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BIOLOGICAL PRODUCTION OF ETHANOL FROM COAL

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SUMMARY

The effects of temperature on the growth and uptake of CO by *C. ljundahlii* were studied in batch culture. A maximum specific growth rate of $0.095h^{-1}$ was found at 37°C, a normal optimum temperature for the growth of anaerobic bacteria. A maximum specific uptake rate of 0.0095 mmol/mg•h was found at a slightly higher temperature of 40°C. CSTR studies were carried out measuring the effects of agitation rate on culture performance. As expected, growth was best at the higher agitation rates until nutrient limitation occurred. A maximum ethanol concentration of 3.5 g/L occurred at an agitation rate of 460 rpm, with an accompanying acetate concentration of 2.5 g/L. Near nutrient limitation appeared to be best for increased ethanol production. Finally, cell recycle studies continued in conjunction with a CSTR. An ethanol concentration of 6 g/L with zero acetate production was obtained. The addition of nutrients such as $(NH_4)3PO_4$ with the hope of increasing both cell and product ethanol concentrations will be tried next in the CSTR with cell recycle.

INTRODUCTION

Clostridium ljungdahlii, a strictly anaerobic bacterium capable of converting CO, CO_2 and H_2 to ethanol and acetate by the equations:

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \qquad (1)$$

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \quad (2)$$

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \quad (3)$$

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (4)$$

was originally isolated from animal waste at the University of Arkansas. Although the native strain was found to produce significantly more acetate than ethanol, research efforts at the University of Arkansas have resulted in product streams from batch and continuous reactors that have reversed this trend. As was reported previously (PETC Report No. 2-3-91, March-June 1991), CSTR studies at a liquid flow rate of 100 mL/d and an agitation rate of 400 rpm have resulted in an ethanol concentration of 2.5 g/L and an acetate concentration of 1.5 g/L. Preliminary results in a CSTR with cell recycle has shown an ethanol concentration of more than 6 g/L and a product ratio of 100 g ethanol/g acetate.

Research is continuing in attempting to increase both the ethanol concentration and product ratio from the *C. ljungdahlii* fermentation. Both batch and continuous reactors are being used for this purpose. The purpose of this report is three-fold. First, the results of batch fermentations carried out at various temperatures between 25 and 40°C are presented. Specific growth and production rates are compared at the various temperatures. Secondly, the results of continuous stirred tank reactor studies are presented, where cell concentration and product concentrations are shown as a function of agitation rate. Finally, the performance of *C. ljungdahlii* in a CSTR with cell recycle is presented and discussed.

EFFECT OF TEMPERATURE ON GROWTH AND PRODUCT FORMATION BY *C. LJUNGDAHLII*

Temperature is known to affect both the growth and formation of products over a fairly narrow temperature range. Mesophilic organisms have an approximate operating temperature range of 10-47°C, with typical optimum temperatures of 30-45°C. Thermophilic organisms have a higher temperature operating range of 40-80°C, with an optimum of 55-75°C (Stanier *et al.* 1970). *C. ljungdahlii* is a mesophile. Organisms usually continue to live at temperatures below their operating temperature range with significantly decreased metabolic activity, but usually die at temperatures only a few degrees above their operating range.

The growth and formation of ethanol and acetate by *C. ljungdahlii* were monitored over a temperature range of 35 to 40°C, with all other variables except pH held constant. Specific growth and production rates were then calculated for the exponential growth phases at each temperature. These rates were then compared at the various temperatures, along with the ratios of ethanol to acetate.

Equipment and Procedures

C. ljungdahlii was grown on basal medium without yeast extract and one-half the normal concentration of B-vitamins. This medium is the same medium as employed in recently reported studies (PETC report No. 2-3-91, March-June 1991). The medium was reduced with 2.5% cysteine-HCl solution. All fermentations were carried out in 150 mL serum stoppered bottles, the medium occupying a volume of 50 mL.

The gas phase consisted of 65% CO, 24% H_2 and 11% CO_2 in each of the bottles. The bottles were pressurized to 10 psig with this synthetic

synthesis gas initially and when replenishing the gas supply during the fermentation. All bottles were incubated in a horixontal position shaken in a New Brunswick shaker incubator at 100 rpm.

Results and Discussion

Typical cell concentration and total product (ethanol plus acetate) concentration profiles at a temperature of 34°C are shown in Figures 1 and 2. As is noted, two different pH levels were studied at this temperature, pH 4.5 and pH 5.0. The pH level reported is the time average pH value over the exponential growth period, defined as:

$$\text{pH avg} = \frac{\int \text{pH } dt}{\Delta t} \tag{5}$$

where Δt is defined by the straight line data points used in obtaining the specific growth rate.

As is noted in Figures 1 and 2, pH had only a small effect on cell concentration and total product concentration. However, as was shown and discussed previously, the pH did affect the product ratio, with lower pH levels resulting in higher ethanol: acetate ratios (data not shown). The maximum call concentration reached at 34°C was approximately 350 mg/L, reached after 50-60 h of fermentation (see Figure 1). The maximum product concentration at 34°C was obtained at the end of the fermentation period, reaching a combined ethanol plus acetate concentration of nearly 90 mmol/L (see Figure 2).

Similar profiles to Figures 1 and 2 at all fermentation temperatures were then used to calculate the specific growth and production rates in the exponential growth phase. This procedure is demonstrated in Figures 3 and 4 for a temperature of 34°C and pH levels of 4.5 and 5.0. The specific growth rate, μ, is defined in the exponential growth phase as:

$$\mu = \frac{1}{X} \frac{dX}{dt} \tag{6}$$

Separating variables and integrating Equation (6) yields:

$$\int_{X_o}^{X} \frac{dX}{X} = \mu \int_{o}^{t} dt \tag{7}$$

and,

$$\ln \frac{X}{X_o} = \ln X - \ln X_o = \mu t \tag{8}$$

Thus, a plot of ln X as a function of time should yield a straight line with a slope equal to μ in the exponential growth phase. As is shown in Figure 3, a straight line is obtained in the exponential growth phase at 34°C for both pH levels. The specific growth rate is 0.064 h^{-1} at pH 4.5 and 0.082 h^{-1} at pH 5.0.

The specific production rate, ν, is defined as:

$$\nu = \frac{1}{X} \frac{dP}{dt} \tag{9}$$

Rearranging Equation (9) yields:

$$dP = \nu X \, dt \tag{10}$$

Integration of Equation (10) yields:

$$P - P_o = \nu \int_{o}^{t} X \, dt \tag{11}$$

where P_o, the initial product concentration, equals zero. Thus, a plot of the total product concentration, P, as a function of the time integral of cell concentration, $\int_{o}^{t} X \, dt$, should yield a straight line over the exponential growth region with a slope equal to the specific production rate, ν. As is noted in Figure 4, the specific growth rate at 34°C and pH 4.5 is 0.0068 mmol/mg·h, and at pH 5.0 is 0.0065 mmol/mg·h.

A plot of specific growth rate and specific production rate as a function of temperature (regardless of pH) is shown in Figure 5. As is noted, both the average specific growth rate and the average specific production rate are seen to reach a maximum at a particular temperature. The maximum specific growth rate was 0.095 h^{-1} at about 37°C. The maximum specific production rate was 0.0095 mmol/mg•h at about 40°C. Thus, the best temperature for growth is at a typical optimum for mesophilic bacteria, while production is favored at a slightly higher temperature. It should be noted that the product ratio (ethanol:acetate) was not significantly affected by temperature, but was affected by fermentation pH as expected.

