BIOLOGICAL PRODUCTION OF ETHANOL FROM COAL

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SUMMARY

The fermentation pH has been observed to be the key parameter affecting the ratio of ethanol to acetate produced by Clostridium ljungdahlii. The effects of controlled pH on cell growth and product formation by C. ljungdahlii were measured. It was found that cell concentration and acetate concentration increased with pH, while the ethanol concentration was highest at the lowest pH. The molar product ratio of ethanol to acetate was 0.74 at pH 4.0, 0.39 at pH 4.5 and 0.12 at pH 5.0. Future experiments will concentrate on studying other important parameters such as agitation rate and nutrients concentration with controlled pH as a prelude to continuous reactor studies.

INTRODUCTION

Clostridium ljungdahlii, a bacterial strain isolated from animal waste in the University of Arkansas laboratories, is capable of converting CO, CO2, and H2 in synthesis gas to ethanol and acetate by the reactions:

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\begin{align*}
6 \text{ CO} + 3 \text{ H}_2\text{O} & \rightarrow \text{C}_2\text{H}_5\text{OH} + 4 \text{ CO}_2 \\
2 \text{ CO}_2 + 6 \text{ H}_2 & \rightarrow \text{C}_2\text{H}_5\text{OH} + 3 \text{ H}_2\text{O} \\
4 \text{ CO} + 2 \text{ H}_2\text{O} & \rightarrow \text{CH}_3\text{COOH} + 2 \text{ CO}_2 \\
2 \text{ CO}_2 + 4 \text{ H}_2 & \rightarrow \text{CH}_3\text{COOH} + 2 \text{ H}_2\text{O}
\end{align*}
\]

Acetate is produced in conjunction with growth and energy production by the bacterium, so that its production is favored over ethanol, which requires
energy for formation. In fact, early batch experiments performed just after isolation showed a 20:1 molar product ratio of acetate to ethanol.

Several strategies have been employed in other clostridial fermentations, particularly the acetone-butanol fermentation by _C. acetobutylicum_, which have led to increases in the selectivity of solvents versus acids. Studies in the acetone-butanol fermentation have shown that high solvent yields can be obtained by adjusting growth-limiting factors such as phosphate, nitrogen, etc. in a range which allows some growth but not under optimum conditions (1-3). Other researchers have shown that the presence of reducing agents in the liquid medium brought about an increase in solvent formation in the product stream at the expense of acid formation (4-8). Finally, the connection between sporulation and solventogenesis has been the subject of extensive studies both in batch culture (9-11) and _chemostat_ culture (3,12).

The conditions that cause the onset of solventogenesis vary among the clostridial strains. In the case of _C. thermosaccharolyticum_, significant ethanol production and elongated cell formation both require a combined signal of a specific carbon source (L-arabinose or L-xylose), lower pH and a restricted supply rate of the energy source (13). In the case of _C. acetobutylicum_, switching to the solventogenic phase and differentiation to the clostridial stage requires the presence of glucose and nitrogen, a low pH and minimum acetate and butyrate concentrations. It has also been observed that, in general, solventogenesis can be induced in batch culture using carbon sources that tend to reduce the bacterial growth rate (14).

Many of the techniques described above have been used successfully in producing ethanol in favor of acetate from CO, CO₂, and H₂ by _C. ljungdahlii_. The addition of low concentrations (30 ppm) of reducing agent to the liquid
medium in batch culture resulted in the production of equimolar amounts of ethanol and acetate. Also, the use of a medium containing low concentrations (0.01%) of yeast extract, the use of a minimal medium containing only salts and vitamins and the addition of high partial pressures of H₂ in the gas phase favored ethanol as a product over acetate. Finally, a pH shift in the medium from 5.0 to 4.0 resulted in greater ethanol production in favor of acetate production.

In analyzing the results of the studies with C. ljungdahlii, it was observed that pH manipulation appeared to be the most significant parameter affecting product ratio. All other variables (nutrients concentration, reducing agent addition, etc.) were certainly important, but seemed to be overshadowed by the effects of pH shift. A study of the effects of controlled pH on product ratio and bacterial growth was thus warranted.

