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Proceedings of the 13th Annual Biochemical Engineering Symposium

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13th
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Peter J. Reilly
Editor
April 23, 1983

Department of Chemical Engineering
College of Engineering
Iowa State University, Ames, Iowa

ISU-ERI-Ames 83277
Proceedings of the
Thirteenth Annual Biochemical
Engineering Symposium:

April 23, 1983

Peter J. Reilly
Editor

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PROCEEDINGS OF THE THIRTEENTH
BIOCHEMICAL ENGINEERING SYMPOSIUM

The symposium reported here was the thirteenth of a series devoted to talks by students on their biochemical engineering research. The first, third, fifth, ninth, and twelfth were at Kansas State University, the second and fourth were at the University of Nebraska-Lincoln, the sixth was in Kansas City and was hosted by Iowa State University, the seventh and tenth were at Iowa State, and the eighth and eleventh were at the University of Missouri-Columbia and Colorado State University, respectively. All symposia have been followed by proceedings edited by faculty of the host institution. Because final publication usually takes place elsewhere, papers here are brief, and often cover research in progress.

Attending this meeting at Iowa State were Khalif Ahmed, Kate M.V. Baptie, Marfa S. Bertrán, C. J. Huang, Nikhil Mehta, Vincent G. Murphy, and Wayne A. Nakagawa from Colorado State University, Seble W. Afework, Nam Wae Choi, Kyle Dybing, Larry E. Erickson, L. T. Fan, Mahendra M. Gharapuray, Bumshik Hong, C. H. Lee, Hyeon Yong Lee, Chetan D. Mehta, Debasish Neogi, Mehmet Durdu Oner, Snehal A. Patel, Sujeet Shenoi, Bamidele O. Solomon, and Steven D. Tessendorf from Kansas State University, Rakesh Bajpai and Chen Jen Wong from the University of Missouri-Columbia, and B. Douglas Brown, Judy Brown, Michael K. Dowd, Kenneth H. Hsu, Michael M. Meagher, Peter J. Reilly, Etienne J.-M. Selosse, Michael R. Sierks, and Bernard Y. Tao from Iowa State University.

Inquiries on the research conducted at these schools should be directed to Professors Rakesh Bajpai, Larry E. Erickson, Vincent G. Murphy, or Peter J. Reilly.

Peter J. Reilly
Editor
Thirteenth Annual Biochemical Engineering Symposium
Iowa State University
Ames, Iowa 50011

Saturday, April 23, 1983

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Growth, Byeon Y. Lee, Kansas State University  103
INTRODUCTION

When microorganisms are grown in multisubstrates, such as molasses and wood hydrolyzate, a phenomenon of diauxic growth and sequential substrate utilization is often encountered. The growth phenomenon, termed diauxie by Monod (1), is typically characterized by a two-phase growth curve in which growth on glucose corresponds to the first phase of growth and growth on the secondary substrate corresponds to the second phase.

Two mechanisms that have been studied in greatest detail are enzyme repression and enzyme inhibition (2-5). These two mechanisms can be physiologically distinguishable. Either enzymes necessary for the utilization of various substrates are not synthesized by the microbial cell in the presence of a repressing substrate (enzyme repression) or these catabolic enzymes are formed but remain inactive (enzyme inhibition). Both responses lead to diauxic growth and sequential substrate utilization.

The objective of the study presented here was to investigate which regulatory mechanism is responsible for the diauxic phenomenon in Cl. acetobutylicum.

MATERIALS AND METHODS

Microorganism

The strain, Cl. acetobutylicum, was used.

Media

A medium, containing 140 g/l of sorghum molasses, and 2 g/l of (NH₄)₂SO₄, was used for butanol fermentation. A basal medium with the composition as shown in Table 1 was used for investigating mechanisms. A 20 g/l of glucose, fructose, or sucrose was added to the basal medium as the sole carbon source.
Cultivation

A 10 ml of corn meal medium was used to activate the maintenance culture stored at 5°C. The inoculated corn meal medium was heat-shocked to germinate the spores in boiling water for 60 seconds, and was immediately cooled in a water bath. The culture was then incubated at 37°C for 18 hours. Inoculum was prepared by adding the growing culture on corn mash to 140 ml of the molasses or synthetic medium in a 300 ml flask. Fermentation was performed for 72 hours at 37°C in a 5 l jar fermentor (New Brunswick Scientific Co., Inc. Model 19) containing 2.85 l of the medium and 150 ml of the inoculum. The initial pH was adjusted to 6.0. The pH was maintained at 5.0 by adding 5N sodium hydroxide during fermentation.

Analytical methods

Methods for analyzing substrates and products are described below.

(a) Sugars: Sucrose, glucose and fructose were monitored using a Varian Model 5000 high pressure liquid chromatograph (HPLC).
(b) Solvents: Concentration of the products were assayed using a Hewlett Packard Model 5710A gas chromatograph.
(c) Biomass: Cells were harvested by the Millipore technique (0.5 μm filter) and dried overnight at 105°C.

RESULTS AND DISCUSSION

As indicated in Fig. 1, glucose and fructose were consumed essentially in parallel at the initial stage. As time progressed, the rate of glucose consumption became increasingly greater than that of fructose. In other words, the sugar consumption pattern shifted from a parallel one to a sequential. Note that fructose was consumed rapidly after fructose disappeared completely. Sucrose started to be consumed almost immediately after the disappearance of fructose. It appears that the utilization of sucrose was inhibited by glucose and fructose.

The pattern of cell growth indicates that growth is closely related to the sugar consumption. No transitional period could be detected after depletion of glucose in the cell growth pattern. However, the biomass concentration decreased immediately after fructose was consumed completely. The cell growth was divided by a distinct lag period. The reduction in the biomass concentration during the transitional period was due to cell autolysis. The second exponential growth was at the expense of sucrose.

In the butanol-acetone fermentation from sorghum molasses, little butanol was produced during the lag period in the biomass concentration, which led to a low product yield (fig. 2). The product formation and yield apparently were influenced by the diauxie phenomenon. On the
other hand, no transitional period was detected in the biomass concentra-
tion in an inverted sorghum molasses medium (Fig. 3). Hence, the
total solvent production increased by as much as 50%.

To investigate the mechanism responsible for the diauxie phenomenon,
进一步 experiments were carried out with model systems. In the first
experiment cells were cultured in a fructose medium and glucose was
introduced 18 hours later (Fig. 4). Introduction of glucose quickly
lessened the rate of fructose utilization. When glucose was totally
consumed, a new rate of fructose utilization was established. The
ability to consume fructose was quickly restored once glucose was
consumed completely. The rate of fructose consumption was as high as
that established before the addition of glucose. The results seem to
indicate that the rate of fructose elimination from the medium is
proportional to the concentration of fructose hydrolyzing enzymes
formed before the addition of glucose. Thus, it appears that these
enzymes remained inactive when glucose was consumed.

In the second experiment, cells were grown in a sucrose medium for
about 20 hours at which time glucose was introduced into the reaction
mixture (Fig. 5). Whereupon, the sucrose consumption ceased quickly,
signifying sucrose activity was inhibited by glucose. The ability to
consume sucrose was also quickly restored after glucose was consumed
completely. The quick inhibitory effect of glucose and fast recovery
of the ability to consume sucrose indicate that catabolite repression is
not the regulatory mechanism in this system.

In the third experiment, a sucrose-fructose system was used as
shown in Fig. 6. the pattern of sugar utilization is similar to that
in Fig. 5.

The evidence obtained appears to support the notion that enzyme
inhibition rather than catabolite repression is the main control mechanism
for the diauxy in Cl. acetobutylicum.

REFERENCES

   37, 531.
Table 1. Composition of the basal medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{K}_2\text{HPO}_4$</td>
<td>0.75 g</td>
</tr>
<tr>
<td>$\text{KH}_2\text{PO}_4$</td>
<td>0.75 g</td>
</tr>
<tr>
<td>$\text{MgSO}_4$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$</td>
<td>0.01 g</td>
</tr>
<tr>
<td>$\text{MnSO}_4 \cdot \text{H}_2\text{O}$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>$\text{NaCl}$</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.5 g</td>
</tr>
<tr>
<td>$(\text{NH}_4)_2\text{SO}_4$</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Resazurin</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Fig. 1. Relationship between the pattern of sugar consumption and biomass concentration during butanol-acetone fermentation of the sorghum molasses by \( \text{C. acetobutylicum} \) ATCC 4259.
Fig. 2. Biomass and solvent concentrations during butanol-acetone fermentation of the sorghum molasses medium by *C. acetobutylicum* ATCC 4259.
Fig. 3. Biomass and solvent concentrations during butanol-acetone fermentation of the inverted sorghum molasses medium by *Clostridium acetobutylicum* ATCC 4259.
Fig. 4. Consumption of glucose and fructose by *Clostridium acetobutylicum* ATCC 4259.
Fig. 5. Consumption of glucose and sucrose by \textit{Cl. acetobutylicum} ATCC 4259.
Fig. 6. Consumption of fructose and sucrose by *Clostridium acetobutylicum* ATCC 4259.
THE EFFECTS OF DILUTION RATE ON
THE KINETICS OF ANAEROBIC ACIDOGENESIS

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Colorado State University
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INTRODUCTION

Anaerobic digestion is a heterogeneous bacterial fermentation in which complex organic materials are converted into carbon dioxide and methane. This process has been used to stabilize the sludges produced from domestic wastewater treatment for decades. Anaerobic digestion offers several significant advantages including a high conversion of available organic carbon to gaseous end products, low bacterial growth yield as a result of minimal energy available for microbial growth, and generation of product gas high in methane content.

Despite its many attractive features, anaerobic digestion has not yet been fully accepted due to its process instability. This problem may be attributed to the multiphase nature of the process and the complexity of the heterogeneous bacteria involved (1). As shown in Fig. 1, four different bacterial groups are recognized as being responsible for carrying out the fermentation. The H₂-producing acetogenic bacteria grow in harmony with the methanogenic bacteria. Indeed, the methanogens must remove the hydrogen produced by the former to render the thermodynamic conditions for growth favorable for their syntrophs. The hydrolysis and acidogenesis are usually performed by fermentative bacteria. Therefore, the process stability depends on maintenance of a delicate biochemical balance between the fast-growing acidogens and the more fastidious methanogens. If this delicate balance is upset, acidogens may overwhelm methanogens to a point where the environment is altered adversely and the process fails.

Since these two dominant groups of organisms differ greatly with respect to physiology, nutritional requirements, growth and metabolic characteristics, and sensitivity to environmental stresses, the concept of controlling process stability by phase separation of the two groups has been developed (2). In such a manner, optimum environments can be provided for both groups and the substrate loading rates to each group can be controlled, thus giving enhanced substrate utilization and process reliability (3). This procedure would also allow for the development and implementation of associated instrumental monitoring and automatic control techniques.
The successful application of the phase-separation concept depends on the feasibility of physically separating and maintaining cultures of the dominant groups of facultative anaerobic and strict anaerobic microorganisms. This may be accomplished by using kinetic control on each phase by operational adjustment of the dilution rates. Therefore, the objective of this study was to investigate the effects of dilution rate on the kinetics of anaerobic acidogenesis.

THEORETICAL ASPECTS OF CHEMOSTAT OPERATION

In a continuous flow, completely mixed fermenter without recycle, there is a continuous pressure for biological solids to increase due to growth and to decrease due to cells being washed out of the vessel. Therefore, the change in cell mass can be expressed in terms of a mass balance.

\[ V \frac{dx}{dt} = V (\mu x) - F x \]

where \( V \) is the working volume of the fermenter, \( x \) is the cell mass concentration in the fermenter, \( t \) is the time, \( \mu \) is the specific growth rate of the cells (mass increase per unit mass per unit time), and \( F \) is the volumetric flow entering and leaving the fermenter. It is assumed that the entering stream contains no cells.

At steady state, the mass balance reduces to

\[ \mu = \frac{F}{V} = D \]

where \( D \) is known as the dilution rate of fermenter. This identity is a very important one. It states that the specific growth rate is equal to the dilution rate and, therefore, is subject to hydraulic control. The influent flow rate to the fermenter is then a very important physical parameter which can exert a considerable effect on the biological system. This fact has obvious engineering significance, since \( F \) is a system parameter which is more subject to engineering control than are the biological parameters.

Combining the above steady state equation with the Monod model for the relationship between specific growth rate and substrate concentration, we obtain

\[ D = \mu = \frac{S}{\mu_m K_s + S} \]

where \( S \) is the substrate concentration in the fermenter and \( \mu_m \) and \( K_s \) are parameters of the model, which are known as the maximum specific growth rate and the saturation constant, respectively. This equation may now be rearranged to give

\[ \frac{1}{D} = \frac{K_s}{\mu_m} (\frac{1}{S}) + \frac{1}{\mu_m} \]
The above equation is derived for a pure culture, but it can also be applied to a heterogeneous system of constant composition. If species group A is dominant at a dilution rate $D_1$, then the observed data point ($1/D_1$, $1/S_1$) will fall on a line defined by the equation

$$\frac{1}{D_1} = \frac{K_{SA}}{\mu mA} \left(\frac{1}{S_1}\right) + \frac{1}{\mu mA}$$

as illustrated in Fig. 2. However, if the dilution rate is increased to $D_2$, which is higher than the net specific growth rate of the dominant species group A, the observed data point ($1/D_2$, $1/S_2$) will be defined by a different linear function incorporating the biological parameters, $\mu_{MB}$ and $K_{SB}$, of species group B, as also illustrated in Fig. 2.

**MATERIALS AND METHODS**

Initially, 2.0 liter of seed taken from a 5.0 gal stock digester was mixed with 1.0 liter of (MIT) nutrient medium containing about 35 g/L glucose. The stock digester from which the seed was drawn is kept at 50°C and fed weekly with a slurry of cattle manure. The mixture of seed and medium was kept at 37°C and pH 7.0 for a period of one week. During this time, 100 ml aliquots of broth were replaced with a like volume of medium whenever gas production ceased. To acclimate acidogenic bacteria, semi-continuous culture was then initiated by daily replacing about 5% (v/v) of the broth with fresh medium and controlling the pH at 6.0. When steady state (as indicated by a constant level of reducing sugar in the broth) was reached, chemostatic operation was begun.

In the chemostatic runs, the temperature, pH and stirring rate were held constant at 37°C, 6.0 and 200 rpm, respectively. At first, the inlet flow of medium and outlet flow of broth were set at 0.3 L/hr to give a dilution rate of 0.10 hr$^{-1}$ for the 3.0 liter fermenter. After about 15 hydraulic retention times, the system was assumed to be at steady state, and the inlet and outlet concentrations of glucose and the concentration of suspended solids in the outlet were recorded. (The glucose concentrations were determined by the dinitrosoalicylic acid assay for reducing sugar.) The flow rates were then increased in a stepwise manner to achieve higher dilution rates, with similar data being recorded after about 15 hydraulic retention times at each dilution rate. The apparatus used in these runs is shown schematically in Fig. 3.

**RESULTS**

The steady state data for the three dilution rates used in the continuous, completely mixed fermentations are summarized in Table 1 and illustrated in Fig. 4. From these limited data, a linear function
between 1/D and 1/S can be drawn using the two points for the higher dilution rates. The third point, which was obtained at the lowest dilution rate 0.10 hr⁻¹, is excluded because any line that is drawn using all three points would indicate a value of \( \mu_m \) that is less than 0.30 hr⁻¹, the value for highest dilution rate. Therefore, some additional selection for faster growing acidogens must have occurred as the dilution rate was increased above 0.10 hr⁻¹.

From the straight line drawn through the two points for the higher dilution rates, the following parameter values can be determined:

\[
\mu_m = 0.323 \text{ hr}^{-1} \\
K_s = 0.494 \text{ g/L}
\]

This value for the maximum specific growth rate is about the same as that determined by Cohen et al. (5), but higher than the value of 0.27 hr⁻¹ reported by Massey (3).

**Table 1. Summary of experimental data**

<table>
<thead>
<tr>
<th>Dilution rate (hr⁻¹)</th>
<th>Glucose concentration (g/L)</th>
<th>Suspended solids (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inlet</td>
<td>Outlet</td>
</tr>
<tr>
<td>0.10</td>
<td>36.2</td>
<td>0.054</td>
</tr>
<tr>
<td>0.20</td>
<td>35.1</td>
<td>0.805</td>
</tr>
<tr>
<td>0.30</td>
<td>37.2</td>
<td>0.590</td>
</tr>
</tbody>
</table>

**FUTURE WORK**

Due to the limited nature of the data collected in this study, the following additional experiments will be attempted in the future:

1. Batch fermentations inoculated with cells obtained from continuous runs at different dilution rates will be used to derive sets of kinetic parameters for the individual groups of bacteria in order to test the selectivity accomplished by increasing dilution rate.

