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ETHANOL PRODUCTION BY *ZYMOMONAS MOBILIS* IN A TAPERED-BED BIOREACTOR

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ABSTRACT

The fermentation of glucose to ethanol by the bacterium *Zymomonas mobilis* was studied. A tapered bioreactor containing an anion exchange resin, to which bacteria were attached, was fluidized by feed solution at 35°C. Short residence times characteristic of the experimental tapered reactor were necessary to model various differential segments of a hypothetical large-scale reactor. A feed concentration of 10% glucose as well as glucose/ethanol mixtures were studied. Ethanol production ranged from 29 g/liter-h for a feed containing 2% weight per volume glucose to 64 g/liter-h for a 10% glucose feed. Development of biomass concentration-measurement techniques was begun. Caustic stripping, DNA analysis, and dehydrogenase determinations were attempted, but results were inconclusive.

Contents

1. SUMMARY

The demand for inexpensive ethanol in large quantities has led directly to the reexamination of the fermentation by yeast, the traditional means of producing ethanol from sugars. The bacterium *Zymomonas mobilis*, currently under study at Oak Ridge National Laboratory, has shown a several-fold improvement in productivity over yeast. The overall objective of this study was to initiate reactor studies. The reactor selected was a tapered-tube fluidized-bed reactor, in which the bacteria were attached to an anionic ion exchange resin.

Because the fluid flow necessary to cause fluidization is too light to allow for significant depletion of the substrate in a single pass, it was necessary to treat the reactor as a series of differential slices at discrete points in a hypothetical large industrial bioreactor. Column feed, glucose, and ethanol concentrations were varied to model four distinct points; the change of glucose and ethanol concentrations in the system was measured as was the rate of CO_2 production. No ethanol inhibition of the microorganism was observed. Ethanol production rate varied from 29 g/liter-h of reactor volume to 64 g/liter-h.

A second objective was to determine a method for estimating the loading (g biomass, dry basis/liter of reactor) of *z. mobilis* on the anion exchange resin. Three procedures were examined: caustic stripping, DNA, and dehydrogenase determination. The primary difficulty in all cases was to differentiate the biomass from the support particles. Of the three methods, caustic stripping was the poorest because caustic caused resin-degradation. The DNA and dehydrogenase experiments gave qualitative indications of biomass attachment to the ion exchange resin, but further development of these methods is needed before quantitative results can be obtained.

2. INTRODUCTION

2.1 Background

Interest in the use of ethanol as a fuel or chemical feedstock has grown in recent years. Before the ethanol production from renewable natural products can become an economic reality, a fast, efficient, fermentation process must be developed. Fermentation is currently conducted primarily by the beverage industry, where batch processing is utilized to maintain product quality and taste. The batch process is inherently inefficient, particularly in the latter stages, because of the depletion of substrate and because of the inhibition of the process by high ethanol concentrations. The presence of significant ethanol levels (e.g., greater than 5% w/v) in the batch mixture is sufficient to inhibit the activity of the microorganisms.

2.2 Previous Work

2.2.1 Continuous Fermentation

While flavor is the important factor in beverage industry processes, the economics of ethanol use as a fuel depends primarily on substrate cost, fermentation rate, and yield. Therefore, most studies directed at reducing ethanol's inhibiting effects have centered around continuous flow systems. Rosen (6) described a Danish plant in which molasses feed equivalent to 12% w/v sugar was fed to two sequential, stirred-tank fermenters, with an ethanol productivity of 4 g/liter-h. In this configuration, the full inhibitory effect was experienced only in the second fermenter.

2.2.2 Use of Cell Recycle

One problem associated with a continuous-flow fermenter is the loss of active biomass through its being continuously flushed from the system with the product. Cysewski and Wilke (4) explored the use of a cell recycle system to increase the biomass loading of a continuous-stirred-tank fermenter. At atmospheric pressure, an ethanol productivity of 7.0 g/liter-h was obtained. By holding all factors in the system constant and by introducing cell recycle, the amount of biomass in the system increased four-fold, and the ethanol productivity increased to 28 g/liter-h. Increasing the cell density brings forth alcohol inhibition as a limiting factor. Cysewski and Wilke conducted further fermentations under reduced pressure (50 mm Hg) so that the ethanol produced would be continuously removed from the fermentation broth, eliminating inhibiting effects. Maximum continuous productivities of 40 g/liter-h without cell recycle, and 82 g/liter-h with recycle, were reported with a condensate produced containing 16-20% ethanol. The cost penalties incurred by this process include operation of the vacuum and recycle systems, compression of CO_2 generated up to atmospheric pressure, and the heat input to the fermenter which was needed to supply the latent heat of the evaporating water and ethanol. Of course, the fact that the ethanol product is more concentrated is a positive factor in fuel production.

2.2.3 Fixed Microorganisms

The use of fixed active-biomass fractions in a continuous-flow column fermenter provides a means of maintaining a high biomass loading without cell recycle. Alcohol toxicity effects are only a factor at the top of the column, where the full alcohol concentration has been achieved. Griffith and Compere (5) report column effluents containing 14-15% w/v ethanol and ethanol productivities of 20-80 g/liter-h using fixed yeast cells. This suggests that fixed-cell systems are capable of as high a productivity as a low-pressure system with recycle without the added costs of a vacuum system.

2.3 Use of *Zymomonas mobilis* Bacteria

In all the studies discussed above, the yeast *Saccharomyces cerevisiae* (baker's yeast) was used as the microorganism. Recently, Arcuri, Worden, and Shumate (1) studied the use of *Z. mobilis* (a bacterium) for ethanol fermentation. A packed bed containing *Z. mobilis* fixed in borosilicate-glass pads yielded an ethanol productivity of 85.5 g/liter-h. They also examined a tapered fluidized bed of flocculated bacteria. The advantages of a tapered reactor have been reported by Scott and Hancher (7). Chief among these is the ability to operate under a wide range of flow rates without particle elutriation and with limited backmixing. Arcuri *et al.* reported ethanol productivities of 132 and 120 g/liter-h for this flow reactor using 5% and 10% w/v glucose feeds, respectively (1).

