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Proceedings of the Sixteenth Annual Biochemical Engineering Symposium

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PROCEEDINGS OF THE SIXTEENTH ANNUAL
BIOCHEMICAL ENGINEERING SYMPOSIUM

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FOREWORD

This volume represents the proceedings of the Sixteenth Annual Biochemical Engineering Symposium held at Kansas State University on April 26, 1986. Some of the papers describe the progress of ongoing projects, and others contain the results of completed projects. Only brief summaries are given of many of the papers that will be published in full elsewhere. The table of contents gives the name of the student who presented the work at the conference. Requests for further information on the presented papers may be directed to their respective advisors, namely, Dr. Bruce Dale, Dr. Jim Linden or Dr. R. P. Tengerdy at Colorado State University, Dr. Chuck Glatz or Dr. Peter Reilly at Iowa State University, Dr. Rob Davis or Dr. Dhinakar S. Kompala at the University of Colorado, Dr. Rakesh Bajpai at the University of Missouri, and Dr. L. E. Erickson or Dr. L. T. Fan at Kansas State University.

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END PRODUCT INHIBITION OF THE ACETONE-BUTANOL FERMENTATION

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INTRODUCTION

The acetone-butanol fermentation of Clostridium acetobutylicum consists of two distinct phases when grown in batch culture. Initially, organic acids, primarily acetate and butyrate, are produced. These products build up to inhibitory levels and the metabolism of the bacteria shifts to the production of neutral solvents, of which butanol and acetone form the major portion. Concentrations of solvents on the order of 2% w/v bring a halt to metabolism and further production (1). These low titres of product in the fermentation broth lead to high recovery costs. With conventional distillation technology, recovery of butanol from fermentation broth containing less than 3-4% w/v of this product is less than optimal in terms of net energy recovery (2). This paper will discuss the biochemistry of butanol inhibition of Clostridium acetobutylicum. The experimental procedures and results for this study have been reported in detail in Kuhn and Linden (3).

Costa and Moreira (4) have studied inhibition of growth of C. acetobutylicum by its major end products. At levels produced during fermentation, ethanol and acetone were found to be noninhibitory. Butanol was the only product which accumulated to levels which resulted in greater than 50% growth inhibition. Acetic acid and butyric acid were inhibitory to a lesser extent at levels observed during fermentation. Butanol is generally regarded as the major end product responsible for bringing a premature end to C. acetobutylicum fermentations. This alcohol has been shown to affect many cellular processes and properties in C. acetobutylicum (Fig. 1) including cell growth, cellular ATP levels (5), uptake of glucose (1,5-7), xylose (6) and alanine (8), membrane-bound ATPase activity (8), the effectiveness of the cell membrane as a permeability barrier (7) and the ability to maintain a transmembrane pH gradient (5,9). Linden and Moreira (8) have correlated the concentrations of ethanol, butanol and hexanol needed to cause 50% inhibition of cell growth and membrane associated enzyme activity with their octanol/water partitioning coefficients, indicating a hydrophobic site of action. An obvious candidate for this target site is the cell membrane. Indeed, butanol has been shown to interact with the membranes of C. acetobutylicum affecting physical and biological properties. Upon addition to lipid dispersions from C. acetobutylicum, butanol has been shown to cause an increase in membrane fluidity (10) and a decrease in temperature and enthalpy of lipid phase transitions (11). These results are indicative of the disorganizing effects of butanol on the lipid bilayer.
RESULTS AND DISCUSSION

One of the primary functions of biological membranes is to act as a permeability barrier between the cytoplasm and the environment. Many reports in the literature show that alcohols can cause biological membranes to become leaky (12-17). Recently, Hutkins and Kashket (7) have reported that butanol inhibits the uptake of glucose in \textit{C. acetobutylicum} by causing the cell membrane to become leaky to phosphoenolpyruvate and sugar phosphates. We have demonstrated that butanol causes the cell membrane of \textit{C. acetobutylicum} to become leaky to protons.

An interior alkaline pH gradient across the cell membrane has been shown to be necessary for growth and survival of \textit{C. acetobutylicum} (5,9,18). At toxic levels of butanol, the ability to maintain this gradient is destroyed (5,9). This butanol-induced collapse of the ΔpH could result from an increased influx or a decreased efflux of protons across the cell membrane or a combination of these two mechanisms (Fig. 2). H⁺-ATPase, a membrane bound enzyme which catalyzes the expulsion of protons from the interior of the cell, is known to be necessary for the control of internal pH (5,18) and has been shown to be inhibited by growth inhibiting butanol concentrations (5,8). Our results indicate that butanol also causes the cell membrane to become leaky, causing increased influx of protons. Figure 3 shows the kinetics of butanol-induced proton influx in \textit{C. acetobutylicum}. The cells were resuspended to ten times original density in 150 mM KCl. Butanol was added at time zero and the resultant influx of protons is expressed as a rise in the extracellular pH. The effects of butanol on proton influx correlate well with effects on growth. At the subinhibitory butanol concentration of 0.05 M there was little increase in proton influx over control cultures. Between this concentration and 0.2 M butanol, there was a dose dependent increase in proton influx corresponding to a linear decrease in growth rate. At the toxic butanol concentration of 0.25 M there was a dramatic increase in butanol-induced proton influx. The close correlation between effects of butanol on proton influx and cell growth are further illustrated in Fig. 4. Addition of the toxic butanol concentration of 0.25 M caused a two order of magnitude increase in passive proton influx over control cultures.

Increasing temperature, like alcohols, decrease the stability of the lipid bilayer and have been shown to decrease alcohol tolerance in many microorganisms (19-22). Figure 5 shows the effect of temperature on butanol tolerance in \textit{C. acetobutylicum}. As expected, increasing temperature increased the sensitivity of this bacteria to inhibition by butanol. Butanol was also seen to cause a decrease in the optimum temperature of growth in \textit{C. acetobutylicum} (Fig. 6). The optimum growth temperature for the control cultures (no added butanol) was about 37°C while the presence of 0.15 M added butanol caused the temperature optimum to drop to 30°C.
Growth in the presence of alcohols has been shown to elicit a variety of changes in membrane fatty acid composition in a wide range of microorganisms (10,23-27). We have studied the effect of altering the cell membrane fatty acid composition on butanol tolerance in C. acetobutylicum. This was accomplished through growth in a biotin deficient medium with various exogenously supplied fatty acids. Biotin is a necessary cofactor in the biosynthesis of fatty acids. Table 1 lists the results from growth rate inhibition experiments with control (with biotin added), palmitate supplemented and elaidate supplemented cultures at various temperatures. The butanol tolerance of elaidate supplemented cultures did not differ significantly from control cultures. At normal growth temperatures, however, palmitate supplemented cultures were significantly more sensitive to butanol. These trends were also observed in growth yield experiments at 35°C (Fig. 7). Ingram and coworkers obtained similar results for the effects of fatty acid supplementation on ethanol tolerance of E. coli (22). The hypersensitivity of palmitate grown cells to ethanol was attributed to their susceptibility to membrane leakiness. This may also be the case with C. acetobutylicum. Further research should elucidate the functional relationship between membrane fatty acid composition and butanol tolerance in this bacteria. This information will be useful in the rational design of strains with high butanol tolerance and ultimately high productivity.

CONCLUSIONS

The cell membrane is the major target for the toxic effects of butanol on C. acetobutylicum. Butanol was shown to cause the cell membrane to become leaky to protons. The effects of butanol on passive proton influx correlated well with effects on cell growth. This result, as well as the recent findings of Hutkins and Kashket showing that butanol caused the cell membrane to become leaky to PEP and sugar phosphates (7), indicates that butanol causes a general breakdown in the effectiveness of the cell membrane as a permeability barrier. This may be the basis of butanol toxicity in this bacteria. Increasing temperature was shown to have a deleterious effect of butanol tolerance. Butanol tolerance was also affected by the fatty acid composition of the cell membrane. Cells supplemented with palmitic acid were hypersensitive to butanol while those supplemented with elaidic acid had tolerance to butanol similar to control cultures.

ACKNOWLEDGEMENTS

This work was supported by the E.C.U.T. program of the U.S. Dept. of Energy, subcontract no. 957209 administered by the Jet Propulsion Laboratory, Pasadena, CA.
REFERENCES

Figure 1. Effects of butanol on *Clostridium acetobutylicum*

1) Inhibits growth and metabolism.
2) Lowers cellular ATP levels.
3) Inhibits nutrient uptake.
   - Glucose
   - Xylose
   - Alanine
4) Inhibits $\text{H}^+\text{-ATPase}$ activity.
5) Enhances passive proton influx.
6) Inhibits ability to maintain transmembrane pH gradient.
7) Increases membrane fluidity and lowers temperature and enthalpy of membrane phase transitions.
Figure 2. Schematic of proton flux across the cell membrane.

Figure 3. Effect of butanol on passive proton influx into cells of C. acetobutylicum.
Figure 4. Effects of butanol on specific initial rate of proton influx and specific growth rate of \textit{C. acetobutylicum} at 35 \textdegree C.
Figure 5. Effect of temperature on butanol tolerance of *C. acetobutylicum*.

Figure 6. Arrhenius plot of *C. acetobutylicum* grown with 0 M, 0.1 M and 0.15 M added butanol.
Figure 7. Effect of fatty acid supplementation on butanol tolerance of *C. acetobutylicum*.
Table 1. Effect of temperature and butanol concentration on specific growth rate inhibition for various membrane modifications

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<th>Supplemented Fatty Acid</th>
<th>Growth Rate Inhibition Parameter*</th>
<th>Temperature (M)</th>
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<tr>
<td>Control</td>
<td>Half Maximal</td>
<td>30 C 35 C 40 C</td>
</tr>
<tr>
<td></td>
<td>Zero Growth</td>
<td>0.17 0.14 0.12</td>
</tr>
<tr>
<td>Palmitate</td>
<td>Half Maximal</td>
<td>0.13 0.13 0.12</td>
</tr>
<tr>
<td></td>
<td>Zero Growth</td>
<td>0.21 0.19 0.18</td>
</tr>
<tr>
<td>Elaidate</td>
<td>Half Maximal</td>
<td>0.18 0.14 0.11</td>
</tr>
<tr>
<td></td>
<td>Zero Growth</td>
<td>0.27 0.21 0.18</td>
</tr>
</tbody>
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* Extrapolated values of concentrations of butanol which caused the indicated growth rate.
INTRODUCTION

Cheese whey which contains lactose as the main sugar can be categorized into acid or sweet whey. Gillies [1] has reported that different fermentation patterns could be observed from fermentations of acid whey and of sweet whey under the same conditions. Even when whey of same type but from different sources were used, significantly different fermentation profiles were observed as shown in Figure 1 [2]. Here, Fermentation using Kluyveromyces marxianus CBS 397 upon Mozzarella whey developed a more sluggish profile than that upon Cheddar whey. It has been noted that these sweet wheys had distinctively different compositions of monosaccharides glucose and galactose. Cheddar whey contained only small amounts of galactose while Mozzarella whey had both glucose and galactose. These different profiles have been ascribed to the presence of monosaccharides, especially of glucose [2], although the possibility that some undefined components in these wheys may have effects is yet to be explored. A later study [3] has also shown that a considerable variety in the fermentation patterns upon lactose and upon whey was due to the presence of monosaccharides.

A further investigation was, therefore, conducted to examine the interactively kinetic effects of glucose, galactose and lactose upon the rates of growth, metabolism and product formation. A logistic growth model was set up in an attempt to explain the effect of lactose upon cell growth characteristics.
MATERIALS AND METHODS

Microorganism: Yeast Kluyveromyces marxianus CBS 397 was used in this study. The cells were maintained on Yeast and Mold agar (Difco Laboratories, Detroit, MI, USA) slants at 4°C, to be inoculated as required.

Cheese Whey: Spray dried sweet cheese whey powder (Sigma Chemical Co., St. Louis, Missouri) was used as the major substrate. Pure glucose, galactose and lactose supplied by the same company were used as the other carbohydrate sources.

Fermentation Media: Solutions of whey powders were deproteinized as previously described [2]. These were supplemented with glucose, galactose, and/or lactose at desired levels. Deproteinized clear media were not supplemented with any nutrients. The semisynthetic media were prepared according to the composition given by Tu et al. [2].

Inoculum Preparation: Inoculum media (about 100 ml) were prepared in the same fashion as the fermentation media except that only whey powder was used and its concentrations were 25 g/L. A loopful of cells was transferred into this media, which was then incubated in a shaker at 30°C and 200 rpm for 24 hours. For anaerobic fermentations, 100 ml inoculum was added into 1 liter broth. For shaken-flask fermentations 5 ml of inoculum was added into 200 mls. medium, under sterile conditions.

Fermentation: All the anaerobic fermentations were conducted in 1 liter fermenters at a pH 4.5, 30°C and 500 rpm. The shaken-flask fermentations were carried out in 1 liter Erlenmeyer flasks containing 200 ml of solutions at an initial pH 4.5. The temperature was maintained at 30°C and the shaker speed was 200 rpm.

Analytic Methods: Analyses for biomass, lactose, glucose, galactose, and ethanol were carried out using the procedures previously described [2].
RESULTS AND DISCUSSION

Effect of Lactose

Shaken-flask fermentations of 100 g/L CWP (Cheese Whey Powder) solutions supplemented with different amounts of pure lactose were conducted. Results of only two of these are displayed here in Figures 2 and 3. As expected, fermentations having higher lactose concentrations produced higher ethanol levels in broth and also lasted longer. An interpretation of these results can be made in the form of a mathematical model of cell growth and product formation.

Since the controlling nutrients were not well defined in this system, logistic law was used to describe the growth of cells. Product formation was assumed to be dependent upon the concentration of lactose (Monod's model) and ethanol (non-competitive inhibition). Thus the governing equations can be written as

Biomass production

$$\frac{dX}{dt} = \mu_m [1 - \left(\frac{X}{X_m}\right)] X$$ \hspace{1cm} (1)

Ethanol production

$$\frac{dP}{dt} = \nu_m \left[\frac{S}{(K_s + S)} \right] \left[\frac{X_p}{(K_p + P)}\right]$$ \hspace{1cm} (2)

Substrate uptake

$$\frac{dS}{dt} = - \left(\frac{1}{Y_p/s}\right) \left(\frac{dP}{dt}\right) - \left(\frac{1}{Y_x/s}\right) \left(\frac{dX}{dt}\right)$$ \hspace{1cm} (3)

where

- $X$ = biomass concentration, g DW/L.
- $S$ = lactose concentration, g/L.
- $P$ = product concentration, g/L.
- $t$ = time, h.
- $\mu_m$ = maximum specific growth rate, h$^{-1}$.
- $\nu_m$ = maximum specific product formation rate, g/g.h.
- $X_m$ = maximum biomass concentration, g DW/L.
- $Y_{p/s}$ = yield coefficient for product, dimensionless.
- $Y_{x/s}$ = yield coefficient for biomass, dimensionless.

The kinetic parameters ($\mu_m, \nu_m, K_p$) were estimated with the help of Nelder-Mead optimization procedure by fitting the solution of equations (1-3) in the experimental observations. The parameters obtained by this procedure are listed in Table I. Since $S \gg K_p$, $K_p$ was set at a low value. It was found that $\mu_m$ and $X_m$
for all cases changed slightly as the amount of added lactose increased. Accordingly, one may expect a slight influence of lactose upon growth of cells. On the other hand, production parameters were same for all the experiments suggesting no influence of lactose upon formation of product.

Table I. Calculated kinetic parameters for shaken-flask fermentation of 100 g/L CWP plus lactose at pH 4.5 and 30°C.

<table>
<thead>
<tr>
<th>Media</th>
<th>$\mu_m$ (h)</th>
<th>$X_m$ (g/L)</th>
<th>$v_m$ (g/g h)</th>
<th>$K_{ip}$ (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.40</td>
<td>3.7</td>
<td>1.13</td>
<td>19.4</td>
</tr>
<tr>
<td>+ 20 g/L</td>
<td>0.40</td>
<td>3.7</td>
<td>1.13</td>
<td>19.4</td>
</tr>
<tr>
<td>+ 60 g/L</td>
<td>0.40</td>
<td>3.4</td>
<td>1.13</td>
<td>19.4</td>
</tr>
<tr>
<td>+ 100 g/L</td>
<td>0.37</td>
<td>3.2</td>
<td>1.13</td>
<td>19.4</td>
</tr>
<tr>
<td>+ 120 g/L</td>
<td>0.37</td>
<td>3.2</td>
<td>1.13</td>
<td>19.4</td>
</tr>
</tbody>
</table>

Effect of Glucose:

It is has been well known that for anaerobic fermentations in a mixture of glucose and lactose by E. coli, the presence of glucose represses synthesis of inductive lactose permease and $\beta$-galactosidase system [4]. Thus, a sequential uptake of glucose and lactose may be expected. For shaken-flask fermentations of glucose - lactose mixture by K. marxianus CBS 397, however, the uptake behavior of multiple substrates is somewhat different and dependent on the ratio of cheese whey powder (CWP) to glucose.

Two different ratios of CWP to glucose were used in medium for shaken-flask fermentations. Their results are presented in Figures 4 and 5. While the consumption of sugars appears to be sequential or simultaneous depending upon the ratio of the two sugars, biomass growth was continuous with only a single lag-phase at the beginning of the experiments. $\beta$-galactosidase activity was measured according to the method of Toda et al. [5] in the cells from different phases in the batch experiments. No definite evidence of repression of the activity
was observed under the circumstances exhibiting sequential sugar uptake (Figure 5). Evidently the activity of β-galactosidase might be inhibited, but not repressed, by the presence of glucose or any other inhibitor(s) present in CWP. The theory concerning glucose mediated inhibition is supported by the fact that the case of simultaneous sugar consumption (Figure 4) is characterized by substantial changes in the uptake rates of glucose and lactose from the beginning to the end. It is also supported by the continuous culture data reported in Figure 6. Although the residual concentrations of both the sugars in steady state broths were high enough to affect induction/repression, none was observed. Also, the lactose consumption rates at low glucose levels were higher than those at high glucose concentrations.

There is some evidence to suggest that the sequential/simultaneous uptake pattern may be related to the amount of complex nitrogen present in the broth. Note that the experiments reported in Figures 4 and 5 were conducted without any external supplementation. Hence, higher CWP concentrations corresponded to higher nitrogen availability to the cells. Accordingly, experiments were conducted with synthetic media containing different amounts of yeast extract in the broth. These results are presented in Figures 7(a & b). The experiment with higher yeast extract concentration in the broth showed profiles akin to sequential uptake. Hence, yeast extract must be influencing the lactose uptake/metabolism in a manner similar to the way CWP-concentration does.

The phenomena of sequential uptake of glucose and lactose by K. marxianus is not limited to shaken flask cultures. Figures 8(a & b) show the results of two experiments involving CWP with or without glucose under anaerobic conditions. Although the biomass and product formation profiles are not influenced by the presence of glucose, lactose consumption profile is. The effect of glucose upon lactose uptake is summarized by the results presented in Figure 9 for two different CWP concentrations. In all the cases, glucose was associated with strong inhibition of lactose uptake.

**Effect of Galactose:**

Figures 9 also show the lactose consumption profiles for two different levels of CWP supplemented with different amounts of galactose in shaken-flask fermentations. It has been found that glucose exerted a much stronger inhibition than did galactose for both cases. For a mixture of CWP, glucose, and galactose, the coupled inhibitory effects by both monosaccharides is approximately additive.
CONCLUSIONS

Ethanol fermentations of cheese whey solutions, which contains glucose and galactose, by K. marxianus CBS 397 are affected by the presence of glucose and galactose. Effect of Lactose on the cell growth and product formation is not significant even at a high lactose concentration. The effect of glucose on lactose uptake is much stronger than that of galactose. Sequential uptake behavior for the broth having only CWP and glucose can be observed at a higher ratio of CWP to glucose.

REFERENCES


Figure 1: Ethanol production profiles in fermentations of two different sweet sheys. P: Ethanol concentration.

Figure 2: Shaken-flask fermentation of 100 g/L CWP supplemented with 20 g/L lactose. Points represent experimental data and the solid lines are results of simulation. L: Lactose, P: Ethanol, X: Biomass.
Figure 3: Shaken-flask fermentation of 100 g/L CWP supplemented with 60 g/L lactose. Points represent experimental data and the solid lines are results of simulation. L: Lactose, P: Ethanol, X: Biomass.

Figure 4: Shaken-flask fermentation of 25 g/L CWP supplemented with 20 g/L glucose. L: Lactose, G: Glucose, P: Ethanol, X: Biomass.
Figure 5: Shaken-flask fermentation of 250 g/L CWP supplemented with 20 g/L glucose. L: Lactose, G: Glucose, P: Ethanol, X: Biomass.

Figure 6: Results of a chemostat fed with a synthetic medium having 20 g/L glucose supplemented with 20 g/L yeast extract. G: Glucose, L: Lactose, P: Ethanol, Gy: Glycerol, X: Biomass.
Figure 7a: Batch fermentation of a mixture of glucose and lactose in a synthetic medium having 0.5 g/L yeast extract. L: Lactose, G: Glucose, P: Ethanol, Gy: Glycerol, X: Biomass.

Figure 7b: Batch fermentation of a mixture of glucose and lactose in a synthetic medium having 2.0 g/L yeast extract. L: Lactose, G: Glucose, P: Ethanol Gy: Glycerol, X: Biomass.
Figure 8a: Batch fermentation of 100 g/L CWP supplemented with 20 g/L glucose. L: Lactose, P: Ethanol, G: Glucose, X: Biomass.

Figure 8b: Batch fermentation of 100 g/L CWP. L: Lactose, P: Ethanol, X: Biomass.
Figure 9a: Effect of monosaccharides upon lactose uptake in a 25 g/L CWP fermentation in Shaken-flasks.

Figure 9b: Effect of monosaccharides upon lactose uptake in 250 g/L CWP fermentation in Shaken-flasks.
EXTRACTION AND FERMENTATION OF ENSILED SWEET SORGHUM

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Introduction

Sweet sorghum is an alternative biomass source being considered for its potential use in the production of chemical feedstocks and liquid fuels. It can produce high yields of sugars and cellulose with relatively low input costs (1). It is also drought tolerant and can be grown in most regions of the United States (2).

An important consideration in the use of renewable resources is the seasonal availability and storability of the raw material. The availability of fresh crops is constrained by the growing season and their rapid deterioration restricts their use to only a short portion of the year. Therefore, for a process using biomass as a feedstock, to operate year round, a reliable preservation method must be employed. Studies by Linden et al. have shown ensiling to be an effective and inexpensive preservation method for sweet sorghum (3,4,5).

The purpose of this research was to recover the fermentable sugars from the ensiled sweet sorghum in a manner compatible for use as a fermentation medium; e.g. extractions should not dilute the fermentable sugars to concentrations less than 6% (w/v). Samples of ensilage extracts were then fermented to evaluate product yields and to determine if inhibitors or other detrimental substances were formed during the ensiling or extraction steps.

MATERIALS AND METHODS

Extraction Studies:

Sweet sorghum of the Rio variety was collected from CSU's Agronomy Research Center by a field harvester. It was then mixed with ensiling inoculum (0.5 g/kg wet sorghum) containing Lactobacillus plantarum and Streptococcus faecium (Pioneer Silabac 1177). Ensiling was conducted in plastic bags. Air was displaced by the sublimation of dry ice placed into the bottom of the bag before the chopped sorghum was added.

After ten months, the ensiled sweet sorghum was extracted using a pilot scale Bruniche-Olsen continuous countercurrent diffuser (fig. 1) loaned to CSU by the Beet Sugar Development Foundation (Fort Collins, CO). This unit, a model of the continuous slope diffusers typically used in the sugar beet industry, consisted of a round bottomed copper trough (6 inches wide and 4 feet long) containing a single interrupted scroll with...
blades approximately 5.5 inches in diameter. The unit was mounted with a slope of one degree. Auxiliary equipment included a constant speed belt which delivered feed to the lower end of the diffuser and a peristaltic pump which fed preheated water into the trough bottom at the higher end of the extraction unit. Diffusion juice exited through a perforated wiped plate at the lower end, while pulp (exhausted sorghum) was collected from a chute at the higher end as it was kicked out of the unit by the action of the rotating scroll. Temperature was maintained by three jackets, to which the steam flow was controlled by two thermocouples mounted between the three sections of scrolls in the sorghum mass.

The retention time of the sweet sorghum in the diffuser was determined by the time required for a certain amount of ensilage dyed with crystal violet to pass through the unit. Dispersion of the dyed samples occurred because of the mixing action of the scroll during passage from the lower end of the unit to the higher end. The fixed rotation rate of the scroll resulted in a retention time between 90 and 120 minutes.

A set of experiments was performed to determine the effect of temperature and liquid-solid ratio (L/S; the mass of feed water to the mass of feed ensilage) on the fermentable sugar recovery. The temperatures studied were 50°C and 70°C with L/S ratios varying between 5/1 and 1/1 for each temperature. During the runs for a given extraction temperature, 0.6 kg of sweet sorghum ensilage (feed ensilage) was placed on 4 feet of the feed belt, corresponding to a solid feedrate of 23.5 kg/min. For a given L/S ratio, when no change in the dissolved solids content of the diffusion juice was observed (using a Bausch and Lomb Abbe-3L refractometer) juice and pulp samples were collected in tared containers for a specified amount of time. After sampling, the L/S ratio was changed by altering the water feedrate. Representative samples of the feed ensilage, pulp, and juice were analyzed for sugar and organic acid concentration by HPLC analysis described below.

Fermentation:
A one liter working volume batch fermentation was conducted using the 70°C juice as the substrate. As the juice from the diffuser did not have suitable fermentable sugar concentrations, it was concentrated prior to fermentation using a Buchi 110 rotar evaporator. The Clostridium acetobutylicum fermentation was conducted under anaerobic conditions at 37°C with a 100 rpm agitation rate. The pH was controlled at 5.0, after dropping from an initial value of 6.5. During the 96 hour fermentation, samples were taken periodically to determine changes in the substrate and product concentrations using the HPLC analysis described below.

Analytical Methods:
Samples from the extractions and fermentation were analyzed using a Waters Associate HPLC with a Biorad organic acid column (HPX-87H). Twenty microliters of sample were carried through the
47°C column by 0.008 N H₂SO₄ at a flowrate of 0.6 ml/min, which corresponded to a system pressure of 1000 psig. A refractive index detector was used to detect peaks.

The feed ensilage and pulp samples were prepared for analysis by packing a syringe with sorghum and pressing out the juice using a Carver laboratory press. Liquid samples were centrifuged four minutes in an Eppendorf microcentrifuge and then filtered through 0.45 micron filters.

RESULTS AND DISCUSSION

Extraction Studies:

Figure 2 shows the sugars and organic acids found in the ensiled sweet sorghum. The stachyose peak includes raffinose, stachyose and other oligosaccharides, while the fructose peak includes xylose, fructose, mannitol, arabinose and other possible pentoses. The sugars are natural components of the sweet sorghum, whereas presence of mannitol results from the Lactobacillus metabolism of fructose, and presence of lactic acid, acetic acid, propionic acid, and ethanol are from the Lactobacillus metabolism of glucose. For this paper the first four components were grouped together as sugars.

Figure 3 shows the sugar concentration in the pulp. As the L/S increased the pulp sugar concentration decreased. This was due to the increased concentration gradient created by more water at the higher L/S ratios. The scatter in the data was due to the unsteady state nature of the diffuser.

Since the feed ensilage sugar concentration varied (180 mg/g dry wt. and 140 mg/g dry wt. for extraction temperatures of 50°C and 70°C respectively), a way to compare the effects of temperature and L/S ratio is to define an extraction efficiency. By defining a sugar extraction efficiency based on pulp, (the fraction of sugar removed from the feed ensilage) the diffuser was 90% efficient or greater (fig. 4). As the L/S ratio increased, the pulp sugar decreased implying more sugar was extracted. Therefore, extraction efficiency increased with increasing L/S ratio. Figure 3 shows as the L/S ratio decreased the pulp sugar concentration at 70°C approached that at 50°C. This, plus the fact of the lower feed ensilage concentration during the 70°C extraction runs, accounts for the sharper decrease in efficiency with decreasing L/S ratio for the 70°C runs.