2. The distribution pattern of volatile fatty acids will be obtained for continuous runs at different dilution rates to optimize the two-phase anaerobic digestion.
3. Different loading rates will be used to determine the effects of this variable on the kinetic parameters for various groups of bacteria.

REFERENCES


FIGURE 1. Mechanism of anaerobic digestion.

FIGURE 2. Hypothetical Lineweaver-Burk plots for chemostatic culture of two groups of microorganisms with different specific growth rates.
FIGURE 3. Schematic diagram of acidogenesis fermentation system.

FIGURE 4. Lineweaver-Burk Plot of experimental data on chemostatic acidogenesis.

Slope = 1.528 hr·g/L

$K_S = 0.494$ g/L

$\mu_m = 0.323$ hr$^{-1}$

$\frac{1}{\mu_m} = 3.10$ hr

$$ \frac{1}{S} \text{, L/g} $$
ETHANOL PRODUCTION BY ZYMOMONAS MOBILIS IN ANAEROBIC GLUCOSE-LIMITED CULTURE: A YIELD STUDY

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INTRODUCTION

The true growth yield and the maintenance coefficient are important in understanding microbial processes. Recently, the concept of material and energy balance regularities were used and maintenance coefficient, \( m_e \), true growth yield, \( \eta_{\text{max}} \), and true product yield, \( \xi_p \), for aerobic cultures have been studied by Erickson and coworkers [1-8].

In this work, consideration is given to anaerobic processes, especially to ethanol production. Based on earlier work [9] equations are presented and used to estimate the maintenance parameter, \( m_e \) and true growth yield, \( \eta_{\text{max}} \), for several experimental and literature data. (where the only available electron acceptor is ethanol).

The same concept and similar equations may be used to estimate the parameters, \( \alpha_e \) and \( \beta_e \), for non-growth and growth associated product formation, respectively. Anaerobic processes have been treated previously by Roels and coworkers [10,11].

THEORY

Based on the available electron concept, an available electron balance for anaerobic processes can be written as follows [5,9];

\[
\eta + \xi_p = 1.0 \tag{1}
\]

where \( \eta \) is the fraction of available electrons of organic substrate which is incorporated into biomass, and \( \xi_p \) is the fraction of available electrons of organic substrate which is incorporated into extracellular product.

The carbon balance is:

\[
y_c + z + d = 1.0 \tag{2}
\]

where \( y_c \), \( z \) and \( d \) are the fractions of substrate carbon incorporated into biomass, extracellular product, and \( CO_2 \), respectively.

When ethanol produced in treated as the only available electron acceptor, the following equations can be written to estimate the maintenance parameter, \( m_e \) and the true growth yield, \( \eta_{\text{max}} \), [9]

\[
\frac{1}{\eta} = \frac{1}{\eta_{\text{max}}} + \frac{m_e}{\mu} \tag{3}
\]
\[
\frac{n + \xi_p}{n} = \frac{1}{\eta_{\text{max}}} + \frac{m_e}{\mu} \tag{4}
\]

\[
\frac{y_c + z + d}{n} = \frac{1}{\eta_{\text{max}}} + \frac{m_e}{\mu} \tag{5}
\]

where \(\mu\) is the specific growth rate, hr\(^{-1}\). Each of the Equations (3), (4) and (5) can be used to estimate \(m_e\) and \(\eta_{\text{max}}\). Since different experimental errors are involved in different experimental measurement procedures, one will obtain different sets of estimates of \(m_e\) and \(\eta_{\text{max}}\) from each of these equations. Using some statistical techniques, this problem can be overcome and better estimates of parameters can be obtained [8,12]. In this work the covariance adjustment technique [12] was used for this purpose.

Maintenance coefficient, \(m_{\text{th}}\), and growth yields \(\eta_{\text{th}}\) and \(\eta_{\text{th}}^\text{max}\), which are based on free energy, can be calculated from \(m_e\), \(n\), and \(\eta_{\text{max}}\) with the following equation:

\[
m_{\text{th}} = \frac{(g_s - g_p) m_e}{g_b} \tag{6}
\]

\[
\eta_{\text{th}} = \frac{g_b \cdot n}{g_s - g_p \xi_p} \tag{7}
\]

\[
\eta_{\text{th}}^\text{max} = \frac{g_b \cdot \eta_{\text{max}}}{g_s - g_p (1 - \eta_{\text{max}})} \tag{8}
\]

where the numerical values of the free energy relative to \(\text{CO}_2\), \(\text{NH}_3\) (aq.) and \(\text{H}_2\text{O} (l)\) are [9]

\[
g_b = 114.07 \text{ KJ/eq. avail. electron (for biomass)}
\]

\[
g_s = 119.667 \text{ KJ/eq. avail. electron (for glucose)}
\]

\[
g_p = 109.875 \text{ KJ/eq. avail. electron (for ethanol)}.
\]

One can also calculate theoretical values of \(\eta_{\text{max}}\) and \(m_e\) by using the following equations:

\[
\eta_{\text{max}} = \frac{\sigma_b Y_b \gamma_{\text{max}}}{12 \ \text{ATP}} \tag{9}
\]

\[
m_e = \frac{12 \cdot \delta \cdot m_{\text{ATP}}}{\sigma_b Y_b} \tag{10}
\]
where,
\[ \sigma_b = 0.462 \text{ (wt. fraction of carbon in biomass)} \]
\[ Y_b = 4.291 \text{ (reductance degree of biomass, number of eq. avail. electrons/g atom carbon in biomass)} \]
\[ Y_{\text{max}}^{\text{ATP}} = \text{maximum biomass yield based on ATP, g biomass/g mole ATP.} \]
\[ m_{\text{ATP}} = \text{moles of ATP expended per g of cells per hour for maintenance, moles ATP/g-hr.} \]
\[ \delta = \text{number of eq. avail. electrons transferred to ethanol to produce 1 g mole of ATP from ADP. Value of } \delta \text{ is 12 for yeasts and 24 for Zymomonos mobilis.} \]

Recently, it was found that product formation parameters, \( \alpha \) and \( \beta \), in the following equation:
\[ \frac{1}{X} \frac{dP}{dt} = \alpha + \beta \mu \] (11)
can be estimated, similar to \( \bar{m}_e \), and \( \eta_{\text{max}} \), by using the following equations:
\[ \frac{\mu}{\eta} \xi_p = \alpha_e + \beta_e \mu \] (12)
\[ \frac{\mu}{\eta} (1 - \eta) = \alpha_e + \beta_e \mu \] (13)
\[ \frac{\mu}{\eta} (y + d + z - \eta) = \alpha_e + \beta_e \mu \] (14)

where,
\[ \alpha_e = \text{non-growth related product formation parameter, eq. avail. electron of product per eq. avail. electron of biomass.} \]
\[ \beta_e = \text{growth related product formation parameter, eq. avail. electron of product per eq. avail. electron of biomass per hour.} \]

Comparing equations (3), (4), (5) and (12), (13), (14) the following relationships can be written:
\[ \alpha_e = m_e \] (15)
\[ \beta_e = \frac{1}{\eta_{\text{max}}} - 1 \] (16)

Therefore, one can either use equations (12), (13) and (14) to estimate \( \alpha \) and \( \beta \) or if estimates of \( \bar{m}_e \) and \( \eta_{\text{max}} \) are obtained previously, equation (15) and (16) can be used to calculate \( \alpha_e \) and \( \beta_e \) directly from \( m_e \) and \( \eta_{\text{max}} \) values. Roels [10,11] has also reviewed the relationships among these parameters.

**EXPERIMENTAL PROCEDURE**

Two anaerobic glucose-limited batch fermentation runs were performed with *Zymomonos mobilis*, ATCC-10988.
Samples were collected every 30 minutes and analyzed for biomass, glucose and ethanol concentrations. Also, volume of broth and effluent gas CO₂ concentrations were recorded during sampling procedure. For details of experimental procedures, refer to Öner [13].

RESULTS AND DISCUSSION

Available electron and carbon balance results for Runs 1 and 2 are given in Tables 1 and 2, respectively. During early and late periods of the process, carbon and available electron balances showed significant inconsistency. Decreased accuracy of glucose and ethanol analysis at low concentrations and the transfer rate of CO₂ from liquid to gas phase may be partially responsible for these inconsistencies. Also, the differentiation procedure involved in instantaneous balances of biomass, ethanol and glucose data, would have contributed to these errors.

Although some inconsistencies were observed with instantaneous balances, reasonably good consistency was obtained for the integrated balances as shown in Table 3.

Tables 4 and 5 show point and 95% confidence interval estimates of \(\mu\) and \(\eta_{\text{max}}\) for Runs 1 and 2, respectively. First three sets of results were obtained from individual equation (Eq's (3), (4) and (5)). Last two sets of results, combination estimates, were obtained by using the covariate adjustment technique [10].

For Run 1, following estimates were chosen as best estimates (based on smallest \(J\) values); 3.8793 \(\leq \mu \leq 4.0873\) and 0.1280 \(\leq \eta_{\text{max}} \leq 0.2592\) Form I \(3.8092 \leq \mu \leq 4.5607\) and 0.1794 \(\leq \eta_{\text{max}} \leq 0.6642\) Form II. Similarly for Run 2, 1.2423 \(\leq \mu \leq 2.3387, 0.0590 \leq \eta_{\text{max}} \leq 0.1429\) and 1.6023 \(\leq \mu \leq 2.9705, 0.0916 \leq \eta_{\text{max}} \leq 0.3622\) were chosen as best estimates for form I and II, respectively. These estimates, where both covariates are included, correspond to maximum likelihood estimates (MLE).

The theoretical maximum value of \(\eta_{\text{max}}\) for Zymomonas mobilis was calculated as 0.165 from Equation (9) by using \(\delta = 24\) eq. avail. electron transferred to ethanol/mole ATP produced and \(\gamma_{\text{ATP}} = 28.8\) g cell/mole ATP. Therefore, \(\eta_{\text{max}}\) values less than 0.165 can be accepted as significant and acceptable results.

Several data sets from the literature were also analyzed and results are summarized on Table 6. As it is seen from the results, Zymomonas mobilis has a much higher maintenance coefficient and lower true growth yield than Saccharomyces cerevisiae. These properties, which are related to the ability of this organism to produce ethanol rapidly, are desirable for Zymomonas mobilis.

The values of the maintenance coefficient in Table 6, which are estimated from the experimental results presented in Tables I and II, are in good agreement with those of other workers; however, the estimates of true growth yield appear to be larger than those of other workers.

Estimated values of the product formation kinetic parameters, \(\alpha\) and \(\beta\), are presented in Table 6. Since the maximum growth rate of Zymomonas mobilis
is approximately 0.2 hr\(^{-1}\), comparison of \(\alpha\) and \(\beta\) values shows that in most cases, more than 50% of the ethanol production is non-growth associated or related to the maintenance requirement.

CONCLUSIONS

Available electron and carbon balances can be used to check consistency of the data collected during anaerobic fermentation processes. These consistency tests show that the experimental data is not very consistent.

It is possible to obtain better estimates of maintenance parameter, \(m_e\), and true growth yield, \(\eta_{\text{max}}\), by using all the available experimental data with suitable statistical techniques.

Parameters \(\alpha\) and \(\beta\) in the product formation model can be obtained directly from estimated values of \(m_e\) and \(\eta_{\text{max}}\).

The higher maintenance requirement and lower growth yield than conventional yeasts are unique to \textit{Zymomonas mobilis} and they are desirable properties which are related to the ability of this organism to produce ethanol rapidly.

ACKNOWLEDGEMENT

This work was supported in part by National Science Foundation Grant Nos. CPE 79-18202 and CPE 81-20039.

REFERENCES


Table I. Examination of data consistency using instantaneous available electron and carbon balances for growth of *Zymomonas mobilis* on glucose, under anaerobic condition; Run #1.

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<th>( \eta + \xi_p )</th>
<th>( y_c )</th>
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Table II. Examination of data consistency using instantaneous available electron and carbon balances for growth of *Zymomonas mobilis* on glucose, under anaerobic conditions; Run #2.

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Table III. Examination of data consistency using integrated available electron and carbon balances for anaerobic growth of *Zymomonas mobilis* on glucose in batch cultures.

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Table IV. Point and 95% confidence interval estimates of the maintenance parameter, \( m_e \), and the biomass yield, \( n_{max} \), for Run #1.

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<th>Equation and Method</th>
<th>Form*</th>
<th>Maintenance Parameter</th>
<th>True Biomass Yield</th>
<th>Covariates Included</th>
<th>J</th>
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</table>

\( a \) L.R. refers to linear regression.

* Form I: Equations (3), (4), (5) as they are. Form II: Equations (3), (4), (5) were multiplied by \( u \).

For Form I, the eigenvalues are 8874.01 and 178.473 for \( z_2 \) and \( z_3 \), respectively; for Form II the eigenvalues are 48.9215 and 2.9577 for \( z_2 \) and \( z_3 \), respectively.
Table V. Point and 95% confidence interval estimates of the maintenance parameter, $m_e$, and the true biomass yield, $\eta_{\text{max}}$, for Run #2.

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<th>Equation and Method</th>
<th>Form*</th>
<th>Maintenance Parameter</th>
<th>True Biomass Yield</th>
<th>Covariates Included</th>
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</thead>
<tbody>
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<td></td>
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<td></td>
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<td>0.7389, 1.5579</td>
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<td>0.0451, 0.0911</td>
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<td>Eq. (5), L.R.</td>
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<tr>
<td>Technique</td>
<td></td>
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<td>0.0398, 0.0780</td>
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* L.R. refers to linear regression.

Form I: Equations (3), (4), (5) as they are. Form II: Equations (3), (4), (5) were multiplied by $\mu$.

For Form I, the eigenvalues are 612.834 and 1126.65 for $z_2$ and $z_3$, respectively; for Form II, the eigenvalues are 9.91749 and 4.91059 for $z_2$ and $z_3$ respectively.
### Table VI. Comparison of point and 95% confidence interval estimates of $m_e$, $\eta_{\text{max}}$, $\alpha_e$ and $\beta_e$ obtained from some literature data.

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<th>$m_e$ and $\alpha_e$ (hr$^{-1}$)</th>
<th>$\eta_{\text{max}}$</th>
<th>$\beta_e$</th>
<th>Microorganism</th>
<th>Substrate</th>
<th>Source of Data</th>
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<td>0.896[0.663, 1.130]</td>
<td>0.164[0.127, 0.231]</td>
<td>5.107[3.329, 6.874]</td>
<td>Saccharomyces cerevisiae NRRL, Y-132</td>
<td>Glucose</td>
<td>Ghose et al. [14]</td>
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<td>-0.006[-0.060, 0.047]</td>
<td>0.088[0.072, 0.113]</td>
<td>10.377[7.889, 13.870]</td>
<td>Saccharomyces cerevisiae CBS A26</td>
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<td>Dekkers et al. [15]</td>
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<td>1.970[1.410, 2.520]</td>
<td>0.037[0.031, 0.047]</td>
<td>26.027[20.277, 31.258]</td>
<td>Zymomonas Mobilis</td>
<td>Sucrose</td>
<td>Lee et al. [16]</td>
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<td>3.264[2.459, 4.069]</td>
<td>0.078[0.057, 0.122]</td>
<td>11.879[7.211, 16.548]</td>
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<td>Glucose</td>
<td>Lee et al. [16]</td>
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<td>3.694[3.061, 4.328]</td>
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<td>Lee et al. [16]</td>
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<td>4.645[4.033, 5.257]</td>
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<td>Lee et al. [16]</td>
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<td>4.112[3.685, 4.537]</td>
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<td>Lee et al. [16]</td>
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<td>3.9833[3.8793, 4.0873]</td>
<td>0.1713[0.1280, 0.2592]</td>
<td>4.8377[2.8580, 6.8125]</td>
<td>Zymomonas Mobilis ATCC-10988</td>
<td>Glucose</td>
<td>Run 1 (This work)</td>
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<tr>
<td>1.791[1.242, 2.339]</td>
<td>0.0835[0.059, 0.143]</td>
<td>10.976[5.998, 15.949]</td>
<td>Zymomonas Mobilis ATCC-10988</td>
<td>Glucose</td>
<td>Run 2 (This work)</td>
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INTRODUCTION

Many recent cases of toxic shock syndrome, a newly described disease, have been linked by statistical inference to the use of tampons, and specifically to tampons of high absorbance. One of these products, Procter & Gamble's Rely®, has received much attention, and was withdrawn from the market several years ago. However, to this point no explanation of the cause of toxic shock syndrome, either in general or associated specifically with tampons, has been accepted by the scientific community.

