output E-particles from the roll machine.

The three matrixes, $R_e$, $R_m$ and $A$, characterize a roll machine. $R_e$ is the size transition matrix for E-particles; it takes the form

\[
R_e = [R_{e1} \ R_{e2} \ ... \ R_{ei} \ ... \ R_{en}]^T
\]

where
The row vector, \( R_{ei} \), represents the size distribution of the E-particles produced from i-sized E-particles or M-particles. \( R_m \) is size transition matrix for M-particles; it is expressed as

\[
R_m = \begin{bmatrix} R_{m1} & R_{m2} & \cdots & R_{mi} & \cdots & R_{mn} \end{bmatrix}^T
\]

where

\[
R_{mi} = \begin{bmatrix} R_{mil} & R_{mi2} & \cdots & R_{mij} & \cdots & R_{min} \end{bmatrix}
\]

The row vector, \( R_{mi} \), corresponds to the size distribution of the M-particles produced from the i-sized M-particles. \( A \) represents the fractions of the E-particles generated from the M-particles; it takes the form

\[
A = \text{diag} [ a_1 \ a_2 \ \ldots \ a_i \ \ldots \ a_n ]
\]

Diagonal element \( a_i \) represents the ratio of the amount of E-particles produced from i-sized M-particles to that of i-sized M-particles before grinding.

The matrixes, \( E \) and \( M \), express the size distributions of E-particles and M-particles at the input of the roll machine, respectively. The multiplication of \( E \) and \( R_e \) gives the size distribution of E-particles produced from the E-particles, \( E_e \), i.e.,

\[
E_e = ER_e
\]

The multiplication of \( M \), \( A \) and \( R_e \) yields the size distribution of E-particles produced from M-particles, \( E_m \), i.e.,

\[
E_m = MAR_e
\]

The multiplication of \( M \), \( (1-A) \), and \( R_m \) leads to the size distribution of M-particles produced, \( M_m \), i.e.,

\[
M_m = M(1-A)R_m
\]

where \( I \) is a unit matrix. The size distribution of E-particles and M-particles at the output, denoted by \( E_o \) and \( M_o \), respectively, can be evaluated as follows:

\[
E_o = E_e + E_m = ER_e + MAR_e
\]

\[
M_o = M_m = M(1-A)R_m
\]

**Sifter**

A sifter serves to separate one particle stream into several streams depending mainly on particle sizes. It consists of several units; each unit is composed of a number of sieves with the same characteristics. Sieves in a different unit may have different characteristics, and the number of sieves in a unit may vary. When a particle stream enters the first unit of the sifter, consisting of the first, second and third sieves, it is separated by the first sieve into two streams, over stream and through stream, as demonstrated in Figure 6. The over stream proceeds to the second sieve, and is separated again into an over stream and a through stream; the process is repeated once more. The over stream from the last sieve in a unit exits as one of the output streams of the sifter. The through streams from the first, second and third sieves are combined before they are fed to the next unit. Therefore, the overall performance of a sifter can be determined by combining the characteristics of all component sieves.
A purifier separates a particle stream into several particle streams depending mainly on particle densities. The schematics of a purifier is shown in Figure 7. Air flows upward through the purifier by suction. Thus, only those particles with relatively high densities tend to pass through sieves. Since endosperm is heavier than bran, E-particles or M-particles with relatively high endosperm content leave the purifier in the through streams, and the M-particles with relatively high bran content leave it in the over streams. As in the modeling of a sifter, the overall performance of a purifier can be determined by combining the characteristics of all component sieves.

The characteristics of a sieve can be depicted by the fraction of particles passing through it; the fraction is shown in Figure 8. If the sizes of particles are much smaller than the mesh opening of the sieve, the fraction of the particles passing through the sieve is nearly 1. On the other hand, if the sizes of particles are much larger than the mesh opening, the fraction is negligible.

Figure 9 illustrates the fractions of particles passing through a sieve of a sifter. The solid line represents the fraction for pure endosperm particles, and the dotted line represents the fraction for pure bran particles. Note that the latter is not for M-particles.

Figure 10 illustrates the fraction of particles passing through a sieve of a purifier. As can be observed in the figure, the fraction of light bran particles passing through it is much smaller than that of heavy endosperm particles. The matrixes specifying input streams and the sieve’s characteristics are given below.

The size distribution of input E-particles is expressed as

\[
E = [e_1 e_2 \ldots e_i \ldots e_n]
\]  

and that of input M-particles as

\[
M = [m_1 m_2 \ldots m_i \ldots m_n]
\]

The bran fractions of input M-particles are expressed as elements in the matrix,

\[
B = [b_1 b_2 \ldots b_i \ldots b_n]
\]

The passing fractions of E-particles with various size indexes are expressed as elements in the matrix,

\[
S_e = [S_{e1} S_{e2} \ldots S_{ei} \ldots S_{en}]
\]

and those of bran particles as

\[
S_b = [S_{b1} S_{b2} \ldots S_{bi} \ldots S_{bn}]
\]

The fraction of i-sized M-particles passing through the sieve, denoted by \(S_{mi}\), is calculated by the following equation;

\[
S_{mi} = S_{ei} - (S_{ei} - S_{bi})b_i
\]

The physical significance of this equation is illustrated in Figure 11; it relates the bran fraction of particles to the fraction of the particles passing through the sieve. When the bran fraction is 0, the particles are pure endosperm particles. The fraction of particles with a size index of i passing through the sieve is \(S_{ei}\). When the bran fraction is 1, the particles in it are pure bran particles whose passing fraction is \(S_{bi}\). In between, we have M-particles, whose bran fraction is \(b_i\). The fraction of M-particles passing through the sieve is \(S_{mi}\).
The multiplication of the amount of i-sized input E-particles, \(e_i\), and their passing fraction, \(S_{ei}\), gives the amount of these particles passing through the sieve, \(e_{ti}\), i.e.,
\[
e_{ti} = e_i S_{ei}
\] (20)

We can construct a matrix \(E_t\) to represent the size distribution of E-particles in the through stream as
\[
E_t = \begin{bmatrix} e_{t1} & e_{t2} & \cdots & e_{tn} \end{bmatrix}
\] (21)

The multiplication of the amount of i-sized input M-particles, \(m_i\), and their passing fraction, \(S_{mi}\), gives the amount of these particles passing through the sieve, \(m_{ti}\), i.e.,
\[
m_{ti} = m_i S_{mi}
\] (22)

The matrix representing the size distribution of M-particles in the through stream, \(M_t\), can be expressed as
\[
M_t = \begin{bmatrix} m_{t1} & m_{t2} & \cdots & m_{tn} \end{bmatrix}
\] (23)

The size distributions of E-particles and M-particles in the over streams, denoted by \(E_0\) and \(M_0\), respectively, can be calculated by subtracting the size distributions of these particles in the through streams from those of input streams, i.e.,
\[
E_0 = E - E_t
\]
\[
M_0 = M - M_t
\] (24) (25)

**CONCLUDING REMARKS**

A mathematical model has been constructed to simulate the performance of a flour milling system. The model visualizes that particle streams of two different types pass through a processing unit. The model consists of a series of transition matrixes representing processing units; these transition matrixes can be determined through experiments. The proposed model provides a systematic approach for the design of a flour milling system to replace the current ad hoc approach.

**REFERENCES**


Figure 1. Indexing of particle sizes.

Figure 2. Size distribution of E-particles.

Figure 3. Size distribution of M-particles.

Figure 4. Fraction of bran content.
Figure 5. Functioning of the roll machine.
Figure 6. Schematic representation of the sifter.

Figure 7. Schematic representation of the purifier.
Figure 8. Fraction of particles passing through a sieve.

Figure 9. Fraction of particles passing through a sieve of the sifter.

Figure 10. Fraction of particles passing through a sieve of the sifter.

Figure 11. Bran fraction vs passing fraction.
A NOVEL SOLUTION TO THE PROBLEM OF PLASMID SEGREGATION IN CONTINUOUS BACTERIAL FERMENTATIONS

Kimberly L. Henry and Robert H. Davis
Department of Chemical Engineering
University of Colorado
Boulder, Colorado 80309-0424

Austin L. Taylor
Department of Immunology and Microbiology
University of Colorado Health Sciences Center
Denver, Colorado 80262-0175

ABSTRACT

Selective recycle based on flocculation and sedimentation properties has been shown to successfully maintain slower-growing microorganisms in continuous bioreactors, in spite of competition from faster-growing organisms (1,2). This novel method for continuous fermentations can overcome problems of contamination and plasmid loss due to segregation. The focus of this paper is to summarize recent work done with bacterial cultures, and to briefly discuss the construction of new bacterial plasmids with controllable promoters which will allow for continuous maintenance of a plasmid-containing strain without the extra metabolic burden of producing floc-forming proteins during the fermentation stage.

INTRODUCTION

Selective recycle for the maintenance of recombinant cells in a continuous reactor was first proposed by Ollis (3) who developed a theory which predicts the degree of selection necessary in a recycle stream. However, he did not comment extensively on a method for achieving the selectivity. Stephanopoulos et al. (4) noted that plasmid-bearing cells contain more protein and are larger than segregants, and proposed that the two types of cells could be separated by differential sedimentation. Due to the small sizes and relative densities of the cells, such a separation is expected to be difficult and perhaps impractical for large-scale continuous fermentations. They were, however, able to successfully maintain a yeast culture in the presence of a bacterial culture using inclined settling to achieve selective recycle, but they did not attempt to separate recombinant cells from host cells of the same species (5).

In our laboratory, we are selectively separating two phenotypes of the same microbial species by having one of the phenotypes be flocculent. When these cells flocculate, the resulting flocs or clumps of many cells settle much faster than do the unflocculated cells. The two strains may therefore be separated on the basis of the difference in their sedimentation velocities. One way that bacterial cells form flocs is due to an hydrophobic pili-pili interaction (6). Pili are proteinaceous surface appendages which are assembled by the polymerization of pilin, a protein that is rich in nonpolar amino acid side chains. When cells over-produce pili, they form aggregates or flocs as large as a few millimeters in diameter. Since pilus production is under direct genetic control, it may be exploited
to control flocculation and, therefore, to achieve selective separation and recycle based on flocculation and sedimentation.

SELECTIVE RECYCLE REACTOR THEORY

Selective recycle reactor theory for two competitive species, one faster-growing and nonflocculent, and one slower-growing and flocculent, was developed by Davis and Parnham (1) and is summarized here. A schematic of the reactor is shown in Figure 1. The reactor is continuous with two product lines. One passes through an inclined settler (or other suitable separator), where the desired flocculent cells are separated from the undesired nonflocculent cells. A concentrated stream enriched in the flocculent cells exits the bottom of the settler and is recycled back to the reactor, while a diluted stream containing primarily nonflocculent cells is removed from the top of the settler and discarded. The other product line acts as a level controller and withdraws cell broth directly from the bioreactor.

Figure 1: Schematic of a selective recycle reactor and separator.
Unsteady mass balances about the entire system (reactor and cell separator) are:

\[
\frac{dX^+}{dt} = (\mu^+ - \beta^+ D)X^+ - \mu^+ pX^+ \tag{1}
\]

\[
\frac{dX^-}{dt} = (\mu^- - \beta^- D)X^- + \mu^- pX^- \tag{2}
\]

where \( \mu \) is the specific growth rate, \( X \) is the cell concentration in the fermentor, \( D = Qf/V \) is the dilution rate, \( p \) is the segregation probability, and \( \beta \) is an effective cell dilution factor accounting for the direct product line and defined by

\[
\beta^+ = 1 - (1 - \gamma^+) \frac{Qo}{Qf} ; \quad \beta^- = 1 - (1 - \gamma^-) \frac{Qo}{Qf} \tag{3}
\]

The quantity \( \gamma \) is the cell dilution factor, defined as the ratio of the cell concentration of a given strain in the diluted stream exiting the inclined settler to that entering it (if the reactor is well stirred, the cell concentration entering the settler is the same as that in the reactor). The desired strain is denoted as (+), while (-) denotes the undesired strain. Equations (1) and (2) assume sterile feed, negligible cell maintenance and death terms, and negligible cell growth in the cell separator. The latter assumption is a reasonable approximation when the separator volume is small compared to the fermentor volume, which is the case for the experiments described later in this paper. It is also assumed that there are no physiological differences between the recycle cells and those in the bioreactor. Monod kinetics were assumed, with the specific growth rates having the following forms:

\[
\mu^- = \frac{\mu_{m} S}{K^- + S} ; \quad \mu^+ = \frac{\mu_{m}^+ S}{K^+ + S} \tag{4}
\]

where \( S \) is the limiting substrate concentration in the fermentor. The final equation needed is the mass balance on the limiting substrate:

\[
\frac{dS}{dt} = D(S_o - S) - \frac{\mu^+ X^+}{Y^+} - \frac{\mu^- X^-}{Y^-} \tag{5}
\]

where \( S_o \) is the limiting substrate concentration in the feed, and \( Y^+ \) and \( Y^- \) are the yield coefficients for cell growth.

There exists a point of stable coexistence for the two strains which can be found from Equations (1) and (2) by assuming steady state and equating the dilution rates for the (+) and (-) strains, yielding:

\[
\frac{\beta^- \mu^+}{\beta^+ \mu^-} (1-p) = 1 + p \frac{\mu^+ X^+}{\mu^- X^-} \tag{6}
\]
The coexistence ratio, \( G = \beta \mu^+ (1-p)/\beta \mu^- \), determines whether a selective recycle reactor will fail (become 100% undesired cells) or not. For nonsegregating cultures \((p=0)\), complete washout of the desired (+) strain occurs if \( G < 1 \), whereas complete washout of the undesired (-) strain occurs if \( G > 1 \). Coexistence (metastable) is possible only if \( G = 1 \). In contrast, stable coexistence for segregating cultures \((p \neq 0)\) occurs for all \( G > 1 \). The mass ratio, \( X^+/X^- \), at steady state is given by Equation (6) and increases with increasing \( G \). For \( G < 1 \), however, complete washout of the desired strain \((X^+ = 0)\) occurs for all possible values of the segregation probability, \( 0 \leq p \leq 1 \). Note that \( p \) is defined as the fraction of the daughter cells that are of the plasmid-free (-) phenotype, when their parent cells are of the plasmid-bearing (+) phenotype. Thus, \( p \) is a measure of the frequency of plasmid shedding, and it generally has a value of a few percent, or less.

INCLINED SETTLER THEORY

An inclined settler is used in this reactor scheme to selectively separate and recycle cells. This settler design, when compared to conventional settlers, enhances the sedimentation rate of particles or cells due to an increase in available settling area. Not only can flocs of cells settle onto the bottom of the vessel, but they can also settle onto the upward-facing inclined wall. These flocs then form a thin sediment layer which slides down to the bottom of the vessel due to gravity. Details of inclined settler theory and enhanced sedimentation are reviewed by Davis and Acrivos (7).

Figure 1 includes a schematic of a rectangular inclined settler. The equation for the volumetric rate of production of fluid which is free from particles or flocs of cells that settle with a vertical settling velocity \( v \) is:

\[
Q(v) = vw(L \sin \theta + b \cos \theta)
\]  

(7)

where \( L \) is the length of the inclined wall, \( w \) is the width of the inclined wall, \( b \) is the spacing between the plates of the settler, and \( \theta \) is the angle of inclination of the settler from the vertical.

The inclined settler can be used continuously to separate two kinds of bacteria, if they have different sedimentation properties. In this work, flocs of cells settle at an average sedimentation velocity which is approximately 100-fold greater than that of single cells. This difference allows, for example, flocculent plasmid-bearing cells, which are on the order of 0.1-1 mm in diameter, to be separated from nonflocculent plasmid-free cells.

It is necessary to determine the distribution of settling velocities in order to design an inclined settler for the selective recycle reactor. Davis, et al. (8) used Equation (7) along with mass balances to analyze inclined settlers operating continuously to separate faster-settling particles from slower-settling particles, predicting that:
The quantity $v_0$ is the cutoff sedimentation velocity for flocs or particles reaching the overflow and is given by $Q(v_0) = Q_0$. All flocs with settling velocities of $v \geq v_0$ will settle and be recycled to the reactor, whereas a portion of those with $v < v_0$ will be carried out in the overflow. $P(v)$ is the normalized probability density function representing the distribution of settling velocities of particles fed into the settler. It can be experimentally determined using the sedimentation/light extinction apparatus designed by Davis and Hunt (9).

**MATERIALS AND METHODS**

**Microbial Strains and Plasmids** - *E. coli* strain ORN103 (*recA lacU169* derivative of P678-54) was the host bacterial strain for the plasmid used in this study. The plasmid pORN108 is a 15.6 kb (kilobase) fragment and is a regulatory mutant of the *pi* operon inserted into the vector pACYC184 (10). It confers resistance to both chloramphenicol and kanamycin and was used for all fermentation experiments. Its isolation and characterization is described by Orndorff and Falkow (6). The average plasmid copy number for the plasmid pORN108, when it is present in the strain ORN103, was experimentally determined to be 3.8 copies per cell (11). The plasmid pSH2 (12) contains the entire *pi* operon and was used for the cloning work. All cloning vectors were purchased from Pharmacia, Inc.

**Experimental Determination of Reactor Design Parameters** - The parameters needed to design and run a continuous fermentor were determined in batch shake flask experiments. The values of $\mu$, $\mu^+$, $\gamma^-$, $\gamma^+$, and $p$ were found to be $1.14 \pm 0.12$ hr$^{-1}$, $0.72 \pm 0.12$ hr$^{-1}$, 0.43, 0.48, and 0.03 $\pm$ 0.01, respectively, where the confidence levels are 90%, when given. M9CA medium (6 g Na$_2$HPO$_4$, 3 g KH$_2$PO$_4$, 0.5 g NaCl, 1 g NH$_4$Cl, 2 g casamino acids, 2 ml 1M MgSO$_4$, 0.1 ml 1M CaCl$_2$, 2 g glucose, 4.1 ml 1% L-leucine, 4.1 ml 4% L-proline, and 0.166 ml 0.1% Vitamin B1, in 1 liter of water) was used for all experiments. A value of 0.004 g/l was assumed for the Monod constant, as is typical for *E. coli* strains growing in glucose-limited medium (13).

**Continuous Fermentations** - Continuous experiments were performed in a 500-series LH fermentor which has dissolved oxygen, pH, temperature, and foam controls. A 1 l vessel was used with a 0.6 l working volume and the settler was a rectangular glass tube with $L = 51$ cm, $b = 0.5$ cm and $w = 5.0$ cm. The angle of inclination from the vertical was set at 30°. The design of the settler was based on Equation (8) using experimentally determined settling velocity distributions measured with a sedimentation/light extinction apparatus (9), with the design goal of $\gamma^-$ close to unity (all undesired cells passing through the separator without settling) and $\gamma^+$ close to zero (all desired cells settling in the separator and subsequently recycled).

The experiments were started as batch fermentations, with antibiotics added to maintain the desired cells, from an inoculum of plasmid-containing cells. When stationary phase was reached, continuous operation was started, with medium that did not contain antibiotics. Samples were withdrawn periodically from both the fermentor
and settler overflow. The fraction of plasmid-bearing cells was determined by diluting the cells in mannose to disperse the flocs before plating them on LB medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 liter of water). After the colonies had grown on the plates, they were transferred, using cotton velvet, to both an LB plate without antibiotics to determine the number of plasmid-containing cells, and one with antibiotics to check the efficiency of the transfer.

**Plasmid Construction** - All plasmids were constructed using standard molecular biology techniques (i.e. gel electrophoresis, plasmid transformations and plasmid purifications). The exact procedures for which are found in Henry (11).

![Graph showing fraction of plasmid-bearing cells (F+) over time](image)

**Figure 2:** Washout of the plasmid-bearing strain without recycle, and maintenance of the plasmid-bearing strain with selective recycle.

**FERMENTATION RESULTS**

The results of two continuous fermentations are shown in Figure 2. The plasmid-containing cells are slower-growing, and they also have a finite possibility of losing a plasmid, which results in an increased amount of segregant (-) cells. The first experiment depicted was without recycle and demonstrated that the plasmid-bearing strain is washed out of the reactor. The dilution rate was set at 0.45 hr⁻¹, and the value of $G$ was calculated to be 0.61, which correctly predicts the washout of the desired strain. The second experiment shown in the figure is with recycle, and the plasmid-bearing strain was maintained as dominant in the reactor for a period of about 180 hours, after which the fermentation was stopped. For this experiment, the dilution rate was 0.16 hr⁻¹, and the value of $G$ was calculated to be 1.33. The dilution factors were measured to be $\gamma^+ = 0.45$ and $\gamma^- = 1.0$. Again there is very
good agreement between experiment and theory. These experiments demonstrate that slower-growing microorganisms can be maintained in a continuous fermentation by selective recycle in spite of competition from faster-growing microorganisms, and that this strategy can even overcome such problems as plasmid instability.

A third experiment demonstrates the power of the selective recycle reactor (Figure 3). The reactor was allowed to fail ($G < 1$) to the point where it contained about 20% plasmid-bearing cells and 80% segregants. At this time ($t = 0$ on the graph), the dilution rate was lowered, which caused the value of $\gamma^+$ to decrease due to the increased holdup time in the settler, placing the system in favorable conditions for recovery ($G > 1$). This shows that selective recycle can be used to recover a failing reactor which would otherwise have to be shut down. The theoretical curve and the data again agree well.

![Experimental Data vs Theoretical Curve](image)

Figure 3: Recovery of the plasmid-bearing strain with selective recycle.

PLASMID CONSTRUCTION

Current research involves developing the capability to artificially control the genes responsible for flocculation in bacteria. This would allow the cells to grow without overproducing pili in the fermentor. They would then enter the inclined settler where the $pil$ operon would be induced, causing the formation of flocs and allowing the separation of plasmid-bearing cells from segregant cells. The advantage of this strategy is that the plasmid-bearing cells in the main fermentor would then not be subject to the extra metabolic burden of overproducing pili. This burden causes a significant reduction in growth rate.
Figure 4: Summary of cloning strategies used to construct inducible plasmids (Kb = kilobases).
The cloning strategy used to make the new constructs is shown in Figure 4. Briefly, the desired part of the pil operon (pil operon without its operator and promoter) was isolated by performing partial restriction enzyme cuts with SalI and AccI, from the plasmid pSH2. It was placed in the vector pUC19, which contains the lac promoter. These cells can grow in the absence of lactose and the pil operon is then repressed. When lactose or IPTG (a more efficient inducer) is added to the bacterial culture, the cells begin to produce pili. The problem with this promoter is that it is inhibited by glucose, which is the limiting substrate used in the fermentation experiments. However, this construct showed that the desired part of the pil operon was isolated. It also allowed for easier subsequent cloning, since unique restriction enzyme sites (Sal I and Hind III) exist in this new construct around the pil operon. This eliminates performing partial restriction enzyme digests, which are difficult to do experimentally.

The desired part of the pil operon was then isolated from the new construct and inserted into the vector pDR540, which contains the tac promoter, at the BamHI site. This vector is also induced by the addition of IPTG, but is not catabolically repressed by glucose. The pil operon has also been placed behind a temperature sensitive promoter, λPL. This plasmid is repressed at low temperatures (approximately 30°C); when the temperature is raised (to approximately 40°C), the repressor protein is altered, allowing the pil operon to be expressed.

All of the plasmids described above overproduce pili, which leads to flocculation, when there is no repressor protein present, but the rate at which the pili form on the cell surfaces after induction is still being determined. The chemically induced promoter and the temperature sensitive promoter will be compared to determine which one is less lethal to the cells after repeated turning on and off of the pil operon.

CONCLUDING REMARKS

A selective recycle reactor has been shown to continuously maintain flocculent, unstable plasmid-bearing bacterial cells in spite of competition from the faster-growing, segregant cells. Selective recycle reactor theory agrees well with the experiments, and the utility of the stable maintenance ratio, G, has been demonstrated. The value of this ratio allows a researcher to determine in advance whether or not a selective recycle reactor will be able to maintain a slower-growing microorganism as dominant when in competition with a faster-growing organism. Three plasmids have been constructed to artificially control the pil operon and thus flocculation. Current research involves determining the rate at which pili are formed on the cell surface after induction. Once this is known, experiments will be performed with these inducible cells in a selective recycle reactor.

ACKNOWLEDGMENTS

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REFERENCES


Modeling of Embryonic Growth in Avian and Reptile Eggs

C. L. Krause
R. C. Seagrave
Department of Chemical Engineering

R. A. Ackerman
Department of Zoology

Iowa State University
Ames, Iowa 50011

Abstract

An attempt to model the growth of avian and reptile embryos, based totally on environmental conditions, has been conducted. Classical chemical engineering principles, such as heat and mass transfer and dimensionless analysis, have been applied in order to elucidate the significant characteristics of development. Mass and energy balances have been written treating the egg as a "black box" which produces heat and transfers water to and from its surroundings. Solution of the differential equations fits the mass data surprisingly well. Also, a system of differential equations has been developed based on a modification of autocatalytic kinetics to describe the growth of the embryo and the consumption of the stored materials (egg yolk and white). Parameters are then fitted to the data, and heat generation can be determined.

1 Introduction

The effects of environment upon energy conversion within biological systems has been of great interest. The egg of avian and reptile species provides a near ideal example to study for several reasons. First, the shell of the egg provides a defined system boundary. Second, a limited number of interactions with the environment can occur through the shell, which is not true with many other organisms, such as mammals. This makes modeling a much easier task since a few number of processes need to be described. Third, the environment can be made constant over time. Hence, the model can be easily tested. Finally, data are readily available, especially avian eggs, and particularly on chickens, due to the poultry industry.

The modeling was done in two stages. The first deals with exchange through the shell between the whole egg and environment, treating the embryo and the rest of the egg contents as one entity. This may be labeled as external interactions. Heat transfer, water loss, and respiratory gas exchange are the predominant mechanisms involved. The second stage of modeling is internal interactions, that is, the conversion of egg contents (egg white and yolk) into embryo. This is the transfer of lipids, proteins, carbohydrates, and minerals into the embryo, where they are converted into biomass or energy.
2 Model Development

2.1 External Interactions

In order to model external interactions of the egg, it is necessary to understand more about the transport processes of the egg. Descriptions of avian embryo development can be found in texts by Romanoff [10] and Freeman and Vince [4]. A diagram of the typical avian egg can be found in Figure 1. Oxygen and carbon dioxide are carried to and from the embryo by the chorioallantoic membrane, much like the placenta in mammals. Within the embryo, oxygen and fuel (mainly from the yolk) react to form heat, carbon dioxide, water, and waste products. Water is lost to the environment by diffusion through the shell due to a partial pressure difference. The heat generated is lost at a rate dependent upon the egg and the environment. An air cell within the egg appears due to the loss of mass from the egg. Reptile eggs are very similar to avian eggs except in a couple of ways. First, reptile eggs are generally buried in moist earth, therefore, it is possible that they gain moisture. Second, their shells are soft, which allows them to either contract not allowing the air pocket to form or expand if water is gained from a very moist soil.

To continue, more should be know about how the respiratory gas exchange can affect the mass of the egg. The exchange of oxygen and carbon dioxide will alter the mass of the egg depending on the relative amounts of each. One way to define a ratio between these two is the respiratory quotient, \( R.Q. \), defined as:

\[
R.Q. = \frac{\text{volume of } CO_2 \text{ produced}}{\text{volume of } O_2 \text{ consumed}}
\]

The \( R.Q. \) is dependent upon the type of fuel that is being metabolized due to the stoichiometry of the reaction. The change in mass, \( \Delta M \), associated with the respiratory gas exchange for a specific type of fuel can be defined as:

\[
\Delta M = \frac{\text{mass of } O_2 \text{ consumed} - \text{mass of } CO_2 \text{ produced}}{\text{mass of fuel consumed}}
\]

Typical values for the \( R.Q. \) and \( \Delta M \) for different fuel types are presented in Table 1. As can be seen, if a combination of these fuel types were consumed, such that a \( R.Q. \) of 0.72 was associated with this diet, no mass change due to the respiratory gases would occur. The \( R.Q. \) is often measured and for most eggs is in the range of 0.70 to 0.80. Hence, respiratory gas exchange can be neglected in the mass balance, assuming that \( R.Q. \) is 0.72 or close enough to be insignificant.