Figure 5 shows the sugar concentration in the diffusion juice. As the L/S ratio increased the sugar concentration in the juice decreased. This was due to the dilution effect of the increased L/S ratio. As mentioned earlier, the diffuser was in an unsteady state condition. This not only accounts for the scatter in the data, but also partly for the higher sugar concentrations of the 70°C juice. Material balances indicate a greater accumulation term for the 50°C extraction runs versus the
Figure 4 showed that approximately the same percentage of sugar was removed from the feed ensilage for both temperatures. Therefore, the lower accumulation term for the 70°C runs accounts for the increased sugar concentration of the juice at that temperature.

Figure 5 also shows that the sugar in the diffusion juice was not of a fermentable concentration. The density of the diffusion juices was approximately 1 g/ml. Therefore, the highest sugar concentration of juice obtained was around 30 g/l (3% w/v).

Figure 6 shows the sugar extraction efficiency based on juice; the amount of sugar in the juice divided by that in the feed ensilage. Efficiency increased with L/S ratio. This was because as the L/S ratio increased, more sugar was extracted from the feed ensilage, as shown by the decreasing pulp sugar concentrations (fig. 3). Therefore, more sugar came out in the juice with increasing L/S ratio, thereby increasing the efficiency. Temperature had little effect on the efficiency.

**Fermentation:**

Figure 7 shows the time course of the Clostridium acetobutylicum fermentation on the juice extract from the 70°C runs. From Figure 7a, glucose, lactic acid, and fructose and other pentoses, were all utilized around the same time frame, 24 to 40 hours; glucose 0 to 30 hours, lactic acid, 24 to 40 hours, and fructose, 30 to 36 hours. Stachyose and other oligosaccharides were consumed after 40 hours. The main point is that C. acetobutylicum was capable of utilizing lactic acid, a by-product of the ensiling, as a substrate.

Figure 7b shows the time course for the products. Butyric acid was produced as the substrates were utilized (24 hours) and leveled off at about 36 hours, the end of substrate consumption. Butanol production started and leveled off a little later, 30 and 40 hours respectively, but was still in the time frame of substrate utilization. Slight increases in product concentration after 40 hours were due to oligosaccharide consumption, while larger increases were probably due to HPLC error.

For this particular fermentation, the yield coefficient \( (Y_{p/x}) \) was 0.46. The problem was that high levels of butanol were not formed. This was because the fermentation never seemed to reach the acid break point typically found in C. acetobutylicum fermentations.

**CONCLUSIONS**

Temperature had little effect on the extraction efficiency of the diffuser. The sugar concentration of the juice was not suitable for direct fermentations; juice samples needed to be concentrated first. The Clostridium acetobutylicum fermentation gave a good yield coefficient, but butanol concentrations were low.
REFERENCES


FIGURE 1
SCHEMATIC OF THE DIFFUSER AND AUXILIARY EQUIPMENT

FIGURE 2
COMPOSITION OF SWEET SORGHUM ENSILAGE

CONCENTRATION (MG/G DRY WT.)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mg/g dry wt.)</th>
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<tbody>
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FIGURE 3
PULP SUGAR CONCENTRATION

CONCENTRATION (MG/6 DRY WT.)

FIGURE 4
SUGAR EXTRACTION EFFICIENCY BASED ON PULP

% EFFICIENCY
FIGURE 5
SUGAR CONCENTRATION OF JUICE

CONCENTRATION (MG/6 JUICE)

FIGURE 6
SUGAR EXTRACTION EFFICIENCY BASED ON JUICE

% EFFICIENCY

BASED: SUGAR IN JUICE / SUGAR IN FEED ENZYMES
FIGURE 7
TIME COURSE OF THE CLOSTRIDIUM ACETOBUTYLCUM
FERMENTATION ON 70 C EXTRACT JUICE

A: SUBSTRATES

<table>
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0 20 40 60 80 100 HOURS

♦ LACTATE  △ STACHYOSE  × FRUCTOSE

□ SUCROSE  + GLUCOSE

B: PRODUCTS

<table>
<thead>
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</table>

0 20 40 60 80 100 HOURS

♦ ARABINOSE  ♦ ACETATE  × BUTYRATE  ◀ BuOH
REMOVAL OF NUCLEIC ACIDS FROM BAKERS' YEAST

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INTRODUCTION

Historically, fermentation has been used to produce alcohol, antibiotics, and a variety of other biological materials. With the advent of genetic engineering, fermentation is being used to produce a more extensive range of products. Microorganisms are provided with raw materials in the form of nutrients and precursors which they convert into more valuable materials. Some of these products are secreted into the fermentation broth. Others are retained in the cell. For products remaining in the cell after fermentation, a system of release and recovery is necessary.

Enzymatic hydrolysis or cell homogenization can be used to release the desired product, which is usually a protein. Other cell constituents are released along with the proteins. Among these are nucleic acids.

Microorganisms contain from 1-25% nucleic acids on a dry mass basis (1). The nucleic acids are needed for protein formation, and make up a larger proportion of the cell during growth periods than in periods of little or no growth. Most of the nucleic acids are ribonucleic acids (RNA), which are single stranded biopolymers containing negatively charged phosphate groups along the backbone of the molecule. (See Figure 1.) In the cell, RNA is often attached by covalent or by electrostatic interactions to proteins (2). Even after release from the cell, some nucleic acids and proteins continue to be associated, so that a step designed to recover proteins may recover nucleic acids as well.

Other nucleic acids are released without associated proteins. Because of their linearity and their high molecular
weight, they increase the solution viscosity. The increased viscosity inhibits the extraction of material from the cell homogenate.

Various methods have been used to remove nucleic acids. Base-catalyzed hydrolysis, enzymatic hydrolysis, and chemical extraction have been used (3), but base-catalysed and enzymatic hydrolysis lead to protein denaturation and protein loss, while chemical extraction methods leave a chemical residue. Precipitating agents such as metal salts (4), protamine sulfate (5), or streptomycin sulfate (6) cause protein denaturation. These cationic agents also lack the selectivity that is desirable in removing nucleic acids.

However, Atkinson and Jack (7) investigated the precipitation of nucleic acids from homogenized and from lysed cell suspensions using polyethyleneimine (PEI). PEI, shown in Figure 2, is a branched cationic polyelectrolyte. It derives its cationic character from the association of protons with nitrogen atoms. Lowering the pH of the solution will increase the overall positive charge on the polymer.

Working with Micrococcus lysodeikticus, Bacillus stearothermophilus, and Escherichia coli, Atkinson and Jack found little or no protein precipitation or enzyme degradation when 95% of the nucleic acids were precipitated with PEI. Changes in pH in the range from 4 to 9 had no effect on the nucleic acid removal. Varying the ionic strength also appeared to have little effect in the range from 1 to 500 mM.

PRECIPITATION MECHANISM

Precipitation prepares nucleic acids for removal by filtration, centrifugation, or by other solid-liquid separation processes. Large, strong, dense aggregates are more easily removed from solution than small, weak, aggregates of low density. The precipitating conditions influence the size, strength, and density of the floc, and can be set to optimize the rate of removal. An understanding of the precipitation process aids in determining the desired conditions.

Formation of a solid precipitate is thought to be a three-step process (8). First, a PEI-nucleic acid complex is formed due to the interaction of the positively charged imine groups of the PEI and the negatively charged phosphate groups of the RNA. This complex is brought into contact with other complexes by Brownian motion, forming a primary particle. Aggregation of these primary particles leads to the formation of flocs.

Several mechanisms for floc formation have been suggested. Three that have been proposed are the charge neutralization model, the patch model, and the bridging model.
In the charge neutralization model (9), the primary particles that are formed have little or no residual charge. When two or more particles are brought into contact, they are held together by van der Waals forces. Aggregation continues as other particles are brought into contact. Growth is limited by forces acting on the floc surface. These forces are produced by turbulent shear, pressure fluctuations, and particle collisions.

The patch model (10) suggests that although there may be little or no overall charge on the primary particle, regions of high PEI concentration and areas of high nucleic acid are present at the particle surface, producing localized positive and negative regions. When primary particles approach one another, the positively charged region on one is attracted to the negatively charged area on the other. As this proceeds, the floc grows, until restricted by the forces mentioned above.

In the bridging model (9), the primary particles are charged. One segment of the polyelectrolyte adsorbs to the surface of the primary particle. The free end (or ends) of the polyelectrolyte adsorbs to one or more other particles, increasing the floc size until it is restricted by other forces (such as turbulent shear).

EXPERIMENTAL

One pound of compressed yeast (Red Star compressed yeast, Universal Foods Corporation, Milwaukee, Wisconsin) was suspended in two liters of a 0.15 M NaCl, 4 mM K₂HPO₄ solution (11), and cooled to 4°C. The resulting suspension was homogenized with two passes through a Gaulin 15T-6TM lab homogenizer (Gaulin Corporation, Everett, Massachusetts) at a pressure of 550 kgf/cm². The homogenate was centrifuged at 20,000g (DuPont Sorvall RC-5 Superspeed Refrigerated Centrifuge) for forty minutes to remove cell debris and unbroken cells.

The supernatant from the cell extract was adjusted from a pH of 5.8 to pH 6.0 with 3 M HCl. Varying amounts of a 5% PEI (Aldrich Chemical Company, 50,000-60,000 daltons, adjusted to pH 6.0 with a hydrochloric acid solution) were added to 100 ml samples of the cell extract and mixed with a magnetic stirrer. (The pH of the solution was kept constant with the addition of hydrochloric acid.)

The addition of PEI produced a solid phase which was removed by centrifugation at 20,000g for forty minutes. The resulting supernatant was assayed for nucleic acid and protein content by the orcinol (12) and biuret (13) methods, after using a modified Schneider (12) extraction.

To determine the nucleic acid removal at pH 8.0, the cell extract was adjusted to pH 8.0 with 1 M NaOH. The procedure otherwise remained the same.
RESULTS AND DISCUSSION

The effect of increasing PEI dosage on the precipitation of nucleic acids and proteins at pH 6.0 and pH 8.0 is shown in Figure 3. The higher degree of protonation of PEI at the lower pH increases its effectiveness in removing nucleic acids. The linear relationship that is seen between polymer dosage and nucleic acid content can be explained as a charge neutralization effect between the PEI and the nucleic acid. Bloomfield et al. (15) reported intramolecular condensation of DNA molecules in a dilute solution when 89-90% of the phosphate charges on the DNA were neutralized by polycations. In a more concentrated solution, this condensation would probably correspond to intermolecular condensation, or precipitation. Using Bloomfield's observation that the neutralization of nine of ten negative (RNA) charges produces condensation, a PEI dose of 0.54 mg/g yeast is expected to condense 46.4 percent of the RNA in solution. It was found experimentally that this dose precipitated 46 percent of the RNA.

This result, however, does not account for the presence of proteins in solution. Proteins are coprecipitated with nucleic acids, as illustrated in Figure 3. The linear relationship between the polymer dosage and the amount of protein precipitated ends when the protein loss nears twenty percent. The proteins which are coprecipitated may be attached to the nucleic acids by electrostatic interactions and brought out of solution when the nucleic acids are precipitated, or the proteins may be negatively charged and neutralized by the addition of PEI.

Light microscopy of the aggregates indicated that their size ranged from five to fifty microns. This range agrees well with aggregates of other biopolymer-polyelectrolyte systems.

FUTURE WORK

In order to further characterize the action of PEI on nucleic acids and proteins, more work needs to be done. The use of HPLC and isoelectric focusing will help determine if there is a selective nature to the precipitation of proteins and nucleic acids. The activity of invertase will be measured before and after the addition of PEI. This will help indicate if the addition of PEI causes denaturation, inactivation, or precipitation of the enzyme.

An electronic particle counter will be used to determine the particle size distribution of the flocks prepared under selected mixing, pH, and polymer dosing conditions. Scanning electron microscopy will provide a means to observe the particles and characterize them by shape and structure.

The work to be completed will provide insights into the precipitation process which will be important in designing processes and equipment for nucleic acid removal.
SUMMARY
Nucleic acid removal by polyethyleneimine precipitation is a practical step in the downstream processing of biological materials produced by fermentation. Optimal polymer dosing can lead to the removal of over 95% of the nucleic acids. The loss of protein by coprecipitation can be reduced by proper control of the polymer dose and the precipitating conditions.

The mechanism of flocculation may be clarified by the study of the nucleic acid-PEI system. Charge neutralization certainly plays a part in the process. Further investigation can lead to increased understanding of the nature of polyelectrolyte precipitation.

ACKNOWLEDGEMENTS
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REFERENCES


8. K.M. Clark, personal communication.


Figure 1. Ribonucleic acid. The bases may be adenine, cytosine, guanine, or uracil.
Figure 2. Polyethyleneimine, in protonated form.
Figure 3. The effect of polymer dosage on nucleic acid and protein precipitation. * and ◇ represent the nucleic acid removed at pH 6 and pH 8, respectively, and □ and △ represent the protein lost at pH 6 and pH 8.
Modeling the Effects of Plasmid Replication and Product Expression on the Growth Rate of Recombinant Bacteria

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July 6, 1986

1 Introduction

Novel biochemical techniques employing recombinant DNA technology have been developed which induce microorganisms to synthesize normally non-indigenous compounds. The gene for a specific protein of interest is transferred into a host cell, where it serves as a template for protein synthesis. A popular technique splices the gene into a plasmid and inserts the plasmid into a host cell. Plasmid replication, transcription, and translation occurs by way of the host cell's "machinery". Thus, for the expression of foreign protein, it is necessary for the host cell to be able to accommodate the foreign plasmid. Certain hosts are better able to maintain plasmids, likewise certain plasmids are more stable than others in a given host.

Over-production of a foreign protein can be accomplished by multiplying the number of plasmids in a host cell and by inserting strong promoter regions on the plasmid at the gene of the desired protein. In this way, foreign protein may be synthesized by simply growing the recombinant bacteria in batch or continuous cultures. However, the host cell can shed the plasmid, leaving a cell deficient in product formation. Also, segregation can occur where a plasmid-bearing mother cell divides into one plasmid-bearing cell and one plasmid-deficient cell. Regardless, expression of foreign protein will occur as long as there are cells in the culture with the functioning plasmid.

In addition, the plasmid-bearing cells have an added "metabolic burden" since plasmid replication, transcription, and translation is performed with the host cell's "machinery" and energy. This burden can tax the cell sufficiently to slow its overall growth. This effect increases with the copy number of the plasmid and the extent to which the cell produces the foreign protein. Pierce (1985) [6] found that E. coli cells which produced foreign protein at abnormally high values grew 40% slower than the wild type cells.
The cells in a chemostat initially containing only the recombinant strain will not all be identical, instead there will be a distribution of plasmid copy number. Those with fewer plasmids have a higher probability of shedding or improperly segregating their plasmids yielding the wild-type strain which has no "metabolic burden". Thus, in a short while the population could be overrun by the non-protein producing wild type cells. For example, Pinches et al. (1985) [7] found that after 28 generations the number of *Bacillus subtilis* cells not containing plasmids rose to nearly 40%. They found that the recombinant strain was at most 16% slower growing than the wild-type.

### 2 Modeling

It is desirable to predict the effect of the metabolic burden on the growth rate so that processes can be designed which minimize or circumvent this instability. This paper discusses our initial efforts to calculate the growth rate of recombinant cells. More specifically, our efforts have focused on the metabolic burden placed on the cells both by the presence of multi-copy plasmids and the expression of foreign protein. These two factors have been considered independently in order to identify the more burdensome process: replication or transcription and translation.

If there is little or no significant metabolic strain due solely to the self-replicating plasmids a two-stage chemostat could be employed which minimizes the overall plasmid instability (Siegel, 1985)[11]. The first stage can be devoted to recombinant cell growth by repressing the foreign protein expression. Chemostat instability due only to the presence of a multi-copy plasmid will result from a growth rate difference between recombinant and wild-type strains. Repression of protein synthesis can be accomplished using controllable promoters which serve as protein expression 'on-off' switches. In the system used by Remaut (1981) [8], the host *E. coli* K12 ΔH1Δtrp chromosome contains the temperature-sensitive phage λc1857 repressor. This repressor blocks transcription of the cloned gene from the pl promoter carried on the recombinant plasmids.

The second stage is dedicated to protein expression. Wild-type cells will be formed due to improper plasmid segregation and shedding, however a steady state level of foreign protein expression could be achieved since the recombinant strain will constantly be introduced by the exit of the first stage. In the Remaut system, increasing the culture temperature above 38°C denatures the repressor and causes expression from the pl promoter on the plasmid. Protein production after derepression in batch systems was stable for 70 minutes to 5 hours, depending on the plasmid.
3 Existing Models

One model recently proposed by Lee (1985) \cite{5} incorporates terms for the plasmid copy number and the extent of foreign protein expression:

\[
\mu_{rec} = \mu_{wt} \left[ 1 - \frac{G}{G_{\text{max}}} \right]^m \left[ 1 - \frac{p}{p_{\text{max}}} \right]^n \left[ \frac{s}{K_s + s} \right]
\]

In this model, \(\mu_{rec}\) and \(\mu_{wt}\) are the growth rates of the recombinant and wild type strains, respectively. \(G\) and \(G_{\text{max}}\) are the plasmid content and maximum plasmid content. \(p\) and \(p_{\text{max}}\) represent the existing foreign protein level and its maximum. The substrate, \(s\), and saturation constant, \(K_s\), are in the typical Monod form. There is no biochemical basis for the determination of the constants \(m\) and \(n\). The specific growth rate of the wild type cell is required as a parameter. This is an empirical model which does not identify the specific causes of the metabolic burden. It can be used, however, to separate the effects of replication from transcription and translation.

At the opposite extreme, the Cornell model for a single cell \textit{E. coli} has been adapted to include terms for the presence of plasmids and the expression of foreign protein (Shuler, to be publ.). This model is quite detailed biochemically in that it describes the metabolism and catabolism of 28 cellular components with 35 differential equations. The success of this model for determining a wild type cell's activity has been well documented (Domach, 1984)\cite{1}.

4 Model Development

Our model does not require the wild type maximum growth rate, instead we calculate the growth rate based on the extracellular substrate concentration and levels of nine major intracellular component pools. These components include: protein, \(P\); foreign protein, \(P_f\); chromosomal DNA, \(G\); plasmid DNA, \(G_f\); ribosomes, \(R\); lipids, \(L\); nucleotides, \(N\); amino acids, \(A\); and internal substrate and metabolites, \(S\). A figure depicting the model pools and
External substrate, Cs, passes the cell membrane and becomes part of the internal substrate pool, S. This substrate pool contains all glycolysis and citric acid cycle intermediates. The material leaving this pool flows to one of three compartments: lipids, amino acids, and nucleotides. The lipid pool consists of all cellular membrane material and precursors. There are no significant depletions of this pool in our model.

The amino acids are depleted by the formation of lipids, proteins, and nucleotides. The consumption of amino acids by lipid formation is minor in comparison. The nucleotide pool consists of both deoxyribose and ribose moieties, therefore nucleotide pool depletion occurs by the production of rRNA and DNA, both chromosomal and plasmid. Turnover of the amino acid, nucleotide, protein, and ribosomal RNA pools is included.

The synthesis rate of any component depends upon the concentration of its principle reactants. Protein production also depends upon the ribosomal RNA level, and foreign protein production on the plasmid level as well.

The model consists of a differential equation for each pooled constituent. Each differ-
The differential equation consists of a rate of synthesis term and the appropriate terms for depletion or turnover rate. The rate expressions are of the Monod form with parameters consisting of a maximum rate and a saturation constant. Therefore, the rate of formation of a particular constituent depends upon other constituent concentrations with the order varying between zero and one. Stoichiometric coefficients maintain the material balance between components.

A unique feature of our model is the direct calculation of the instantaneous specific growth rate. The levels of all constituents are expressed in mass fraction or gm. component per gm. dry cell mass. Thus, the instantaneous growth rate is simply the summation of all the net synthesis terms for each component pool.

Since our equation dimensionality is not consistent with the majority of biochemical engineering literature, a detailed explanation of one differential equation follows. The rate of formation of chromosomal DNA depends upon the nucleotide level and itself (more replication forks imply a higher overall replication rate). The following equation is expressed in grams/unit vol.-time.

$$\frac{d(GC)}{dt} = \mu_{\text{max}} \left( \frac{N}{K_N + N} \right) GC$$  \hspace{1cm} (1)

$C$ is the cell mass per unit volume, $G$ the mass fraction of DNA (gm/gm cell mass), $N$ the nucleotide mass fraction. In batch cultures the following expression for specific growth rate is commonly used:

$$\frac{dC}{dt} = \mu C$$  \hspace{1cm} (2)

The specific growth rate is given by $\mu$. By imparting the chain rule on the left-hand side in eq. 1 and substituting eq. 2 into eq. 1 the following result is obtained which is listed in Table 1. with the remaining model equations.

$$\frac{dG}{dt} = \mu_{\text{max}} \left( \frac{N}{K_N + N} \right) G - \mu G$$  \hspace{1cm} (3)

A fourth order Runge-Kutta algorithm was used to solve this set of equations.

### 5 Results and Significance

All model simulations were performed using the batch culture equations at a fixed external substrate concentration of 10 gm/liter. Figure 2 illustrates the exponential increase in total mass over time for the wild type cell. The specific growth rate calculated was $0.91 \text{ hr}^{-1}$.

Figure 3 depicts the same increase in cell mass over time for a recombinant cell with approximately 50 plasmids per cell. There is no expression of foreign protein, and no
difference was found in the calculated growth rate: 0.91 hr\(^{-1}\). Thus, there appears to be no significant metabolic burden placed on the cells due to the maintenance of a fifty copy per cell plasmid. The significance of this simulation is that the two-stage chemostat described previously should have a stable first stage constantly introducing the recombinant strain into the second stage.

In figure 4, foreign protein was expressed at 5% of total protein. The calculated growth rate dropped to 0.87 hr\(^{-1}\). For comparison, this figure includes the previous two simulations. It is apparent that more time is required for the total mass to accumulate in the culture with foreign protein expression.

This demonstrates that a chemostat with sterile feed would be unstable, eventually containing only wild type cells. However, a stable first stage producing a feed with the proper recombinant strain to the second stage should produce a stable reactor system, continually producing the foreign protein.

..6 Summary

A model has been developed which predicts the drop in growth rate experienced by bacteria due to the added "metabolic burden" placed on cells by the replication of foreign plasmids and expression of foreign protein. This model calculates the growth rate of bacteria from the levels of external substrate and nine internal constituent pools. Nine differential equations have been included.

Future enhancements of this model will include simulation of complete batch and continuous fermentations. Recent papers have indicated that the metabolic burden is most severe at low dilution rates in continuous cultures (Pinches, 1985\[7\] and Seo, 1985\[9\]). Apparently plasmid copy number and the expression of foreign protein is highest at low dilution rates. Future simulations should be able to account for these trends.

References


Table 1: Model Equations

\[
\frac{dS}{dt} = R_s - \left[ \beta_1 \frac{dA}{dt_s} + \beta_2 \frac{dN}{dt_s} + \beta_3 \frac{dL}{dt_s} + \mu S \right]
\]

\[
R_s = \nu \left[ \frac{C_s}{K_{ts} + C_s} \right] \frac{K_s}{K_s + S^n}
\]

\[
\frac{dA}{dt} = k_1 \left[ \frac{K_a}{K_a + A} \right] \left[ \frac{S}{K_a + S} \right] - K_T \left[ \frac{K_{TA}}{K_{TA} + S} \right] A
- \varepsilon_1 \frac{dN}{dt_s} - \gamma_1 \frac{dP}{dt_s} - \varepsilon_2 \frac{dL}{dt_s} - \mu A
\]

\[
\frac{dN}{dt} = k_2 \left[ \frac{K_N}{K_N + N} \right] \left[ \frac{A}{K_{NA} + A} \right] \left[ \frac{S}{K_{NS} + S} \right] - K_{TN} \left[ \frac{K_{TN}}{K_{TN} + S} \right] N
- \frac{dG}{dt_s} - \frac{dG_f}{dt_s} - \frac{dR}{dt_s} - \mu N
\]

\[
\frac{dP}{dt} = \mu_1 \left[ \frac{A}{K_{PA} + A} \right] R - K_{TP} P - K_{TP}^l \left[ \frac{k_{TS}}{K_{TS} + S} \right] P - \mu P
\]

\[
\frac{dG}{dt} = \mu_2 \left[ \frac{N}{K_{GN} + N} \right] G - \mu G
\]

\[
\frac{dR}{dt} = \mu_6 \left[ \frac{N}{K_{RN} + N} \right] - K_{TR} R - \mu R
\]

\[
\frac{dP_f}{dt} = \mu_4 \left[ \frac{A}{K_{PA} + A} \right] RG_f - K_{TP} P_f - K_{TP}^l \left[ \frac{K_{TP}}{K_{TP} + S} \right] - \mu P_f
\]

\[
\frac{dG_f}{dt} = \mu_5 \left[ \frac{N}{K_{GN} + N} \right] G_f - \mu G_f
\]

\[
\frac{dL}{dt} = \mu_3 \left[ \frac{K_{LS}}{K_{LS} + S} \right] \left[ \frac{A}{K_{LA} + A} \right] - \mu L
\]

<table>
<thead>
<tr>
<th>Component</th>
<th>Meaning</th>
<th>Component</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
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<td>Substrate</td>
<td>R</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>A</td>
<td>Amino Acids</td>
<td>P</td>
<td>Foreign Protein</td>
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<tr>
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<td>G</td>
<td>DNA</td>
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<td>\gamma</td>
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</tr>
<tr>
<td>\mu</td>
<td>Maximum Synthesis Rates</td>
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</table>
Figure 2.

Total Cell Mass - Wild Type & Plasmids

Figure 3.

Total Cell Mass - Wild Type & Recombinant

Figure 4.
Indirect Estimates of Cell Concentrations 
in Mass Cultivation of Bacterial Cells

by

Andrew J. Fischer
&
Rakesh Bajpai

Chemical Engineering Department
University of Missouri-Columbia
ABSTRACT

Indirect estimation of cell concentrations has produced a methodology from which a model can achieve excellent predictions of growth rates and concentrations provided there is an absence of product formation. Existing methods fail in accurate estimation when acetic acid or similar substances are produced. The similarities in molar ratios of carbon, hydrogen, nitrogen and oxygen of the carbon-energy substrate (glucose) with either a microbial product or the biomass under investigation result in the algorithm failing to recognize, and account for, product formation in estimation of the system. This discussion will center around the accuracy of prediction the estimation method can achieve.

Introduction

Microbial systems can provide a wide spectrum of valuable substances, from bulk chemicals to nutritional additives to biologically active agents. A vital economic consideration for processes involving microbial output is this productivity or the overall rate of formation of the desired products per unit volume of the system. Product formation is a function of the proportion of active cells present in the system and the specific activity of each individual cell. Manipulation of the environment surrounding the organisms greatly influences both of these factors. Environmental control is accomplished through controlled addition of specific nutrients with the awareness of broth-characteristics of concentrations of cells, glucose,
essential and non-essential nutrients and the possible products formed by the cells. Indirect methods utilizing elemental balance techniques have been developed for measuring these characteristic properties because on-line sensors are either unavailable or not generally applicable. Using these methods, microbial reactions may be represented by the general stoichiometric equation as follows:

\[
\text{substrate} \quad \text{biomass} \quad \text{product} \\
CH_kO_mN_n + bO_2 + cNH_3 \quad \gamma_CCH_aO_bN_\delta + zCH_aO_bN_\delta + dCO_2 + eH_2O \quad (1)
\]

where only one microbial product, other than carbon dioxide is being considered. These elemental compositions have been normalized to a single c-atom without any loss of generality. The stoichiometric coefficients (b, c, \(\gamma_C\), z, d, and e) are the unknowns that are generally not constant over the duration of fermentations and must be determined at any time to define the microbial system.