Recently, Tierno (1) and Hanna (2) have suggested that one absorbant in Rely tampons, a lightly crosslinked carboxymethylcellulose in the form of fibers and present in tampons as small chips, plays a role in the occurrence of toxic shock syndrome. According to them, this material, designated as CLD-2®, can be broken down by microbes found in the vagina during menstruation, and this process encourages the production of toxin by Staphylococcus aureus. Tierno (1) demonstrated one part of this chain by mixing loopfuls of each of a number of enteric microorganisms with 0.4 mL distilled water and adding four chips of CLD-2. The chips absorb the water and form a gel at the bottom of the test tube. In some cases an increase in mobility of the gel was noticed and attributed to degradation of the CLD-2 fibers by cellulase activity. Hanna (2) extended Tierno's experiments with many of the same species, but also with a number of new ones, by conducting the mobility test and in addition by measuring the concentration of reducing sugars, supposedly produced by microbial action, after incubation of the culture with CLD-2 chips. Finally, Tierno et al. (3) added 7.5 mg of CLD-2 chips to 0.5 mL of a solution of commercially obtained cellulase or β-glucosidase. They again attributed the resulting increase in mobility and reducing sugar to enzyme-catalyzed hydrolysis of the CLD-2 fibers.

There appear to be some difficulties with these results. Preliminary experimentation by Meagher (4) reproduced Tierno's mobility results (1) with some of his organisms but not with others. In general Meagher found that reducing sugar levels decreased during incubation, indicating that reducing sugars carried in with the microbial inoculum or with the CLD-2 was being metabolized during incubation to a greater extent than new reducing material was being formed. This suggested that Hanna's results (2) were also questionable. Meagher was unable to find any production of glucose or oligosaccharides by HPLC in his preliminary work.

Because of the importance of the issue and the lack of reproducibility of much of Tierno and Hanna's data, a more extensive investigation of hydrolysis of cellulosic materials by some of Tierno's strains was undertaken. This included culturing them on a standard microbiological growth medium to which CLD-2 or substances known to induce cellulase or β-glucosidase activity had been added. Following this, supernatant solutions as well as preparations of disrupted cells were assayed for activity on α-cellulose, carboxymethylcellulose, p-nitrophenyl-β-D-glucopyranoside, and cellobiose to determine whether any cellulase or
ß-glucosidase had been formed. The preliminary results of this study are presented in the pages that follow.

MATERIALS AND METHODS

Cultures

We received from Procter & Gamble eleven cultures of bacteria and two of yeast from thirteen different species, passed to them by Tierno, supposedly from among those he had tested for activity on CLD-2 (1). Of the species received by us, four had at least some cultures that were reported by Tierno to increase the mobility of gels formed by CLD-2 in water: Klebsiella oxytoca (2 positive, 0 negative cultures), Klebsiella pneumoniae (4 positive, 1 negative), Proteus mirabilis (1 positive, 1 negative), and Serratia marcescens (2 positive, 0 negative). Therefore, two of the cultures sent to us, representatives of Klebsiella oxytoca and Serratia marcescens, surely had activity in Tierno's hands. He reported that the other nine species, Acinetobacter anitratus, Bacillus subtilis, Candida albicans, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus faecium, Streptococcus G, and Torulopsis glabrata, had no cultures with activity. Hanna (2) tested six of these species, K. oxytoca, K. pneumoniae, P. mirabilis, S. marcescens, S. aureus, and S. epidermidis, though not necessarily the same cultures tested by Tierno, and found like Tierno that the first four had some strains that could increase mobility and/or reducing sugar.

The cultures were transferred to fresh slants, containing 3% trypticase soy broth (BBL 11768, Lot E3DKL1, containing 0.25% w/v glucose) in 1.7% Difco Bacto-Agar, every six weeks. The slants were incubated at 37°C for 24 h and then stored at 4°C.

Inoculum

One loopful of culture was added to 5 mL of 3% trypticase soy broth in 20 x 150 mm loosely capped culture tubes. The tubes were incubated at 37°C for 24 h and then used as inoculum for growth tests.

Growth Tests

In order to promote production of cellulases or ß-glucosidase, the cultures were grown in 3% trypticase soy broth alone or with one of six different substrates added. The latter included lactose, cellobiose, amorphous cellulose (Schleicher & Schuell 286), crystalline cellulose (Schleicher & Schuell 144) and carboxymethyl-cellulose (Sigma C-8758, Lot 42F-0438, low viscosity)(all 0.25% w/v), and (0.125% w/v) CLD-2 chips (degree of substitution between 0.63 and 0.92 per unit glucose).

Cultures were grown in 250 mL flasks containing 50 mL of substrate. A 10% inoculum was added. The flasks were capped with polyurethane sponges and incubated at 37°C with shaking at 100 rpm. Incubation was continued until growth had stopped, as indicated by a decrease or levelling off of optical density, which was checked every 12 h. In all cases glucose had decreased to undetectable levels well before the end of incubation. The flasks were then stored at 4°C.
Optical Density

Optical density of the broth during growth tests was measured on a Gilford Stasar II visible spectrophotometer with a 10 mm pathlength cuvette at 420 nm. Samples were diluted to keep the reading below 0.2.

Sample Preparation

The culture media were centrifuged in 50 mL centrifuge tubes on a Sorvall RC-5 high speed centrifuge at 12,000 rpm for 20 min at 4°C. The broth was separated and the cells were rinsed twice with 0.05 M phosphate buffer, pH 7.3. The cells were resuspended in 50 mL of the same phosphate buffer, and were then sonicated with a Heat Systems Cell Disrupter Model W-375 until at least a 20% decrease in optical density occurred, so that intracellular enzymes would be released.

Enzyme Assays

Cellulase and carboxymethylcellulase (CMCase) activities were assayed in a similar manner. In both assays, 200 μL of enzyme sample (broth or sonicated cells) were placed in 20 x 150 mm screw cap tubes and 1 mL of 0.1% (w/v) α-cellulose (Sigma C-8002, Lot 22F-0540) or CMC (as before) in phosphate buffer (pH 7.3) was added. The tubes were incubated at 37°C. Increases in reducing sugar from a 5 min to a 1 h sample was determined by the Somogyi-Nelson (5,6) method using a wavelength of 500 nm. One unit (1 IU) was the activity liberating 1 μmol of reducing sugar as glucose per min. The limit of detectability for these assays was 0.02 IU/mL.

β-Glucosidase activity was determined by two methods. The first used p-nitrophenyl-β-D-glucopyranoside (PNGP) as a substrate. A 1 mL sample of the enzyme solution was added to 1 mL of 4 mM PNGP dissolved in phosphate buffer and incubated at 37°C for 3.0 h. The reaction was stopped by addition of 2 mL of 20% (w/v) Na2CO3. The activity present was determined by measuring absorbance of released p-nitrophenol at 400 nm. Net absorbance was that of the reaction sample minus that of a blank sample taken immediately after addition of substrate. One unit (1 IU) was the activity liberating 1 μmol/min of p-nitrophenol under these conditions. The limit of detectability of this assay was 9 x 10⁻⁶ IU/mL.

The second method to determine β-glucosidase activity was to use cellobiose as a substrate and analyze for glucose using a Beckman Model ERA-1002 glucose analyzer. In it, 1 mL of enzyme solution and 1 mL of 1% (w/v) cellobiose in phosphate buffer were incubated at 37°C for 3.0 h. One unit (1 IU) was the activity liberating 1 μmol/min of glucose; the detectability limit was 1.85 x 10⁻³ IU/mL.

RESULTS

Two of the 13 cultures tested, A. anitratus and P. mirabilis, had activity on α-cellulose. They and one other, K. oxytoca, were active on CMC (Table 1). No particular substrate of the six tested was a marked inducer; however, it appears that CLD-2 may be a repressor, as no culture grown with it was active on α-cellulose, and only K. oxytoca and P. mirabilis attacked CMC. No detectible activity was present in the sonicated cell preparations of any culture.
All 13 cultures were grown on lactose, cellobiose, and CLD-2 and assayed for β-glucosidase using PNGP and cellobiose as substrates. All but three species, *P. mirabilis*, *S. agalactiae*, and *S. faecium*, attacked PNGP, and all but these three and *Streptococcus G* were active on cellobiose, usually at much higher levels (Table 2). Again CLD-2 appeared to be a repressor, as only five cultures grown on it attacked PNGP, and none exceeded the detectability limit of activity on cellobiose. Most of the β-glucosidase activity was present in the cells and was released by sonication, though appreciable activity was found in the broth, presumably mainly liberated upon cell lysis. Table 2 presents highest activities found in either broth or cell suspensions; the source of the data is noted.

Optical density measurements may be used to buttress the enzyme assay results. Increased values over those with trypase soy broth were found with many of the organisms grown on lactose and cellobiose and some grown on CLD-2 that had β-glucosidase activity. Little increase in optical density was observed with cultures on amorphous or crystalline cellulose or on CMC. In addition, it appears that many cultures with β-glucosidase activity grown on lactose and cellobiose had lower reducing sugar levels at the end of the fermentation.

**DISCUSSION**

The results presented here in general do not confirm those of Tierno (1) and Hanna (2). Of the four organisms they reported as being able to break down CLD-2, only two, *P. mirabilis* and *K. oxytoca*, have cellulase activity. A third species, *A. anitratus*, in which Tierno could not find activity, in fact does produce a cellulase.

Ten of the thirteen species tested here can produce a β-glucosidase. However, in many cases this enzyme does not appear if CLD-2 is a substrate.

At least one endo-cellulase and a β-glucosidase are necessary to degrade CLD-2 or other cellulosics to monomeric units. Just two species, *K. oxytoca* and *A. anitratus* of the thirteen investigated in this work can do this by themselves, and neither they nor any other species, including *S. aureus*, is active on cellobiose, a major cellulase product, if grown on media containing CLD-2. In addition, as pointed out by Reese et al. (7), one does not expect β-glucosidase by itself to be capable of attacking long-chain cellulosic molecules such as those found in CLD-2, as stated by Tierno et al. (3). Therefore, the results presented here do not confirm the claims of Tierno and Hanna that enteric bacteria found in the vagina are able to break down CLD-2 to nourish *S. aureus*.

An alternate explanation for their observation of a decrease of viscosity of CLD-2 gels in the presence of some microbial cultures is that these cultures increase the ionic strength of the medium, causing CLD-2 fibers to dehydrate and decrease in size. Such an effect has been noted by Grignon and Scallan (8) with this material.

**ACKNOWLEDGMENT**

The author is grateful for the help of Mark Kieras with the experimental work. This project was supported by Dinsmore & Shohl, Cincinnati, Ohio, and by the Engineering Research Institute, Iowa State University.
REFERENCES

4. M. M. Meagher, Iowa State University, unpublished research.
Table 1. Activity of bacterial and yeast strains on α-cellulose.

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<th>Cellobiose</th>
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<th>Crystalline cellulose</th>
<th>CMC</th>
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Table 2. Activity of bacterial and yeast strains on carboxymethylcellulose.

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<th>Lactose</th>
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Table 3. Activity of bacterial and yeast strains on p-nitrophenyl-β-D-glucopyranoside (PNPG) and cellobiose.

<table>
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<th>Added substrate</th>
<th>Activity on PNPG, IU/mL, $\times 10^5$</th>
<th>Activity on cellobiose, IU/mL, $\times 10^3$</th>
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<td>9.7</td>
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<tr>
<td>Bacillus subtilis</td>
<td>4.0</td>
<td>15</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>100</td>
<td>450*</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>28</td>
<td>19</td>
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<tr>
<td>Klebsiella pneumoniae</td>
<td>7.2</td>
<td>-</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
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<td>-</td>
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<tr>
<td>Serratia marcescens</td>
<td>2.0</td>
<td>11</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>5.8</td>
<td>4.7</td>
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<tr>
<td>Staphylococcus epidermidis</td>
<td>1.1</td>
<td>71*</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
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<td>-</td>
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<tr>
<td>Streptococcus faecium</td>
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<td>-</td>
</tr>
<tr>
<td>Streptococcus G</td>
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<td>14</td>
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<tr>
<td>Torulopsis glabrata</td>
<td>41</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*Supernatant values. All others are of sonicated cell preparations.
THE CELULASE SYSTEM OF CHAETOMIUM CELLULOLYTICUM

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

Introduction

There is a strong ongoing interest in cellulases due to the worldwide effort to utilize lignocellulosic materials, a renewable resource, as a source of food and fuel. Cellulases of a large number of microorganisms have been studied(1). However, even now one of the critical areas of research(2) is the search for new strains and the study of their cellulase enzyme systems.

Cellulases reportedly(2) have low activities and the extent of conversion of the substrate is also usually low. The major reasons attributed to this are:
1. Celluloses are insoluble and recalcitrant to hydrolysis.
2. Synergistic action of cellulases is needed for hydrolysis.
3. Enzyme inactivation
4. Product inhibition
5. Increasing resistance of the residual substrate.

Chaetomium cellulolyticum is a thermotolerant cellulolytic fungus which has been shown to be more optimal for protein production than Trichoderma reesei(3). It has also shown one of the highest rates of protein production among cellulolytic microorganisms(4). Its growth on various substrates for production of single cell protein has been studied by several groups(5-9).

This paper will report on the growth of C. cellulolyticum on glucose and lactose, as well as growth and enzyme production on Avicel PH 102 (FMC Corp., Philadelphia, Penn.), a purified microcrystalline cellulose. It will also discuss the denaturation kinetics of the cellulases of C. cellulolyticum, in order to get a better understanding of the role of enzyme inactivation in decreasing the rate and extent of cellulose hydrolysis.

Materials and Methods

Fermentation Conditions: Shake flask fermentations were carried out in 500 ml flasks using a working volume of 50 ml. During these fermentations, the flasks were placed in a rotary incubator operated at 37 C and 250 rpm. Larger scale fermentations were carried out in 7 or 14 liter fermenters (5 or 10 liter working volume). The fermenters were maintained at 37 C and were mechanically agitated at 250 rpm. In all cases Mandels and Weber medium(10) was used to provide supplemental nutrients. The shake flask fermentations were buffered at pH 4.8 using 0.05M citrate buffer. The large scale fermentations were unbuffered; however, the pH of the medium in this case was automatically controlled at 4.8 by a Fermentation Design (Allentown, Pa.) model PH-1001 pH controller. Carbon in the form of glucose, lactose or Avicel PH 102 was added at 1% (w/v) as substrate.
Inoculum Preparation: A culture of *C. cellulolyticum* (ATCC 32319) was routinely maintained on Rabbit Food Agar slants. A spore suspension washed from the slants was serially cultured two times in shake flask fermentations using glucose as the carbon source. After 48 hours, mycelium from the first culture was separated by centrifugation. The mycelium was then resuspended in sterile distilled water and aseptically broken up in a blender. This suspension containing approximately 0.5% dry weight of mycelium was used on a 20% volume basis to inoculate a second batch of shake flasks. Mycelium from the exponential growth phase of the second culture was separated by centrifugation. The mycelium was washed three times with sterile distilled water, broken up into small hyphal bits using a blender, and resuspended in distilled water. Aliquots of this mycelial suspension, which contained approximately 0.5% dry weight mycelium, were used on a 4% (v/v) basis to inoculate the fermentations.

Growth and Enzyme Production Studies: These were carried out in shake flask fermentations with glucose, lactose, and Avicel as the substrates. One shake flask was removed approximately every 8 hours. It was cooled quickly and stored at −5°C. After thawing, the contents of the flask were centrifuged and the solids separated from the broth. Assays were run on the broth to determine the filter paper activity and the aryl β-glucosidase activity.