The mass and energy balances on the egg can now be written neglecting changes due to respiratory gases [1, 2]. Water is the only material being transported across the shell that needs to be modeled. The rate at which the egg accumulates mass is equal to the rate of water transported into the egg due to

<table>
<thead>
<tr>
<th>Fuel Type</th>
<th>( R.Q. )</th>
<th>( \Delta M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids</td>
<td>0.71</td>
<td>0.09</td>
</tr>
<tr>
<td>Proteins</td>
<td>0.80</td>
<td>-0.15</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>1.00</td>
<td>-0.40</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Table 1: Respiratory Gas Exchange Relationships.
Data taken or calculated from Seagrave [11].
the partial pressure difference across the shell:

\[ \frac{dm}{dt} = KA(P_a - P) \]

The rate at which the egg accumulates energy is the sum of the rates of energy transported by the water exchange, heat exchange with the environment, and heat generated by metabolism:

\[ mC_p \frac{dT}{dt} = H_s KA(P_a - P) + HA(T_a - T) + \mathcal{G} \]

This model can be applied to eggs exposed to air, soil, or both. In the case of partially buried eggs, the heat and mass transfer coefficients are averaged. It has been found that the limiting resistance to heat transfer is the film external to the egg. Heat transfer coefficients for air are well known. However, the soil heat transfer coefficient is estimated from the thermal diffusivity of the soil, by using a Nusselt number of 2 (the pure conduction limit). Also, correlations for the surface area of the egg and the product of the mass transfer coefficient and surface area are known. These correlations can be expressed as:

\[ A = (AS)m^{ES} \]
\[ KA = (AKA)m^{EKA} \]
\[ H_s = 2.0 \left( \frac{k_s}{d} \right) \]

Substitutions for the vapor pressure of water can be made. The first is a linearized (by Taylor Series) Antoine Equation, which allows the vapor pressure to be expressed in terms of temperature. Also, the activity of water in the egg, \( r \), and the relative humidity of the environment, \( s \), can be used to simplify the equations. The partial pressures can be written as:

\[ P_a = s P_a^{sat} \]
\[ P = r P_a^{sat} \left[ 1 + \frac{B(T - T_a)}{(T_a + C)^2} \right] \]

The mass and energy balance equations can be made dimensionless by making the following definitions:

\[ \Theta_M = \frac{m}{m_o} \]
\[ \Theta_T = \frac{H_{A_o}(T - T_a)}{\mathcal{G}_r} \]
\[ \xi = \frac{t}{\tau} \]

The reference heat generation, \( \mathcal{G}_r \), is usually taken as the heat generation maximum or the heat generation at the time of hatching. When the substitutions for the heat and mass transfer coefficients, the partial pressures, and the definitions of the dimensionless numbers are made and the equations simplified, they become:

\[ N_{TS} \Theta_M \frac{d\Theta_T}{d\xi} = -\Theta_M^{ES} + N_T \Theta_M^{EKA} \Theta_T - N_H + \frac{\mathcal{G}}{\mathcal{G}_r} \]
\[ \frac{d\Theta_M}{d\xi} = -\Theta_M^{EKA} N_M (N_H + N_T \Theta_T) \]
where:

\[ N_{TS} = \frac{m_0 C_p}{H_A o \tau} = \text{Stored Energy} \]  
\[ N_T = \frac{H_1 (KA)_o P_{e} B r}{HA (T_a + C)^2} = \text{Conducted Energy} \]  
\[ N_H = \frac{H_0 (KA)_o P_{e} (r - s)}{G_e} = \text{Vaporized Energy} \]  
\[ N_M = \frac{\tau G_e}{m_0 H_e} = \text{Generated Energy} \]  
\[ \frac{m_0}{m_0 H_e} = \text{Vaporized Energy} \]

This external model has a few shortcomings. First, the heat generation must be known \textit{a priori}. This results in the model not being totally dependent on the environmental conditions. Second, the model treats the entire egg as one compartment. It does not give any indication of the status of the embryo.

### 2.2 Internal Interactions

Several theories on how to model growth have been proposed [5]. One is based on monomolecular autocatalytic kinetics and was supported by Robertson [8]. He believed that a specific chemical species was responsible for activating growth. Furthermore, this species followed monomolecular autocatalytic kinetics and had a concentration proportional to the mass of the organism. The rate of growth of an organism could be expressed as:

\[ \frac{d m_s}{d t} = - K m_s (m_{s,_{max}} - m_s) \]

This type of equation yields an "S" shaped curve typical of biological growth. Hence, it is capable of giving good fits to the data.

However, several inadequacies and problems exist with this model. First, this rate limiting growth species has never been identified. Second, during growth, the concentration is not proportional to mass for almost all chemical species and would not be expected of the growth limiting chemical species. Third, there are no temperature effects or compositional and qualitative food supply effects built into this model. Finally, the constants for one species are not applicable to another species. Combined, these problems disallow any predictive power of the model.

I am proposing that the egg be divided into two compartments: the embryo (including the embryonic membranes) and the food supply (egg white and yolk). Further, the food supply is made up of four components or types of nutrients: lipids, proteins, carbohydrates, and minerals. It has been shown that the embryo will reach a similar dry weight regardless of how much water, within a reasonable amount, is lost by the egg [3]. Therefore, the dry weight of the embryo is chosen as being more indicative of embryonic growth and is used throughout the internal model. A large body of chicken data exists and has been collected by Romanoff [9] into a single source. It is this collection that is used throughout the rest of this document as data. The shape of the curves for these food components and the embryo can be described, at least in part, by "S" curves as can be seen by Figure 2. The rate of absorption of the \( i \)th food component can be described as:

\[ \frac{d S_i}{d t} = K_i m_e (S_i - S_{i_e}) \]

The term \( S_{i_e} \) refers to the amount of the food supply that is not available to the embryo by absorption through the membranes. A few days before many
species are born, the yolk sac is drawn into the embryo. After hatching, the yolk sac, now called the spare yolk, can be digested by the intestines of the chick. The spare yolk provides the chick with partial nutrition for several days after hatching. It is possible that certain food components are made unavailable to the embryo so that they may be reserved for the chick.

Once a food component is absorbed into the embryo, a fraction, \( h_i \), is converted into biomass, while the remainder is degraded into heat and end products. The rate of growth of the embryo with membranes is the sum of the food components used for biomass:

\[
\frac{dm_e}{dt} = \sum h_i \frac{dS_i}{dt}
\]

The heat generation can then be estimated by the sum of the products of the heat of metabolism, \( H_m \), and the rate of metabolism of a component:

\[
G = \sum H_m (1 - h_i) \frac{dS_i}{dt}
\]

The heats of metabolism can be found in Seagrave [11]. Also, the rates of oxygen consumption and carbon dioxide production can be modeled because it is now known what type of fuel is being metabolized by the egg at a given time.

The external and internal interaction models can be combined. The internal model will yield heat generation, respiratory gas consumption and production, and mass of the embryo plus the membranes. The external model using the heat generation function from the internal model will yield mass and temperature profiles for the entire egg. Now the egg can be described solely from initial conditions and environmental factors, such as, heat and mass transfer coefficients and humidity.

### 3 Results and Discussion

Typical values for the four dimensionless numbers can be found in Table 2, where turkey and chicken are avian species and Chrysemys and Spalerosis are reptile species. It is apparent that values for \( N_M \) and \( N_{TS} \) are of a similar magnitude for all species, while a few orders of magnitude difference occur in \( N_T \) and \( N_H \) between avian and reptile species. These dimensionless numbers point out the large significance of the environment upon the reptiles, more so than the aves. Values for \( N_M \) are approximately equal to unity. If not known, a value for \( G_r \) can be calculated by setting \( N_M \) to one and rearranging, that is:

\[
G_r = \frac{m_o H_v}{\tau}
\]

Figures 3 and 4 show the results of the external model for two reptile species. The model represents real life both qualitatively and quantitatively. Figure 5

<table>
<thead>
<tr>
<th></th>
<th>( N_T )</th>
<th>( N_M )</th>
<th>( N_{TS} )</th>
<th>( N_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>0.020</td>
<td>1.75</td>
<td>1.13E-3</td>
<td>0.31</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.014</td>
<td>1.01</td>
<td>1.78E-3</td>
<td>0.25</td>
</tr>
<tr>
<td>Chrysemys</td>
<td>2.57</td>
<td>0.78</td>
<td>0.45E-3</td>
<td>225</td>
</tr>
<tr>
<td>Spalerosis</td>
<td>3.46</td>
<td>0.83</td>
<td>1.01E-3</td>
<td>165</td>
</tr>
</tbody>
</table>

Table 2: Typical Values for the Dimensionless Numbers.
is a more interesting example. Here the turtle *Chrysemys picta* is incubated with three different soil moisture contents with half the egg exposed to the soil and the other half exposed to air. The model demonstrated its usefulness by accurately predicting the moisture effect upon egg mass. It correctly predicted a gain or loss in the mass of the egg in the appropriate situations.

For the internal interaction model, twelve parameters (each food component has a $K_i$, $S_{i*}$, and $h_i$) are needed. The standard chicken, *Gallus gallus*, has had enough observations on embryonic development to provide an extensive database to fit all twelve parameters. Hence, *Gallus gallus* is the only species to which the internal model has been applied. To simplify the parameter fitting computations, the $S_{i*}$ parameters were estimated from observation of the data as being the value where the “S” curve flattens out. Also, the $h_i$ parameters were estimated as the amount of a component present in the embryo at the time of hatch divided by the total amount absorbed of that component over the length of incubation. Finally, the $K_i$ parameters were found by a least squares fit to the data. The parameters found in this manner for the standard chicken are presented as Table 3. This table demonstrates by looking at the values for $h_i$ that most of the protein is being used for biomass and the lipid for energy. It should be noted that lipids and proteins compose over 90% of the food supply and the carbohydrates very little. Thus, the assumption that the respiratory quotient is near that of lipids should be appropriate.

The parameters from Table 3 are used to solve the system of differential equations of the internal model. The results are shown in Figure 6. Also, the heat generation as predicted by the model is compared to that of the data in Figure 7. The model fits very well up until day 18. However, on or about day 18 the chicken embryo begins to feed by a different mechanism, that of swallowing food rather than membrane absorption. This model makes no attempt at modeling this second form of feeding and is restricted to only the first 18 days of chicken embryo development.

Finally, the heat generation function of the internal model can be used by the external model to predict the egg mass. Also, the metabolic information produced by the internal model can be used to correct for any mass changes due to the exchange of respiratory gases. The solution of the mass and energy balances for the whole egg can be found in Figure 8. The combination of the two models produces an excellent fit to the standard chicken egg data.

<table>
<thead>
<tr>
<th></th>
<th>$K_i \left( g \text{ day}^{-1} \right)$</th>
<th>$S_{i*} \left( g \right)$</th>
<th>$h_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>0.0381</td>
<td>1.00</td>
<td>0.442</td>
</tr>
<tr>
<td>Protein</td>
<td>0.0866</td>
<td>3.00</td>
<td>0.932</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>0.1027</td>
<td>0.14</td>
<td>0.690</td>
</tr>
<tr>
<td>Mineral</td>
<td>0.0686</td>
<td>0.17</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Table 3: internal Interaction Model Parameters.

### 4 Conclusions

The external interaction model has been demonstrated as being capable of fitting whole egg weight for a variety of species and environmental conditions. In addition, dimensionless numbers have been identified in the mass and energy balances. These dimensionless numbers have been useful in describing not only the differences between avian and reptile eggs but also the environmental influences. However, this model needs a heat generation function given in order to
solve the differential equations.

The internal interaction model has shown the usefulness of compartmentalizing the egg into a living tissue compartment and a food compartment, with the food composing of four components. This model has described the growth of the embryo, the consumption of food components, and the generation of heat. This heat generation function can then be used in the external model to eliminate any a priori knowledge other than initial conditions and environmental parameters. This internal model has only been applied to the standard chicken due to the lack of extensive data sets needed for other species. However, due to the basic similarities between eggs, it is hoped that once put in dimensionless form, the parameters of the standard chicken will be appropriate for other avian species and that other sets of parameters will fit classes of reptiles, such as turtles, lizards, etc.

In the future, the combination of models could be applied to other species as more data becomes available to prove the extent of the usefulness of these models. Also, the water within the egg has not been modeled but shall be in the future. Water content information is helpful in describing the condition of the embryo. All in all, this method of modeling the growth of embryos within eggs has achieved promising results in predicting embryo and egg mass during development based solely on external conditions.

References


Nomenclature

\( A \) surface area of the egg

\( A_0 \) initial surface area of the egg

\( (AKA) \) constant in mass transfer-mass correlation

\( (AS) \) constant in surface area-mass correlation

\( B \) Antoine Equation constant for water

\( C \) Antoine Equation constant for water

\( C_p \) heat capacity of the egg

\( d \) characteristic diameter of the egg

\( EKA \) constant in mass transfer-mass correlation

\( ES \) constant in surface area-mass correlation

\( h_i \) fraction of food component \( i \) converted to biomass

\( H \) heat generation of the egg

\( H_{m_i} \) heat of metabolism of food component \( i \)

\( H_s \) latent heat of vaporization of water at the environmental temperature

\( Q \) heat of metabolism of food component \( i \)

\( Q_r \) reference heat generation of the egg

\( k \) thermal diffusivity of soil

\( K \) proportionality constant

\( K_i \) proportionality constant for component \( i \)

\( KA \) product of mass transfer coefficient-surface area

\( (KA)_0 \) initial product of mass transfer coefficient-surface area

\( m \) mass of whole egg

\( m_a \) mass of embryo plus membranes

\( m_0 \) initial mass of the whole egg

\( m_i \) mass of an organism

\( m_{max} \) maximum obtainable mass of an organism

\( N_H \) dimensionless \( \frac{\text{vaporized energy}}{\text{generated energy}} \)

\( N_M \) dimensionless \( \frac{\text{generated energy}}{\text{stored vaporization energy}} \)

\( N_T \) dimensionless \( \frac{\text{vaporized energy}}{\text{conduct ed energy}} \)

\( N_{TS} \) dimensionless \( \frac{\text{stored energy}}{\text{conducted energy}} \)

\( P \) water vapor pressure within the egg

\( P_a \) water vapor pressure in the environment

\( P^w_a \) saturated water vapor pressure at the environmental temperature

\( r \) activity of water in the egg

\( R.Q. \) respiratory quotient

\( s \) relative humidity of water in the environment

\( S_i \) mass of food component \( i \)

\( S_{i,a} \) mass of unavailable food component \( i \)

\( t \) time

\( T \) temperature of the egg

\( T_a \) temperature of the environment

\( \Delta M \) mass change associated with respiration

\( \Theta_M \) dimensionless whole egg mass

\( \Theta_T \) dimensionless egg temperature

\( \xi \) dimensionless time

\( \tau \) incubation time
Figure 1: Diagram of developing avian egg from Rahn et al [7].

Figure 2: Standard chicken development curves for embryo and food supply demonstrating the occurrence of the "S" shape.

Figure 3: Results of the external interaction model applied to the Elapho.

Figure 4: Results of the external interaction model applied to the Dipsosaurus.
Figure 5: Results of the external interaction model applied to *Chrysemys picta* for several different soil moisture contents [1]. Data from Packard [6].

Figure 6: Results of the internal interaction model applied to the standard chicken.

Figure 7: Heat generation as predicted by the internal interaction model for the standard chicken.

Figure 8: The results of using both models in conjunction to predict whole egg mass.
In situ bioremediation of contaminated soil is an innovative and cost effective treatment technology. This technology exploits the capability of naturally occurring microorganisms to decompose toxic substances deposited in a soil bed. It can be applied to the cleanup of organic sludge where organic compounds with high molecular weights are adsorbed on the soil particles. To aerobically operate the biodegradation process, water containing oxygen is allowed to flow through the soil bed. The flow behavior of water through the soil bed is very similar to that observed in bioremediation of contaminated groundwater.

Several mathematical models have been proposed for simulating in situ bioremediation of contaminated groundwater (Borden and Bedient, 1986; Molz et al., 1986; Kosson et al., 1987; Lee et al., 1988). These models consider that contaminants are initially in the bulk liquid and the fate of the contaminants can be described by an adsorption-reaction process. Moreover, the models assume that a local adsorption-desorption equilibrium is rapidly established for the contaminants between the liquid and solid phases (Valocchi, 1985). Nevertheless, in biodegradation of the contaminants adsorbed in a soil bed, the rate of contaminant migration from the soil particles to the bulk liquid is generally controlled by the rate of contaminant transport within the pore network; thus, the local equilibrium assumption may be inappropriate. In the present work, a nonequilibrium model has been developed for simulating bioremediation of contaminated soil.

MODEL DEVELOPMENT

Organic contaminants are initially deposited in a soil bed. Water is allowed to flow through the bed continuously, thereby saturating the bed. The dissolved oxygen in the water effects aerobic biodegradation. By consuming substrate, including all contaminants, oxygen and other nutrients, naturally occurring microorganisms grow both in the solid phase as immobile microcolonies, which are clusters of microorganisms attached to the surface of soil particles, and in the liquid phase as suspended microorganisms.

The following major assumptions are made in deriving the model for bioremediation of contaminated soil. (a) Water in interstices or pores of the soil bed is the liquid phase and the remaining part of the bed is the solid phase. No gas phase exists because the bed is saturated with water; (b) only three components, substrate, oxygen and biomass are involved in biodegradation; (c) macroscopically, one dimensional flow prevails through
the liquid phase. The void fraction in any cross-section of the soil bed is constant, and thus, the pore velocity of water is constant; (d) no convective flow and dispersion occur in the solid phase; (e) the microcolonies in the solid phase are attached to the surface of soil particles, i.e., the interface between the solid and liquid phases, where the supply of oxygen is more effective than the inside of soil particles; (f) the biodegradation by microcolonies takes place at the interface between the liquid and solid phases; in other words, no reaction proceeds in the bulk of the solid phase; and (g) the concentration gradients across this liquid layer, adjacent to the interface between the liquid and solid phases, are negligible, and thus, the concentrations of substrate and oxygen extracted by the microcolonies are equal to those in the bulk liquid.

The schematic diagram of the transport and biodegradation in a controlled volume is given in Fig. 1. The mass balance of component i in the liquid phase gives rise to

\[
\frac{\partial c_i}{\partial t} = \frac{\varepsilon A(\Delta x)}{} \left( - \frac{\partial c_i}{\partial x} + v c_i \right) |_{x} - \varepsilon A(-\frac{\partial c_i}{\partial x} + v c_i) |_{x+\Delta x} + \varepsilon A(\Delta x) r_i^L + A(\Delta x) a_{ij}^L
\]

where subscript i is s, o or b, standing for substrate, oxygen or biomass, respectively. Dividing both sides of equation (1) by A\Delta x and letting \Delta x approach to zero give

\[
\frac{\partial c_i}{\partial t} = \frac{\varepsilon^2 c_i}{\varepsilon x^2} - \varepsilon \frac{\partial c_i}{\partial x} + \varepsilon r_i^L - a_{ij}^L
\]

The corresponding mass balance in the solid phase yields

\[
\rho A(\Delta x) \frac{\partial q_i}{\partial t} = A(\Delta x) a_{ij}^s
\]

This equation can be simplified to

\[
\rho \frac{\partial q_i}{\partial t} = a_{ij}^s
\]

The rate of mass transfer of component i from the liquid phase to the interface must be equal to the sum of the rate of its transfer from the interface to the bulk of the solid phase and the rate of its consumption at the interface, i.e.,

\[
a_{ij}^L = a_{ij}^s + \rho (-r_{ij}^s)
\]

Substituting this equation into equation (2) leads to

\[
\varepsilon \frac{\partial c_i}{\partial t} = \varepsilon E \frac{\partial^2 c_i}{\partial x^2} - \varepsilon v \frac{\partial c_i}{\partial x} + \varepsilon r_i^L + \rho r_{ij}^s - a_{ij}^s
\]

Equations (4) and (6) are the general transport equations for component i in the solid and liquid phases, respectively.
In bioremediation of contaminated soil, the substrate is initially deposited in soil particles, and the rate of substrate desorption from the soil particles to the liquid phase is generally controlled by transport within the pore network; thus, the concentration of substrate in the liquid phase is not in equilibrium with that in the solid phase. In terms of the film model, the mass flux of the substrate, \( j_s^* \), in equations (4) and (6) can be expressed as

\[
(-j_s^*) = k'(q_s - q_s^*)
\]  

(7)

where

\[
q_s^* = K_{ds} C
\]  

(8)

Substituting this expression into equation (7) yields

\[
(-j_s^*) = k\left(\frac{q_s}{K_{ds}} - C_s\right)
\]  

(9)

where

\[
k_s = k'K_{ds}
\]  

(10)

Substitution of equation (9) into equations (6) and (4) results, respectively, in

\[
\frac{\partial c_s}{\partial t} = \frac{\partial^2 c_s}{\partial x^2} - \frac{\partial c_s}{\partial x} + \epsilon_r L + \rho r_s + ak_s (\frac{q_s}{K_{ds}} - C_s)
\]  

(11)

\[
\frac{\partial q_s}{\partial t} = -ak_s (\frac{q_s}{K_{ds}} - C_s)
\]  

(12)

The flux of oxygen, \( j_o^* \), is negligible because the solid phase hardly adsorbs oxygen; thus,

\[
\frac{\partial q_o}{\partial t} = 0
\]  

(13)

\[
\frac{\partial c_o}{\partial t} = \frac{\partial^2 c_o}{\partial x^2} - \frac{\partial c_o}{\partial x} + \epsilon_r L + \rho r_o
\]  

(14)

The rates of exchange between biomass in the form of immobile microcolonies and that in the form of suspended microorganisms are not controlled by transport within the pore network of the solid phase because the microcolonies are mainly at the interface between the liquid and solid phases. Thus, a local adsorption-desorption equilibrium exists, which can be expressed as

\[
q_b = K_{db} C_b
\]  

(15)

Substituting this equation into equation (4) and combining the resultant expression with equation (6) lead to a single expression, i.e.,

\[
\frac{\partial c_b}{\partial t} = \frac{\partial^2 c_b}{\partial x^2} - \frac{\partial c_b}{\partial x} + \epsilon_r L + \rho r_b
\]  

(16)

where
This expression is termed as the retardation factor of biomass.

The reaction terms in equations (11), (14) and (16) can be expressed in terms of the Monod model (see, e.g., Bailey and Ollis, 1987). The rate of biomass growth in the form of the suspended microorganisms in the liquid phase, \( r_b^L \), is expressed as

\[
    r_b^L = \mu_m \frac{C_s}{b(K_s + C_s)} \left( \frac{C_o}{K_o + C_o} \right) - k_d C_b
\]

where the first term on the right-hand side is for the growth and the second term is for the decay. The rate of biomass growth in the form of the microcolonies at the interface, \( r_b^S_f \), is expressed as

\[
    r_b^S_f = \mu_s b \frac{C_s}{K_s + C_s} \left( \frac{C_o}{K_o + C_o} \right) - k_d q_b
\]

where \( q_b \) is the concentration of microcolonies at the interface, based on the mass of the solid phase. Note that as stated in assumption (g), the concentrations of substrate and oxygen extracted by the microcolonies are equal to those in the bulk of the liquid. Similarly, the rate of substrate degradation by the suspended microorganisms in the liquid phase, \( -r_s^L \), is

\[
    (-r_s^L) = \frac{\mu_m}{Y_s} \frac{C_s}{b(K_s + C_s)} \left( \frac{C_o}{K_o + C_o} \right)
\]

The rate of substrate degradation by the microcolonies at the interface, \( -r_s^S_f \), is

\[
    (-r_s^S_f) = \frac{\mu_m}{Y_s} \frac{C_s}{b(K_s + C_s)} \left( \frac{C_o}{K_o + C_o} \right)
\]

The rate of oxygen consumption in the bulk of the liquid phase, \( -r_o^L \), and that at the interface, \( -r_o^S_f \), are expressed, respectively, as

\[
    (-r_o^L) = \frac{\mu_m}{Y_o} \frac{C_s}{b(K_s + C_s)} \left( \frac{C_o}{K_o + C_o} \right)
\]

\[
    (-r_o^S_f) = \frac{\mu_m}{Y_o} \frac{C_s}{b(K_s + C_s)} \left( \frac{C_o}{K_o + C_o} \right)
\]

Substituting the above kinetic expressions and the equilibrium relation of biomass, equation (15), into equations (11), (14) and (16) gives rise, respectively, to

\[
    \frac{\partial C_s}{\partial t} = E \frac{\partial^2 C_s}{\partial x^2} - v \frac{\partial C_s}{\partial x} + \frac{k_a q_s}{\epsilon} (K_s - C_s) - \frac{\mu_m}{Y_s} R_b b \frac{C_s}{K_s + C_s} \left( \frac{C_o}{K_o + C_o} \right)
\]

\[
    \frac{\partial C_o}{\partial t} = E \frac{\partial^2 C_o}{\partial x^2} - v \frac{\partial C_o}{\partial x} - \frac{\mu_m}{Y_o} R_b b \frac{C_s}{K_s + C_s} \left( \frac{C_o}{K_o + C_o} \right)
\]
These three equations together with equation (12) rewritten as
\[ \frac{\partial q_s}{\partial t} = - \frac{k_a q_s}{\rho (K_{ds} - C_s)} \] (27)
constitute a nonequilibrium model.

DIMENSIONAL ANALYSIS

To better understand the effects of the model parameters on the solution, it is desirable to rewrite equations (24) through (27) in dimensionless form. For this purpose, the following dimensionless variables are defined.

\[ \Theta = \frac{t v_c}{L}, \quad X = \frac{x}{L}, \quad \bar{C}_s = \frac{C_s}{C_{so}} \]

\[ \bar{C}_o = \frac{C_o}{C_{of}}, \quad \bar{C}_b = \frac{C_b R_b}{C_{so} R_f V_s}, \quad \bar{q}_s = \frac{q_s}{K_{ds}} \]

In these definitions, \( C_o \) is the concentration of oxygen in the feed solution, and \( C_{so} \) is the concentration of substrate in the liquid phase which would be in equilibrium with the initial concentration of substrate in the solid phase, \( q_{so} \). Substitution of the dimensionless variables into equations (24) through (27) results, respectively, in

\[ \frac{\partial \bar{C}_s}{\partial \Theta} = \frac{1}{P_e} \frac{\partial^2 \bar{C}_s}{\partial \Theta^2} - \frac{\partial \bar{C}_s}{\partial \Theta} + St_m (\bar{q}_s - \bar{C}_s) - N_{r,1} \bar{C}_b \bar{C}_o \]

(28)

\[ \frac{\partial \bar{C}_o}{\partial \Theta} = \frac{1}{P_e} \frac{\partial^2 \bar{C}_o}{\partial \Theta^2} - \frac{\partial \bar{C}_o}{\partial \Theta} - N_{r,1} \bar{C}_o \bar{C}_b \]

(29)

\[ \frac{\partial \bar{C}_b}{\partial \Theta} = \frac{1}{R_b P_e} \frac{\partial^2 \bar{C}_b}{\partial \Theta^2} - \frac{1}{R_b} \frac{\partial \bar{C}_b}{\partial \Theta} + N_{r,1} \bar{C}_b \bar{C}_o \bar{C}_s \]

(30)

\[ \frac{\partial \bar{q}_s}{\partial \Theta} = - \frac{St_m}{R_s - 1} (\bar{q}_s - \bar{C}_s) \]

(31)

where

\[ P_e = \frac{L v_c}{\varepsilon}, \quad N_{r,1} = \frac{\mu m L}{\nu}, \quad N_{r,2} = \frac{k_d L}{\nu} \]

\[ S_{m} = \frac{k_A L}{\nu \varepsilon}, \quad R_s = 1 + \frac{\rho K_{ds}}{\varepsilon}, \quad K = \frac{S}{C_{so}} \]
Among the above dimensionless numbers, \( N_r \) and \( N_g \) are the reaction unit for the growth and decay, respectively, \( \text{St} \) is the Stanton number for mass transfer; \( R \) is the retardation factor for substrate; and \( W \) is the oxygen supply factor.

RESULTS AND DISCUSSIONS

The model equations developed in the preceding section consist of three convection-dispersion partial differential equations (PDE's) and one ordinary differential equation (ODE). The model equations have been solved with a recently developed numerical method, the three-point backward finite difference method (Wu et al., 1989a). Numerical simulation for an once-through operation, where water supply oxygen is permitted to flow through the bed only once, has been conducted subject to the following initial and boundary conditions:

\[
\begin{align*}
\text{At } \Theta = 0, & \quad \bar{C}_s(0,X) = 1.00, \quad \bar{q}_s(0,X) = 1.00 \\
& \quad \bar{C}_o(0,X) = 0.05, \quad \bar{C}_b(0,X) = 0.01 \\
\text{At } X = 0, & \quad \bar{C}_i(0,0) = \frac{1}{Pe} \left( \frac{\partial \bar{C}_i}{\partial X} \right)_0, \quad i = a, o, b \\
& \quad \bar{C}_s(0,0) = 0.00, \quad \bar{C}_o(0,0) = 1.00, \quad \bar{C}_b(0,0) = 0.01 \\
\text{At } X = 1, & \quad \frac{\partial \bar{C}_i}{\partial X} = 0, \quad i = s, o, b
\end{align*}
\]

The effects of model parameters on the solution have been analyzed with the emphasis on the Stanton number, \( \text{St} \), the retardation factor of substrate, \( R_s \), and the oxygen supply number \( W \) (Wu, 1989). The dynamic simulation of the recycle of the unreacted substrate to the top of the bed has also been conducted (Wu et al., 1989b). Figure 2 shows the concentration profiles for the once-through operation. The parameters used are given in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N_r,1 )</td>
<td>12.0</td>
</tr>
<tr>
<td>( N_r,2 )</td>
<td>0.2</td>
</tr>
<tr>
<td>( K_s )</td>
<td>3.0</td>
</tr>
<tr>
<td>( K_o )</td>
<td>0.05</td>
</tr>
<tr>
<td>( Pe )</td>
<td>100</td>
</tr>
<tr>
<td>( W )</td>
<td>12.5</td>
</tr>
<tr>
<td>( R_s )</td>
<td>20</td>
</tr>
<tr>
<td>( R_b )</td>
<td>50</td>
</tr>
<tr>
<td>( \text{St}_m )</td>
<td>8</td>
</tr>
</tbody>
</table>

CONCLUDING REMARKS

A nonequilibrium model for biodegradation of contaminants adsorbed in a soil bed has been developed. The effects of the insufficient oxygen supply,
the growth of biomass and the resistance to contaminant migration on the rate of biodegradation have been examined with the model. The model equations, consisting of three convection-dispersion partial differential equations and one ordinary differential equation, have been solved with the three-point backward finite difference method. The dynamic simulation provides insight into the kinetics and transport phenomena involved in biodegradation processes.

