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Biological Production of Ethanol from Coal Synthesis Gas

Medium Development Studies

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ABSTRACT

The anaerobic bacteria *Clostridium ljungdahlii* produces ethanol and acetate from CO, CO_2, and H_2 in synthesis gas. Early studies with the bacterium showed that relatively high concentrations of ethanol could be produced by lowering the fermentation pH and eliminating yeast extract from the medium in favor of a defined medium. This article presents the results from a medium development study based on the aerobic bacterium *Escherichia coli*. The results of continuous-reactor studies in a continuously stirred tank reactor (CSTR) with and without cell recycle are shown to demonstrate the utility of this improved medium.

Index Entries: *C. ljungdahlii;* ethanol; acetate; synthesis gas; carbon monoxide; hydrogen.

INTRODUCTION

The gasification of coal produces a synthesis gas that consists of more than 50% H_2 and CO. The raw gas has a low to medium energy content, with a heating value of 160–450 BTU/SCF, depending on whether air or oxygen is used during gasification (*1*). Following quenching and purification, the synthesis gas contains 25–35% H_2, 40–65% CO, 1–20% CO_2, 0–7% CH_4, and small quantities of sulfur gases.

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CO, CO_2, and H_2 in synthesis gas may be used as substrates for the biological production of fuels and chemicals. The bacterium *Rhodospirillum rubrum* uses CO and water in producing H_2 and CO_2 (*2,3*). Bacteria, such as *Peptostreptococcus productus* (*4*) and *Eubacterium limosum* (*5*), convert CO, CO_2, and H_2 to acetate. Butanol may be produced from CO using the bacterium *Butyribacterium methylotrophicum* (*6*), and ethanol may be produced from CO, CO_2, and H_2 by *Clostridium ljungdahlii* (*7,8*).

The anaerobic bacterium *C. ljungdahlii* produces ethanol and acetate from CO, CO_2, and H_2 by the following equations (*8*):

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

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

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

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

The bacterium was isolated from animal waste, with the "wild" strain producing predominantly acetate in favor of ethanol. In fact, early studies showed an ethanol-to-acetate product ratio of 0.05 mol/mol and an ethanol concentration of <0.1 g/L (*8*). A major research effort was undertaken to improve the product ratio and increase the ethanol concentration. Studies at decreased pH (to 4.0) and with yeast extract removed from the liquid medium resulted in product ratios of 0.8, and an ethanol concentration of 1.8 g/L in a continuously stirred tank reactor (CSTR) (*9*).

The purpose of this article is to show the results of a medium constitutent study that has resulted in significant improvements in both the product ratio and ethanol concentration. A history of medium development is presented, as well as results of CSTR studies and a CSTR study with cell recycle.

MATERIALS AND METHODS

Organism and Medium

Clostridium ljungdahlii, Strain PETC, ATCC 49587, was originally isolated from chicken waste in the University of Arkansas laboratories, and later identified and characterized by R. S. Tanner, University of Oklahoma, Department of Botany and Microbiology. The stock culture was stored in a nonshaking incubator (Precision Scientific, Chicago, IL) at pH 5 and 37°C on a basal medium (shown as original medium in Table 1), and synthesis gas (65% CO, 25% H_2, and 10% CO_2 at 10 psig) and regassed every 2 wk. It was transferred to fresh medium monthly.

The working culture, which served as the seed culture for the experiments, was maintained on basal medium (without yeast extract) and

Table 1
Basal Medium Comparison (in mg/L)