The purpose of this report is to present results from an ongoing study investigating the effects of pH and other parameters in combination on growth and product formation by C. ljungdahlii. In particular, the effects of controlled pH over a pH range of 4.0 to 5.0 on bacterial growth, ethanol concentration and cell concentration are presented and discussed. These results will be followed by results on the combined effect of reaction pH, nutrients concentration, agitation rate, etc. on growth and product formation in the coming months, as well as the effects of these variables in continuous cell recycle and immobilized cell reactors.

EQUIPMENT AND PROCEDURES

The batch reactor is traditionally the best reactor for use in comparing the effects of different parameters on cell growth and product formation. The batch reactor is easy to operate and yields a large amount of useful data in a
relatively short period of time. A typical batch reactor used in the fermentation of gas phase reactants such as CO, CO₂, and H₂ is the serum bottle, sealed with a butyl rubber stopper to ensure anaerobic conditions.

One of the problems associated with the serum bottle reactor is pH control when an acidic product is formed. Buffering with standard buffer solutions has not proven to be effective in controlling this problem. A chemostat (or batch stirred tank vessel) can be operated with pH control, but the vessel cannot be pressurized above atmospheric pressure.

A solution to this problem is the use of a batch reactor with continuous gas flow. This "fed-batch" reactor yields quantitative data from liquid phase analyses at constant pH, but the data obtained from the gas phase is somewhat inconsistent because the gas phase is being continually replaced. However, if cell concentration and product concentration are the measured variables of concern (as opposed to gas phase conversions), the "fed-batch" reaction system can be the reactor of choice. This type of reactor was thus used for the study of the effects of reaction pH on cell growth and product formation.

Reaction Vessel

The "fed-batch" reactor consisted of a 1L New Brunswick Bioflo Model C30 chemostat equipped with automatic pH control and pump modules. The gas flow rate to the reactor was continuous, and controlled with a rotameter and needle valve.

Medium

The medium for C. ljungdahlii consisted of basal medium supplemented with 0.02 percent yeast extract. No sodium bicarbonate was added for pH control.
Feed Gas

The gas feed to the reactor was a mixture with the following approximate composition:

- Hydrogen 20 mol percent
- Argon 15 mol percent
- Carbon Monoxide 55 mol percent
- Carbon Dioxide 10 mol percent

As mentioned previously, the gas was fed continuously to batch liquid in the reactor.

Inoculum

The inoculum for reactor start-up was prepared in a 1.2 L serum bottle containing 250 mL of 0.01 percent yeast extract. The seed bottle was inoculated with a 7-10 day old culture maintained in a shaker incubator. The seed was regassed and the pH adjusted to 4.5 to maintain good growth conditions (typically on days 3, 6 and 9). The seed culture was then used as the inoculum after 7 to 10 days.

Reactor Operation

Detailed procedures for reactor start-up and sampling are appended.

RESULTS AND DISCUSSION

Cell concentration, ethanol concentration and acetate concentration profiles for the fed batch runs at three pH levels are shown in Figures 1-3, respectively. As was mentioned previously, gas phase conversion profiles are not shown due to the scatter of the data obtained in this type of reaction system. Figure 1 clearly shows that high pH levels favor cell production over lower pH levels, although the profiles were nearly identical at pH 4.5 and pH 5.0. The maximum cell concentration at pH 5.0 was about 490 mg/L after 650 hr.
and the maximum at pH 4.0 was only 330 mg/L after 470 hr. Figure 2 shows nearly identical ethanol concentration profiles at pH 4.0 and pH 4.5 (if the two data points at 220 and 260 hr for pH 4.5 are excluded). Furthermore, the profile run at pH 5.0 yielded a much lower maximum ethanol concentration than at pH 4.0 and pH 4.5. The maximum ethanol concentration at pH 5.0 was 0.8 g/L after only 180 hr and the maximum at pH 4.5 was about 1.8 g/L after 300 hr. The ethanol concentration was quite steady after reaching a maximum at a given pH level.

Figure 3 shows that the acetate concentration increased significantly with pH, reaching a maximum of 4.0 g/L after 470 hr at pH 4.0, a maximum of 6.0 g/L after 230 hr at pH 4.5 and a maximum of about 13 g/L after 400 hr at pH 5.0.