The solid fraction was washed with distilled water and resuspended in 25 ml distilled water. 5 ml of this suspension was transferred into an aluminum boat and dried for 24 hours at 80°C to determine the mycelial dry weight. Another 5 ml of the suspension was used to find the nitrogen content of the cell mass by the micro-Kjeldahl method (11). In the case of Avicel fermentations, the mycelial dry weight was found by determining the nitrogen content of the solid sample by the micro-Kjeldahl method and then referring to a standard calibration graph of mycelial dry weight versus nitrogen content.

Enzyme Denaturation Studies: The enzyme used in these studies was obtained from large scale fermentations of Avicel with *C. cellulolyticum* lasting approximately 96 hours. The broth from these fermentations was filtered through glass wool and then concentrated by ultrafiltering with a UM-10 membrane (Amicon Corp., Lexington, Mass.) The concentrated enzyme was diluted to a final activity of 1.0 Filter Paper Unit (FPU) per ml with 0.05 M citrate buffer (pH 4.8) and stored at 4°C.

Test tubes containing 1 ml of this enzyme were maintained at the appropriate temperature either by refrigeration (4°C and 15°C) or by setting them in a hot water bath. In the case of 4 and 15°C, one test tube was removed every 24 hours and immediately cooled with ice and stored at −5°C. At higher temperatures a test tube containing the enzyme solution was removed every 60 minutes, cooled, and stored at −5°C. Filter paper assays were done on the samples after thawing.

Filter Paper Activity: A 1 ml sample of supernatant was incubated with 1 ml of 0.05M citrate buffer (pH 4.8) and 50 mg of Whatman no. 1 filter paper for 60 minutes at 50°C. At the end of this period, the reducing sugar content was assayed by the dinitrosalicylic acid method (12) using glucose as the standard. A unit of enzyme activity was defined as 1 mg/ml of reducing sugars formed as glucose equivalent under these conditions.
Aryl β-glucosidase activity: A 0.5 ml sample of supernatent was incubated with 0.5 ml of 2 mM p-nitrophenol-β-D-glucopyranoside for 30 minutes at 50 C. The reaction was stopped by addition of 2 ml of 1 M Na₂CO₃. The solution was assayed for p-nitrophenol concentration by reading the absorbance of the solution at 412 nm. The aryl β-glucosidase activity was defined as moles per ml per minute of p-nitrophenol released under these conditions.

Results and Conclusions

The results of the shake flask fermentations are shown in Figures 1 and 2. A maximum cell mass yield of 60% (w/w) was obtained after 25 hours when the microorganism was grown on glucose. With Avicel as the substrate, a lag phase of about 8 hours was observed. In this case, the maximum cell mass yield of 45% (w/w) did not occur until 90 hours after the start of the fermentation. As shown in Figure 2, C. cellulolyticum produces relatively low levels of filter paper activity. However, it does produce much higher levels of aryl β-glucosidase activity than Trichoderma reesei QM 6a, QM 9414 and RUT C-30 (13,14,15).

The thermal decay of enzyme activity was studied over the temperature range of 4 to 60 C. Over a ten day period, the filter paper activity showed no decay at 4 C and 15 C. The decay over a 10 hour period at temperatures ranging from 37 to 60 C is shown in Figure 3. These data were modelled as a first order process:

\[ E = E_e \exp(-kT) \]

where \( E \) is the enzyme activity (FFU/ml) at time \( t \) (hours), \( E_e \) is the initial enzyme activity and \( k \) is the first order deactivation rate constant (hours⁻¹). The lines drawn in Figure 3 represent the best fits of the model to the data. Table 1 gives the \( k \) values obtained at various temperatures. Figure 4 shows an Arrhenius plot, \( \ln k \) versus \( 1/T(K) \), of these values. From this plot, an activation energy of 37.0 kcal was determined for the enzyme deactivation process.

<table>
<thead>
<tr>
<th>T (C)</th>
<th>( k ) (hours⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>0.013</td>
</tr>
<tr>
<td>50</td>
<td>0.0326</td>
</tr>
<tr>
<td>55</td>
<td>0.0871</td>
</tr>
<tr>
<td>60</td>
<td>0.1780</td>
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References


FIGURE 1. Growth of *C. cellulolyticum* in shake flask fermentation with various substrates.
FIGURE 2. Development of extracellular enzyme activity (units per ml) during shake flask fermentation of C. cellulolyticum with Avicel as the substrate.
FIGURE 3. Decay of cellulase activity at various temperatures. Enzyme was obtained from submerged culture of *C. cellulolyticum* on Avicel.
FIGURE 4. Arrhenius plot of relationship between first order rate constant for enzyme decay $k$ and absolute temperature $T$. 
DNA MEASUREMENT AS A TOOL FOR ESTIMATING BIOMASS CONCENTRATION IN THE PRESENCE OF INTERFERING SOLIDS

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Manhattan, Kansas 66506

INTRODUCTION

The estimation of biomass concentration in complex media which contain solids presents considerable problems at the industrial level [1]. The measurement of biomass concentration is needed for modelling of growth and parameter estimation [2-11].

In this work DNA analysis on the total culture was used to estimate the biomass concentration in the presence of solids. In preparing the media, unfiltered glucose obtained by enzymatic hydrolysis of corn dust was used.

The data collected were analyzed using a multivariate statistical procedure called covariate adjustment. A comparison between the true biomass energetic yields and maintenance coefficients for the growth of Candida utilis on filtered and unfiltered glucose was made [11].

THEORY

The reparameterized forms of Pirt's model have been used by Erickson and coworkers [2-9]. These equations may be written in the following forms.

Form I.

\[ x_1 = \frac{\mu}{\eta} = m_e + \frac{\mu}{\eta_{\text{max}}} + \varepsilon_1 \]  
\[ x_2 = \frac{\mu}{\eta} (\eta + \varepsilon) = m_e + \frac{\mu}{\eta_{\text{max}}} + \varepsilon_2 \]  
\[ x_3 = \frac{\mu}{\eta} (\gamma_c + d) = m_e + \frac{\mu}{\eta_{\text{max}}} + \varepsilon_3 \]  
\[ x_4 = \frac{\mu}{\eta_{\text{N}_2}} = m_e + \frac{\mu}{\eta_{\text{max}}} + \varepsilon_4 \]
Form II of the equations is obtained from Form I by dividing every term by the specific growth rate $\mu$. In the equations, $\mu$ is the specific growth rate, $\eta$ is biomass energetic yield using direct or indirect biomass measurements, and $\eta_{N_2}$ is biomass energetic yield using nitrogen measurements; $\epsilon$, $y_c$ and $d$ are, respectively, the fractions of available electrons transferred to oxygen, substrate carbon incorporated into biomass and substrate carbon evolved as carbon dioxide; $m_e$ is the maintenance coefficient; $\eta_{\text{max}}$ is the true biomass energetic yield; and $\epsilon_i$ ($i = 1, \ldots, 4$) are the random errors associated with models (1-4). From the equations, eight different least square estimates of the parameters are possible and two combined point and interval estimates can be obtained, the first used Form I of equations (1-4) while the second uses Form II of equations (1-4). The multivariate statistical procedure used in the analysis is called the covariate adjustment technique [7, 8, 9].

Consider equations (1-4) for microbial growth. We may use the model

$$\bar{x}_i = \frac{1}{4} \sum_{k=1}^{4} x_{ki} = \frac{1}{\eta_{\text{max}}} \mu_i + m_e + \text{error}$$

(i = 1, 2, \ldots, N) \hspace{1cm} (5)

to estimate the unknown parameters but then the information contained in $x_{1i}$, \ldots, $x_{4i}$ may not be efficiently utilized. Hence, an appropriately chosen set of covariates $z_{1i}$, \ldots, $z_{ci}$ ($0 \leq c \leq 3$) may be used in model (5). These covariates have zero means and are linear functions of $x_{1i}$, \ldots, $x_{4i}$.

This yields the model

$$\bar{x}_i = \frac{1}{\eta_{\text{max}}} \mu_i + m_e + \sum_{j=1}^{c} a_j z_{ji} + \text{error}$$

(6)

For more details of the procedure see Solomon et al. [7, 9], Yang et al. [8] and Solomon [10].

**MATERIALS AND METHODS**

**Fermentor**

The bench-scale fermentor, model 19, produced by New Brunswick Scientific Co. was used. The operating temperature, pH, foam control, feeding of fermentor, continuous monitoring of the exit $O_2$ and $CO_2$ percentages were as described in the earlier batch/fed-batch work [9].

**Corn Dust**

The corn dust used was supplied by the U.S.D.A. Grain Marketing Research Laboratory, Manhattan, Kansas 66502. The sample used was obtained from 786-43 class I corn dust which was a corn cyclone tailing dust from dust bin collected June 23, 1978.

**Organism and Cultivation Methods**

*Candida utilis* ATCC - 1084 was used throughout this study. The hydrolysis of the corn dust used in the experiment and the growth media
preparations are as described by Solomon et al. [4]. The batch/fed-batch operations for the growth on unfiltered glucose is identical to that employed in an earlier work [9].

**Analytical Methods**

For detail of analytical procedures see Solomon et al. [11].

As described in earlier work [9], two methods were used to obtain the specific growth rate (Methods I and II); the specific rates of glucose and nitrogen consumption were determined as described earlier [9].

From the indirect measurements of biomass concentration, \( X_{DNA} \) (g/1), oxygen consumption rate, \( Q_{O_2} \) (moles \( O_2 \)/g biomass h), carbon dioxide evolution rate, \( Q_{CO_2} \) (mole \( CO_2 \)/g biomass h), substrate consumption rate, \( Q_s \) (g substrate/g biomass h), and nitrogen consumption rate \( Q_{N_2} \) (g nitrogen/g biomass h), the calculations of the values of \( \eta, \varepsilon, \gamma_c, d \), and \( \eta_{N_2} \) were carried out as discussed in earlier studies [4, 9].

**RESULTS AND DISCUSSIONS**

Tables (1 - 4) contain the estimates of the true biomass energetic yield, \( \eta_{max} \), and maintenance parameter, \( m_e \), from the analysis of data set 1-20-83 when \( \mu \) was obtained by Methods I and II and for Forms I and II of equations (1 - 4). In all of the analysis the average values for \( \sigma_b, \gamma_b \) and \( Q \) were used [9, 10].

The first four estimates shown on each of the tables are the least square estimates using substrate and biomass (DNA) data, oxygen and biomass data, carbon dioxide and biomass data and nitrogen, biomass and substrate data respectively. Due to measurement errors, different parameter estimates are obtained. The next four estimates are obtained using equations (6). These estimates are combined estimates using all the measured data. As discussed in earlier works [9, 10], the magnitude of the interval estimates, the correlation coefficient, \( \rho \), \( \xi \), and \( \sigma^{2/4}/(n-2) \) (n-1) can be examined to choose the best combined estimates.

Contained in table 5 are the best combined point and 95% confidence intervals estimates for the growth of *Candida utilis* from Tables 1 - 4. For data set 1-20-83, the only biomass estimate used is from DNA measurement. These results may be compared to earlier work where *Candida utilis* was grown on glucose and both cell dry weight and DNA were measured. A t-test of the biomass energetic yield, \( \eta_{max} \), showed no significant difference between estimates obtained for growth on filtered and unfiltered glucose; however, generally the maintenance coefficients for growth in the presence of solids are significantly higher than those for growth on filtered glucose. Average values of \( \eta_{max} = 0.617 \) and \( m_e = 0.088 \) hr\(^{-1} \) are obtained for growth in the presence of solids. These values may be compared to the average values of \( \eta_{max} = 0.633 \) and \( m_e = 0.027 \) for growth.
on filtered glucose [11]. The increased value of maintenance coefficient may be due to obstructions caused by the solids to the diffusional processes from the liquid medium to the biomass surface.

CONCLUSIONS

Based on the consistency of the data and the values of the estimated true biomass energetic yield, $\eta_{\text{max}}$, DNA measurement has been shown to be a good way to determine biomass concentration in complex media which contain solids.

The multivariate statistical procedure (covariate adjustment) employed in the analysis of the data is an efficient method which is easy to program using the SAS packages [12].

ACKNOWLEDGEMENT

This work was partly supported by NSF Grants CPE 79-18202 and CPE 81-20039.

REFERENCES


Table 1. Data Set 1-20-83. Estimates of True Biomass Energetic Yield and Maintenance Parameters for Batch/Fed-Batch Growth of *Candida utilis* on Glucose.†

<table>
<thead>
<tr>
<th>Data</th>
<th>Covariates Included</th>
<th>( \eta_{\text{max}} )</th>
<th>( m_e ) hr(^{-1} )</th>
<th>( \rho^2 ) (n-r-c-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Point Interval</td>
<td>Point Interval</td>
<td></td>
</tr>
<tr>
<td>( Q_S, \mu )</td>
<td>--</td>
<td>0.628 (0.517, 0.795)</td>
<td>0.127 (0.040, 0.214)</td>
<td>--</td>
</tr>
<tr>
<td>( Q_{O_2}, \mu )</td>
<td>--</td>
<td>0.655 (0.605, 0.715)</td>
<td>0.091 (0.059, 0.123)</td>
<td>--</td>
</tr>
<tr>
<td>( Q_{CO_2}, \mu )</td>
<td>--</td>
<td>0.790 (0.713, 0.887)</td>
<td>0.094 (0.059, 0.129)</td>
<td>--</td>
</tr>
<tr>
<td>( Q_{N_2}, \mu )</td>
<td>--</td>
<td>0.354 (0.293, 0.449)</td>
<td>0.017 (-0.137, 0.171)</td>
<td>--</td>
</tr>
<tr>
<td>( Q_S, Q_{O_2}, Q_{CO_2}, Q_{N_2}, z_1, z_2, z_3 )</td>
<td></td>
<td>0.566 (0.476, 0.697)</td>
<td>0.078 (0.048, 0.111)</td>
<td>6.471 * 10(^{-5} )</td>
</tr>
<tr>
<td>( Q_S, Q_{O_2}, Q_{CO_2}, Q_{N_2}, z_1, z_2 )</td>
<td></td>
<td>0.661 (0.591, 0.748)</td>
<td>0.086 (0.052, 0.120)</td>
<td>6.94 * 10(^{-5} )</td>
</tr>
<tr>
<td>( Q_S, Q_{O_2}, Q_{CO_2}, Q_{N_2}, z_1 )</td>
<td></td>
<td>0.686 (0.603, 0.795)</td>
<td>0.100 (0.063, 0.137)</td>
<td>8.852 * 10(^{-5} )</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>0.555 (0.498, 0.627)</td>
<td>0.082 (0.029, 0.135)</td>
<td>1.818 * 10(^{-4} )</td>
</tr>
</tbody>
</table>

† Method I of obtaining \( \eta \), Form I of equations and DNA measurement used in analysis.

\( \rho_{x,z_1} = -0.817, \quad \rho_{x,z_2} = -0.331, \quad \rho_{x,z_3} = -0.331, \quad \rho_{z_1z_2} = 0.886, \quad \rho_{z_1z_3} = 0.356, \quad \rho_{z_2z_3} = -0.674, \quad \rho_{z_2z_3} = -0.333 \)
Table 2. Data Set 1-20-83. Estimates of True Biomass Energetic Yield and Maintenance Parameters for Batch/Fed-Batch Growth of Candida utilis on Glucose.†

<table>
<thead>
<tr>
<th>Data</th>
<th>Covariates Included</th>
<th>$\eta_{\text{max}}$</th>
<th>$m_e$, $\text{hr}^{-1}$</th>
<th>$\hat{\sigma}^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_S$, $\mu$</td>
<td>--</td>
<td>0.739 (0.585, 1.004)</td>
<td>0.166 (0.129, 0.202)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{O_2}$, $\mu$</td>
<td>--</td>
<td>0.759 (0.632, 0.950)</td>
<td>0.124 (0.097, 0.151)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{CO_2}$, $\mu$</td>
<td>--</td>
<td>0.945 (0.757, 1.258)</td>
<td>0.128 (0.101, 0.155)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{N_2}$, $\mu$</td>
<td>--</td>
<td>0.317 (0.277, 0.373)</td>
<td>-0.038 (-0.086, 0.011)</td>
<td>--</td>
</tr>
<tr>
<td>$b_{Q_S}$, $Q_{O_2}$, $Q_{CO_2}$, $Q_{N_2}$, $z_1$, $z_2$, $z_3$, $\mu$</td>
<td>$z_1$, $z_2$, $z_3$</td>
<td>0.421 (0.330, 0.581)</td>
<td>0.050 (0.010, 0.090)</td>
<td>3.504 * $10^{-3}$</td>
</tr>
<tr>
<td>$b_{Q_S}$, $Q_{O_2}$, $Q_{CO_2}$, $Q_{N_2}$, $z_1$, $z_2$</td>
<td>$z_1$, $z_2$</td>
<td>0.557 (0.473, 0.676)</td>
<td>0.076 (0.046, 0.109)</td>
<td>3.883 * $10^{-3}$</td>
</tr>
<tr>
<td>$b_{Q_S}$, $Q_{O_2}$, $Q_{CO_2}$, $Q_{N_2}$, $z_1$</td>
<td>$z_1$</td>
<td>0.550 (0.466, 0.670)</td>
<td>0.086 (0.055, 0.117)</td>
<td>3.939 * $10^{-3}$</td>
</tr>
<tr>
<td>$b_{Q_S}$, $Q_{O_2}$, $Q_{CO_2}$, $Q_{N_2}$</td>
<td>--</td>
<td>0.582 (0.525, 0.652)</td>
<td>0.095 (0.076, 0.124)</td>
<td>3.674 * $10^{-3}$</td>
</tr>
</tbody>
</table>

† Method I of obtaining $\mu$, Form II of equations and DNA measurement used in analysis.