It is of considerable interest to hypothesize on the possible reasons for the high efficiency of *Z. mobilis*. Both *S. cerevisiae* and *Z. mobilis* ferment glucose to ethanol according to the following stoichiometry:

$$C_6H_{12}O_6 \text{ (glucose)} \longrightarrow 2CO_2\uparrow + 2C_2H_5OH(\Delta H = -56 \text{ kcal/mol}) \quad (1)$$

Neither of these types of organisms is perfectly efficient in its production of ethanol. Therefore, the effective stoichiometry results in somewhat less than 2 moles of ethanol being produced for every mole of glucose consumed.

The energy transfer system of living matter involves the dephosphorylation of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). The energy produced by this reaction is equivalent to 8 kcal/mol, and it is this energy which is used in the biosynthetic processes of the cell. Similarly, 8 kcal/mol are required to phosphorylate ADP to produce ATP, and this energy is obtained from catabolic processes such as that of Eq. (1).

S. cerevisiae ferments glucose to ethanol via the Embden-Meyerhof pathway, whereas *Z. mobilis* utilizes the Entner-Doudoroff mechanism. The two pathways yield the same products, with the exception that the former yields 2 moles of ATP per mole of glucose fermented, and the latter yields only 1 mole. Thus, of the 56 kcal/mol of glucose released, *S. cerevisiae* obtains the equivalent of 16 kcal in the form of ATP, and *Z. mobilis* obtains 8 kcal, with the remainder released as heat. Thus, to obtain the same amount of energy for cellular activity, *Z. mobilis* must ferment twice as much glucose as the yeast, and thus produce nearly twice as much ethanol.

Z. mobilis is similarly inefficient in its cellular growth processes. As was mentioned earlier, some of the glucose consumed by the organisms is converted to CO_2 and ethanol, and some is used for cellular growth. *S. cerevisiae* grows to the extent of 21 g of cell material per mole of glucose fermented, while *Z. mobilis* only grows by 8.6 g of cell material per mole of glucose fermented (8). Furthermore, only 48% of the increase in cellular carbon results from glucose uptake; the remainder comes from yeast extract

included in the feed as a nitrogen source (9). As a result, *z. mobilis* only consumes about 2% of the glucose substrate for cellular growth and ferments the remaining 98%.

These characteristics of *z. mobilis* in using glucose substrate for cellular energy and cell growth make the bacterium industrially attractive; and this, combined with the high ethanol productivities reported by Arcuri *et al.* (1), provided motivation for further examination of *z. mobilis*.

2.4 Objectives

The purpose of this project was to lay the groundwork for future work with *z. mobilis*. Two specific objectives were established:

1. To study the ethanol production rates achievable in a tapered fluidized-bed bioreactor as a function of feed composition. The specific program that was pursued involved bacteria which were attached to ion-exchange-resin particles.

2. To recommend methods for measuring biomass loading within the reactor which would be applicable to subsequent studies.

2.5 Method of Approach

The first objective, to maintain a sufficient flow to fluidize the resin and to attain a substantial conversion of the glucose in the feed, is in direct conflict. To fluidize the solids in the tapered bioreactor, a liquid residence time of just under 3 min was necessary. The fermenter used by Griffith and Compere (5) required residence times of 2 to 8 h. Even if the enhanced productivity of Arcuri *et al.* (1) could be achieved using *z. mobilis*, an industrial fermenter would still require a residence time which is an order of magnitude longer than is available in the laboratory reactor. A 10% glucose solution is a typical feed concentration for such a reactor. To simulate the fermentation process in an industrial-scale reactor or chain of reactors, a number of feed compositions was selected representing differential slices of an overall process. Four mixtures, each containing the glucose and ethanol equivalent of a partially fermented 10% glucose solution, were fed to the column. In this manner the experimental apparatus was used to model various points along the length of the industrial reactor. Based on the results of the experiments, a prediction of the behavior of a full-scale system would be possible.

The second objective was to suggest a method of determining the biomass loading on the ion-exchange resin. To date there has been no measurement of biomass loading on this support. A number of methods for distinguishing biomass from the support particles have been suggested, and a preliminary investigation of these approaches was attempted.

3. EXPERIMENTAL APPARATUS AND PROCEDURE

3.1 Ethanol Production-Rate Experiment

The experimental apparatus used to obtain ethanol production data for *Z. mobilis* is shown in Figs. 1 and 2. The feed mixture which is maintained at 2-4°C is purified from a 100-liter container at 0.062 liter/min. The feed flow is metered through a peristaltic pump into a pasteurizer, in which the feed is sterilized at 90°C. The fluid then passes into a cooler in which the temperature is reduced to about 20°C before entering the column preheater. Circulating water systems maintain the preheater and column at 30 and 35 ± 1°C, respectively.

