Ethanol Production by *Zymomonas mobilis* in Fed-Batch Fermentations

R. W. Silman
Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, * Peoria, Illinois 61604

Accepted for publication August 16, 1983

The production of ethanol by *Zymomonas mobilis* NRRL B-4286 was studied in fed-batch cultures. Initial percent (w/v) glucose, rate of feed, and quantity of 50% (w/v) glucose feed were varied. Glucose inhibition of growth rate occurred at concentrations greater than 8% (w/v). Feed was begun after 4 h incubation. Feed volume was ca. 36% of starting batch volume to get ca. 10% (w/v) ethanol at harvest. The range of feed rates studied varied from 16% batch volume/h (glucose concentration increased to an inhibitory level) to 4% batch volume/h (glucose concentration dropped rapidly to zero and was limiting). Increasing feed volume to 46% of starting volume at the best feed rate (ca. 10% feed volume/h) increased final ethanol concentration to 11.3% (w/v). However, the resultant increase in fermentation time from ca. 21 to 29 h decreased ethanol volumetric productivity from 5.2 to 4.6 g/L h.

**INTRODUCTION**

Production of fermentation products is possible by batch, fed-batch, continuous culture, and immobilized cells. The literature on ethanol production with *Zymomonas mobilis* shows little recent study of the fed-batch technique. This is surprising, since this technique has the advantage that the deleterious effects of initial high substrate levels can be avoided. This inhibitory effect is well known in the genus *Zymomonas*. The fed-batch technique also is useful when starting up continuous cultures. This work reports a study of ethanol production by *Z. mobilis* in a fed-batch fermentation with the emphasis upon increasing volumetric productivity. The goal was to make at least 10% (w/v) ethanol as fast as possible with complete glucose consumption. It was decided to limit the number of fed-batch experiments by always using 10% (v/v) of 24-h-old inoculum grown in standard medium with 12% (w/v) glucose and 50% (w/v) glucose in the feed.

The study actually began with batch fermentations (not reported in detail) in which yeast extract and glucose concentrations were varied to determine optimum starting conditions. Greater than 1% (w/v) yeast extract did not increase cell production. With up to 8% (w/v) glucose there was no inhibition of growth rate nor was ethanol production reduced during the first 5–6 h fermentation. As glucose level was increased above 8% lag phases became longer and rates of growth became less. At 20% (w/v) glucose there was neither growth nor ethanol production by 11 h and only 3% (w/v) ethanol by 24 h. Also, in order to prevent too rapid exhaustion of initial glucose, it was found necessary to begin feeding at 4 h.

The effects of feed rate and total feed used upon various characteristics of fed batch fermentations is the main subject of the remainder of this article.

**EXPERIMENTAL**

**Culture**

*Zymomonas mobilis* NRRL B-4286, which is synonymous with culture CP-1 of deLima et al., was used throughout the work.

**Inoculum Buildup and Media**

Inoculum buildup scheme and media used are presented in Table I. A lyophilized culture was opened to initiate every fed-batch fermentation. All cultures were incubated at 30°C. Test-tube and 300-mL Erlenmeyer flask cultures were static. Fernbach flask cultures were incubated on a rotary shaker at 100 rpm; closures were a two-hole rubber stopper with two 8-mm-o.d. × 5-cm-long glass tubes plugged with nonabsorbent cotton. After inoculation and immediately before incubation of all shaken cultures, CO₂ was used to purge the air space above the culture media.

**Fermentations**

Fed-batch fermentations were conducted in 50-L New Brunswick Fermacell fermentors. Medium B (Table I) at 8% (w/v) glucose was used throughout. The glucose part of the medium was steam sterilized in the fermentor at 70% of the final volume. The nitrogen part of the me-
Table I. Inoculum buildup for 20-plus-L fed batch.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>20 liter Medium B at 8% glucose in fermentor</td>
<td></td>
</tr>
</tbody>
</table>

Medium A: 2% glucose, 1% yeast extract, 0.5% peptone.