The elemental balances for carbon, oxygen, hydrogen and nitrogen, available from the stoichiometric equation, yield four relationships between the unknown coefficients:

\[
\begin{align*}
C: \quad 1 &= \gamma_C + Z + d \\
H: \quad k &= \gamma_C + Z + e - 3c \quad (2) \\
O: \quad m &= \gamma_C + Z + d + e - 2b \\
N: \quad n &= \gamma_C + Z - c
\end{align*}
\]

Other macroscopic relationships, equation (3), can be established for the oxygen uptake rate (\(QO_2\)), carbon dioxide evolution rate (\(QCO_2\)), and ammonia consumption rate (\(QNH_3\)) to
complete a system of six equations with the six unknown coefficients that will define the system at any moment. This technique requires on-line measurement of the consumption and evolution rates while not requiring any prior knowledge or model of the characteristic behavior of the microorganism.

\[
\frac{Q_s}{M_s} = \frac{QO_2}{b} = \frac{QCO_2}{d} = \frac{QNH_3}{c} = \frac{Q_B}{V_c \cdot M_B} = \frac{Q_P}{Z \cdot M_p}
\]

(3)

Important limitations arise in this analysis when certain specific products are formed by the microbial agents, or when certain specific substrates are utilized for growth. These situations result in inter-dependence of the six equations mentioned above and thus singularity, causing an inability to determine the stoichiometric coefficients [3]. This happens whenever

i) the degree of reductance of the product 
\( \nu_p (\nu_p = 4 + \alpha - 2\beta - 3\delta) \) is equal to that of substrate. \( \nu_s (\nu_s = 4 + k - 2m - 3n) \).

ii) the degree of reductance of the biomass 
\( \nu_B (\nu_B = 4 + \alpha - 2\beta - 3\delta) \) approaches \( \nu_s \).

Organic acids (CH₂O) illustrate the problem noted in the first situation. These acids are the primary product in many fermentations and have a reductance equal to that of a common substrate, glucose, thus preventing analysis by the technique above. The reductance of biomass lies in the range of 4.1 - 4.3 and therefore approaches the reductance of the substrate ( \( \nu_s = 4.0 \)). Again, the analysis cannot be accomplished due to the associated errors of measurement of \( QO_2, QCO_2 \) and \( QNH_3 \).
Situations that will allow the analysis for the stoichiometric coefficients to be successful involve the use of non-carbohydrate substrate or the production of some other metabolite such as ethanol (CH$_3$OH). Fermentations involving no metabolite production ($Q_p = 0$) during biomass production on glucose submit to utilization of only one of the gas consumption or evolution rates ($QO_2/QCO_2$) to determine unique solutions for the coefficients. Therefore, another on-line measurable macroscopic relationship must be evaluated. There are two possible routes to successful analysis of the system. The first involves the oxygen uptake rate related to the biomass production [1]. The other possibility is relating the alkali consumption to biomass production [3]. Both may also be related to organic acid production to predict its evolution at any time. This paper investigates the possibility of predicting biomass production by relating the oxygen uptake rate during the growth of $E$. coli cells on glucose.

**Theoretical Background**

Smith [9] investigated growth of $E$. coli K-12 (ATCC 23716) in batch and continuous cultures and proposed the following model for cell growth and product (acetic acid) formation:

$$\mu = \mu_{\text{max}} \cdot \frac{S}{K_S + S} \cdot \frac{K_p}{K_p + P}$$

(4a)

$$\mu = 0 \quad \text{when } \mu \leq 0.52 \text{ hr}^{-1}$$

$$= 0.81 (\mu - 0.52) \quad \mu \leq 0.52 \text{ hr}^{-1}$$

(4b)
During fed-batch culture, the governing equations can be written as:

\[
\frac{dX}{dt} = \mu x - \frac{F}{V} x \tag{5a}
\]

\[
\frac{dP}{dt} = \mu x - \frac{F}{V} p \tag{5b}
\]

\[
\frac{dS}{dt} = -\frac{\mu x}{V_{x/s}} - \frac{v x}{V_{p/s}} + \frac{F}{V} (S^f - S) - m_x x \tag{5c}
\]

\[
\frac{dV}{dt} = F \tag{5d}
\]

\[
\frac{dCO_2}{dt} = K_{La} (CO_2^* - CO_2) - \frac{\mu x}{V_{x/0}} - \frac{v x}{V_{p/0}} - m_o x + \frac{F}{V} (CO_2^f - CO_2) \tag{5e}
\]

where yield ratios are used to relate oxygen and substrate consumption to biomass and product formation. As shown in Figure 1, the validity of the growth model is verified during simulations with slow feeding in which no product formation takes place.

Dissolved gas concentrations rapidly achieve pseudo-steady states, thus the derivative in equation (5e) can be equated to zero and neglecting the dilution term,

\[
K_{La} (CO_2^* - CO_2) = \frac{\mu x}{V_{x/0}} + \frac{v x}{V_{p/0}} + m_o x \tag{6}
\]
Equation (6) represents the oxygen uptake rate ($Q_{O_2}$) of the cells related to growth and product formation as follows:

$$Q_{O_2} = \frac{\mu X}{V_{x/o}} + \frac{\nu X}{V_{p/o}} + m_0 X$$  \hspace{1cm} (7)

Similarly, the carbon dioxide evolution rate ($Q_{CO_2}$) can be related to growth and product formation by:

$$Q_{CO_2} = \frac{\mu X}{V_{x/co_2}} + \frac{\nu X}{V_{p/co_2}} + m_{co_2} X$$ \hspace{1cm} (8)

Biomass and product formation rates ($Q_B$ and $Q_P$ respectively) may then be determined. When both the yield and maintenance factors ($Y_{x/o}, m_0$) are constant, equation (7) along with (2) and (3) and the measurements of $Q_{O_2}$ or $Q_{NH_3}$ will provide for the evaluation of the stoichiometric coefficients. This will in turn yield $Q_B$ and $Q_P$ since:

$$Q_B = \mu X$$

$$Q_P = \nu X$$ \hspace{1cm} (9)

Because of the inclusion of the maintenance term ($m_0 X$) in (7) the solutions must be obtained in conjunction with (9) that requires simultaneous integration to update the biomass and product concentrations ($X$ and $P$). If the yield and maintenance factors can be related to state variables such as $X$, $P$, $S$, CO$_2$, pH or temperature there is no need for them to remain constant. Again simultaneous integration of (5) to update the relevant state variables will be required.
When there is no product formation, equations (7) or (8) may be substituted into (5a) resulting in:

$$\frac{dx}{dt} = (Q_0 - m_0X) \frac{X}{V} - F X$$

(10)

which in differential form yields:

$$X_t = X_{t-\Delta t} \left[(Q_0 - m_0X_{t-\Delta t}) \frac{X_t}{V} - \frac{F}{V} X_{t-\Delta t}\right]$$

(11)

where \(\Delta t\) is assumed to be small so that \(Q_0\) and \(X\) would not change significantly. During low density, slow feeding cultivations the dilution effect may be neglected.

**Materials and Methods**

**Microorganisms:** *E. Coli* K12 (ATCC 23716) was investigated in all experiments. The cells were maintained on nutrient agar slants (Difco, Inc., Detroit, MI.) at 40°C.

**Media:** The modified growth media of Shiloach and Bauer [7] reported by Smith and Bajpai [6] was utilized. Experiments presented here used the modified composition given in Table 1.
### TABLE 1. Modified Media Composition

#### A. Batch composition.

<table>
<thead>
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<th>Concentration (g/l)</th>
</tr>
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<tbody>
<tr>
<td>glucose</td>
<td>5.0</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.5</td>
</tr>
<tr>
<td>(NH$_4$)$_2$HPO$_4$</td>
<td>3.5</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.35</td>
</tr>
<tr>
<td>yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>FeSO$_4$·4H$_2$O</td>
<td>0.333</td>
</tr>
<tr>
<td>ZnSO$_4$·4H$_2$O</td>
<td>0.192</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>0.168</td>
</tr>
<tr>
<td>NaMoO$_4$·2H$_2$O</td>
<td>0.168</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>0.084</td>
</tr>
<tr>
<td>CuCl$_2$·2H$_2$O</td>
<td>0.084</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.084</td>
</tr>
</tbody>
</table>

#### B. Feed composition.

<table>
<thead>
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<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>400.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>94.0</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>5.54</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>1.33</td>
</tr>
</tbody>
</table>

The experiments were conducted in a 14 liter New Brunswick Microfirm bioreactor fitted with controls for agitation, aeration, pH, temperature, and dissolved oxygen. Inlet gas flow rates were monitored with a mass flow meter, while exit gases were dried and analyzed with a paramagnetic oxygen analyzer and an infra-red carbon dioxide analyzer for online exit gas analysis. Data from gas analyzers, mass flow meter, dissolved oxygen probe, and load cells under the feed tanks were monitored by a data acquisition unit (ISAAC 2000, Cyborg Corp. MA) connected to an IBM-XT microcomputer. Sampling frequency was every 10 seconds, and all values were smoothed with a sixth order polynomial filter before analysis.
Results and Discussion

The most crucial parameters in the governing equations (5) were observed to be the yield and maintenance factors. Based on a number of experiments conducted with different modifications of Shiloach and Bauer media [7], the yields were found to depend on the media and the conditions of growth. Figure 2 illustrates the biomass profile for an experiment in which glucose was fed at several different constant rates, after batch completion. Experimental data taken between 7 and 15 hours was used to estimate $Y_{X/s}$ and $m_s$ in equations (4, 5a, 5c) with a non-linear least squares parameter optimization method. Since no acetic acid production was observed only $Y_{X/s}$ was evaluated and found to be 0.35 (gDW cells /g glucose consumed). $m_s$ values between 0.001 to 0.01 (g glucose /g DW cells/hr.) did not influence this simulation outcome. The model equations (4, 5a, 5c) were solved for the same 8 hour period to generate smooth values of $X$ and $dx/dt$ every half hour. These values were substituted into equations (7) and (8) to estimate the parameters $Y_{X/o}$, $m_0$, $Y_{X/CO_2}$, and $m_{CO_2}$. The factors were then utilized in equation (11) which must hold during zero product formation. The characteristic equation with the estimated values included was evaluated using on-line measured values of $QO_2$ at sampling intervals of 10 seconds. The predicted profile is presented in Figure 3 as well as the measured dry weight data. The fit is considered to be excellent.
The constancy of the yield and maintenance factors could not be assured unless they could predict other independent biomass profiles. Therefore another feeding strategy was investigated. In this experiment, the batch portion was immediately followed by an exponential glucose feeding. The feeding rate of the substrate solution was increased every minute according to the following characteristic equation:

\[ F(t) = \frac{X(tp)}{s} \frac{V(to)}{Yx/s} \exp[\mu(t-to)] \]  

(12)

where \( \mu \) was held constant at 0.2 hr\(^{-1}\) Figure 4 illustrates the predicted profile (using parameters from the experiment shown in Figure 2) as well as the dry weight data. Again the fit is considered excellent.

**Conclusions**

*E. Coli* growth on glucose can be monitored by the on-line measurement of the oxygen uptake rate. Although the inlet gas was switched from air to pure oxygen, to prevent oxygen limitation, the parameters did not change and estimation was still possible with equation (11).

The procedure outlined here can effectively predict the critical constants necessary to estimate biomass production in suitably designed fed-batch experiments. In these experiments acetic acid was not produced and thus its effect has not been determined.

Experiments designed to grow these cells at higher growth rates, causing acetic acid accumulation, will allow the determination of its affect, and the possibility of on-line estimation using the characteristic equations in (5).
Figure 1: Profile of growth with no product formation.

Figure 2: Averaged growth profile with dry weight data points.

Figure 3: Continuously estimated growth profile with dry weight data points.

Figure 4: Continuously estimated growth profile during exponential feed with dry weight data points.
Nomenclature

b moles of oxygen consumed per mole substrate consumed.
c moles of ammonia consumed per mole substrate consumed.
\( \text{CO}_2 \) dissolved oxygen concentration (mmoles/liter).
\( \text{CO}_2^f \) dissolved oxygen concentration in feed (mmole/liter).
\( \text{Co}_2^* \) solubility of oxygen at exit gas oxygen partial pressure (mmoles/liter).
d moles carbon dioxide produced per mole substrate consumed.
e moles water produced per mole substrate consumed.
F feed rate of nutrient solution (liters/hr.).
K_P constant of growth inhibition by product (g/liter).
K_S Monod's constant for growth (g/liter).
K_{La} volumetric mass transfer coefficient (hr.\(^{-1}\)).
m_{CO}_2 maintenance production of CO\(_2\) (mmoles/g DW cells/hr.).
m_{O}_2 maintenance consumption of oxygen (mmoles/g DW cells/hr.).
m_S maintenance consumption of sugar (g/g DW cells/hr.).
M_C molecular weight of cell mass having one carbon atom.
M_P molecular weight of product having one carbon atom.
M_S molecular weight of substrate having one carbon atom.
Q_B biomass production rate (g DW cells/liter/hr.).
Q_{CO}_2 carbon dioxide evolution rate (mmoles/liter/hr.).
Q_{NH}_3 ammonia consumption rate (mmoles/liter/hr.).
Q_{O}_2 oxygen consumption rate (mmoles/liter/hr.).
Q_p product formation rate (g/liter/hr.).
Q_s substrate consumption rate (moles/liter/hr.).
P product concentration (g/liter).
S glucose concentration in broth (g/liter).
S_f glucose concentration in feed solution (g/liter).
\( t \)  
\( t \) time (hr.).

\( X \)  
\( X \) biomass concentration (gDW cells/liter).

\( V \)  
\( V \) volume of liquid (liters).

\( Y \)  
\( Y \) yield factors.

\( Y_c \)  
\( Y_c \) normalized moles of biomass produced per mole of substrate consumed.

\( z \)  
\( z \) normalized moles of product formed per mole of substrate consumed.

\( \mu \)  
\( \mu \) specific growth rate (hr.\(^{-1}\)).

\( \mu_{\text{max}} \)  
\( \mu_{\text{max}} \) maximum specific growth rate (hr.\(^{-1}\)).

\( \nu \)  
\( \nu \) specific product formation rate (g/g DW cells/hr.).

\( \nu_B \)  
\( \nu_B \) degree of reductance of product.

\( \nu_P \)  
\( \nu_P \) degree of reductance of product.

\( \nu_S \)  
\( \nu_S \) degree of reductance of substrate.
References


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A MATHEMATICAL MODEL FOR LIQUID RECIRCULATION IN AIRLIFT COLUMNS

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INTRODUCTION

Airlift fermentors can be designed in one of two basic configurations. Either the upflowing gassed liquid and the downflowing liquid are separated by a draft tube or a baffle plate suspended inside the reactor (internal loop), or the two streams flow in separate pipes connected at the top and bottom (external loop). It has been shown that, because of the introduction of gas, a strong liquid circulation pattern is developed. The liquid circulation rate is considered to be one of the key parameters in the design and scale-up of the airlift reactor. It affects mixing characteristics of the reactor, gas hold-up, volumetric mass and heat transfer coefficients and uniformity of reaction temperature and cell concentration; thus, it affects the overall performance of the reactor.

Fundamental studies of airlift reactors have been reported by Hatch (1), Hatch et al. (2), and Orazem and Erickson (3), among others. The problem of modeling an airlift reactor has been considered by Hatch (1), Ho et al. (4) and Merchuk et al. (5). The main difficulty in such modeling is the lack of information on the hydrodynamics of the airlift reactor.

One of the earliest studies of the draft-tube airlift column was by Lamont (6) who predicted the liquid circulation rates in a Pachuca tank using airlift pump theory. In this semiempirical model the energy transferred as the air expands in rising through the vessel is corrected for energy dissipation due to bubble slip and the remainder gives rise to velocity heads associated with liquid flow in the vessel. Whalley and Davidson (7) also modeled the liquid circulation in a shallow bubble column based on an energy balance method and showed that the liquid circulation velocities calculated by the energy balance method are closer to reality than those predicted by pressure balance. More recently, Jones (8) proposed a simple model based on airlift pump theory to predict liquid circulation velocities in draft-tube bubble columns. Reasonable agreement was found only for draft-tubes ≤ 0.121 m in diameter at gas flow rates ≤ 0.0004 m³/s; significant deviations were found with larger draft-tube diameter.

The primary objective of this study was to develop a mathematical model based on the macroscopic energy balance and determine the applicability of this model for the prediction of liquid circulation velocities in airlift fermentors.

THEORY

The basis of the energy balance method is to equate:
(1) the rate of energy input to the fermentor by the gas to
(2) the rate of energy dissipation by the fluid motion.
For buoyancy-driven two phase flows, the supply of energy results from introduction of the dispersed phase. The energy input rate for gas-liquid dispersion is given by the following equation:

\[ E_1 = QP [\ln(1+p_L g H_L / P)] \]  

(1)

where

- \( E_1 \): energy input rate (W),
- \( Q \): volumetric gas flow rate (m³/s),
- \( P \): pressure at the top of the column (N/m²),
- \( \rho_L \): density of the liquid phase (kg/m³),
- \( H_L \): ungassed liquid height (m),
- \( g \): acceleration due to gravity (m/s²).

The energy dissipation in an airlift column is due to the following factors:

1. Energy dissipation in the upflow zone due to wakes behind the bubbles, \( E_{d1} \).
2. Energy dissipation in the top and bottom regions due to liquid turnaround and other resistance to liquid recirculation in the column, \( E_{d2} \).
3. Energy dissipation in the downflow zone due to the upflow motion of bubbles with respect to the liquid, \( E_{d3} \).
4. Energy dissipation due to the viscous drag at the walls of the column, \( E_{d4} \).

An order of magnitude calculation for a 15 cm diameter column indicates that the dissipation due to viscous drag, \( E_{d4} \) makes up only 1 to 2% of overall energy input for liquid viscosities up to 20 mPa s. Therefore, the energy dissipation due to this mode is negligible.

The energy dissipation rate, \( E_{d1} \) in the bubble wakes can be evaluated by considering a bubble cloud with uniform fractional hold-up \( E_{G1} \), ungassed liquid height \( H_L \), liquid density \( \rho_L \), horizontal cross-sectional area in the upflow zone \( A_1 \), and liquid and gas superficial velocities \( U_{L1} \) and \( U_G \). \( E_{d1} \) is the result of three terms, namely the rate of loss of pressure energy of the liquid = \( A_1 \rho_L g H_L U_{L1} \), the rate of loss of pressure energy of the gas = \( A_1 \rho_L g H_L U_G \), and the rate of gain of potential energy of the liquid = \( A_1 \rho_L g H_L U_{L1} (1-E_{G1}) \), giving

\[ E_{d1} = A_1 \rho_L g H_L E_{G1} [U_G/E_{G1} - U_{L1} / (1-E_{G1})] \]  

(2)

The slip velocity, the relative velocity of the bubbles with respect to the liquid, is equal to \( U_G/E_{G1} - U_{L1} / (1-E_{G1}) \). Using Turner's assumption that slip velocity is equal to the terminal velocity of bubbles rising in a stagnant liquid, \( V_{b\infty} \), gives

\[ E_{d1} = A_1 \rho_L g H_L E_{G1} V_{b\infty} \]  

(3)

As might be expected, gas and liquid flow rates do not appear in this equation; the energy dissipation due to wakes behind the bubbles is mainly determined by the gas hold-up.

Evaluation of the energy dissipation \( E_{d2} \) in the airlift column gives
\[ E_{d2} = \sum_{i=1}^{2} \frac{1}{2} \rho_L V_i^3 e_i A_i (1-E_{G1}) \]  \hspace{1cm} (4)

where \( e_1 \) : friction loss factor due to liquid turnaround and other resistance to liquid recirculation in the column,
\( V_i \) : interstitial liquid velocity = \( U_{Li}/(1-E_{G1}) \) (m/s),
\( A_i \) : horizontal cross-sectional area (m²),
1,2 : represent the upflow zone and downflow zone, respectively.

The energy dissipation \( E_{d3} \) can be evaluated by considering the macroscopic energy changes of the liquid between the top and bottom regions of the downflow section. If we neglect the change of kinetic energy, \( E_{d3} \) is the result of two terms, namely the rate of loss of potential energy = \( A_2 \rho_L g H_{L} V_2 \) and the rate of gain of pressure energy = \( A_2 \rho_L g H_{L} (1-E_{G2}) V_2 \), giving

\[ E_{d3} = A_2 \rho_L g H_{L} E_{G2} V_2 \]  \hspace{1cm} (5)

The energy balance, therefore, takes the following form:

\[ E_i = E_{d1} + E_{d2} + E_{d3} \]  \hspace{1cm} (6)

If we substitute the expressions of \( E_{d1} \), \( E_{d2} \), and \( E_{d3} \) into Equation (6) we get

\[ E_i = A_1 \rho_L g H_{L} E_{G1} V_{dmo} + \sum_{i=1}^{2} \frac{1}{2} \rho_L V_i^3 e_i A_i (1-E_{G1}) + A_2 \rho_L g H_{L} E_{G2} V_2 \] (7)

Since there is no net liquid flow from the column the primary liquid volumetric flowrates in upflow and downflow zones are equal and so from continuity:

\[ V_1 A_1 (1-E_{G1}) = V_2 A_2 (1-E_{G2}) \]  \hspace{1cm} (8)

Hence, the interstitial liquid velocities \( V_1 \) and \( V_2 \) in a given geometry of airlift column may now be predicted from Equations (7) and (8) for a given volumetric gas flow rate, \( Q \), and ungassed liquid height, \( H_{L} \), provided the friction loss factors, \( e_1 \) and \( e_2 \), are known and the characteristic bubble rise velocity, \( V_{dmo} \), and gas hold-ups, \( E_{G1} \) and \( E_{G2} \), can be either estimated from appropriate correlations or measured from the experiment.

The bubble rise velocity, \( V_{dmo} \), can be predicted by the method of Mendelson (10), for example, from the expression:

\[ V_{dmo} = \left[ \frac{2 \sigma / \rho_L d_l^2 + g d_l^2}{2} \right]^{1/2} \] (9)

where \( \sigma \) is the surface tension and \( d_l \) is the equivalent bubble diameter in the gas-liquid system. It can be shown that the bubble rise velocity calculated from
Equation (9) is approximately equal to 0.23 m/s in the range of equivalent bubble diameter from 0.002 m to 0.01 m for air-water system. Since under normal operating conditions the diameters of most of the bubbles generated in an airlift column lie in this range, we assume that the terminal rise velocity of bubbles in the range of interest (bubblly flow regime) is independent of bubble size and equal to 0.23 m/s. The friction loss factors, \( \varepsilon_1 \) and \( \varepsilon_2 \), can be determined by one of two methods: (a) simultaneous solution of the macroscopic balances or (b) experimental measurement. Theoretically, these values will vary with flow condition and column configuration. However, due to the lack of information of the experimental data for \( \varepsilon_1 \) and \( \varepsilon_2 \) we will assume the friction loss factors are constants and roughly equal to 2 to 4 depending on the resistance to liquid recirculation in the system.

**DISCUSSION**

The energy balance model described above was solved numerically using Newton-Raphson iteration and was tested by comparing the liquid circulation velocities predicted by this model with the experimental data reported in the literature from three different types of airlift columns.

**Draft-Tube Airlift Column**

The experimental data obtained by Jones (8) in draft-tube airlift column where the liquid medium was tap water were tested. The results are shown in Table 1. Since Jones did not measure the gas hold-up in the annulus region, we use the empirical equation proposed by Bello et al. (11) which relates the gas hold-up in the downflow zone of the draft-tube airlift column to that in the upflow zone by the following equation:

\[
E_{G2} = 0.89E_{G1}
\]  

(10)

Table 1 indicates that good agreement between predicted and experimentally measured liquid circulation velocity was obtained with a maximum error of about 8%.

**External-Loop Airlift Column**

Merchuk and Stein (12) also measured liquid circulation velocities in an external-loop airlift column. The comparison of the predicted and experimental liquid circulation velocities is shown in Table 2. Since they used a gas-liquid separator in the top section of the airlift device to prevent gas from recirculating through the downcomer the gas hold-up \( E_{G2} \) is negligible and the energy dissipation rate \( E_{d3} \) is therefore zero. In addition to a liquid flowmeter they also placed many small diameter tubes which acted as straightening vanes in the upper portion of the downcomer for the purpose of changing the resistance to liquid recirculation. They also installed the gas sparger in such a way that it acted as an additional flow obstruction in the riser. Consequently, the friction loss factors are increased from 2.0 for the previous analysis in draft-tube airlift column to 3.4 for both \( \varepsilon_1 \) and \( \varepsilon_2 \) in order to take the additional flow resistance into account. It can be seen from Table 2 that the predicted values of liquid circulation velocity are in good agreement with the experimental data over the entire range of superficial gas velocities examined. For their system
$E_{d2}$ is larger than $E_{d1}$ because the circulation velocities are larger.

**Split-Cylinder Airlift Column**

The liquid circulation velocities predicted by the energy balance model for the split-cylinder airlift column were compared with those estimated by Patel (13) for two different systems: tap water and salt water (0.6% NaCl). The results are shown in Tables 3 and 4. It can be seen from Tables 3 and 4 that the predicted values of liquid circulation velocity are close to those estimated by Patel. However, the average error between the predicted and the estimated values in tap water is about 16.7% which is much larger than those in the previous analysis for draft-tube and external-loop airlift columns. This is probably due to the fact that the values we compared with are not from experimental measurement but from another estimation based on the photographic results of the bubble size distribution in the downflow zone of the airlift column which indeed will have larger error compared to the direct measurement. Besides, the measurement of gas hold-up in the split-cylinder airlift column were done manually by measuring the ungassed liquid height and the average dispersed height between the upflow and downflow zones. The gas hold-up obtained in this way is the average hold-up which can not theoretically represent the actual hold-up in the upflow and downflow zones, respectively. Therefore, the estimation of liquid circulation velocity based on the average gas hold-up is subject to error especially when the interstitial liquid velocity is significantly different from the terminal rise velocity of bubbles.

**CONCLUSIONS**

1. The energy balance model gives satisfactory estimates of liquid circulation velocity in three different types of airlift columns.

2. This model is general and applicable over a wide range of superficial gas velocities.

3. In bubbly flow regime, the liquid circulation velocity can be estimated directly from the knowledge of the operating condition and gas hold-up.

**ACKNOWLEDGMENT**

This work was partially supported by National Science Foundation grant CBT-8317967.
REFERENCES


Table 1

Comparison of liquid velocities estimated from energy balance model with the experimental data obtained by Jones (1985) in draft-tube airlift column, where we assume $e_1 = e_2 = 2.0$.

<table>
<thead>
<tr>
<th>$U_G$ (m/s)</th>
<th>$E_{G1}$</th>
<th>$U_{L2}$</th>
<th>$E_1$</th>
<th>$E_{d1}$</th>
<th>$E_{d2}$</th>
<th>$E_{d3}$</th>
<th>$V_{b-}$ (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0134 0.0149 0.0167 0.0179 0.0194 0.0209 0.0224 0.0239</td>
<td>0.024 0.021 0.025 0.030 0.032 0.034 0.036 0.039</td>
<td>0.0159 0.0165 0.0174 0.0176 0.0182 0.0186 0.0188 0.0186</td>
<td>0.120 0.131 0.141 0.151 0.160 0.170 0.182 0.193</td>
<td>0.25 0.28 0.34 0.35 0.35 0.38 0.41 0.42</td>
<td>1.31 1.47 1.69 1.82 2.00 2.18 2.36 2.53</td>
<td>0.23 0.23 0.23 0.23 0.23 0.23 0.23 0.23</td>
<td></td>
</tr>
</tbody>
</table>

* The values in the first and second rows of $E_{G1}$ under each operating condition represent the gas hold-up in the upflow and downflow zones, respectively.

** The first value of $U_{L2}$ is the superficial liquid velocity estimated from the energy balance model, and the second value of $U_{L2}$ in parenthesis is the experimental data obtained by Jones (1985).
Table 2

Comparison of liquid velocities estimated from energy balance model with the experimental data obtained by Merchuk and Stein in external-loop airlift column, where we assume $e_1 = e_2 = 3.4$.