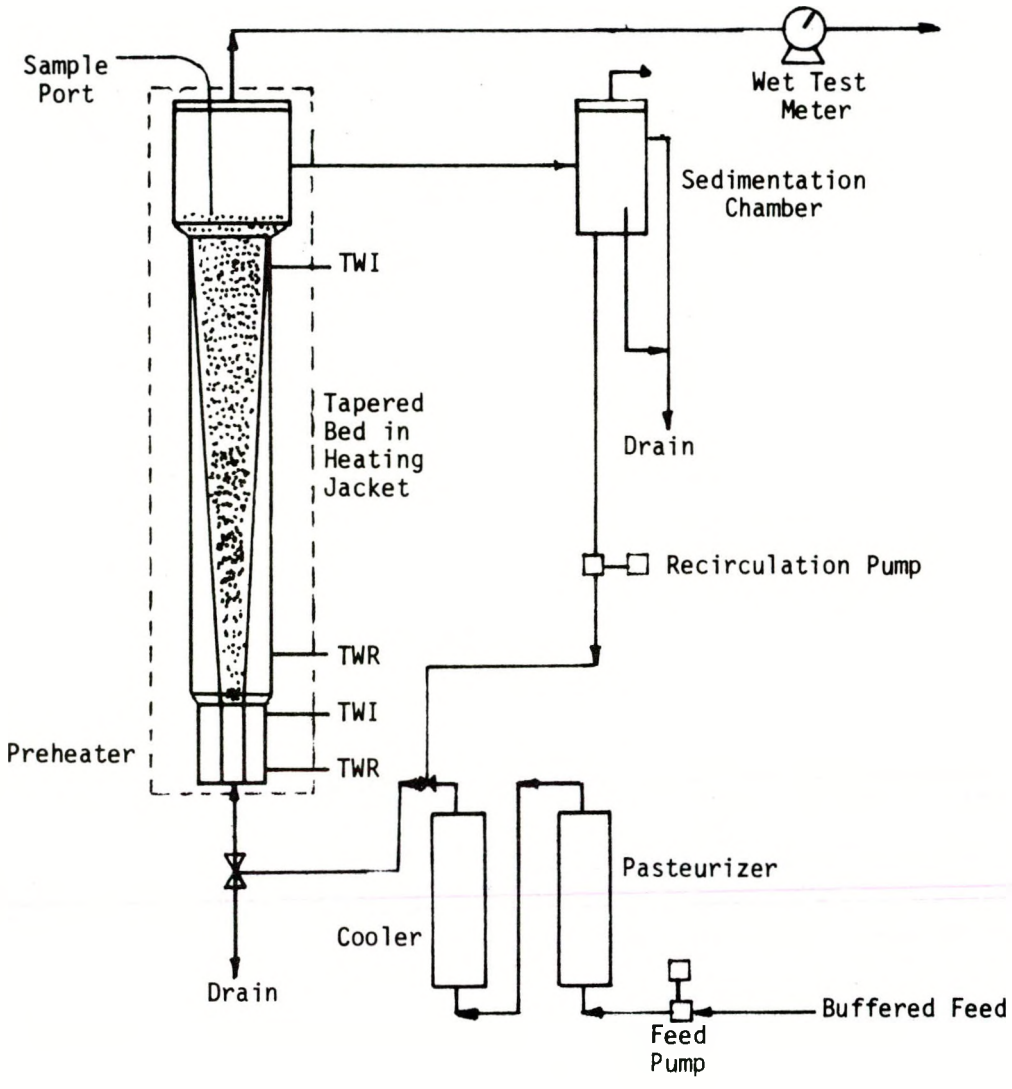
The prepared feed enters the bottom of the tapered fluidized-bed reactor (see Fig. 2). The flow rate in the bed must be sufficient to maintain fluidization of the solids throughout the expanding section. Hence a residence time in the bed of 3 min was selected. Product vapors from the top of the reactor and sedimentation chamber are vented after passing through a wet-test meter. Liquid effluent spills into the sedimentation chamber, where some biomass is recycled and some sent to the drain. In this experiment, the recycle feature was not used. Liquid samples were taken from the drain line.

The column was charged with Rohm and Haas IRA-900 anion exchange resin, filled with an autoclaved 5% w/v glucose solution containing 0.5% yeast extract ($DIFC_0$), and innoculated with a culture of *Z. mobilis* (ATCC 10988) grown in the same feed medium. The system was operated on total recycle for 24 h, after which a 10% w/v glucose in double-distilled water feed was introduced. This substrate also contained 0.3% yeast extract, 0.01 M pH = 7 phosphate buffer solution and 0.005% w/v Dow-Corning silicone antifoam Emulsion B. An initial feed rate of 0.005 liter/min was used, and the flow increased to the final 62 ml/min over a 7-h period.

Liquid effluent samples were drawn periodically, centrifuged at 10,000 rpm for 10 min, and assayed for glucose and ethanol by the methods of Bostick and Burtis (2) and Tiffiny *et al.* (10), respectively. The production of CO_2 was monitored using a wet-test meter. When the column effluent composition gave indication of having stabilized, a new feed was introduced.

The new feeds were intended to represent partially fermented 10% glucose solutions. The stoichiometry of the fermentation [Eq. (1)] is such that the ethanol concentration on a weight basis will be slightly more than half of the glucose consumed (92 g ethanol produced/180 g of glucose). *Z. mobilis*, as mentioned before, ferments almost 98% of the glucose in the feed to ethanol and CO_2, with only small amounts converted to other products or diverted for cell growth. Therefore the theoretical yield was used for feedstock-preparation calculations.