**NOTATION**

\[ a = \text{interfacial area per unit volume of the soil bed, } L^2/L^3 \]

\[ C_i = \text{concentration of component } i \text{ in the liquid phase, } M/L^3 \]

\[ C_{of} = \text{concentration of oxygen in the feed solution, } M/L^3 \]

\[ E = \text{dispersion coefficient, } L^2/t \]

\[ J_i^L = \text{transport flux from the liquid phase to the interface, } M/L^2/t \]

\[ J_i^s = \text{transport flux from the interface to the bulk of the solid phase, } M/L^2/t \]

\[ k_d = \text{reaction rate constant for the decay of biomass, } t^{-1} \]

\[ k_s = \text{mass transfer coefficient of substrate, } L/t \]

\[ K_o = \text{saturation constant of oxygen, } M/L^3 \]

\[ K_s = \text{saturation constant of substrate, } M/L^3 \]

\[ K_{di} = \text{dimensionless linear isotherm partition coefficient of component } i \]

\[ L = \text{depth of the contaminated soil bed, } L \]

\[ q_i = \text{concentration of component } i \text{ in the solid phase, } M/M \text{ dry soil} \]

\[ r_i^L = \text{reaction rate in the liquid phase, } M/L^3/t \]

\[ r_i^s = \text{reaction rate at the interface, } M/M \text{ dry soil/t} \]

\[ R_i = \text{retardation factor for component } i \]

\[ v = \text{pore velocity of the liquid, } L/t \]

\[ t = \text{time, } t \]

\[ x = \text{vertical position, } L \]

\[ Y_o = \text{yield factor of oxygen} \]

\[ Y_s = \text{yield factor of substrate} \]
Greek letters

\( \rho \) = bulk density of the soil bed, \( \text{M dry soil/L}^3 \)

\( \varepsilon \) = volumetric fraction of the bulk of the liquid

\( \mu_m \) = maximum specific growth rate of biomass, \( \text{t}^{-1} \)

REFERENCES


Figure 1. Schematic diagram of transport and biodegradation in a controlled volume.
Figure 2. Concentration profiles for the once-through operation at θ=3.
Introduction

The manipulation of shear rate, temperature and moisture content in the processing of starches can produce a variety of end products. Low moisture systems are used where puffing and expansion are desired. Excess water systems (>70%) are used to gelatinize and paste starch. The interplay of the three variables, shear rate, temperature and moisture content, with starch molecules results in end products that are functionally very different. An understanding of the effects of these parameters is essential if product quality is to be predictable.

Literature Review

The starch granule is considered a semi-crystalline spherulite consisting of crystalline and amorphous regions. Chains with a degree of polymerization (DP) of 50-60 run continuously from one phase to the other. The crystalline phase is due to parallel clusters of short chains (DP=15-20) packed side by side. These chains could be either the external amylopectin chains (the principle crystalline component) or parts of the amylose molecules (less important) which, when folded, can give analogous parallel chain structures (Biliaderis et al., 1980). Crystallinity of the granules is generally around 15-35%. Amorphous regions are those where chain folding or multiple branching prevent the formation of ordered polymer structures.

The starch granule is an organized ring structure. Amylopectin molecules are oriented perpendicular to these rings and to the outer surface of the granule. They are aligned along an imaginary axis extending from the hilum of the granule radially towards the edge of the granule. Portions of those long branched molecules are in crystal formations perpendicular to the rings. It is the regular orientation of the crystalline and amorphous regions that gives the granule its characteristic birefringent pattern known as the maltese cross. The amorphous regions contain most of the amylose molecules and sections of amylopectin molecules not in crystallite formation. Absorbed water will hydrate the amorphous areas and is
able to move freely within the granule (Hoseney, Zeleznak, and Yost, 1986). This "softening" of the amorphous region occurs at a temperature known as the glass transition temperature (Zeleznak and Hoseney, 1987). Crystallite disruption occurs at the melting temperature. This process starts at the hilum and extends outward because the crystal formations under the most stress disrupt first (Hoseney, Zeleznak, and Yost, 1986).

The gelatinization endotherms obtained with the DSC (differential scanning calorimeter) represent the difference between the endothermic energy associated with the transition (melting of the crystallites, granule swelling and denaturation) and the exothermic energy associated with the transition (hydration of starch molecules and formation of the amylose-lipid complex) (Biliaderis et al., 1980; Yost, Burkhard, and Hoseney, 1986).

Materials and Methods

Torque Rheometer Procedure

High amylopectin (90%) corn starch (Sigma) was mixed with deionized water to acquire 45, 50, and 55% (wet basis) moisture doughs. Below 40% moisture the torque went too high to measure, and above 60% moisture the torque was too low to measure. Each dough was mixed in a 50 gram sigma type Brabender mixer that was held at a constant temperature by recirculating water from a hot water bath through an external jacket on the mixer. Two different temperatures were tested, 74°C and 85°C. The gelatinization temperature of amylopectin was found to be 74°C. Below this temperature there was no viscosity change during mixing. A second temperature of 85°C was chosen as it was between 74°C and 100°C, the maximum temperature achievable with a hot water bath. Mixer speeds of 100 and 200 RPM were used. Samples were taken from the mixer at timed intervals. Torque was continually monitored on a strip chart recorder attached to the Brabender torque rheometer.

DSC Procedure

Samples taken during the torque rheometer tests were immediately prepared to be tested for the degree of gelatinization that had occurred during mixing. 1-2 mg samples of the dough were weighed into DuPont DSC pans. Deionized water was added to bring the total sample weight within an 18-22 mg range (excess moisture
conditions). The pans were hermetically sealed and allowed to sit overnight. They were then heated on a DuPont 910 differential scanning calorimeter from 0-100 °C at 5 °C/minute. The results were analyzed with a DuPont 1090 thermal analyzer.

Results

A graph of the 200 RPM mixer speed torque rheometer results can be seen in Figure 1. There was a downward shift in the torque curves and the gelatinization values in the 100 RPM tests, but the relative results of both mixer speed tests were the same. Since shear rate was constant (Figure 1), torque is proportional to viscosity. From Figure 1 it can be seen that when moisture content and shear rate are held constant, the higher torque curve is always the one obtained under the higher temperature condition. Clearly something is happening to the basic components of the dough as normally viscosity decreases when temperature increases.

![Figure 1. Torque vs. time results for 45, 50, and 55% moisture amylopectin doughs mixed at 200 RPM speed at 74 C and 85 C.](image)

Table 1 lists the amount of gelatinization that occurred in the samples from each run. The first mixer samples in each run were taken after 10 minutes. Average values and the ranges that were averaged are listed. The values increased and decreased randomly over time; there were no trends within a run. However, there were trends between the runs. Gelatinization increased as temperature and moisture content increased.
The degree of gelatinization that was to occur during a run occurred within the first 10 minutes.

The amount of energy needed to complete gelatinization was found by integrating the curves from the DSC test results. Percent gelatinization was calculated by dividing the curve integration for an untreated sample into the difference between the curve integrations for untreated and test samples.

From Table 1 it can be seen that the amount of gelatinization occurring increases when the temperature is increased. The first sample was taken from the mixer after 10 minutes. The total amount of gelatinization that was to occur for each run had occurred by this time.

Table 1. Results of DSC gelatinization scans of amylopectin doughs mixed at 200 RPM.

<table>
<thead>
<tr>
<th>Mixing Conditions Moisture (%)/Temp(°C)/RPM</th>
<th>% Gelatinization (Range Averaged)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45/74/200</td>
<td>63 (60–65)</td>
</tr>
<tr>
<td>45/85/200</td>
<td>95 (84–100)</td>
</tr>
<tr>
<td>50/74/200</td>
<td>74 (70–80)</td>
</tr>
<tr>
<td>50/85/200</td>
<td>99 (97–100)</td>
</tr>
<tr>
<td>55/74/200</td>
<td>90 (85–93)</td>
</tr>
<tr>
<td>55/85/200</td>
<td>100 (all=100)</td>
</tr>
</tbody>
</table>

The initial viscosity spikes (Figure 2) recorded by the torque rheometer correlate with the time during which gelatinization is occurring. This process includes initial hydration of the starch granules and subsequent disruption of the granule with concomitant release of the water soluble amylose and amylopectin molecules. High molecular weight polymers unfolding and binding water and enlarged granules could explain this initial viscosity spike.
A regression analysis was done using viscosity and gelatinization as the dependent variables and moisture content, temperature and rpm as the independent variables. Results showed that gelatinization was most dependent on temperature; moisture and rpm followed in order of importance. Viscosity was most dependent on moisture with temperature and rpm being the next and the least important variables, respectively.

Summary and Conclusions

The gelatinization process occurs at the same time an initial viscosity spike is observed in the torque rheometer curves. Hydration of starch granules, one of the first steps to occur in the gelatinization process, could explain this spike.

Normally viscosity decreases as temperature increases. Results reported here show that amylopectin starch dough viscosity increases with increasing temperature when moisture content and shear rate are held constant. The percent gelatinization is shown here to also increase with temperature. Regression analysis determined that gelatinization is highly temperature dependent. The upward shift in viscosity curves with increasing temperature could be related to the gelatinization phenomena. Granule enlargement and release of large water soluble polymers could explain, at least partially, increased viscosity with increased temperature.
REFERENCES


Analysis of Two-Stage Recombinant Bacterial Fermentations Using a Structured Kinetic Model

Fudu Miao and Dhinakar S. Kompala
Department of Chemical Engineering
University of Colorado
Boulder, Colorado 80309-0424

June 30, 1989

Abstract

To enhance plasmid stability in recombinant *Escherichia coli* cultures, a two-stage fermentation has been utilized in which the bacterial growth phase is separated from the gene expression phase. The growth rate difference between plasmid-bearing cells and plasmid-free cells is minimized due to repression of the foreign gene in the first stage, where the plasmid-bearing cells can be maintained for a long time. In the second stage, the gene is strongly expressed by introducing inducers, yielding a large amount of foreign protein. This two-stage fermentation strategy for recombinant fermentation leads to longer plasmid stability and higher productivity than a single stage fermentation.

A structured kinetic model for recombinant fermentation has been employed which predicts that plasmids can be maintained for a long time. The simulations provide us with a thorough understanding of this process and are very useful to the development of large scale production of foreign proteins in *E. coli*.

Introduction

With the advent of recombinant DNA technology, plasmids have been widely used as tools for producing gene products. The plasmids which have been engineered are transformed into host microorganisms so that the genes encoding for the desired proteins can be expressed inside the cells. In this way, continuous production of foreign proteins can be achieved by maintaining plasmid-bearing cells. For the cultures of recombinant microorganisms, however, plasmid instability, or plasmid loss, will severely deteriorate fermentation performances. One reason is uneven plasmid segregation which produces a few plasmid-free cells[1,2]. That is, during dividing of plasmid-harboring cells, there is a probability that a few daughter cells do not receive any plasmid from their parent cells since the plasmids are usually randomly segregated. These plasmid-free cells generated are able to grow faster than the plasmid-harboring cells in which the synthesis of foreign protein causes an extra metabolic burden for cell growth[3]. Therefore, during fermentations, the fraction of
plasmid-free cells will continuously increase, and the plasmid-bearing microorganisms will be eventually dominated by those plasmid-free cells, thus the bioreactor becomes totally nonproductive.

To enhance gene products, the plasmids containing promoters, such as trp, lac and tac, are widely employed. When the promoters are strongly induced, the genes for foreign proteins are turned on and large amounts of foreign proteins are synthesized. In this case, the growth-rate differential between plasmid-bearing cells and plasmid-free cells could be so distinct that plasmids will be rapidly lost, particularly for the cells with low plasmid copy numbers. This phenomenon could be more severe in continuous cultures of recombinant microorganisms due to the washout of the plasmid-harboring cells. Certainly, plasmid loss could be overcome by using selective media that contain antibiotics. But it is impractical for large scale fermentations because the utilization of antibiotics is expensive.

Then, a new method is needed to stabilize plasmids for the cultures of recombinant microorganisms in the absence of antibiotics. A two-stage fermentation strategy has been proposed by Ryu and his co-workers [4,5] in recent years. They suggested that the cell growth phase can be separated from the gene expression phase by using two fermentors. The gene encoding for the foreign protein on the plasmid is repressed in the first stage so that the growth rate difference between plasmid-bearing cells and plasmid-free cells is minimized. Continuously providing plasmid-bearing cells to the second stage can greatly increase plasmid stability, where the foreign protein is synthesized by turning on the gene expression, which can be accomplished by either shifting temperature or introducing inducers.

In this paper, the performance of the two-stage continuous cultures of recombinant bacteria using a structured kinetic model for cell growth is analyzed. Based on this model, some operating parameters, such as inducer concentration and dilution rates, are optimized for maximizing the cloned gene product. The analysis is mainly focused on expression systems involving controllable promoters, but this methodology of modeling is also applicable to the systems in which temperature-sensitive repressors are used.

For two-stage continuous cultures of recombinant bacteria, the sudden shifting of the plasmid-bearing cells from the gene-repressed state to the gene-expressed state will dramatically change the intracellular composition so that the cells need to adjust their composition to adapt to the environmental change. Therefore, the growth rates of the plasmid-bearing cells vary with residence time in the second stage. The observed cell growth rate of the plasmid-bearing cell population is contributed by various cells with different cell ages.

The average retention time and the growth rate are determined by dilution rate, which usually affects the plasmid copy numbers. Therefore the dilution rate is an important parameter affecting plasmid stability and foreign protein productivity. The optimal operating parameters have been discussed by simulating the two-stage fermentation.
Reactor Model

The two-stage fermentation is depicted in Figure 1, which illustrates that the cell growth phase is separated from the gene expression phase in two fermentors. The feed ($D_1$) to the first fermentor is the sterile nutrient medium for cell growth. The outlet ($D_{12}$) from the first stage is continuously fed into the second stage where the induction of the cloned gene is carried out by the inducer (e.g., isopropyl thiogalactopyranoside). An extra medium input, $D_{02}$, is necessary in order to supply nutrient for producing the foreign protein and maintaining the cell growth. Obviously, the overall dilution rate ($D_2$) of the second fermentor is the sum of $D_{12}$ and $D_{02}$.

A model describing cell growth in the first stage can be written as follows based on mass balance:

\[
\frac{dX_1^+}{dt} = \mu_1^+(1 - \nu_1)X_1^+ - D_1X_1^+ \quad (1)
\]

\[
\frac{dX_1^-}{dt} = \mu_1^-X_1^- + \nu_1\mu_1^+X_1^+ - D_1X_1^- \quad (2)
\]

and

\[
\frac{dS_1}{dt} = D_1S_0 - \frac{1}{Y_{e/s}}(\mu_1^+X_1^+ + \mu_1^-X_1^-) - D_1S_1 \quad (3)
\]

where we have assumed that the yield coefficient, $Y_{e/s}$, of plasmid-bearing cells equals that of plasmid-free cells. $X_1^+$ and $X_1^-$ denote the cell mass of the plasmid-bearing cells and the plasmid-free cells, and $\mu_1^+$ and $\mu_1^-$ are the corresponding specific growth rates. $S_1$ and $S_0$ represent the growth-limiting substrate concentrations in the first stage and in the feed respectively. $\nu_1$ is the segregational coefficient from the plasmid-bearing cells to the plasmid-free cells during cell division. Its value could be calculated using the following equation:

\[
\nu_1 = 1 - \frac{\ln(2 - \delta)}{\ln(2)} \quad (4)
\]

where $\delta = 2C_n^n(\frac{1}{2})^n(\frac{1}{2})^0$, $n$ is the average plasmid copy number when the cells are about to divide and its value can be found using the same method as in Bentley and Kompala[7].

For the second fermentor, the growth rate of an individual plasmid-bearing cell is residence time dependent. The shifting of the cells from the first stage to the induced stage causes a shock for cell growth, so that the growth rate varies with time. Thus, the mass balance equations for the second stage are formulated as follows:

\[
\frac{dX_2^+}{dt} = D_{12}X_1^+ + (1 - \nu_2)\int_0^t \hat{\mu}_2^+(\alpha)\left[dX_2^+(\alpha)\right]d\alpha - D_2X_2^+ \quad (5)
\]

\[
\frac{dX_2^-}{dt} = D_{12}X_1^- + \mu_2^-X_2^- + \nu_2\int_0^t \hat{\mu}_2^+(\alpha)\left[dX_2^+(\alpha)\right]d\alpha - D_2X_2^- \quad (6)
\]

and

\[
\frac{dS_2}{dt} = D_{12}S_1 + D_{02}S_0 - D_2S_2 - \frac{1}{Y_{e/s}}\int_0^t \hat{\mu}_2^+(\alpha)\left[dX_2^+(\alpha)\right]d\alpha - \frac{1}{Y_{e/s}}\mu_2^-X_2^- \quad (7)
\]
where \( \frac{dX^+(\alpha)}{da} \) is the residence time distribution density of the plasmid-bearing cells, as defined by the following equation:

\[
\int_0^t \left[ \frac{dX^+(\alpha)}{da} \right] da = X^+_2
\]

where \( dX^+_2(\alpha) \) is the cell mass with the age from \( \alpha \) to \( \alpha + da \).

The mass balance equation for a differential unit of cells \( dX^+_2(\alpha) \) with the residence time \( \alpha \) in the second stage is:

\[
\frac{d[dX^+_2(\alpha)]}{da} = [\mu^+_2(1 - \nu_2) - D_2] dX^+_2(\alpha)
\]

Integrating the above equation, we have the following equation for calculating the residence time distribution density:

\[
\frac{dX^+_2(\alpha)}{da} = D_{12} X^+_1 e^{\int_0^\alpha [\mu^+_2(\alpha)(1 - \nu_2) - D_2] da} da
\]

Substitution of the above relationship into equations (5), (6) and (7) yields

\[
\frac{dX^+_2}{dt} = D_{12} X^+_1 - D_2 X^+_2 + (1 - \nu_2) D_{12} X^+_1 \int_0^t \mu^+_2(\alpha) e^{\int_0^\alpha [\mu^+_2(\alpha)(1 - \nu_2) - D_2] da} da \]

\[
\frac{dX^-_2}{dt} = D_{12} X^-_1 - D_2 X^-_2 + \nu_2 D_{12} X^-_1 \int_0^t \mu^-_2(\alpha) e^{\int_0^\alpha [\mu^-_2(\alpha)(1 - \nu_2) - D_2] da} da
\]

and

\[
\frac{dS_2}{dt} = D_{12} S_1 + D_{02} S_0 - D_3 S_2 - \frac{1}{Y_{2/s}} \int_0^t \mu^+_2(\alpha) e^{\int_0^\alpha [\mu^+_2(\alpha)(1 - \nu_2) - D_2] da} da - \frac{1}{Y_{2/s}} \mu^-_2 X^-_2
\]

From equations (11-13), the variations of both plasmid-bearing cells and plasmid-free cells with time can be computed. It should be noted that \( \mu^+_2(\alpha) \) denotes the growth rate of the plasmid-bearing cells with the residence time \( \alpha \) in the second stage.

An important variable in our analysis is the foreign protein level in the plasmid-bearing cells. It can be calculated by the following equation:

\[
\frac{dP_{f2}}{dt} = \frac{D_{12} X^+_1 P_{f1}}{X^+_2} - \frac{P_{f2}}{X^+_2} \frac{dX^+_2}{dt} - D_3 P_{f2}
\]

\[
+ (1 - \nu_2) \frac{D_{12} X^+_1}{X^+_2} \int_0^t \mu^+_2(\alpha) P_{f2}(\alpha) e^{\int_0^\alpha [\mu^+_2(\alpha)(1 - \nu_2) - D_2] da} da
\]
where $P_{f2}(a)$ is the mass fraction of the foreign protein in the plasmid-bearing cells with residence time $a$. The productivity of the two-stage fermentation may be calculated by the following equation:

$$P = D_2 X_f^+ P_{f2}$$

From equations (1) to (14), the plasmid stability for a two-stage fermentation system can be simulated. The problem remaining is how to compute the cell growth rates. Here a structured kinetic model developed by Bentley and Kompala[6,7] is utilized. In that model, eight constituent pools have been defined for the plasmid-bearing cells. They are amino acids, nucleotides, lipids, ribosomes, natural protein, foreign protein, chromosome DNA and foreign DNA.

In this structured kinetic model, a very important parameter is $\mu_4$, the maximum synthesis rate of the foreign protein [7], which can be related to inducer concentration and type of the inducer used. The corresponding relationship can be determined from experiments. Generally speaking, the value of $\mu_4$ increases with increasing inducer concentration and promoter strength. When all the foreign genes are completely repressed, that is, there is no synthesis of foreign protein, $\mu_4$ is identical to zero. This may be the ideal situation, since for many gene expression systems foreign proteins are still synthesized in small amounts, even though inducers are not present. To simplify our analysis, we assume that the promoter is completely repressed in the first stage.

Simulations of Two-Stage Fermentations

Figure 2 shows the simulation of a single stage continuous culture of the recombinant bacteria. It indicates that the plasmid stability decreases with increasing $\mu_4$ (the values of $\mu_4$ are marked in the figure), or say, increasing inducer concentration. For the gene expression systems regulated by control operons, the foreign genes are more strongly expressed at higher level of inducer, yielding more foreign protein. Increasing the rate of foreign protein synthesis gives rise to more metabolic burden for cell growth, thereby enlarging the growth rate difference between plasmid-bearing cells and plasmid-free cells. However, if the gene encoding for the foreign protein is completely repressed, the synthesis of foreign protein is shut off and the value of $\mu_4$ corresponds to zero, the plasmid-bearing cells can almost grow as fast as the plasmid-free cells can. In this case, plasmid loss is strongly suppressed, thus the plasmid-bearing cells can be stabilized for a very long time.

In two-stage fermentations, the growth rate of the plasmid-bearing cells dramatically changes due to the sudden induction of the cloned gene, as illustrated in Figure 3. Here the dilution rates, $D_1$, $D_{12}$ and $D_{02}$, are 0.3, 0.2 and 0.3 hr$^{-1}$ respectively. At pseudo-steady state, that is, both the intracellular composition and the total cell mass almost do not change with time, the growth rate of the plasmid-bearing cells, $\mu_1^+$, is approximately equal to the dilution rate $D_1$ in the first stage. When the plasmid-bearing cells are suddenly induced, $\mu_4$ is switched from zero to a positive value (e.g. 6000 hr$^{-1}$), the growth rate rapidly decreases and gradually recovers. For a highly induced system (high $\mu_4$), the shock
in growth rate is so serious that the recovery time could be very long. Therefore, when we calculate the overall growth rate, \( \mu^+ \), consideration should be made to compute the cell growth rates \( \mu^+ (\alpha) \) with different residence times in the second fermentor since the growth rates of plasmid-bearing cells vary with time. But, for the slightly expressed systems (low \( \mu_4 \)), the shock is not so distinct.

The variation of foreign protein productivity with \( \mu_4 \) is shown in Figure 4. In this simulation the dilution rate of the first stage is chosen to be 0.3 hr\(^{-1} \), and \( D_{02} \) and \( D_{12} \) are 0.3 and 0.2 respectively. The main factors influencing protein productivity are plasmid stability and intracellular foreign protein level. For a strongly induced system, the fraction of plasmid-bearing cells in the second stage is low while the plasmid-free cells do not produce protein, so that it is impossible to have high productivity even though the foreign protein level in the plasmid-bearing cells is high. Inversely, for a slightly induced system, the productivity will also be low due to the slow synthesis rate of the foreign protein even though the plasmids are stable. Thus, there is an optimal \( \mu_4 \) (inducer concentration) at which the proper combination of plasmid stability with foreign protein level yields the highest productivity. This behavior can be seen in Figure 5, which shows that when \( \mu_4 \) is near 4000 hr\(^{-1} \) the fraction of the plasmid-bearing cells is about as high as 0.9. With slight increase of \( \mu_4 \), the plasmid stability decreases rapidly, which is due to the increase in growth rate difference. However, the fraction of plasmid-bearing cells can not reach to zero even at very high value of \( \mu_4 \), since the first stage provides the second stage with the plasmid-bearing cells. This is the main advantage of the two-stage fermentation over a single stage fermentation.

The effect of the dilution rate, \( D_{02} \), on the mass fraction of the foreign protein and the productivity is shown in Figure 6. Here, the dilution rate \( D_{12} \) is fixed at 0.2 hr\(^{-1} \). It shows that there is an optimal dilution rate (\( D_{02} \) is approximately equal to 0.3 hr\(^{-1} \)) at which the mass fraction of the foreign protein for the total cells is maximized. But, at this dilution rate, the productivity is not at the maximum because the overall dilution rate \( D_2 \) increases with increasing the dilution rate \( D_{02} \), so that productivity further increases with \( D_{02} \) and arrives at the maximum when \( D_{02} \) is about 0.5 hr\(^{-1} \).

The effect of \( D_1 \) on the plasmid stability for the first fermentor is not distinct in a completely repressed system, particularly for the cells having high plasmid copy numbers. But, for a partially repressed system, high dilution rate \( D_1 \) is recommended since the plasmid stability is higher at higher dilution rate for most of the \( E. coli \) cells.

Conclusions

1. The simulations of the reactor model show that plasmid stability can be maintained using two-stage fermentation strategy so that continuous production of foreign protein can be achieved.

2. Suddenly shifting bacteria from the first stage to the second stage gives a shock in growth rate that significantly reduces the growth rate of bacteria, and the recovery time
increases with increasing inducer concentration.

3. There is an optimal inducer concentration as well as an optimal dilution rate at which the productivity of foreign protein can be maximized.

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References


Figure 1. Schematic Diagram of Two-stage Fermentation

Figure 2. Effect of $\mu$ on Plasmid Stability
Figure 3. The Variation of Growth Rate of Plasmid-Bearing Cells with Residence Time

Figure 4. Effect of $\mu_\alpha$ on Productivity of Foreign Protein
Figure 5. The effect of $\mu_a$ on plasmid Stability in the second stage

Figure 6. Effect of DO2 on Productivity of Foreign Protein
Lactic Acid Fermentation from Enzyme-Thinned Starch
by Lactobacillus amylovorus

Department of Chemical Engineering
University of Missouri-Columbia
Columbia, MO 65201

Introduction

Lactic acid has been increasingly-produced year after year [1] and it is consider as a one of the commodity chemicals [2]. In industrial, generally, soluble sugar is fermented to produce lactic acid and glucose is the most popular one [1,3,4]. Glucose is obtained through liquefaction and saccharification of starch [5]. Hence, if starch could be used directly or with minimal pretreatment, the cost for raw material could reduced and thus lower the final cost of lactic acid produced. This is the reason why starch was chosen as the fermentation substrate in this work.

Lactobacillus amylovorus utilizes starch to product lactic acid [6]. This study focused on environmental factors governing the direct fermentation of corn-starch to lactic acid. The effects of nutrients, temperature, pH, and buffer source on the rate of product formation were investigated.

The maximum lactic acid levels obtained in the batch process was 166 g/L and 98% conversion from starch to lactic acid was reached. The rate of lactic acid production from starch compared favorably with that from glucose by Lactobacillus delbrueckii.

Materials and Methods

Microorganisms

Two starch-utilizing lactic acid bacteria were used in these studies. Lactobacillus amylovorus NRRL B-4542 was obtained from the Northern Regional Research Center in Peoria, Illinois and SMC-32 was isolated in the Waste Management Laboratory of University of Missouri-Columbia. A glucose-utilizing strain, Lactobacillus delbrueckii NRRL B-445 was also studied and obtained from the Northern Regional Research Center.