<table>
<thead>
<tr>
<th>$U_G$ (m/s)</th>
<th>$E_{fl}$ (m/s)</th>
<th>$U_{ll}$ (m/s)</th>
<th>$E_i$ (W)</th>
<th>$E_{d1}$</th>
<th>$E_{d2}$</th>
<th>$E_{d3}$</th>
<th>$V_{b,oo}$ (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0469</td>
<td>0.049</td>
<td>0.663</td>
<td>24.18</td>
<td>6.89</td>
<td>17.29</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(0.610)</td>
<td></td>
<td>(28.5%)</td>
<td>(71.5%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
<tr>
<td>0.0782</td>
<td>0.076</td>
<td>0.774</td>
<td>40.32</td>
<td>10.74</td>
<td>29.58</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(0.750)</td>
<td></td>
<td>(26.6%)</td>
<td>(73.4%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
<tr>
<td>0.1095</td>
<td>0.100</td>
<td>0.853</td>
<td>56.45</td>
<td>14.05</td>
<td>42.40</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(0.855)</td>
<td></td>
<td>(24.9%)</td>
<td>(75.1%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
<tr>
<td>0.1407</td>
<td>0.118</td>
<td>0.920</td>
<td>72.54</td>
<td>16.65</td>
<td>55.89</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(0.915)</td>
<td></td>
<td>(23.0%)</td>
<td>(77.0%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
<tr>
<td>0.1720</td>
<td>0.136</td>
<td>0.972</td>
<td>88.68</td>
<td>19.13</td>
<td>69.55</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(0.980)</td>
<td></td>
<td>(21.6%)</td>
<td>(78.4%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
<tr>
<td>0.2033</td>
<td>0.154</td>
<td>1.014</td>
<td>104.81</td>
<td>21.67</td>
<td>83.14</td>
<td>0.00</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>(1.045)</td>
<td></td>
<td>(20.7%)</td>
<td>(79.3%)</td>
<td>(0.0%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Comparison of liquid velocities estimated from energy balance model with those estimated by Patel (1985) in split-cylinder airlift column for tap-water system, where we assume \( e_1 = e_2 = 2.0 \).

<table>
<thead>
<tr>
<th>( U_G ) (m/s)</th>
<th>( E_{G1} ) (m/s)</th>
<th>( U_{L1} )</th>
<th>( E_1 ) (W)</th>
<th>( E_{d1} )</th>
<th>( E_{d2} )</th>
<th>( E_{d3} )</th>
<th>( V_{b=} ) (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0259</td>
<td>0.065</td>
<td>0.134</td>
<td>2.42</td>
<td>1.46</td>
<td>0.05</td>
<td>0.91</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.065</td>
<td>(0.180)</td>
<td></td>
<td>(60.4%)</td>
<td>(2.1%)</td>
<td>(37.5%)</td>
<td></td>
</tr>
<tr>
<td>0.0517</td>
<td>0.079</td>
<td>0.296</td>
<td>4.84</td>
<td>1.79</td>
<td>0.56</td>
<td>2.49</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.079</td>
<td>(0.260)</td>
<td></td>
<td>(36.9%)</td>
<td>(11.5%)</td>
<td>(51.6%)</td>
<td></td>
</tr>
<tr>
<td>0.0776</td>
<td>0.099</td>
<td>0.365</td>
<td>7.25</td>
<td>2.23</td>
<td>1.09</td>
<td>3.93</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>0.099</td>
<td>(0.330)</td>
<td></td>
<td>(30.8%)</td>
<td>(15.0%)</td>
<td>(54.2%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4

Comparison of liquid velocities estimated from energy balance model with those estimated by Patel (1985) in split-cylinder airlift column for salt-water system, where we assume \( e_1 = e_2 = 2.0 \).

<table>
<thead>
<tr>
<th>( U_G ) (m/s)</th>
<th>( E_{G1} ) (m/s)</th>
<th>( U_{L1} ) (m/s)</th>
<th>( E_1 ) (W)</th>
<th>( E_{d1} )</th>
<th>( E_{d2} )</th>
<th>( E_{d3} )</th>
<th>( V_{b=} ) (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0259</td>
<td>0.056</td>
<td>0.187</td>
<td>2.42</td>
<td>1.20</td>
<td>0.13</td>
<td>1.08</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.056</td>
<td>(0.187)</td>
<td></td>
<td>(49.7%)</td>
<td>(5.6%)</td>
<td>(44.8%)</td>
<td></td>
</tr>
<tr>
<td>0.0517</td>
<td>0.089</td>
<td>0.262</td>
<td>4.84</td>
<td>1.93</td>
<td>0.39</td>
<td>2.51</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.089</td>
<td>(0.284)</td>
<td></td>
<td>(39.8%)</td>
<td>(8.2%)</td>
<td>(52.0%)</td>
<td></td>
</tr>
<tr>
<td>0.0776</td>
<td>0.111</td>
<td>0.328</td>
<td>7.25</td>
<td>2.41</td>
<td>0.81</td>
<td>4.03</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>0.111</td>
<td>(0.346)</td>
<td></td>
<td>(33.2%)</td>
<td>(11.2%)</td>
<td>(55.6%)</td>
<td></td>
</tr>
</tbody>
</table>

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Characterization of imperfect mixing of batch reactors by two compartment model

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Columbia, Missouri 65211

(A) Introduction
Although ideal mixing can be established in a small reactor, it is almost impossible to hold, during scale up, exactly the same mixing conditions as in the small scale. The impact of this difference in mixing quality upon outcome of the scale up process determines if the expectations of laboratory optima can be duplicated under new conditions. It is, therefore, necessary to characterize mixing in vessels of different scales and to quantitatively evaluate its impact upon the extent of reactions. In doing so, the attention in this article will be focused on the characterization of nonideal behavior of a biological batch reactor. This problem was examined by Bajpai and Reuss (1) with circulation-time distribution model in which the reactor was divided into two zones, that is, micromixing in the vicinity of impeller and macromixing away from impeller region. Although simulation of their model to characterize the reactor gave a good agreement with the known behavior of a nonideal reactor, they experienced enormous computational difficulties during the computer simulation. Khang and Levenspiel (3) proposed a CSTR's in series model in order to reconstruct the mixing behavior in a one-impeller batch reactor, and proved it to be a satisfactory model by experimental observation. However, if this model is extended to a multiple-impeller system involving the reaction kinetic processes, the computation is very involved.

Therefore, the purpose of this paper is to present a simpler two compartment model to explain the global behavior of a batch reactor. The following will be discussed, (a) the mathematical formulation of a two compartment model, (b) parameters of the model, and (c) predictions of the known behavior of microbial oxygen-uptake kinetics with the help of the proposed model.

(B) Formulation of Two compartment Model

(1) One impeller model
A single-impeller reactor can be conceptually divided into two compartments, as used by Bajpai and Reuss (1). One of these may be a zone around the impeller which is characterized by high energy dissipated rates and turbulence. This is similar to the micromixing zone defined by Bajpai and Reuss (1). The other compartment will then compose of the rest of reactor volume. This zone has considerably reduced energy dissipation and is dominated by convective flows. This concept is represented in Figure 1. For the sake of simplicity, it is assumed that both of zones are well mixed. For the zone 2 (Figure 1) in particular, this
assumption may be quite gross. These zones are assumed to be connected by an exchange flow, $q$, that will be related to impeller discharge.

For the case of a non-reactive tracer introduced into zone 1, the component material balances may be written as

$$\frac{dA_1}{dt} = \frac{D}{\chi} (A_2 - A_1)$$
$$\frac{dA_2}{dt} = \frac{D}{1 - \chi} (A_1 - A_2)$$

(1)

Initial conditions for this case are

$$A_1(0) = A_0/\chi = \frac{Q_{\text{tracer}}}{V_L} \chi$$ \text{ and } $$A_2(0) = 0.0$$

where $Q_{\text{tracer}}$ is the amount of tracer introduced in zone 1. Solutions of these equations result in

$$\frac{A_1}{A_0} = 1 + \frac{1 - \chi}{\chi} \exp(-Dt \left(\frac{1}{\chi} + \frac{1}{1 - \chi}\right))$$

and

$$\frac{A_2}{A_0} = 1 - \exp(-Dt \left(\frac{1}{\chi} + \frac{1}{1 - \chi}\right))$$

where $D = \frac{q}{V_L}$, $\chi = \frac{v_1}{V_L}$

(2)

Here $A_0$ ($Q_{\text{tracer}}/V_L$) represents the uniform tracer concentration in reactor. Equations (2) are graphically represented in Figure 2 and one notices a monotonous change of tracer concentration in the two zones. This behavior is experimentally observed only in vessels having high viscosity fluids or those with low speeds of agitation. In other circumstances, oscillatory concentration profiles are obtained that are better represented by the tanks-in-series model of Khang and Levenspiel (3) as shown in Figure 3. In such cases the number of tanks-in-series, $N$, will be larger than 2. Note that the model presented in Figure 1 is a special case ($N=2$) of Khang and Levenspiel's model and it will be shown that this model does represent the essential trends of global behavior of oxygen uptake kinetics in bioreactor.

(2) Multiple impellers

The two compartment model presented in Figure 1 can be extended to multiple impellers by assuming two zones for each impeller. For the case of two impellers, it means a four compartment representation of Figure 4. Compartments 1 and 3 are
the two impeller regions and 2 and 4 are the two non-impeller regions. Note that only the non-impeller regions exchange fluids at a rate of \( q_1 \) to cause inter-impeller mixing. Further, it was assumed that the impellers are identical and they share the liquid equally between them. This assumptions are reasonable for symmetrically placed impellers.

For tracer injection in region 1, the governing equations are

\[
\frac{d A_1}{dt} = \frac{D}{\alpha} (A_2 - A_1)
\]
\[
\frac{d A_2}{dt} = \frac{D}{0.5 - \alpha} (A_1 - A_2) + \frac{\beta}{0.5 - \alpha} (A_3 - A_4)
\]
\[
\frac{d A_3}{dt} = \frac{D}{0.5 - \alpha} (A_4 - A_3)
\]
\[
\frac{d A_4}{dt} = \frac{D}{0.5 - \alpha} (A_3 - A_4) + \frac{\beta}{0.5 - \alpha} (A_2 - A_4)
\]

Initial conditions for this case are

\[ A_1(0) = \frac{q_{\text{tracer}}}{\alpha V_L} \quad \text{and} \quad A_2(0) = A_3(0) = A_4(0) = 0.0 \]

Its solutions for regions 1 and 3 are

\[
\frac{A_1}{A_0} = 1 + \frac{0.5 - \alpha}{\alpha} \exp(-Dt \left( \frac{1}{\alpha} + \frac{1}{0.5 - \alpha} \right))
\]
\[
+ \left( 1 + \frac{0.5 - \alpha}{\alpha} \right) \left( x + y - \frac{2\beta + 1}{0.5 - \alpha} \right) \frac{1}{2y} \exp(-t(x+y))
\]
\[
+ (y-x+ \frac{2\beta + 1}{0.5 - \alpha}) \frac{1}{2y} \exp(-t(x-y))
\]

\[
\frac{A_3}{A_0} = 1 - \frac{0.5 - \alpha}{\alpha} \exp(-Dt \left( \frac{1}{\alpha} + \frac{1}{0.5 - \alpha} \right))
\]
\[
+ \frac{1}{(2y)(\frac{D}{\alpha} + \frac{D}{0.5 - \alpha} -x-y))} \exp(-t(x+y))/(x+y)
\]
\[
- \exp(-t(x-y))/(x-y)
\]

where \( D = q/V_L \), \( \alpha = v_1/V_L \), \( \beta = q_1/q \),

\[
x = \frac{D}{2} \left( \frac{1}{\alpha} + \frac{2\beta + 1}{0.5 - \alpha} \right)
\]
\[
y = \frac{D}{2} \left( \frac{-2x^2}{D} - \frac{8\beta}{(0.5 - \alpha)} \right)^{0.5}
\]
Equations (4) are represented in Figure 5 wherein the simulated tracer concentration profile for region 3 is plotted. This represents simulations for a case where tracer is injected in the vicinity of one impeller and measurements are made around a different impeller. The solid line represents $\frac{A_t}{A_0}$ from equation (4). If the more rigorous Khang and Levenspiel's model (Figure 3) is extended to two identical impeller case, the dotted line in Figure 5 is plotted. Noticeable is the considerable reduction in oscillations compared to Figure 3. In our contention, the two compartment model (Figure 4) is quite indistinguishable from Khang and Levenspiel's model.

In fact an analysis of Khang and Levenspiel's model ($N > 2$) shows that the influence of the number of tanks-in-series ($N$) upon tracer profiles becomes considerably milder as the number of impellers increases. Hence, two compartment model may be considered satisfactory for multi-impeller systems.

(C) Simulation of oxygen uptake kinetics

In order to check the validity of and to take advantage of the model presented, oxygen uptake by microorganisms in a single impeller batch reactor was simulated. For the purpose of simulation, the reactor is again divided into two regions as shown in Figure 6, where 1 is impeller region in which most of the gas-liquid mass transfer takes place and where 2 is non-impeller region.

Parameters in this model include the exchange flow rate, $q$, and the volumes of the two regions. In the absence of any correlation for these parameters, a method is suggested below in which information available from the established circulation time distribution model (3, 4) is used to generate parameter values.

(1) Volume of impeller region

Since the energy introduced is dissipated mostly in the highly turbulent impeller region, following can be assumed.

$$V_L \times \varepsilon = V_1 \times \varepsilon_{\text{max}} \quad (5)$$

By using the relation,

$$\varepsilon_{\text{max}} = 0.5 \frac{2}{\pi} (\frac{d_i}{D_T})^3 \quad (6)$$

Volume of impeller region may be calculated.

$$\alpha = \frac{V_1}{V_L} = 2 (\frac{d_i}{D_T})^3 \quad (7)$$

(2) Prediction of the exchange flow rate

In order to calculate the exchange flow rate, mixing time of the two compartment model is equated to the mixing time predicted by Khang and Levenspiel's model.
According to Khang and Levenspiel (3)

\[ \Theta_{\text{mix}} = \frac{\ln(2/A_m)}{k_A} \]  

where \( k_A \) is related to operating conditions as

\[ 0.5 = \frac{n}{k_A} (d_i/D_T)^{2.3} \]  

\( A_m \) is the degree of homogeniety (typically 0.05, Figure 3) to be achieved for calculation of mixing time.

According to the two compartment model, time required to achieve the same degree of homogeniety, \( A_m \), is as

\[ Q = -\left( \alpha (1-\alpha) V_L \right)/q \]  

From equations (8) and (10)

\[ q = -V_L \alpha (1-\alpha) k_A \frac{\ln(A_m \alpha)}{\ln(2/A_m)} \]  

The exchange parameter thus calculated was plotted as a function of \( d_i/D_T \) and resulted into a simplified correlation of the following type

\[ -q/Q = 1.78 (d_i/D_T)^{1.3} \]  

where \( Q \) is the circulation rate given by

\[ Q = V_L/\Theta_{\text{circulation}} \]  

(3) Simulation

Reuss et al. (5) have suggested that the uptake of oxygen in filamentous broths may be modelled as the basis of diffusion reaction in hypothetical elements of initial eddy sizes. Accordingly, the specific oxygen uptake rate can be written as
\[ Q_{o2} = \frac{Q_{o2}^{max} C}{k_m + C} x \quad (14) \]

In the context of model presented in Figure 6, the value of \( \gamma \) will be different in two regions due to the differences in bulk dissolved oxygen concentrations. In this case the governing material balance equations may be written as

\[
\frac{dC_1}{dt} = \frac{D}{\alpha} (C_2 - C_1) + \frac{k_L \alpha}{\alpha} (C_1^* - C_1) \\
- \eta_1 Q_{o2}^{max} \frac{C_1}{k_m + C_1} x
\]

\[
\frac{dC_2}{dt} = \frac{D}{1 - \alpha} (C_1 - C_2) - \eta_2 Q_{o2}^{max} \frac{C_2}{k_m + C_2} x
\]

\[
\frac{d \gamma_{out}}{dt} = \left( \frac{\gamma_{in}}{g} - \frac{\gamma_{out}}{g} \right) \frac{k_L \alpha}{\varepsilon g} (C_1^* - C_1) \quad (15)
\]

Simulations were conducted for different values of power introduced per unit mass of liquid in reactor and the total specific uptake rate was calculated by volume averaging the individual region uptake rates. This rate is plotted in Figure 7 as a function of energy dissipation rate. The parameter for the different curves is the impeller to tank diameter ratio. We notice that the model predicts the smaller impeller to be better, as is also experimentally observed in mycelial broths. As the volume of the reactor increases, the influence of impeller to tank diameter ratio diminishes, which is also an experimentally corroborated fact. The same type of behavior was also predicted for a different biomass concentration also. As a result it may be concluded that the two compartment model proposed here satisfactorily describes several important kinetic phenomena observed in single and multiple impeller systems.

(D) Conclusion

A single impeller batch reactor exhibits an oscillatory pattern of tracer concentration having decaying amplitude when measurements are conducted in the vicinity of impeller. The two compartment model proposed here predicts only decaying amplitude but no oscillation in such a case. However, as the number of impellers increases, the prediction of this simple model approaches those of more realistic circulation models. Even for
a single impeller system, the model predicted patterns of oxygen uptake kinetic that are similar to those observed in practice.

(E) Recommendation

Although two compartment model is proved to lead to a very close approximation for the simulation of the oxygen uptake kinetics, it is still necessary to compare these with experimental data. It'll be worthwhile to develop correlations to estimate the exchange flow rate and volume of each compartment suggested in this work or to find a new method of correlating those parameters by other variables.

References

6. Tanner, R.D., S. Pohani, I. J. Dunn, and J.R. Bourne, American Chemical Society 184th meeting, Division of Microbial and Biotechnology, Kansas City, Mo. (1982)

Nomenclature

A ; tracer concentration, (moles/m3)
Am ; degree of homogeneity
A0 ; initial tracer concentration (moles/m3)
C ; oxygen concentration (moles/m3)
D ; dilution rate (q/VL), (1/sec)
d ; impeller diameter (m)
D0 ; reactor diameter (m)
M; energy dissipation (watt/kg)
ka ; gas-liquid mass transfer coefficient, (1/sec)
km ; correlation constant, (0.0013 mole/m3)
Q ; circulation flow rates (m3/sec)
q ; exchange flow rate between compartments, (m3/sec)
Q02 ; oxygen uptake, (mole/Kg sec)
R ; gas constant (m3 Atm/mole K)
T ; temperature (K)
t ; time (sec)
v ; volume of compartment (m3)
vg ; gas flow rate (m3/sec)
V ; total reactor volume (m3)
xb ; biomass concentration, (Kg/m3)
Yg ; partial pressure of oxygen in and out (Atm)
; mixing time (sec)
; density of liquid medium (Kg/m3)
; effectiveness factor of uptake
; hold up of gas in reactor
; volume ratio of impeller region to total volume
; ratio of exchange flow rate
Figure 1: One impeller two compartment model

Figure 2: Tracer trajectory in one impeller batch reactor
Figure 3; Tracer trajectory in one impeller batch reactor

deaying amplitude

oscillation
Two compartment model

Experimentally observed behavior

Figure 5: Tracer trajectory in two impeller batch reactor
Figure 6: Two compartment model of oxygen uptake reaction.

Figure 7: Oxygen uptake rate vs. Energy dissipation.
FIRST ORDER BREAKAGE MODEL
FOR THE DEGRADATION OF PULLULAN IN THE BATCH FERMENTOR

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INTRODUCTION

Over the last 40 years polymers produced by biological methods have received increasing attention from industry and the public. One of the main reasons for this is, unlike petrochemical plastics, all polymers of biological origin are biodegradable. Concern for the environment makes biodegradable plastics attractive alternatives to many of the nondegradable plastics in present use. Additionally, the processes for making biological polymers do relatively little harm to the environment.

The majority of the biopolymers of current interest for industrial production are polysaccharides. One such polymer is pullulan. Pullulan is an extracellular polysaccharide produced by Aureobasidium pullulans (formerly known as Pullularia pullulans). The microorganism is commonly known as a "black yeast" (Cooke, 1962). To the brewing industry it is a contaminant that can blacken the brew during fermentation. A. pullulans often propagates on trees and painted surfaces and commonly can be isolated from soil where plant material is degrading.

The polymer, pullulan, is clear, colorless, odorless, water soluble, and provides no calories upon consumption by humans. No toxic effects from pullulan have been detected. The high solubility of pullulan in water limits the use of the polymer in many areas, but also opens the possibility for many new polymer applications. One of pullulan's most important properties is that in the crystalline form it has a very low oxygen permeability.

A. pullulans produces pullulan from many carbon based substrates, including glucose, sucrose, fructose, maltose, xylose, lactose, galactose, arabinose, and rhamnose (Imshetskii et al., 1981). To produce pullulan in financially viable, high concentration, A. pullulan is grown in aerated, well mixed aqueous solution with a carbohydrate carbon source of starch hydrolyzate (Kato, 1975) or sucrose (Shipman and Fan, 1978).

The organism forms pullan's monomer, maltotriose, from the carbohydrate and links the monomer together to form pullulan, which is released into the medium (Catley, 1970). Maltotriose consists of three glucose molecules joined by two $\alpha(1-4)$ bonds. The maltotriose units are linked by $\alpha(1-6)$ bonds (see Fig. 1a). Intermittent tetraose units are also present in this straight-chain polymer (see Fig. 1b).

The tetraose units provide cites for enzymatic degradation of the polymer by amylolytic attack. The degradation occurs in the fermentation medium because A. pullulans produces extracellular $\alpha$-amylase along with extracellular pullulan. The degradation causes the average molecular weight
Figure 1. (a) Polymaltotriose structure of pullulan polymer. ○ Glucose residue, α(1→6)glucosidic linkage, α-α(1→4)glucosidic linkage. (b) Inclusion of maltotetraose unit within pullulan polymer.
of the pullulan to decrease as production proceeds in batch fermentation (Catley, 1970).

The molecular weight distribution of pullulan affects the quality and, consequently, the applications of the polymer. The objective of the present study is to develop a model which describes the change in the molecular weight distribution of pullulan as a function of time in the batch fermentor.

BREAKAGE MODEL

A. Cell free broth.

Before examining the system in the batch fermentor, it is expedient to develop a model which describes the degradation of pullulan by α-amylase in a cell free broth. Such a broth could be obtained from an immobilized culture of \( \text{A. pullulans} \).

For this model, it is assumed that α-amylase cleaves randomly at the tetraose units, and tetraose units are distributed evenly throughout the pullulan molecules. From these assumptions it can be stated that the probability that degradation occurs in a specific polymeric molecule is only dependent on the number of bonds the molecule contains. It is further assumed that the number of bonds broken over the entire reaction period is negligible compared to the number of available bonds, and the concentration of free α-amylase is many times greater than the concentration of bound α-amylase. Consequently, the continuous degradation of pullulan can be modelled as a binary chain breakage process. In this process two and only two shorter chains are created from a single breakage, and higher order breakages are considered to be simultaneous binary reactions. Thus we can treat the degradation reaction as first order:

\[
\begin{align*}
  k_j & \rightarrow c_1 + c_{j-1} \\
  k_j & \rightarrow c_2 + c_{j-2} \\
  & \quad \vdots \\
  & \quad \vdots \\
  k & \rightarrow c_{j-1} + c_1
\end{align*}
\]

(1)

where

\[
\begin{align*}
  c_j & = \text{molar concentration of pullulan of length } j \\
  k & = \text{first order rate constant for degradation}
\end{align*}
\]

Applying a mass balance for pullulan molecules of chain length \( i \):
rate of accumulation = rate of disappearance of polymer of length i due to degradation + rate of production of polymer of chain length i due to breakage of polymer of greater chain length
dc_i \over dt = -(i-1)k c_i + 2k \sum_{j=i+1}^{A} c_j \quad (3)

Where A is the longest chain length in the system. Only one parameter, k, need be determined.

B. System within batch fermentor.

The concentration of pullulan and α-amylase are assumed to be proportional throughout the period of fermentation. Thus it can be stated that the reaction rate is constant throughout the period of fermentation. To adapt equation (3) to this system the following terms are defined as

c_i(\tau) = \text{molar concentration of molecules of length } i \text{ and age } \tau

\tau = \text{time since a molecule was released into the broth by a cell; age}

Replacing c_i in equation (3) with c_i(\tau) gives

dc_i(\tau) \over d\tau = -(i-1)k c_i(\tau) + 2k \sum_{j=i+1}^{A} c_j(\tau) \quad (4)

To describe the total mass concentration of polymer at any time, t, \rho_T(n) is defined as

\rho_T(n) = \sum_{m=0}^{n} \rho(m) \quad (5)

where

\rho(m) = \text{mass concentration of polymer of age } \tau

Likewise for a specific molecular length of i, the total mass concentration at time t is defined:

\rho_i(n) = \sum_{m=0}^{n} \rho(m)\omega_i(m) \quad (6)

where

\omega_i(m) = \text{mass fraction of polymer with chain length } i \text{ from all polymer of age } \tau
Discretizing time

\[ t = n\Delta t \quad (7) \]
\[ \tau = m\Delta t \quad (8) \]

where

\( n = \text{number of time intervals since the onset of fermentation} \)
\( m = \text{number of time intervals that have passed since a molecule was generated by a cell} \)

Applying discrete time to equation (4) according to equations (7) and (8) produces,

\[
\frac{\Delta c_i(m)}{\Delta \tau} = -(i-1)k c_i(m) + 2k \sum_{j=i+1}^{1} c_j(m) \quad (9)
\]

Equations (6) and (9) are related by

\[
\omega_i(m) = \frac{M_i}{\rho(m)} c_i(m) \quad (10)
\]

where

\( M_i = \text{molecular weight of polymer of chain length } i \)

From the prior statement that the reaction can be treated as first order, the reaction can be expressed as

\[
\frac{dn}{dt} = -kn \quad (11)
\]

where

\( n = \text{total number of bonds available for attack by } \alpha\text{-amylase}. \)

Additionally,

\[
n = N - \frac{N}{\bar{M}_n} \quad (12)
\]

where

\( N = \text{number of "monomer units" (A "monomer unit" is a segment of polymer which contains uninterrupted triose molecules. The "monomer units" are linked by tetraose units.)} \)
\( \bar{M}_n = \text{Number mean average molecular weight} \)
Integrating equation (11) produces

\[ kt = \ln \frac{n^0}{n} \]  

(13)

Substituting equation (12) into equation (13)

\[ kt = \ln \frac{N - \frac{N}{\bar{M}_n}}{N - \frac{N}{\bar{M}_n}} \]

(14)

\[ k = \frac{1}{t} \ln 1 - \frac{1}{\bar{M}_n} - \frac{1}{t} \ln 1 - \frac{1}{\bar{M}_n} \]  

(15)

Since \( \bar{M}_n^0 \gg 1 \), and \( \bar{M}_n \gg 1 \), equation (15) simplifies to

\[ k = \frac{1}{t} \left( \frac{1}{\bar{M}_n} - \frac{1}{\bar{M}_n^0} \right) \]  

(16)

Applying equation (16) to equation (9) and substituting in the definition of \( \bar{M}_n \) produces

\[ \frac{\Delta c_j(m)}{\Delta T} = \frac{1}{T} \sum_{k=1}^{l} \frac{c_k}{M_k} - \frac{1}{\bar{M}_n^0} \left[ (i-1)c_i(m) + 2 \sum_{j=i+1}^{l} c_j(m) \right] \]  

\[ \sum_{k=1}^{l} c_k \frac{M_k}{\bar{M}_n} \]  

(17)

Equation (17) can be used to describe the system in conjunction with equations (6) and (10), where \( \Delta t \) need be specified and the molecular weight distribution need be specified at \( T = 0 \) (accomplished by calculating first sample back to time of initiation of fermentation, \( t = 0 \)). One parameter, \( k \), need be determined by numerical method.