The five feeds tested were, in chronological order:



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EXPERIMENTAL APPARATUS FOR TAPERED FLUIDIZED BED

DATE	DRAWN BY	FILE NO.	FIG.
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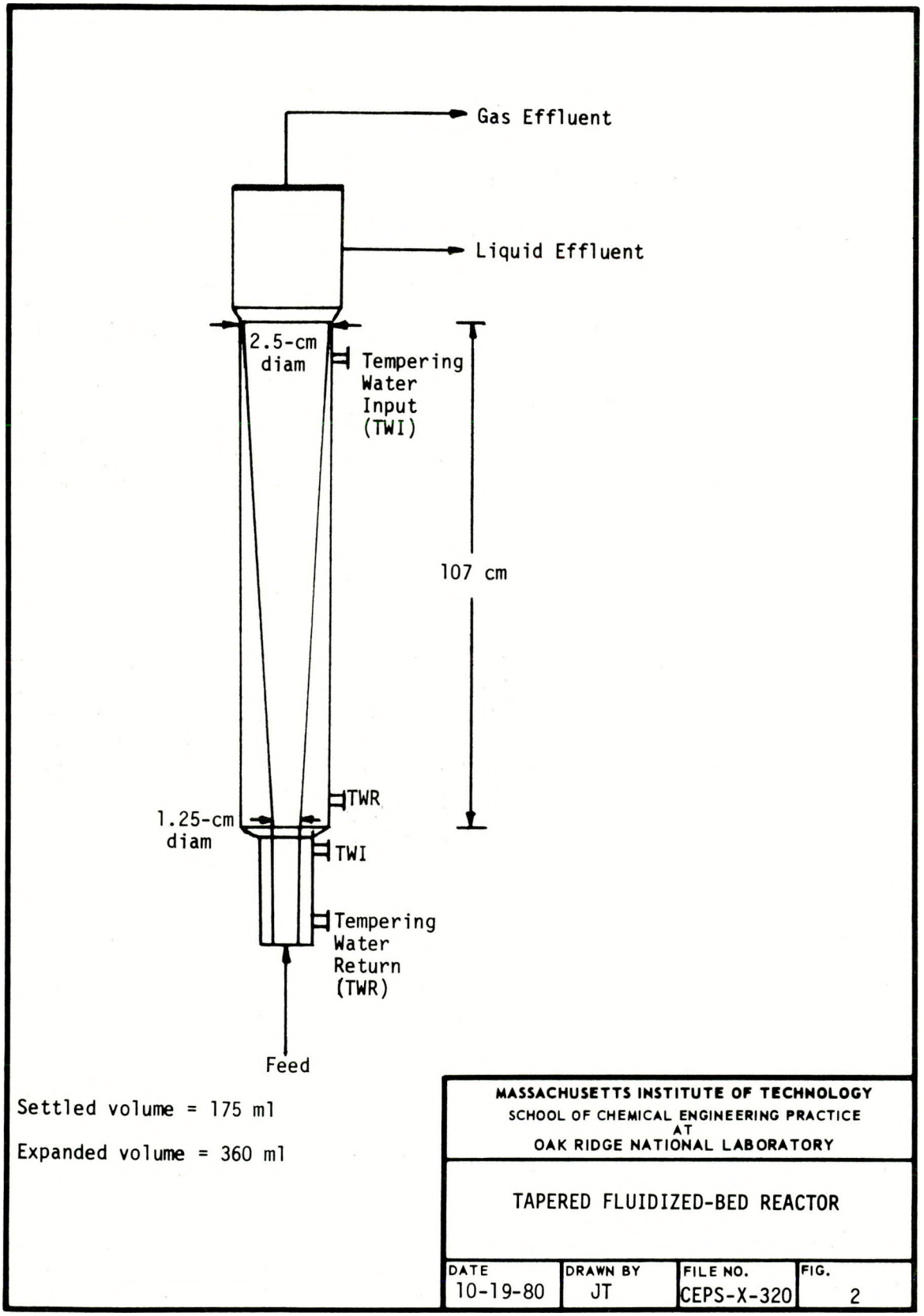
Gas Effluent
Liquid Effluent
2.5-cm diam
Tempering Water Input (TWI)
107 cm
TWR
1.25-cm diam
TWI
Tempering Water Return (TWR)
Feed
Settled volume = 175 ml
Expanded volume = 360 ml
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TAPERED FLUIDIZED-BED REACTOR
DATE 10-19-80
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FILE NO. CEPS-X-320
FIG. 2

1. 10 wt % glucose
2. 8 wt % glucose, 1 wt % ethanol
3. 5 wt % glucose, 2.5 wt % ethanol
4. 2 wt % glucose, 4 wt % ethanol
5. 10 wt % glucose

All feeds contained the same levels of yeast extract, buffer, and surfactants. The reintroduction of the 10% feed was intended as a check of the reproducibility of the initial production rate and also as a check on the viability of the bacteria at the end of the experimental period. A gas effluent sample was taken just before column shutdown and analyzed by gas chromatography, using a column capable of separating air (nitrogen, oxygen, and hydrogen), carbon dioxide, methane, and other organic vapors. The presence of methane and other organics, aside from ethanol, would be a clear indication that competitive reactions were occurring and that other bacteria had contaminated the original *Z. mobilis* biomass.

3.2 Biomass-Loading Measurement

The importance of maintaining a high biomass concentration in a fermenter is shown by the effect of cell-recycle systems on ethanol productivities. Biomass-loading measurements may, among other things, prove useful in the comparison of various support particles used in fluidized-bed reactors. The key to measuring loading is in differentiating between the microorganisms and the support. For relatively inert supports, such as borosilicate glass, this was fairly straightforward. A dry weight would be obtained for the coated support, the biomass ashed at elevated temperature, and the weight change noted.

Ion exchange resin was used as a support particle for this experiment. While exchange resins offer some potential advantages over other supports, they are comparatively labile and would decompose if subjected to an ashing temperature. A number of analytical procedures were proposed and the ones which showed promise were investigated. These procedures fall into two categories.

3.2.1 Direct Measurement

These techniques involve the physical separation of the biomass and support and the comparison of support dry-weights before and after the separation. The stripping techniques considered involved the use of caustic, enzymes, detergents, and chelators. Of these methods, the first is used when coal is used as a support material; therefore, it was tried in this project. Resin samples were dried overnight at 40°C and weighed. These were then soaked for one day in sodium hydroxide solutions ranging from

0.5 to 5 M, washed, and soaked overnight in 1 N hydrochloric acid to return the resin to the chloride form. After drying, the weight change would represent the quantity of dry biomass stripped and could be related to the loading (g dry biomass/volume of reactor).