The wide variation in the data presented in Figure 5 is due mainly to the variation of pH during fermentations. This variation is illustrated in Figure 6, where the specific growth and production rates at 34°C are plotted as a function of the time average pH (see Equation 5). As is noted, the specific growth rate was seen to increase with increasing pH. This result was expected since higher pH levels were shown earlier to be more suitable for both growth and acetate production. The specific production rate, on the other hand, was shown to be rather low at pH 3.75, but was essentially constant at the higher pH levels. The bacterium was shown earlier to function well at pH levels of 4.0 and above, and the total product formation (ethanol plus acetate) was shown to be essentially constant with pH. Ethanol production, of course, was favored at lower pH levels.

CONTINUOUS STIRRED TANK REACTOR STUDIES

A continuous stirred tank reactor with both continuous liquid and gas feed was used to demonstrate synthesis gas conversion to ethanol by *C. ljungdahlii* in continuous culture. The principles learned in earlier batch culture studies for maximizing ethanol production (low operating pH, no yeast

extract in the medium and limited B-vitamins in the medium) were applied in these CSTR studies. A wide range of important variables are to be studied in the CSTR, including liquid flow rate, agitation rate and gas flow rate. The purpose of these parametric studies is to determine the importance of these variables on the values of cell and product concentrations, yields and productivities. This report concentrates on the effects of agitation rate on CSTR performance.

A New Brunswick Bioflo Model C30 chemostat with automatic pH control and pump modules was used for the continuous fermentation studies. The flow of synthesis gas from a high pressure cylinder into the reactor was regulated using a rotameter in conjunction with a needle valve. Liquid medium was pumped from a 13 L glass feed reservoir using a Masterflex pump equipped with an on/off timer for enhanced control of the low flow rates required. The medium flowed into the reactor (350 mL total volume) and eventually exited into a 22 L product recovery vessel. A schematic of this CSTR system was shown previously (Clausen and Gaddy, 1990).

The liquid medium employed in the studies was a basal medium described previously (PETC report 2-2-91). The gas fed to the reactor was a synthetic gas mixture containing 20 mol percent H_2, 15 percent Ar, 55 percent CO and 10 percent CO_2. Details on inoculum preparation, CSTR start-up and CSTR sampling have been shown earlier.

Results and Discussion

Fermentation profiles of cell concentration, ethanol concentration and acetate concentration as a function of time were obtained in the CSTR at each agitation rate. During these studies the agitation rate was varied between 300 and 480 rpm, the gas flow rate was held at 0.02-0.15 mmol/min and the

liquid flow rate was held constant at 200 mL/d. The resulting concentration profiles were then used to calculate cell and product yields, specific uptake rates and specific productivities as a function of time. Finally, steady state values of the concentrations, yields, rates and productivities were plotted as a function of liquid flow rate in order to observe trends in the experimental data as a function of liquid flow rate.

Figure 7 and 8 show typical cell concentration and product concentration profiles in the CSTR at increasing agitation rates from 300 to 480 rpm. Time plotted on the abscissa ranged from 500 to 2500 h, since these data are part of a longer term study considering several variables in the CSTR. As may be noted in Figure 7, the steady-state cell concentration generally increased with increasing time (agitation rate), showing a concentration of 120 mg/L at 500 h and a value of nearly 500 mg/L at 2000 h. The cell concentration dropped slightly at the end of the study, probably due to the nutrient limitation. Thus, as expected, an increase in agitation rate (shown with increasing time) brought about an increase in the steady state cell concentration through increased mass transfer. Figure 8 presents product (ethanol and acetate) concentration profiles for the same agitation rate study. The steady state ethanol concentrations were quite low until a time of 1500 h where the ethanol concentration increased dramatically with time. At a time of 2200 h (agitation rate of 460 rpm) the ethanol concentration was 3.6 g/L. The steady-state acetate concentrations increased steadily with time, up to a time of 1800 h. At this point the acetate concentration was nearly constant at 2.8 g/L.

The cell yield, $Y_{X/S}$, is shown as a function of time for the various agitation rates in Figure 9. As is noted, the yield remained essentially

constant at about 0.2-0.25 mg cells/mol substrate. In fact, it was found that the cell yield was essentially unaffected by agitation rate over the range tested in the study. Figure 10 presents the product yield from substrate, $Y_{P/S}$, as a function of time for the agitation rates. As with the cell yield, the product yield was found to be essentially independent of liquid flow rate, having a value of approximately 0.4 mol of carbon in the products per mol of carbon in the substrate.

Finally, specific uptake rates in mmol CO/mg cells hr and specific productivities in mmol product/mg cells hr are shown in Figure 11 for the agitation rates. The specific uptake rate was found to vary from 0.01 to 0.03 mmol/mg h during the study. The specific productivity increased from 0.004 to 0.007 mmol/mg•h with increasing agitation rate.

A compilation of the CSTR data as a function of liquid flow rate is presented in Figures 12-14. Figure 12 shows the effects of agitation rate on cell density achieved in the CSTR. As is noted, the cell density increased along a rather gentle curve reaching a maximum of about 500 mg/L at agitation rates of 430 and above. The cell density fell slightly at an agitation rate of 480 rpm. This leveling off and decrease in cell density is undoubtedly due to a limitation of nutrients for growth. A richer nutrient mix should result in higher cell concentrations, but could, at the same time, result in acetate production in favor of ethanol.

Figure 13 shows the effects of agitation rate on the ethanol and acetate concentrations in g/L. A low agitation rates ($\leq$ 360 rpm), the ethanol concentration remained low at a level of approximately 0.2 g/L. The corresponding acetate concentration was seen to increase over this increasing agitation rate period, reaching a concentration of 2 g/L. It is quite

possible that the agitation rate was not high enough to stress the culture with regard to dissolved CO concentration such that acetate production was favored over ethanol production.

At higher agitation rates (≥ 420 rpm), the ethanol concentration increased with agitation rate while the acetate concentration remained nearly constant. At an agitation rate of 460 rpm, the ethanol concentration was nearly 3.5 g/L and the acetate concentration was about 2.5 g/L. When the agitation rate was increased to 480 rpm, the ethanol concentration fell slightly, probably due to nutrient limitation of the culture. As was noted earlier in Figure 12, the cell concentration at 480 rpm also decreased slightly from earlier levels.

It should be noted that the higher ethanol concentrations and product ratios occurred when the culture was near nutrient limitation as indicated by the constant cell concentration in Figure 12. This follows well with previously reported information stating that culture stress could be at least partially responsible for increased ethanol production.

The specific productivity (total product) is plotted as a function of agitation rate in Figure 14. Specific productivity gently increased with agitation rate, reaching a maximum of 0.0065 mmol product/mg•h, attained at an agitation rate of 480 rpm. The more CO available to the cells, the higher the productivity. The key to continuous operation appears to be a high productivity system with high ethanol concentrations and high ethanol to acetate product ratios. Future continuous reactor studies need to concentrate on this important aspect.

THE USE OF CELL RECYCLE IN THE CSTR

A cell recycle apparatus was used in conjunction with the standard CSTR. This apparatus was described earlier (see PETC Report 2-3-91, March-June 1991), and was used as a method to increase the cell concentration inside the reactor. This is particularly important since product formation with *C. ljungdahlii* has been shown to be proportional to the cell concentration inside the reactor.

Fermentations were carried out in a 1.6 L CSTR with cell recycle. The total liquid volume in the reactor was 1.0 L, consisting of basal medium without yeast extract and one-half B-vitamins. The temperature of the reactor was held constant at 37°C and the agitation rate was 400 rpm. The gas flow rate was 16.5 mL/min and the liquid flow rate was 300 mL/d.