CONCLUSIONS

1. Computer simulation of the present model shall be developed.

2. The present model need be tested with experimental data.

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REFERENCES


SYNTHESIS AND NUCLEAR MAGNETIC RESONANCE OF $^{13}$C-LABELED AMYLOPECTIN AND MALTOOLIGOSACCHARIDES

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INTRODUCTION

Carbon-13 nuclear magnetic resonance ($^{13}$C n.m.r.) is a very useful tool in identifying the structure of biopolymers and studying the catalytic mechanisms of enzymes. Information on both structural linkages and tertiary conformation can be obtained from n.m.r. spectra. The low natural abundance of the $^{13}$C isotope, however, makes high-resolution spectra difficult to obtain. Very long scan times and high material concentrations are usually required. For biopolymers such as polysaccharides, solubility can further limit the signal strength and degree of spectral resolution.

To overcome these constraints, $^{13}$C-enriched monosaccharides have been synthesized and very precise spectra are available. However, we know of no commercially available oligo- or polysaccharides that contain residues enriched in $^{13}$C. We therefore enzymatically polymerized $\alpha$-D-$[1-^{13}$C]-glucopyranosyl fluoride with amylosucrase to produce the correspondingly labeled amylopectin, and formed maltotetraose from the latter by enzymatic hydrolysis with pullulanase and a maltotetraose-producing amylase.

Other isotopically labeled glucose species (e.g. $^{14}$C or $^3$H) can be incorporated into $\alpha$-D-(1→4)-linked polysaccharides by the same technique. The polymers and the various oligosaccharides formed from them by various hydrolytic processes can be used for studies of carbohydrate structure and carbohydrase mechanisms. As a first step in such a study, we present the n.m.r. spectra of $1-^{13}$C-labeled amylopectin, maltotetraose, maltotriose, and maltose.

RESULTS

Specifically labeled $1-^{13}$C-enriched D-glucose was acetylated with acetic anhydride in anhydrous pyridine. Following crystallization from anhydrous methanol, the pentacetate was reacted with anhydrous hydrogen fluoride. After extraction into chloroform and removal of solvent, the resulting tetra-O-acetyl-$\alpha$-D[$1-^{13}$C]-glucopyranosyl fluoride was deacetylated with sodium methoxide in anhydrous methanol to produce $\alpha$-D[$1-^{13}$C]-glucopyranosyl fluoride. Amylosucrase from Neisseria perflava produced a branched polysaccharide with $\alpha$-D-(1→4) glycosidic linkages and $\alpha$-D-(1→6)-initiated branches. Repeated use of a maltotetraose-producing amylase from Pseudomonas stutzeri, which cleaves $\alpha$-D-(1→4) glycosidic linkages, gave $[1-^{13}$C]-maltotetraose. Incubation with pullulanase, which cleaves $\alpha$-D-(1→6) glycosidic bonds in glycogen and amylopectin, was used to increase the overall yield, which was 25% by weight based on D-[1$^{13}$C]-glucose.
$^{13}$C n.m.r. spectra from labeled amylopectin, maltotetraose, maltotriose, and maltose agree with previously presented assignments (Fig. 1). However, due to the enhanced level of $^{13}$C in the saccharides, we were able to observe distinct chemical shifts for the three different $\alpha$-(1→4) linkages in labeled maltotetraose. The bond nearest the reducing end yields a fused doublet at 100.83 ppm, with the cause of splitting unknown. The middle bond gives a peak at 101.00 ppm, while that associated with the bond at the nonreducing is at 101.09 ppm. N.m.r. spectra from labeled maltose and maltotriose show similar chemical shifts for the corresponding bonds. These shifts are apparently due to conformational differences among the linkages in solution$^{10}$. These results demonstrate that n.m.r. spectroscopy of 1-$^{13}$C-enriched malto-oligosaccharides is a very sensitive probe into their structure and conformation.

**EXPERIMENTAL**

T.l.c. was used to determine the oligosaccharide composition of reaction mixtures. Whatman (Clifton, N. J., U.S.A.) K5F silica plates were irrigated with one ascent of methanol-ethyl acetate-water (40:37:23, v/v/v). Carbohydrates were visualized by spraying with sulfuric acid-methanol (1:4, v/v) and heating at 120°C for 5 min. $^{13}$C n.m.r. spectra were obtained using a Nicolet NT-300 Fourier-transform spectrometer (75 MHz) in the proton-decoupled mode with $^2$H$_2$O solvent at pH 7.0. Peak assignments were relative to an external tetramethylsilane standard. Maltotetraose concentration was determined by reducing value analysis with the potassium ferricyanide method, calibrated with maltose standards$^{11}$. D-[1-$^{13}$C]-glucose (99.7 atom% enriched) was purchased from MSD Isotopes (Rahway, N. J., U.S.A.). Amylosucrase was obtained from *N. perflava* using the methods of Okada and Hehre$^{12}$. Maltotetraose-producing amylase was obtained from *P. stutzeri* following Robyt and Ackerman$^9$. Pullulanase was purchased from Enzyme Development Corp. (New York, N.Y., U.S.A.) and purified by ammonium sulfate precipitation (50% saturation) followed by fractionation on a Sephadex G-75 column.

$\alpha$-D-[1-$^{13}$C]-glucopyranosyl fluoride was prepared using the methods described by Hehre et al.$^{13}$, except that D-[1-$^{13}$C]-glucose was used instead of D-glucose and the reaction temperature was -196°C instead of -20°C.

Amylosucrase (0.41 IU) was added to $\alpha$-D-[1-$^{13}$]-glucopyranosyl fluoride (0.23 mmol) in 4 mL of buffer (50 mM sodium maleate, pH 7.0, 0.02% sodium azide) and incubated at 35°C for 48 h. The pH was maintained at 7.0 by the addition of dilute sodium hydroxide (approx. 0.1N). The resulting polysaccharide was precipitated with two volumes of ethanol, recovered by centrifugation, and dried in vacuo at 30°C. The yield was approximately 50% by weight of $\alpha$-D-[1-$^{13}$C]-glucopyranosyl fluoride.

The formation of maltotetraose from the polysaccharide was done in two steps. First, the polysaccharide was redissolved in 1 mL of buffer (pH 7.0) and *P. stutzeri* amylase (0.07 IU) was added, followed by incubation at 35°C for 24 h. Pure maltotetraose was recovered in about 12% yield from the supernatant of a 50%
ethanol precipitation of the mixture following centrifugation. This procedure was repeated three times more, giving an overall maltotetraose yield of about 30% by weight based on initial polysaccharide. The precipitated limit dextrin was dried and resuspended in buffer (50 mM sodium maleate, pH 4.0, 0.02% sodium azide) and incubated with pullulanase (0.014 IU) for 24 h at 35°C. Following the pullulanase treatment, more amylase (0.014 IU) was added, the pH was adjusted to 7.0, and the solution was incubated and maltotetraose was recovered as before. The final overall yield of maltotetraose was about 40% by weight of the initial amount of polysaccharide.

Maltotetraose purity was tested by t.l.c. and by analysis of the products from hydrolysis with porcine pancreatic α-amylase, which gave primarily maltose with very slight traces of glucose and maltotriose. The n.m.r. spectra of maltotetraose indicated only α-(1→4) linkages (Fig. 1).

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REFERENCES


Fig. 1. N.m.r. spectra of 1-\textsuperscript{13}C-labeled amylopectin, maltotetraose, maltotriose, and maltose. Peaks derived from \(\alpha\)- and \(\beta\)-anomeric forms of oligosaccharides appear at approximately 93 and 97 p.p.m., respectively.
INTRODUCTION

Fungal growth, both in solid substrate fermentation (SSF) and in submerged fermentation (SF), requires an active and fairly large amount of starter culture (inoculum) to initiate a rapid and uniform growth without competitive contamination from faster growing bacteria and yeast (1,2). Traditionally inoculum is prepared in submerged fermentation (3). This requires additional fermentation equipment, extra steps and time in the process flow and is costly.

With fungal cultures, there is a possibility to use spore suspensions for inoculation, but the time necessary for spore germination and enzyme induction usually slows down the main fermentation process (3). It is more advantageous to use germinated spores or mycelial cultures that are already induced and in optimal physiological condition for rapid growth. Such fungal starter cultures may be prepared most advantageously in solid substrate, because the germinated spores or mycelia can survive and thus stored for a long time and the solid material can be handled and dosaged conveniently in industrial fermentations. A good example for such a starter culture is the traditional mushroom spawn.

Starter culture preparation in conventional SSF suffers from the limitations of this process: the inhomogeneity of the substrate impedes mass transfer and makes difficult the control of fermentation parameters (temperature, moisture content, partial pressure of O₂). The consistency of the 70-80% moisture containing substrate makes mixing and heat removal difficult, especially in larger fermenters (4).
In this paper a novel method is described for the preparation of a fungal starter culture in a gas fluidized bed reactor. The unique feature of such a reactor is the spatial separation of each spore bearing carrier particle suspended in a moisture saturated air stream allowing optimal conditions for spore germination. The main points of the paper are: 1) the selection of the optimal carrier particle that at high relative humidity (necessary for fungal growth) has a low water content (necessary for fluidization), thus allowing optimal conditions for spore germination; 2) finding the optimum conditions for spore attachment and germination in the gas fluidized bed reactor.

MATERIALS AND METHODS

Substrate

Furfural bran, the residue from corn cob after acid hydrolysis and extraction of furfural was obtained from Nitrochem Works, Péti, Hungary in a granular form. The chemical composition of furfural bran is: lignin 50%, cellulose 41%, crude protein 4%, ash 3%, and hemicellulose 2%. For the experiments the granules were sieved to a 0.4-0.6 mm size range having a bulk density of 442 kg/m³, a total porosity of 0.73 and internal porosity of 0.19. For some experiments the granules were coated with 1% w/w carboxymethyl cellulose (CMC) by soaking the granules in 10 g/l CMC solution then drying at 105°C. For control, untreated ground corn cob (Anderson's Grit-O-Cob, The Andersons, Maumee, Ohio) with 0.84-1.41 mm particle size range was used.

Gas fluidized bed reactors

Jacketed glass or plexiglass columns of 36 to 80 mm inside diameter (ID) and 250-500 mm length with perforated bottom plates of 0.05-0.25 mm openings were used. A perforated disk air distributor, placed under the bottom plate, served for fluidization. To avoid vapor condensation, the whole apparatus, except the air compressor, was housed in a controlled environment chamber. The nonfluidizable light particles (spores, mycelial and substrate fragments or condensed water) were collected in separate cyclons. A typical arrangement is shown in Fig. 1.

Analytical procedures

Water adsorption isotherms were determined by the stationary water adsorption method in constant relative humidity atmospheres over distilled water (100%), saturated KNO₃ (90%), KCl (82%) and NaCl (75%) solutions (5). Moisture content was measured gravimetrically by drying at 105°C for constant weight. Spore germination was followed by scanning electron microscopy.
Culture

The cellulolytic fungus Chaetomium globosum (isolated at the U. of Technical Sciences, Budapest, Hungary) was grown at 28°C on modified Czapek agar slants (3). The spores were washed from a fourteen-day agar slant culture with sterile water. This spore suspension adjusted to a predetermined spore count was used in the fluidization experiments.

RESULTS

Fluidization of furfural bran granules

Water adsorption and flowability

The stationary water adsorption isotherms of furfural bran and control ground corn cob taken at 37°C are shown in Fig. 2. The adsorption velocity was unusually rapid, reached equilibrium in 4-7 days. Equilibrium water content \( W^* \) at 100% relative humidity was 21%. At equilibrium the microcapillaries of the particles are filled with water and along the inner pores and outer surface the partial vapor pressure approximates the saturation value. The moisture content above \( W^* \) is considered as "free water" disponible for fungal growth (4,7). Fungal growth is thus possible on furfural bran at a relatively low moisture content, slightly above equilibrium.

The flowability of the 0.4-0.6 mm size granules determined by the angle of repose of a free flowing granule pile does not change significantly with the increase of the water content up to 35%. With the 1% CMC coated granules the angle of repose increased sharply above 30% water content, indicating that CMC increases particle cohesion only above this water content (Fig. 3).

Fluidization properties of furfural bran

Dry granules were placed in columns of 36 and 80 mm ID at resting bed heights of \( y = 0.1, 0.2 \) and 0.4 m. The moist (100% ϕ) airstream caused significant bed expansion before reaching minimal fluidization velocity (Fig. 4). The measured pressure drops, extrapolated for \( y = 1.0 \) m for uniform comparison, agree well with the calculated theoretical values (Fig. 4).

The flow pattern was irregular with channels and bubbles in the wider columns. In the 36 mm ID column gas plugs filled the entire cross section, causing superficial pulsation and increased expansion of the bed. The minimal fluidization velocity, \( u_{mf} = 0.14 \) m/s, calculated from an equation valid at Reynolds numbers less than 5 (6), agreed well with experimental values measured at \( y/D \geq 5 \) bed height/diameter ratios. On the other hand, regular flow pattern can be observed at \( y/D < 1.5 \) ratios.
Spore germination in the gas fluidized bed reactor

The porous furfural bran granules provide extensive surfaces for spore attachment, and the inner pores provide protection for the developing hyphae from shearing forces. The electronmicrograph of a dry granule is shown in Fig. 5. Spore attachment and germination may be helped by additives that can be coated on the porous granules. The cross section of a CMC coated granule is shown in Fig. 6. CMC coats not only the outer surface but the surfaces of the inner pores, acting as an adhesive for spore attachment and also inducing cellulases effectively, being in direct contact with germinating spores. A 55x250 mm jacketed glass fluidized bed reactor was filled with 60g dry sterilized granules previously coated with 0.6g CMC, then supplemented with 20 mL of a solution containing 0.1 g/L Tween 80 and 9.0x10^6 spores/mL in Czapek-Dox salt solution. The initial water content was 25%, this decreased to 23.1% during 3 days of fluidization at 28°C. The initial fluidization velocity was 0.12 m/s; this was gradually increased to 0.205 m/s to maintain fluidity. Apparently the developing hyphae caused an increased cohesion of the granules which had to be compensated by increased shearing forces. The pressure drop did not increase significantly from the initial 250 Pa.

The electronmicrographs of the fluidized granules reveal uniform hyphal development starting from the pores and terminating close to the outside surfaces (Fig. 7). This hyphal development is radically different from the one observed in a stationary culture, which is characterized by branching hyphae growing away from the surface (Fig. 8). The fluidized granules with germinated spores were stored for nine months in a dry state and retained their viability and vigor for starter culture use, initiating rapid and abundant growth in a stationary solid substrate fermentation on fresh furfurool bran.

DISCUSSION

Gas fluidized bed technology may offer the following advantages over conventional SSF or SF technologies for fungal starter culture production: 1) Fungal spores attached to appropriate carriers may germinate faster, with a more vigorous and uniform growth than in conventional SSF, approaching or equaling conditions in SF; 2) special enzyme inducers, such as CMC for cellulases, would be effective in very low concentration because they would be in direct contact with fungal spores; 3) the starter culture is ready for use or storage after fluidization without further processing.
An essential condition for the preparation of a gas fluidized starter culture is the selection of the appropriate carrier that has a low equilibrium water content at a high relative humidity and for this reason is dry enough for fluidization yet contains enough free water for fungal growth. Furfural bran, a commercial byproduct available in large quantities, has an equilibrium water content of 21% at 100% relative humidity and retains its flowability up to 35% moisture content in the presence of "free water", thus providing a comfortable range in which both fluidization and optimal fungal growth is possible. Since furfural bran is chemically processed, its lignocellulose structure is readily available for enzyme attack without pretreatment, in contrast to most natural lignocellulosic substrates.

From the standpoint of the user, the above starter culture preparation has the advantages of long term storability in a dry state and direct applicability in a ready-mix form.

REFERENCES
Fig. 1. Gas fluidized bed reactor assembly: 1 - sterile air filter; 2 - reactor 3, 6 - cyclons; 4 - airflow meter; 5 - air filter; 7 - gas pump; 8 - air saturation bubbling column; 9 - regulator valve.

Fig. 2. Water vapor adsorption isotherms at 37°C. O = granulated furfural bran, 0.4-0.6 mm particle size; Δ = Anderson's Grit-0-Cob, 0.84-1.41 mm particle size.
Fig. 3. Flowability of furfural bran granules. 
Δ - untreated granules; 0 - 1% carboxymethyl cellulose coated granules.

Fig. 4. Fluidization properties of furfural bran granules. D - reactor diameter [mm]; y - resting bed height [m].
Fig. 5. Electronmicrograph of dry furfural bran granule; magnification is 290X.

Fig. 6. Electronmicrograph of a cut surface of carboxymethyl cellulose coated dry granule; magnification is 570X.
Fig. 7. Electronmicrograph of fungal growth in the gas fluidized bed reactor; magnification is a) 270X; b) 3000X.

Fig. 8. Electronmicrograph of fungal growth in stationary culture; magnification is 230X.
Yeast Flocculation and Sedimentation

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Introduction

Microbial flocculation is the aggregation of unicellular microorganisms into larger, relatively stable multicellular masses (see Figure 1). In general, these flocs settle out of suspension due to gravity much faster than do single cells. This phenomenon has been used for centuries in the brewing industry. More recently, the advent of genetic engineering has increased the attention given to yeast, single-celled eukaryotic organisms, as recombinant DNA hosts. Many yeast have the ability to flocculate. Utilizing flocculent strains would allow for the simple separation of yeast from media and for the development of continuous fermentation strategies.

In order to model and design new biochemical processes based on flocculent organisms, i.e. yeast, a quantitative understanding of flocculation is necessary. The various methods of quantifying this phenomenon that are discussed in the literature include microscopic enumeration using hemacytometry, subjective visual estimation, simple sedimentation studies, and sedimentation studies coupled with light extinction techniques. Recently, this latter technique has gained popularity because it is both simple and powerful. The yeast cells, suspended in growth media or buffer, are placed in an optical cuvette, gently agitated and inserted into a colorimeter. A measure of the degree of flocculation is made by observing the change in turbidity with time as the yeast flocs sediment out of suspension. Until recently, however, there had been no theory relating the change in turbidity to the degree of flocculation. In this paper, a sedimentation/light extinction experiment is described which, when combined with sedimentation theory, yields the floc size distribution and the floc settling velocity distribution.

Many factors are known to affect yeast flocculation. Among these are genetics, physiological age, cations, temperature, pH, proteins, agitation, aeration, sugars, and alcohols. Even though yeast flocculation has been studied for over sixty years, the exact relationship between these factors and the mechanism for flocculation is not completely understood. One of the most studied factors is the need for divalent cations, primarily calcium. The dependence of flocculation on the presence of calcium cations has been shown repeatedly, as described by Lindquist (1953), Gilliland (1957), Mill (1964a,b), Taylor and Orton (1973), and Stewart (1975). The sedimentation/light extinction technique described in this paper has been used to quantify the relationship between calcium cation concentration, pH, and yeast flocculation.

Structure of Yeast Flocs

The important properties of yeast flocs depend not only on the number of cells contained in the floc but also upon the packing structure of the cells. Based upon the knowledge of colloidal aggregates (Mandelbrot, 1982), one would expect the yeast flocs to be fractal in structure. Fractals have the property that the number of particles composing the aggregate increases in proportion to the geometric diameter raised to some power less than three:

$$N = a D^f$$  \(1\)
where \( f \) is known as the fractal dimension, \( D \) is the geometric diameter, and \( \alpha \) is a constant of proportionality that is inversely related to the size of the particles composing the aggregate. In general, smaller values of \( f \) are characteristic of more loosely branched and open floc structures.

Microscopic observation of flocs from many different strains of yeast have confirmed that yeast flocs are fractal in structure. From log-log plots of the aggregation number versus the geometric floc diameter, Hunt (1985) found that \( 1.75 < f < 2.25 \pm 0.15 \) at the 90\% confidence level.

**Experimental Methods and Materials**

The sedimentation/light extinction apparatus developed in our laboratory and which is capable of measuring the floc settling velocity distribution is shown in Figure 2. A yeast suspension is placed in an optical cuvette with a 1 cm path length and gently agitated so that all flocs are randomly distributed but not so as to break the flocs. The yeast flocs then settle due to gravity past a thin monochromatic slit of light placed a distance \( h \) below the liquid-air interface and produced by passing a 2mW He-Ne laser through a set of cylindrical lenses. The transmitted light is then focused onto a photodiode, and the intensity is continuously recorded on a stripchart recorder.

The light absorbance is proportional to the cell concentration according to a form of Beer's law (Herdan, 1960):

\[
A = k c
\]

(2)

where \( A \) is the absorbance, \( c \) is the number of cells per unit volume, and \( k \) is an extinction coefficient proportional to the light path length. This relationship is valid if the cell concentration is low enough to prevent multiple scattering from becoming significant. Davis and Hunt (1986) showed that the light extinction coefficient decreased markedly with an increased degree of flocculation. This results from the fact that, as flocculation increases, a higher percentage of the cells in the floc are in the shadows of other cells and do not absorb any light. In order to account for this decrease in the light extinction coefficient, an empirical relationship that correlated the data for all yeast suspensions was found:

\[
k = k' \langle N^{-1/3} \rangle
\]

(3)

where the brackets denote the average value of all flocs in the light beam, and \( k' \) is independent of floc size.

Four strains of yeast were used in these studies: ATCC 58231 (nonflocculent), ATCC 46785 (mildly flocculent), ATCC 58230 (moderately flocculent) and ATCC 48869 (highly flocculent). Growth and sampling conditions were reported in a previous paper (Davis and Hunt, 1986). The floc aggregation distribution was found for all yeast strains. In order to validate the technique, sample results were compared with more tedious microscopic enumeration using a hemacytometer. Three of the yeast strains, 58231, 58230, and 46785, were also used in studies where additional calcium was added to the media after logarithmic growth had stopped and where the pH was adjusted to various values. The sedimentation/light extinction experiment and model were then used to measure the aggregation number distributions.
Theoretical Analysis

A detailed analysis and development of the method for determining the floc settling velocity distribution and size distribution from the light extinction data was performed by Davis and Hunt (1986). An outline of this method follows. The cumulative settling velocity distribution function $F(v)$ is defined to be the fraction of cells in flocs that have a settling velocity less than or equal to $v$. The cumulative distribution can be calculated from the absorbance versus time data, obtained by the sedimentation/light extinction experiment:

$$F(v = h/t) = c(t)/c_0$$ (4)

where $c(t)$ is the concentration of cells in the beam at time $t$ and $c_0$ is the initial concentration of cells in the suspension. Combining equations (2), (3), and (4) gives the following relationship:

$$F(v = h/t) = \frac{A(t) \langle N^{-1/3}(0) \rangle}{A(0) \langle N^{-1/3}(t) \rangle}$$ (5)

where $A(t)$ is the light absorbance at time $t$ after the start of sedimentation and $\langle N^{-1/3} \rangle$ is the average of $N^{-1/3}$ for the flocs in the light beam at time $t$. The value for $k$ divides out of the expression, eliminating the need for tedious calibration when changing to a new organism. The ratio $\langle N^{-1/3}(0) \rangle / \langle N^{-1/3}(t) \rangle$ is not known a priori, so an iterative procedure is required. For the first iteration, this ratio is set equal to unity for all $t$.

The normalized probability density function of settling velocities is given by the relationship:

$$P(v) = dF/dv$$ (6)

In this model, Stokes law is used to relate the settling velocity of the flocs to the aggregation number $N$. Stokes law for the sedimentation velocity of a rigid sphere is:

$$v = \left( \rho_s - \rho \right) D_s^1 g / 18 \mu$$

where $\rho_s$ is the density of the sphere, $\rho$ is the density of the fluid, $D_s$ is the diameter of the sphere, $g$ is the gravitational constant, and $\mu$ is the viscosity. In this simple analysis, the geometric diameter of the floc is substituted for $D_s$, and $\rho_s$ is set equal to the apparent density of the floc. The latter may be calculated from volume averaging the density of the cells and the fluid surrounding them in the floc:

$$\rho_s = \rho + 6N \frac{V_c(\rho - \rho_c)}{(3\pi D_s^3)}$$ (8)

where the average volume of a yeast cell was measured to be $V_c = 9.2 \times 10^{-11} \text{ cm}^3$ and the wet density of the yeast cells was found to be $\rho_c = 1.11 \text{ g cm}^{-3}$ (Hunt, 1985).

Combining equations (1), (7), and (8), the desired relationship between floc settling velocity and aggregation number is obtained:

$$v = \gamma N^\beta$$ (9a)

where

$$\gamma = \alpha^{1/f} \frac{V_c (\rho_c - \rho)}{(3\pi \mu)}$$ and $$\beta = (f - 1)/f$$ (9b)
The distribution of floc aggregation numbers can be found easily using the fact that there is a one to one relationship between velocity and aggregation number; it follows that \( P(N)dN = P(v)dv \). Differentiating equation (8) and substituting into this latter expression yields:

\[
P(N) = \gamma B N^{(B-1)} P(v)
\]  
(10)

It is also necessary to calculate a new value for \( \langle N^{-1/3}(t) \rangle \) to be used in the subsequent iteration:

\[
\langle N^{-1/3}(t) \rangle = \int_0^{N(t)} N^{-1/3} P(N)dN \int_0^{N(t)} N P(N)dN
\]  
(11)

where \( N(t) \) is the aggregation number of the largest floc in the light beam at time \( t \), as determined by the equation \( v = h/t \) and equation (8).

Results and Discussion

The results for the strain ATCC 46785 are presented in Figures 3-5 in order to illustrate the sedimentation/light extinction technique. The light absorbance is plotted versus elapsed time in Figure 3. The point where the slope of the curve changes from zero to a negative number corresponds to the time required by the largest flocs to fall the distance \( h \). The absorbance then decreases as first larger and then smaller flocs settle past the beam. The absorbance becomes zero after all cells have sedimented out of the fluid, and the slope of the curve once again becomes zero. Equations (4) and (5) were then used to calculate the probability density function of the floc settling velocities shown in Figure 4. The probability distribution function of the aggregation number is shown in Figure 5 and was calculated using equations (6) and (7). The dashed line corresponds to the first iteration which used a value of unity for the ratio \( \langle N^{-1/3}(0) \rangle / \langle N^{-1/3}(t) \rangle \). The solid line corresponds to the second iteration. Further iterations showed no further change. The agreement between the sedimentation/light extinction experiment and the hemacytometer count denoted by the histogram in Figure 5 is very good. The sedimentation/light extinction technique yielded values of \( N = 8.8 \) for the median and \( V = 61\% \) for the coefficient of quartile variation, whereas these values are \( N = 6.9 \) and \( V = 53\% \), respectively, for the hemacytometer data. Results for other strains may be found in Davis and Hunt (1986).

The sedimentation/light extinction technique was applied to the measurement of floc size distributions for yeast suspensions to which additional calcium was added. Normal calcium ion concentration in the media was 0.68 mM. Increasing the amount of calcium ions (Ca = 2.92 mM) in the media at normal pH (pH = 2.7) had little effect on the floc aggregation number distribution for all three strains tested. This supports the findings of Mill (1964a,b) that maximum flocculation occurred at a calcium ion concentration of 0.200 mM. Saturation kinetics govern flocculation in this regime since there are only a limited number of calcium binding sites on the yeast cell surface, and additional calcium cannot interact with the cell walls.

When the above experiment was combined with a rapid pH switch, some interesting results were produced. If the pH was raised to a high basicity (pH = 7.4) after the addition of calcium, the floc aggregation distribution was altered significantly for all strains. The aggregation number distribution for ATCC 46785 is shown in Figure 6. The median aggregation number increased from \( N = 12 \) to \( N = 48 \). For the yeast ATCC 58230, the median value of \( N \) increased from 24 to 37, and for ATCC 58231 (initially nonflocculent) the median value of \( N \) increased approximately from 2 to 100. A synergistic effect between pH and calcium was also reported by
Porter and MacAulay (1965). They offered no explanation for their findings. One possible explanation that we set forth for this phenomenon is that an increased pH causes protons to be stripped from carboxyl or phosphate groups on the cell surfaces, resulting in an increase in the number of negatively charged sites. The divalent calcium ion can then form additional bridge bonds between yeast cells, resulting in an increase in flocculation.