3.2.2 Indirect Measurement

These involve the measurement of chemical species of living matter not found in the resin support. The quantity of material found must then be related to its equivalent in terms of dry biomass. Suggested techniques included assays of nitrogen, ATP, DNA, and dehydrogenase. The latter two appeared to be the most straightforward and hence were pursued. Both assays are colorimetric, with DNA using the diphenylamine procedure, and dehydrogenase being assayed by the iodonitrotetrazolium violet/formazan method. Control samples for both methods included clean resin, coated resin supernatant, and standard solutions.

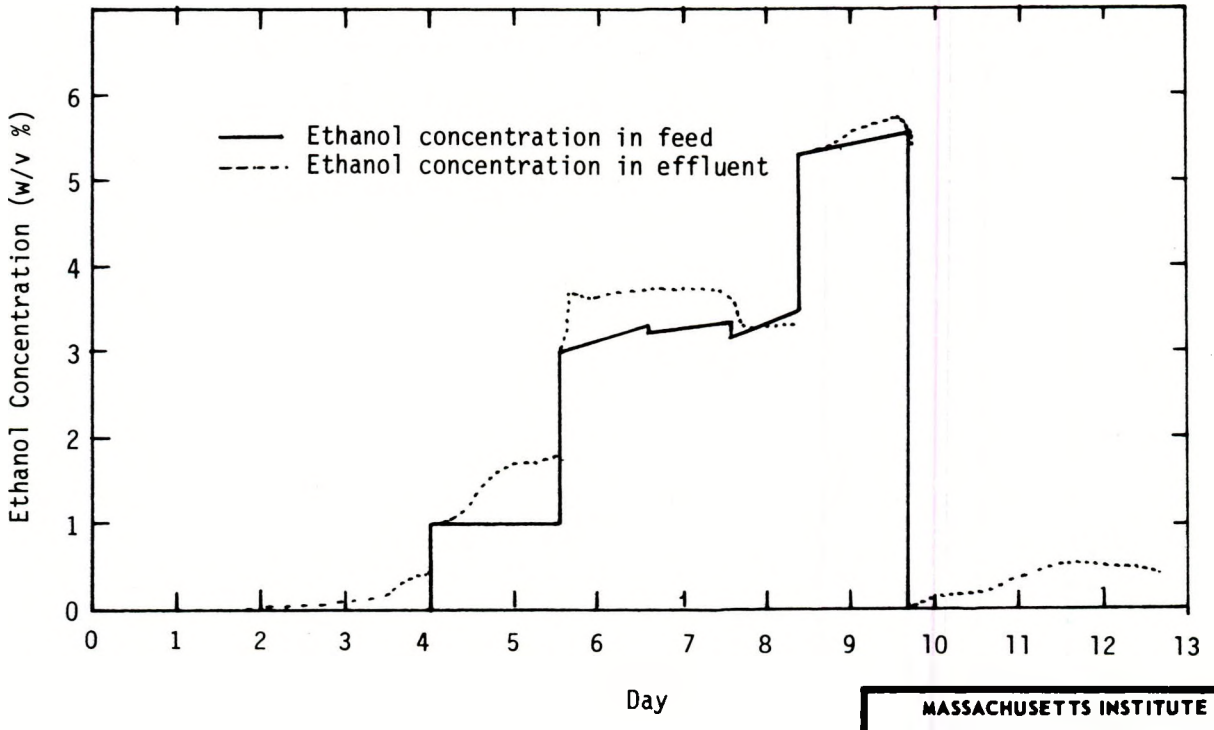
4. RESULTS AND DISCUSSION

4.1 Ethanol Production-Rate Experiment

A history of the ethanol content (wt %) of the fermenter feed and effluent from innoculation at day zero until the end of the series of experiments is shown in Fig. 3. The solid lines indicate the ethanol assay of the feed, while the dotted lines represent effluent concentration. The experiments were operated until a steady state was indicated. This was achieved after 4 days with the first feed (10% glucose), while the lowest glucose-concentration feed (2% glucose, 4% ethanol) came to steady state within one day.

During the experimentation, a variation in the ethanol concentration of the feed was noted. Feed samples were drawn at the time each 100-liter batch (a 26-h supply) was put into use. The feed was stirred during make-up and just before use but was not continuously mixed. Beginning with the use of 5% glucose, 3.5% ethanol feeds, two feed samples were taken from each batch, one just after the batch was put on-line and the other just before the run was discontinued. No end-of-run samples were taken in the first two runs. A gradient in ethanol concentration was in fact found to exist in all feeds tested. This gradient amounted to as much as a 0.3% w/v increase in ethanol content of the feed over the feed initially prepared.

It should be noted that the ethanol concentrations in the 5% glucose and the 2% glucose feeds were much higher than was desired. In the first case, while the intended concentration was 2.5%, we actually obtained 3.5%. In the latter case we obtained an ethanol concentration of 5.5% where our intention was to feed a mixture containing 4.0%. The ramp in the feed concentration and the apparent error in the feed concentration remain largely unexplained.