Figures 15 and 16 show cell concentration and product concentration profiles for the CSTR with cell recycle. In these experiments, the CO conversion was rather low at about 20%. As is shown in Figure 15, the maximum cell concentration reached was 600 mg/L (ignoring the single data point of 750 mg/L), a value that was essentially constant for nearly 150 h of fermentation time. The product concentrations, shown in Figure 16, behaved somewhat differently. At a time of 250 h (the beginning of the maximum cell concentration), the ethanol concentration was about 4 g/L and the acetate concentration was nearly 2 g/L. At a later fermentation time of 400 h, however, the ethanol concentration reached 6 g/L with a corresponding zero acetate concentration. The product ratio thus increased from 2.0 at 250 h to an infinite value at 400 h. Ethanol production as the only product has thus been shown in a continuous reaction vessel.

It should be noted that, at a time of 450 h, the cell concentration and ethanol concentration fell. It is believed that the fermentation became nutrient limited, so that nutrient addition (perhaps $(NH_4)_3PO_4$ might be beneficial to both growth and product formation. This hypothesis will be tested further in the coming months.

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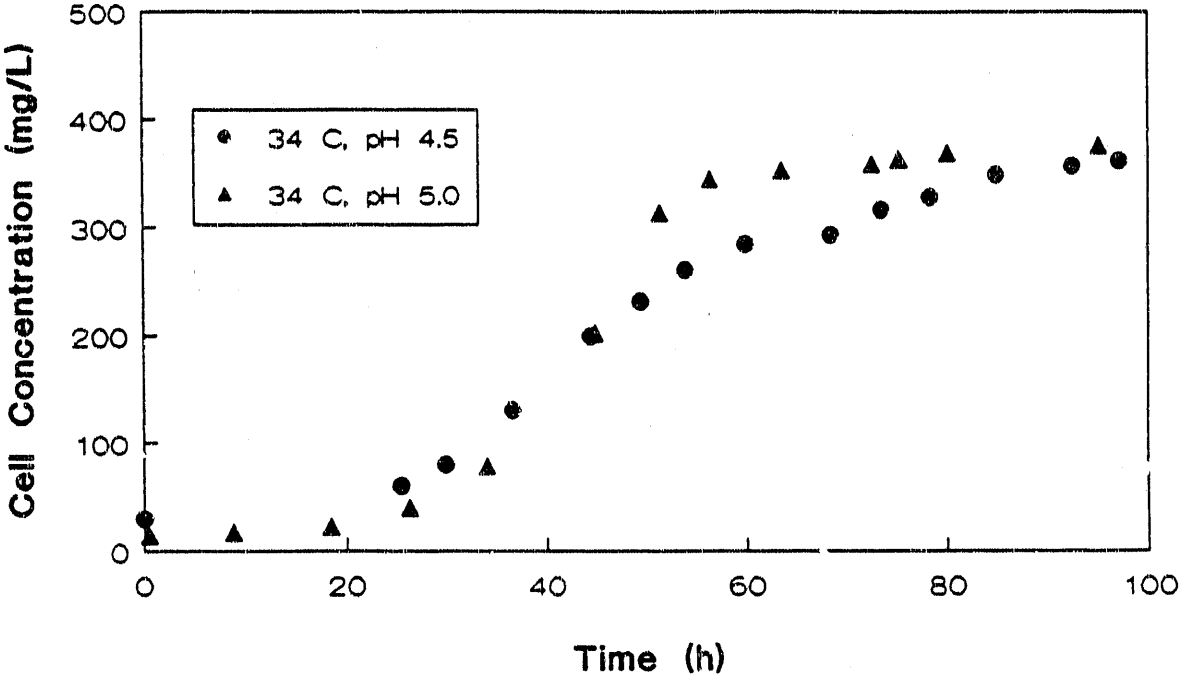

Figure 1. Cell Concentration Profiles for *C. ljungdahlii* in Batch; Basal Medium (No Yeast Extract, 50 Percent of the Standard B Vitamins) at 34 Degrees C.

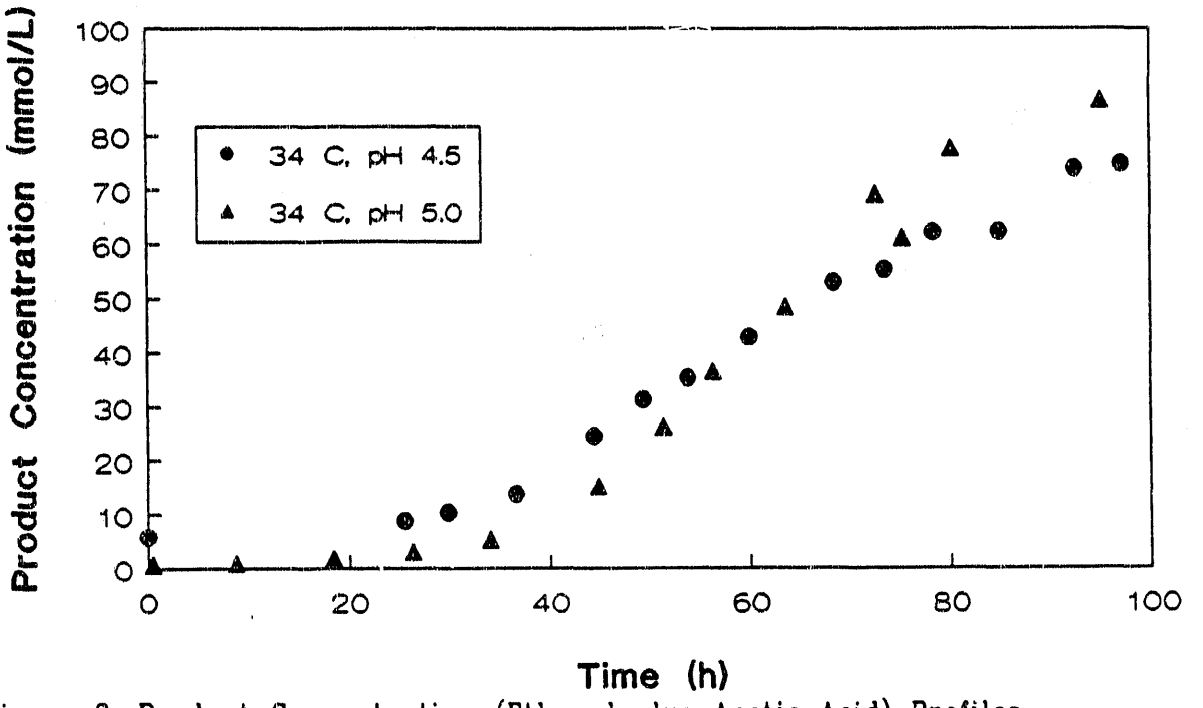

Figure 2. Product Concentration (Ethanol plus Acetic Acid) Profiles for *C. ljungdahlii* in Batch; Basal Medium (No Yeast Extract, 50 Percent of the Standard B Vitamins) at 34 Degrees C.