In comparing Figures 1-3, it is seen that the acetate concentration continued to increase after the maximum ethanol concentration was reached, indicating that, late in the fermentation, substrate was converted mainly to acetate. Also, it appears that acetate production was more closely related to growth and ethanol production was not growth related. This latter observation is consistent with results reported with other clostridia, where acid production was shown to be growth associated and ethanol production was shown to be non-growth associated.

The ethanol to acetate molar product ratio at the maximum ethanol concentration was 0.74 at pH 4.0, 0.39 at pH 4.5 and 0.12 at pH 5.0. The addition of other controlled parameters such as nutrients concentration, agitation rate, H₂ addition, etc. should increase this ratio significantly.
REFERENCES


APPENDIX
"Fed Batch" Reactor Start-up

**REACTOR:** Disconnect the reactor at the quick connect fitting near the inlet gas filter and clamp the tubing between the filter and the reactor to preclude backup of liquid into the filter during autoclaving. Remove the sodium hydroxide injection needle and disconnect the pH probe cable at the back of the pH control module and the gas outlet tubing prior to the check valve. Set the reactor on its stainless steel support plate and clean as needed.

**MEDIA:** Prepare 400 mL of 0.01 percent basal medium (for one reactor). Rinse the reactor with 20 mL of medium, then charge 350 mL of medium to the reactor. Close the reactor, plugging the gas outlet tubing with a glass wool filter (this will allow the reactor to breathe during autoclaving). Autoclave the assembled reactor, including the reactor, pH probe, thermometer, overflow flask and support plate for 30 min at 120°C.

**PURGING:** Remove the hot reactor from the autoclave, dry the support plate and install the assembly on the Bioflo. Immediately begin purging the reactor with nitrogen using a sterile cotton filter and needle inserted in the gas sample port. Connect the synthesis gas line to the reactor and open the valves before releasing the clamp on the tubing between the filter and the reactor. Adjust the synthesis gas flow to the maximum flow that registers on the rotameter (about 50 on the rotameter scale). Maintain nitrogen flow for 10 to 15 min; then stop the nitrogen flow and connect the outlet check valve. Maintain the gas purge until the medium has cooled to room temperature and the reactor gas analysis is the same as that for the feed gas. Reduce the gas flow and start gentle agitation (about 200 rpm). Reduce the cooled medium with 7.0 mL of 2.5 percent cysteine hydrochloride solution (1.0 mL per 50 mL of medium). The cooled, reduced medium should be pale yellow at the experimental pH.

**INOCULUM:** Use 40 mL of the prepared seed culture. Connect the pH controller and pump module using a sterile needle for 3 M sodium hydroxide injection, and adjust pH. Hydrochloric acid solution can be used to lower pH using the basic program, CORRPH, or the pH can be allowed to drop as growth occurs. Agitation rate should remain low with only slight entrainment of the overhead gas until growth is established in the reactor.
"Fed Batch" Reactor Sampling

GAS COMPOSITION: The gas phase compositions were determined by gas chromatography. Feed gas samples were drawn with a gas syringe, 0.8 mL typical sample size.

LIQUID SAMPLE: Flush a 5.0 mL syringe fitted with a sterile 22 gauge needle several times in the overhead vapor space of the reactor. Draw 3.0 mL of the overhead gas and inject it into the liquid sample dip tube to clear the stagnating liquid. Draw 2.6 mL of the medium for liquid analysis.

OPTICAL DENSITY: Optical density was determined with a Spectronic 21 spectrophotometer at 580 nm versus a water blank. The cell density in mg/L was 473.9 times the OD.

PRODUCT CONCENTRATIONS: Concentrations of ethanol and acetic acid were determined by gas chromatography. A n-propanol internal standard was mixed 1:10 volume parts with the raw sample for injection into the gas chromatograph.
Figure 1. Cell Concentration Profiles for \textit{C. ljungdahlii} on CO, CO$_2$, and H$_2$ at Various Controlled pH Levels in a Fed Batch Reactor
Figure 2. Ethanol Concentration Profiles for *C. ljungdahlii* on CO, CO₂, and H₂ at Various Controlled pH Levels in a Fed Batch Reactor.
Figure 3. Acetate Concentration Profiles for *C. ljungdahlii* on CO, CO₃ and H₂ at Various Controlled pH Levels in a Fed Batch Reactor

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