$\rho_{x.z_1} = -0.757$, $\rho_{x.z_2} = 0.611$, $\rho_{x.z_3} = -0.792$, $\rho_{z_1z_2} = -0.416$, $\rho_{z_1z_3} = 0.599$, $\rho_{z_2z_3} = -0.3023$
Table 3. Data Set 1-20-83. Estimates of True Biomass Energetic Yield and Maintenance Parameters for Batch/Fed-Batch Growth of *Candida utilis* on Glucose.†

<table>
<thead>
<tr>
<th>Data</th>
<th>Covariates Included</th>
<th>$\eta_{\text{max}}$</th>
<th>$m_e$, hr⁻¹</th>
<th>$\sigma^2$ (n-r-c-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_S, \mu$</td>
<td>--</td>
<td>0.621 (0.488, 0.855)</td>
<td>0.118 (0.006, 0.230)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{O_2}, \mu$</td>
<td>--</td>
<td>0.645 (0.590, 0.711)</td>
<td>0.084 (0.047, 0.121)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{CO_2}, \mu$</td>
<td>--</td>
<td>0.786 (0.701, 0.894)</td>
<td>0.092 (0.053, 0.131)</td>
<td>--</td>
</tr>
<tr>
<td>$Q_{N_2}, \mu$</td>
<td>--</td>
<td>0.348 (0.283, 0.446)</td>
<td>0.0061 (-0.160, 0.173)</td>
<td>--</td>
</tr>
<tr>
<td>$bQ_S, Q_{O_2}, Q_{CO_2}, Q_{N_2}, z_1, z_2, z_3$</td>
<td>$z_1$, $z_2$</td>
<td>0.519 (0.448, 0.615)</td>
<td>0.062 (0.031, 0.093)</td>
<td>5.076 * 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>$z_1$</td>
<td>0.636 (0.573, 0.716)</td>
<td>0.076 (0.042, 0.110)</td>
<td>6.59 * 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>$z_3$</td>
<td>0.640 (0.551, 0.764)</td>
<td>0.091 (0.043, 0.139)</td>
<td>1.316 * 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>0.546 (0.487, 0.621)</td>
<td>0.075 (-0.019, 0.131)</td>
<td>1.82 * 10⁻⁴</td>
</tr>
</tbody>
</table>

† Method II of obtaining $\mu$, Form I of equations and DNA measurement used in analysis.

$b \rho_{x.z_1} = -0.772, \quad \rho_{x.z_2} = -0.0125, \quad \rho_{x.z_3} = -0.8775, \quad \rho_{z_1z_2} = 0.196, \quad \rho_{z_1z_3} = 0.650, \quad \rho_{z_2z_3} = 0.0771$
Table 4. Data Set 1-20-83. Estimates of True Biomass Energetic Yield and Maintenance Parameters for Batch/Fed-Batch Growth of Candida utilis on Glucose.†

<table>
<thead>
<tr>
<th>Data</th>
<th>Covariates Included</th>
<th>$n_{\text{max}}$</th>
<th>$m_{e \text{, hr}^{-1}}$</th>
<th>$\sigma^2$</th>
<th>(n-r-c-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_s$, $\mu$</td>
<td>--</td>
<td>0.759 (0.597, 1.043)</td>
<td>0.166 (0.128, 0.205)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>$Q_{O_2}$, $\mu$</td>
<td>--</td>
<td>0.772 (0.639, 0.975)</td>
<td>0.125 (0.096, 0.154)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>$Q_{CO_2}$, $\mu$</td>
<td>--</td>
<td>0.958 (0.763, 1.286)</td>
<td>0.128 (0.100, 0.156)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>$Q_{N_2}$, $\mu$</td>
<td>--</td>
<td>0.317 (0.277, 0.372)</td>
<td>-0.043 (0.093, 0.007)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>$b_{Q_s}$, $Q_{O_2}$, $Q_{CO_2}$, $Q_{N_2}$, $z_1$, $z_2$, $z_3$</td>
<td>$z_1$, $z_2$, $z_3$</td>
<td>0.452 (0.342, 0.667)</td>
<td>0.058 (0.013, 0.103)</td>
<td>4.707 $\times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>$\mu$</td>
<td>$z_1$, $z_2$</td>
<td>0.564 (0.470, 0.703)</td>
<td>0.077 (0.040, 0.114)</td>
<td>4.717 $\times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>$z_1$</td>
<td></td>
<td>0.555 (0.464, 0.690)</td>
<td>0.085 (0.050, 0.120)</td>
<td>4.595 $\times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>--</td>
<td></td>
<td>0.588 (0.526, 0.665)</td>
<td>0.094 (0.074, 0.115)</td>
<td>4.250 $\times 10^{-3}$</td>
<td></td>
</tr>
</tbody>
</table>

† Method II of obtaining, Form II of equations and DNA measurement used in analysis.

$b_{Q_s} = -0.751, b_{Q_{O_2}} = 0.545, b_{Q_{CO_2}} = -0.751, b_{Q_{N_2}} = -0.378, b_{z_1} = 0.588, b_{z_2} = -0.265$
Table 5. Data set 1-20-83. Estimates of true biomass energetic yield and maintenance parameters for the growth of *Candida utilis* on unfiltered glucose from corn dust using DNA as a measure of biomass.

<table>
<thead>
<tr>
<th>Data</th>
<th>Method of obtaining $\mu$</th>
<th>Form of equation</th>
<th>$n_{\text{max}}$</th>
<th>$m_g (\text{hr}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Point Interval</td>
<td>Point Interval</td>
</tr>
<tr>
<td>$Q_S, Q_{O_2}, Q_{CO_2}, Q_{N_2}, \mu$</td>
<td>I</td>
<td>I</td>
<td>0.661 [0.591, 0.748]</td>
<td>0.086 [0.052, 0.120]</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>0.582 [0.525, 0.652]</td>
<td>0.095 [0.076, 0.114]</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>I</td>
<td>0.636 [0.573, 0.716]</td>
<td>0.076 [0.042, 0.110]</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>II</td>
<td>0.588 [0.526, 0.665]</td>
<td>0.094 [0.074, 0.115]</td>
</tr>
</tbody>
</table>
THE EFFECT OF CELLULOSE CRYSTALLINITY ON ENZYMATIC HYDROLYSIS

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

INTRODUCTION

It is well known that only a small fraction of the annual growth of cellulosic materials is being utilized. Since cellulose may be considered as the most abundant renewable natural resource available for the obtaining of food, fuel, and chemicals, and because of the high demand for these products, the efficient utilization of cellulosic materials is essential. In particular, economical hydrolysis of cellulose is a major goal of research due to its central role in the cellulose conversion processes, as stated by Humphrey (1).

There are two principal deterrents to the cellulose hydrolysis process: the insolubility of the lignocarbohydrate complex and the highly crystalline nature of cellulose. Therefore, several chemical, physical, and biological pretreatments have been developed not only to disrupt the lignin-hemicellulose cellulose complex, but also to reduce cellulose crystallinity in order to increase the rate and extent of cellulose hydrolysis.

The peculiar characteristics of cellulose structure make this natural fibrous, highly hygroscopic and crystalline polysaccharide a difficult substrate to degrade. The influence of cellulose structure is much more significant for enzymatic hydrolysis than for acid hydrolysis, probably due to the different sizes and characteristics of the active agents: the large enzyme complex acting very specifically during enzymatic hydrolysis compared with the small hydrogen ion cleaving acetal bonds of any kind during acid hydrolysis (2). Therefore, for enzymatic cellulose hydrolysis, the rate and extent of hydrolysis not only depends on the structure of the substrate, but also on the structure and the mode of cellulase action which is extremely complex and not fully understood (3). Another problem during enzymatic hydrolysis is the slow diffusion of the products such as cellobiose and glucose from the reaction site that produce high local concentration with consequent enzyme inhibition (4,5).

Among all the factors which affect the rate of enzymatic hydrolysis, the surface area and crystallinity of cellulose have been considered the most important. However, some authors have claimed that the rate of enzymatic hydrolysis depends primarily on the degree of crystallinity of cellulose structure (6,7). It has been amply proved that as crystallinity decreases cellulose becomes more accessible to enzymatic attack (6,7,8,9,10), as is also deduced from our results.
Studying the crystalline structure of the cellulose residues after acid hydrolysis, several authors showed that during acid hydrolysis there is a partial conversion of amorphous cellulose into crystalline cellulose, a process which is called recrystallization (11,12,13,14). We found a similar behavior for enzymatic hydrolysis of different cellulose materials.

MATERIALS AND METHODS

Cellulose materials from three different sources were used:

- Cotton linter pulp sample (SR-210 from Buckeye Cellulose Corp.) with 99% alpha cellulose content and DP=2,400.
- Foley fluffs wood pulp (SR P-2774 from Buckeye Cellulose Corp.), a highly purified Kraft pulp from Southern slash pine, with 91% alpha cellulose content and DP=1,850.
- Southern pine Kraft pulp (SR WP-2894 from Buckeye Cellulose Corp.) with 97.7% alpha cellulose content and DP=1,500-1,620.

Cellulose samples with different low degree of crystallinity were prepared by a physical mechanical procedure: Wiley milling followed by ball milling treatment for diverse periods of time.

In order to estimate the crystalline-amorphous ratio of all cellulose materials prepared, duplicate x-ray diffractograms from each of the samples were taken using a General Electric XRD-6 diffractometer. The x-ray diffractograms from all the samples were analyzed by using the procedure of Segal and others (15).

Enzymatic Hydrolysis of Cellulose. Three samples with different crystallinity indices were selected from each of the three groups of decrystallized cellulose materials obtained. Duplicate samples of each of the nine selected cellulosics were subjected to enzyme hydrolysis. About 0.5 g of each cellulose sample was accurately weighed, immersed in 10 ml cellulase-cellobiase enzyme solution at pH=4.8, and incubated at 50°C with shaking. The enzyme solution used was prepared by dissolving 3 g cellulase (NOVO SP122) and 1.5 g cellobiase (NOVO 250 L) in 100 ml 0.1 M citrate buffer.

Buffer Treatment of Cellulose. Duplicate samples of each of the same nine cellulose materials selected for enzyme hydrolysis were immersed in 0.1 M citrate buffer solution, pH=4.8, and shaken at 50°C during 48 hrs.
Sugar Yield Determination. Total reducing sugar yields were determined at different times of hydrolysis by the dinitrosalicylic acid (DNSA) technique of Miller (16) using glucose as the standard. Glucose yields were also determined by the oxygen rate method making use of a Beckman Analyzer. All sugar determinations were made on duplicate samples.

Crystallinity Determination of Cellulose residues. The cellulose residues obtained after enzymatic hydrolysis, as well as the celluloses treated with only buffer solution at the same pH, temperature, and time conditions, were centrifuged, washed with ethanol, and air dried. Then, crystallinity indices were estimated by the same x-ray method used for the determination of cellulose crystallinity before treatments.

RESULTS AND CONCLUSIONS

Grinding treatments produced cellulose materials of very small particle size with consequent decrease in their crystallinities. The finer the particle was ground, the greater the content of amorphous cellulose. Figure 1 illustrates the relationship found between crystallinity index and ball milling time for the three different cellulose materials used. All the curves showed the same trend: a rapid almost linear decrease in crystallinity during the first hours of grinding.

It was found that ground samples with low degree of crystallinity were much more accessible to enzymatic attack than those of high crystallinity. The clear influence of initial cellulose crystallinity on sugar yield, determined by DNSA technique, after enzymatic hydrolysis is observed in Figure 2, Figure 3, and Figure 4. The lower the crystallinity of the initial cellulose, the higher the sugar yield obtained. For each cellulose material, the plots show the same relationship between sugar yield and hydrolysis time: a very rapid increase in sugar production during the first 48 hours followed by a steady but more gradual increase. A similar tendency was observed from the results obtained by the glucose analyzer procedure. An examination of Figure 5, where glucose yield data from cotton linters and Foley Fluff cellulose samples with different crystallinity indices were plotted together, revealed that accessibility of cellulose seems to be highly dependent on degree of crystallinity rather than on the source of the cellulose material.

Crystallinity index data before and after treatments are shown in Tables 1, 2 and 3 for cotton linter, Foley Fluff, and Southern pine cellulose samples respectively. As the crystallinity values obtained after buffer treatments, shown in the second columns of the tables, are greater than those found for the initial cellulose samples, it is evident that some cellulose recrystallization occurred. But, as these values are at the same time smaller than those obtained after enzymatic hydrolysis, it is concluded that both processes: cellulose recrystallization and selective enzyme attack to the amorphous regions of cellulose were probably occurring simultaneously during enzymatic hydrolysis of cellulose.
REFERENCES

Table 1
Crystallinity of Cotton Linter Cellulose Samples.

Percent Crystallinity Index

<table>
<thead>
<tr>
<th>Starting Cellulose</th>
<th>Cellulose After Buffer Treatment</th>
<th>Cellulose After Enzyme Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td>63</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>32</td>
<td>45</td>
<td>76</td>
</tr>
</tbody>
</table>

Table 2
Crystallinity of Foley Fluff Cellulose Samples.

Percent Crystallinity Index

<table>
<thead>
<tr>
<th>Starting Cellulose</th>
<th>Cellulose After Buffer Treatment</th>
<th>Cellulose After Enzyme Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>63</td>
<td>70</td>
</tr>
<tr>
<td>29</td>
<td>51</td>
<td>69</td>
</tr>
<tr>
<td>_a</td>
<td>42</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 3
Crystallinity of Southern Pine Cellulose Samples.

Percent Crystallinity Index

<table>
<thead>
<tr>
<th>Starting Cellulose</th>
<th>Cellulose After Buffer Treatment</th>
<th>Cellulose After Enzyme Hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>62</td>
<td>73</td>
</tr>
<tr>
<td>35</td>
<td>54</td>
<td>59</td>
</tr>
<tr>
<td>_a</td>
<td>51</td>
<td>68</td>
</tr>
</tbody>
</table>

\(^a\) Not determined; no diffraction intensities detected.
FIGURE 1. Percent Cr.I. of cellulose as a function of ball milling time.
FIGURE 2. Percent glucose yield as a function of hydrolysis time for cotton linter cellulose samples of different initial crystallinity indices.
FIGURE 3. Percent glucose yield as a function of hydrolysis time for foley fluff cellulose samples of different initial crystallinity indices.

Initial Crystallinity Indices:

- △ Cr. I. < 20 %
- □ Cr. I. 29 %
- ○ Cr. I. 56 %
FIGURE 4. Percent glucose yield as a function of hydrolysis time for southern pine cellulose samples of different initial crystallinity indices.
FIGURE 5. Percent glucose yield vs hydrolysis time for cellulose samples of different initial crystallinity indices.
INTRODUCTION

Separation of sugars on chemically-bonded amino phases has been intensively investigated since its first application by Linden and Lawhead (1) and Palmer (2). The former reported retention times of fructose, glucose, sucrose, melibiose, and the trisaccharides 1-kestose, neokestose, 6-kestose, and raffinose with a mobile phase of 75%, 80%, 85%, or 90% (v/v) acetonitrile in water. They used a Waters μBondapak column, not further specified. Palmer (2) used a μBondapak/carbohydrate column for separation of the cellooligosaccharides up to cellopentaose, as well as sucrose, maltose, lactose, and five monosaccharides. Again an acetonitrile-water mixture was employed as the mobile phase.