	Original medium	Defined basal medium	Designed medium
Pfennig minerals			
KH_2PO_4	500	500	-
$MgCl_2 \cdot 6H_2O$	330	330	500
NaCl	400	440	200
NH_4Cl	400	400	-
$CaCl_2 \cdot 2H_2O$	50	50	200
Pfennig trace metals			
$ZnSO_4 \cdot 7H_2O$	0.1	0.1	1
$MnCl_2 \cdot 4H_2O$	0.03	0.03	0.3
H_3BO_3	0.3	0.3	3
$CoCl_2 \cdot 6H_2O$	0.2	0.2	2
$CuCl_2 \cdot H_2O$	0.01	0.01	0.1
$NiCl_2 \cdot 6H_2O$	0.02	0.02	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.03	0.03	0.3
$FeCl_2 \cdot 4H_2O$	1.5	1.5	15
Na_2SeO_3	0.01	0.01	0.1
B-vitamins			
Biotin	0.1	0.05	0.106
Folic acid	0.1	0.05	0.005
Pyridoxal-HCl	0.05	0.025	0.0025
Lipoic acid	0.3	0.15	0.015
Riboflavin	0.25	0.125	0.0125
Thiamine-HCl	0.25	0.125	0.266
Ca-D-pantothenate	0.25	0.125	0.413
Cyanocobalamin	0.25	0.125	0.0125
P-aminobenzoic acid	0.25	0.125	0.0125
Nicotinic acid	0.25	0.125	0.0125
Supplements			
Yeast extract	1000	-	-
$(NH_4)_2HPO_4$	-	-	2000
H_3PO_4	-	-	1.5
NaH_2CO_3	2500	-	-
KCl	-	-	150

synthesis gas in a shaker incubator (New Brunswick Scientific, Edison, NJ) at 36°C. Fresh gas was supplied every other day, and the culture was transferred weekly using approx 15% inoculum. The cultures were kept for 2 wk after inoculation and used as seed for experiments during the second week.

Equipment and Procedures

Batch-Culture Experiments

Medium preparation was carried out under a nitrogen atmosphere as described by Hungate (*10*) and Ljungdahl and Wiegel (*11*). Batch experiments were carried out in serum stoppered bottles with working vol of 158 mL or 1080 mL (Wheaton Scientific, Millville, NJ). After steam sterilization at 121°C for 20 min, the gas phase was replaced with synthesis gas and pressurized to 10 psig prior to reduction with 2 mL of 2.5% cysteine HCl/100 mL of medium. All experiments were performed in the shaker incubator at 36°C with an agitation rate of 150 rpm. The pH was "controlled" by a calculated addition of NaOH at sample time.

Continuous-Culture Experiments

Continuous experiments were performed in Bioflo fermenters (New Brunswick Scientific, Edison, NJ), modified for anaerobic operation. The medium was prepared in a 13.5-L Pyrex™ carboy (New Brunswick Scientific) and steam sterilized at 15 psig for 45 min. Cooling and removal of oxygen from the autoclaved medium were accomplished by bubbling sterile N_2 through the medium. The cooled medium was reduced with 10–25 mL of 2.5% cysteine-HCl/L medium. Cultures were initially grown in the fermenters as batch cultures with a continuous gas supply of H_2, CO, CO_2, and Ar (20/55/10/15%) before the liquid feed was started. Once started, the fermenters were operated with a continuous gas and liquid supply. The temperature and pH were controlled at 36°C and 4.5, respectively, and the agitation rate was varied from 300–500 rpm. For the CSTR operating with cell recycle, 90% of the liquid effluent was removed through a 0.45-μm hollow fiber filter module (AG Technology Corporation, Needham, MA), with the remainder pumped out under level control.

Analytical Procedures

Cell concentrations (in mg/L) were determined by comparing optical density readings at 580 nm in a Bausch and Lomb (Milton Roy Company, Rochester, NY) Spectronic 21 spectrophotometer with a standard calibration curve. Gas compositions were obtained by gas chromatography (Model: Sigma 300, Perkin-Elmer, Norwalk, CT) with a 1.8 m × 3 mm Carbosphere (Alltech, Deerfield, IL) 60/80 mesh, column at 100°C. Liquid analyses were performed by gas chromatography (Model: HP 5859 Series II, Hewlett-Packard, Avondale, PA) on previously acidified samples in a 1.5 m × 3 mm column packed with a Porapak QS (Alltech), 100/120 mesh column at 190°C. 1-Propanol was used as the internal standard during liquid-phase analysis after first verifying that 1-propanol was not present as a product.

RESULTS AND DISCUSSION

Medium Development

C. ljungdahlii was initially isolated using a basal medium formulated by Pfennig (*12,13*) (*see* Table 1, original medium). Growth of *C. ljungdahlii* on this basal medium gave <500 mg/L cells, with the production of <10 g/L combined ethanol and acetate with long batch fermentation times. Product formation was strongly related to growth. Thus, the successful enhancement of product concentrations was clearly dependent on increasing the cell concentration. Attempts to increase cell concentration by simply doubling the medium component concentrations resulted in severe inhibition of growth, probably because of a hypertonic solution. The nutritional requirements were subsequently evaluated using reductions in the medium component concentrations.