Lactobacillus amylovorus and SMC-32 stock cultures were maintained in a 10% (w/v) starch modified Friedman's medium (Table 1), and the Lactobacillus delbrueckii stock culture was maintained in 5% (w/v) glucose unmodified Friedman's medium [7]. Stock cultures were incubated at 37°C and transferred every two weeks.

In preparation for an experiment, stock cultures (3 drops) were transferred to 3 ml of either 10% starch or 5% glucose Friedman's medium. After 24 hours this was repeated. From that point, 5% inoculum was used in successive transfers that were 24 hours apart until the final medium was inoculated.

Medium and Culture Conditions

Unless stated otherwise, the medium used in experiments was Friedman's medium modified to contain 10% enzyme-thinned starch. For shake flasks, the buffer was 3.3g CaCO₃ chips in 15 ml
of medium. For the fermentor, 5N NaOH was used to control the pH.

Table 1: Modified Friedman's Medium (Friedman and Gaden 1970):

<table>
<thead>
<tr>
<th>(1) mineral solution:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO$_4$$\cdot$7H$_2$O</td>
<td>1.225 g/L</td>
</tr>
<tr>
<td>MnSO$_4$$\cdot$H$_2$O</td>
<td>0.0336 g/L</td>
</tr>
<tr>
<td>FeSO$_4$$\cdot$7H$_2$O</td>
<td>0.0584 g/L</td>
</tr>
<tr>
<td>CH$_3$COONa$$\cdot$3H$_2$O</td>
<td>1.658 g/L</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.5 g/L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(2) nitrogen source</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3% yeast extract</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(3) carbon sources</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>variable*</td>
<td></td>
</tr>
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</table>

*Original medium included 5% glucose as a carbon source, but enzyme-thinned starch of variable concentration was used in these studies.

The starch in these studies was gelatinized and enzyme-thinned. Pearl starch (A. E. Staley, food grade) was mixed with distilled water to make 20 liter of 30% (w/v) solution. The pH was adjusted to pH 7.0 with 5N NaOH and 1.2g of bacterial a-amylase (Amano; Bacillus subtilis, 176000 BAU/gm) in 80 ml of 0.2% CaCl$_2$·2H$_2$O solution was added. The mixture was heated in a steam jacketed kettle (Groen, Model TDB/7) at a stirring dial of 2. The temperature was increased from room temperature to 87°C over a 24 minute period and then held constant for 10 minutes. The enzyme-thinned starch was then allowed to air cool for 10 minutes and dispensed into 600 ml bottles and stored in a freezer. Microscopic examination indicated that all of the starch grains were completely gelatinized and disrupted. The final concentration of the enzyme-thinned starch solution was 300.6 g/L (30% w/v) as determined by drying 10 ml solution in an oven at 95°C for 24 hours.

Most studies were carried out in shake flasks and incubated in a temperature controlled shaker (Psycrotherm; New Brunswick Scientific Co. Inc.) operated at 150 rpm. The fermentor used was a New Brunswick Scientific Co. Inc. Model C30 bench top chemostat with a Model pH-40 pH controller. The working volume of the fermentor was 1.2 liters with agitated at 400 rpm.

Unless stated otherwise, the incubation temperature in all studies was 37°C. Media preparation, inoculation and incubation were carried out using the anaerobic technique developed by Hungate [8].

Assay Procedures

Lactic acid was analyzed by HPLC using a Series 4 Perkin Elmer equipped with an RI detector (Model LC 25) and a computing integrator (Model LC 100). The column used was a Polypore H 10nm (4.6X220 mm; Brownlee Labs, Inc) operated at room temperature. The mobile phase was H$_2$SO$_4$ at pH 2 and the flow rate was 0.2 ml/min. When necessary, samples were heated to redissolve calcium lactate crystals.

Starch and oligosaccharides were analyzed also by HPLC with a carbohydrate column
(AMINEX PHX 42A, serial 2770; Bio-Rad) at 75°C. Mobile phase used was water and flow rate was 0.4 ml/min.

VFAs were detected by gas chromatography (Model 3700; Varian) with flame ionization detector. The column was a Chromosorb 101 column (1.8 x 0.004m; Supelco Co.) at 180°C. The carrier gas was N₂ at flow rate 80 ml/min. The sample was acidified with 0.2 ml of 2N HCl for 1 ml of centrifuged (13000 rpm for 30 minutes) sample.

Cell numbers were determined by viable plate count on modified Friedman's medium with 1.5% agar and 10% starch. Agar plates were incubated in an anaerobic gas jar for three days before counting.

**Results and Discussion**

The work done in this study can be split into three parts. The first one was comparison of lactic acid production by a glucose-utilizing and two starch-utilizing organisms. The second was nutritional studies. It included that effect of substrate concentrations, different kinds of nitrogen sources, concentrations of the yeast extract, concentrations of mineral solution and supplement of some growth factors. The third was study of operating conditions which included temperature and pH of culture broth.

**Comparison of Lactic Acid Production by Different Strains**

The experiments were conducted with 15% initial starch concentration for SMC-32 (37°C and pH 5.5), 10% initial starch concentration for *L. amyllovorus* (37°C and pH 5.5), and 15% initial glucose concentration for *L. delbrueckii* (45°C and pH 5.5). While the rate of lactic acid production by *L. amyllovorus* from enzyme-thinned starch compared favorably to *L. delbrueckii* B445 grown from glucose (Figure 1), SMC-32 had the worst performance. It had a long lag period (40 hours), a slow lactic acid production rate during the log phase and the lowest conversion (around 50%) in the final stage.

The total amount of lactic acid produced by *L. delbrueckii* and by *L. amyllovorus* were close to theoretical value. A significant amount of starch was left in medium by SMC-32.

Since the different organisms may have different optimum operating conditions, this was not a strict comparison of the potential of the organisms. It did, however, clearly suggest that *L. amyllovorus* can convert starch to lactic acid in a quantitative fashion, to a high conversion, and to a high lactic acid concentration. Hence, this organism was chosen for further studies with starch to lactic acid fermentation.

**Nutritional Studies**

The effects of three major kinds of nutrition (carbohydrate, nitrogen source, and minerals) on lactic acid production were identified in the modified Friedman's medium. The modified Friedman's medium was also added another growth factors to find out whether the rate of lactic acid production would be stimulated by these growth factors.

**Concentration of Enzyme-Thinned Starch**

In order to determine the effect of starch concentration on the lactic acid production, 7.5%, 10%, 12%, 15% or 20% enzyme-thinned starch was added to Friedman's medium as a
carbon source.

These studies were conducted in shaken flasks at 37°C and pH 6.0 (initial pH of broth). Amount of residual starch was measured by iodine test at the end of each experiment. The results showed that up to 15% starch was completely consumed by L. amylovorus (Figure 2). But residual starch was observed with 20% initial starch concentration. The conversion of starch to lactic acid was changed from a constant value of 92% (approximated) to 75% (approximated). It is well known [7,9] that lactic acid inhibits bacterial metabolism. Yet, it was not clear from these experiments whether the incomplete conversion of starch was solely due to lactic acid accumulation. pH in these experiments was controlled by calcium; as a result of calcium lactate crystallized at high lactic acid concentration which may have caused additional toxicity.

Nitrogen Sources

Nitrogen source is one of the most important nutrients for bacterial growth, especially, for lactic acid bacteria because of their under-developed biosynthetic capability [4]. Ten different nitrogen sources were tested to find the best one for lactic acid production by L. amylovorus. The concentration of nitrogen sources were calculated to give an approximately equivalent amount of nitrogen content; if the nitrogen content were unknown, 3% (w/v) of the nitrogen source was added. The amounts used were (NH₄)₂HPO₄ (Mallinckrodt), 1.36%; (NH₄)₂SO₄ (Sigma), 1.36%; cottonseed hydrolysate (lot # 8NH0#; Sheffield), 3.48%; neopeptone (Difco), 3%; corn steep liquor (Sigma), 1.36%; trypticase (BBL), 2.64%; pepticase (Sheffield), 2.32%; phytone peptone (papaic digest of soybean meal; BBL), 3%; distiller grains and solubles (Sigma), 3%; or yeast extract (Difco), 3%.

Inorganic nitrogen sources were not utilized by the cell (Figure 3). Phytone peptone, cottonseed hydrolysate, neopeptone, and yeast extract resulted in the highest lactic acid levels. Starch was consumed completely in five days with these four best nitrogen sources. While cottonseed hydrolysate resulted in the highest final concentration of lactic acid and is a relatively inexpensive raw material, the level of impurities in the cottonseed hydrolysate would increase purification cost. Yeast extract was chosen as the nitrogen source for the following studies because of its general availability and widespread use by other researchers.

Concentration of Yeast Extract

The concentration of yeast extract in starch Friedman's medium was changed from 0.5%, 1.5% to 3% (w/v) in order to determine the optimum level of yeast extract.

There was no significant difference in final lactic acid concentration by addition of different concentrations of yeast extract (Figure 4); however lactic acid production rate increased with increasing yeast extract concentration. The total lactic acid production rate in 3% yeast extract was four times faster than in 0.5% yeast extract.

Yeast extract apparently contains a growth factor which increases growth rate and L. amylovorus required large amount of these growth factors for better lactic acid production. This requirement for high yeast extract levels will increase the cost of raw materials, and make purification more difficult.

Concentration of Mineral Solution
The mineral solution concentration in Friedman’s medium was modified to be 0.5, or 1.5 fold of the original medium. The lactic acid production curves for these three levels of minerals in Friedman’s medium were nearly identical (Figure 5). Hence, it can be concluded that the modified Friedman’s medium contains more than sufficient quantities of mineral nutrients.

**Stimulation Studies**

In this study, 1 ml of vitamin complex solution, 1 ml of trace metal solution, 1 ml of VFA solution, and 0.1 ml of hemin solution (Table 2) were added to the modified Friedman’s medium (per 100 ml medium) contained 0.5% yeast extract.

The requirement for high concentrations of yeast extract could possibly be reduced by addition of known nutrients to the medium, thus reducing the cost of raw materials. Unfortunately, addition of VFA, vitamins, hemin and trace metals into the medium did not increase the rate of lactic acid production significantly (Figure 6). It is possible that one component may have been stimulatory and another component inhibitory. This should be tested in future studies by single addition experiments.

**Investigation of Operating Conditions**

Each organism has its own optimum operating condition. Some of this information for strain *L. amylovorus* were given by previous investigator (Nakamura 1981), but not in enough detail. The main works done in this section were to give a detailed picture concerning the effects of temperature and pH.

**Temperature**

Optimum temperature for *L. amylovorus* was investigated in 5% starch Friedman’s medium incubated in the shaker at 30, 35, 40, 45, and 50°C. The concentration of starch in the broth was reduced from 10% to 5% to eliminate any possible interference due to calcium lactate crystallization in the final stages of the fermentation.

*L. amylovorus* was highly sensitive to the changes in temperature out of its optimum temperature range (Figure 7). The lactic acid production rate increased as temperature increased from 30°C to 40°C, remained the same between 40°C and 45°C, and decreased dramatically above 45°C. Maximum lactic acid production rate was around 3.8 g/l-hr. From these studies, the optimum temperature appeared to be between 40°C and 45°C. Similar results have been reported by Nakamura (1981).

**pH**

Optimum pH for *L. amylovorus* was determined in the fermentor with pH maintained at 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 with 5N NaOH. Maximum lactic acid production rate was obtained between pH 5 to 6 (Figure 8). It decreased only slightly at 4.5, but was greatly reduced at pH 4.0 and 6.5. Hence pH value of 4.5 appears to be a desirable operating pH, since a low pH is beneficial in reducing the probability of contamination.

In pH uncontrolled fermentations supplemented with CaCO₃, the pH of broth dropped from 6 to 4 (data not shown) during the initial part of fermentation. CaCO₃ prevented a drop lower than pH 4. Yet, there was no significant difference in lactic acid production rates in pH-controlled fermentation and in uncontrolled shake flask culture buffered by CaCO₃ (Figure 9). The pH-
controlled data in Figure 9 were corrected for dilution by base addition. Possible reasons for such as an anomalous behavior may be the broad pH optima for this organism, as well as the possibility that lactic acid is less toxic to growth of cells than it is to its own formation. Another possibility is that calcium has a modulating influence upon pH effect.

**Fermentation Under Optimum Operating Conditions**

Since there was no difference in product formation rate between the shake flask buffer with CaCO₃ and the fermentor with pH controlled by NaOH, the shake flask system was chosen for the optimum condition experiment. The temperature optimum is between 40° to 45°C and although a high fermentation temperature reduces the chance of contamination, it requires more energy to maintain a high temperature system than low temperature. Since less contamination can also be achieved by low pH in the system, 40°C was chosen for fermentation under optimum conditions. Modified Friedman's medium with 3% yeast extract and 10% enzyme-thinned starch was used in this study.

Bacterial cells started lysing after 16 hours (Figure 10), probably due to the accumulation of toxic material and the depletion of energy source. Oligosaccharides larger than four glucose unit were enzymatically broken down to the smaller oligosaccharides during the fermentation (Figure 11). The concentrations of glucose, maltose, maltotriose and maltotetrose increased initially and then decreased (Figure 13). The increased concentrations can be explained by these four oligosaccharides being the products of starch broken down by α-amylase (Nakamura 1981) and the enzymatic breakdown of starch being faster than bacterial consumption rate. Later, all of the oligosaccharides were depleted by bacterial consumption. No oligosaccharides were left in the broth after 26 hours of fermentation. Small amounts of acetic acid (2.38 g/L) were produced by this organism. Conversion of glucose to lactic acid was 98.1% in this experiment.

**Conclusions**

Organic nitrogen sources are necessary for better lactic acid formation. Phytone peptone, cottonseed hydrolysate, neopeptone and yeast extract are the best ones among the ten different kinds of nitrogen sources tested. High concentrations (3%) of yeast extract is required for the fastest production rate. This is the primary disadvantage from an economical point of view. Addition of VFA, vitamins, hemin, and trace metals didn't improve significantly the rate of lactic acid production in 0.5% yeast extract Friedman's medium. Temperature optimum is between 40°C and 45°C. Maximum lactic acid production occurs between pH 5.0 and pH 6.0. There was no difference between CaCO₃ and 5N NaOH as buffer, although pH dropped to pH 4 when CaCO₃ was used. Lactic acid formation from starch is comparable to *L. delbrueckii* from glucose.

**Table 2 : Compositions of Vitamin, Hemin, VFA, and Trace Metal Solution.**

<table>
<thead>
<tr>
<th>(1) Vitamin mixture stock solution</th>
<th></th>
</tr>
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<tr>
<td>Thiamine HCl (vitamin B₁, Aneurine)</td>
<td>20.0 mg</td>
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<tr>
<td>D-Pantothenic Acid (Calcium salt)</td>
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</tr>
<tr>
<td>Niacinamide (Nicotinaamide, Nicotinic Acid Amide)</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>Component</td>
<td>Amount</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Pyridoxine, monohydrochloride (vitamin B6, Pyridoxal)</td>
<td>20.0 mg</td>
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<tr>
<td>p-Aminobenzoic Acid (PABA)</td>
<td>1.0 mg</td>
</tr>
<tr>
<td>d-Biotin (vitamin H)</td>
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</tr>
<tr>
<td>Folic acid (Pteroylglutamic acid)</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>Cyanocobalmin (vitamin B12)</td>
<td>0.2 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

(2) Hemin solution

- Hemin: 50 mg
- NaOH: 1 N for 1 ml
- Distilled H2O: 100 ml

(3) VFA solution (Volatile Fatty Acid Solution)

- Acetic acid: 16.0 ml
- Propionic acid: 6.7 ml
- n-Butyric acid: 4.1 ml
- i-Butyric acid: 0.84 ml
- n-Valeric acid: 0.98 ml
- i-Valeric acid: 0.98 ml
- z-Methyl Butanoic Acid: 0.98 ml
- Distilled water: up to 100 ml

(4) Trace metal solution:

- EDTA-di Na: 500 mg
- FeSO4·7H2O: 200 mg
- ZnSO4·7H2O: 10 mg
- MnCl2·4H2O: 3 mg
- H3BO3: 30 mg
- CoCl2·6H2O: 20 mg
- CuCl2·2H2O: 1 mg
- NiCl2·6H2O: 2 mg
- Na2MoO4·2H2O: 3 mg

References


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**Figure 1:**
Comparison of lactic acid production by starch-utilizing (*L. amylovorus*, SMC-32), and glucose-utilizing (*L. delbrueckii*) cultures.

**Figure 2:**
Effect of enzyme-thinned starch concentration on conversion to lactic acid by *L. amylovorus* (each data point represent the arithmetic mean of duplicate experiment)
Figure 3: Lactic acid production by *L. amylovorus* with different kinds of nitrogen sources. Abbreviation are as follows: AS : (NH₄)₂SO₄, ADP : (NH₄)₂HPO₄, DGS : distiller grain and solubles, PTC : pepticase, PPT : phyton peptone, CS : cotton seed hydrolysate, CSL : corn steep liquor, TPT : trypicase, NPT : neopeptone, YE : yeast extract. (each data point represents the arithmetic mean of triplicate experimental measurements)

Figure 4: Effect of yeast extract concentration on lactic acid production by *L. amylovorus*. (each data point represents the arithmetic mean of duplicate experimental measurements)

Figure 5: Effect of mineral solution concentration on lactic acid production by *L. amylovorus*. (each data point represents the arithmetic mean of duplicate experimental measurements and control data was obtained from yeast extract concentration studies with 3% yeast extract)

Figure 6: Effect of addition of growth factors on lactic acid production by *L. amylovorus*. (each data point represents the arithmetic mean of duplicate experimental measurements and control data was from yeast extract concentration studies with 0.5% yeast extract)

Figure 7: Effect of temperature on lactic acid production by *L. amylovorus*. (each data point represents the arithmetic mean of duplicate experimental measurements)
Figure 8:  
Effect of pH on lactic acid production by *L. amylovorus*. Comparison of lactic acid production by *L. amylovorus* using different buffers. (data points represented total lactic acid amount which was calculated from pH 6 experiment and arithmetic mean of duplicate experimental measurements of 3% yeast extract with CaCO$_3$ buffer.)

Figure 9:  
Cell growth and lactic acid production by *L. amylovorus* under optimum operating conditions of fermentation.

Figure 10:  
Starch, maltoheptose, maltose, and glucose utilization by *L. amylovorus* under optimum operating conditions of fermentation.
Solubilization of Preoxidized Texas Lignite by Cell Free Broths of *Penicillium* Strains

Richard T. Moolick, M. Nazmul Karim*, James C. Linden and Brian L. Burback

Department of Agricultural and Chemical Engineering
Colorado State University
Fort Collins, Colorado 80523

ABSTRACT

Significant solubilization of preoxidized Texas Lignite was achieved using cell-free filtered fermentation broths of the *Penicillium* strains RTM-2B and RTM-2C. Sodium azide was discovered to strongly inhibit the solubilizing activity of the broths. Further experiments with inhibitors identified the process as enzymatic in nature and suggestive of peroxidase or oxidase activities. The kinetics of the process were studied and a diffusion limitation was identified.

INTRODUCTION

Cohen and Gabriele¹ found that *Polyporus versicolor* and *Portia monticola* were capable of significantly degrading some low-ranked coals. Ward² isolated fungal cultures from a weathered

* Person to whom correspondence should be addressed.
coal outcrop which had the capacity for solubilizing weathered low-ranked coals. One of these cultures, RWL-5, was the parent culture from which the strains RTM-2B and RTM-2C were isolated.

Wondrack et al. examined the enzymatic attack of lignin peroxidase on water soluble coal polymers from North Dakota lignite and German subbituminous coal.

This project evaluated the solubilization of preoxidized Texas lignite by the extracellular products of RTM-2B and RTM-2C to determine whether the solubilization process was alkaline as with Streptomyces species or enzymatic.

MATERIALS AND METHODS

RWL-5, RTM-2B and RTM-2C were grown in 500 ml shake flasks containing 100 ml Czapek Dox broth. The cultures were incubated in a shaker incubator for 8 days at 30 C and 150 rpm. Cell-free broths of each culture were obtained by filtering the broths through 0.45 μm membranes. The broths were divided into 20 ml scintillation vials to which 200 mg of TXL35W had been added. The vials were incubated at room temperature for 4 days. A control consisting of Czapek Dox broth with sodium azide and coal was incubated under the same conditions as the cell-free broths.

A time based kinetics study using the cell-free broth of RTM-2B was performed by preparing a series of vials containing 100 mg of TXL35W and 20 ml of broth. Vials were sampled
periodically to determine the degree of solubilization at various times.

To characterize the agent in the cell-free broth that solubilizes the coal, vials of cell-free broth were prepared as above. One fourth of the vials were heated in a water bath to 100 °C for 2 hours to deactivate any heat labile agents present. A second set treated with mercuric chloride (1 mM) to deactivate any enzymes present. A third set was treated with sodium azide (1 mM). The fourth set was not treated. All vials were allowed to reach room temperature before 100 mg of TXL35W was added to each vial. After 4 days static incubation at ambient temperature, the degree of coal solubilization was measured as above.

RESULTS AND DISCUSSION

The cell-free broths of RTM-2B and RTM-2C solubilized between 36 and 54 percent of pretreated lignite in two separate experiments. (Figure 1). The broth of RWL-5 exhibited 3 percent solubilization, which was only slightly better than 2 percent in the control. Additional experiments to which sodium azide was added to the broths to prevent microbial growth, were conducted to verify these results. The azide was added due to the observed growth of bacteria and fungi in the cell-free broth. None of the broths exhibited significant solubilization. A letter listing sodium azide as a "potent inhibitor of glucose oxidase" led to the exclusion of sodium azide as a preservative.
Between 24 and 72 hours, the solubilization rate was approximately constant at 0.59 grams per liter - day ($r^2=0.998$) using 100 mg TXL35W per 15 ml of broth (6.67 g/l) (Figure 2). The solubilization rates were 0.35 grams per liter - day ($r^2=0.986$) before and 0.18 grams per liter - day ($r^2=0.994$) after this period. Rates were obtained by least squares regression of the data for each timespan. The 0.35 gl$^{-1}$d$^{-1}$ average rate between 0 and 24 hours was due to a lag phase caused by the difficulty of wetting the coal particle surfaces. The rate reduction after 72 hours was possibly due to a gradual deactivation of solubilizing activities by product inhibition or by activity degradation.

The effect of initial solid coal concentration on the solubilization rate was measured at 48 hours. The rate changed with respect to initial substrate concentration in a manner consistent with Michaelis-Menten kinetics (Figure 3). Using the fortran package MNFIT, an estimation of the Michaelis-Menten parameters ($V_{max} = 0.71d^{-1}; K_m = 5.792 g/l^{-1}$) was obtained. Figure 4 compares the solution of the Nelder-Mead iterative fit with the actual data (correlation coefficient = 0.955).

Following a suggestion by Bajpai, a modified Michaelis-Menten model was used. The model equation, shown below, provided a significantly better fit of the data (Figure 5). This model accounts for the limitations caused by diffusion of enzyme to the coal surface and/or diffusion of the products away from the surface at low substrate concentrations.
\[
V = \frac{V_{\text{max}} S^n}{K_m + S^n}
\]

where \( V \) is the solubilization velocity, \( V_{\text{max}} \) is the maximum solubilization velocity, \( S \) is the initial coal concentration and \( n \) is the factor to compensate for diffusion limitations. For this model, \( V_{\text{max}} = 0.49 \text{ d}^{-1}, \) \( K_m = 5.792 \text{ gl}^{-1} \) and \( n = 1.85. \)

To verify if the solubilizing activities were enzymatic in nature, an experimental series was conducted to determine if the broth activities were affected by heat or chemical inhibitors. Addition of mercuric chloride or sodium azide both at 1 mM strongly inhibited the coal solubilizing activity (Figure 6). Boiling the broth at 96° C for two hours, also, significantly reduced the activity. The untreated broth solubilized approximately 40 percent of the coal while the boiled broth exhibited an 94 percent reduction in solubilizing activity relative to untreated broth. The activities of the chemically treated broths were reduced by 99 percent.

The cell-free fermentation broths of RTM-2B and RTM-2C had greater solubilization activities than RWL-5. The activity of the RTM-2B broth was inhibited by NaN₃ and HgCl₂. Heat reduced the activity significantly. RTM-2B and RTM-2C produced the lignite solubilizing enzymes constitutively, suggesting that the enzymes were fairly non-specific.
REFERENCES CITED


5. Mason, Marc, Private communication with L. Henk, Yellow Springs Instrument Co., Inc., Yellow Springs, OH 45387, June 1, 1984

Figure 1: Comparison of the degrees of solubilization of TXL35W by cell-free fermentation broths of RWL-5, RTM-2B and RTM-2C at 96 hrs. Run 1 and Run 2 confirmed significant solubilizing activity by RTM-2B and RTM-2C.

Figure 2: Solubilization of TXL35W by cell-free broth of RTM-2B as a function of time.
Figure 3: Effect of initial coal concentration on the rate of solubilization of TXL35W by cell-free broth of RTM-2B as measured at 48 hrs.

Figure 4: Nelder-Mead fit of the effect of coal concentration on TXL35W solubilization rates.
Figure 5: Diffusion limited Monod model of the effect of coal concentration on TXL35W solubilization rates by cell-free broth of RTM-2B.

Figure 6: Inhibition of coal solubilizing activities of cell-free broth of RTM-2B by 100°C, sodium azide and mercuric chloride.
Separation of Proteins from Polyelectrolytes by Ultrafiltration

Andrea G. Bozzano and Charles E. Glatz
Iowa State University
Dept. of Chemical Engineering
Ames, IA 50011

May 1, 1989

Abstract

Polyelectrolyte precipitation can be a simple and effective method for concentration and fractionation of protein mixtures. Following precipitation, separation of polyelectrolytes from the proteins can be effected by ultrafiltration.

Precipitations of bovine serum albumin and egg white protein solutions were carried out using polyacrylic acid as a precipitant. The precipitate was resolubilized and ultrafiltered in a stirred cell. Polyacrylic acid was completely retained during ultrafiltration. Permeation of proteins was found to be dependent on parameters such as pH and ionic strength, which control the protein/polyelectrolyte complexation, and stir rate, which controls concentration polarization. Typical BSA rejection coefficients for membranes with MWCO of $3 \times 10^5$ daltons were $\approx 0.2$.

INTRODUCTION

In today's biotechnology industry, a typical process may involve the production of an enzyme by fermentation. In such a process, a large part of the cost of production of proteins is generally associated not with the fermentation step, but with the many downstream processing operations required to purify and concentrate the target enzyme $^{1}$.

One of the many downstream separation steps which can be used following fermentation is that of polyelectrolyte (PE) precipitation. This involves the precipitation of charged proteins by aggregation with oppositely charged, high molecular weight polymers. PE precipitation is an excellent method for protein recovery as it can selectively precipitate proteins from a solution and isolate them in the solid phase, thus achieving both concentration and purification. Also, PE precipitation is easily scaled-up, it can be carried out continuously, it is relatively inexpensive, and it retains the enzymatic activity of proteins.

In some cases, subsequent removal of the polyelectrolyte may be required. Because of the large difference in the molecular weights of proteins and polyelectrolytes, removal of the latter can, in some cases, be carried out by ultrafiltration. Ingham et al. $^{2}$ and Papamichael and Kula $^{3}$ have used ultrafiltration to recover polyethylene glycol (PEG) from a PEG Bovine Serum Albumin (BSA) mixture (PEG was recovered in the permeate due to its small size). The goal of this research has been to develop a strategy of using UF membranes to recover proteins in the permeate and polyelectrolytes in the retentate.
ULTRAFILTRATION THEORY

UF is a technique for the separation of molecules on the basis of size. It is a pressure driven process in which solvent and smaller solutes are forced through a semipermeable membrane which retains larger molecules.

An important parameter for studying the rejection behavior of membranes is the rejection coefficient (\(R\)), defined as the fraction of molecules of a given species retained by the membrane:

\[
R = 1 - \frac{C_P}{C_R}
\]

Using a mass balance across the membrane and the above definition, we can get \(R\) in a form involving feed and permeate concentrations only:

\[
R = \frac{\ln \left( \frac{C_o V_o - C_P V_P}{V_o C_o} \right)}{\ln \left( \frac{V_o}{V_o - V_P} \right)}
\]

Chemical manufacturers generally characterize membranes by means of a nominal molecular weight cutoff (MWCO). The MWCO is defined to be the size of the smallest molecule to have a given retention (generally about .9) by the membrane. The rejection coefficient used to define a MWCO is generally arbitrarily chosen by manufacturers. For UF, available MWCO’s range from 1000 to \(1 \times 10^6\) daltons, with corresponding membrane pore diameters ranging from 10 to 200 A. Because the MWCO of a membrane is generally found using “arbitrary” globular proteins, it is generally not an accurate indicator of the rejection behavior of all molecules. In practice, rejection behavior for specific molecules must be found by trial and error [5]. This is especially true of nonglobular macromolecules such as polyelectrolytes.