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ETHANOL CONCENTRATION WITH TIME			
DATE 10-19-80	DRAWN BY JT	FILE NO. CEPS-X-320	FIG. 3

The rate of CO_2 gas generation as a function of time is given in Fig. 4. Generally speaking, the CO_2 production follows the ethanol output; however, it tends to be somewhat more variable. In calculating a balance between CO_2 and ethanol production, one must account for dissolved CO_2. The medium used in these experiments was a complex buffered liquid. Therefore, a comparison of the two rates is subject to rather substantial inaccuracy. For instance, in the first run, the maximum CO_2 rate is 4.5 liters of gas per hour or approximately 8 g/h. If one assumes that the medium is water and calculates the CO_2 dissolved in the exit fluid which would be in equilibrium with the pure CO_2 gas phase, one would find 4.5 g/h leaving in that stream. Hence the total CO_2 effluent is 12.4 g/h or approximately 67 g/liter of reactor/h. This number compares quite well with the number obtained for ethanol (64 g/liter-h).

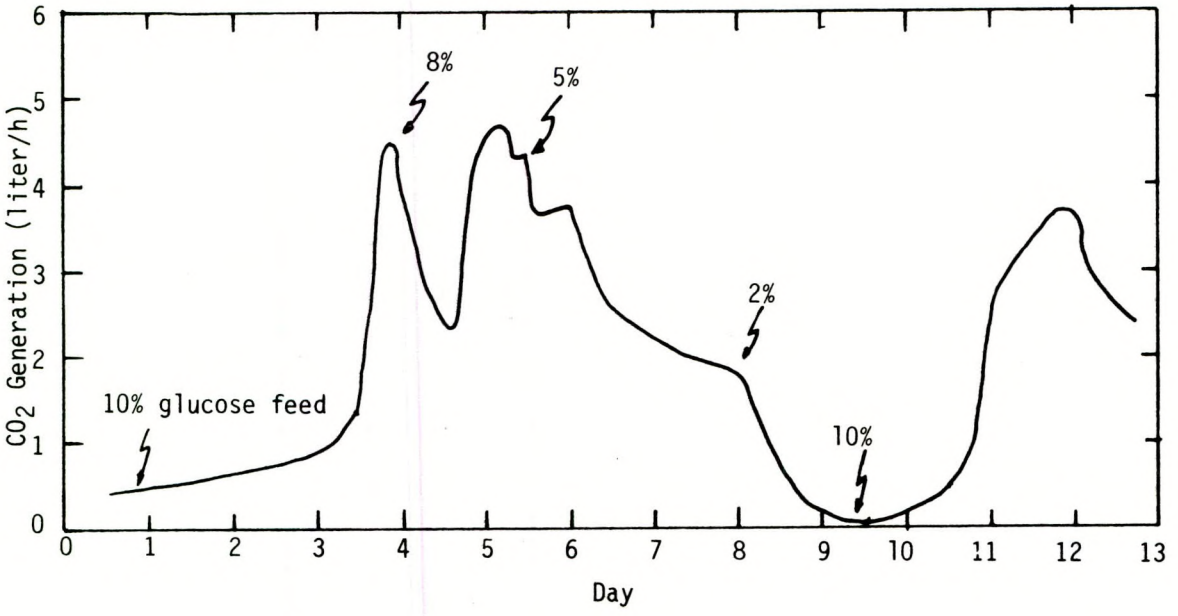
If one assumes that the dissolved-CO_2 number is nearly correct and that it accounts for 24 g/liter-h of CO_2 synthesized, one would predict that there would be very little undissolved gas produced in the 2% glucose-feed experiment. Figure 4 confirms this supposition.

Another indication of dissolution is the pH behavior of the fluid. The double-distilled water used in feed preparation had a pH betwen 5.5 and 6.0. Prepared-feed pH was approximately 6.2, suggesting that the water acidity was sufficient to break the buffering used. Column-effluent pH varied between 4.2 and 4.8, implying substantial amounts of dissolved carbon dioxide.

Each time the feed composition was changed, the system experienced a shock. This can be seen from the ethanol-content plot (Fig. 3) and especially from a plot of CO_2 production rate with time (Fig. 4). An acclimation time of as much as one or two days is required in some cases for the system to adjust to a new feed. The changes in feed concentrations from batch to batch and within a batch in the case of the ethanol ramp may have resulted in the system being continuously inhibited, in the same manner in which substrate changes affect a batch system. The ethanol analysis (Fig. 3) indicates that its rate of production becomes steady more rapidly than does CO_2 generation (Fig. 4). This may be an artifact of bubble formation and disengagement.

Another potential source of difficulty was the fact that the ethanol assay was found to be accurate only to within about ±10%. When higher ethanol concentrations were used in the feed, the measured difference between ethanol in the feed and in the effluent was often of the same magnitude as the analytical error. On day 8 the effluent level fell below the feed ethanol concentration. Since feed and effluent samples do not correspond exactly in time, the error is impossible to uncover. Since this phenomenon occurred primarily at the high end of the concentration range, there is substantial reason to suspect the analytical procedure. A similar phenomenon was observed for the glucose analysis, with the difference between inlet and effluent concentration often less than the observed analytical error.

One possible source of error in the measurement of the CO_2 production rate would be the competition from other organisms which might produce



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CO_2 GENERATION WITH TIME			
DATE 10-19-80	DRAWN BY JT	FILE NO. CEPS-X-320	FIG. 4

hydrogen. Chromatographic analysis of the effluent gas indicated that other materials accounted for only about 2% of the products of reaction. It is likely that gas dissolved in the cold feed accounts for most of this material. As we noted earlier, the CO_2 generation rate followed the same general trend as the ethanol-production data. However, the CO_2 data tended to fluctuate substantially more than ethanol. For example, after day 12, ethanol concentration in the effluent tapers off slightly; CO_2 production drops substantially. This is possibly the result of transient dissolution phenomena which were not noted earlier or perhaps is the result of the aging or contamination of the biomass.

Despite the errors and uncertainties noted in the ethanol-, glucose-, and CO_2-generation data, a trend in the ethanol-production data is evident. The rate of ethanol productivities (μ) is plotted as a function of glucose concentration in the feed (5) in Fig. 5. Since the number of runs is severely limited, only an indication of a model may be attempted. Bacterial reactions are normally modeled using Monod kinetics. The data in this case are clearly not diffusion-controlled but follow reaction control.