The reduction of the gross components of the basal medium (yeast extract, minerals, and so forth) resulted in the elimination of yeast extract as a required component, the identification of the trace metals solution as the growth-limiting component, and the reduction of the required B-vitamin concentration by a factor of six. The reduction of the B-vitamin concentration and elimination of yeast extract diminished growth potential slightly, but caused a significant increase in the ethanol-to-acetate product ratio. Furthermore, the basal medium without yeast extract is totally defined with chemical composition as given in Table 1 (*see* defined basal medium). Further studies concentrated on the individual B-vitamin requirements of *C. ljungdahlii*, and indicated that biotin, thiamine-HCl, and Ca-D-pantothenate are the only required B-vitamins.

Definition of the medium composition allowed the application of elemental mass balances to the growth of *C. ljungdahlii* for analysis of the growth potential of the basal medium and design of the production medium. These mass balances assume that the chemicals fed to the fermentation are fixed in the cells in some "stoichiometry" that defines a limiting nutrient with all other nutrients being in excess. All excess nutrients remain in the liquid medium. The stoichiometry of growth is defined by the elemental composition of the organism, as yet undetermined for *C. ljungdahlii*. The elemental composition of *Escherichia coli* as given by Bailey and Ollis (*14*) was used to evaluate the growth potential of the basal medium. *E. coli* as an aerobic and unrelated species was expected to be a poor model, but was chosen because it was conveniently available. Similar data were sought for other species of *Clostridium*, but were not found.

Growth potential of the medium was evaluated by a comparison of the elemental composition of the defined medium with the elemental composition of *E. coli* (Table 2). The maximum cell mass was calculated by assuming each element in its turn to be limiting; the lowest calculated cell

Table 2
Predicted Maximum Cell Concentration for Different Media

	E. coli % dry weight	Predicted maximum cell concentration,[a] mg/L	
		Defined basal medium	Designed medium
Element			
N	14	1014	3142
P	3	3728	15496
S	1	8948	4518
K	1	14118	7784
Na	1	24481	12334
Ca	0.5	3051	10797
Mg	0.5	7751	11839
Cl	0.5	147549	101732
Fe	0.2	207	2086
Others[b]			
Biotin		2219	4518
Thiamine-HCl		2214	4534
Ca-D-pantothenate		1416	4578

[a]Based on the elemental analysis of *E. coli*.
[b]Based on B-vitamin studies.

mass is the predicted growth potential and defines the expected limiting nutrient. Furthermore, the ratio of cell mass calculated for each element to the predicted growth potential defines the excess of a particular nutrient. The results of the authors' B-vitamin study are included in Table 2. The predicted growth potential is about half of the growth observed in the experiment (207 vs 400 mg/L), but the limiting element is predicted to be iron. Iron is added as part of the trace metals that were found to be the limiting component of the basal medium. This initial success portends the utility of the model.

An analysis of the basal medium (Table 2) reveals huge excesses of most of the elements. The *E. coli* analysis was thus used to formulate a more balanced production medium in which cations containing required nutrients were matched with nutrient-contributing anions. The resulting designed medium is defined in Table 1 and analyzed in Table 2. The particular form-ulation given is for the feed to a continuously stirred-tank reactor (CSTR) and has a predicted growth potential of 2100 mg/L cells, about 10 times the predicted growth in the basal medium. This growth is again predicted to be limited by the trace metals, reported as iron. However, as previously mentioned, the growth potential based on iron concentration in the basal medium was half of the growth observed. Thus,