Figure 1 shows typical flux vs. pressure behavior in a stirred cell UF apparatus [4]. For UF of a pure solvent, the only resistance to flux is that imparted by the membrane. Since the membrane resistance is constant, increases in flux are proportional to increases in the driving force \(\Delta P\).

When solute is introduced into the system, the resistance to flux is no longer constant: as solute is carried towards the membrane, a concentration gradient is formed (see fig 2 ). This causes a phenomenon known as concentration polarization, a result of which is that the osmotic pressure at the membrane is increased due to the higher solute concentration at the membrane interface. The increase in osmotic pressure causes the driving force to decrease, therefore producing a decline in flux. The pressure-dependent region of the flux vs. \(\Delta P\) curve (fig. 1) illustrates concentration-polarized and non-polarized behaviors in ultrafiltration.

As the driving force is increased further, the solute concentration at the membrane interface rises as well. This can sometimes cause the solute to form a gel layer on the membrane. The gel layer generally causes decreased flux and may in some cases affect the rejection behavior of the membrane by retaining molecules which would normally be permeated. The presence of a gel layer gives rise to the mass transfer controlled region of the curve, also known as pressure independent. Once the gel layer is formed on the membrane, any increases in \(\Delta P\) will not result in increases in flux, but in a thickening or compression of the gel layer [4].
MATERIALS AND METHODS

Materials

Polyacrylic acid was obtained from Polysciences, Inc. (Warrington, PA) and had a mass average molecular weight of $4 \times 10^6$ daltons. Dried egg white solids were obtained from Henningsen Foods, Inc. (White Plains, NY). The composition of egg white is shown in Table 1 [6]. Standard solutions of bovine serum albumin and lyophilyzed pure egg white proteins were purchased from Sigma Chemical Company (St. Louis, MO).

The ultrafiltration apparatus used was a stirred cell device (Amicon, Cherry Hill, CA, model 8400). Ultrafiltration membranes were obtained from Amicon (type YM100, MWCO=1 x 10^5) and from Pharmacia LKB (Piscataway, NJ; membrane types Ω100, MWCO=1 x 10^5 and Ω300, MWCO=3 x 10^5). All membranes used are termed "low protein binding".

Analyses

Total protein analysis was carried out by the biuret method [7] and standardized using BSA. Lysozyme was assayed by measuring the rate of lysis of a suspension of *M. Lysodeikticus* cells [8]. Egg white proteins were also individually and qualitatively analyzed for by ion-exchange liquid chromatography using an Aquapore CX-300 cation exchange column (Pierce: Rockford, IL). A qualitative PAA analysis was carried out for the egg white ultrafiltrations by simply adjusting the pH to conditions favorable for precipitation and visually checking for precipitated particles. A quantitative PAA analysis (standardized with PAA) was used for BSA ultrafiltrations by addition of a known amount of lysozyme solution and subsequent measurement of solution turbidity.

Experimental Procedure

Egg White

Egg white solutions were prepared in acetate buffer (pH=5.4, I=.07M) at a total protein concentration of about 4 mg/mL. Precipitation was carried out by addition of PAA at a dosage of 7.5 mg per g total protein. The precipitate was separated by means of centrifugation (1x10^4 g, 40 min.) and resolubilized after addition of NaOH to pH 11. After pH and ionic strength adjustments, ultrafiltration was carried out on 150 mL of feed. The qualitative PAA, biuret, and lysozyme assays were performed on the permeate and feed. HPLC analysis was carried out in some cases.

Bovine Serum Albumin

Bovine Serum Albumin was diluted to 10 mg, mL prior to precipitation. PAA dosage used was 20 mg PAA per g protein. As with egg white, the precipitate was separated and resuspended to a concentration of 4 mg/mL. Ultrafiltration was carried out in the same way as with egg white. The quantitative PAA and biuret total protein assays were done on feed and ultrafiltrate.

Experimental Strategy

The emphasis of this work was on understanding what mechanisms govern the separation of protein PAA solutions. Stir rate, ionic strength and pH were all varied in an effort to optimize the rejection behavior of proteins and polyelectrolytes. Optimization of the flux performance of the ultrafiltration was of secondary importance.
RESULTS AND DISCUSSION

Egg White Ultrafiltrations

Ultrafiltrations of egg white proteins were carried out with MWCO=1×10^5 membranes (YM100 and OM100). Typical results from these runs are shown in fig 3. As can be seen in the graph, lysozyme was the only protein to be completely permeated by the membrane. Ovalbumin was partially retained, and conalbumin completely rejected. Enzymatic activity was completely retained as demonstrated by the lysozyme activity assay. Also, PAA was completely retained by the membranes.

High rejection was not expected for ovalbumin (MW=45000 daltons) and conalbumin (MW=76000 daltons), as Amicon literature for YM100 membranes indicated low rejection for proteins as heavy as 67000 daltons [9]. In order to explain the high retention of ovalbumin and conalbumin, it was hypothesized that protein-protein [2] or protein-polyelectrolyte complexation may have been taking place; such complexation would have caused the formation of large complexes and would have prevented some of the proteins from filtering through the membrane. To test this hypothesis, ultrafiltrations of pure proteins were carried out. These runs indicate that ovalbumin and conalbumin were highly retained even in the absence of PAA or other proteins (see table 2). Higher ionic strength conditions (.2M NaCl added) were used in order to break up protein-protein or protein-polyelectrolyte complexes [2]. Figure 4 shows that increased ionic strength had little effect on rejection of proteins.

In conclusion, we have seen that only small proteins (lysozyme) can be easily separated from PAA using MWCO=1×10^5 membranes. High protein rejection in egg white ultrafiltration is due not to protein-protein or protein-polyelectrolyte binding but to the impermeability of the membrane to the individual proteins.

Bovine Serum Albumin

After having achieved the separation of only the smaller egg white proteins from PAA, it was decided to use a higher MWCO membrane to carry out separations of larger proteins. In order to facilitate analysis, experimentation was carried out using a single protein system. Bovine serum albumin was chosen since it was a fairly large, easily analyzable protein.

Initial runs with BSA were carried out with MWCO=1×10^5 in order to check the BSA rejection behavior against that of egg white. As expected, retention of BSA was quite high, approximately 99%. PAA was completely retained. To get permeation of BSA, membranes with MWCO=3×10^5 (OM300) were used.

Runs with the higher MWCO membranes were carried out at two different pH values, pH=7.5. and pH=11 (see figure 5). The pH values were chosen as the lowest pH needed to get redissolution of the precipitate, and the highest pH allowed for use with the membrane. As can be seen in the diagram, the rejection coefficient for pH=7.5 (R=0.2) is quite a bit higher than for pH=11 (R=0). This seems to indicate that at the lower pH, protein-polyelectrolyte interactions may have been preventing the total permeation of protein.

Once again, in order to see if binding was present, runs were carried out at both high and low ionic strengths (see figs 6 and 7). The high ionic strength runs showed lower protein retention than the low ionic strength ones. This is further indication of the presence of a protein PAA complex.
as the higher salt concentration should break up such a complex and give a lower protein retention.

The pH value at which protein/PAA binding takes place was found by means of an experiment involving the titration of a PAA/BSA solution with dilute acid (see fig. 8). As the pH is lowered, solution turbidity is monitored with a spectrophotometer. This experiment showed that complexation is not present until a lower pH value for higher ionic strength, a behavior previously seen by Dubin [10]. In fact, at the conditions used in the UF runs (pH=7.5), quite a bit of complexation is taking place for the low ionic strength run. Complexation is reduced but not entirely eliminated when ionic strength is raised to .2M NaCl.

Resistance to flux

Ultrafiltrations were carried out at varied stir rate to characterize the types of flux resistance present during ultrafiltration. Figures 6,7 show a dependence of $R$ on stir rate when operating at low ionic strength. As can be seen in figure 6, $R$ is quite high when no stirring is used and decreases as stir rate increases. However, retention decreases only up to a stir rate of about 200 rpm, at which point $R$ levels off.

The dependence of $R$ on stir rate may be caused by the presence of a gel layer on the membrane, which was observed on the membrane after ultrafiltration. This layer apparently contributes to the exclusion of proteins from the membrane, and is present only at stir rates lower than 200 rpm. Note that although $R$ exhibits stir dependence only up to 200 rpm, the ultrafiltration flux is always dependent on stirring, evidence of concentration polarization in all the runs.

In the high ionic strength runs, $R$ is independent of stir rate. It is not well understood why $R$ is affected by the gel layer only in the case of low ionic strength. One possible explanation is that an equilibrium is involved:

$$K = \frac{[Prot] [PAA]}{Prot \cdot PAA}$$

in which $K$ is lowest at low ionic strength. The protein concentration, [Prot], would be higher inside the gel than in the bulk, causing an increase in the concentration of the electrostatic complex, Prot-PAA. The increase in [Prot-PAA] would bind some of the free protein and increase retention. Because of the dependence of $K$ on I, this effect would be greatest at low ionic strength. Another possibility is that crosslinking of the complexes inside the gel may be occurring due to the low ionic strength interactions between proteins and PAA. This could reduce the size of the voids in the gel and act as a physical barrier to protein molecules.

CONCLUSIONS

Separation of PAA from proteins was successfully carried out using two different membranes for different size proteins. Proteins as small as lysozyme could be permeated with a membrane MWCO of $1 \times 10^5$, whereas larger proteins, such as BSA, required the use of MWCO=$3 \times 10^5$. Also, it was found that ultrafiltration conditions must be chosen to prevent protein/PAA complexation. When operation is at conditions which promote complexation, the gel layer which forms on the membrane can increase retention of proteins.
References


Tables

Table 1: Main components of egg white [6].

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<th>nominal amount (%)</th>
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<td>54</td>
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<tr>
<td>Conalbumin</td>
<td>76600</td>
<td>13</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>28800</td>
<td>11</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14700</td>
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Table 2: Retention of pure EW proteins

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<td>Conalbumin</td>
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</tr>
<tr>
<td>Ovalbumin</td>
<td>.4</td>
</tr>
<tr>
<td>Lysozyme</td>
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MODELLING OF ULTRAFILTRATION PROCESSES

Figure 1: Flux vs. transmembrane pressure behavior in an ultrafiltration process. Also note effect of other parameters [4].
Figure 2: Phenomena of concentration polarization and gel polarization in ultrafiltration [4].

Figure 3: Ion exchange liquid chromatography of egg white ultrafiltration retentate (top) and permeate (bottom). Peaks are ovalbumin (0.5 min), conalbumin (0.7 min), and lysozyme (11 min).

Figure 4: Effect of ionic strength on retention of egg white proteins. pH = 11, stir rate = 200 rpm.

Figure 5: Effect of pH on retention of BSA. pH = 11.0, no salt added. stir rate = 200 rpm.
Figure 6: Effect of stir rate on flux and retention of BSA (no salt added). △: R(BSA); ●: flux (mL/min · cm²); □: R(PAA).

Figure 7: Effect of stir rate on flux and retention of BSA (0.2M NaCl added). △: R(BSA); ●: flux (mL/min · cm²); □: R(PAA).

Figure 8: Complexation of BSA with PAA vs. pH. Turbidity of solution was measured as absorbance (λ=436nm). △, □: I=0.0M added NaCl; ●, ○: I=0.2M added NaCl.
Growth Estimation and Modeling
of *Rhizopus oligosporus* in
Solid State Fermentation

Doohyun Ryoo¹, Vincent G. Murphy¹, M. Nazmul Karim¹, R. P. Tengerdy²
¹Dept. of Agri. & Chem. Engineering
²Dept. of Microbiology, Colorado State University, Fort Collins, Colorado 80523 USA

SUMMARY

*Rhizopus oligosporus* was grown on the corn substrate enriched with minerals. A barrel type reactor, which was connected to a computer-coupled data acquisition system, was used. The relationship between carbon dioxide evolution rate ($Q_{\text{CO}_2}$) and specific cell growth rate ($\mu$) was established. The stoichiometric equations for the catabolic and anabolic reactions were derived from balance equations of four elements (C, H, O and N) and the relationship between $Q_{\text{CO}_2}$ and $\mu$. Modeling equations for cell growth, water content and total weight of dry matter were developed. Values predicted by the modeling equations were compared with experimental values. The logistic model provided good results for the entire period of fermentation. Calculated values of water content were slightly overestimated because of water condensation on the reactor wall. The trend of dry matter consumption was well represented by modeling.

INTRODUCTION

Although solid state fermentation (SSF) has been used from ancient times in the production of enzymes and fermented foods and in composting, it has not generated much interest in modern time until recently. The inherent difficulties in estimating growth and in controlling the temperature and the moisture content of the substrate have hindered the industrialization of this old technique. The lack of sensors and the
nonhomogeneity of the substrate are major obstacles to the control of SSF and to the measurement of microbial growth and substrate water content.

During last decade, attention in fermentation control research has been focused on the development of an indirect estimation procedure that utilizes growth related process data such as carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR) \(^2\,^5\). To constrain the variance of the estimated process variables, Kalman filtering has been applied\(^2\,^5\). However, most research has dealt with submerged fermentation. Cell growth and product accumulation have been estimated through exit gas analysis, which is appropriate for computer control.

The overall purpose of our research is to develop on-line estimation and control techniques that optimize SSF. Kinetic studies of SSF have not resulted in great progress to date because of the technical difficulties in chemical analysis of cell mass and substrate consumption. In the present study, we propose a model for SSF of *Rhizopus oligosporus* growth, substrate utilization and water content. This model is used to estimate the state of the system by an integration method.

**MATERIALS AND METHODS**

**Microorganism**

*R. oligosporus* (NRRL 2710) with rice starch was used in this study. A commercial culture was purchased and stored in a refrigerator. The activity of inoculum was not changed after 3-4 months at 3-4 °C.

**Media**

Flour-free yellow corn grit (#25 mesh particle size) was used. Corn grit (300 g) was soaked for 3-4 hours with 300 ml of mineral medium which had the following composition (in g/100 mL): \((\text{NH}_4)_2\text{SO}_4\), 7.59; urea, 4.09; NaH\(_3\)PO\(_4\)·H\(_2\)O, 1.726; MgSO\(_4\)·7H\(_2\)O, 0.059; KCl, 0.159; CaCl\(_2\), 0.059; FeSO\(_4\)·7H\(_2\)O, 0.137; alanine, 0.045. This wet material was cooked for 40 minutes at 110 °C in an autoclave. The autoclaved corn was then adjusted to the desired moisture content with water.
Culture conditions

The moisture content of the substrate was adjusted to 40-65% wet weight by the addition of tap water. The pH of water and the mineral medium was preadjusted to 3.5. A commercial starter culture of *Rhizopus oligosporus* spores was mixed with moistened substrate on a 10% w/w basis. The culture was then grown at 37 °C for two days.

Analytical methods

Wet samples (4-5 g) were taken from the reactor with a cork perforator. A 2 g portion of the wet sample was diluted with 20 mL of 0.5% NaCl solution and mixed in a shaker for 3 hours. This diluted sample was centrifuged at 3000 rpm for 10 min to separate the liquid and the solids. The liquid was removed and used for the analysis of sugar and pH. Glucose and reducing sugar concentrations were measured by a glucose analyzer (Yellow Springs Instruments Model 27) and the DNSA method respectively.

The remainder of the wet sample was dried for more than 24 hours at 105 °C to determine the water and protein contents. The water content was determined gravimetrically by drying the sample in an oven. Protein concentration was determined by the micro-Kjeldahl method after trichloroacetic acid (TCA) precipitation.

Fermentation equipment

A schematic diagram of the apparatus used for the SSF procedure is shown in Figure 1. The fermenter was made from acrylic plastic (20cm (D) X 25cm (L)). Fermentation substrate was placed on a grid to facilitate aeration. Temperature of the substrate was measured at three positions to get an average temperature. The temperature of the substrate was controlled by controlling the incubator temperature. The inlet air was first humidified by passing it through hot water (60-70 °C) and then saturated by draining off the excess water in the saturator located in the incubator. The relative humidity and temperature of the inlet and outlet air were measured with hygroscopic humidity sensors and thermocouples.
The aeration rate was set at 6 L/min with a mass flow controller. The exit air was vented to the outside, but a part of this was passed through a drying column and a CO\textsubscript{2} analyzer. An IBM-compatible personal computer with an Omega Control data acquisition system (WB-AIA-B) was used for data acquisition and control.

**RESULTS AND DISCUSSION**

**Cell growth estimation**

Because of the difficulties involved in determination of cell mass in solid cultures, protein content (measured by the micro-Kjeldal method) was used to estimate cell concentration. The cell specific growth rate was then correlated with carbon dioxide evolution rate.

In Figure 2, the relationship between the specific carbon dioxide evolution rate \((Q_{CO2})\) and the specific growth rate \((\mu)\) of *R. oligosporus* at different initial water contents is shown. A reasonably good linear correlation was found. A linear regression method was used to establish the relationship between \(\mu, Q_{CO2}, m_{CO2}\) (maintenance coefficient for carbon dioxide) and \(Y_{X/CO2}\) (yield coefficient).

\[
Q_{CO2} = \frac{1}{Y_{X/CO2}} \mu + m_{CO2} \quad (1)
\]

From this equation, the yield coefficient \(Y_{X/CO2}\) and the maintenance coefficient were determined to be 19.61 [g cell/mol CO\textsubscript{2}] and 0.00197 [mol CO\textsubscript{2}/g·cell·hr] respectively.

**The stoichiometric equations**

To estimate the yield coefficients \((Y_{H2O/X} \text{ and } Y_{O2/CO2})\), the stoichiometric equation for cell growth of *R. oligosporus* on starch was considered. The anabolic reaction for the cell growth without product formation is
where \(a, b\) and \(c\) are known parameters that were determined to be \(CH_{1.781}O_{0.636}N_{0.079}\) for \(R.\ oligosporus\). If parameters \(f_1-f_5\) are calculated, the yield coefficients can be determined. To calculate five unknown parameters, five independent equations or relations should be given. The element balance equations for \(C, H, O\) and \(N\) and one more equation or relation can be used for this purpose. The most convenient additional relationship is the one between specific growth rate and carbon dioxide evolution rate. The yield coefficient, \(Y_{x/CO_2}\), can expressed in terms of the stoichiometric constants as follows.

\[
Y_{x/CO_2} = \frac{f_3 \cdot \text{(unit weight of cell)}}{f_5}
= \frac{19.61 \text{ [g cell/mol CO}_2]}{}
\]

So \(f_3\) and \(f_5\) have the following relationship.

\[
f_5 - 1.278 f_3 = 0
\]

Thus, parameters \(f_1-f_5\) can be obtained by solving the following matrix equation.

\[
\begin{bmatrix}
0 & 0 & 1 & 0 & 1 \\
-3 & 0 & 1.781 & -2 & 0 \\
0 & -2 & 0.636 & 1 & 2 \\
1 & 0 & -0.079 & 0 & 0 \\
0 & 0 & -1.278 & 0 & 1
\end{bmatrix}
\begin{bmatrix}
f_1 \\
f_2 \\
f_3 \\
f_4 \\
f_5
\end{bmatrix}
= \begin{bmatrix}
6 \\
10 \\
5 \\
0 \\
0
\end{bmatrix}
\]

The resulting stoichiometric equation for anabolism is

\[
(1/n)(C_6H_{10}O_5)_n + 0.208 NH_3 + 3.187 O_2
\rightarrow 2.634 CH_{1.781}O_{0.636}N_{0.079} + 2.967 H_2O + 3.366 CO_2
\]
We assume that *R. oligosporus* utilizes starch as a carbon source and that corn grit is composed primarily of starch. Equation (2) is based on the assumption that the change in intermediate products from the cells can be neglected. Because the maximum concentration of reducing sugar was less than 40 mg/g dry matter, we are able to use this assumption.

**Modeling of the process**

The batch fermentation process was modeled by the following non-linear differential equations.

\[
\frac{dx_1}{dt} = \mu_m (1-x_1/x_m)x_1 \tag{8}
\]

\[
\frac{dx_2}{dt} = \left\{ F(H_{in}-H_{out}) + Y H_2O/X (dx_1/dt) + m H_2O x_1 x_3 - x_2 (dx_3/dt) \right\}/x_3 \tag{9}
\]

\[
\frac{dx_3}{dt} = \mu_m (1-x_1/x_m)x_3 Y CO_2/x (\Delta M|_{anabolism}) \\
+ m CO_2 x_1 x_3 (\Delta M|_{catabolism}) \tag{10}
\]

where $x_1(t)$ is the biomass concentration (g cell/g dry matter)

$x_2(t)$ is the water content (g water/g dry matter)

$x_3(t)$ is the total dry matter weight (g)

$\mu_m$ is the maximum specific growth rate (hr$^{-1}$)

$x_m$ is the maximum fungal growth (g cell/g dry matter)

$H_{in}$ is the absolute humidity of inlet air (g/L)

$H_{out}$ is the absolute humidity of outlet air (g/L)

$Y$ is the yield coefficient (g/g)

$m$ is the maintenance coefficient (g/g hr)

$F$ is the air flow rate (L/hr)

$M$ is the weight of total dry matter at time t (g)

$\Delta M|_{anabolism}$ is the specific weight of matter consumed by anabolism ($\Delta g$ matter/g CO$_2$ evolved)

$\Delta M|_{catabolism}$ is the specific weight of matter consumed by anabolism ($\Delta g$ matter/g CO$_2$ evolved)
In Equation (8), a logistic model was used for the simulation of \( R. \) *oligosporus* growth. The maximum fungal growth \( (x_m) \) and the maximum specific growth rate \( (\mu_m) \) were derived from experimental results. The specific weight of the matter consumed by anabolism \( (\Delta M_{\text{anabolism}}) \) is estimated from the difference between the amount of cells produced and the amount of the substrate consumed from Equation (3). Starch and ammonia are converted to the cell mass. The specific weight difference is

\[
\Delta M_{\text{anabolism}} = Y_{X/CO_2} - Y_{\text{matter/CO}_2} \quad [\text{g matter/g CO}_2 \text{ evolved}] \quad (11)
\]

The specific weight consumed by catabolism is determined from the amount of starch consumed in Equation (7). The starch is consumed and produces \( \text{CO}_2 \) and \( \text{H}_2\text{O} \). The specific weight of the matter consumed by catabolism is

\[
\Delta M_{\text{catabolism}} = -0.6136 \quad [\text{g matter/g CO}_2 \text{ evolved}] \quad (12)
\]

The maintenance coefficient for water was calculated from the stoichiometric relationship of Equation (7) and \( m_{\text{CO}_2} \).

\[
m_{\text{H}_2\text{O}} = m_{\text{CO}_2}^{5/6} \quad (13)
\]

The temperature of the inlet and outlet air was monitored with thermocouples. The Antoine equation was used to calculate the saturation vapor pressure at a certain temperature:

\[
P_{\text{vap}} = \exp \{A + B/(T + C)\} \quad [\text{mmHg}] \quad (14)
\]

where \( P_{\text{vap}} \) is the saturated vapor pressure. For water, the constants \( A, B \) and \( C \) are 18.3036, -3816.44 and -46.13 respectively. If the air is assumed to be saturated at the given temperature, the absolute humidity can be calculated as follows:

\[
H = \{P_{\text{vap}}/(676.2-P_{\text{vap}})\}(18/22.4)(273/T) \quad [\text{g H}_2\text{O/L air}] \quad (15)
\]
Equation (15) contains correction terms for altitude and temperature. Equations (8)-(10) were solved by the Runge-Kutta-Gill method with renewal of the absolute humidity of inlet and outlet air.

The time course of cell growth is shown in Figure 3. The logistic model gave a good estimation of cell mass over most of the range. Water content per unit wet matter is shown in Figure 4. Although the simulated values deviate somewhat from the experimental values, they represent the whole range of the fermentation reasonably well. Overestimation during the later fermentation period probably comes from the condensation of water on the reactor wall. Because the temperature of the incubator was usually lower than the inside temperature of the fermenter, water condensed on the wall and collected at the bottom of the reactor. In Figure 5, the weight variation of the dry matter is shown. The trend of the weight variation is well represented by our model. The deviation of the total weight of dry matter during the later period of fermentation may also be explained by the water condensation on the fermenter wall.

REFERENCES

Fig. 1. Schematic diagram of solid state fermentation system for kinetic study. 1, fermenter; 2, thermocouples; 3, humidity sensors; 4, heat humidifier; 5, saturator; 6, incubator; 7, CO2 analyzer; 8, drying column.

Fig. 2. The relation between CO2 evolution rate ($Q_{CO2}$) and specific cell growth rate ($\mu$). Moisture content were 66% (■), 60% (△) and 54% (□) on a wet basis. All runs were at 37°C.
Fig. 3. Cell growth measurement and estimation. Solid line and represent the results of modeling and experiment at 66% (w/w) initial moisture content and 37°C.

Fig. 4. Water content measurement and estimation. Solid line and ■ represent the results of modeling and experiment at 66%(w/w) initial moisture content and 37°C.
Fig. 5. Dry matter weight measurement and estimation. Solid line and ■ represent the result of modeling and experiment at 66% and 37°C.
Simulation of Ethanol Fermentations from Sugars in Cheese Whey

Chen-Jen Wang and Rakesh K. Bajpai
Department of Chemical Engineering
University of Missouri-Columbia
Columbia, Missouri 65201

Abstract

The anaerobic fermentations of multiple sugars in cheese whey and in semi-synthetic media by Kluyveromyces marxianus CBS 397 were simulated by a cybernetic model in which product formation was incorporated. The concept of cell maintenance was modified in light of the energy-generating nature of product formation. The model successfully simulated ethanol fermentations from batch to transient-continuous cultures, from single substrate to multiple substrates, and from semi-synthetic media to CWP solutions.

I. Introduction

Fermentations from reconstituted cheese whey (CWP) may result in extracellular accumulation of glucose and galactose in addition to the major lactose, depending on the source of whey, the initial concentration of CWP, and the operating conditions [1,2]. In the meantime, cheese whey is often either spiked with additional sugars to raise ethanol production [3], or used as the fluid portion in fermentations of agricultural products [4-5]. As a result, fermentation from whey may involve the presence of multiple substrates in broth.

Although there are quite a number of experimental reports on ethanol fermentation from whey, no mathematical model has been presented. The lack of model is primarily due to the complex nature of the multiple-substrate system. This may be resolved by the cybernetic approach [6,7].

II. Mathematical Model

Ramkrishna and coworkers [6,7] have successfully modelled the growth of bacteria upon a mixture of carbon substrates without consideration of the formation of energy-rich products. These models consider that cellular metabolism of a given substrate is controlled by a key enzyme whose synthesis and activity is modulated by appropriate cybernetic variables (ɛ_i and δ_i as defined in Nomenclature), respectively. Since the control of substrate utilization in yeasts is also achieved via induction/repression of enzyme synthesis coupled with inhibition/activation of those already synthesized, fermentations involving yeasts can be modelled in a similar way.

However, unlike the aerobic growth of bacteria, anaerobic growth of yeasts involves both cell growth and ethanol production. A debate arises as to whether product formation and cellular growth involve a single common enzyme or two separate key enzymes. In order to resolve this question, it is necessary to understand the interactions between growth and ethanol production in yeasts.

Under unsteered conditions (i.e., in absence of bisulfite or salt stress) of anaerobic growth of yeasts, the dinucleotides (NAD) reduced by metabolism of sugar are regenerated during reduction of pyruvate to ethanol. Thus, ethanol is the major product formed under these conditions [8]. The ATP produced during the metabolism is utilized to support growth of cells,
the extent of which depends on the coupling between catabolic and anabolic processes. This is manifested also in the observation that ethanol production rate is often growth-related in batch cultures [9]. Hence, it should be possible to use a single common key enzyme for growth as well as the ethanol production process. Under this circumstance, each substrate will be associated with a single set of cybernetic variables (e_i, δ_i). Another cybernetic variable, δ_M, is associated with maintenance-related activities [7].