The above authors, along with others (3-9), have investigated sugars of either varying chain length or of different configurations such as glucose and fructose, or sucrose and melibiose. Nikolov (10) to this point has been the only individual to study the separation of glucodi- and trisaccharides; he used only the Waters μBondapak column and did not work with all the members of the series.

This paper will report the separation by HPLC of all the glucodisaccharides, except α,β-trehalose, and of seven glucotrisaccharides, using three columns with chemically-bonded amino phases: the Waters μBondapak, the Supelcosil LC-NH₂, and the Du Pont Zorbax NH₂.

MATERIALS AND METHODS

Three types of amino columns were used. The first was a Waters (Milford, MA) μBondapak NH₂ column, 300 mm long and 3.9 mm i.d., with chemically bound amino groups attached to irregularly shaped silica particles with an average diameter of 10 μm. The second was a Supelcosil (Supelco, Bellefonte, PA) LC-NH₂ column, 250 mm long and 4.6 mm i.d., with 5 μm spherically shaped silica particles. The third was a Zorbax NH₂ (Du Pont Instruments, Wilmington, DE) column, 250 mm long and 4.6 mm i.d., with 7 μm spherically shaped silica particles. In all three cases a 100 mm long, 4.6 i.d., precolumn packed with Whatman (Clifton, NJ) CO: PELL PAC was used. The eluant was 23°C acetonitrile-water at various concentrations flowing at 1 mL/min. Samples were 1% carbohydrate in water, and 10 μL portions were usually injected. The liquid chromatograph was a Waters ALC 201.

Among the disaccharides, α,α-trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), β-cellobiose (4-O-β-D-glucopyranosyl-β-D-glucose), and β-isomaltose (6-O-α-D-glucopyranosyl-β-D-glucose) were supplied by Sigma (St. Louis, MO). β-Maltose (4-O-α-D-glucopyranosyl-β-D-glucose) was obtained from J. T. Baker (Phillipsburg, NJ). α-Kojibiose (2-O-α-D-glucopyranosyl-α-D-glucose) and α-sophorose (2-O-β-D-glucopyranosyl-α-D-glucose) were purchased from Adams (Round Lake, IL). The following compounds were gifts: nigerose (3-O-α-D-glucopyranosyl-D-glucose) from Professor John F. Robyt of Iowa State and Dr. Elwyn T. Reese of the U. S. Army.
Natick Research and Development Laboratories, laminaribiose (3-O-β-D-glucopyranosyl-D-glucose) from Dr. Reese, and β,β-trehalose (β-D-glucopyranosyl-β-D-glucopyranoside) from Dr. Riaz Khan of the Tate & Lyle Group Research and Development Laboratories, Reading, England.

Among the trisaccharides, maltotriose (4-O-α-D-glucopyranosyl-D-maltose) and isomaltotriose (62-O-α-D-glucopyranosyl-D-isomaltose) were obtained from Sigma. Cellotriose (42-O-β-D-glucopyranosyl-D-cellobiose) was purchased from V-Labs, Inc. (Covington, LA). Panose (62-O-β-D-glucopyranosyl-D-maltose) was purchased from BDH Chemicals Ltd. (Poole, England) and was also a gift from Dr. Robyt. Isopanose (42-O-α-D-glucopyranosyl-D-isomaltose) was a gift from Dr. Bent Stig Enevoldsen of the Carlsberg Research Center (Copenhagen, Denmark) and Dr. Robyt. 62-α-D-glucopyranosyl-D-nigerose was a gift from Dr. Robyt.

RESULTS AND DISCUSSION

Three different acetonitrile-water mixtures (69:31, 72:28 and 75:25 (v/v)) were employed with the µBondapak column. The acetonitrile compositions for the Supelcosil column were 69%, 72%, 75%, and 77% (v/v). The Zorbax column was used to produce the chromatograms in this report, and further work to obtain carbohydrate retention times is now underway with it.

Preliminary experiments indicate that there are no appreciable differences in separation between the Supelcosil and Zorbax columns, and that both yield better separations than the µBondapak column. Each carbohydrate eluted as a single peak from all three columns. Resolutions between adjacent peaks, defined as the difference in their retention times divided by their average baseline peak width:

\[ R_s = \frac{t_1 - t_2}{(w_1 + w_2)/2} \]

are shown in Tables 1 and 2 for the µBondapak and Supelcosil columns, along with retention times and peak widths.

The retention times of the sugars follow as set pattern over varying acetonitrile concentrations. Disaccharides elute before trisaccharides. The order of elution for the disaccharides does not vary appreciably from column to column: compounds with 1,3-bonds, followed by those with 1,4-, β-1,2-, α,α-, α-1,2-, β,β-, and 1,6-bonds (Fig. 1). For all reducing disaccharides of the same linkage, the β-bonded compound elutes before the α-bonded compound, except where the glucosidic link is from the primary hydroxyl (the 1,6-bond); therefore isomaltose elutes before gentiobiose. The trisaccharides follow the same pattern: The two 1,3-linked compounds elute first, with laminaritriose (β-linked) exiting before nigerotriose. The next compound is maltotriose (1,4) followed by 62-α-D-glucopyranosyl-D-nigerose, then isopanose and panose, and finally isomaltotriose.

The ability to resolve peaks differs greatly between columns with larger irregularly-shaped particles, such as the µBondapak, and those with smaller
particles. This difference is apparent if we compare the separation of the \(\beta\)-linked disaccharides by the \(\mu\)Bondapak column (Fig. 2), and the Zorbax column (Fig. 3). Laminaribiose and \(\beta\),\(\beta\)-trehalose are better resolved from cellobiose and sophorose by the latter.

The reason the carbohydrates elute in the above order is not completely understood, but preliminary observations with molecular models indicate that the later-eluting compounds appear to have more freedom of rotation about the glucosidic bond, and therefore more opportunity to hydrogen bond to the carrier. If we look at the structure of \(\beta\)-maltose (Fig. 4), there are two bonds (\(\phi\), \(\psi\), a secondary linkage) for the glucose moieties to rotate. On the other hand, \(\beta\)-gentiobiose (Fig. 5) has three bonds (\(\phi\), \(\psi\), \(\omega\), a primary linkage).

One last point of interest is that the \(\beta\)-series is more easily resolved than the \(\alpha\)-series under identical conditions using the same column (Figs. 6 and 7). The reason, obviously, is a difference in conformations; however, at this point we are unable to determine specifics.

In conclusion, we have looked at the separation of a series of glucodisaccharides and glucotrisaccharides. The only difference between compounds of the same length is the linkage between the glucose molecules. This indicates that conformation of the sugar in solution determines its retention time, with the sugars linked through primary hydroxyls having a greater ability to interact with the carrier than do secondary-linked carbohydrates. Much work remains to understand why the secondary-linked sugars elute as they do. We hope that more work with models will help to solve this problem.

ACKNOWLEDGMENT

I would like to thank Drs. Enevoldsen, Khan, Robyt and Reese for the gifts of di- and trisaccharides. Also, I would like to thank Zivko Nikolov for his discussions.

This project was supported by National Science Foundation grants CPE-8022895 and CPE-8101102 and by the Engineering Research Institute, Iowa State University.
REFERENCES

Table 1. Separation of di- and trisaccharides by HPLC at varying acetonitrile-water mixtures with a Waters μBondapak column.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Acetonitrile Concentration, % (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>( t_i ) (min)</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>8.13</td>
</tr>
<tr>
<td>Nigerose</td>
<td>8.55</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>8.64</td>
</tr>
<tr>
<td>Maltose</td>
<td>8.82</td>
</tr>
<tr>
<td>Sophorose</td>
<td>8.89</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>9.01</td>
</tr>
<tr>
<td>α,α-Trehalose</td>
<td>9.37</td>
</tr>
<tr>
<td>Isomaltose</td>
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</tr>
<tr>
<td>Gentiobiose</td>
<td>10.18</td>
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<td>Laminaritriose</td>
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<td>Maltotriose</td>
<td>12.23</td>
</tr>
<tr>
<td>Glc-α-1,6-Glc-α-1,3-Glc</td>
<td>12.87</td>
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<tr>
<td>Panose</td>
<td>13.77</td>
</tr>
<tr>
<td>Isopanose</td>
<td>13.77</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>15.48</td>
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Table 2. Separation of di- and trisaccharides by HPLC at varying acetonitrile-water mixtures with a Supelco Supelcosil LC-NH$_2$ column.

<table>
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<tr>
<th>Carbohydrate</th>
<th>Acetonitrile Concentration, % (v/v)</th>
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<th>77</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$t_i$ (min)</td>
<td>$w_i$ (min)</td>
<td>$R_s$</td>
<td>$t_i$ (min)</td>
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<td>8.49</td>
<td>0.80</td>
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<td>0.80</td>
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<td>0.61</td>
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</tr>
<tr>
<td>Kojibiose</td>
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<td>8.78</td>
<td>0.78</td>
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<td>1.00</td>
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<td>11.76</td>
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<td>Gentiobiose</td>
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<td>0.83</td>
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<td>Glc-$\alpha$-1,6-Glc-$\alpha$-1,3-Glc</td>
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<td>12.31</td>
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<td>17.01</td>
<td>1.54</td>
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<td>1.01</td>
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<td>1.16</td>
<td>18.00</td>
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<td></td>
<td>13.95</td>
<td>1.28</td>
<td>20.29</td>
<td>1.89</td>
</tr>
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</table>
Figure 1. Elution order for glucodi- and glucotrisaccharides from a 5 μm diameter particle Supelcosil LC-NH₂ column.
Column: 300 mm x 4.6 mm Waters µBondapak NH₂
Mobile Phase: 77/23 (v/v) CH₃CN/H₂O
Pressure: 425 psig
Flow: 1.0 mL/min
Detector: Refractive Index 8X
Injection: 10 µL

Figure 2. Elution pattern of disaccharides from a µBondapak column.
Column: 250 mm x 4.6 mm Zorbax NH2
Mobile Phase: 77/23 (v/v) CH3CN/H2O
Pressure: 700 psig
Flow: 1.0 mL/min
Detector: Refractive Index 8X
Injection: 10 µL

Figure 3. Elution pattern of di- and trisaccharides from a Zorbax column.
Figure 4. Constitution and atom numbering for \( \beta \)-maltose (4-O-\( \alpha \)-glucopyranosyl-\( \beta \)-glucopyranose). (11).
Figure 5. Constitution and atom numbering for β-gentiobiose (6-O-β-D-glucopyranosyl-β-D-glucopyranose). (12).
Column: 250 mm x 4.6 mm Zorbax NH₂
Mobile Phase: 77/23 (v/v) CH₃CH₂/H₂O
Pressure: 700 psig
Flow: 1.0 mL/min
Detector: Refractive Index 8X
Injection: 10 µL

Figure 6. Elution pattern of β-linked di- and trisaccharides from a Zorbax column.
Figure 7. Elution pattern of α-linked di- and trisaccharides from a Zorbax column.
DYNAMICS OF BUBBLE SIZE DISTRIBUTIONS IN AIR-LIFT FERMENTORS

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INTRODUCTION

In the pharmaceutical industry, the productivity of fermentations is frequently limited by the transfer of oxygen from the gas phase to the organism in the liquid. Three important factors affect the rate of mass transfer in gas-liquid contactors, \( k_L \), the liquid phase mass transfer coefficient, \( a \), the interfacial area and the degree of liquid mixing in the system. In systems that are well mixed, the mass transfer coefficient \( k_L \) is not greatly affected by liquid hydrodynamics, changes in the interfacial area generally control the mass transfer rate.

Two phenomena, bubble break-up and coalescence, control the bubble size distribution and thus the interfacial area. Bubble break-up is controlled by the liquid phase hydrodynamics and is affected by the physical properties of the liquid, the power input to the system and by the sparger design in some cases. Bhavaraju et al. (1978) have suggested that at low and moderate viscosities bubble break-up is related to liquid-phase turbulence and is independent of conditions at the sparger orifice.

The purpose of this paper is to study the mechanism involved in bubble break-up with the aid of bubble size distribution measurements in an air-lift fermentor. The effects of liquid viscosity, electrolyte and power input on bubble size distribution are also examined.

THEORY

Hinze (1955) first predicted mechanisms for droplet break-up in various flow fields. He suggested that droplet break-up is the result of viscous shear forces and/or turbulent pressure fluctuations. He also pointed out that in dispersions where drops are much larger than the Kolmogoroff microscale, \( \eta \), dynamic pressure forces rather than viscous shear forces control the breakage processes. If we assume that bubble break-up is caused by dynamic pressure forces and opposed by surface tension forces, then at the point of breakage, these forces must balance:

\[
\frac{-2}{\rho_g u^2} = k \frac{4\sigma}{d_{\text{max}}}
\]  

(1)
where $d_{\text{max}}$ is the maximum stable bubble diameter in this flow field, and $u^2$ is the mean square value of the fluctuating velocity over a distance $d_{\text{max}}$. This balance leads to the prediction of a critical Weber number:

$$W_{ec} = \frac{\rho c u d_{\text{max}}}{2\sigma}$$  \hspace{1cm} (2)

Under highly turbulent conditions bubble break-up can be modeled by isotropic turbulence theory. For this case of turbulence the main contribution to the kinetic energy, which is available for the bubble breakage, is made by the fluctuations in the wave length region where the Kolmogoroff energy distribution law is valid. This is because that the maximum sized turbulent eddies which can cause the bubble breakup are assumed to lie in the inertial subrange. In this inertial subrange the three-dimensional wave-number spectrum $E(k)$ is solely determined by the energy dissipation rate $\varepsilon$, and can be expressed as

$$E(k) = \alpha \varepsilon \frac{2}{3} k^{-5/3}$$  \hspace{1cm} (3)

Since the energy content of this spectrum between wave number $k$ and $k + dk$, is $E(k)dk$, one finds

$$u^2 = \frac{\int E(k)dk}{\int E(k)dk} = \int \frac{\alpha \varepsilon^{2/3} k^{-5/3}}{1/d_{\text{max}}} dk$$  \hspace{1cm} (4)

Integration of Eq. (4) gives

$$u^2 = c_1 \varepsilon^{2/3} d_{\text{max}}^{2/3}$$  \hspace{1cm} (5)

where $c_1 = 1.5\alpha$; $c_1$ is approximately equal to 2.0 according to Batchelor (1951).
If we assume that the viscous shear force is not important, then we obtain from Eq. (2)

\[ \frac{\rho_c d_{\text{max}}}{2} c_1 (\varepsilon d_{\text{max}})^{2/3} = \text{const.} \]

or

\[ d_{\text{max}} = C (\sigma/\rho_c)^{3/5} \varepsilon^{-2/5} \]  

(6)

On introducing dimensionless groups into Eq. (6) we get

\[ \frac{\rho_c \sigma d_{\text{max}}}{\mu_c^5} = C (\frac{c}{\rho_c \sigma^4})^{2/5} \]  

(7)

This simple result also follows directly from dimensional reasoning once it has become evident that only the quantities \( \rho_c \), \( \sigma \), and \( \varepsilon \) determine the size of the largest bubbles.

**MATERIALS AND METHODS**

A cylindrical airlift fermentor has been constructed with optically-flat glass panels for bubble size distribution analysis. The fermentor is 136 cm high, 15 cm diameter and air is introduced on the upflow side through 38 1.6 mm holes (see Fig. 1). Experiments were conducted for four different systems: air-tap water, air-salt water (0.6% NaCl), air-CMC solution (0.5%) and air-CMC solution (0.8%). Surface tension measurements were made using a Fisher Surface Tensiomat Model 21. Table 1 gives the properties of materials used.

Power input was estimated by applying steady state macroscopic energy balance to the gas phase in the upflow zone assuming ideal gas behavior and neglecting frictional losses and potential difference. The power input, \( \bar{p} \), is then given by the following equation:

\[ \bar{p} = Q_G \rho_g \left[ \frac{R_T}{M_g} \ln(P_1/P_2) \right] \]  

(8)
where $Q_G$ is volumetric gas flow rate, $R$ is the gas constant, $T$ is absolute temperature, $M_G$ is the gas molecular weight, $P_1$ and $P_2$ are pressures at gas sparger and top of fermentor respectively, and $\rho_G$ is gas density. The energy dissipation rate per unit mass of liquid, $\varepsilon$, is given by

$$
\varepsilon = \frac{\dot{p}}{V_L \rho_c}
$$

where $V_L$ is the liquid volume in the upflow zone, and $\rho_c$ is the continuous phase density.