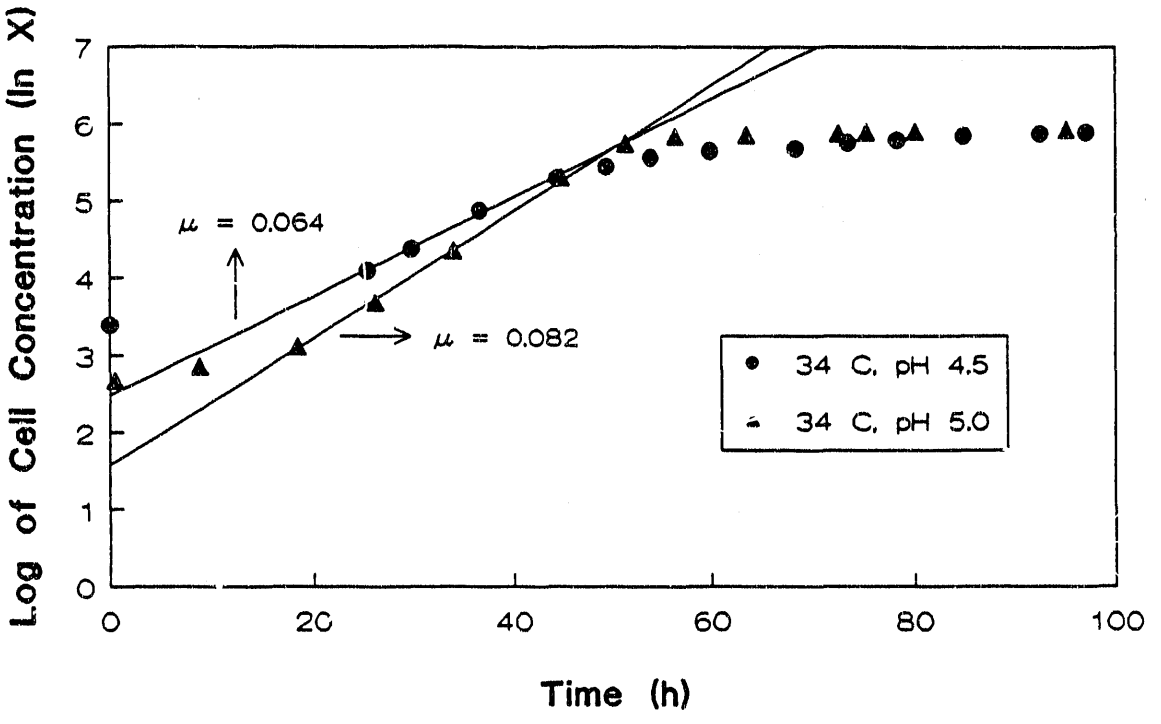

Figure 3. Determination of Specific Growth Rate (μ) During Exponential Growth of *C. ljungdahlii* in Batch Culture.

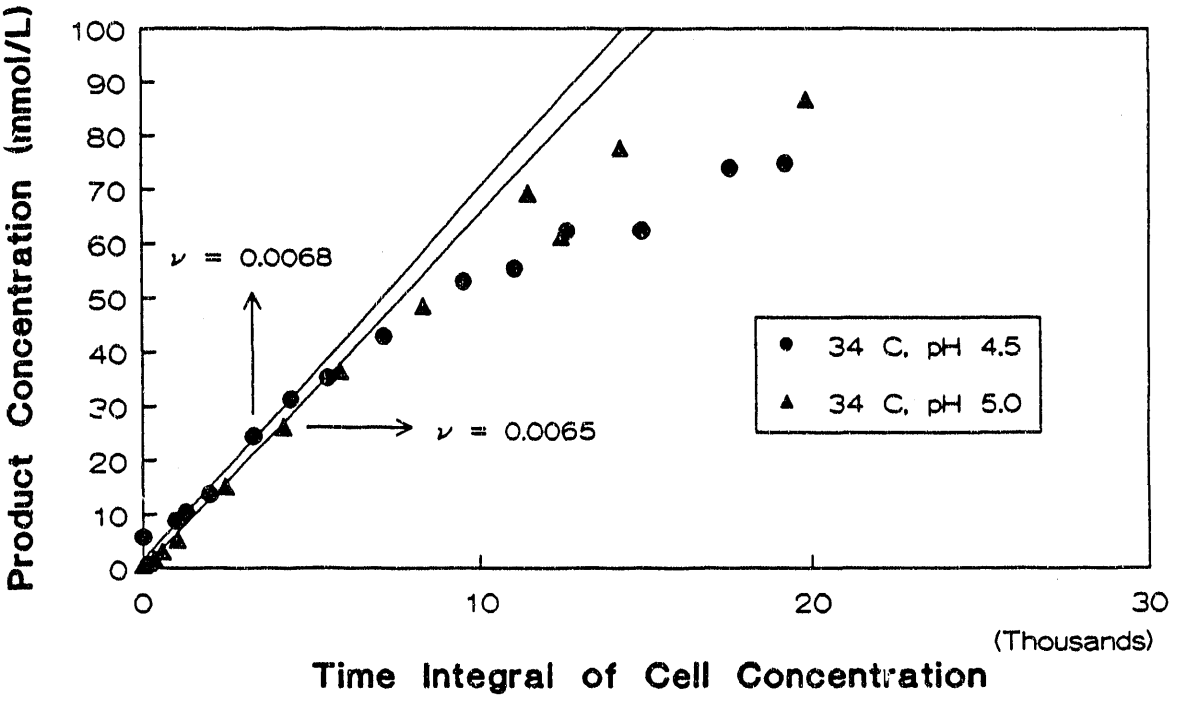

Figure 4. Determination of Specific Production Rate (ν) During Exponential Growth of *C. ljungdahlii* in Batch Culture.

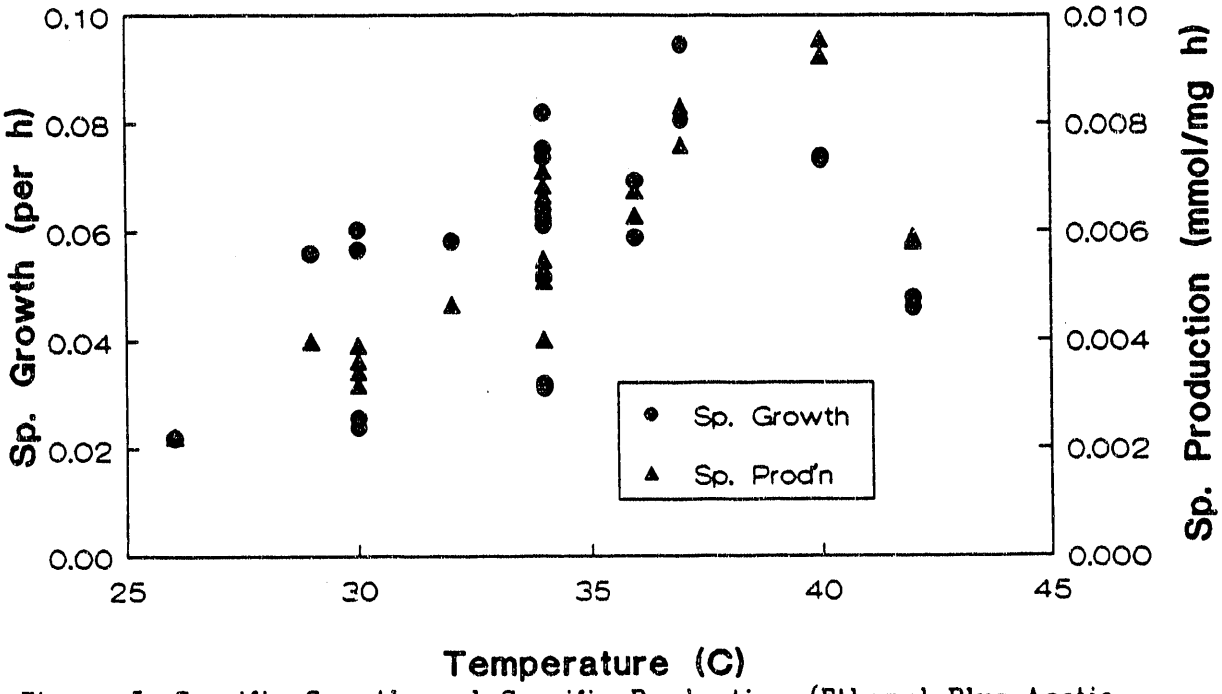

Figure 5. Specific Growth and Specific Production (Ethanol Plus Acetic Acid) Rates for *C. ljungdahlii* at Various Temperatures in Batch Culture.