Summary

The sedimentation/light extinction technique described above is a reliable alternative to tedious hemacytometer enumeration for quantifying yeast flocculation. Moreover, in addition to providing the size and number of cells in the floc, it provides the distribution of floc settling velocities. This information would be essential in designing cell separators and continuous fermentors such as fluidized beds. The technique is also easy to perform, and the calculations can be easily programmed into a personal computer. As shown by the results in the section on the synergistic effect of calcium and pH, the sedimentation/light extinction technique can be a powerful tool for quantifying the effects of flocculation promoters and inhibitors.

Acknowledgments

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References


Figure 1. Moderate floc size *S. cerevisiae* ATCC 46785. (A) single floc  (B) distribution of flocs

Figure 2. Experimental apparatus. (A) laser power supply  
(B) He-Ne laser  (C) lenses  (D) optical cuvette  
(E) photodiode  (F) photodiode power meter  
(G) strip chart recorder
Figure 3. Sedimentation curve for ATCC 46785

Figure 4. Probability distribution function of settling velocities for ATCC 46785

Figure 5. Probability distribution function of aggregation numbers for ATCC 46785

Figure 6. Calcium and pH effects on the probability density function of aggregation numbers for ATCC 46785
INTRODUCTION.

The solid substrate fermentation (SSF) technique, which essentially consists of substrate uptake by a microbial population in the absence of free water (1), has received increased attention in Western research laboratories recently. Simplicity, low energy requirements and capital investment, and absence of wastewater output as compared with submerged fermentations (SFs), are some advantageous features. However, heat build-up, difficult moisture control, fermentation slowness and scale-up problems have limited the industrial application of the technique (2,3).

Protein-enriched fermented food or feed (PEFF) is a new acronym to designate those food or agricultural materials on which microbial protein has upgraded their total protein content using a non-protein nitrogen (NPN) source (4). SSF processes are generally involved in the manufacture of PEFFs. In many cases, pre-gelatinized starches from agricultural commodities are used as carbon source for mold growth (4).

On the other hand, extrusion-cooking processing of starch-based foods has gained widespread popularity (5). During such a process, starch is subjected to simultaneous gelatinization and dextrinization while protein is denatured. In addition, the food matrix can also be expanded increasing its internal porosity. However, the relationships between extrusion cooking pretreatment of starchy substrates and the performance of SSFs of these materials have not been elucidated. Therefore, this paper reports some preliminary results on the SSF of corn extrudates, selected as a model starchy material.

MATERIALS AND METHODS.

Microorganism: The mold Rhizopus oligosporus (NRRL 2710), supplied as spores mixed with rice starch powder by GEM Cultures, Fort Bragg, CA was used.

Corn: The corn subjected to extrusion cooking was provided by Illinois Cereal Mills, Inc., Paris, IL. It consists of a coarse granulation, flour-free yellow grit (#25) made from degermed corn. A typical composition (in wt. %) is: Moisture 13.5; protein 7.2; ash 0.25; fat 0.7; fiber 0.3 and starch 78.0.
Mineral Medium: A solution of nutrients (100 ml) containing (in g): (NH₄)₂SO₄, 7.5; Urea, 4.0; NaH₂PO₄, 1.5; MgSO₄·7H₂O, 0.5; KCl, 0.15; CaCl₂, 0.05; FeSO₄, 0.075; was added to 100 g of extruded corn (6).

Extrusion Cooking Pretreatment: A C.W. Brabender single-screw extruder, 3/4" screw diameter was used. Corn grits were subjected to two sets of extrusion cooking conditions. A High Shear Corn Extrudate (HSCE) was produced at 177 °C Barrel Temperature (Tb); Initial Moisture (Mi, % w.b.) 21; Screw Speed (Ns, r.p.m.) 90, and 3:1 Screw Compression Ratio (SCR). On the other hand a Low Shear Corn Extrudate (LSCE) was the result of the following conditions: Tb, 147 °C; Mi, 16% w.b.; Ns, 70 r.p.m. and SCR 1:1. A constant die-diameter of 0.5 cm was maintained, and a summary of the physico-chemical characteristics of both extrudates samples are shown in Table 1.

Fermentations: Four microfermentors as that shown in Fig. 1 were submerged in a thermostated bath. They represented 2 duplicates of each set of conditions. Moistened air (98 % relative humidity) was circulated through the bed of each microfermentor, comprised by inoculated extrudate pellets. Fermentation conditions were as follows: Temperature (Tr): 37 °C; initial moisture (Mi): 55 % w.b.; aeration flow rate: 1.0 st. ml air/(g d.w. min); particle size (Tyler # 4): 4.75 mm; initial pH: 4.2 (adjusted with lactic acid solution); inoculum size 5.1x10⁶ (spores/g corn extrud.). Air flowmeters by Gilmont Instruments and Matheson Gas Products, up to 370 and 270 ml/min respectively were used. The spore powder was added to the dry extrudate pellets after grinding with a manual chopper and sieving. Mixing of spores was aided by rotating the solids in a closed beaker. Due to density and water absorption differences between the extrudate samples, equal weights and depths of substrates in the microfermentors were not possible. During about 2 days head-space gas samples were frequently taken from each fermentor and analyzed for carbon dioxide.

Analytical Methods: Kjeldahl protein of fermented extrudates were determined after trichloroacetic acid (TCA) treatment to wash out residual NPN compounds. Carbon dioxide evolved during fermentations were analyzed using a Gow Mac gas chromatograph, Series 550 (Gow Mac Instrum. Co., Madison N.J.), with a coaxial CTR1 double column (Alltech Assoc., Deerfield, IL). Water absorption and water solubility indices (WAI & WSI) respectively, were determined according to the method of Anderson et al. (7). Bulk density of extrudates were measured weighing ground material before tapping it into a 250-ml graduated cylinder. Extrudate porosities (total and internal) were calculated using apparent and bulk densities, and equation provided by Launay and Lisch (8). Moisture was determined by oven-drying unfermented samples at 70 °C and fermented samples at 105 °C during 24 hrs.

RESULTS AND DISCUSSION.

High-shear extrusion cooking conditions increased internal porosity of corn substrate, as compared to the milder extrusion conditions of the LSCE sample (Table 1, Figs. 2 & 3). Internal expansion forces create a more opened structure in the corn matrix that would facilitate the attachment of growing hyphae when inoculated externally. This is shown in Fig. 4, suggesting larger area and space availability for mold growth. The hyphae cannot readily penetrate the more compact mass of the low-shear corn substrate. From having a
more free growth at surfaces with a better air exposure (upper left side in Fig. 5), the aerial hyphae progressively disappear towards the upper right side of the corn pellet. The micrograph on Fig. 6 presents a magnified detail of the hyphal tips crawling into the unexpanded corn matrix on the right side. This poses a limitation for growth since enzymes responsible for nutrients conversion are located at those tips. These observations are consistent with fermentation results (Table 2) where a significant protein enrichment by the mold on the HSCE substrate, as compared to that obtained with the LSCE, is shown. Furthermore, material loss during fermentation of HSCE doubled that with LSCE, and a negligible protein variation between fermented LSCE and unfermented corn grits was apparent. On the other hand, the amount of CO$_2$ evolved during HSCE fermentation was larger and produced at higher rate than for the other substrate. However, a sooner CO$_2$ production decline was evident for HSCE, it might be due to variations in aeration rates.

These results suggest extrusion cooking pretreatment of starch-based substrates as an attractive way to overcome mass transfer limitations in SSFs. In addition, an internal colonization of the substrate can be attempted by coextruding the cereal with the fed spore powder at conditions such that a substantial spore population survives (i.e. lower temperature and moisture). In such manner the extruder, used as a spore mixing device would promote a more uniform internal inoculation and attachment to internal pellet surfaces, and hence would tend to increase packing densities (9) of fungal biomass.

WAI and WSI are popularly used characterization parameters for food extrudates. WAI is related to gel-forming capabilities of starches while WSI has to do with the amount of smaller carbohydrate molecules or dextrinization. Higher starch water-soluble solids/gel solids ratios presented by the most extensively extrusion-cooked corn substrate (HSCE) seem to promote mold growth (Tables 1 & 2). A less predominant effect of starch gelatinization is therefore inferred. However, the significant protein enrichment observed was an effect of summed contributions of both physical expansion and physicochemical transformations of substrate components which simultaneously take place during extrusion. Therefore, further investigation is needed and a next paper should include a mathematical model correlating the fermentation performance with the physico-chemical characteristics of the corn extrudates.

CONCLUSIONS.

The extrusion cooking pretreatment of corn substrate may improve mold growth and promotes protein enrichment in solid substrate fermentation. Higher internal porosities of extrudate pellets may overcome mass transfer limitations when they are used as substrate in solid state fermentation. Increased starch solubles in extruded substrate seem to enhance mold growth. The use of a cooking extruder as a mixing device for survived mold spores in the extruded substrate, in order to promote internal as well as external colonization of each pellet, is suggested.

ACKNOWLEDGMENT.

The partial support by the Colorado Cooperative State Research Service under Project # 153831 is acknowledged.
REFERENCES.


<table>
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<tr>
<th></th>
<th>HIGH SHEAR EXTRUDATE</th>
<th>LOW SHEAR EXTRUDATE</th>
<th>RAW CORN GRITS</th>
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<tr>
<td>DIAMETER (cm)</td>
<td>0.69</td>
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<tr>
<td>fine powder</td>
<td>0.75</td>
<td>0.79</td>
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<tr>
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<td>0.14</td>
<td>0.59</td>
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<tr>
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<td></td>
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<td>size # 4</td>
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</tr>
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<td>(g gel/g sample)</td>
<td>6.57</td>
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<td>WATER SOLUBILITY INDEX</td>
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<tr>
<td>g soluble solids/g sample</td>
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<td>g soluble solids/g gel solids</td>
<td>0.125</td>
<td>0.042</td>
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Figure 1. Microfermentor
Figure 2. Scanning electron micrograph of the low shear corn extrudate (LSCE) showing restricted internal air voids. Magnification: x48.6. Unit length: 1 mm.

Figure 3. Scanning electron micrograph of the high shear corn extrudate (HSCE) showing larger internal spaces and details of internal surfaces. Magnification: x48.6. Unit length: 1 mm.
### TABLE 2. SOLID SUBSTRATE FERMENTATIONS

<table>
<thead>
<tr>
<th></th>
<th>Fermented High Shear Extrudate</th>
<th>Fermented Low Shear Extrudate</th>
<th>Unfermented Corn Grits</th>
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<td><strong>Bed Depth (cm)</strong></td>
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<td>2.7</td>
<td>-</td>
</tr>
<tr>
<td><strong>Extrudate Weight (g)</strong></td>
<td>3.5</td>
<td>12.0</td>
<td>-</td>
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<td>55.0</td>
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<td>7.2</td>
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<tr>
<td>Final</td>
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<td><strong>Material Loss</strong></td>
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<td>(g solids)</td>
<td>(g d.w.)</td>
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<td>10.58</td>
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<tr>
<td>(g/g d.w.)</td>
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<tr>
<td><strong>Kjeldahl Protein Gain</strong></td>
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<tr>
<td>(g protein / (g material lost))</td>
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Figure 4. Scanning electron micrograph of the fermented HSCE, showing aerial hyphae. Magnification: x48.6. Unit length: 1 mm.

Figure 5. View of a fermented LSCE pellet, presenting dense aerial hyphae (upper left) and growth-free core of unexpanded extrudate (upper right). Magnification: x19.5. Unit length: 1 mm.
Figure 6. Magnified SEM detail of the growth - non-growth interface of the fermented LSCE pellet shown in Fig. 5. Magnification x163. Unit length 1 mm.

Figure 7. Carbon Dioxide evolution during fermentation of corn extrudates
Optimum Design of a Hollow Fiber Mammalian Cell Reactor

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Fort Collins, Colorado 80521

Introduction

The large-scale culture of mammalian cells is a viable method for the production of many important pharmaceuticals. Among these are vaccines, monoclonal antibodies, growth hormones, and interferons (1). Most mammalian cells are anchorage-dependent, that is, they require a surface on which to attach (2). The primary criterion for an efficient reactor system is, therefore, a high surface area to volume ratio. Hollow fiber reactors satisfy this criterion and have found widespread use for large-scale cell culture (3). In these systems, a bundle of hollow polymeric fibers is enclosed in a cylindrical shell, much like a shell and tube heat exchanger. Cells attach and grow on the outside of the fibers and are supplied by nutrient solution which flows through the lumen of the fibers. We have developed and modeled a high surface area hollow fiber reactor. Overall reactor cell density is shown to be a function of fiber spacing and optimum fiber spacing is calculated. Preliminary experimental data confirm the model.

Reactor Design

Hollow fiber reactors generally employ a tightly packed fiber bundle. Cells grow in a monolayer and the total attachment area is limited to the surface area of the fibers. We have chosen a reactor system which is patterned after cell growth in vivo. The fibers are spaced a uniform distance apart and cells grow on an inter-fiber matrix. Multilayer growth is realized since the available attachment area is greatly increased. Collagen, the major protein component in connective tissue, is employed as the inter-fiber matrix. Figure 1 shows a mouse fibroblast cell (3T3) immobilized in a collagen matrix. Optimal fiber spacing must be determined to ensure maximal overall cell density.

Hollow fibers with a 100,000 dalton molecular weight cutoff and a 450 micron O.D. were provided by the Celanese Corporation. The fibers were spaced uniformly in a 1 inch O.D. polycarbonate shell and potted with H.B. Fuller 2187 polyurethane resin. Cells were mixed with a collagen (or agar) gel and inoculated into the reactor at a density of about $10^6$ cells/ml. Gibco MEM medium was perfused and
recirculated from a 500 ml reservoir. The entire system was maintained at 37°C in a CO₂ incubator.

**Model**

The system is modeled by considering an annular region of cells surrounding an individual fiber (Figure 2). The continuity equations are written for the tube side of the fiber, the fiber wall, and the cell suspension annulus, with the appropriate flux continuities as boundary conditions (4). Substrate consumption is assumed to proceed at a zero order rate.

The three coupled P.D.E.'s are solved by an implicit finite difference scheme (5). The initial profile (reactor inlet) is solved with a first order discretization; subsequent steps are calculated with a Crank-Nicolson discretization. The resulting concentration profile is a function of radial and axial position. Figure 5 shows a substrate concentration profile. The model will predict the axial reactor length at which the substrate concentration falls to zero for a given density of cells. The reactor is diffusion limited at that point. Alternatively, the maximal local cell density can be predicted for a reactor of given length.

**Kinetic and Transport Data**

Successful optimization of the reactor hinges on the procurement of accurate kinetic data. Figure 3 shows batch data of cell number, glucose consumption and lactate production for a culture of Ehrlich Ascites Tumor (EAT) cells. It is interesting to note that when glucose is depleted lactate is consumed as a carbon source (6). Glucose consumption and lactate production rates may be calculated from the growth curve. These are shown in Figure 4. It is clear from this plot that metabolism is highly anaerobic in the early stages of a culture (glc and lac rates high) while at later stages it becomes predominately aerobic (glc and lac rates low). The following maximal rates have been determined:

- glucose consumption $- 6.9 \times 10^{-11}$ mg/ml sec
- oxygen consumption $- 1.1 \times 10^{-12}$ mg/ml sec
- lactate production $- 5.4 \times 10^{-11}$ mg/ml sec

Comparison of the maximal glucose and lactate rates shows that metabolism is never entirely anaerobic since two lactate molecules would be obtained from each glucose molecule consumed.
Transport data are also needed to realize optimum reactor design. In particular, the diffusivity of various substrates and waste products through the cell matrix must be determined. We have developed a simple technique for the measurement of diffusivities through a cell layer. The basis of this technique is the observation that diffusion through a dilute gel is nearly as rapid as diffusion through pure water (7). Cells may be immobilized in a collagen, agar, or alginate gel, thus imparting a high degree of rigidity to the cell mass while hindering solute diffusion only to a small extent. Stirred bath diffusion measurements as outlined by Crank (8) may now be performed since the cell-gel matrix has sufficient integrity to withstand vigorous stirring in a solution which it contacts. This technique is particularly attractive for the determination of oxygen diffusivity.

The following are results for solute diffusivity through a \(3 \times 10^8\) cells per ml matrix of EAT cells at 37°C (cm\(^2\)/sec).

- glucose - \(6.0 \times 10^{-6}\)
- urea - \(1.3 \times 10^{-5}\)
- lactate - \(6.9 \times 10^{-6}\)
- oxygen - \(\sim 1 \times 10^{-5}\)

These kinetic and transport data indicate that at cell densities of \(3 \times 10^5\) and above, lactate accumulation will be inhibitory to cell growth. At later stages in the culture oxygen delivery will be limiting. It has been shown that lactate accumulation can be greatly reduced by supply of an alternate carbon source such as fructose (1). The delivery of oxygen in cell matrices has not been addressed to date.

**Optimization**

It is obvious that a large spacing between fibers will not permit dense cell growth. A close-packed bundle of fibers will support a high cell density; however, the overall reactor density may not be high since a very large portion of the volume will be occupied by fibers. Figure 6 shows a schematic of a reactor cross section with fibers spaced a uniform distance (s) in both radial and angular directions. Equations for the number of fibers (N) and the overall reactor density (p) are presented. The scheme for determining optimum fiber spacing is as follows:

1) Choose a fiber spacing s - calculate N.

2) Use finite difference algorithm to determine maximum local cell density \(p_1\).

3) Calculate \(p_g\) from N and \(p_1\).

In this manner, overall reactor density is determined as a
function of fiber spacing. Based on our preliminary kinetic and transport data, we have determined that optimal fiber spacing is between 20 and 40 microns for the Celanese fibers. The optimal spacing will of course vary depending on the specific fiber employed.

Conclusions

A high surface area hollow fiber reactor for mammalian cells has been developed. Attachment area is increased by use of a collagen or agar matrix between the fibers. The system is modeled with an implicit finite difference algorithm and the resulting solution is a function of both radial and axial position. Kinetic and diffusivity data may be obtained and are used to optimize the fiber spacing. Our results show that close packing is not an optimal design; fiber spacings of 20 to 40 microns will result in higher overall reactor density. In the early stages of a culture lactate accumulation may be a problem but may be remedied by the use of an alternate carbon source. At later stages oxygen delivery is limiting. It is clear that development of an oxygen carrier is critical for the attainment of high density cell cultures.

References

Region 1 \[ 2u \left[ 1-(r/R_i)^2 \right] \frac{\partial C}{\partial z} = \frac{D_m}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) \] \[ \frac{\partial C}{\partial r} = 0 \quad r=0 \]

Region 2 \[ \frac{D_m}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) = 0 \]

Region 3 \[ \frac{D_m}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) = V \]

Figure 1  Mouse Fibroblast cell immobilized in collagen

Figure 2  Mathematical model of an individual fiber. Region 1 (0 to \( R_i \)) is the tube side of the fiber, region 2 (\( R_i \) to \( R_o \)) is the fiber wall, and region 3 (\( R_o \) to \( MP \)) is the cell suspension annulus. Laminar flow velocity profile is used for the convective term in region 1.
Figure 3
Batch data for EA1 cell growth.

Figure 4
Glucose and lactate rates for EAT cell
Figure 5  Solution of concentration profile by finite difference algorithm

\[ N = \frac{\pi R_s}{R_f (2s+2)} \left[ R_s + R_f (2s+2) \right] \]

\[ \rho_g = \rho_L \left[ 1 - N \left( \frac{R_f}{R_s} \right)^2 \right] \]

Figure 6  Cross section of reactor showing fiber spacing. Equations for number of fibers (N) and overall density (\( \rho_g \)).
GAS CHROMATOGRAPHY AND NUCLEAR MAGNETIC RESONANCE OF TRIFLUOROACETYLATED CARBOHYDRATES

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INTRODUCTION

This report is the sixth in a series on the gas chromatography (GC) and high-performance liquid chromatography (HPLC) of oligosaccharides. In the first article capillary column GC of trimethylsilyl (TMS) disaccharides was described. The next three reported the HPLC of disaccharides and trisaccharides on amine-bonded silica columns and the apparent mechanism of HPLC on these columns. These reports made it evident that analytical problems existed and that development of a new technique was needed for separating complex mixtures of trisaccharides and larger oligosaccharides.

Selosse and Reilly separated the anomers of thirteen trisaccharides by GC using derivatization with N-methyl-bis(trifluoroacetamide) (MBTFA). This method permits a better separation and discrimination of the trifluoroacetyl (TFA) saccharides in capillary column GC than any previously developed because of the high volatility obtained with MBTFA derivatization. This high volatility is a result of replacing the saccharide's hydroxyl groups with heavier but less interactive TFA groups. Without derivatization, the amount of hydrogen bonding between hydroxyl groups of the saccharides makes their boiling point so high that they decompose upon GC before they can boil (or melt, in some cases). Perhaps the most important conclusion from the use of MBTFA is that capillary GC of longer TFA oligosaccharides facilitates discrimination of the location of branch points in compounds having the same backbones.

Selosse and Reilly also discussed correlations between the separation of reducing and nonreducing saccharides. One such comparison indicated that concentrations of the anomic peaks produced upon GC do not correspond to the underivatized anemonic concentrations. In addition, they found that proton nuclear magnetic resonance (NMR) of a few TFA saccharides indicated that the major chromatographic peaks of these reducing TFA saccharides corresponded to \(\alpha\)-anomers, in direct contradiction to known mutarotation equilibria.

This paper presents work in two areas: (1) The determination of retention times and concentrations of the anemonic peaks of TFA saccharides produced by GC, and the comparison of the latter with concentrations of fully mutarotated, underivatized saccharide anomers, and (2) the determination of structures of TFA saccharides and their anomers by proton NMR. To accomplish this, the anemonic concentrations of 33 TFA mono-, di-, and trisaccharides were measured by capillary column GC and were compared to values cited in the literature. In addition, the anemonic structures of 17 TFA saccharides were defined by careful interpretation of proton NMR spectra.
EXPERIMENTAL

Carbohydrates

Nine monosaccharides were studied: D-glucose, D-mannose, D-galactose, L-sorbose, L-fucose, L-rhamnose, D-xylose, D-lyxose, and L-arabinose. All but L-rhamnose were submitted to GC and all but L-sorbose were analyzed by NMR.

Eighteen disaccharides were derivatized and analyzed by GC: α, α-trehalose [Glc-α-(1,1)-Glc], α, β-trehalose [Glc-α-(1,1)-β-Glc], β,β-trehalose [Glc-β-(1,1)-β-Glc], sophorose [Glc-β-(1,2)-Glc], nigerose [Glc-α-(1,3)-Glc], laminaribiose [Glc-β-(1,3)-Glc], maltose [Glc-α-(1,4)-Glc], cellobiose [Glc-β-(1,4)-Glc], leucrose [Glc-α-(1,5)-Fru], lactose [Galp-β-(1,4)-Glc], melibiose [Galp-α-(1,6)-Glc], sucrose [Fru-β-(2,1)-α-Glc], turanose [Glc-α-(1,3)-Fru], palatinose [Glc-α-(1,6)-Fru], lactulose [Galp-β-(1,4)-Fru], and xylobiose [Xyl-β-(1,4)-Xyl]. Eight of these, sophorose, nigerose, maltose, cellobiose, isomaltose, gentiobiose, lactose, and sucrose, were submitted to NMR.

Seven trisaccharides were derivatized and analyzed by GC: maltotriose [Glc-α-(1,4)-Glc-α-(1,4)-Glc], panose [Glc-α-(1,6)-Glc-α-(1,4)-Glc], isopanose [Glc-α-(1,4)-Glc-α-(1,6)-Glc], isomaltotriose [Glc-α-(1,6)-Glc-α-(1,6)-Glc], melezitose [Glc-α-(1,3)-Fru-β-(2,1)-α-Glc], raffinose [Galp-α-(1,6)-Glc-α-(2,1)-β-Fru], and xylotriose [Xyl-β-(1,4)-Xyl-β-(1,4)-Xyl]. Isomaltotriose was analyzed by NMR.

Regents

MBTFA and silylation-grade pyridine were obtained from Pierce (Rockford, IL, U.S.A.). Dichloromethane-d₂ (99.6 + atom % D) came from Aldrich (Milwaukee, WI, U.S.A.).

Derivatization

For gas chromatography, between 1 and 3 mg of the saccharide were dissolved in 0.5 ml pyridine; after a 24-h period at 40°C for equilibration, the pyridine/saccharide solution was derivatized by the method of Sullivan and Schewe by adding 0.5 ml of MBTFA and heating the mixture at 65°C for 1 h. For NMR, between 10 and 25 mg disaccharide were dissolved in 1 ml pyridine and, after derivatization, 1 ml of MBTFA was added.

Gas Chromatography

A Hewlett-Packard (Palo Alto, CA, U.S.A.) 5890A gas chromatograph coupled to a Hewlett-Packard 3492A integrator was used for the analysis of the derivatized samples. A fused-silica capillary column manufactured by J & W Scientific (Rancho Cordova, CA, U.S.A.), 30 m x 0.26 mm I.D. and coated with a 0.1 µm film of DB-5 liquid phase, was used.

The column oven was held at constant 100, 150, and 190°C for mono-, di-, and trisaccharides, respectively. Injector and flame-ionization detector temperatures were 220°C and 240°C, respectively. Helium carrier gas flow was 0.204 m/s measured by methane; a 1:100 splitting ratio was used.

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Analysis by GC provided a chromatogram, the relative retention time, and the relative concentration of each TFA saccharide anomer.

Nuclear Magnetic Resonance

To concentrate each TFA saccharide, a Duo-Seal vacuum pump (Welch Scientific, Chicago, U.S.A.) in series with a glass tube-in-shell heat exchanger submerged in a dry ice/acetone trap was used to pull off most of the pyridine, MBTFA, and MBTFA byproducts. The concentrated TFA saccharide was dissolved with 0.8 ml of deuterated dichloromethane; 0.6 ml of the resulting solution was transferred into a size 5, WM-300 NMR tube.

Proton NMR spectroscopy was conducted using a Bruker (Los Angeles, U.S.A.) WM-300 spectrophotometer. This provided the spectrum of each TFA saccharide and the relative height and the frequency (chemical shift) of each peak with respect to tetramethylsilane (TMS).

RESULTS AND DISCUSSION

Gas Chromatography

The retention times and anomic concentrations upon capillary GC for TFA mono-, di-, and trisaccharides are reported in Tables 1a, 1b, and 1c, respectively. Several of the anomic concentrations for TFA monosaccharides are compared with fully mutarotated, nonderivatized monosaccharides in aqueous solution reported by Isbell and Pigman6 (Table 2). In all cases, the concentrations of TFA saccharide α-anomers were greater than for nonderivatized saccharides.

Two explanations can be offered for trifluoracetylation favoring the α-anomer: the effects of derivatization with MBTFA and the use of different solvents.

During derivatization by MBTFA, the equilibrium of the anomic concentrations is shifted by conformational changes; after derivatization, the anomic concentrations are fixed in a new anomic form. These changes are produced by the strain of the new bulky TFA groups attaching to the ring and by the anomic effect. The anomic effect is caused by conformational change due to dipole-dipole interaction between the highly electronegative TFA group and the ring oxygen atom6. The polar TFA group on the primary carbon tends to switch to the axial position, which stabilizes the α-anomer.

Also, the anomic effect varies inversely with the dielectric constant of the solvent6. The dielectric constant of pyridine is fairly small relative to water, 12.3 vs. 78.5, respectively, at 25°C. Therefore the anomic effect contributes to a greater concentration of the α-anomers of TFA saccharides in pyridine solution.
Nuclear Magnetic Resonance

The anomeric peak frequencies, relative heights, and amount of splitting upon proton NMR of the TFA mono-, di-, and trisaccharides in deuterated dichloromethane are reported in Tables 3a and 3b.

Analysis of the data in Table 3 indicates that the major anomeric peaks occurring upon proton NMR are $\alpha$- and $\beta$- anomers. However, in some cases the $\beta$-anomer is so distorted that it appears by NMR to be an $\alpha$- anomer. Typically an $\alpha$-anomer is downfield (higher ppm), larger in height, and has a smaller peak split (3-4 Hz vs. 5-7 Hz) than a $\beta$-anomer. A distorted $\beta$-peak having the appearance of an $\alpha$-peak tends to have peak splits of 4-5 Hz and in some cases to be shifted downfield close to the true $\alpha$-anomeric peak.