As a result, the model equations for fermentation of multiple substrates by yeasts can be expressed as [10]:

specific cell growth rate:

\[
\dot{r}_X = \sum_{i=1}^{n} \frac{\mu_i^* e_i S_i}{K_{SX,i} + S_i + S^2/K_{IX,i}} \left(1 + \frac{1}{1 + P/K_{Pi,i} + P^2/K_{Pi,2,i}}\right) \delta_i
\]

(1)

specific product formation rate:

\[
\dot{r}_P = \sum_{i=1}^{n} \frac{v_i^* e_i S_i}{K_{SP,i} + S_i + S^2/K_{IP,i}} \left(\frac{K_{Pi,i}}{K_{Pi,i} + P}\right) \delta_i
\]

(2)

Specific formation rate of enzyme:

\[
\dot{r}_{e,i} = \left(\frac{\alpha_i S_i}{K_{SX,i} + S_i + S^2/K_{IX,i}}\right) \left(1 + \frac{1}{1 + S_i/K_{P1,i} + S^2/K_{P2,i}}\right) e_i - e_i \left(\beta_i + \frac{1}{X} \frac{dX}{dt}\right) + a_i
\]

(3)

Specific substrate consumption rate:

\[
\dot{r}_{S,i} = \frac{\dot{r}_X,i \delta_i}{Y_{X/S_i}} + \frac{\dot{r}_P,i \delta_i}{Y_{P/S_i}} + \frac{\phi_{M,i}^* e_i S_i}{K_{SM,i} + S_i} \delta_M
\]

(4)

where

\[
\mu_i^* = \mu_i^{\text{max}} \left(\frac{\mu_i^{\text{max}} + \beta_i}{\alpha_i + a_i}\right), \quad \phi_{M,i}^* = \phi_{M,i}^{\text{max}} \left(\frac{\mu_i^{\text{max}} + \beta_i}{\alpha_i + a_i}\right), \quad v_i^* = v_i^{\text{max}} \left(\frac{\mu_i^{\text{max}} + \beta_i}{\alpha_i + a_i}\right)
\]

The cybernetic variable ε_i [6] represents induction/repression of synthesis of key enzyme e_i and is given by

\[
\varepsilon_i = \frac{\dot{r}_{X,i}}{\sum_{j=1}^{n} \dot{r}_{X,j}}
\]

(5)

The cybernetic variable δ_i [6] represents the inhibition/activation of enzyme activity and is given by
\[ \delta_i = \frac{r_{X,i}}{\max (r_{X,j})} \]  

(6)

In cybernetic models, \( \delta_M \) is considered as a measure of cellular control of activity of a key enzyme for maintenance functions. Turner et al. [7] have suggested a linear functional dependence of \( \delta_M \) on specific growth rate in their modelling. Similar dependence has been suggested by the experimental data of Neijssel and Tempest [11] and used in Pirt's model [12]. However, all of these studies have involved growth process only. For a product formation system, especially that involving ethanol that results in net ATP production, product formation rate should also affect the maintenance metabolism. This is in agreement with cybernetic perspective [6] according to which the cells aspire to achieve maximum return (growth rate) by efficiently allocating the resources to the metabolic function. Under anaerobic conditions, the cells cannot achieve such a cybernetic goal without a continuous regeneration of NAD via ethanol production. As a result, cell growth and ethanol production are interrelated in the sense of cybernetics. This dependence of \( \delta_M \) on specific growth and product formation rates has been derived [10] and the expression for a single-substrate environment is

\[ \delta_M = \left( 1 - \frac{r_X}{\mu_{\text{max}}} \right) \left( 1 - \frac{r_P}{v_{\text{max}}} \right) \]  

(7)

For multiple substrate systems, an analogous function for \( \delta_M \) is suggested as [10]

\[ \delta_M = \left( 1 - \frac{\sum_{j=1}^{n} r_{X,j} \delta_j}{\sum_{j=1}^{n} \mu_{\text{max}} e_j} \right) \left( 1 - \frac{\sum_{j=1}^{n} r_{P,j} \delta_j}{\sum_{j=1}^{n} v_{\text{max}} e_j} \right) \]  

(8)

III. Materials and Methods

Data for fermentations of cheese whey to ethanol have been reported elsewhere [2] and were used in this study for simulation. Experimental conditions for fermentations of semi-synthetic media were kept the same as those reported before [2] except the composition of supplemental nutrients as shown in Table 1.

Analytical methods were also the same as previously described [2]. However, samples containing less than 0.3 g/L glucose or lactose were analyzed by the Nelson-Somogyi method [13]. In a mixture of glucose and lactose at low concentrations, glucose content was analyzed by the glucose analyzer (Yellow Springs Instrument Co., Inc., Ohio), and lactose concentration was determined by subtracting glucose concentration from the total sugar concentration obtained from the Nelson-Somogyi method.

For determination of cell concentration, 5 ml samples were vacuum-filtered on preweighed 0.2 \( \mu \)m cellulose-acetate membrane filter (Gelman Sciences, Michigan). The solids were washed once with 5 ml distilled water and dried along with the filter in a microwave oven for 5 minutes at full power (500 watts). The solids were cooled in a desiccator for 10 minutes before weighing.
IV. Results and Discussion

IV.1. Simulation of Single-substrate System

The kinetic parameters for single sugars were estimated from steady-state continuous culture data [10] and are listed in the Table 2. Figure 1 shows the experimental and computed data for fermentations involving glucose and lactose at two different feed concentration levels.

Batch fermentations were simulated using estimated kinetic parameters. Figure 2 shows the experimental and the computed results of simulation. Generally, the experimental data are in good agreement with model predictions. Evidently, the cybernetic model with kinetic parameters obtained from continuous cultures can be used to satisfactorily simulate batch fermentations.

Transients in chemostat were studied by changing feed sugar concentrations at constant dilution rate. Both shift-up and shift-down experiments were conducted and the concentrations of cell mass (X), sugar (S), and product (P) were monitored during shift from one steady state to another. These transient results are plotted in Figure 3 as discrete points. For fermentations of glucose, the employed concentrations of sugar in feed media were 5 g/L and 20 g/L, and the constant dilution rate was set at 0.144 hr⁻¹. For lactose, they were 6.4 g/L and 20 g/L along with a constant dilution rate of 0.146 hr⁻¹.

For both types of transient experiments, the observed concentrations of cell mass, sugar, and ethanol are generally in agreement with the model predictions. The model predicted a minimum in the profile of sugar concentration during shift-downs; it predicted a maximum during shift-ups. These are also shown by the experimental data. Experimental measured sugar concentrations during shift-down are marked by a slower response compared to those from model predictions. For shift-up experiments, experimental results show higher maxima than model predictions. Both of these phenomena suggest that the activities of 'key enzyme' in model predictions are higher than the real levels. The concentrations of cell mass and ethanol in the model are not so sensitive to the precise enzyme levels and, therefore, show a good agreement with the experimental observations.

These delays and differences will not be observed in batch cultures and in steady-state continuous cultures. In batch cultures, the initial concentrations of key enzyme were assumed in order to obtain the best simulation results. The real process (or the time period) to reach these levels was ignored although they are dependent on culture history. In steady-state continuous cultures, cells have ample time to synthesize enough enzymes to effectively utilize sugar for growth.

The present model involving enzyme synthesis essentially introduces an inertia in cellular response to environmental changes. This structure improves the model performance during transients in continuous culture, but still falls short of successfully emulating system response under these conditions. Incorporation of "more structures" in the form of the amount of RNA polymerase, its affinity to promoter [14], synthesis and activation of ribosomes, etc. [15] may be required to successfully predict the effect of large changes in system behavior during transients.

IV.2. Simulation of Two-Substrate System

The usefulness of cybernetic models is best realized in predicting the behavior of substrate utilization in a mixed-substrate environment. The kinetic parameters obtained from fermentations on single substrates can be used to simulate ethanol fermentation from a mixture of substrates.

A batch fermentation was conducted with a mixture of 10 g/L glucose and 10 g/L lactose. The experimental results are plotted in Figure 4a. A sequential consumption of the two sugars was observed. Cell growth and ethanol production profiles showed an intermediate lag between two distinct phases. The model also predicted a diauxic growth on glucose and lactose. Glucose which supported faster growth rate, as expected, was consumed first,
followed by the utilization of lactose.

Continuous culture studies involving ethanol production from mixed substrates were also conducted with a mixture of glucose (10 g/L) and lactose (10 g/L). Steady-state values of X, P, and S were measured at different dilution rates and the data are presented in Figure 4b. Parameter values used in the simulation were again those for single sugars listed in Table 2. The steady state results were obtained by simulating the dynamic equations for long periods (about 500 hours) until steady state was achieved.

The model satisfactorily predicted fermentor performance. In contrast to the sequential uptake in batch fermentation, a simultaneous uptake of both sugars was observed at dilution rates below 0.4 hr⁻¹. Above this value, only glucose was consumed. This switch in sugar-uptake pattern was also predicted by the cybernetic model utilizing the information obtained from single sugar experiments.

IV.3. Simulation of fermentations on Cheese Whey

In these fermentations, no nutrient supplementation was done [10]. Hence, the growth characteristics may be different from those on glucose and lactose presented earlier. Therefore, new estimates of certain parameters (μ, YX/S, YP/S, and ϕM) were made from experimental data. The rest of the parameters were kept the same as those identified previously in Table 2.

Figures 4c and 4d show batch fermentation data for two different concentrations of cheese whey powder (CWP). The experimental results for 100 g/L CWP were used to obtain new estimates of growth parameters on lactose in CWP. The estimated parameters along with all the others are also tabulated in Table 2.

Any nutritional variation appears to affect only the maximum specific growth rate, maintenance, and yield parameters, as shown by a good match between predictions (solid lines) and experimental observations, shown in Figure 4c. The same set of parameters were used to simulate the fermentations with 25 g/L CWP. This is presented in Figure 4d. Again an excellent match is observed for 25 g/L CWP experiment.

To verify the applicability of cybernetic models to this complex system, batch fermentations were carried out with a mixture of cheese whey and glucose. In these experiments, 100 g/L CWP solutions were spiked with 10 g/L and 20 g/L glucose, respectively. No additional nutrient supplement was provided. The experimental results are shown in Figure 5.

Since these fermentations were also not supplemented with nutrient, the growth parameters for glucose can be different from those in Table 2. Hence, one of the experiments (Figure 5a) was used to estimate μ, YX/S, and ϕM on glucose. The other parameters for glucose and the parameters for lactose were kept the same as those in Table 2. These estimated parameters are also listed in Table 2 and used to simulate the experiment of a mixture of 100 g/L CWP and 20 g/L. The predictions are presented in Figure 5b as solid lines and an excellent agreement is obtained with experimental data.

V. Conclusion

The extended cybernetic approach can satisfactorily model the fermentations of cheese whey/semisynthetic media to ethanol under diverse experimental conditions. These include single substrates and multiple substrates.
VI. Nomenclature

\[ \alpha, \beta \] enzyme synthesis and degradation constants
\[ \gamma_{\text{max}} \] maximum specific rate of product formation
\[ \mu_{\text{max}} \] maximum specific rate of cell growth
\[ \phi_{\text{M}} \] maximum specific substrate consumption rate for maintenance
\[ \delta \] cybernetic variable for inhibition/activation of enzyme activity
\[ \varepsilon \] cybernetic variable for induction/repression of enzyme synthesis
\[ \delta_{\text{M}} \] variable for the degree of uncoupling between catabolism and anabolism
\[ a \] basal enzyme production
\[ e \] key enzyme level
\[ K_{\text{IX},i} \] substrate inhibition constant for cell growth
\[ K_{\text{IP},i} \] substrate inhibition constant for product formation
\[ K_{P} \] product inhibition constant for product formation
\[ K_{P1,i}, K_{P2,i} \] product inhibition constants for cell growth
\[ K_{S\text{X},i} \] substrate saturation constant for cell growth
\[ K_{SP,i} \] substrate saturation constant for product formation
\[ K_{SM,i} \] substrate saturation constant for maintenance
\[ P, S, X \] ethanol, sugar, and cell concentrations, respectively

VII. Reference

Table 1. Composition of semi-synthetic medium

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Composition (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar</td>
<td>5-20</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>4.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2.038</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.334</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.122</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.012</td>
</tr>
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<td>CaCl₂·2H₂O</td>
<td>0.007</td>
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Table 2. Kinetic Parameters

<table>
<thead>
<tr>
<th>parameter</th>
<th>glucose*</th>
<th>lactose@</th>
<th>glucose*</th>
<th>CWP**</th>
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<tbody>
<tr>
<td>µₑ</td>
<td>425</td>
<td>293</td>
<td>180</td>
<td>93</td>
</tr>
<tr>
<td>µ_max (hr⁻¹)</td>
<td>0.628</td>
<td>0.517</td>
<td>0.414</td>
<td>0.281</td>
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<tr>
<td>Kᵢₓ (g/L)</td>
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<td>0.094</td>
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<tr>
<td>Kᵢᵧ (g/L)</td>
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<td>370</td>
<td>10,000</td>
<td>370</td>
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<tr>
<td>Kₚ₁ (g/L)</td>
<td>7.8</td>
<td>12.1</td>
<td>7.8</td>
<td>12.1</td>
</tr>
<tr>
<td>Kₚ₂ (g²/L²)</td>
<td>330</td>
<td>330</td>
<td>330</td>
<td>330</td>
</tr>
<tr>
<td>νₑ</td>
<td>1037</td>
<td>867</td>
<td>688</td>
<td>506</td>
</tr>
<tr>
<td>ν_max [g/(g DW-hr)]</td>
<td>1.531</td>
<td>1.531</td>
<td>1.531</td>
<td>1.531</td>
</tr>
<tr>
<td>Kₛₚ (g/L)</td>
<td>0.050</td>
<td>0.088</td>
<td>0.05</td>
<td>0.088</td>
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<tr>
<td>Kᵢₚ (g/L)</td>
<td>10,000</td>
<td>250</td>
<td>10,000</td>
<td>250</td>
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<tr>
<td>Kₚ (g/L)</td>
<td>24.9</td>
<td>33.0</td>
<td>24.9</td>
<td>33.0</td>
</tr>
<tr>
<td>Φₚₘₐₓ [g/(g DW-hr)]</td>
<td>1.7</td>
<td>4.0</td>
<td>2.3</td>
<td>20.0</td>
</tr>
<tr>
<td>Φₘ [g/(g DW-hr)]</td>
<td>0.0025</td>
<td>0.007</td>
<td>0.005</td>
<td>0.06</td>
</tr>
<tr>
<td>Kₛₘ (g/L)</td>
<td>10⁻⁶</td>
<td>10⁻⁶</td>
<td>10⁻⁶</td>
<td>10⁻⁶</td>
</tr>
<tr>
<td>Yₓₛ (g DW/g S)</td>
<td>0.957</td>
<td>0.839</td>
<td>0.532</td>
<td>0.433</td>
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<tr>
<td>Yₚₛ (g P/g S)</td>
<td>0.510</td>
<td>0.538</td>
<td>0.510</td>
<td>0.445</td>
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</table>

µₑ, νₑ, and Φₘ are in unit of [g DW/(hr·unit enzyme activity)].
# estimated from continuous cultures on 5 and 20 g/L glucose
@ estimated from continuous cultures on 6.4 and 20 g/L lactose
* estimated from batch culture on 100 g/L CWP plus 10 g/L glucose
** estimated from batch culture on 100 g/L CWP
Figure 1. Experimental data and model predictions for continuous cultures (a) of 5 g/L glucose, (b) of 20 g/L glucose, (c) of 6.4 g/L lactose, and (d) of 20 g/L lactose. 

[●, lactose; □, glucose; ○, cell mass; ▲, ethanol]. Solid lines represent simulation results.
Figure 2. Experimental data and model predictions for batch fermentations (a) of 5 g/L glucose, (b) of 20 g/L glucose, (c) of 6.4 g/L lactose, and (d) of 20 g/L lactose. [●, lactose; □, glucose; ○, cell mass; △, ethanol]. Solid lines represent simulation results.
Figure 3. Experimental data and model predictions for transients in chemostats (a) with shift-up from 5 to 20 g/L glucose and (b) with shift-down from 20 to 5 g/L glucose at a dilution rate of 0.144 hr⁻¹, and (c) with shift-up from 6.4 to 20 g/L lactose and (d) with shift-down from 20 to 6.4 g/L lactose at a dilution rate of 0.146 hr⁻¹. [ ○, lactose; □, glucose; ○, cell mass; ▲, ethanol]. Solid lines represent simulation results.
Figure 4. Experimental data and model predictions for (a) batch fermentation and (b) chemostat of a mixture of glucose (10 g/L) and lactose (10 g/L), and for batch fermentations (c) of 100 g/L CWP and (d) of 25 g/L CWP. [ ●, lactose; □, glucose; ○, cell mass; △, ethanol]. Solid lines represent simulation results.
Figure 5. Experimental data and model predictions for batch fermentations (a) of 100 g/L CWP plus 10 g/L glucose and (b) of 100 g/L CWP plus 20 g/L glucose.

[•, lactose; ○, cell mass; △, ethanol]. Solid lines represent simulation results.
Studies on Protoplast Fusion of B. licheniformis

Beiqian Shi
Department of Biochemistry, Kansas State University
Manhattan, Kansas 66506

ABSTRACT

The fusant TR 8501 was obtained by protoplast fusion of two selected mutant strains, B. licheniformis Dx-12 (Tc<sup>r</sup>StRif<sup>f</sup>) and B. licheniformis r-1 (Tc<sup>f</sup>Str Rif<sup>r</sup>). Dx-12 came from B. licheniformis Dx2709 by induced mutation, and it produced more alkaline protease than the others. The optimum conditions for protoplast preparation are lysozyme concentration 0.70 mg/ml, pH 6.7, temperature 42°C, time 1.0 hour. The efficiency of regeneration is about 10%. The fusion of protoplasts is carried out in SMM buffer (which contained 0.5 M sucrose, 0.2 M maleic acid, 20 mM MgCl<sub>2</sub>) containing 30% polyethene glycol. The fusion frequency is 3.5x10<sup>-4</sup>.

The stability of fusants through transfer incubation continuing for ten generations is 6%. These fusants could grow in medium containing tetracycline (10 unit/ml), rifampin (10 unit/ml) and streptomycin (50 unit/ml). The shape of cells and colonies of fusants are different from their parents. Because fusant TR 8501 had higher yield of alkaline protease, it may be useful in the fermentation industry. Therefore, protoplast fusion could be considered a good way for breeding strains.

INTRODUCTION

Of the microorganism improvement methods, the mutation and sexual recombination are the most effective ways for changing and renewing the microorganisms. The mutagenesis method is a classical one for screening the highly producing strains (1,10), but it takes a lot of work and it is hard to improve further when "saturation" is reached after many times of treatment. Recombination can cause great changes of a gene and consequently its behaviors. However, its frequency is very low and besides that the transformation and transduction of hereditary substances from one particular donor to acceptor in the microorganisms are not common.

The protoplast fusion technique makes the recombination of genes occur even in the absence of mating, transformation and transduction. It has the advantage of large amplitude and high frequency of recombination, being easily combined with other breeding methods (4,13). Therefore, as a high potential and new breeding method, it has a promising future in fermentation. In 1976, after the success of fusion of the respective cells of Bacillus megaterium and Bacillus subtilis, many scientists made a lot of investigations about the fusion of protoplast of bacteria, actinomycete and molds (2,3,5-9,12,14). In these experiments, the optimum conditions of release, regeneration and fusion of protoplast of B. licheniformis were obtained, and also the protoplast with high yield of alkaline protease, which is important product in fermentation industry.
MATERIALS AND METHODS

Chemical Reagents—Unless otherwise noted, all chemicals were reagent grade and were obtained from commercial sources. Lysozyme was purchased from Shanghai Institute of Biochemistry (Academia Sinica). Polyethylene glycol was from Koch Light Lab., Ltd. England.

Bacterial Strains and Growth Conditions—B. licheniformis strain Dx2709 was the wild type. Mutant strain Dx-12 (Tc\(^5\),Str\(^5\),Rif\(^5\)) was obtained by mutagenization with UV from Dx2709. The spores of Dx2709 were suspended in sterile water and treated under UV for 30 minutes, then inoculated to broth medium containing 5 unit/ml each of tetracycline, erythromycin, kanamycin, and streptomycin. After shaking incubation for 18 hours at 37°C, cells were streaked on 2% agar medium containing tetracycline, erythromycin, or both, then mutant strain Dx-12 was obtained and stored at 0°C. Another mutant strain r-1 (Tc\(^5\),Str\(^5\),Rif\(^5\)) was provided by the Microbiology Group, East China Institute of Chemical Technology.

The basal medium containing tryptone, yeast-extract and NaCl, described previously (14), was used as the broth medium. Cells were grown aerobically with shaking for 18 hours at 37°C in 20 ml of broth medium. SMM buffer which contained 0.5 M sucrose, 0.2 M maleic acid, and 20 mM MgCl\(_2\), adjusted pH 6.7 before sterilization, was used in the experiment.

Preparation and Regeneration of protoplasts — After incubation aerobically for 18 hours at 37°C, the bacterial cells were collected by centrifugation at 4000 rpm for 15 minutes, and the pellets were washed in 5 ml of 0.15 M NaCl (by centrifugation at 4000 rpm for 10 minutes). The pellets were resuspended with appropriate concentration of lysozyme (in SMM buffer), and then incubated for a definite time at a definite temperature. Lysozyme was removed by centrifugation at 4000 rpm for 15 minutes and the pellets were washed twice in SMM. 2 ml of SMM was added to resuspend the pellets, and the supernatant was collected carefully by centrifugation at 1000 rpm for 5 minutes. The supernatant containing protoplasts was diluted in 0.3 M sucrose and spread on high-osmolarity broth medium containing 2% agar, 0.3 M NaCl. After incubation for 18 hours at 37°C regenerated colonies and the bacteria without broken walls were obtained.

The supernatant was diluted in the same volume of sterile water and spread on high-osmolarity medium to get the number of bacteria without broken walls.

Protoplast Fusion and Fusant Detection — Appropriate concentration of Dx-12 and r-1 protoplast were mixed in a centrifuge tube, and 30% PEG (in SMM) was added. After incubation in a water-bath for 3 minute at 42°C, PEG was removed by centrifugation at 4000 rpm for 15 minutes and pellets were washed in SMM. SMM was used to resuspend the pellets and dilute the protoplast, and then the protoplasts were spread on high-osmolarity medium without antibiotics and high-osmolarity medium containing 10 unit/ml (0.506 mg/ml) tetracycline, 50 unit/ml (4.045 mg/ml) streptomycin and 10 unit/ml (0.278 mg/ml) rifampin. After incubation for 18 hours at 37°C, the fusants and the regenerated colonies of their parents were obtained. Calculation of the efficiencies of
Segregation of Fusants -- The cells of fusants were diluted with sterile water and spread on high-osmolarity medium without antibiotics and high-osmolarity medium containing 10 unit/ml tetracycline, 50 unit/ml streptomycin and 10 unit/ml rifampin, and then incubated for 18 hours at 37°C. By comparison of the colony numbers on two different kinds of medium, the heterokaryon phenomenon and restoring mutation could be excluded and the pure fusants were obtained.

The fusants were checked on high-osmolarity medium containing the antibiotics.

Fermentation Testing -- Cells were grown aerobically with shaking for 40 hours at 37°C in 20 ml of medium containing 6% maize powder, 3.5% powder of soybean cake and 0.2% Na₂HP₃O₆ (before sterilization, adjusted pH 7.5-7.8). The protease activity of fermentation was measured by Folin method (11).

Enzyme activity = K x OD value x dilution of the enzyme
In this experiment: K = 95

RESULTS AND DISCUSSION

1. Obtaining the Drug-resistant Strains

As shown in Figure 1, after treating by UV for 30 minutes, there was about 50% survival.

The bacteria which were treated by UV for 30 minutes were spread on the plate containing streptomycin, tetracycline, and rifampin to check their characteristics of drug-resistance. After five generations, it was proved that their markers were stable (as shown in Table I), therefore, the two mutants r-1: Str⁵TcRiR and Dx-12: Str⁵TcRiR⁶ were used as the parents for protoplast fusion.

2. Optimum Conditions of Protoplast Release

a) Lysozyme Concentration

As shown in Figure 2, if the temperature for breaking the wall was 42°C, and incubation time was 1 hour, then optimum lysozyme concentration was 0.7 mg/ml, and repeated tests show that the conclusion was reliable.

The yield of protoplasts was increased with increasing the lysozyme concentration. When the lysozyme concentration was 0.7 mg/ml, the number of protoplasts was maximum; at this time, the quantified substrate had already made the lysozyme be fully saturated. Further increasing of the enzyme concentration had no beneficial effects on the yield of the protoplasts. Too high lysozyme concentration caused the excessive wall-breaking, so that the protoplast was permeable to the outside, and the number of the protoplasts decreased. It was seen from the result with microscope that when the lysozyme
concentration was 0.7 mg/ml, the protoplasts were a distinct globular body, while at 0.9 mg/ml, the protoplasts were relatively indistinct, which showed that some protoplasts were permeable to the outside.

b) Temperature for breaking the wall

As shown in Figure 3, if the concentration of lysozyme was 0.5 mg/ml, and the incubation time was 1 hour, then the optimum temperature was 42°C. It was known by the result with the microscopy that at this temperature the protoplast was most distinct, and rarely carried by the bacteria with unbroken walls, i.e. the walls were broken completely.

There were two effects of temperature on the enzyme promotion reaction: one was that when the temperature rose, the speed of reaction also increased; another was that the enzyme was gradually denatured with increasing temperature so that the speed of reaction decreased by reducing the activity of enzyme; therefore, the optimum temperature of enzyme reaction was the equilibrium results of these two processes. When the temperature was lower than 42°C, the former effect was dominant and the yield of protoplasts increased with increasing the temperature. It reached maximum amount at 42°C. When the temperature was higher than 42°C, the latter effect was dominant and the activity of lysozyme was lost rapidly, and then the yield of the protoplasts reduced quickly.

c) Incubation time

As shown in Figure 4, if the concentration of lysozyme was 0.5 mg/ml, and temperature for breaking the wall was 42°C, then the optimum time was 1 hour. The polysaccharide hydrolysis of the cell walls of the cell walls by the lysozyme required an appropriate time depending on strains. At the beginning, the yield of the protoplasts increased with increasing the time, and it reached maximum about 1 hour. If the incubation time was further prolonged, because the cytoplasm membrane was sensitive to the environment some of the released protoplast were broken and died making the final amount of protoplasts decreased.

d) Centrifugation Speed

As shown in Figure 5, if the centrifugation time was 5 minutes, and the centrifugation velocities were different, the numbers of protoplast obtained were quite different. It was seen from Figure 5 that the yields of protoplast obtained at 500 rpm and 1,000 rpm were almost the same, but it could be seen from the result of microscopy that the centrifugation speed of 500 rpm could not make the bacteria be fully separated from the protoplast; and with the centrifugation speed of 1,000 rpm, the most of the bacteria were precipitated. With the continuously increasing of the centrifugation speed, some protoplasts were also precipitated by the centrifugation so that the numbers of protoplasts in the supernatant were significantly decreased. Thus, the optimum centrifugation speed for removing the bacteria was 1,000 rpm.

e) SMM buffer with different pH value
As shown in Figure 6, if the concentration of lysozyme was 0.7 mg/ml, the incubation temperature was 42°C, and the incubation time was 1 hour, SMM buffer with different pH may affect the yield of the protoplasts. The optimum pH was 6.7.

3. Regeneration of Protoplast

a) Effect of different high-osmolarity media on the regeneration

By comparing the regeneration results of protoplast on high-osmolarity 0.3 M sucrose, 0.5 M sucrose, or 0.3 M NaCl, it was discovered that their regeneration rates were almost the same, but the regeneration of colonies on the medium containing sucrose exhibited easily flowing and viscous dew drop shape, and they were not easy to pick up. But the regeneration of colonies on the medium containing NaCl was similar to the colony morphology on the ordinary medium; if it was appropriate diluted, the single white colony could be obtained, so the medium containing 0.3 M NaCl was used as regeneration medium.

b) Regeneration of protoplasts

With the concentration of lysozyme 0.7 mg/ml, incubation temperature of 42°C, and incubation time of 1 hour, yield of regenerated colonies was calculated after incubation for 18 hours.

It was seen from Table II, with respect to Dx-12 and Dx2709, the number of regenerated colonies diluted with water and that diluted with sucrose were almost the same. The protoplasts of the two strains were not sensitive to water. If they were suspended in water for 30 minutes, they did not lyse. But r-1 was sensitive to such treatment.

4. Fusion of Protoplast

During the promotion of fusion by PEG, the cytoplasm of two cells and the whole chromosomes were combined together and formed a homogeneous structure, i.e. the fusion of protoplast occurred. The fusion sequence began at the coagulation and deformation of protoplast caused by the serious dewatering, the protein particles within the membrane were shifted in position and coagulated, and consequently the disturbance and recombination of the lipid molecules caused the cytoplasm membrane at the contact to be partially fused, the very small cytoplasm bridges were formed, and then gradually enlarged, and two protoplasts were finally fused (4).

A direct plating method may be used for detecting the fusion recombinant (13). The fusants were obtained on the high-osmolarity medium containing three kinds of antibiotics. The fusants and their parents Dx-12, r-1 were checked on CM plate containing different antibiotics for ten generations. It was proved that the heredity was relatively stable.

\[
\text{fusion frequency} = \frac{\text{# of colonies on the regeneration medium containing three kinds of antibiotics at a specific concentration}}{\text{# of colonies on the regeneration medium not containing antibiotics at a definite concentration}}
\]
5. Segregation and instability

Of the generation of fusants, there were about over 937 primary fusants which were either heterokaryons, or the restoring mutations occurred, they could be gradually separated into the parents shaped types. Only 6% were stable fusion recombinants.