The Monod model (11) is a relationship between productivity and substrate concentration which is based on the assumption that no product inhibition is made:

$$\mu = \mu_{max}\left(\frac{S}{K_S + S}\right) \tag{2}$$

where

μ = productivity, g/liter-h

S = substrate concentration, wt %

μ_{max} and K_S = adjustable parameters

To statistically analyze the data, it is necessary to examine the inverse of Eq. (2).

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_S}{\mu_{max}}\left(\frac{1}{S}\right) \tag{3}$$

A linear-regression analysis was performed on the five experiments which were carried out, and it was found that:

μ_{max} = 97.3 g/liter-min

K_S = 4.66% glucose

The coefficient of correlation was 0.86. Obviously, insufficient data are available to permit firm conclusions; however, when the data are compared with the regression-analysis results (Fig. 5), it can be inferred from the shape of the curve and the nature of the data that ethanol is not inhibiting the process.

The point representing an 8% glucose, 1% ethanol feed falls rather far from the regression curve. It is possible that a gradient existed in the ethanol feed concentration. No measurement was made of this run's final feed concentration; but, if a gradient of the same magnitude as was measured in the 2 and 5% glucose feeds is assumed in the 8% case, a corrected ethanol productivity may be calculated which aligns itself more closely with the other data. It must be stated though that the CO_2 data for the 8% glucose run appears to be the highest of all the runs (68.5 g/liter-h). The lower of the two productivities measured for a 10% glucose feed represents the initial feed supplied to the column and is perhaps low because adequate time was not allowed for the system to achieve steady state.

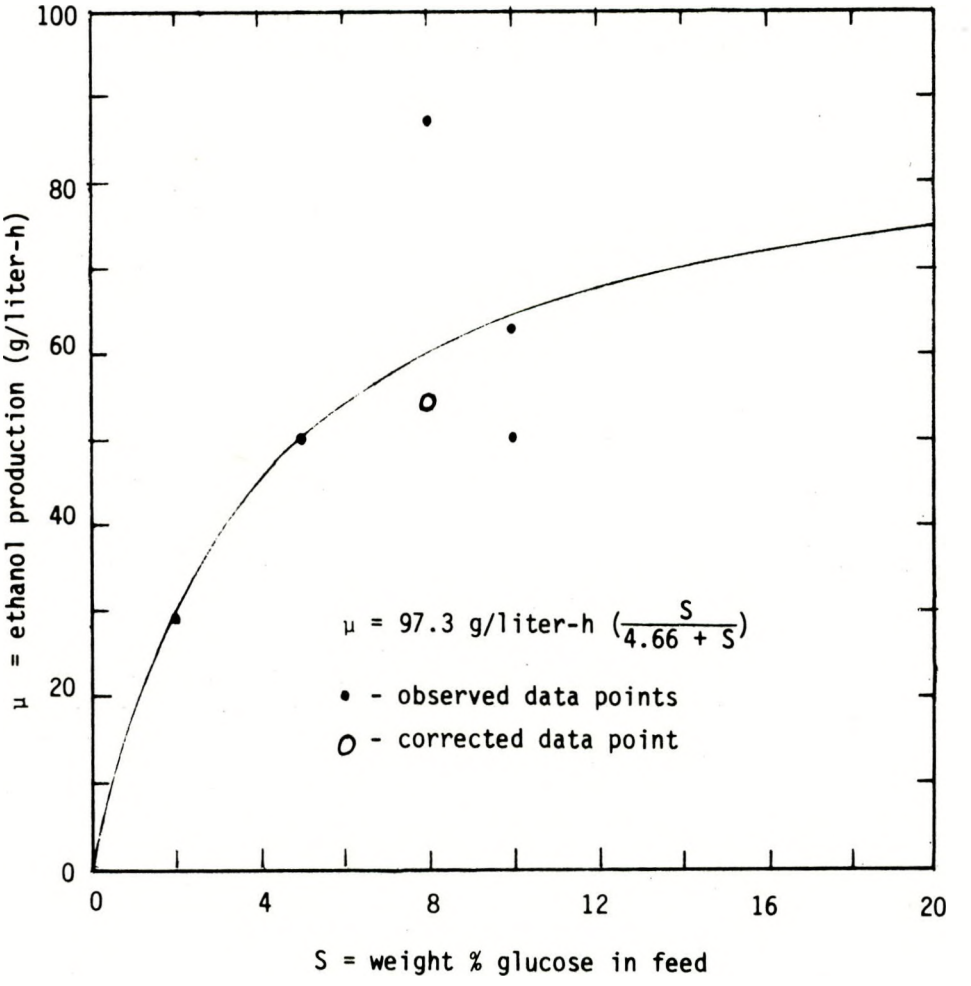
The Monod-model representation in Fig. 5 does not quantitatively describe the system studied, and the values used for μ_{max} and K_S must be considered crude estimates until further data are acquired. However, the shape of the curve does fit the data reasonably well, suggesting that substrate limitation, rather than produced toxicity, is the predominant influence in the system. This and the measured ethanol productivities of 29 g/liter-h for a 2% glucose feed and 64 g/liter-h for the second 10% glucose feed are considered the significant results of this portion of the project.

4.2 Biomass-Loading Measurements

In Sect. 3.2, possible methods of measuring biomass loading were discussed. Time limitations prevented a complete survey of these methods; however, three methods which showed the most promise were studied. The samples studied were resin-attached biomass collected from the reactor at the end of the experimental program.