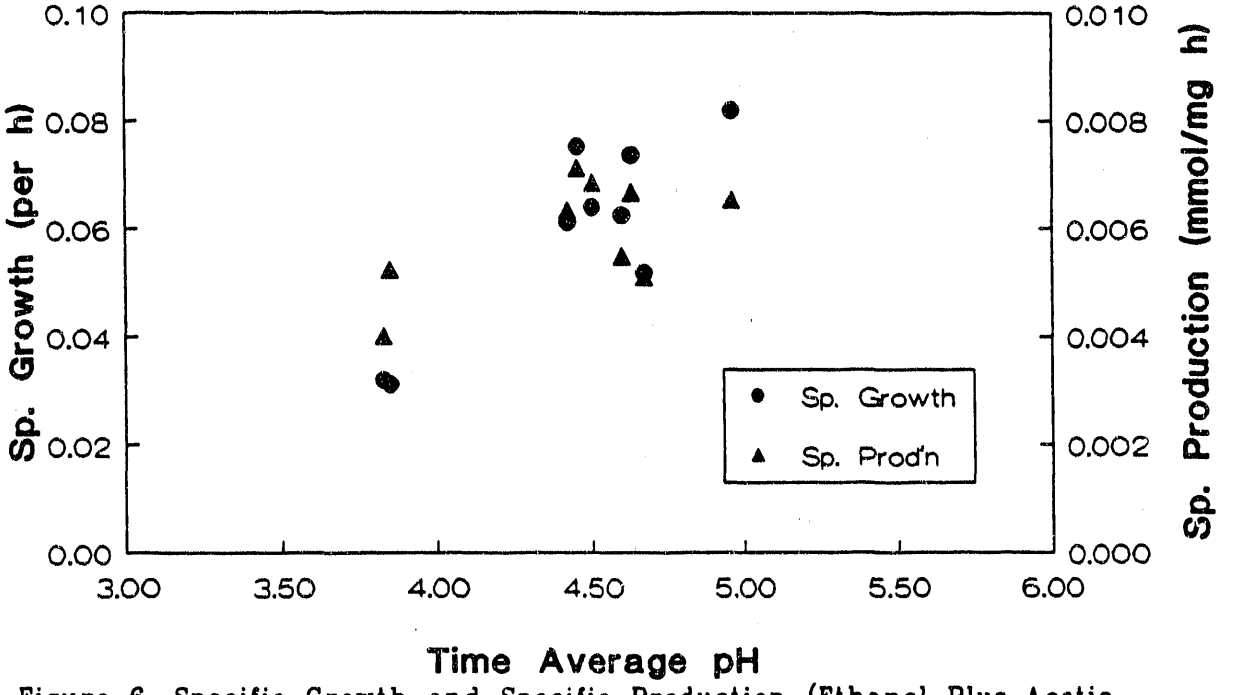

Figure 6. Specific Growth and Specific Production (Ethanol Plus Acetic Acid) Rates for *C. ljungdahlii* at Varying Average pH in Batch Culture at 34 Degrees C.

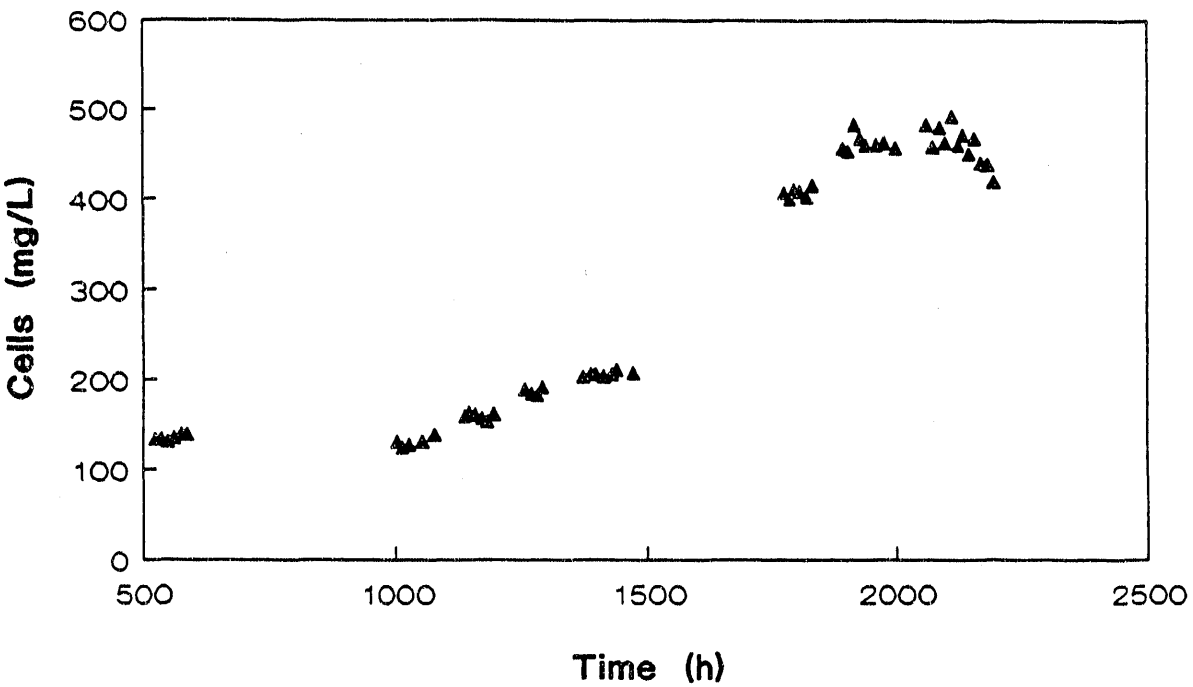

Figure 7. Cell Concentration Profile for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

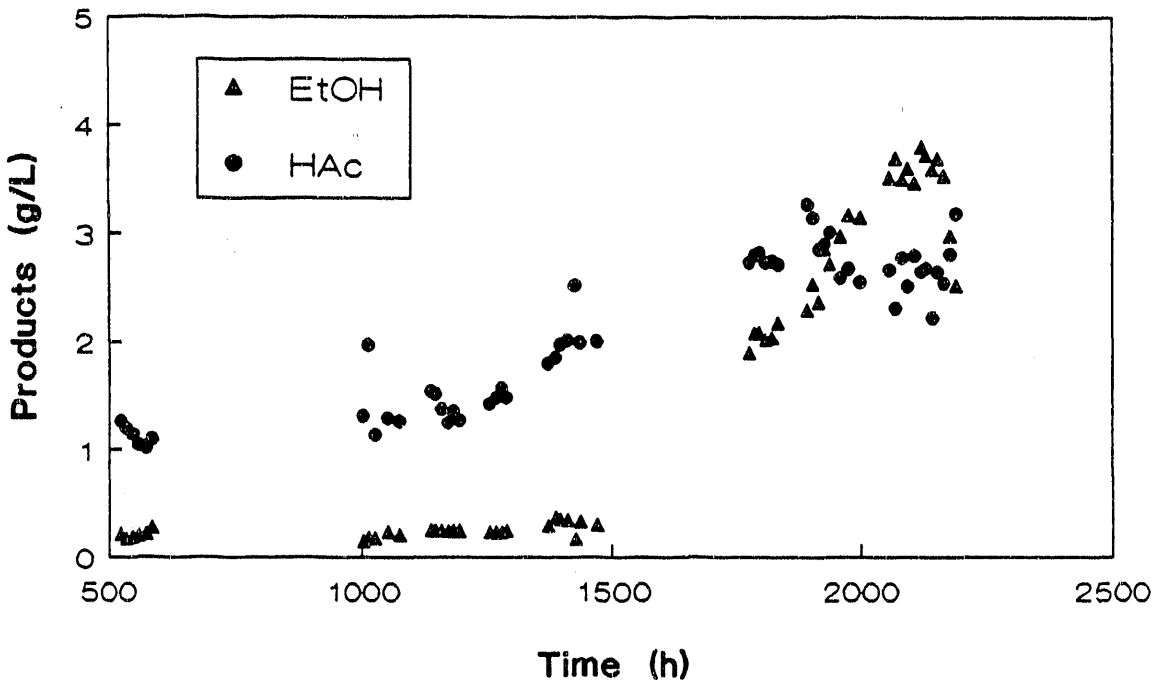

Figure 8. Product Concentration Profile for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