A direct photographic method for bubble size distribution analysis has been developed in which negatives are scanned and reduced to histographic data by means of a Bausch & Lomb Omnicron system. The maximum stable bubble diameter $d_{\text{max}}$ was determined from the cumulative bubble size distribution by taking $d_{\text{max}} = D_{95}$ where $D_{95}$ is the value for which 95% by volume is contained in the drops with $d < D_{95}$.

**DISCUSSION OF RESULTS**

The bubble size distributions are shown in Figs. 2 to 5 for the four systems we examined. The probability density functions are obtained by numerically differentiating cumulative volume distributions and passing them through a digital triangular filter:

$$
f_i = \left( f_{i-2} + 3f_{i-1} + 5f_i + 3f_{i+1} + f_{i+2} \right) / 13
$$

A smoothing filter has the advantage that while discontinuities in the curve are removed, only relatively small dispersion of the data occurs. The basic characteristics of the curve are preserved. Comparison of the original data with the data after smoothing is shown in Fig. 6.

As can be seen in Fig. 2, the bubble size decreases with increasing air flow rate for tap water system. This is because an increase in dynamic pressure forces as a result of a higher air flow rate causes the bubble size reduction. But if we further increase the air flow rate to 90 ft$^3$/hr the bubble size distribution does not change too much. This is due to an increase of bubble coalescence frequency at higher gas flow rates which tends to offset the effect of bubble breakup resulting from an increase of dynamic pressure forces.

The effect of electrolyte on bubble size distribution is shown in Fig. 3 for salt water systems. A striking difference can be seen by comparing the bubble size distributions of the tap water and salt water systems.
The bubble size of salt water system is still reduced by a significant extent for each window we examined when the air flow rate is increased to 90 ft³/hr. This is probably due to the effect of ionic strength which can prevent bubbles from coalescing and thus reduce bubble size even at higher gas flow rates.

The effect of viscosity on bubble size distribution is shown in Fig. 4 and Fig. 5 for CMC solutions. As can be seen from these figures, the bubble size does not change too much with the air flow rate; moreover, for CMC solution with concentration 0.8% the bubble size increases with air flow rate. This can be explained by the decrease of bubble break-up in high viscosity systems and the domination of bubble coalescence.

An illustration of the influence of energy input on the critical bubble size is shown in Fig. 7 for the four different systems we examined. As can be seen from this figure, the critical bubble size is proportional to the -0.38 power of energy input which is very close to the theoretical value -2/5. A comparison of the specific interfacial area for four different systems is shown in Fig. 8. The specific interfacial area for salt water system is significantly higher than the other three systems except at the lowest air flow rate. This is due to the combined effect of the increase in gas hold-up and the decrease of the mean bubble diameter with power input.

CONCLUSIONS

i) Bubble break-up can occur in low viscosity systems by dynamic pressure fluctuations in the vicinity of the bubble.

ii) For high viscosity systems the power input is less efficient with respect to the bubble breakage process.

iii) The addition of electrolyte may significantly increase the specific interfacial area.

iv) The maximum stable bubble diameter is roughly proportional to the -2/5 power of energy input.

REFERENCES


ACKNOWLEDGMENT

This work was partially supported by the National Science Foundation, Grant CPE-8108799.
Figure 1. Plexi-glass airlift column used in the experiments (all dimensions are in millimeters).
Table 1. Properties of materials used (at 25°C)

<table>
<thead>
<tr>
<th>Materials</th>
<th>ρ, g/cm³</th>
<th>μ or μ_s, cP</th>
<th>σ, dyne/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap Water</td>
<td>0.989</td>
<td>0.890</td>
<td>64.889</td>
</tr>
<tr>
<td>Salt Water</td>
<td>0.991</td>
<td>0.902</td>
<td>55.380</td>
</tr>
<tr>
<td>CMC (0.5%)</td>
<td>0.994</td>
<td>6.20 - 6.66</td>
<td>68.700</td>
</tr>
<tr>
<td>CMC (0.8%)</td>
<td>0.993</td>
<td>13.40 - 15.90</td>
<td>65.450</td>
</tr>
</tbody>
</table>

Figure 2. Bubble size distribution for air-water system at window #4 for various air flow rates. [□: F = 30 ft³/hr, ◇: F = 60 ft³/hr, Δ: F = 90 ft³/hr]
Figure 3. Bubble size distribution for air-salt water system at window #4 for various air flow rates. [□: F = 30 ft³/hr, ○: F = 60 ft³/hr, △: F = 90 ft³/hr]

Figure 4. Bubble size distribution for air-CMC solution (0.5%) at window #4 for various air flow rates. [□: F = 30 ft³/hr, ○: F = 60 ft³/hr, △: F = 90 ft³/hr]
Figure 5. Bubble size distribution for air-CMC solution (0.8%) at window #4 for various air flow rates. [□: F = 30 ft³/hr, ▼: F = 60 ft³/hr, △: F = 90 ft³/hr, ◇: F = 120 ft³/hr]

Figure 6. The effect of smoothing on bubble size distribution using a digital filter. (system: air-water @ 60 ft³/hr of air/observed at window #4) [———: data after smoothing, ◇: original data]
Figure 7. Variation of critical bubble size with energy input.

Figure 8. Influence of superficial gas velocity on specific interfacial area at window #4.
A Thermal Coagulation Study of Alfalfa Leaf Proteins by Differential Scanning Calorimeter

Khalif Ahmed and Bruce Dale

Differential scanning calorimeter (DSC) is a technique which measures changes in heat flow to a sample as a function of temperature. It has been widely used in studies of both synthetic and natural polymers. Natural polymers studies by DSC include cellulose, starch, fats and proteins (1,2,3,4). Coagulation is the ultimate stage of aggregation undergone by protein molecules under denaturing conditions, where hydrophobic bond formation is probably enhanced. Proteins may coagulate in solution due to heat or salt effects or changes in pH(5).

DSC is finding wider application in more heterogeneous food systems. The physicochemical changes in whey protein concentrates (WPC) and β-lactoglobulin in solution during heating at various acid pH values and ionic strengths have been reported (6,7). The changes in beef proteins during meat processing into sausage batter have also been monitored by DSC (2).

Several reports have also appeared using this calorimetric technique on plant proteins. Thermal transitions have been observed in complex multicomponent proteins of legumes such as soybean and cowpea, among others (8). The effects of heat and pH treatments on plant proteins following various processing schemes have also been determined with DSC (9).

This study was undertaken in an attempt to elucidate the denaturation and coagulation behavior of alfalfa (Medicago sativa) leaf juice under varying conditions which included:

(a) content of various sugars,
(b) sample heating rate,
(c) pH,
(d) ionic strength, and
(e) content of divalent cations.

Materials and Methods

Midbloom to late alfalfa plants were collected in a plastic bag from the CSU Agronomy Research Center and were brought to our laboratory within 15 minutes of harvest. The leaves together with the petioles were hand picked and kept on ice (0 - 4°C) through the process. The leaves were then homogenized in a chilled Waring blender. The temperature of the leaf homogenate was monitored and did not exceed 10°C. The homogenate was placed on a triple layer of cheese cloth and squeezed manually to extract the protein-rich green solution. The protein-rich solution was kept in an ice bath during extraction and was then divided into several 10 mL aliquots. The aliquots were centrifuged at 2000 x g at 0°C for 7 minutes to remove particulate material, e.g. unruptured, macerated leaf tissue.

The supernatant was carefully decanted into a clean test tube and the sediment was discarded. The pH of the aliquots of various collections was measured and ranged from 5.8 to 6.1. One ml of the supernatant was then weighed and the remaining aliquots were lyophilized after freezing in an acetone-dry ice bath. The freeze-dried material was scrapped off the tube and weighed again. The dry solid content per ml was determined by the difference
between pre- and post-lyophilization weights. Total nitrogen was determined by the micro kjeldahl method (N x 6.25) [AOAC Methods 1980].

A 10 percent protein concentrate sample (w/w) was prepared by dissolving 0.1 gm of lyophilized material (20 weight % protein, dry basis) in 0.1 ml of distilled water. In experiments to determine pH effects, the pH of extracted protein-rich juice was adjusted to 5 or 8.5 with 20 percent phosphoric acid or 0.5 M potassium hydroxide while in an ice bath prior to freezing and lyophilization. The samples thus were later redissolved as above in pH 5 or pH 8.5 aqueous solutions and scanned. This step was necessary due to the buffering capacity of the leaf proteins homogenate. For the study of ionic strength effects, other lyophilized samples were dissolved in distilled water with various concentrations of KCl, NaCl, CaCl₂, ZnCl₂, or with sugars including fructose, ribose, sucrose and glucose. After dissolving the sample in water, it was mixed well. Such reconstituted protein samples were extremely viscous.

**Differential Scanning Calorimetry**

A DuPont Model 1090 thermal analyzer with a 910 differential scanning calorimeter was used in this study. The thermal denaturations were conducted at a machine sensitivity of 0.05 milliwatts/cm. The heating rate was set at 10°C/min, unless otherwise indicated, and the temperature of the scan ranged from 20-100°C. The calibration coefficient E, and calorimeter temperatures were determined using previously weighed indium over a scanning range of 20-200°C.

A sample of redissolved alfalfa protein weighing 9-18 milligrams (net weight) was deposited into a preweighed aluminum DSC pan and hermetically sealed and weighed to an accuracy of 0.010 mg. An empty sealed reference pan was used to stabilize the baseline (2).

Peak areas were measured with a planimeter after drawing a straight line joining the pre- and post-denaturation shoulders of each peak. To determine the enthalpy of the reaction, \( \Delta H \), the following formula was used (9):

\[
\Delta H = \left( \frac{A}{MC} \right) \times (60 \text{ BE } \Delta qs)
\]

where these variables are as follows:

- \( \Delta H \) = enthalpy of the reaction (mcal mg⁻¹)
- A = peak area (in²)
- M = sample mass (mg)
- C = sample concentration (% w/w)
- P = protein concentration of sample (%)
- B = time base (min in⁻¹)
- E = cell calibration coefficient
- \( \Delta qs \) = Y-axis range (mcal s⁻¹ in⁻¹)

The recent addition of data analysis hardware and software to our system will allow more accurate measurement of these variables in the future.
Results and Discussion

Figure 1 shows a composite thermogram of lyophilized alfalfa leaf juice, dissolved in distilled water to 10% (w/w) protein content and then scanned at heating rates of 2.5, 10 and 15.5°C/min. The solution was not buffered. Three endothermic peaks with denaturation temperatures of approximately 57°C, 70°C and 80°C are shown. These peaks were consistently present at different heating rates in all thermograms. The variations in the size of the peaks reflects variations in the sample mass scanned in each case. The denaturation temperature ($T_d$) is the temperature where the maximum rate of heat absorption by the sample occurs. Our general observation that denaturation temperature are not affected by scanning rate is in agreement with the work of others (10).

Several investigators have reported temperatures of 50°C to 60°C for the chloroplastic (green) protein fraction of alfalfa while reported denaturation temperatures for the soluble (white) alfalfa proteins range from 70°C to 80°C (11, 12). The endothermic peak at $T_d$ 57°C obtained by DSC in this study apparently corresponds to the chloroplastic proteins reported in the literature. The peak at $T_d$ 80°C corresponds to the soluble alfalfa proteins or white fraction. Although the heterogeneity of alfalfa leaf proteins is well known, we believe this is the first time that such clear evidence for their differing thermal behavior has been found. In addition, these three well-separated peaks hold out the possibility of a further fractionation of the alfalfa leaf proteins by a three-stage heating approach versus the one-stage and two-stage approaches in common use if some method can be found to separate the proteins.

Effect of Sugar Content

The content of monomeric or dimeric sugars in the alfalfa leaf cytoplasm ranges from approximately 1 to 10 percent of the total dry weight (13). Lyophilized leaf proteins were dissolved in distilled water containing concentrations of glucose, fructose, sucrose and ribulose up to ten times their normal concentration in alfalfa and subjected to DSC analysis. These levels had no effect on either the temperature or enthalpy of denaturation (Figure 2). As before, variation in peak size can be accounted for by difference in the mass of sample scanned.

Effect of Salt and Divalent Cations

The effects of NaNCl concentration on leaf protein denaturation can be seen in Figure 3. A solution of 0.23 M NaNCl or 0.46 M NaNCl (Figures 3a and 3c) had no effect on the endothermic peaks of alfalfa. However, the 0.92 M NaNCl (Figure 3b) resulted in the disappearance of the chloroplastic peak but did not affect the two higher temperature peaks. This indicates the sensitivity of the chloroplastic proteins to denaturation by high NaNCl levels.

KCl concentrations of 0.05 M, 0.23 M and 0.46 M had little or no effect on any of the endothermic peaks (Figure 4), but 0.92 M KCl diminished or eliminated the chloroplastic peak with no obvious effect on the two soluble
protein peaks. Thus, there was no apparent difference between effects of NaCl and KCl on the thermal denaturation of alfalfa leaf proteins. This is in agreement with data of Quinn and co-workers on meat proteins where ionic strength alone, and not the specific salt, was responsible for the effect on denaturation temperature (2). The important implication of these results is that stream recycle with the associated salt build up can be expected to alter the protein coagulation behavior.

The effect of various concentrations of CaCl₂ is shown in Figure 5. At 0.05 and 0.1 M CaCl₂, the size of the peak at 80°C was decreased by approximately half. The two lower peaks were generally unaffected. However, it appears that the lower peak Tₐ moved down a few degrees to about 54°C, while the middle peak may have moved up slightly. Thus, the sharpness of the separation between the chloroplastic and cytoplasmic proteins might be somewhat improved by addition of relatively small amounts of CaCl₂. Lyophilized leaf protein redissolved in 0.2 M CaCl₂ exhibited no chloroplastic peak. The size of the 80°C was also reduced by over 50 percent. It is evident that the divalent cation calcium has a much greater effect on the denaturation of alfalfa protein than the monovalent cations, sodium or potassium. The system is undoubtedly complex, but it seems likely that this result is at least partly due to intercalating of Ca²⁺ between dicarboxylic or hydroxyl amino acid residues of a protein molecule which results in the protein denaturation more readily than when exposed to KCl or NaCl.

Effect of pH

The pH effect on the thermograms of alfalfa leaf juice is shown in Figure 6. The pH of the samples was adjusted prior to the DSC scan. At pH 5 only one peak with a Tₐ of 57°C and 70°C are absent, i.e. these proteins are apparently denatured by the low pH prior to the scan. At pH 6 endothermic peaks with Tₐ's of 80°C and 54°C are visible although the lower peak is greatly diminished in size. The size of both peaks is increased 20 percent as compared to the control thermograms. Since the pH of the unbuffered alfalfa protein solution is about 5.8, the result at pH 6.0 may be due to the KOH used to adjust pH. This warrants further investigation. At pH 8.5 a much larger endothermic at 70°C is observed, while the 57°C peak disappears. The peak at 80°C is slightly diminished. There is at least one possible explanation for the increase in the size of the 70°C peak. The alkaline pH has caused a change in the thermal behavior of the proteins which usually denature at 57°C and has shifted the entire peak upscale to superimpose itself on the proteins which initially denature at 70°C. Certainly, the large peak at 70°C supports the idea that pH 8.5 is useful for thermal denaturation of alfalfa leaf proteins.

Conclusions

(1) The denaturations of alfalfa leaf proteins as evidenced by DSC is strongly affected by pH and calcium ions content.
(2) Ionic strength also affects the denaturation behavior but the effect is not as strong as are the pH and Ca⁺⁺ ions.
(3) Concentrations of sample sugars and heating rates have minimal effect on denaturation of the proteins.
(4) There are 3 major protein moities present.
(5) None of the changes induced by denaturing agents are reversible.