4.2.1 Caustic Stripping

To assess the damage to the ion exchange resin which might be caused by caustic, samples of clean, dry resin were first subjected to the caustic stripping treatment (24 h at various NaOH concentrations). After stripping, the resin was returned to the acid form by soaking in 1 M HCl. The results shown in Table 1 suggest that the technique possibly degrades the resin. As a result, more work is required on the method before it can be used to differentiate between biomass and support particles.



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COMPARISON OF DATA WITH MONOD MODEL			
DATE 10-19-80	DRAWN BY JT	FILE NO. CEPS-X-320	FIG. 5

Table 1. Caustic Stripping Results

Sample No.	Conc. of NaOH Used (M)	Change in Resin Weight Due to Treatment (%)
1	0	-12.8
2	0.5	-11.9
3	1	-13.5
4	2	-20.4
5	5	+15.6

4.2.2 DNA Analysis

Coated-resin samples were taken from the reactor bed and analyzed for DNA. Standards were prepared using salmon-sperm DNA. The results of the three samples analyzed are given in Table 2. Clean resin was treated first to obtain a blank DNA measurement for the support. Resin supernatant was analyzed to measure the effect of the supernatant clinging to the coated resin.

Table 2. Biomass Loading by DNA Analysis

Sample	DNA Measured (μg/ml)	Biomass Loading (g cells/liter of reactor)
Clean resin	0	0
Resin supernatant	375	0.01
Coated resin	4500	0.13

Biomass loading was calculated using a settled bed volume of 175 ml, an expanded-bed volume of 360 ml, and a *Z. mobilis* DNA content of 2.7% on a dry basis (9).

The standard solutions used were blue after incubation, as were the resin supernatant samples. The coated resin samples, however, were reddish-brown. The cause of this coloration and its effect on the spectrophotometric analysis are not known. This phenomenon must be studied before the usefulness of the DNA method can be assessed.

4.2.3 Dehydrogenase Determination

Results for the dehydrogenase-measurement experiment are given in preliminary form in Table 3. Clean-resin and coated-resin supernatant samples were once again included in the analysis. In addition, a sample of a *z. mobilis* culture containing 1.9×10^8 cells was analyzed. One variable in the INT method to be determined was the incubation time required for full color development. Although iodonitrotetrazolium-formazan standards were prepared, these would give valid results only if proper incubation time had been allowed. The *z. mobilis* culture, on the other hand, could be used as a standard at any time. Thus, the results in Table 3 are given in terms of absorbance, which can be related to dehydrogenase levels and thus to biomass loading once the relationship between them has been determined.

Table 3. Biomass Loading - Dehydrogenase Determination (Absorbance at 484 nm)

Sample	Incubation Time		
	30 min.	60 min.	90 min.
Clean resin	0.14	0.15	0.233
Resin supernatant	0.116	0.20	0.08
	0.153	0.192	0.08
z. mobilis culture	0.23	0.33	0.40
	0.285	0.45	0.40
Coated resin	0.37	0.403	0.42
	0.51	-	0.415

Although it was not possible to obtain a firm evaluation of the biomass loading on the coated resin, it is evident from the results in Table 3 that the supernatant contains little biomass. Also, a comparison of absorbance for the *z. mobilis* culture and the coated material gives a rough equivalence between the two results. Since clean resin appears to give a substantial blank, it would appear that the biomass loading of the resin is substantially less than the culture (perhaps about one-half). However, sampling of the coated resin was subject to significant error. For instance, difficulty in obtaining a constant volume of resin plus problems related to inhomogeneity of flocculated bacteria in the supernatant clinging to the resin are probable causes of some of the scatter of the data in Table 3. One further conclusion can be drawn: the clean resin alone represents a baseline for the coated-resin assay, but the experiment shows that a dehydrogenase determination will detect the presence of bacteria attached to the support. No color interference from the resin was noted.

5. CONCLUSIONS

1. Ethanol productivities observed ranged from 29 g/liter-h for a feed containing 2% glucose and a 5.5% (w/v as analyzed) ethanol to 64 g/liter-h for a 10% glucose feed.

2. *Z. mobilis* in a tapered, fluidized-bed bioreactor was not observed to be ethanol-inhibited up to 5.5%(w/v) ethanol.

3. An unexplained ethanol gradient developed in an unstirred feed solution.

4. The inhibitory effect expected from the change in feed composition as a result of the ethanol gradient suggests that observed productivities are conservative. Certainly they are substantially below those reported by Arcuri *et al.* (1).

5. Because of the preliminary nature of the experimental work on biomass loading, the results for the three methods examined are inconclusive.

6. RECOMMENDATIONS

1. This study was intended to give preliminary results aimed eventually at an optimal reactor design. Nothing found in this investigation indicates that this process should be discontinued. Some areas for future work might be: flow rate variation, substrate concentration, pH, and sterilization studies.

2. The observed time gradient in ethanol content of the feed and the unexplained ethanol content (see Sect. 4.1) of the 2 and 5% glucose feeds must be corrected. At least one of these runs must be repeated, with continuous stirring of the feed.

3. Only the most preliminary study of biomass loading was completed. The DNA and dehydrogenase analyses require further evaluation. Cell-stripping with other materials, such as enzymes, detergents, and chelators, should be investigated.

7. ACKNOWLEDGMENT

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8. APPENDIX

8.1 Location of Original Data

The original data are located in ORNL Databook A-6487-G, pp. 50-75, on file in the Chemical Technology Division, Bldg. 4505, ORNL. The calculation files are located at the MIT School of Chemical Engineering Practice, Bldg. 1505, ORNL.

8.2 Nomenclature

K_S adjustable parameter, wt %

S substrate concentration, wt %

μ ethanol production rate, g/liter-h

μ_{max} adjustable parameter, g/liter-h

8.3 Literature References

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