The distinction between $\alpha$-anomeric peaks and the $\beta$-anomeric peaks that closely resemble them was based upon the realization that conformational change occurred when the saccharide was derivatized with MBTFA. The distortion of the $\beta$-anomers is caused by the introduction of the TFA groups, and this is caused by the anomeric effect discussed earlier. The conformational change is thought to reduce the Karplus angle, the arc separating the lone hydrogen atoms attached one each to the 1- and 2-carbons. A rotation in the Karplus angle would give the $\beta$-anomer the appearance of an $\alpha$-anomer by decreasing its peak split by 1-2 Hz. It also follows that if a conformational change influences splitting, it would also influence the chemical shift of the anomer.

CONCLUSION

The discrepancy between anomeric concentrations of saccharides and TFA saccharides observed by Selosse and Reilly has been described. This discrepancy results from derivatization by MBTFA and from the use of different solvents. In all cases, the results indicate that the concentrations of $\alpha$-anomer were greater for TFA saccharides than for nonderivatized saccharides. The anomeric effect was used to explain these results; from this it can be predicted that derivatization by MBTFA and the use of pyridine as the solvent introduces conformational changes in the saccharides which favor the $\alpha$-anomer.

A preliminary analysis by Selosse and Reilly indicated that the major chromatographic peaks of a number of reducing TFA saccharides correspond to $\alpha$- anomers, in direct contradiction of known mutarotation equilibria. This study corrects the preliminary appraisal and indicates that the contradictory extra $\alpha$-anomeric peak is actually a distorted $\beta$-anomeric peak, which occurs because of conformational changes brought about by trifluoracetylation.
REFERENCES


Table 1a. Retention times and anomeric concentrations for TFA monosaccharides by capillary column GC.

<table>
<thead>
<tr>
<th>TFA sugar</th>
<th>Retention time (min)</th>
<th>Anomeric conc. (%)</th>
<th>TFA sugar</th>
<th>Retention time (min)</th>
<th>Anomeric conc. (%)</th>
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<tbody>
<tr>
<td>D-glucose</td>
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<td>D-mannose</td>
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<td>L-arabinose</td>
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Table 1b. Retention times and anomeric concentrations for TFA disaccharides by capillary column GC.

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<th>TFA sugar</th>
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<th>Anomeric conc. (%)</th>
<th>TFA sugar</th>
<th>Retention time (min)</th>
<th>Anomeric conc. (%)</th>
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<td>α,α-trehalose</td>
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Table 1c. Retention times and anomeric concentrations for TFA trisaccharides by capillary column GC.

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Table 2. Equilibrium proportions of anomeric concentrations for TFA saccharides in pyridine, and saccharides in water.

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<td>D-glucose</td>
<td>0.4876</td>
<td>0.4786</td>
<td>0.374</td>
</tr>
<tr>
<td>D-mannose</td>
<td>0.8100</td>
<td>0.1630</td>
<td>0.689</td>
</tr>
<tr>
<td>D-galactose</td>
<td>0.6792</td>
<td>several</td>
<td>0.314</td>
</tr>
<tr>
<td>D-xylose</td>
<td>0.4922</td>
<td>0.4635</td>
<td>0.321</td>
</tr>
<tr>
<td>D-lyxose</td>
<td>0.8089</td>
<td>0.1911</td>
<td>0.797</td>
</tr>
</tbody>
</table>
Table 3a. Anomeric peaks of TFA monosaccharides in deuterated dichloromethane by proton NMR.

<table>
<thead>
<tr>
<th>TFA sugar</th>
<th>Relative peak heights</th>
<th>Peak splitting (Hz)</th>
<th>Chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>2486/2334</td>
<td>3.74</td>
<td>6.712/6.725</td>
</tr>
<tr>
<td></td>
<td>722/680</td>
<td>7.92</td>
<td>6.252/6.271</td>
</tr>
<tr>
<td>D-galactose</td>
<td>2825/2868</td>
<td>3.66</td>
<td>6.775/6.786</td>
</tr>
<tr>
<td></td>
<td>275/275</td>
<td>4.33</td>
<td>6.736/6.751</td>
</tr>
<tr>
<td></td>
<td>824</td>
<td>--</td>
<td>6.611</td>
</tr>
<tr>
<td></td>
<td>221/210</td>
<td>7.84</td>
<td>6.245/6.270</td>
</tr>
<tr>
<td>D-mannose</td>
<td>1397</td>
<td>--</td>
<td>6.508</td>
</tr>
<tr>
<td></td>
<td>200?</td>
<td>--</td>
<td>6.38?</td>
</tr>
<tr>
<td>L-fucose</td>
<td>19580/19586</td>
<td>3.62</td>
<td>6.639/6.651</td>
</tr>
<tr>
<td></td>
<td>5581</td>
<td>--</td>
<td>6.513</td>
</tr>
<tr>
<td></td>
<td>633/591</td>
<td>3.71</td>
<td>6.442/6.455</td>
</tr>
<tr>
<td>L-rhamnose</td>
<td>1576</td>
<td>--</td>
<td>6.481</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>--</td>
<td>6.454</td>
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<tr>
<td></td>
<td>286</td>
<td>--</td>
<td>6.397</td>
</tr>
<tr>
<td>D-xylose</td>
<td>470/460</td>
<td>3.66</td>
<td>6.648/6.660</td>
</tr>
<tr>
<td></td>
<td>149/143</td>
<td>3.36</td>
<td>6.298/6.309</td>
</tr>
<tr>
<td>D-lyxose</td>
<td>256/211</td>
<td>4.57</td>
<td>6.684/6.699</td>
</tr>
<tr>
<td></td>
<td>1417/1235</td>
<td>3.93</td>
<td>6.562/6.575</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>--</td>
<td>6.475</td>
</tr>
<tr>
<td></td>
<td>2595/2941</td>
<td>1.38</td>
<td>6.422/6.426</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>4828/4915</td>
<td>3.36</td>
<td>6.737/6.749</td>
</tr>
<tr>
<td></td>
<td>4109</td>
<td>--</td>
<td>6.594</td>
</tr>
<tr>
<td></td>
<td>203</td>
<td>--</td>
<td>6.574</td>
</tr>
<tr>
<td></td>
<td>100?/100?</td>
<td>3?</td>
<td>6.523/6.534</td>
</tr>
<tr>
<td></td>
<td>250?</td>
<td>--</td>
<td>6.419</td>
</tr>
<tr>
<td></td>
<td>200?/200?</td>
<td>--</td>
<td>6.164/6.186</td>
</tr>
</tbody>
</table>
Table 3b. Anomeric peaks of TFA di and trisaccharides in deuterated dichloromethane by proton NMR.

<table>
<thead>
<tr>
<th>TFA sugar</th>
<th>Relative peak heights</th>
<th>Peak splitting (Hz)</th>
<th>Chemical shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sophorose</td>
<td>164/185</td>
<td>4.06</td>
<td>6.666/6.680</td>
</tr>
<tr>
<td></td>
<td>69/64</td>
<td>7.65</td>
<td>6.102/6.128</td>
</tr>
<tr>
<td>nigerose</td>
<td>2907/2555</td>
<td>3.83</td>
<td>6.611/6.624</td>
</tr>
<tr>
<td></td>
<td>1892/1655</td>
<td>7.38</td>
<td>6.131/6.156</td>
</tr>
<tr>
<td>maltose</td>
<td>1554/1607</td>
<td>3.69</td>
<td>6.646/6.659</td>
</tr>
<tr>
<td></td>
<td>106/107</td>
<td>7.69</td>
<td>6.296/6.322</td>
</tr>
<tr>
<td>cellobiose</td>
<td>369/320</td>
<td>3.15</td>
<td>6.526/6.540</td>
</tr>
<tr>
<td></td>
<td>679/612</td>
<td>7.94</td>
<td>6.044/6.070</td>
</tr>
<tr>
<td>isomaltose</td>
<td>246/250</td>
<td>3.71</td>
<td>6.662/6.674</td>
</tr>
<tr>
<td>gentiobiose</td>
<td>210/205</td>
<td>4.40</td>
<td>6.696/6.710</td>
</tr>
<tr>
<td></td>
<td>268/267</td>
<td>8.03</td>
<td>6.224/6.251</td>
</tr>
<tr>
<td>lactose</td>
<td>756/857</td>
<td>3.69</td>
<td>6.534/6.547</td>
</tr>
<tr>
<td></td>
<td>150?/140?</td>
<td>7?</td>
<td>6.049/6.078</td>
</tr>
<tr>
<td>sucrose</td>
<td>no peaks between 6.1 and 7 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>isomaltotriose</td>
<td>41/26</td>
<td>4.40</td>
<td>6.648/6.662</td>
</tr>
</tbody>
</table>
**INTRODUCTION**

It is important to understand photosynthetic microbial conversion processes in supplying good quality of single cell protein and treating wastewater with blue-green algae, *Spirulina platensis* [1 - 15]. Kinetic and bioenergetic models for these processes are considered to express the relationship of light intensity with specific growth rate and bioenergetic yield. A linear model which combines a kinetic model with a bioenergetic model is also presented. Kinetic growth factors are considered with respect to light intensity in optimizing culture systems. The photobioreactor is designed to have a relatively uniform light profile and specific growth rate inside the vessel under turbidostat cultivation.

Blue-green algae, *Spirulina platensis* was cultivated under turbidostat conditions. The composition of media was defined by Kratz and Myers [7] except for using sodium bicarbonate and urea as carbon and nitrogen source, respectively. The pH of media was adjusted to 9.2 by adding 0.5N NaOH. Under carbon limited conditions continuous chemostat cultivation was employed. Average light intensities were reported as mean values of light intensities measured at front and center of a rectangular vessel designed to give a uniform light profile by illuminating light from both sides of the vessel [15].

Under turbidostat operations, dilution rate, D of the system is equal to the average specific growth rate. The consistency of the experiment can be checked by carbon balance as follows:

\[
\frac{D\sigma_b q}{24} + \frac{Q_c}{12} X = \frac{DX_b}{12} \tag{1}
\]

where \( q \) is the ratio of g-mole nitrogen to g-mole carbon in biomass, \( Q_c \) is the specific consumption rate of bicarbonate ion as carbon source, \( \sigma_b \) is weight fraction carbon of algae (assumed to be 0.4766 [2]), \( X \) is the cell concentration and \( V \) is the liquid volume. The value of biomass energetic yield can be obtained from

\[
\eta_{kcal} = \frac{DX_b Y_b Q_o}{12I_a A} \tag{2}
\]

where \( A \) is the total illuminating area of the vessel, \( I_a = I_{in} - I_{out} \) is the amount of light absorbed. Values of \( Y_b \) and \( Q_o \) are assumed to be 4.402 and...
26.95 kcal per equiv. available electron, respectively [2]. The carbon balance is also used to obtain bioenergetic yield,

\[
\eta_C = \frac{\Delta X_a qV}{(\frac{24}{24} + Q_{c,v})Y_b Q_0 I_a}
\]

(3)

based upon measurements of light and bicarbonate ions.

Several kinetic models are considered to express the relationship between mean light intensity and average specific growth rate. The Monod model is

\[
\mu = \frac{\mu_{\text{max}} I_{\text{Avg}}}{K_I + I_{\text{Avg}}}
\]

(4)

The Aiba model [8] which includes the photoinhibition term at high light intensity is

\[
\mu = \frac{\mu_{\text{max}} I_{\text{Avg}}}{(K_I + I_{\text{Avg}})^{2}}
\]

(5)

The Bannister model [9] is

\[
\mu = \frac{\mu_{\text{max}} I_{\text{Avg}}}{(K_I + I_{\text{Avg}})^{1/m}}
\]

(6)

where \(K_I\) is the saturation constant and \(K_I\) is the photoinhibition term. The average value of light intensity is obtained by

\[
I_{\text{avg}} = \frac{(I_{\text{in}} + I_{\text{out}}) + 2I_C}{2}
\]

(7)

where \(I_C\) is the light intensity directionally measured at the center. A modified form of the Aiba model in which the middle term in the denominator is eliminated was also considered; that is,

\[
\mu = \frac{I_{\text{avg}}}{K_1 + K_2 I_{\text{avg}}^2}
\]

(8)

where \(K_1 = K_I / \mu_{\text{max}}\) and \(K_2 = K_I / \mu_{\text{max}}^2\). These equations are utilized to estimate kinetic parameters and to fit the experimental data from light-limited experiments. A linear model for the light-limited region is also tested. The model which combines a bioenergetic model with kinetics [15] is
or allowing for a nonzero intercept

\[ \mu = \alpha I_{\text{Avg}} + \beta \]  

Other kinetic considerations for the growth of *Spirulina platensis*, for example, pH, temperature and concentration of bicarbonate ion, on bioenergetic yield and specific growth rate are also investigated at several light intensities.

**RESULTS AND DISCUSSION**

Figure 1 shows the variation of specific growth rate and bioenergetic yield with light intensity at \( X = 0.04(\text{g/L}) \). It is apparent that both bioenergetic yield and growth rate are functions of light intensity. Specific growth rate shows a saturation value at about 35 \( \text{W/m}^2 \), and decreases at higher light intensity because of photoinhibition. It is interesting that higher bioenergetic yield is obtained at lower light intensity and values of growth yield decrease as intensity of light increases. A similar phenomena was also observed by Oswald [6]. This can be partly explained by the inverse relationship between chlorophyll content and light intensity; in other words, higher amounts of chlorophyll are synthesized at lower light intensity [10], which results in more effective utilization of input light energy.

Table I contains results of estimating kinetic parameters using Equations (4-10) from data in Fig. 1. The graphical fit of experimental data is shown in Figure 2. Values of maximum specific growth rate for both Monod and Bannister are somewhat higher than other published results of about 0.10 (1/h). Aiba model and modified Aiba model fit very well for the entire range of light intensities because photoinhibition at higher light intensity is considered. All kinetic models can be reasonably fit to the experimental results in the light limited region. Results of estimating kinetic parameters of Monod model using data for bicarbonate limited growth are summarized in Table II. Values of those parameters are relatively constant for the light intensities investigated. Values of maximum specific growth rate are somewhat lower than those from light-limited growth and values of saturation constant are higher than literature data [11].

Figure 3 and 4 show the relationship of dilution rate and bioenergetic yield with temperature for three levels of light intensity. Figure 3 indicates that the optimum temperature for the growth of *Spirulina platensis* is 32-35°C for all ranges of light intensity, which is close to other reported results [12]. The highest dilution rate is observed at medium and high light intensity, however, the highest bioenergetic yield is obtained at the lowest light intensity due to a higher amount of chlorophyll content in the algae.

The results of Arrhenius plotting, to obtain the activation energy of algae gave similar values at the three light intensities; these values are
Table I. Results of estimating parameters for kinetic models from turbidostat data at $X = 0.04$ (g/L).

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameters</th>
<th>Point Estimate</th>
<th>95% Confidence Interval</th>
<th>M.S. (\dagger\dagger)</th>
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</thead>
<tbody>
<tr>
<td>Monad*([9])</td>
<td>$\mu_{\text{max}}(h^{-1})$</td>
<td>0.1617</td>
<td>(0.1380, 0.1854)</td>
<td>4.18\times10^{-5}</td>
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<tr>
<td></td>
<td>$K_1(W/m^2)$</td>
<td>17.04</td>
<td>(10.41, 23.67)</td>
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<tr>
<td>Bannister*([14])</td>
<td>$\mu_{\text{max}}(h^{-1})$</td>
<td>0.2976</td>
<td>(-0.2193, 0.8145)</td>
<td>5.71\times10^{-4}</td>
</tr>
<tr>
<td></td>
<td>$K_1(W/m^2)$</td>
<td>15.01</td>
<td>(7.448, 22.57)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m$</td>
<td>0.4970</td>
<td>(-0.1255, 1.1191)</td>
<td></td>
</tr>
<tr>
<td>Aiba ([13])</td>
<td>$\mu_{\text{max}}(h^{-1})$</td>
<td>5.4849</td>
<td>(-99.0974, 100.067)</td>
<td>5.76\times10^{-5}</td>
</tr>
<tr>
<td></td>
<td>$K_1(W/m^2)$</td>
<td>959.2</td>
<td>(-17625, 19548)</td>
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</tr>
<tr>
<td></td>
<td>$K_1(m^2/W)$</td>
<td>0.5817</td>
<td>(-10.75, 11.912)</td>
<td></td>
</tr>
<tr>
<td>Modified(\dagger)</td>
<td>$K_1^+(Wh/m^2)$</td>
<td>177.9</td>
<td>(158.3, 197.4)</td>
<td>5.29\times10^{-5}</td>
</tr>
<tr>
<td>Aiba</td>
<td>$K_2^+(m^2h/W)$</td>
<td>0.1083</td>
<td>(0.0942, 0.1223)</td>
<td></td>
</tr>
<tr>
<td>Linearity**([4])</td>
<td>$\alpha(m^2/W/h)$</td>
<td>0.0023</td>
<td>(0.0019, 0.0027)</td>
<td>3.46\times10^{-5}</td>
</tr>
<tr>
<td></td>
<td>$\beta(h^{-1})$</td>
<td>0.0254</td>
<td>(0.0143, 0.0364)</td>
<td></td>
</tr>
</tbody>
</table>

* Parameters are estimated with all data except lost five points in Table II \([15]\).
** Parameters are estimated with data of first nine points in Table II for light-limited region \([15]\).
\(\dagger\) $K_1 = K_1/\mu_{\text{max}}$ and $K_2 = K_1/\mu_{\text{max}}$.
\(\dagger\dagger\) Mean Square: the summation of squares of residuals divided by degrees of freedom.
Figure 1. Variation of average specific growth rate and bioenergetic yield with light intensity for turbidostat operation with $X = 0.04$ g/L: 
* specific growth rate; ● $\eta_{_{\text{Kcal}}}$; △ $\eta_{_{C}}$. 

Light Intensity, $I_{\text{Avg}}$, W/m$^2$
Figure 2. Comparison of experimental data relating average specific growth rate to average light intensity for \( X = 0.04 \) g/L with: ———, Monod model; —— ——, Bannister model; —— ——, Aiba model; —— ——, linear model; and —— —— ——, modified Aiba model.
Figure 3. Variation of average specific growth rate (dilution rate) with temperature at three different light intensities: *, 13.043 W/m²; ◇, 26.756 W/m²; ●, 53.750 W/m²; turbidostat operation was employed.
Figure 4. Variation of biomass energetic yield with temperature at three different light intensities: *, 13.043 W/m$^2$; ☼, 26.756 W/m$^2$; ♦, 53.750 W/m$^2$; turbidostat operation was employed.
about 15 to 16 kcal/g-mole which is somewhat higher than the other value of 13.5 kcal/g-mole for Spirulina platensis [14].

The variation of average specific growth rate and bioenergetic yield with pH are presented elsewhere [15]. The optimum value of pH is about 9.0 to 9.5 for both light intensities investigated; this value is close to published results [12].

CONCLUSIONS

A photobioreactor is designed to give relatively uniform light profile and specific growth rate. This system is utilized to investigate the kinetics and bioenergetics of growth of Spirulina platensis. Several kinetic models are presented and the kinetic parameters are estimated. Optimum values of growth factors, such as pH, light intensity, temperature and concentration of bicarbonate ion are obtained using the developed fermentation system. These observed values provide the fundamental information for mass cultivation for producing SCP and treating wastewater.

ACKNOWLEDGEMENT

This work is partially supported by NSF Grant CPZ-8406862.

REFERENCES


Table II. Results of estimating parameters for Monod model from the data under carbon-limited continuous cultivation conditions at different light intensities for *Spirulina platensis*.

<table>
<thead>
<tr>
<th>Light Intensity, $I_{in}$ ($W/m^2$)</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu_{max}$ ($h^{-1}$)</td>
</tr>
<tr>
<td>14.364</td>
<td>0.088 (0.067, 0.129)</td>
</tr>
<tr>
<td>29.241</td>
<td>0.093 (0.080, 0.110)</td>
</tr>
<tr>
<td>51.410</td>
<td>0.089 (0.040, 0.148)</td>
</tr>
<tr>
<td>All</td>
<td>0.090 (0.063, 0.156)</td>
</tr>
</tbody>
</table>
MATHEMATICAL MODELING AND SIMULATION OF BICARBONATE-LIMITED PHOTOSYNTHETIC GROWTH IN CONTINUOUS CULTURE

Craig Curless
Department of Chemical Engineering
Kansas State University
Manhattan, Kansas 66506

Introduction

For carbon-limited photosynthetic growth, Monod models based on various carbon sources have been formulated and experimentally validated. [1-4] Carbon dioxide, bicarbonate ion, and total dissolved inorganic carbon (DIC), respectively, have been selected as the carbon source for these Monod models. It has been shown that under most conditions encountered, equilibrium between carbon species in solution exists, so the carbon source used in the model is immaterial. [5] This is because under equilibrium conditions, model parameters based on one carbon species can easily be converted to those based on another carbon source.

Which carbon source do algae actually consume is a debated question. [6] However, considerable evidence exists that many species of algae can assimilate HCO₃⁻. [7-9] Examples include Scenedesmus, Spirulina and Chlamydomonas. Studies indicate that some of these algae have an active bicarbonate pump and may prefer HCO₃⁻ as a substrate. [10] In these cases, it would be more appropriate to model carbon-limited growth using the bicarbonate concentration. In this paper, a model that describes bicarbonate-limited photosynthetic growth in a chemostat will be presented. This model has been incorporated into a computer simulation program which is applied to systems containing Scenedesmus obliquus.

Theory

The system being modeled consists of a chemostat (perfectly mixed) with both liquid and gas feed streams. Growth of the algae culture in the tank is presumed to be bicarbonate-limited. The temperature and pH within the tank are maintained at fixed levels by controlling devices. Light is assumed to be present at the saturation level throughout the tank. This last assumption is a good one for either dilute cultures or optically thin fermentors.

The kinetics of aqueous carbon chemistry can be completely described by the reactions in Figure 1. [11] At this point, it is worthwhile to compare the magnitudes of the rate constants at 25°C and pH 7:

\[ k_1[H_2O] = 0.039 \text{ sec}^{-1} \]
\[ k_2 = 23.7 \text{ sec}^{-1} \]
\[ k_2[OH^-] = 0.00080 \text{ sec}^{-1} \]
\[ k_2 = 0.00025 \text{ sec}^{-1} \]
\[ k_3 [H^+] = 4700 \text{ sec}^{-1} \]
\[ k_3 = 1.3 \times 10^7 \text{ sec}^{-1} \]
\[ k_4 [OH^-] = 680 \text{ sec}^{-1} \ (20^\circ C) \]
\[ k_{-4} [H_2O] = 1.44 \times 10^6 \text{ sec}^{-1} \ (20^\circ C). \]

It is apparent that reactions 1 and 2 are relatively slow, whereas reactions 3 and 4 are virtually instantaneous. The temperature dependence of the rate constants \( k_1, k_{-1} \) and \( k_2 \) are also given in Figure 1. Because reactions 3 and 4 are so fast, the rate constants for these reactions are available only at a single temperature. To estimate these rate constants at higher temperatures, the value of one constant in each reaction is raised so that the value of the equilibrium constant is correct at this temperature.

The liquid feed is assumed to be at equilibrium with a known total inorganic carbon content at a given temperature and pH. For reaction 1, the equilibrium constant is estimated using values of \( k_1 \) and \( k_{-1} \). All other equilibrium constants were obtained from the text by Stumm and Morgan.[12] Values are given from 0°C to 50°C at 5°C increments. The simulation program will do a linear interpolation of \( \log K_{eq} \) vs \( 1/T \) to estimate equilibrium constants at temperatures in this range not evenly divisible by 5. The effect of ionic strength on equilibrium constants was neglected.

\( \text{CO}_2 \)-transfer from the gas phase to the liquid phase is modeled using a mass-transfer coefficient term defined as

\[ R_{\text{CO}_2} = k_L a ([\text{CO}_2^*] - [\text{CO}_2])v_L \]

where

\[ R_{\text{CO}_2} = \text{rate of transfer of CO}_2 \text{ from gas to liquid (mol/sec)} \]

\[ k_L a = \text{volumetric mass transfer coefficient (sec}^{-1}) \]

\[ v_L = \text{volume of liquid in the tank (m}^3) \]

\[ [\text{CO}_2] = \text{bulk concentration of aqueous CO}_2 \text{ in the tank (mol/m}^3) \]

\[ [\text{CO}_2^*] = \text{concentration of aqueous CO}_2 \text{ that would be in equilibrium with the exiting gas phase CO}_2 \text{ (mol/m}^3). \]
The value of \( k_a \) is somewhat system specific. However, an expression given by Joshi et al. was used as a first estimate:[13]

\[
k_a = P_G^{0.58} V_G^{0.75} D_T / 1000 V_L
\]

where

\[ P_G = \text{gas power (W)} \]
\[ V_G = \text{superficial gas velocity (m/s)} \]
\[ D_T = \text{tank diameter (m)} \]

The gas stream is assumed to be perfectly mixed within the chemostat and the entering molar flow rate is set equal to that flowing out. This last stipulation is reasonable for bubbled air because little \( \text{CO}_2 \) is present to dissolve (0.03%) and oxygen is generated during photosynthesis.

The specific growth rate of the algae is described by a Monod model based on bicarbonate:

\[
\mu_{\text{AVG}} = \frac{\mu_{\text{max}} [\text{HCO}_3^-]}{K_s + [\text{HCO}_3^-]}
\]

where

\[ \mu_{\text{AVG}} = \text{average specific growth rate (hr}^{-1}\text{)} \]
\[ \mu_{\text{max}} = \text{maximum specific growth rate (hr}^{-1}\text{)} \]
\[ K_s = \text{half-saturation constant (mol/m}^3\text{)} \]
\[ [\text{HCO}_3^-] = \text{concentration of bicarbonate in the tank (mol/m}^3\text{)} \]

The rate of consumption of bicarbonate is given by \( \mu_{\text{AVG}} X / Y_c \) where \( X \) is the biomass concentration and \( Y_c \) is the carbon yield to biomass. The value of \( Y_c \) is assumed to be 1 mol of carbon in biomass per mol of bicarbonate consumed. It has been shown that the fraction of carbon in algae on a dry weight basis is constant and near 0.479.[14] Thus, if one knows the amount of carbon consumed by the algae, one can calculate the corresponding increase in biomass.

Now that all of the assumptions made in the modeling procedure have been described, material balances for each form of carbon in the chemostat can be constructed (Figure 2). Under the assumptions presented in this section, for a fixed dilution rate and gas flow rate Figure 2 shows 6 equations with 6 unknowns: [\( \text{CO}_2 \)], [\( \text{HCO}_3^- \)], [\( \text{H}_2\text{CO}_3 \)], [\( \text{CO}_3^- \)], \( p_{\text{CO}_2} \) and \( X \).
X can be solved for explicitly by using a series of substitutions. However, the resulting equation is rather unwieldy. Consequently, the equation is incorporated into a computer program so that repeated calculations can be performed easily.

Results

It has been shown that *S. obliguus* has the ability to assimilate bicarbonate, hence this green algae is a reasonable candidate on which to test the simulation.[15-16] The Monod kinetic parameters for *S. obliguus* have been presented by Goldman et al.: $\mu_{\text{max}} = 1.59$ day$^{-1}$ and $K_s = 0.16$ mg carbon/liter.[3] These parameters were determined using data obtained with a chemostat at 20°C and pH 7.1 - 7.2 using a liquid feed with 1 mM total carbon. Simulations at these conditions indicated that the four carbon species in solution were essentially at equilibrium in the chemostat. Values of $2 \cdot \mu_{\text{max}}$, $\mu_{\text{max}}/2$ and $5 \cdot K_s$, $K_s/5$ were also simulated to verify that carbon equilibrium would be closely approximated even if the kinetic parameters were significantly different.