References

Figure 1. Effect of heating rate on denaturation of alfalfa leaf proteins.
Figure 2. Effect of sugar concentration on the subsequent denaturation of alfalfa leaf proteins.
Figure 3. Effect of NaCl concentration on the subsequent denaturation of alfalfa leaf proteins: (a) Effect of 0.46 M NaCl, (b) Effect of 0.92 M NaCl, (c) Effect of 0.23 M NaCl.
Figure 4. Effect of salt concentration on the subsequent denaturation of alfalfa leaf proteins.
Figure 5. Effect of calcium chloride concentration on the subsequent denaturation of alfalfa leaf proteins.
Figure 6. Effect of pH on the subsequent denaturation of alfalfa leaf proteins.
INTRODUCTION

Because of the need to consider renewable sources of chemicals and energy, there is considerable interest in the growth of algae and photosynthetic bacteria. These microorganisms have a higher conversion efficiency than plants and growth conditions can be more easily controlled. Many papers have been published on light energy conversion by photosynthetic organisms [1, 2, 3, 4].

Several ways may be used to express the yield of these organisms. The conversion efficiency of light to chemical energy in the biomass may be investigated using the concepts of thermodynamics. In this work, carbon, available electron and energy balances are examined. Pirt's yield model is applied in order to estimate the yield and maintenance parameters [5]. Least square estimation is used to obtain the true biomass energetic yield, i.e. the conversion efficiency of light energy, and the maintenance coefficient using a multivariate statistical analysis procedure referred to as the covariate adjustment procedure [6].

THEORY

The efficiency of photosynthesis can be expressed in energetic terms. Material and energy balances and regularities associated with microbial biomass may be used in the yield analysis [7, 8, 9, 10, 11]. The chemical balance equation for photosynthetic growth by microorganisms is

\[ \text{CO}_2 + a\text{NH}_3 + c\text{H}_2\text{O} \xrightarrow{\text{light energy}} \text{CH}_p\text{O}_n\text{N}_q + b\text{O}_2 \]  

where \(\text{CH}_p\text{O}_n\text{N}_q\) is the composition of these atoms in the biomass. Minkevich and Eroshin [12] have found that the weight fraction carbon in the biomass, \(\sigma_b\), the reductance degree, \(\gamma_b\) and energy content per equivalent of available electron in biomass \(Q_o\) are relatively constant. The weight fraction carbon \(\sigma_b\) and reductance degree, \(\gamma_b\), of photosynthetic organisms are similar to the values for other microorganisms; from the data of Aiba and Ogawa [3], \(\sigma_b = 0.47\) and \(\gamma_b = 4.2\) and in earlier work [13], \(\sigma_b = 0.45\) and \(\gamma_b = 4.4\) for valences of \(C = 4\), \(H = 1\), \(O = -2\), and \(N = -3\). These values may be compared to the average values [12] of \(\sigma_b = 0.462\) and \(\gamma_b = 4.291\). The carbon balance
is simple for this case because the carbon consumed as carbon dioxide should be equal to the carbon in the produced biomass when no extracellular products are produced. The carbon balance is

\[ \frac{Q_{\text{CO}_2}}{12} = \frac{\Delta X}{b} \]

or

\[ \frac{12 Q_{\text{CO}_2}}{\Delta X} = 1 \quad (2) \]

The available electron balance can be written as

\[ \gamma_b = 4b, \quad \text{or} \quad \frac{4b}{\gamma_b} = 1 \quad (3) \]

which may also be written in the form

\[ 4 Q_{\text{O}_2} = \frac{\Delta X \gamma_b}{12} \]

The energy balance is

Total light energy input = The energy which is stored in the microbial cells + The energy which leaves because of radiation, conduction, and convection.

or

\[ I_A = \frac{\Delta X \gamma_b Q}{12} + Q_h \]

which becomes

\[ \frac{\Delta X \gamma_b Q}{12 I_A} + \frac{Q_h}{I_{\text{aA}}} = 1 \quad (4) \]

The first term, on the left-hand side in the above equation, is defined as the energetic yield of the biomass, \( \eta_{\text{kcal}} \), and the second term is the fraction of the energy which is lost, \( \varepsilon_h \). Therefore, equation (4) can be expressed as,

\[ \eta_{\text{kcal}} + \varepsilon_h = 1 \quad (5) \]
Besides the direct energetic yield from biomass and light measurements, three other energetic yields can be calculated, based on other measurements. That is, using the available electron, carbon, and energy balances, respectively,

\[
\eta_{O_2} = \frac{4Q_o X_o}{I_a A}, \quad \text{from oxygen and light.}
\]

\[
\eta_{CO_2} = \frac{Q_{CO_2} X_{yb} Q_o}{I_a A}, \quad \text{from carbon dioxide and light.}
\]

\[
\eta_Q = 1 - \frac{Q_h}{I_a A}, \quad \text{from heat evolved and light.}
\]

where \( I_a \) is the incident light energy (kcal/cm\(^2\)/hr), \( A \) is the total area (cm\(^2\)), and \( Q_o \) is the specific rate of oxygen production (mole of \( O_2 \)/g biomass/hr).

Pirt's yield model [5], may be used to estimate the energetic yield on solar energy, \( \eta_{max} \) and the maintenance coefficient, \( m_e \), without any product formation. The model can be written in the following forms:

\[
Y_{1i} = \frac{\mu_i}{\eta_{kcal_i}} = m_e + \frac{\mu_i}{\eta_{max}} \quad (6)
\]

\[
Y_{2i} = \frac{\mu_i}{\eta_{O_2_i}} = m_e + \frac{\mu_i}{\eta_{max}} \quad (7)
\]

\[
Y_{3i} = \frac{\mu_i}{\eta_{CO_2_i}} = m_e + \frac{\mu_i}{\eta_{max}} \quad (8)
\]

\[
Y_{4i} = \frac{\mu_i}{\eta_{Q_i}} = m_e + \frac{\mu_i}{\eta_{max}} \quad (9)
\]

Two forms of equations (6 - 9) are used for parameter estimation. In this paper, equations (6 - 9) are referred to as Form II. Form I is obtained by dividing each term in equations (6 - 9) by \( \mu \).

Four different estimates of the parameters from equations (6 - 9) can be obtained by treating the four equations as multivariate linear models with common parameters. Superficially, it seems appropriate to estimate the parameters based on the following model:
but then the information contained in $\eta_{\text{kcal}}$, $\eta_{O_2}$, $\eta_{CO_2}$, and $\eta_Q$ may not be efficiently utilized. Therefore, a set of covariates is added, $Z_{1i}$, ..., $Z_{qi}$ $(1 < q < 3)$, which are linear combinations of the four dependent variables. This yields a conditional model (conditional on $Z_{1i}$, ..., $Z_{qi}$).

$$
\bar{Y}_{i} = \frac{\mu_i}{4} \left[ \frac{1}{\eta_{\text{kcal}}} + \frac{1}{\eta_{O_2}} + \frac{1}{\eta_{CO_2}} + \frac{1}{\eta_Q} \right] \quad (10)
$$

An example of a full set ($q=3$) of linearly independent covariates that have zero expected value is

$$
Z_{1i} = -3Y_{1i} - Y_{2i} + Y_{3i} + 3Y_{4i} \\
Z_{2i} = -Y_{1i} + 3Y_{2i} - 3Y_{3i} + Y_{4i} \\
Z_{3i} = Y_{1i} - Y_{2i} - Y_{3i} + Y_{4i}
$$

The least square estimates for $m_e$ and $\frac{1}{\eta_{\text{max}}}$, obtained by fitting the conditional model (11), are referred to as the maximum likelihood estimates. Generally, smaller confidence intervals for the parameters may result when the number of useful covariates is decreased without sacrificing an appreciable amount of information. However, when the number of data points are small, the best estimates are frequently obtained when no covariates are included in equation (11). This result is the average of the individual estimates. For more detail, see Yang et al. [14].

**RESULTS AND DISCUSSION**

Table I contains experimental data of Aiba Ogawa [2, 3], and the results of carbon balance analysis and energetic yields for Spirulina platensis. The balance shows that the data is not very consistent. The reductance degree and weight fraction carbon values are based on their experimental data for this organism. The valences of $C = 4$, $H = 1$, $O = -2$, and $N = 0$ were used, to estimate $\gamma_b$ because the source of nitrogen was nitrate. When $N = 0$ is used, the energy required to incorporate the nitrogen into the protein is appropriately considered. Two estimates of biomass energetic yield, $\eta$, are obtained at each dilution rate where both biomass and $CO_2$ are measured.

Table II contains the estimates of the true biomass energetic yield, $\eta_{\text{max}}$ and maintenance coefficient, $m_e$ from the analysis of the data set in Table I by Form I and Form II of equations (6 and 8). All of the estimates are either average or individual estimates. The estimates with covariates are not reported. Since this data set has only a small number of data points, the covariate adjustment technique is not a more efficient method than the average value of the least square estimates. Based on the 95% confidence intervals, the average estimate has the shortest confidence intervals for...
both the true biomass energetic yield and the maintenance coefficient. For
the true biomass energetic yield, the estimates from Form II have shorter
intervals, and for the maintenance coefficient, Form I has shorter intervals.
The estimates with shortest confidence intervals are those with an asterisk
in Table II. It is evident that the maintenance coefficient should not be
less than zero energetically, therefore, the negative value of that must be
due to experimental error.

Table III contains point and 95% confidence interval estimates for the
true biomass energetic yield and maintenance coefficient of Chlorella based
on the data of Pirt [1]. Pirt's results [1] are compared to results which
are calculated, based on the average values of the regularities; that is,
cb = 0.462, yb = 4.291 and Q0 = 26.95 kcal./eq. of available electrons.

The estimates of true growth yield, nmax, are larger in Pirt's work.
He used a value of 22.7 kJ/g dry wt. to estimate the energy content of the
biomass. He also reported the results of biomass elemental composition analy­
sis to be CH1.80N0.163O0.432 and that cb = 0.486. The value of yb = 4.51 and
Q0 = 29.70 kcal/eq. of available electrons for the above data when a valence
of -3 is used for nitrogen. The result of Pirt's experiments represent an
excellent true biomass energetic yield. As has been pointed out elsewhere
[15], these results exceed the maximum expected value of nmax = 0.29 based on
2 photons per equivalent of available electrons (8 photons/mole O2) [16].

The estimates of nmax for the mixed culture are higher than for Chlorella
by itself. The symbiotic growth of bacteria and algae may be a promising
method and algae may be a promising method for converting solar energy to
chemical energy [17].

Table IV contains a summary of several experiments with photosynthetic
organisms. The results appear to show a trend in that the maintenance coe­

cfficient is larger when the true growth yield is smaller.

Burlew [13] has reported a mass yield for Chlorella of 25 g/day/m2 for
outdoor cultivation. Goldman [18] has obtained similar yields.

Further research is needed with photosynthetic cultures in order to
optimize this energy conversion process.

REFERENCES


ACKNOWLEDGMENT

This work was partly supported by NSF Grant CPE 81-20039.
Table I. Data consistency check by carbon balance.
The energetic yield from light and biomass ($\eta_{kcal}$), and from light and carbon dioxide ($\eta_{CO_2}$) for *S. platensis*.

<table>
<thead>
<tr>
<th>dilution rate $\mu$ (hr$^{-1}$)</th>
<th>cell dry weight $x$ (g/l)</th>
<th>$\frac{Q_{CO_2}}{nmole}$</th>
<th>Intensity $I_a \times 10^3$ (kcal/cm$^2$/hr)</th>
<th>$\eta_{kcal}$</th>
<th>$\eta_{CO_2}$</th>
<th>$\frac{12 \ Q_{CO_2}}{D_{g_b}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014</td>
<td>0.272</td>
<td>0.768</td>
<td>1.5</td>
<td>0.0721</td>
<td>0.0900</td>
<td>1.25</td>
</tr>
<tr>
<td>0.022</td>
<td>0.246</td>
<td>1.18</td>
<td>1.44</td>
<td>0.101</td>
<td>0.130</td>
<td>1.29</td>
</tr>
<tr>
<td>0.023</td>
<td>0.177</td>
<td>---</td>
<td>1.25</td>
<td>0.0917</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.029</td>
<td>0.169</td>
<td>1.38</td>
<td>1.21</td>
<td>0.108</td>
<td>0.125</td>
<td>1.15</td>
</tr>
<tr>
<td>0.034</td>
<td>0.122</td>
<td>1.48</td>
<td>1.05</td>
<td>0.105</td>
<td>0.111</td>
<td>1.05</td>
</tr>
<tr>
<td>0.038</td>
<td>0.057</td>
<td>1.62</td>
<td>0.61</td>
<td>0.0982</td>
<td>0.0978</td>
<td>0.996</td>
</tr>
</tbody>
</table>

* $\gamma_b = 4.788$, $\sigma_b = 0.472$, $Q_0 = 27.1$ kcal/eq. a.e.
Table II. The Average and Individual Estimates for the True Growth Energetic Yield for *S. Platensis*.

<table>
<thead>
<tr>
<th>FORM I</th>
<th>FORM II</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\eta_{\text{max}}$</td>
<td>$m_e$</td>
</tr>
<tr>
<td>Average of Both Cases</td>
<td>0.0831, 0.414</td>
</tr>
<tr>
<td></td>
<td>0.138</td>
</tr>
<tr>
<td>$\eta_{\text{kcal}}$</td>
<td></td>
</tr>
<tr>
<td>0.0955, 0.346</td>
<td>*0.00078, 0.178</td>
</tr>
<tr>
<td>0.150</td>
<td>0.0929</td>
</tr>
<tr>
<td>$\eta_{\text{CO}_2}$</td>
<td></td>
</tr>
<tr>
<td>0.0734, 0.519</td>
<td>*-0.0966, 0.166</td>
</tr>
<tr>
<td>0.129</td>
<td>0.0346</td>
</tr>
</tbody>
</table>

*Result with shortest 95% confidence interval. The first line is the 95% confidence interval; the second line is the point estimate.*
Table III. Estimates of the true biomass energetic yield and maintenance coefficient for the data of Pirt [1].

<table>
<thead>
<tr>
<th></th>
<th>Pirt's results</th>
<th>Recalculated results&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \eta_{\text{max}} )</td>
<td>( m_e )</td>
</tr>
<tr>
<td>Chlorella</td>
<td>0.347</td>
<td>0.00088</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed culture (Algae + bacteria)</td>
<td>*[0.427, 0.515]</td>
<td>*[0, 0.0141]</td>
</tr>
<tr>
<td></td>
<td>0.468</td>
<td>0.00132</td>
</tr>
<tr>
<td>Mixed culture (Algae only)</td>
<td>0.468</td>
<td>0.0184</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>*</sup> 95% confidence intervals

<sup>+</sup> Recalculated results are based on the average values of \( a_b = 0.462 \), \( \gamma_b = 4.291 \), and \( Q_0 = 26.95 \) kcal/eq. of available electrons.
Table IV. Comparison of the true biomass energetic yield and maintenance coefficient for photosynthetic organisms.

<table>
<thead>
<tr>
<th>Species</th>
<th>$n_{\text{max}}$</th>
<th>$m_\text{e}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella **</td>
<td>0.27</td>
<td>-0.015</td>
<td>Pirt [1]</td>
</tr>
<tr>
<td>Chlorella, ** mixed culture with bacteria</td>
<td>0.40</td>
<td>0.0029</td>
<td></td>
</tr>
<tr>
<td>Spirulina platensis</td>
<td>0.115</td>
<td>0.0255</td>
<td>Aiba and Ogawa [3]</td>
</tr>
<tr>
<td>Oscillatoria agardhii</td>
<td>0.23</td>
<td>0.00438</td>
<td>Van Liere and Mur [19]</td>
</tr>
<tr>
<td>O. agardhii</td>
<td>0.19</td>
<td>0.0211</td>
<td></td>
</tr>
<tr>
<td>O. agardhii</td>
<td>0.15</td>
<td>0.00667</td>
<td></td>
</tr>
<tr>
<td>Scenedesmus protuberans**</td>
<td>0.18</td>
<td>0.0444</td>
<td>Gons &amp; Mur [19,20]</td>
</tr>
<tr>
<td>S. protuberans**</td>
<td>0.13</td>
<td>0.0692</td>
<td></td>
</tr>
<tr>
<td>S. obliguus</td>
<td>0.04</td>
<td>0.225</td>
<td>Oswald [19,21]</td>
</tr>
<tr>
<td>Chlorella***</td>
<td>0.08</td>
<td>0.187</td>
<td>Myers [19, 22]</td>
</tr>
</tbody>
</table>

** Nitrogen source, urea.

*** Light source, approximate full sun-light.