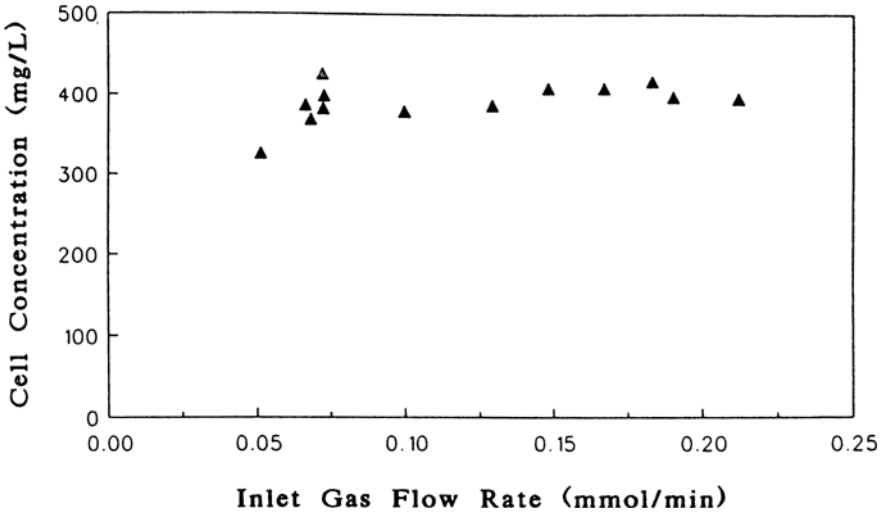

Fig. 1. Cell concentrations obtained in the CSTR using defined basal medium.

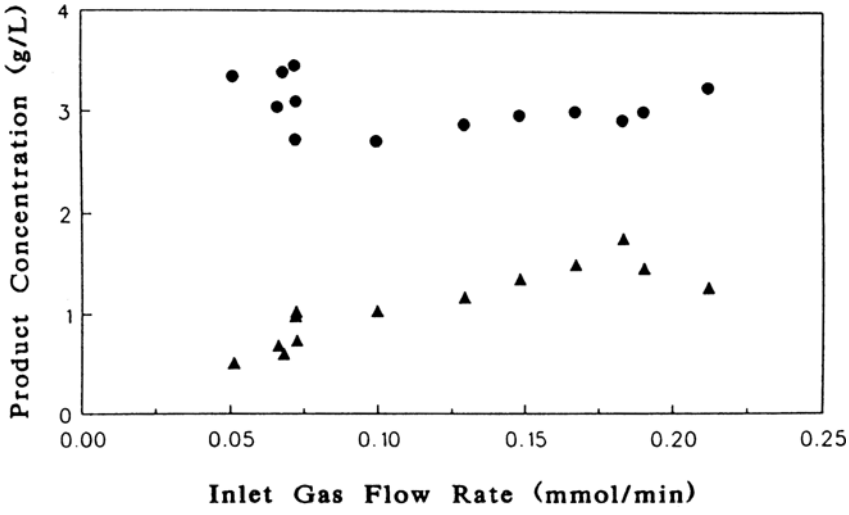

Fig. 2. Product concentrations obtained in the CSTR using defined basal medium—▲ EtOH; ● HAC.

the growth in the designed medium should be limited by nitrogen at 3100 mg/L and indeed appears to be limited by nitrogen in the CSTR.

Culture Performance

Growth, production, and the specific production rate in the CSTR are shown for defined basal medium (Figs. 1-3) and for the designed medium (Figs. 4-6). Growth in the designed medium, although falling well short

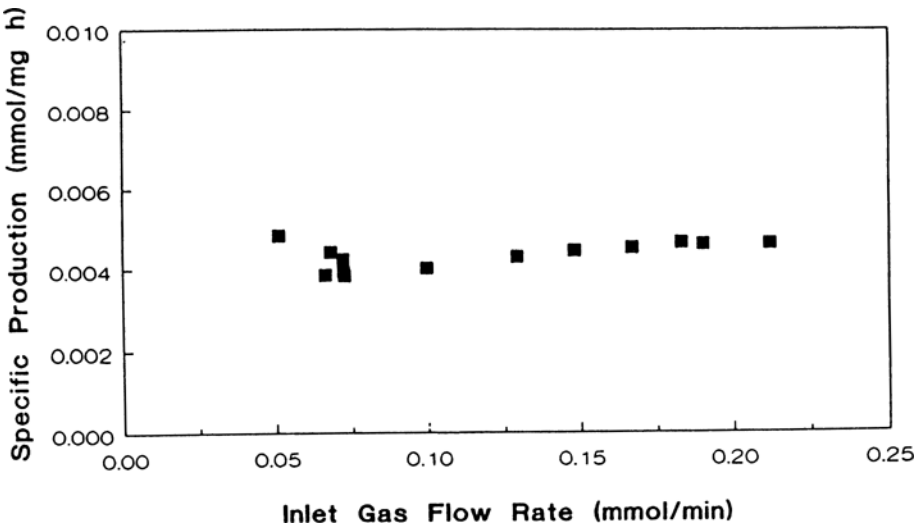

Fig. 3. Specific production rates of *C. ljungdahlii* grown on defined basal medium in the CSTR.