The Monod parameters based on bicarbonate were used in a simulation at the conditions employed in a different experiment by Goldman et al. (Figure 3).[17] These experimental conditions can be found in Table 1. No carbon was present in the liquid feed, so bubbled air served as the carbon source. The mass transfer coefficients presented by Goldman are defined on a different basis than those used in the simulation. Consequently, it was necessary to vary $k_{La}$ in the simulation until a value was found that fit the data well. The simulation used a Henry's Law constant of 0.0389 M atm$^{-1}$ at 20°C.[12]

A value of 0.4/min seems to fit the data best (Figure 3). Figure 4 shows plots of four ratios, each having dilution rate as the independent variable. The dotted line is the fraction of total dissolved and suspended carbon that is present as biomass ($F_C$). The dot-dashed curve shows the fraction of the carbon in the entering gas that does not dissolve ($P_{\text{out}}/P_{\text{in}}$). The solid curve is the ratio of bicarbonate concentration present in the tank to the concentration of bicarbonate that would be in equilibrium with the dissolved CO$_2$ concentration ($[\text{HCO}_3^-]/[\text{HCO}_3^-]$). The other dashed line depicts the ratio of aqueous CO$_2$ to the concentration of dissolved CO$_2$ that would be in equilibrium with the exiting gas ($[\text{CO}_2]/[\text{CO}_2^+]$). Carbonate and carbonic acid concentrations were predicted to be at equilibrium with respect to bicarbonate in the chemostat.

Discussion

There is good agreement in Figure 3 between simulation predictions for $k_{La} = 0.4$/min and the growth results for *S. obliguus* given by Goldman. From the $P_{\text{out}}/P_{\text{in}}$ curve in Figure 4 one can see that for $D = 0.5$ day$^{-1}$, 13% of the entering carbon was used. This is in close agreement with the 14% transfer efficiency obtained by Goldman. As expected, the concentration of aqueous CO$_2$ is far from equilibrium with respect to the gas phase.
However, at higher dilution rates, \([\text{CO}_2]\) approaches this equilibrium value because little biomass is present to draw \(\text{CO}_2\) away from the solution.

From Figure 4 it appears that if \(S. \text{obliguus}\) consumes only bicarbonate under the operating conditions, then aqueous carbon equilibrium is not established. Findenegg found that at pH 5.7, \(S. \text{obliguus}\) which are adapted to air will consume the same proportion of bicarbonate ion as is present in the total carbon pool. The rest of the carbon uptake is in the form of dissolved \(\text{CO}_2\). The simulation predicts that no more than 65% of the total inorganic carbon pool in the chemostat exists as bicarbonate. Thus, it is possible that \(S. \text{obliguus}\) may be obtaining 35% or more of its carbon in the form of \(\text{CO}_2\). This situation would nullify the simulation prediction that a state of nonequilibrium is present in the chemostat and would also account for the observation that biomass output was directly proportional to \(\text{CO}_2\) input. However, at high pH, bicarbonate is present almost to the exclusion of \(\text{CO}_2\). Consequently, at high pH it is more likely that a state of inorganic carbon nonequilibrium could exist in the chemostat.

Conclusions

Some conclusions can be made about chemostats containing carbon-limited algae that consume only bicarbonate. If the culture is air-fed then \(\text{HCO}_3^-\), \(\text{CO}_2^-\), and \(\text{H}_2\text{CO}_3\) levels are maintained at or near equilibrium. However, under air-fed conditions, dissolved \(\text{HCO}_3^-\) and \(\text{CO}_2\) are generally not at chemical equilibrium in the chemostat.

NOMENCLATURE

\[
\begin{align*}
D & = \text{Dilution rate} \\
F_c & = \text{Fraction of suspended and dissolved carbon present as biomass} \\
K_S & = \text{Half-saturation constant for } \text{HCO}_3^- \\
K_j & = \text{Equilibrium constant for aqueous carbon reaction } "j" \\
k_j & = \text{Rate constant for aqueous carbon reaction } "j" \\
k_L & = \text{Mass-transfer coefficient} \\
p_{\text{CO}_2}^{\text{in}} & = \text{Partial pressure of } \text{CO}_2 \text{ entering tank} \\
p_{\text{CO}_2}^{\text{out}} & = \text{Partial pressure of } \text{CO}_2 \text{ exiting tank} \\
pH & = \text{pH in the tank} \\
pH_{\text{feed}} & = \text{pH of the liquid feed}
\end{align*}
\]
\[ Q_{in} = \text{Volumetric flow rate of gas entering tank} \]
\[ Q_{out} = \text{Volumetric flow rate of gas exiting tank} \]
\[ R = \text{Ideal gas constant} \]
\[ T_{\text{liq feed}} = \text{Temperature of the liquid feed} \]
\[ T_{\text{gas in}} = \text{Temperature of gas entering tank} \]
\[ T_{\text{gas out}} = \text{Temperature of gas exiting tank} \]
\[ V_L = \text{Volume of liquid in tank} \]
\[ Y_C = \text{Carbon yield to biomass} \]
\[ [\text{CO}_2^*] = \text{Concentration of aqueous CO}_2 \text{ that would be in equilibrium with the exiting gas stream.} \]
\[ [\text{HCO}_3^-] = \text{Concentration of aqueous HCO}_3^- \text{ that would be in equilibrium with the exiting aqueous CO}_2 \text{ concentration} \]
\[ ["i"] = \text{Aqueous concentration of species } "i" \text{ within the tank (and therefore exiting the tank)} \]
\[ ["i"]_{\text{in}} = \text{Aqueous concentration of species } "i" \text{ in the liquid feed} \]
\[ \mu_{\text{max}} = \text{Maximum specific growth rate of biomass} \]

ACKNOWLEDGMENT

This work is supported in part by National Science Foundation Grant CPE-8406682.

REFERENCES


Table 1. Parameter Values Used in the Simulations

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Parameter</th>
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Figure 1. Aqueous Inorganic Carbon Chemistry

Reactions

1. $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$

2. $\text{CO}_2 + \text{OH}^- \rightleftharpoons \text{HCO}_3^-$

3. $\text{HCO}_3^- + \text{H}^+ \rightleftharpoons \text{H}_2\text{CO}_3$

4. $\text{HCO}_3^- + \text{OH}^- \rightleftharpoons \text{CO}_3^{2-} + \text{H}_2\text{O}$

Equilibrium Constants

$K_1 = k_1/k_{-1}$

$K_2 = k_2/k_{-2}$

$K_3 = k_3/k_{-3}$

$K_4 = k_4/k_{-4}$

Temperature Dependence of Rate Constants

$\begin{align*}
\text{k}_1 \text{[H}_2\text{O}] &= 2.165 \times 10^{10} \exp(-8058.7/T) \text{ (sec}^{-1}) (T[\text{K}])^{11} \\
\text{k}_{-1} &= 1.035 \times 10^{12} \exp(-7300.9/T) \text{ (sec}^{-1}) (T[\text{K}])^{11} \\
\text{k}_2 &= 4.3152 \times 10^{13} \exp(2895.0/T) \text{ (sec}^{-1}) (T[\text{K}])^{18} \\
\text{k}_{-2} &= \frac{k_2}{K_2} \text{ sec}^{-1} \\
\text{k}_3 &= 4.7 \times 10^{10} \text{ M}^{-1} \text{sec}^{-1} \text{ at 25°C}^{19} \\
\text{k}_{-3} &= \frac{k_3}{K_3} \text{ sec}^{-1} \\
\text{k}_4 &= K_4 \cdot k_{-4} \text{ M}^{-1} \text{sec}^{-1} \\
\text{k}_{-4} &= 2.6 \times 10^4 \text{ M}^{-1} \text{sec}^{-1} \text{ at 20°C}^{19}
\end{align*}$

* Dependence of rate constants on ionic strength was not considered.
Figure 2. Material Balances*

**Liquid Phase CO₂ Balance**

\[
\frac{d[C_{O_2}]}{dt} = 0 = k_1 a([C_{O_2}^*] - [C_{O_2}]) + D([C_{O_2}]_{in} - [C_{O_2}]) + k_{-1}[H_2CO_3] - k_1[H_2O][C_{O_2}] + k_2[HCO_3^-] - k_2[OH^-][C_{O_2}]
\]

**Gas Phase CO₂ Balance**

\[
\frac{d}{dt} \frac{v k}{RT} \frac{d[P_{CO_2}]}{dt} = 0 = \frac{Q_{in}^{P_{CO_2}}}{RT_{gas}^{in}} - \frac{Q_{out}^{P_{CO_2}}}{RT_{gas}^{out}} - k_1 a([C_{O_2}^*] - [C_{O_2}])v_L
\]

**CO₃²⁻ Balance**

\[
\frac{d[C_{O_3}^2]}{dt} = 0 = D([C_{O_3}^2]_{in} - [C_{O_3}^2]) + k_4[OH^-][HCO_3^-] - k_4[H_2O][C_{O_3}^2]
\]

**H₂CO₃ Balance**

\[
\frac{d[H_2CO_3]}{dt} = 0 = D([H_2CO_3]_{in} - [H_2CO_3]) + k_1[H_2O][C_{O_2}] - k_{-1}[H_2CO_3] + k_3[H^+][HCO_3^-] - k_3[H_2CO_3]
\]

**Biomass Balance**

\[
\frac{dX}{dt} = 0 = -DX + X \mu_{AVG}
\]

**HCO₃⁻ Balance**

\[
\frac{d[HCO_3^-]}{dt} = 0 = D([HCO_3^-]_{in} - [HCO_3^-]) + k_2[OH^-][C_{O_2}] - k_2[HCO_3^-] - k_{-3}[H_2CO_3] + k_3[H^+][HCO_3^-] + k_4[H_2O][C_{O_3}^2]
\]

\[
-k_4[OH^-][HCO_3^-] - \frac{X \mu_{AVG}}{Y_c}
\]

*See Nomenclature for a description of the symbols.
Figure 3. Comparison of experimental data for *S. obligatus* and simulation prediction for $k_{y} = 0.4$ /min. —— simulation prediction, o o o data from Goldman.[17]

Figure 4. Variation of several ratios with dilution rate for *S. obligatus*. The value of $k_{y}$ is 0.4 /min. —— $P_{c}$, --- $P_{C02} / P_{O2}$, —— $[CO_2]/[CO_2]^*$, —— $[HCO_3^-]/[HCO_3^-]^*$. See nomenclature for a description of variables.
DATA ACQUISITION AND CONTROL OF A ROTARY DRUM SOLID STATE FERMENTOR.

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ABSTRACT

Solid substrate fermentation (SSF) is a developing technology with potential application in the production of foods, feeds as well as higher value fermentation products such as enzymes and pharmaceuticals. Solid substrate fermentation has a number of advantages over submerged fermentation including higher product concentrations, lower capital investment and ease of oxygen transfer. The relative simplicity and low capital investment of SSF make it particularly attractive to developing countries. The key problem with SSF which has thus far severely limited its commercial development is heat buildup due to the exothermic fermentation.

This study aims to extend the state-of-the-art in SSF by a new method of integrated heat and water transfer control. We will permit free evaporation of water from the system into the circulated air to cool the fermentation. The water evaporation will be replaced using a microcomputer-directed system which will control air flows and water addition rates. The microcomputer will provide on-line material and energy balance information which will prove useful in analyzing the system dynamics and in designing improved large scale SSF. If the integrated heat/mass control concept proves valid, we will have removed a major obstacle preventing commercialization of SSF, benefiting the entire fermentation industry.
INTRODUCTION:

Proper utilization of agricultural residues can have an important positive impact on the economic welfare of many developing countries. The agricultural residues in many of these countries are collected, piled and used throughout the year for feeding animals with little or no processing. Farmers of these countries historically apply a solid-state fermentation (SSF) technique by piling up and leaving the residues with no controls under natural conditions. With the exception of Japan and some other Far-Eastern countries, SSF has no large-scale applications. One of the most important obstacles to the large-scale use of SSF is the removal of the heat generated by the exothermic fermentation process.

Water evaporation is inevitable in SSF because of the aeration requirements and metabolic heat production. In this study we propose to turn this inevitable evaporation process into an advantage. We will permit free evaporation of moisture into the flowing air stream. Whenever the fermenter temperature reaches the set point a proportional quantity of water will be added to replace that which was removed by evaporation, thereby controlling the heat buildup. Integrated temperature and moisture control will be accomplished using a microcomputer. The main purpose of this study is to develop the necessary control techniques, hardware and data acquisition systems for temperature/moisture control in SSF.

The state of the art of SSF is reviewed in two 1983 papers (1,2). In using submerged fermentation (SF), the fungus is grown in liquid media. A homogeneous environment throughout the fermenter is made possible via agitation and aeration. Thus the control of SF is rather easy. For many fermentations, however, submerged fermentation becomes less attractive because of the large amounts of water involved and the energy requirements for agitation and separation processes. Chahal (2) observes that the most important factor in obtaining a 20-30% sugar concentration from hydrolysis of cellulosic materials is the level of cellulase activity per unit volume of fermentation medium. This activity can be increased by increasing the cellulose concentration in the broth (3). Nevertheless, it is impossible to handle more than 6% cellulose in SF due to rheological problems. This again makes SF less attractive. Meeting the microorganism's needs for oxygen is also much more difficult in submerged fermentation, while in solid media aeration is less difficult since the fungus is in direct contact with oxygen at the surface and between the particles. In addition to these disadvantages of SF for fungi there is an inherent problem due to the growth nature of the fungi. Fungi produce mycelia which tend to change the medium viscosity. All these problems make fungal growth in SF less attractive.

In his reviews, (4,5), Hesseltine outlines the advantages of SSF:

1. Simple media;
2. Flexibility of the process; batch or continuous i.e., Koji is produced in Japan through continuous solid culturing;
3. In comparison with the product yield, the fermentation equipment occupy a relatively small space because less water is used;
4. Seed tanks are not needed, since in many fungal and natural flora fermentations, spores or cells can be used directly;
5. There is a reduced bacterial contamination problem because of the low moisture level required for maximum fungi product yield;
6. SSF conditions are very close to those under which fungi would grow in nature
7. Sporulation, which appears in static SSF, tends to be absent in agitated SSF;
8. With less substrate used to generate a product, less solvent is needed for the extraction of this product, therefore no large amounts of liquid wastes as in the case of SF are to be disposed of;
9. Due to the interparticle spaces, aeration is quite easy. Aeration is also facilitated by the top-bottom mixing;
10. SSF product yields per gram of media are generally greater than those of SF;
11. And, reproducibility of yields are as good as those of conventional fermenters.

He also mentions the problems of SSF (5):

1. Substrate pretreatment is necessary;
2. Low moisture levels upset most microorganisms. Only a few will grow under these conditions, namely fungi, some yeasts, and Streptomyces;
3. Since we are dealing with a bulky solid with low thermal conductivity, the problem of heat buildup, especially in large fermenters, is one yet to be satisfactorily solved;
4. and, where to place a biosensor in a continuously moving fermenter is a difficult problem. A great deal of innovation is needed to be able to monitor some very important growth parameters such as temperature, moisture, pH, free oxygen, carbon dioxide and product yield in SSF.

PREDICTION OF EVAPORATIVE COOLING NEEDED FOR HEAT REMOVAL

The moisture level is to be kept constant through addition of water droplets to replace the vaporized water. Different authors (6,7) have shown that during microbial growth, there is a constant proportionality between heat generation and oxygen demand. By using appropriate stoichiometric information for glucose combustion (a saccharification ratio of wheat straw equal to 33% and a glucose combustion ratio of 25%) we estimate a required water addition rate of 0.55 kg per kg of dry wheat straw fermented. These stoichiometric parameters can be updated and modified as needed as the work progresses.

Heat must be removed by the dry air flow (at an air inlet temperature of 37°C). If we assume that all the heat present comes only from the microbial metabolism (adiabatic) and an outlet relative humidity in the air of 100%, the dry air flow rate given by the humidity chart is around 500 l/hr per kg dry solids. The superficial air velocity will be 0.25 ft/min.
OPERATION OF AUTOMATED TEMP/MOISTURE CONTROL SYSTEM

The control system depends upon sensors to determine temperature, substrate moisture content, air flow rate and relative humidity and regulates the air flow rate and water addition rate using a microcomputer to manage these operations. SSF will be conducted in a rotary drum fermenter coupled with an evaporative heat control network as shown in Fig. 1 and 2.

INTEGRATED TEMP/MOISTURE CONTROL AND NEW FERMENTER DESIGN

To date, most SSF procedures in use try to control the heat buildup using moistened air. According to some reports this is inefficient even if the air has a 100% relative humidity. To solve this problem we will attempt to turn the unavoidable evaporation into an advantage by using an air with a relative humidity below the equilibrium relative humidity so that the heat is removed through free evaporation of the water. The largest heat sink available in this system is in fact the heat of vaporization of water. The observations of Sato (6) support this approach. Preliminary calculations using the data of Edwards (8) for heat production during SSF of cellulose (3,780 kcal/kg cellulose consumed) also show that the aeration needs of the fungus and the heat removal requirements are in rough balance, i.e., air of ambient relative humidity (approximately 50%) at the fermenter inlet will contain enough water when saturated at the outlet conditions to remove the metabolic heat as well as provide adequate oxygen. A microcomputer (IBM-PC) will monitor the amount of water removed from the system and also the temperature inside the fermenting substrate using a number of thermocouples. The amount of water to be added for controlling heat build-up will be managed by the same microcomputer. Water content will also be regulated through a moisture content sensor. Thus, we will construct a rotary fermenter with water addition and temperature control capabilities.

Some temperature gradients in SSF are likely even with limited agitation (9). Therefore, we must use several thermocouples to take into account these irregularities, particularly in the radial direction in which the percent deviations will probably be greatest. Charles and Gavin (10) also noted significant temperature and oxygen gradients in their 2.54 cm deep tempeh bed. The perforated air pipe in our fermenter will therefore be installed in a way so as to help alleviate the gradients. Our goal is to achieve, as much as possible, a gradientless fermenter to facilitate the analysis of the operating data.

The microcomputer will calculate the water mass balance from the inlet and outlet relative humidities and will regulate the water replenishment rate. This can also be achieved using a dielectric capacitance type moisture sensor. This sensor was developed by Moisture Control System, Vincennes, Indiana and gave satisfactory results for a wheat straw moisture content of 70 to 80% w/w (wet basis). This probe will be used in our project. The water mass balance method has the disadvantage of not accounting for metabolic water, but with this new probe we can continuously monitor bed moisture content.
FIG 1  EXPERIMENTAL APPARATUS WITH MEASUREMENT AND CONTROL SYSTEMS
FIG 2

ROTARY DRUM FERMENTOR CONFIGURATION

SIGHT GLASS

WATER
AIR

LOADING & UNLOADING PORT

TEMP SENSORS

MOISTURE SENSOR

OUTGOING SENSORS' CABLES

STATIC SHAFT

MOIST AIR EXHAUST

DRIVING ROLLER

MOTOR DRIVE & CONTROLLER
SIGNIFICANCE OF THE PROJECT

With the exception of Japan and some other Oriental countries, SSF has no large scale applications. The single most important obstacle to SSF scale up is the heat accumulation due to poor thermal conductivity of the substrate, especially in larger fermenters. Using integrated temperature and moisture control we hope to overcome this obstacle and make SSF a more viable alternative to submerged fermentation for the production of single cell protein for animal feed, and commercial enzymes such as cellulase.

The recent announcement by Genencor researchers (11) that they have developed a secretion system for the products of mammalian genes in filamentous fungi opens the possibility that SSF might be used to produce very high-value products in addition to the relatively low value products mentioned above. Such fungi can, of course, be cultured in submerged fermentation but the advantages of SSF have already been noted, in particular the higher product concentrations available in SSF will be especially important for downstream processing and recovery. Therefore, the proposed research might serve both the very high value product and relatively low value product fermentation industries.

We believe that sophisticated computerized moisture-temperature control has great potential even in a "low-value" technology such as SSF. Moisture-temperature control information developed in this study should provide a solid basis for SSF scaleup, but at the laboratory level we intend to use the control system primarily as a research tool to study the fundamentals of SSF as well as the feasibility of such control systems. In addition, if we are able to gain a better understanding of the factors influencing SSF through innovative techniques, it is worthwhile to consider such sophistication especially with the decreasing cost of microcomputers and microprocessors. One of the long term goals of our research in SSF is to develop predictive models for SSF control based primarily on temperature measurement, an inexpensive and easy variable to measure. Given the intimate relationship between oxygen demand, metabolic water production and temperature rise, an adequate data base (which the computer will facilitate) should help make such models possible. Finally, some unexpected benefits are likely to result with the increasing potential importance of SSF to production of recombinant DNA-derived proteins.
REFERENCES


BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D)

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2,4-Dichlorophenoxyacetic acid (2,4-D) is an aquatic and terrestrial herbicide. It has been selected as a model compound for study because of its wide use and its structural similarity to other toxic and hazardous compounds currently of interest such as other halogenated aromatics and phenolic compounds (1). In addition, 2,4-D is among 38 compounds that the EPA has proposed to add to the list of chemicals used to identify wastes as hazardous and suitable for management under the Resource Conservation and Recovery Act (2).

Organisms capable of degrading 2,4-D have been studied in pure and mixed cultures (3,4). Most studies have been conducted under aerobic conditions. A typical metabolic pathway is shown in Fig. 1 (5).

A number of studies have been conducted in attempts to develop kinetic models for the biodegradation of 2,4-D and related compounds. (6,7-15). These studies have given rise to various biodegradation models, some of which appear to yield conflicting results. Part of the diversity among the proposed models can be attributed to the variability of experimental conditions such as pH, temperature, aeration, supplemental nutrients, culture enrichment, and substrate concentration range, all of which have been identified as significant factors in determining biodegradation rates (4).

Many of the available biodegradation models do not account for any inhibition effects (6,9,11-14). Most of them are the Monod type, characterized by the expression

\[ \mu = \frac{\mu_{\text{max}}[S]}{K_S + [S]} \]  

Figure 2 illustrates the results obtained by Tyler and Finn (6). The top curve is based on the Monod model, which reportedly provides an accurate description of 2,4-D biodegradation up to 2000 mg/L. (2,4-D data points are not shown in the figure). This curve also provides a good fit for 2,4-dichlorophenol (2,4-DCP) up to 25 mg/L, but it does not at higher concentrations.

Considerable uncertainty exists as to where and if 2,4-D itself is actually inhibitory. Some researchers have reported inhibitory effects for 2,4-D at levels of, for example, 35 mg/L and 45 μg/g-soil (7,8,10). Others have successfully employed versions of the Monod model that neglect inhibitory effects as in Fig. 2. The uncertainty about 2,4-D inhibition may be caused in part by the effects of a metabolic product, e.g. 2,4-DCP, that may contribute to or be totally responsible for the observed inhibition.
Several researchers have found 2,4-DCP to be inhibitory at relatively low concentrations, e.g., the 25 mg/L shown in Fig. 2 (6,16,17). In shake flask experiments with Pseudomonas sp. NCIB 9340, 2,4-DCP has been observed to accumulate in the media before being degraded as indicated by Fig. 3. In a series of shake flask experiments with different initial 2,4-D concentrations 2,4-DCP was observed to accumulate to concentrations of 0.1 to 16.9 mg/L as the initial 2,4-D concentration was increased from 52 to 370 mg/L.

Experiments using 1-liter batch fermentations indicate that culture pH is an important factor in determining growth rates and 2,4-DCP accumulation. In the shake flask experiments the pH was not controlled. In flasks where 2,4-DCP accumulation was observed, the pH dropped from an initial pH of 6.8 to approximately 6.5. In the 1-liter batch fermentations pH was controlled with experiments conducted over a pH range of 5.5 to 9.5. Figure 4 demonstrates the relation between pH and specific growth rate. The specific growth rates shown were obtained by taking the slope of ln(biomass concentration) vs time during the exponential growth phase. The maximum growth rate occurred at pH 7.0 and decreased as the pH was increased up to 9.5 where no growth or biodegradation was observed over a period of four days. The only detectable 2,4-DCP accumulation observed in the 1-liter batch fermentations occurred at pH 5.5 where the 2,4-DCP concentration reached 44.5 mg/L. This accumulation of 2,4-DCP appears to have been sufficient to stop the degradation of 2,4-D as indicated by Fig. 5. The specific growth rate shown in Fig. 4 for pH 5.5 is based on growth before significant amounts of 2,4-DCP accumulated. Accumulation of 2,4-DCP is accompanied by a reduction in the biodegradation rate, presumably due to inhibitory effects of 2,4-DCP. Thus, though 2,4-DCP accumulation does not appear to be a factor in the reduction of growth rate in the higher range of pH, it could be important in the low range where it may lead to inhibition kinetics and possibly completely arrest the degradation of 2,4-D. It is not clear why 2,4-DCP accumulation occurred at slightly higher pH in the shake flasks. Additional experiments are planned to further examine the low pH range.

The Haldane model

\[ \mu = \frac{K_1[S]}{(K_2 + [S] + K_3[S]^2)} \] (2)

appears to be the most promising model for the description of inhibitory substrate degradation. Though the Haldane Model does not appear to provide a good description in Fig. 2, numerous other researchers have obtained good fits with the Haldane model for the degradation data of 2,4-D or related compounds such as phenols and benzoate (8,18-20).

The models discussed so far generally relate the target substrate concentration to the growth rate. This approach is not appropriate when substrates are degraded by cometabolism (14,21-23). Microorganisms that cometabolise substrates convert them to organic products without obtaining a significant amount of carbon or energy from the degradation. Schmidt et al. (23) have modeled the biodegradation of organic compounds not supporting
growth. They found that the kinetics of target substrate degradation at concentrations too low to support growth are best described by a first order model or by relations that include the growth of the metabolising population on the growth supporting substrate.

In acclimated cultures, 2,4-D can be degraded even at low concentrations, e.g., 20 μg/L (24). Though degradation occurs with 2,4-D alone, the effects of supplemental nutrients is of interest as a possible means for increasing the biodegradation rate. In several experiments with supplemental nutrients 2,4-D has been observed to be degraded concurrently with nutrient supplements. Figure 6 shows that at 100 and 10 mg/L the time for 2,4-D degradation is significantly reduced by the addition of supplemental nutrients (24). At lower concentrations this effect was less evident. Figure 7 indicates that additional nutrients appear to retard 2,4-D degradation at initial concentrations of 0.14 mg/L (24). Papanastasiou and Maier (8) found that glucose and 2,4-D are mutually inhibitory. Though glucose inhibits the cellular rate of 2,4-D metabolism, the effect can be overcome due to rapidly increasing biomass concentrations resulting from the utilization of glucose. Nutrient supplementation could be especially useful for situations where the target substrate is present in concentrations too low to develop sufficient biomass for rapid degradation.

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Figure 1. Aerobic pathway for 2,4-D biodegradation (I. R. Hill and S. J. L. Wright) (5).

Figure 2. Specific growth rate for Pseudomonas sp. NCIB9340 on 2,4-dichlorophenol, o, o; complete Monod model (for 2,4-D) with $\mu_M = 0.14 \text{ h}^{-1}$, $K_S = 5.1 \text{ mg/L}, ---$; computer fit of Haldane model to 2,4-DCP data with $\mu_M = 0.228 \text{ h}^{-1}$, $K_S = 11.7 \text{ mg/L}$ and $K_I = 35.7 \text{ mg/L}, ---$; o from nephelometric; o from chemostat at pH 7.4 and 25°C (J. E. Tyler and R. K. Finn) (6).
Figure 3. 2,4-D degradation by Pseudomonas sp. NCIB 9340 in shake flasks at T=25 °C and initial pH=6.8; , biomass concentration; +, 2,4-D concentration; △, 2,4-DCP concentration.
Figure 4. Effect of pH on the specific growth rate of Pseudomonas sp. NCIB 9340 in 1-liter batch fermentations at T=25°C.

Figure 5. 2,4-D degradation by Pseudomonas sp. NCIB 9340 in 1-liter batch fermentation at pH=5.5 and T=25°C; ●, 2,4-D concentration; ○, 2,4-DCP concentration.
Figure 6. 2,4-D degradation by an acclimated enrichment culture from a municipal wastewater treatment facility with and without nutrient broth (NB). (C.J. Kim and W.J. Maier) (24).

Figure 7. 2,4-D degradation at low concentration by an acclimated enrichment culture from a municipal wastewater treatment facility with and without nutrient broth (NB). (C.J. Kim and W.J. Maier) (24).


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