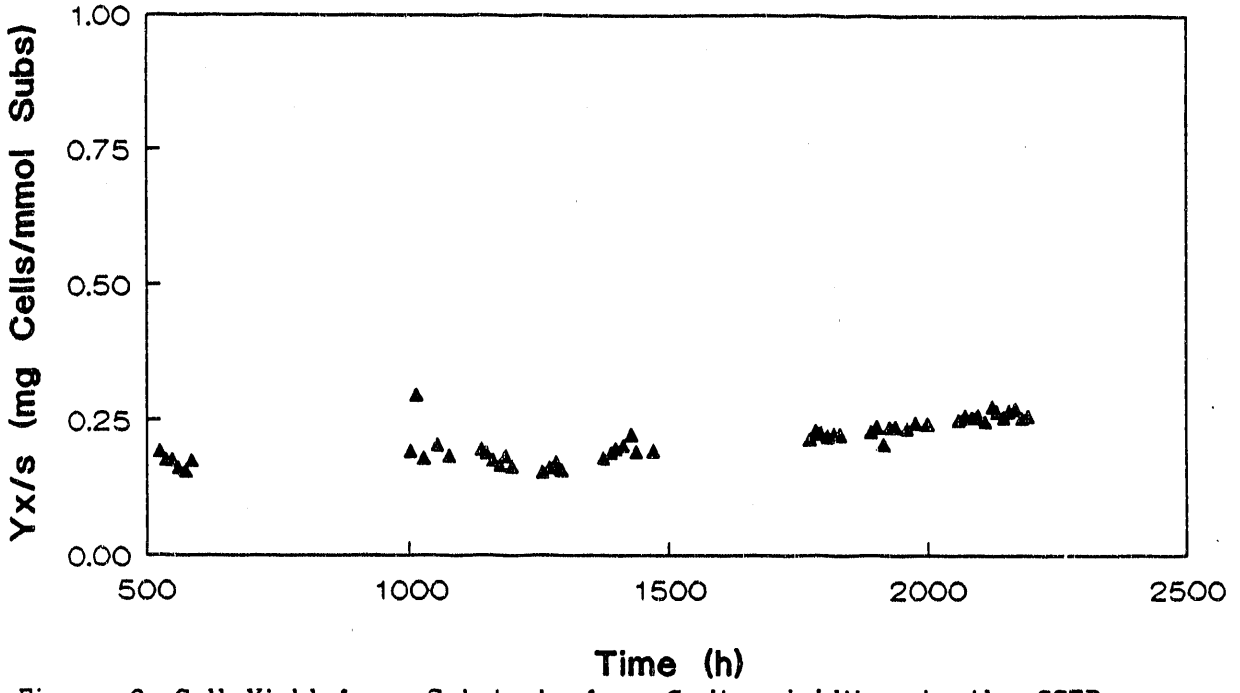

Figure 9. Cell Yield from Substrate for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

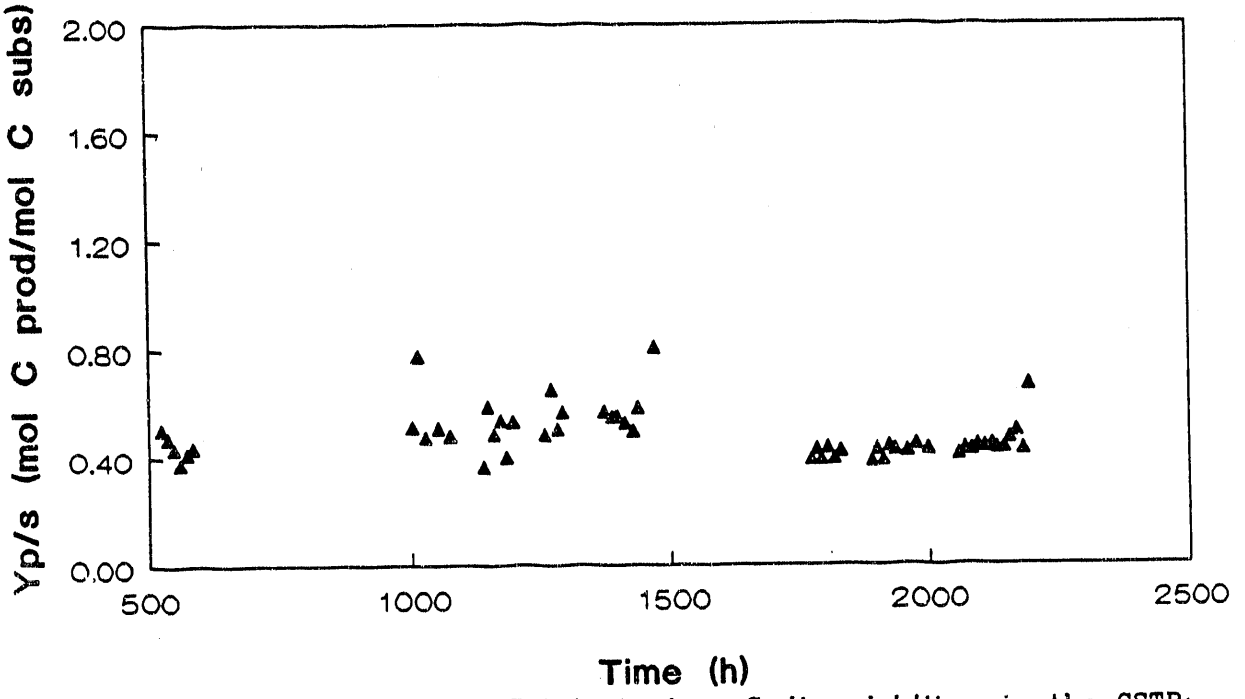

Figure 10. Product Yield from Substrate for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