Medium B: 12% glucose; 0.165% NaH₂PO₄·2H₂O; 0.7% Na₂HPO₄·2H₂O—glucose and MgSO₄·7H₂O separately sterilized.

Merthiolate in distilled water was added to each sample to prevent sample evaporation. Before measurement of the sample volume, glucose and MgSO₄·7H₂O separately sterilized.

Two 2800-mL Fernbach flasks with 1890 mL Medium B at 12% glucose; incubation at 30°C on 100 rpm shaker; 2000 mL; 100 mL sample; 20 liter Medium B at 8% glucose in fermentor.

Analytical

Samples were assayed for dry cell weight, glucose, and ethanol. After measurement of the sample volume and removal of supernatant oil layer, an aliquot of sample was centrifuged. The supernate was used for assays, and the residue was washed with distilled water and centrifuged twice, dried under vacuum at 80°C for 18-24 h and weighed for dry cell weight.

RESULTS

Results comparing fermentations with different feed rates or different feed quantities must be analyzed on a basis of total cell weight, total ethanol produced, and total glucose consumed as well as on concentration. Total amounts were calculated from the assay concentrations, and estimates of fermentation volumes that had first been calculated from average feed rates, sample volumes, and apparent entrainment or evaporative losses for each fermentation. The calculations of total cells, ethanol produced, and glucose consumed were based upon constant initial volume and final volume, i.e., with no sampling before or after feeding.

For example, the actual starting fermentation volume was 20 L and feed was 8 L of 50% (w/v) glucose. After sampling and losses are taken into account, the average calculated values were 19.7 L, 7.1 L, and 56.1%, respectively. Also, the average final volume was 26.7 L and the initial glucose was 7.7%. Actual feed rates were 1.29, 0.82, 0.62, 0.49, and 0.41 L/h, whereas calculated values were 1.22, 0.73, 0.53, 0.44, and 0.34 L/h for the set of runs to study feed rate.

Figures 1, 2, and 3 show smoothed curves based on samples taken at 0.5-h intervals. Cumulative dry cell weight, ethanol produced, and glucose consumed are plotted versus age of the cultures for the fed-batch fermentations fed the same amount of 50% (w/v) glucose but at different rates of flow. Cell growth patterns are similar (Fig. 1), i.e., the growth is exponential until 4-6 h of age. Figure 2 shows that runs at feed rates of 0.62 and 0.82 L/h gave about the same ethanol production rate as the initial glucose was sustained. From the linear patterns of glucose consumption shown in Figure 3, it is apparent that glucose is limiting in runs at 0.49 and 0.41 L/h. The data in Figure 4 confirm that glucose concentration was indeed zero, and therefore limiting, during the times mentioned. The effect of ethanol production by linear glucose consumption due to low feed rate is shown by the linear ethanol increases in Figure 2.

Based upon the preceding runs, the conditions of the run at 0.82 L/h were used except that the quantity of feed was increased to ca. 10 and 12 L for 10- and 12-L actual

![Figure 1](image-url). Cumulative dry cell weight production in fed-batch fermentations at different feed rates.
feed, the calculated feed volumes were 9.0 and 10.7 L, with harvest volumes of 28.6 and 30.3 L. The run with 8 L feed was designed to yield 10% (w/v) final ethanol concentration, whereas the 10- and 12-L feeds were, hopefully, to make 11 and 12% (w/v) ethanol, respectively. The results of these three fed-batch fermentations are compared in Figures 5 and 6, which show glucose concentration and total ethanol produced. As expected, the three runs gave almost identical results for nearly 14 h. The divergence thereafter is the result of continued feeding, shown particularly well in Figure 5. In choosing the best of these three runs, a balance is required between final ethanol concentration, productivity as grams of ethanol per liter volume per hour, and complete consumption of glucose. Final ethanol concentrations of these runs with 8, 10, and 12 L feed were 10.4, 11.3, and 10.7% (w/v), respectively. Productivity was calculated at 20,

Figure 5. Concentration of glucose in fed-batch fermentations fed different quantities of feed at 0.82 L/h.
24.5, and 23 h, the age at which maximum ethanol concentration was attained, and was found to be 5.21, 4.57, and 4.59 g ethanol/L/h, respectively, for the three runs. Corresponding yields of ethanol made from glucose consumed were 50.6, 51.0, and 50.0%. Note that the run with 12 L fed had glucose remaining and basis glucose fed the yield was 42.0%.