6. The Result of Fermentation

It was shown in Table III, although the one of the parents r-1 produced really low yield of protease, the yield of fusant was higher than its parents. Therefore, it was recognized that the fusion of protoplast for obtaining high yield strains was an effective way in fermentation.

7. Observation of the cell morphologies of the parents and fusants, and the difference of colony morphologies

The characteristics of fusants and their parents was shown in Table IV.

CONCLUSIONS

1. The optimum conditions of protoplast preparation was: the concentration of lysozyme was 0.7 mg/ml, the incubation temperature was 42°C, the incubation time was 1 hour, pH of SMM was 6.7 and the centrifugation speed was 1000 rpm.

2. The high-osmolarity medium containing 0.3 M NaCl was used as regeneration medium. Fusants were obtained by the direct plating method, and the fusion frequency was 3.45x10^-4; it was proved that the heredity was relatively stable under the promotion.

3. Through the segregation, the stability of the fusants was about 6%, the cell morphologies of the fusants and that of the colonies were different from their parents, and the fermentation yields of fusants were higher than their parents.

ACKNOWLEDGEMENT

Thanks are due to Prof. Tong of East China Institute of Chemical Technology for his valuable advises and also to Dr. Davis and Dr. Erickson of Kansas State University for their helpful discussion. I am also obliged to Mr. Ding for his patience in typing the paper.

REFERENCES


FIG. 1 RESULT OF UV TREATMENT

FIG. 2 EFFECT OF LYSOZYME CONCENTRATION
FIG. 3 EFFECT OF TEMPERATURE

YIELD OF PROTOPLAST (10^6/μL)

INCUBATION TEMPERATURE (°C)
- Dz-12
+ r-1

FIG. 4 EFFECT OF INCUBATION TIME

YIELD OF PROTOPLAST (10^6/μL)

INCUBATION TIME (hour)
- Dz-12
+ r-1
FIG. 5 EFFECT OF CENTRIFUGATION SPEED

YIELD OF PROTOPLAST (10^6/g)

CENTRIFUGATION SPEED (1000 rpm)

Dx-12

FIG. 6 EFFECT OF pH

YIELD OF PROTOPLAST (10^6/g)

pH

Dx-12
### Table I Strains with Drug-resistant Markers

<table>
<thead>
<tr>
<th>Medium</th>
<th>CM + Tc (10 ( r/ml ))</th>
<th>CM + Str (50 ( r/ml ))</th>
<th>CM + Rif (10 ( r/ml ))</th>
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</thead>
<tbody>
<tr>
<td>Strains</td>
<td>Dx2709</td>
<td>r-1</td>
<td>Dx-1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : growth  
- : no growth  
CM : complete medium

### Table II Regeneration Yield of Protoplast

<table>
<thead>
<tr>
<th>Strains</th>
<th>Dx2709</th>
<th>Dx-12</th>
<th>r-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regenerated colonies diluted (10E-7/ml)</td>
<td>32.25</td>
<td>9.5</td>
<td>4.75</td>
</tr>
<tr>
<td>Regenerated colonies diluted by sucrose (10E-7/ml)</td>
<td>9.0</td>
<td>1.2</td>
<td>0.01</td>
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<tr>
<td>Regenerated colonies diluted by water (10E-7/ml)</td>
<td>9.0</td>
<td>1.1</td>
<td>0.00005</td>
</tr>
<tr>
<td>Regeneration yield (%)</td>
<td>27.9</td>
<td>12.6</td>
<td>0.17</td>
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### Table III The Result of Fermentation

<table>
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<th>TR8501</th>
<th>Dx-12</th>
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<tbody>
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<td>pH</td>
<td>6.7</td>
<td>7.0</td>
<td>6.4</td>
<td>7.0</td>
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<td>dilution</td>
<td>250</td>
<td>250</td>
<td>100</td>
<td>250</td>
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<tr>
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<td>Enzyme activity</td>
<td>8655</td>
<td>5795</td>
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<td>4579</td>
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Table IV Characteristics of Fusant and Their Parents

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<tr>
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<th>Parants Dx-12 r-1</th>
<th>Fusant TR8501</th>
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<tbody>
<tr>
<td>Drug Resistance</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CM+Tc</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CM+Str</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CM+Rif</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Phenotype</td>
<td>thick</td>
<td>thick</td>
<td>thin</td>
</tr>
<tr>
<td></td>
<td>short</td>
<td>short</td>
<td>long</td>
</tr>
<tr>
<td>Spore production</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Colony</td>
<td>dry</td>
<td>dry</td>
<td>wet</td>
</tr>
<tr>
<td></td>
<td>white</td>
<td>white</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>white</td>
</tr>
</tbody>
</table>

Notations: + : growth  Tc: Tetracycline (10 r/ml)
- : no growth        Str: Streptomycin (50 r/ml)
CM: complete medium  Rif: Rifampin (10 r/ml)
CELL SEPARATIONS OF NONDIVIDING AND DIVIDING YEAST USING AN INCLINED SETTLER

Ching-Yuan Lee and Robert H. Davis
Department of Chemical Engineering
University of Colorado
Boulder, CO 80309-0424

Robert A. Sclafani
Department of Biochemistry, Biophysics and Genetics
University of Colorado
Denver, CO 80262-0121

Introduction

Recombinant biotechnology has become a promising way to produce valuable pharmaceuticals and chemicals, such as proteins, hormones and amino acids. The most common mode for manufacture of these products is batch fermentation instead of continuous fermentation. A major problem in switching from batch modes to continuous modes is that of plasmid instability. Since plasmid-free cells grow faster than plasmid-bearing cells, continuous cultures become nonproductive after several generations. Therefore, an important task is to maintain plasmid-bearing cells in the reactor. Since the only way for living cells to lose plasmids is through cell division, a possible way of overcoming the plasmid instability problem is to stop the cell divisions while cells still have plasmids, and then selectively recycle them back to the fermentor in order to maintain the production of the desired products.

In order to achieve the above goal, a preliminary study of the overall performance of an inclined settler in separating different sizes of yeast cells was performed. In this work, several steady-state experiments were carried out. Experimental data were compared with theoretical predictions by the inclined settler theory which was developed based on rigid, spherical particles. Good agreement between experimental results and theoretical predictions was obtained.

Theory of Steady-State Operation

The theory of an inclined settler operated under steady-state and transient conditions is discussed in detail by Davis et al. (1989). Consider steady-state operation of an inclined settler as shown in Figure 1. A fluid suspension with a continuous particle size distribution and a particle volume fraction \( \phi_f \) is fed into the settler at the volumetric flowrate \( Q_f \). The overflow flowrate and underflow flowrate are denoted as \( Q_o \) and \( Q_u \). The subscripts \( f, o, \) and \( u \) refer to the feed, overflow, and underflow streams, respectively.

Steady-state mass balances on total suspension, total particles, and those particles which have settling velocity \( v \) are given as follows:

\[
Q_f = Q_o + Q_u
\]

\[
Q_f \phi_f = Q_o \phi_o + Q_u \phi_u
\]

\[
Q_f \phi_f P_f(v) = Q_o \phi_o P_o(v) + Q_u \phi_u P_u(v)
\]
where $P(v)'s$, the normalized probability density functions, are defined such that $P(v)dv$ is the fraction of particles by volume in a given stream that have settling velocities between $v$ and $v + dv$. $P(v)$ is normalized so that

$$\int_0^\infty P(v)dv = 1 \quad (4)$$

The probability density function in the feed stream, $P_f(v)$, can be determined either directly by allowing a sample to sediment in the presence of an optical density recording device (Davis and Hunt, 1986), or indirectly by measuring the particle size distribution with a particle size analyzer and then using an expression such as Stokes' Law to relate the sedimentation velocity of each particle to its size. The volume fraction of particles, $\phi$, can be determined by the particles' average size and their number concentration, each of which can be obtained from a particle size analyzer.

![Figure 1. Schematic of Experimental Apparatus](image)

The rate of sedimentation in an inclined channel can be described by the so-called PNK theory, which was first proposed by Ponder (1925) and Nakamura and Kuroda (1937). This theory, by assuming that all particles have the same sedimentation velocity, $v$, states that the volumetric rate of production of clarified fluid due to particle sedimentation is equal to the vertical settling velocity of the particles multiplied by the horizontal projected area of the upward-facing surfaces of the channel onto which the particles may settle. By referring to Figure 1, this is given as

$$S(v) = v \cdot w \cdot (L \cdot \sin \theta + b \cdot \cos \theta) \quad (5)$$

where $\theta$ is the angle of inclination of the plates from the vertical, and $b$, $w$, and $L$ are the height, width, and length of the rectangular settler, respectively.
The final mass balance which is required is that on particles entering the overflow stream. Material entering the overflow is comprised of a mixture of clarified fluid, which enters the overflow at the volumetric rate \( S(v) \), and unsettled suspension, which enters the overflow at the volumetric rate \( Q_o - S(v) \). A particle mass balance on the mixing of these two streams yields the following equation

\[
Q_o \phi_o = (Q_o - S(v)) \phi_f
\]

(6)

If \( Q_o < S(v) \), all of the particles settle out of suspension before reaching the overflow, and \( \phi_o = 0 \).

For dilute suspensions in which it is assumed that the particles of different sizes settle without interfering with one another, the above equation may be applied to any given fraction of the particle distribution, with the modification that the total particle volume fraction, \( \phi \), is replaced by the volume fraction of particles having settling velocities between \( v \) and \( v + dv \), which is \( \phi P(v) dv \). Therefore

\[
Q_o \phi_o P_o(v) = (Q_o - S(v)) \phi_f P_f(v) \quad S(v) < Q_o
\]

(7)

\[
P_o(v) = 0 \quad S(v) \geq Q_o
\]

(8)

where \( S(v) \) is given by equation (5) and is now interpreted as the volumetric rate at which suspension devoid of particles with settling velocities greater than or equal to \( v \) is produced as a result of the sedimentation of these particles.

Integrating equation (7) over all particle settling velocities, with the normalization constraint given by equation (4) applied, an expression for the total particle volume fraction in the overflow stream is obtained as

\[
\phi_o = \phi_f \int_0^{v_o} \frac{(Q_o - S(v))}{Q_o} P_f(v) \, dv
\]

(9)

where \( v_o \) is the cutoff sedimentation velocity and is defined by \( S(v_o) = Q_o \). Particles with settling velocities greater than \( v_o \) should settle out of suspension before reaching the overflow. The probability density function for the particles in the overflow stream is obtained by substituting equations (7) and (8) into equation (9)

\[
P_o(v) = \frac{(Q_o - S(v)) P_f(v)}{\int_0^{v_o} (Q_o - S(v)) P_f(v) \, dv} \quad v < v_o
\]

(10)

\[
P_o = 0 \quad v \geq v_o
\]

(11)

Similar expressions for the composition of the underflow stream are obtained by using equations (1)–(3), as described by Davis et al. (1989).

Materials and Methods

In order to test the theory, several steady-state experiments were done in a single-channel inclined settler. The settler is made of glass and has the dimensions of \( L = 60 \) cm, \( w = 4 \) cm, and \( b = 0.5 \) cm. The angle of inclination was typically chosen to be 45 degrees. However, one set of experiments was carried out to test the effect of varying this angle on the performance of the inclined settler. The yeast strain used was \( S. \) cerevisiae 378 with the plasmid pBM746, both obtained from Professor R. A. Sclafani. This strain has a bar1 chromosomal mutation which enables cell divisions to be stopped in the presence of \( \alpha \)-factor, a 13-amino peptide. This strain cannot grow in a-Ura medium if cells lose the plasmids. When cell divisions are stopped, cells begin to increase their sizes and become very irregular in shape. The average sizes of yeast cells were measured by an Elzone 180XY particle size analyzer manufactured by Particle Data, Inc. Typical average equivalent sphere
diameters are 4.5 μm for normal dividing cells and 7.8 μm for nondividing cells. In order to maintain steady-state operation without cell growth, distilled water was used as the suspending fluid. The operating temperature was chosen as 30°C. Observations through a microscope showed that the morphology of yeast cells in water did not have any visible change for 48 hours, which was the time needed for accomplishing both the experiments and data analysis. Results from plating also showed that the viability of yeast cells in water remained the same for this period of time.

The method used to study the agreement between experimental results and theory was to vary overflow flowrate, \( Q_0 \), analyze the overflow samples to obtain \( \phi_o \) and \( P_o(v) \), and compare these quantities with theoretical predictions. The experimental setup is shown schematically in Figure 1. The overflow flowrate, \( Q_o \), was controlled by a pump. The steady-state operation was achieved by recycling the overflow and underflow back to the reservoir. A total of four experiments were done. In the first and second experiments, pure cultures of normal cells and nondividing cells were used, respectively. In the third one, a mixture of equal number of normal cells and nondividing cells was used. The nondividing cells in the third experiment were particularly chosen to be plasmid-bearing cells, and the normal cells were the same host cells but without plasmids. The reason for this was to test if the inclined settler theory can predict, besides \( \phi_o \) and \( P_o(v) \), the fraction of plasmid-bearing cells in the overflow. In the last experiment, normal cells were used for operation at two different angles to show the effect of the angle of inclination on cell separations. For each experiment, approximately three hours were required for steady state to be reached, with the slow step being the equilibrium formation of the sediment deposit on the lower wall of the settler. Samples were taken from the feed reservoir as well as the overflow stream for later analysis.

The samples were analyzed by the Elzone 180XY for cell number concentrations and size distributions. Since the theory requires settling velocity distributions rather than size distributions, Stokes' Law was used to related the two:

\[
v = \frac{k D^2 (\rho_s - \rho) g}{18 \mu}
\]

where \( D \) is the cell diameter measured by the Elzone 180XY, \( \rho_s \) is the cell density, \( \rho \) is the fluid density, \( \mu \) is the fluid viscosity, and \( g \) is the gravitational acceleration constant. A value of 1.13 g/cm\(^3\) was used for \( \rho_s \) (Szlag, 1988). The correction factor \( k \) takes into account wall effects, hindered settling effects, irregularity of cell shapes, and any calibration differences in the diameter measured by the 180XY and the appropriate effective diameter for the Stokes' settling velocity. It was found to be 0.7 by Davis et al. (1989) for spherical particles. The same value was used in this study for normal cells since their shapes are spheroidal with a major to minor axis ratio near unity. For the nondividing cells, a value of 0.5 was chosen in order to account for the fact that their settling velocities are decreased by their irregular shapes under creeping flow conditions.

Results and Discussion

The experimental results for normal yeast, nondividing yeast, and mixed cultures are shown in Figures 2 to 7. The predictions of \( \phi_o \) at different overflow flowrates agree well with experimental data for all three experiments, as shown in Figures 2, 4 and 6. These three plots were non-dimensionalized by dividing \( \phi_o \) by \( \phi_f \) and \( Q_o \) by \( Q \), where \( Q = S(\phi) \) is the cutoff flowrate corresponding to the median size cells in the feed. In all cases, increasing the overflow rate led to a higher concentration of cells reaching the overflow stream. This is because higher overflow rates give less time for the cells to settle out of suspension.
Figure 2. Dimensionless cell concentration in settler overflow as a function of dimensionless overflow rate for normal yeast culture.

Figure 3. Size distribution of cells in overflow and in feed line for normal yeast culture.
Figure 4. Dimensionless cell concentration in settler overflow as a function of dimensionless overflow rate for nondividing yeast culture.

Figure 5. Size distribution of cells in overflow and in feed line for nondividing yeast culture.
Figure 6. Dimensionless cell concentration in settler overflow as a function of dimensionless overflow rate for mixed yeast culture

Figure 7. Size distribution of cells in overflow and in feed line for mixed yeast culture
The agreement between theoretical predictions and experimental results for the size-distributions in the overflow is fairly good. However, as can be seen in Figures 3, 5, and 7, more large cells came out the settler through the overflow than predicted. A possible reason for this rather consistent observation is that a dispersion phenomenon is present. Although the yeast cells are too large for Brownian diffusion to be important, it is expected that the less-familiar phenomenon of hydrodynamic diffusion due to multi-particle interactions is important (Davis and Hassen, 1988). Moreover, since the cells are not exactly spherical, their settling velocities will depend on their orientations relative to the gravity vector. Cells with their major axis normal to the gravity vector will settle slower than predicted by equation (12). Both of these effects will lead to a larger amount of cells reaching the overflow than predicted by our theory.

The effect of cell separation by inclined settling is clearly seen in Figures 7 and 8. For Figure 7, at an overflow flowrate of 0.622 ml/min and an angle of inclination of 45°, the overflow sample contained mainly normal cells, while most of the nondividing cells were recycled back to the reservoir via the underflow. Figure 8 shows that the fraction of cells in the overflow that contained plasmids can be rather precisely predicted by the theory. This fraction is predicted by applying equation (9) independently for the normal cells and the nondividing cells. The fraction of plasmid-bearing (nondividing) cells in the feed reservoir was 0.5. At low overflow rates, these larger cells settle out in the inclined settler, and their fraction in the overflow is much smaller. At high overflow rates, neither of the cell types have time to settle in the inclined channel, and the overflow fraction becomes the same as the feed fraction.

An interesting question in the operation of an inclined settler is, when holding all other parameters constant, how does the angle of inclination affect the performance? This is answered in Figure 9, which shows the overflow concentration for normal yeast at two different settler angles. For the smaller angle, the horizontal projected area for sedimentation is less than that for the larger angle. Therefore, fewer cells have time to settle and the overflow concentration is greater for the smaller angle than for the larger angle at the same overflow rate. In general, inclined settlers are more efficient when the angle of inclination from the vertical is large. The only constraint on this is that θ cannot be so large that the deposit of cells on the lower wall is unable to slide down to the bottom of the settler, in which case the assumption of steady-state operation can no longer hold. From our experience, it is recommended to keep θ less than 60° for rigid spheres, less than 45° for yeast, and less than 30° for bacteria.

According to the derivations of the steady-state theory, when $\phi_0$ and $Q_0$ are properly non-dimensionalized, the result should be independent on the angle of inclination. This is demonstrated to be true by Figure 10.

Concluding Remarks

In this study, we have demonstrated that the theory of inclined settling is applicable to yeast cells. Steady-state experiments showed good agreement between the theoretical predictions and experimental data for both the cell volume fractions and the size distributions in the overflow streams. Experimental results also showed that selective separation of plasmid-bearing and plasmid-free cells was achieved. The results of this study will provide important information to further application of an inclined settler for continuous fermentations involving competition between two cell types.

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Mixed-Culture Experiment

![Graph](image)

Figure 8. Fraction of recombinant yeast cells in overflow as a function of overflow rate for a mixed yeast culture containing 50% recombinant cells in the feed stream

Normal Yeast Culture

![Graph](image)

Figure 9. Cell concentration in settler overflow at two different angles of inclination as a function of overflow rate for a normal yeast culture
Figure 10. Dimensionless cell concentration in settler overflow as a function of dimensionless overflow rate at two different angles of inclination for a normal yeast culture.

References


EFFECTS OF SERUM UPON LOCAL HYDRODYNAMICS WITHIN AN AIRLIFT COLUMN

G. T. Jones, L. E. Erickson, and L. A. Glasgow

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

Introduction

Airlift fermenters have been used for large scale suspension culturing of hybridoma cell lines even though sparging is widely recognized as being detrimental to animal cell viability\(^1\)\(^2\). Currently the physical forces responsible for loss of viability in sparged vessels is unclear. Kilburn and Webb\(^2\) suggested surface active forces at the bubble interface were responsible for poor growth observed in their experiments. They noted damage could be prevented by increasing the serum concentration to 10% or by the addition of a surface-active polymer (0.02% Pluronic F68). A 5 to 10% serum concentration, which is commonly used in cell culture, may affect local hydrodynamics because of the interaction of surface-active proteins with gas-liquid interfaces. Croughan et. al.\(^3\) have shown the size of dissipative eddies is important in determining cell viability for microcarrier fermentations. Thus the local instantaneous microstructure of turbulence has significant impact upon the occurrence of cell damage. The goal of this research is to determine the effect of serum upon local upflow hydrodynamics and investigate local upflow conditions using flow visualization studies.

Handa et. al.\(^4\) investigated gas-liquid interfacial effects on hybridoma viability in a bubble column. They found that cell viability and survival was dependent on several factors; cell line, bubble size and superficial gas velocity. Based upon visualization of the hybridomas during culture they proposed that the bubble disengagement zone is where maximum cell damage is most likely to occur. The disengagement region of an airlift reactor is also likely to be very turbulent, nevertheless, hydrodynamics within airlift and bubble columns are substantially different. Airlift fermenters consist of two distinct fluid zones with usually only one zone sparged by gas to establish a loop circulation pattern, whereas bubble columns do not have a defined circulation path. Attention must also be given to characteristic length scales of dissipative eddies in regard to the use of microcarriers in airlift fermenters. Laser-Doppler velocimetry is a noninvasive technique used to measure local instantaneous liquid velocities. Flow visualization is the simplest method to investigate local conditions and evaluate length scales present.

Flow visualization can provide estimates of the mean velocity, standard deviation of velocity or root-mean-square (rms) velocity, and local shear stresses. The Reynolds' decomposition is commonly used to study turbulence based upon the Navier-Stokes equations. For a
statistically stationary flow the local instantaneous velocity, \( u_i \), is decomposed into a mean flow component, \( U_i \), and a velocity fluctuation, \( u_i' \), about the mean. Typically \( U_i \) is a time averaged value and \( u_i' \) is the standard deviation about the mean or the rms velocity fluctuation. A large number of photographs are required to obtain a time average value of the velocity or a spatial average can be easily obtained from flow visualization experiments assuming that the local velocities have no dependence upon position.

Croughan et al. have shown no growth occurs in stirred tank microcarrier fermentations when dissipative eddy length scales are smaller than the diameter of a microcarrier. Evaluation of dissipative eddy length scales requires an estimate of the dissipation rate of turbulent energy, \( \varepsilon \). The dissipation rate may be estimated using Taylor's large scale inviscid estimate:

\[
\varepsilon = A u_1'^3 / \ell
\]

where \( A \) is a constant to be determined and \( \ell \) is the integral length scale that is characteristic of the system being studied. The length scale of dissipative eddies, \( \eta \), is determined by \( \varepsilon \) and the kinematic viscosity, \( \nu \), with the Kolmogorov equation:

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

Local shear stresses are more important for suspension cultures as dissipative eddy length scales will typically be 5 to 10 times larger than a cell's diameter, thus, a dissipative eddy will entrain a cell. Animal cells are very sensitive to shear stress because unlike bacteria they do not have a cell wall. Large velocity gradients result in high shear stresses and are undesirable.

In the riser of an airlift fermenter liquid is entrained in a bubble's wake resulting in an upflow of liquid; in turn a bubble will rise faster than in quiescent water. This process is limited by the viscous dissipation of energy by the fluid, such that a limit exists for the liquid circulation velocity. The region in the immediate vicinity of a rising bubble would be expected to experience large velocity gradients occurring across the boundary layer at the bubble interface. Clift et al. note that the velocity variation across the boundary layer of a fluid sphere is on the order of \( U/Re^k \), where \( Re \) is the bubble Reynolds number. For example, in quiescent water the terminal velocity of a bubble which is roughly 5 to 10 mm in diameter, which is an oscillating ellipsoid, is approximately 17 to 25 cm/s in contaminated water. Thus, an order of magnitude estimate of the velocity gradient across the boundary layer is 20 to 34 s\(^{-1}\); which is insufficient to damage most cells. For bubbles with diameters of 5 to 10 mm the \( Re \) ranges from approximately 800 to 2000; this is an awkward hydrodynamic region to treat theoretically. Higher \( Re \) allow the use of boundary layer theory to estimate forces in the immediate vicinity of a bubble, whereas, lower \( Re \) allow for numerical solutions. The forces in the vicinity of the gas-liquid interface will be lower than those for a comparable solid sphere. Boundary conditions commonly used at the surface of a fluid drop are the continuity of the tangential velocity, tangential stress and normal stress across the interface. Shear fields
will induce a single cell to rotate exposing the cell membrane to additional forces that are not well characterized.

**Procedure**

Blood was collected from freshly slaughtered cattle and allowed to coagulate overnight at 4°C, the serum was separated from the clot the following day. The serum was centrifuged at 800g for one hour and filtered through a Whatman No. 40 filter paper removing particles larger than 8 μm in diameter. Aliquots of 1.1 liters were frozen and stored at -10°C until use. Adult bovine serum (ABS) is nutritionally different from fetal bovine serum (FBS) commonly used in cell culture, however, we believe little physical difference exists between the use of ABS versus FBS to study bioreactor hydrodynamics. A 5% solution of serum in tap water was used in all experiments involving the use of laser-Doppler velocimetry. Higher serum concentrations were not used because of interference from colloidal particles present in the serum with the light source.

Figure 1 shows the column used for all laser-Doppler velocimeter measurements. The procedures by which measurements were made and data were analyzed have been described elsewhere.

A 3.5 liter acrylic plastic airlift reactor was constructed to provide optically flat surfaces for macrophotography. The corners were filled in and air was introduced through 15 holes 1.6 mm in diameter on one side of the dividing baffle. Focused white light was passed through a 2 mm wide slit to illuminate a planar segment of the fermenter. Deionized water was seeded with 5 to 10 μm diameter glass microballoons to trace eddy paths. An Olympus OM-2 camera equipped with a 50 mm macro lens was used to record the images; the aperture was f2 to provide sufficient light and to minimize the depth of field. Kodak TX Professional film was used for all exposures and developed using HD-110 to provide an ASA of approximately 2000. Exposures were obtained at 1/15 second to provide streaks of sufficient length to accurately estimate local velocities. An experiment using a 5% serum solution was conducted, but "foggy" exposures resulted because of light scatter from colloidal particles.

**Results and Discussion**

Figure 2 displays mean velocities on the upflow side of the column for a superficial gas rate of 1.3 cm/s as a function of the fraction of the total column height above the sparge plate. Tap water and a 5% serum solution are the two systems shown in this figure. The mean velocity of the serum solution is seen to be approximately 12 cm/s whereas the mean velocity of the water system is approximately 18.5 cm/s. One of the problems encountered with the serum system was interference from light scattering centers present; this resulted in very poor signal quality. Approximately 150 to 200 Doppler bursts were used to evaluate the mean and standard deviation from the 5% serum LDV data, however, Boerner et. al.7 indicate that 1200 "good" Doppler bursts are desirable for reproducible mean velocities at low gas holdups. Thus, only rough estimates of the mean velocity result for the serum.
solution system.

Figure 3 displays the rms velocities on the upflow side of the column for a superficial gas rate of 1.3 cm/s as a function of the fraction of total column height above the sparge plate. The rms velocities of the serum solution and tap water systems are approximately 7 and 11 cm/s, respectively. The rms velocity is of greater interest because it can be used to estimate the dissipative eddy length scales present in a reactor. However, the rms velocity is the second statistical moment and thereby requires a greater number of data points than the mean to have a reproducible estimate. Using equations (1) and (2) and assuming the physical properties of the two systems are the same allows an estimate of the ratio of dissipative eddy length scales with the serum system length scale being approximately 1.4 longer than the water system. Assuming that the rms velocities measured are accurate implies that serum does have protective properties against hydrodynamic stress.

Figure 4 shows the mean velocities of tap water and 5% serum solution systems on the upflow side of the column for a superficial gas rate of 2.6 cm/s. The mean velocity of the serum system is seen to be approximately 3.5 cm/s higher than that of the tap water system. The observed difference may simply be due to insufficient data.

Figure 5 shows the rms velocities for the upflow side of the column with tap water and 5% serum solution systems at a superficial gas rate of 2.6 cm/s. The averaged rms velocity for serum is twice the value observed at a specific gas rate of 1.3 cm/s whereas tap water experiences little change. For the low concentrations of serum used in these experiments it is likely serum has little effect upon large scale hydrodynamics. Proteins present in serum act as surface-active polymers interacting with the gas-liquid interfaces and cells. Clift et al. note that surfactants have significant effects upon bubble behavior when compared to pure systems. Nonetheless, little difference in liquid-phase hydrodynamic behavior between the serum and tap water systems can be observed based upon our experimental results.

Photographic analysis of the region immediately below the gas-liquid interface was performed. From the photographs it was observed that the region below the gas-liquid interface is very turbulent with approximately five times the vortices per unit area as observed in the bubble wake. The vortices near the surface appear to be approximately 2/3 the size of vortices seen at the edge of bubble wakes in the central portion of the column. Near the surface large horizontal velocity components are in evidence in contrast to lower sections of the column. Local shear rates range from 40 to 100 s⁻¹, corresponding to shear stresses of 0.04 to 0.1 Nm⁻². The observed local shear stresses in the vicinity of vortex structures are lower than those which result in loss of viability for many cells.