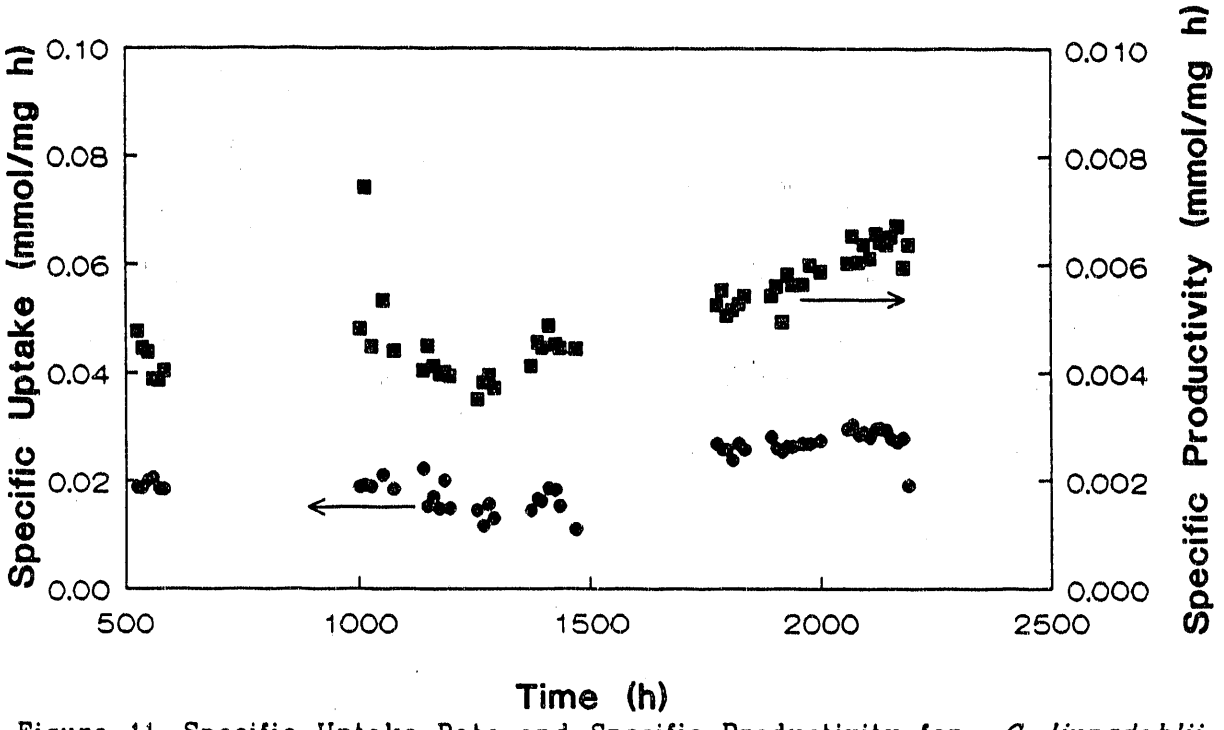

Figure 11. Specific Uptake Rate and Specific Productivity for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

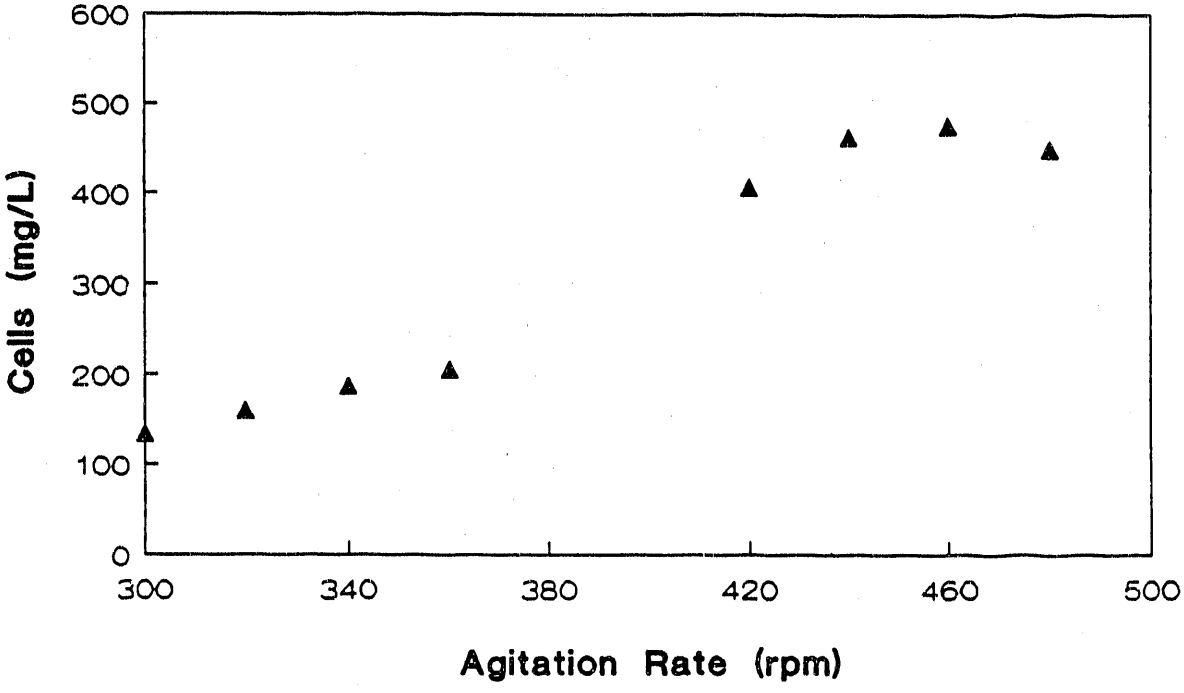

Figure 12. The Effects of Agitation Rate on Cell Concentration Using *C. ljungdahlii* in the CSTR.

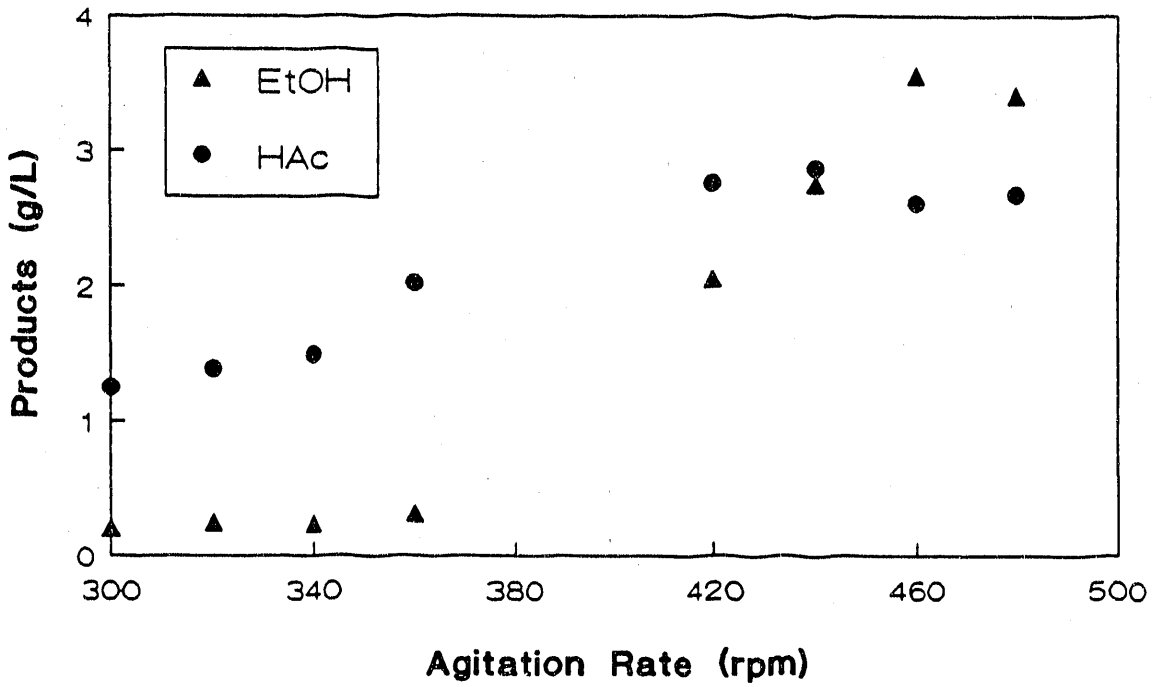

Figure 13. The Effects of Agitation Rate on Product Concentrations Using *C. ljungdahlii* in the CSTR.