**DISCUSSION**

All the fed-batch fermentations reported gave greater volumetric productivity than the 24-h-old batch fermentation run at 20% (w/v) initial glucose that made only 3% (w/v) ethanol at 1.25 g/L/h productivity. The fed-batch fermentation at 0.82 L/h was the most productive run overall. The second most productive fed-batch fermentation was at 0.62 L/h. In the run at 0.62 L/h the ethanol production rate tapered off sooner than in the run at 0.82 L/h. Comparison of Figures 2 and 4 shows the possibility that near depletion of glucose at 12-14 h caused this rate reduction. Another factor may have been that ethanol concentration at 8.8% (w/v) was somewhat inhibitory. However, in the 0.82 L/h run, at 13.5 h the glucose was well above zero (3.0%) when ethanol reached 8.7% (w/v), and less inhibition of ethanol production was evident.

The fact that the feed rate can be too fast was demonstrated by the run at 1.29 L/h, which had both a lower ethanol production rate and a lower glucose consumption rate than did the run at 0.82 L/h (Figs. 2 and 3). The probable cause was the increase of glucose concentration to 10.5% at 10 h where it becomes inhibitory (Fig. 4). The implication is that large additions of glucose in semicontinuous fashion should be avoided, and the feed rate must be properly adjusted for the conditions employed.

In the extended feed fed-batch runs, productivity varied. The run with 8 L fed was the most productive of the three runs; however, the effect in the run with 10 L of higher final percent ethanol (11.3%) on the overall economics of a process should not be neglected. The optimum feed quantity is probably at an intermediate level.

It should be observed that all growth (as measured by dry cell weight) essentially stopped in all runs after 10 h (Fig. 1), whereas glucose consumption and ethanol production continued (Figs. 2 and 3). This is also illustrated by the specific growth rate \( \mu \) and ethanol production rate \( q_p \) data for a composite of the three runs at 0.82 L/h from 0 to 13 h when the feed was stopped for the 8-L run. The calculated average values of \( \mu \) and \( q_p \) with corresponding concentration of ethanol and glucose are given in Table II; they indicate that 1) nearly steady \( q_p \) regardless of decrease in \( \mu \), substantiating the ability of *Z. mobilis* to uncouple growth and ethanol production, 8.9 2) \( \mu \) is an inverse function of ethanol concentration; 3) there is uncertainty as to the nature of the relation between \( q_p \) and glucose or ethanol concentration.

**CONCLUSIONS**

The inhibition of growth and ethanol production by *Z. mobilis* at concentrations of glucose in excess of 8% (w/v) in batch fermentations can be effectively overcome with a fed-batch system of culture while maintaining good yields of ethanol from glucose and volumetric productivity. Glucose can be fed too slowly or too quickly. Too-slow feeding impedes ethanol production by limiting the glucose available for conversion to ethanol, whereas too-fast feeding can so exceed the capacity of the growing culture that the glucose concentration increases, and thereby becomes inhibitory. Ideally, near the very end of the fed-batch fermentation the feed rate should be just fast enough to prevent depletion of glucose. The proper time to stop feeding is a matter of economics of production. Consideration must be given to volumetric productivity, yield, final ethanol concentration, and recovery costs.

The technical assistance of A. Lagoda and T. Larsen in fermentor operation is greatly appreciated. Special thanks is extended to L. Black for the sugar and alcohol analyses.
References