Photographic investigation of bubbles in the central upflow section of an airlift column indicated many small vortices exist near the bubble and the edge of the wake structure. These vortical structures have a scale of about 0.5 mm and the central wake region has decidedly sinuous shape. Estimates of the maximum velocity gradients present in the wake region range from 40 to 70 s⁻¹; these values were normally found in proximity to small vortices or the bubbles themselves. The smallest scale eddies identifiable in the photos have scales of approximately 0.1-0.2 mm indicating that damage to cells grown upon microcarriers may
possibly occur.

Conclusions

From the data collected it appears that there is very little difference in the gross hydrodynamics between tap water and a 5% serum solution in an airlift fermenter. One of the principal problems encountered in these experiments is light dispersion by the serum; this resulted in poor signal quality for the LDV experiments and photographs that were murky. Signal quality may be improved by aseptically filtering collected serum through a 0.1 μm filter, as is done for commercially available FBS, to remove large light scattering particles. Other velocity measurement technologies, such as hot film anemometry, may be less susceptible to interference in a serum system than LDV and flow visualization. Macrophotography indicates that significant shear stresses exist near the gas-liquid interface, bubble surface, and the edge of the wake structure; however, the observed stresses are insufficient by themselves to directly result in cell death. The photos demonstrate that the most turbulent zone of the column is the bubble disengagement region. With the advancement of computational power in recent years, numerical simulation of bubbles at Reynolds numbers of interest should be possible. Modeling of bubble disengagement will allow estimation of local forces and will provide a better understanding of how cells lose viability in sparged vessels.

Acknowledgements

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References


Figure 1. Acrylic plastic airlift column used in the experiments (all dimensions are in millimeters)
Figure 2. Mean velocity of the continuous phase on the upflow side of the column for a specific gas rate of 1.3 cm/s for water and 5% serum systems.

Figure 3. RMS velocity of the continuous phase on the upflow side of the column for a specific gas rate of 1.3 cm/s for water and 5% serum systems.
Figure 4. Mean velocity of the continuous phase on the upflow side of the column for a specific gas rate of 2.6 cm/s for water and 5% serum systems.

Figure 5. RMS velocity of the continuous phase on the upflow side of the column for a specific gas rate of 2.6 cm/s for water and 5% serum systems.
Abstract

This work seeks to determine the optimal system design for the maximization of secreted heterologous protein production in a chemostat cascade, using baker's yeast (S. cerevisiae) as the host. Mutant invertase, the secretion of which is characterized by a seven-fold delay compared to the wild-type, has been chosen as the model protein. This work details the results achieved to date from steady-state optimization studies, using a secretion model developed by Park and Ramirez (1989). The optimum number of chemostat stages required to maximize protein production has been investigated. Single stage results indicate that increasing the degree of recycle and the feed substrate concentration increases the productivity. Two-stage results indicate that a two stage cascade gives superior productivity compared to a single stage without recycle. Provision of cell recycle on both stages yields a higher production than the corresponding system without recycle. Further, the bulk of the total substrate feed and system volume needs to be allocated to the second stage to attain maximum productivity. Currently three stage behavior in the absence and presence of recycle is being investigated.

I. INTRODUCTION

Usage of the secretory pathway for protein production offers several advantages over cytoplasmic release such as:

1. It simplifies downstream processing by allowing protein recovery without cell lysis.

2. It is likely that many proteins when expressed internally (in the cytoplasmic space) will be toxic to the cell to some degree. This will result in selective pressure for a reduction in expression, either through a reduction in copy number or through plasmid rearrangement. It may be possible to avoid these problems by rapid and efficient secretion from yeast [Goodey et al., 1986].

3. Certain proteins cannot be fully activated unless processed through the secretion pathway; e.g., γ-interferon [Simons et al., 1984]. In fact, proteins requiring glycosylation must be processed through the secretory pathway.

4. Overproduction of recombinant protein in E. coli results in the aggregation of the protein product inside the cell in the form of inclusion bodies [Williams et al., 1982; Marston, 1986]. The protein in this form is catalytically inactive, and extensive reprocessing is required to reconstitute the aggregates. In many cases, the recovery of the active protein is incomplete due to improper reaggregation [Georgiou, 1988]. The above problem can be avoided by secretion of soluble active material in yeast.

Heterologous protein secretion is characterized by delayed dynamics [Smith et al., 1985]. The lag in secretion dynamics is caused by the incompatibility of heterologous polypeptides with the secretion machinery of the host cell. The fundamental macromolecular events occurring in the post-translational secretory pathway of yeast have been clearly identified [Novick et al., 1979, 1980, 1981; Esmon et al., 1981]. In the case of yeast, processing at the endoplasmic reticulum or transport of partly glycosylated polypeptide from the endoplasmic reticulum to the golgi complex has been identified as the rate-limiting step for calf-prochymosin using the SUC2 gene as the secretion signal [Smith et al., 1985].
The protein of interest in this work is mutant invertase. The host system SEY 2102-s2I, is constructed by transforming strain SEY 2102, which contains a complete deletion of the chromosomal copy of SUC2 and contains no outer unlinked invertase structural gene (SUC1, SUC3-SUC7), with the integrating plasmid YIp5 into which the SUC2-S2 mutant gene is subcloned. Insertion of the desired gene into the chromosome avoids the structural and segregational instabilities generally associated with plasmid cloning and also enables maintenance of a stable copy number [Hicks et al., 1987]. The invertase mutation, SUC2-S2, is a mutation of the wild-type that converts a thr to an ile at position +64 in the mature protein. This causes a seven-fold delayed secretion of the mutant strain compared to the wild-type invertase [Schauer et al., 1985]. Thus, this conformational change, while not affecting enzymatic activity of the foreign polypeptide, results in delayed processing through the post-translational secretory pathway, which is characteristic of a foreign protein.

Thus SUC2-S2 can be regarded to simulate well a typical cassette of foreign genes and has hence been used in this work.

Invertase, in its active form, resides in the periplasmic space. Thus this work is concerned with the optimisation of large, secreted proteins, which are not released into the culture medium. Obviously, optimising criteria will greatly differ for secreted protein released into the culture medium compared to the case of protein resident in the periplasmic space on account of differences in the mode of protein isolation and recovery.

Fed-batch studies using the above-mentioned host and mutant protein [Ramires and Park, 1988] have yielded the following results for maximising the concentration of secreted foreign protein at the final time corresponding to the attainment of the maximum operating volume:

1. The general solution indicated operation on three singular arcs connected by bang-bang operation. The best assignment of the entire fed-batch running time on each singular arc is determined by optimal control theory.

2. The glucose concentration must change sequentially to optimise host cell growth, protein expression, and secretion of the expressed polypeptides in that order.

This work uses the secretion model developed from fed-batch studies [Ramirez and Park, 1988] to simulate the behavior of a continuous system operating at steady state, with the objective of determining the system configuration required for maximising the productivity of secreted protein.

II. SYSTEM DESCRIPTION

A. Configuration

The system under consideration consists of a cascade of stirred tank reactors operating at steady state. Simulations for ascertaining the system behavior were carried out in the presence and absence of a cell concentration and recycle device like a crossflow microfilter, to study the effect of cell recycle on protein productivity. The schematic diagram of the system is given in Figure 1.

B. Equations

The cell balance equations used in this simulation are:

\[ X_i = \frac{(s_0 - s_i)}{(1 - R_i)(Y_1)} \]

where \( X_i \) and \( s_i \) denote the cell concentration (g/l) and residual substrate concentration (g/l) in stage one respectively. The sugar concentration in the sterile feed stream, \( s_0 \), has been taken as 20 g/l in these studies. \( R_i \) is a recycle parameter defined by:

\[ R_i = \frac{q_1}{F_{01}} \]

where \( q_1 \) (l/h) denotes the permeate rate from stage 1 and \( F_{01} \) (l/h) denotes the rate of inflow of fresh feed to stage 1. Recycle parameters and dilution rates in other stages are defined in a similar manner.

\[ X_i = X_{oi} + \frac{(s_{oi} - s_i)}{Y_i} \quad i > 1 \]
Figure 1: Schematic diagram showing system configuration.
where $X_{oi} \ (g/l)$ is the cell concentration entering the $i$th stage as a mixture of the product stream from the previous stage and fresh feed. Thus,

$$X_{oi} = \frac{F_{i-1}X_{i-1}}{F_{i-1} + F_{0i}} \quad i > 1 \quad (4)$$

where $F_{i-1}$ denotes the flow rate of the stream leaving the $(i-1)$th stage. The entering substrate concentration, $s_{oi}$, is defined similarly. The yield coefficient, $Y_i$ (dimensionless) is evaluated by:

$$Y_i = 58.75(\mu_i^2) + 1.71 \quad i \geq 1 \quad (5)$$

where the growth rate, $\mu_i \ (hr^{-1})$, is given by [Park and Ramirez, 1989]:

$$\mu_i = \frac{21.87(s_i)}{(s_i + 62.5)(s_i + 0.4)} \quad i \geq 1 \quad (6)$$

The residual substrate concentration in the first stage can be obtained by solving the following equation for $s_1 \ (g/l)$:

$$D_1 = \mu_1 = \frac{21.87(s_1)}{(s_1 + 62.5)(s_1 + 0.4)} \quad (7)$$

where $D_1 \ (hr^{-1})$ denotes the stage one dilution rate and is defined by:

$$D_1 = \frac{F_{0i} - q_1}{V_1} = \frac{F_i}{V_1} \quad (8)$$

where $V_1 \ (l)$ denotes the working volume of stage one. The relation $D_1 = \mu_1$ is obtained through a cell balance on the first stage.

The residual substrate concentration in second and subsequent stages can be obtained through an iterative search using the substrate balance equation given below:

$$D_{i-1}s_{i-1}\left(\frac{V_{i-1}}{V_i}\right) - \frac{D_is_i}{1 - R_i} + \frac{F_{0is_0}}{V_i} - \mu_tX_iY_i = 0 \quad (9)$$

where the dilution rate for stage $i$, $D_i$, is given by:

$$D_i = \frac{F_i}{V_i} \quad (10)$$

The balance for total protein, $PT_i \ (activity \ units/l)$, which comprises the mature protein and the partially processed polypeptide still within the secretory pathway, is given by:

$$PT_i = \frac{B_iX_i}{D_i} \quad (11)$$

$$PT_i = \frac{D_{i-1}(\frac{V_{i-1}}{V_i})PT_{i-1} + B_iX_i}{D_i} \quad i > 1 \quad (12)$$

The secretion rate [Park and Ramirez, 1989], denoted by $B_i \ (activity \ units/g/h)$, is given by:

$$B_i = \frac{s_i \exp(-5s_i)}{s_i + 0.1} \quad i \geq 1 \quad (13)$$

The balance for mature, heterologous, secreted protein, $PM_i \ (activity \ units/l)$, is:

$$PM_i = \frac{A_1PT_i}{(D_i + A_1)} \quad (14)$$

$$PM_i = \frac{D_{i-1}PM_{i-1}(\frac{V_{i-1}}{V_i}) + A_1PT_i}{A_i + D_i} \quad i > 1 \quad (15)$$

The expression rate [Park and Ramirez, 1989], denoted by $A_i \ (hr^{-1})$, is given by:

$$A_i = \frac{4.75\mu_i}{\mu_i + 0.12} \quad i \geq 1 \quad (16)$$
C. Basic Constraints

In order to compare the performance of different system configurations on a consistent basis, certain constraints were imposed on the system. These constraints are given below for a $i$-stage cascade:

1. The total system volume was kept constant.
2. The total feed rate was kept constant.
3. The total permeate rate was held constant.

The individual stage volume, feed and permeate rate to a stage were each independently variable for any given $(i-1)$ stages. The feed substrate concentration to each stage was maintained at 20 g/l. The maximum dilution rate in the first stage was selected as 0.21 hr$^{-1}$ [von Meyenburg, 1968] to avoid the deleterious effect of ethanol due to the Crabtree effect [DeDekken, 1968] which results in the suppression of the oxidative metabolic pathway by the fermentative pathway at high dilution rates. Thus the simulations cater to the case of aerobic growth. To avoid cell washout, the maximum dilution rates in the second and subsequent stages was selected as 0.42 hr$^{-1}$.

D. Computation Details

All computations were done in FORTRAN on a VAX/VMS cluster. The objective function to be maximised was the secreted foreign protein productivity $(\text{activity(units)} / (\text{volume})(\text{time}))$.

A preliminary search was carried out using a coarse grid employing nested loops. After localizing the region in which the optimum was located, another search using the IMSL constrained optimization subroutine (DBCONF) was carried out to arrive at the precise optimum.

III. RESULTS

A. One-Stage System

- Protein productivity is maximum at a dilution rate of 0.05 hr$^{-1}$ (Figure 2).
- Cell productivity is maximum at a dilution rate of 0.16 hr$^{-1}$ (Figure 3).
- Since fed-batch studies [Ramirez and Park, 1989] identified two singular arcs emphasizing cell growth and protein expression uniquely, the occurrence of distinct optima for the cell and protein productivity in the continuous case prompted the need to examine the performance of a two stage system. It was...
apparent that to operate only a single stage system would necessitate a compromise between these two factors.

- It was found that cell recycle enhances foreign protein productivity (Figure 2). As mentioned earlier, the cell concentration device contemplated is a microfilter. The maximum degree of recycle allowable would be obtained by an economic analysis accounting for the monetary value of protein and the cost of the discarded permeate.

- Increasing the feed sugar concentration also increases the protein productivity (Figure 4). The maximum possible feed sugar concentration would obviously be determined by the dissolved oxygen transfer capacity of the fermentor (since aerobic conditions are required) and/or power requirements imposed on the motor by dense culture.

B. Two-Stage System

- In the absence of recycle, the optimum productivity is found to occur when 98.2% of the total feed is allocated to the second stage (Figure 5). In the presence of recycle, optimum productivity is achieved when 89% of the total feed is allocated to the second stage (Figure 6). Similarly, in the absence of recycle, 96% of the total volume needs to be allocated to the second stage (Figure 7). In the presence of recycle, the productivity is maximum when stage two volume accounts for 94% of the total volume (Figure 8).

- The maximum protein productivity, obtained with a two-stage system without recycle (3.185 units/L/h), is 7% higher than that obtained with a single chemostat without recycle (2.99 units/L/h). Refer figure 2 and figure 6. Similarly, the productivity yielded by a two-stage system with recycle (3.848 units/L/h) is 16% higher than a similarly configured single chemostat (3.32 units/L/h). Refer figure 2 and figure 6. Thus, as observed in one-stage simulations, operation with recycle gives a higher productivity of foreign protein compared to the non-recycle case for a two-stage system.

- Of the total permeate rejected by the two stage system, over 99 percent comes from the first stage.

IV. DISCUSSION

The performance of a single chemostat proves to be inferior compared to the two-stage system since it is constrained by having to achieve high cell density and protein productivity in the same stage simultaneously. Since fed-batch results indicate that optimization of cell and protein productivities constitute two distinct singular arcs, it is apparent that trying to maximize both in the same stage would necessarily detract from
EFFECT OF FEED SUGAR ON PROTEIN PRODUCTIVITY
ONE-STAGE SYSTEM WITH RECYCLE \( R=0.1 \)

Figure 4: Protein productivity as a function of feed sugar concentration.

PROTEIN PRODUCTION VS. FEED FRACTION TO STAGE TWO
TWO STAGE CASCADE WITHOUT RECYCLE; FEED SUGAR: 20 G/L

Figure 5: Variation of protein productivity in a two-stage system without recycle as a function of fraction of feed allocated to second stage.
**Protein Production vs Fraction Feed to Second Stage**

**Simulation with Recycle: On Both Stages**

![Graph](image)

**Figure 6:** Variation of protein productivity in a two-stage system with recycle as a function of feed allocated to second stage.

**Secreted Protein Productivity vs Stage 1 Volume**

**Two-Stage System Without Recycle**

![Graph](image)

**Figure 7:** Variation of secreted protein productivity in a two-stage system without recycle as a function of fraction of system volume allocated to stage one.
overall protein productivity. This is reinforced by Figures 2 and 3, from where it may be seen that cell and protein productivities are maximised at significantly different dilution rates and hence different culture conditions.

In the two-stage system, the small first stage serves essentially as a means of delivering high-density inoculum to the second stage. This is reinforced by the allocation of almost the entire allowable permeate flux to the first stage, thus indicating a high degree of cell concentration through cell recycle at the point of the maximum protein productivity from the system. Thus the first stage yields high cell density by maximal utilisation of the feed sugar. The high cell density achieved in the first stage chemostat (whose operation is time-independent) is equivalent to the high cell growth rate which constituted the first singular arc of the optimal control policy determined by fed-batch studies [Park and Ramires, 1988]. Whereas the first stage is essentially used to produce cells, the second stage utilises the bulk of the feed to express foreign protein. The protein productivity is enhanced by the high density inoculum coming from the first stage and by the low substrate concentration in the stream from stage one. Since the foreign protein is located in the periplasmic space, the utility of a high density inoculum from the first stage is obvious. Since the expression of the foreign protein is subject to catabolite repression, the low substrate concentration in the stream from stage one serves to achieve a lower overall steady-state residual sugar concentration which causes the expression rate, $B_i$, to be sustained at close to its maximum.

V. CONCLUDING REMARKS

These simulation studies indicate that the first two singular arcs in the fed-batch optimal control policy [Park and Ramires, 1988], which emphasise cell growth and protein expression, manifest themselves distinctively in two separate stages in continuous operation.

Since fed-batch results indicate the presence of three singular arcs, with the last of these emphasising secretion of the expressed protein, investigation of a three-stage system is necessary. This work is currently in progress.

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REFERENCES

References


AN IMPROVED KINETIC-MODEL FOR LACTIC ACID FERMENTATION

Yeh, Paul Li-Hong
Bajpai, Rakesh K.
Iannotti, Eugene, L. 1

Department of Chemical Engineering
University of Missouri - Columbia

INTRODUCTION:

A large number of biologically produced compounds are toxic to the very agents that produce them [1-3]. As a result, the rates of production drop drastically with the accumulation of products in the broth and only dilute solutions of the desired compounds are obtained at the end of fermentations. Accordingly, a significant research effort is underway to find ways to continuously remove the products of fermentation as they are formed [4-13]. The kinetic models needed to analyze these recovery-coupled fermentations must have features that would permit a coupling of the separation process with that of growth and product formation.

This paper deals with modelling of lactic acid fermentation with the objective of coupling microbial reactions with separation schemes. Lactic acid has been chosen for the case study as a member of industrially important category of organic acids that can be used as feed-stocks for chemical industry. Lactic acid may itself serve as a commodity chemical if it can be produced from renewable resources economically [14]. As a fermentation product obtained from carbohydrates, it has a definite advantage over ethanol as formation of lactic acid entails no loss of carbon as carbon dioxide. Any process developed for lactic acid can be easily modified for other organic acids as well.

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1 Department of Agricultural Engineering, University of Missouri - Columbia.
KINETIC MODEL AND PARAMETER ESTIMATION:

Lactic acid causes inhibition of growth of cells and also the process of its own formation by the cells [5,6,15]. It has been shown that undissociated lactic acid is the primary agent responsible for this inhibition [1,2]. In addition, a strong effect of pH of the broth has been reported for this system. Hence, a suitable model must involve the effect of these two important variables. In this fermentation, no other product is produced in significant quantities and, therefore, need to be incorporated in the model.

Kinetics of lactic acid fermentation by Lactobacillus delbrueckii on a glucose-yeast extract medium in batch culture has been reported by Luedeking [16]. These data at constant broth pH have been analyzed in this work to develop a model involving growth and product formation by Lactobacillus delbruckii.

For the equilibrium dissociation reaction

\[ C_3H_8O_3 \rightleftharpoons C_3H_7O_3^- + H^+ \]

it can be shown that

\[ [P_u] = \frac{[P]}{1 + 10^{(pH - pK)}} \]  \hspace{1cm} (1)

where \([P]\) is the total lactic acid concentration and \([P_u]\) is the concentration of undissociated lactic acid.

Assuming Monod's type of dependence of the specific rates of growth and product formation upon the concentration of limiting substrate, we may write

\[ \mu = \mu_{\text{max}} \cdot f([P_u]) \cdot \frac{S}{k_S + S} \]  \hspace{1cm} (2)

and

\[ \nu = \nu_{\text{max}} \cdot g([P_u]) \cdot \frac{S}{k_P + S} \]  \hspace{1cm} (3)

Here, \(f\) and \(g\) represent a postulated non-competitive inhibition of the rate processes by undissociated lactic acid. The nature of these functionalities can be established from the controlled pH-fermentations of Luedeking [16]. The experimental data at controlled pH were processed to calculate the instantaneous values of \(\mu\) and \(\nu\) using a numerical differential procedure suggested by LeDuy and Zajic [17]. Table I shows the original data of Luedeking [16] along with the calculated \(\mu\) and \(\nu\) values for constant pH of 6.0. Similar calculations were
performed for other constant pH fermentations as well. Although Luedeking [16] did not report the concentrations of sugar, these for most of these points may be safely assumed to be far greater than \( K_s \) and \( K_p \). Hence, the calculated values may be utilized to determine the effect of undissociated lactic acid upon growth and product formation. Semi-logarithmic plots of the calculated \( \mu \) and \( \gamma \) values vs. the instantaneous concentrations of undissociated lactic acid were made. Typical plots for each are presented in Figures 1 and 2. From these and many other such plots, it is clear that the nature of equations (2) and (3) should be of the following type:

\[
\mu = \mu_{\text{max}} < P_{\text{min}} \\
= \mu_{\text{max}} \cdot \exp \left( -k \left( [P_u] - P_{\text{min}} \right) \right) [P_u] > P_{\text{min}} \\
\gamma = \gamma_{\text{max}} \cdot \exp \left( -k' \left( [P_u] - P_{\text{min}} \right) \right) \ 	ext{for} [P_u] < P_{\text{min}} \qquad (5)
\]

Here \( P_{\text{min}} \) is the minimum concentration of undissociated lactic acid that causes inhibition of growth and product formation. This parameter \( P_{\text{min}} \) was found to be 0.12 g/L in this work. \( k \) and \( k' \) are the inhibition constants. The parameters \( \mu_{\text{max}} \), \( \gamma_{\text{max}} \), \( k \), and \( k' \) were calculated from such plots. These were found to be functions of pH of broth. The values of parameters calculated from three sets of experimental data presented by Luedeking and Piret [18] are presented in Table II. From these, the following relations were derived:

\[
k = 0.64 + \frac{1.1 \times 10^{-11}}{[H^+]^2} \\
k' = 0.41 + \frac{4.66 \times 10^{-12}}{[H^+]^2} \\
\mu_{\text{max}} = (0.2 + \frac{1.44 \times 10^{6}}{[H^+]} \exp \left( -k P_{\text{min}} \right) \\
\gamma_{\text{max}} = (0.87 + \frac{1.66 \times 10^{6}}{[H^+]} \exp \left( -k' P_{\text{min}} \right) \\
\]

where \([H^+]\) is the concentration of hydrogen ions in the broth. Since the concentrations of cells have been reported by Luedeking [16] in terms of optical density units (UOD), the units of \( \gamma \) and \( \gamma_{\text{max}} \) in equations (5) and (7) are (g lactate/UOD/L/hr).

The values of \( \mu_{\text{max}} \) and \( \gamma_{\text{max}} \) calculated from equations (6) and (7) have been compared with those obtained from the experimental data of Luedeking [16] in Figures 3 and 4, respectively. Considering the fact that data only from three experiments (Table II) were used to estimate the constants in expressions (6) and (7), the agreement is considered very good.
Another test for the validity of the model equations (4 -7) is their capability to adequately predict the experimentally observed profiles of growth and product formation. For batch growth, the governing equations may be written as

\[ \frac{dx}{dt} = \mu x \]  
\[ \frac{dp}{dt} = \gamma x \]  
\[ \frac{ds}{dt} = - \frac{\mu x}{Y_{x/s}} - \frac{\gamma x}{Y_{p/s}} \]  

(8)  
(9)  
(10)

Here, \( \chi \) is the biomass concentration (g/L), \( \pi \) is the concentration of total lactic acid produced by the cells (g/L), and \( \varsigma \) is the concentration of sugar in the broth (g/L). \( Y_{x/s} \) is the yield of biomass from sugar (g DW cells/ g sugar) and \( Y_{p/s} \) is the yield of lactic acid (g lactic acid/ g sugar). In order to use equations (5) and (7) in equations (8-10), it is necessary to convert UOD into (g DW/L). This conversion factor is between 0.4 and 0.7 (g DW/L/UOD) [5,12,18]. In this work, we have assumed it to be 0.5. Hence,

\[ \gamma = 2.0 \gamma \]  

(11)

The results of simulations of equations (8-10) are plotted in Figures 5 and 6. For the sake of comparison, experimental data are also presented as discrete points on these plots. The agreement between experimental observations and the predictions is considered very good. Similar agreements were observed for other experiments as well.

**DISCUSSION:**

The model developed here shows a number of characteristic experimentally observed features of lactic acid fermentations. Although lactic acid profiles at pH higher than 6.0 are not available, the model suggests that a drop in lactic acid production rates may occur. We have indeed seen a drop in lactic acid production at low as well as at high pH values in another study involving direct fermentation of liquified starch to lactic acid by *Lactobacillus amylovorus*.

The model possesses a number of unique features that allow for its coupling with on-line separation processes. As an example of the coupling, simulations are being performed for different separation schemes such as those involving reactive extraction, ion-exchange, and electro-dialysis. These results will be presented in a follow-up paper.
CONCLUSIONS:

The batch data of lactic acid fermentation by Lactobacillus delbrueckii at controlled pH levels in a glucose-yeast extract medium were analysed using a differential analysis. An improved model was developed that closely fitted the observed data. The parameters in the model, which reflected the activity of the culture, were found to be functions of undissociated lactic acid concentration and pH of the broth.

REFERENCES:


Cell growth and lactic acid formation in batch fermentation
(At 45°C, pH controlled at 6.0, on a 5% glucose concentration)

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Table 2  Calculated values of $\mu_{\text{max}}$, $V_{\text{max}}$, $k$ and $k'$ at different pH values

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Figure 1: Specific growth and product formation rates, calculated from experimental data. pH = 4.5. □: data points. Solid line represents the regression curve, equations (4-5).
Figure 2: Specific growth and product formation rates, calculated from experimental data. pH = 6.0. □: data points. Solid line represents the regression curve, equations (4-5).
Figure 3: Maximum specific growth rate of cells at different pH of broth. □: experimental data. —: equations (6-7).

Figure 4: Maximum specific product formation rate at different pH of broth. □: experimental data. —: equations (6-7).
Figure 5: Experimental and simulated profiles of cell dry-weight and lactic acid concentrations at pH = 4.5. ◆, ♦: experimental data. ----: simulation results.

Figure 6: Experimental and simulated profiles of cell dry-weight and lactic acid concentrations at pH = 6.0. ◆, ♦: experimental data. ----: simulation results.
List of Participants:

Iowa State University
Ufuk Bakir
K. B. Bcstawade
Andrea Bazzano
R. Boopathy
Hsiu-Mei Chen
Bipin K. Dalmia
Ali Demirci
Craig Forney
Charles Glatz (Faculty)
Lifuk Gunduz
Lulie Hardwick
Meng H. Heng
Curtis L. Krause
Eduardo Gomez Magueo
Ramanathan Murali
Mark Niederauer
Zivko Nikolov (Faculty)
Peter Reilly (Faculty)
Ilari Suominen
Lourdes Taladriz
Nancy Waletzko
Jing Zeng

Kansas State University
Yiming Chen
Sanjay Dhawan
Larry Erickson (Faculty)
Travis Jones
Bei-gian Shi
Kiyoshi Takahashi
Koranee Tuitemwong
Pravate Tuitemwong
Hengjian Wang
Jianchu Wu

University of Colorado
Ashish Chatterjee
Robert Davis (Faculty)
Debra Hawker
Kim Henry
Jeff Kern
Ching Yuan Lee
Fuche Miao
Kalyan Tadikonda
Huaging Zhon

Washington University
Ales Prokop (Faculty)

University of Missouri - Columbia
Rakesh Bajpai (Faculty)
Pu-Sheng Cheng
Tsair Wang Chung
Fu-Hung Hsieh (Faculty)
Gene Iannotti (Faculty)
Joong Kim
Wei Ming Liu
Shing-Jiang Lue
George W. Preckshot (Faculty)
Ying-Tsung Su
C. J. Wang
Paul Yeh

Non-participating Faculty
M. N. Karim (Colorado State University)
J. C. Linden (Colorado State University)
V. G. Murphy (Colorado State University)
R. P. Tengerdy (Colorado State University)
R. A. Ackerman (Iowa State University)
R. C. Seagrange (Iowa State University)
L. T. Fan (Kansas State University)
L. A. Glasgow (Kansas State University)
D. S. Kompala (University of Colorado)
W. F. Remirez (University of Colorado)