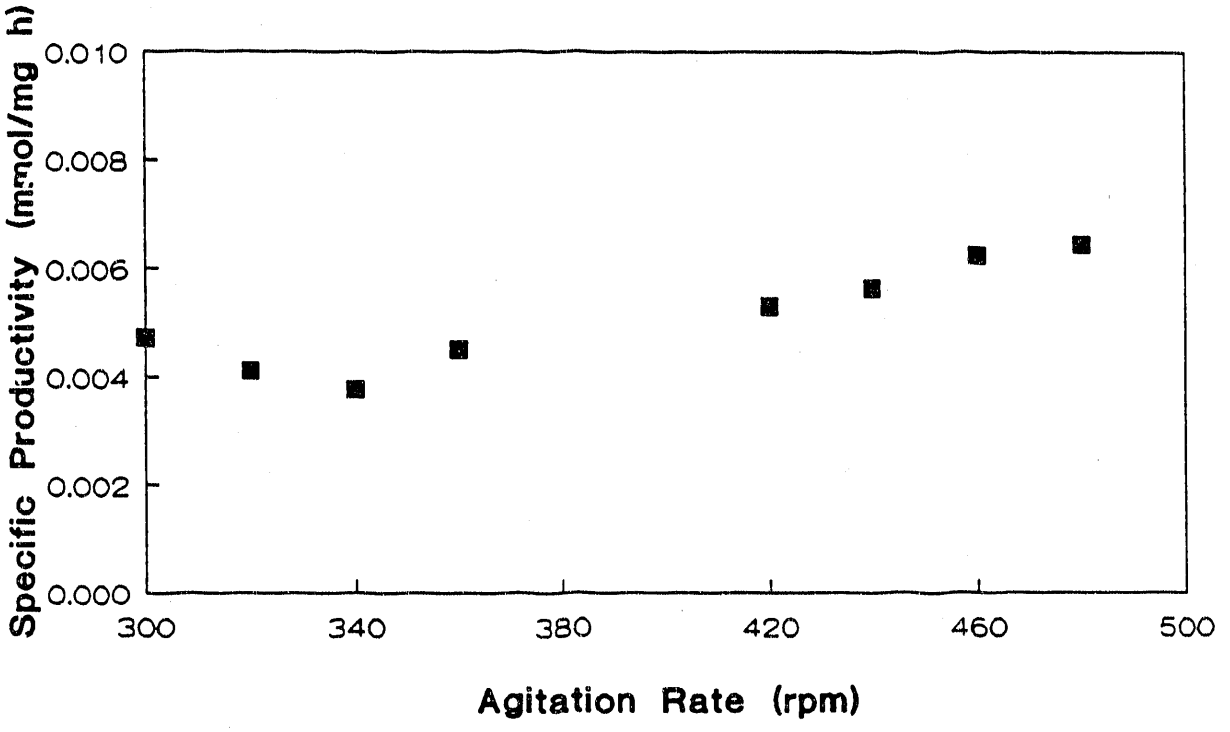

Figure 14. The Effects of Agitation Rate on Specific Productivity Using *C. ljungdahlii* in the CSTR.

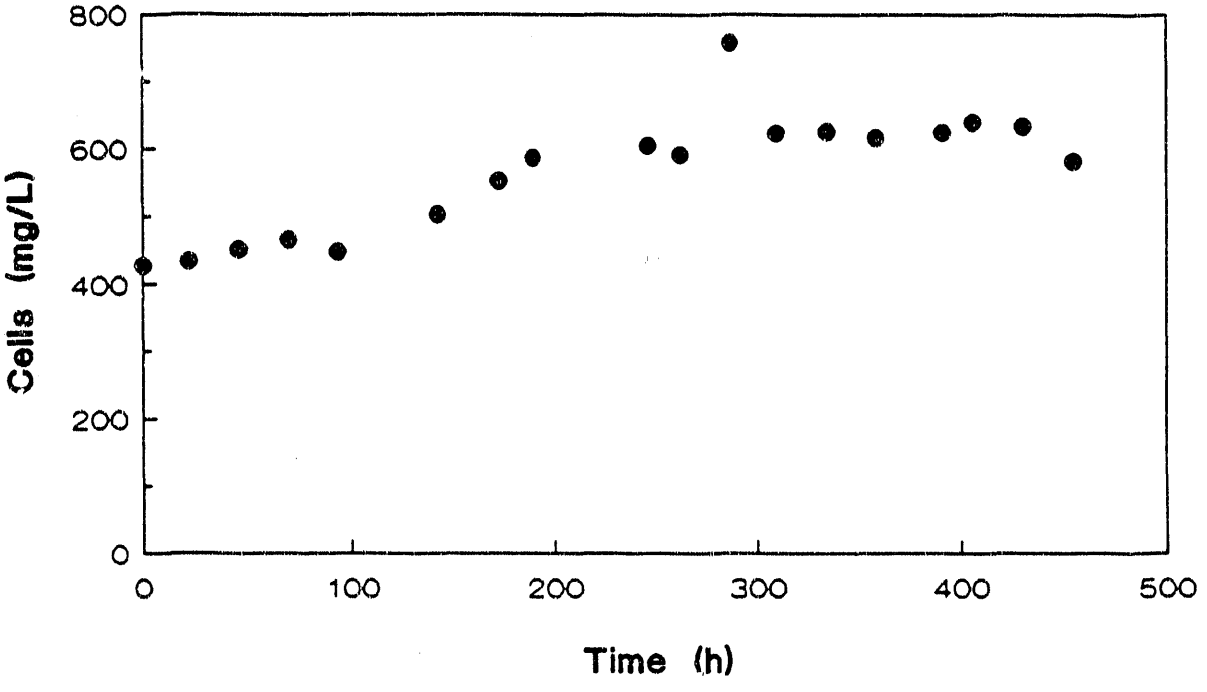

Figure 15. Cell Concentration Profile for *C. ljungdahlii* in the CSTR with Cell Recycle. (Basal Medium Contained no Yeast Extract, one half B-vitamins.)

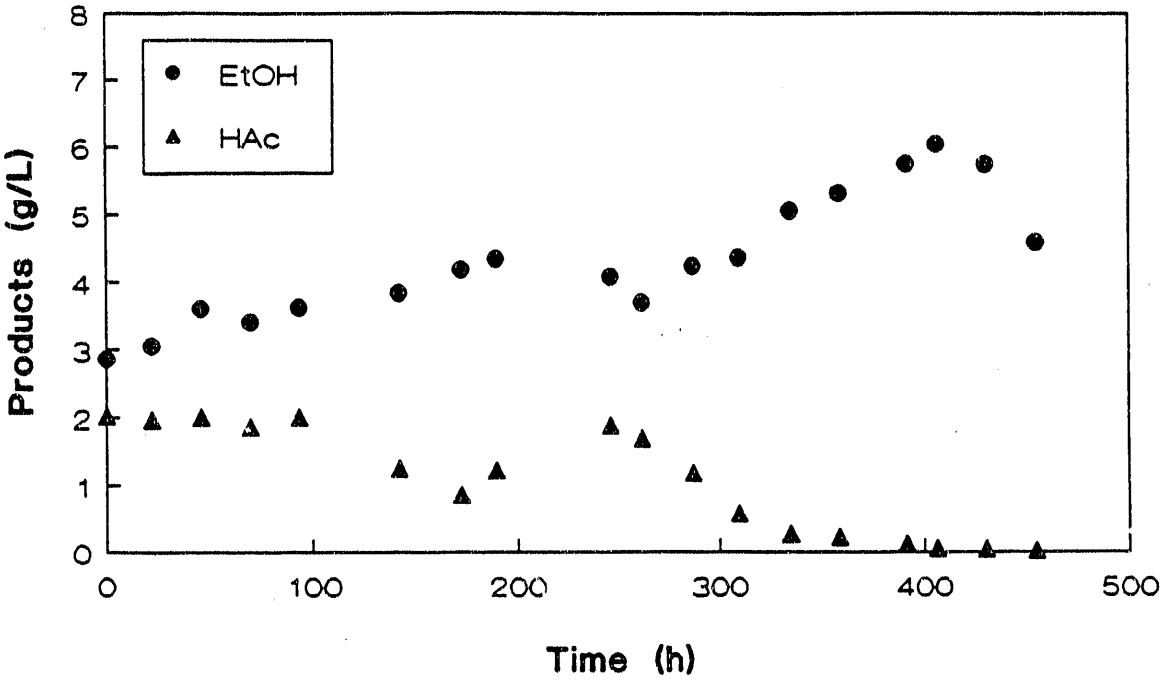

Figure 16. Product Profile for *C. ljungdahlii* in the CSTR with Cell Recycle. (Basal Medium Contained no Yeast Extract, one half B-vitamins.)

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