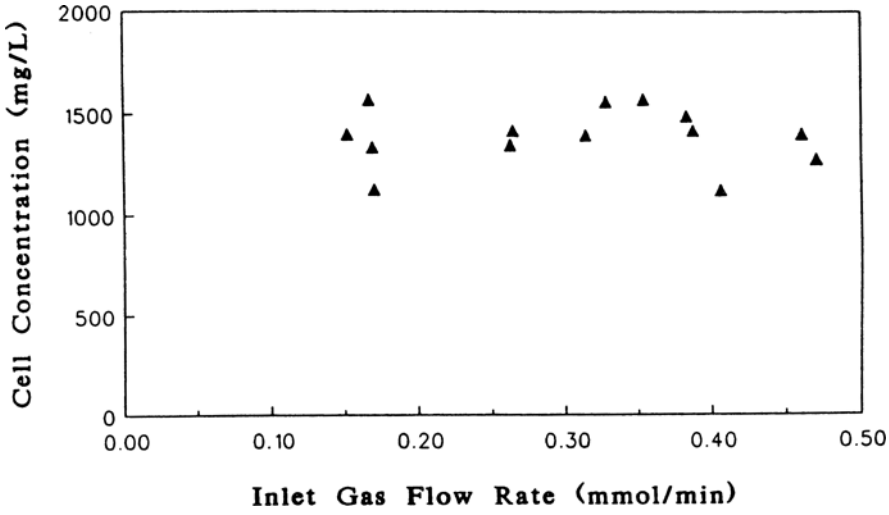

Fig. 4. Cell concentrations obtained in the CSTR using the designed medium.

of the 3100 mg/L of cells expected for nitrogen limitation, was three to four times the level achieved in basal medium (*see* Figs. 1 and 4). The maximum concentration in basal medium was 400 mg/L, and the maximum in the designed medium was 1500 mg/L. The total product concentrations in the CSTR (*see* Figs. 2 and 5) for the designed medium were 5.5 times the concentrations in basal medium on a mass basis and 6.5 times the concen-

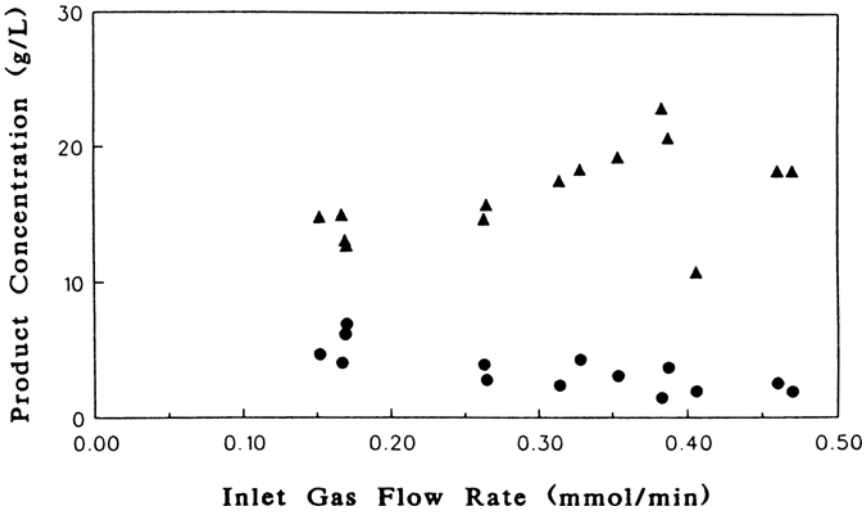

Fig. 5. Product concentrations obtained in the CSTR using the designed medium—▲ EtOH; ● HAC.

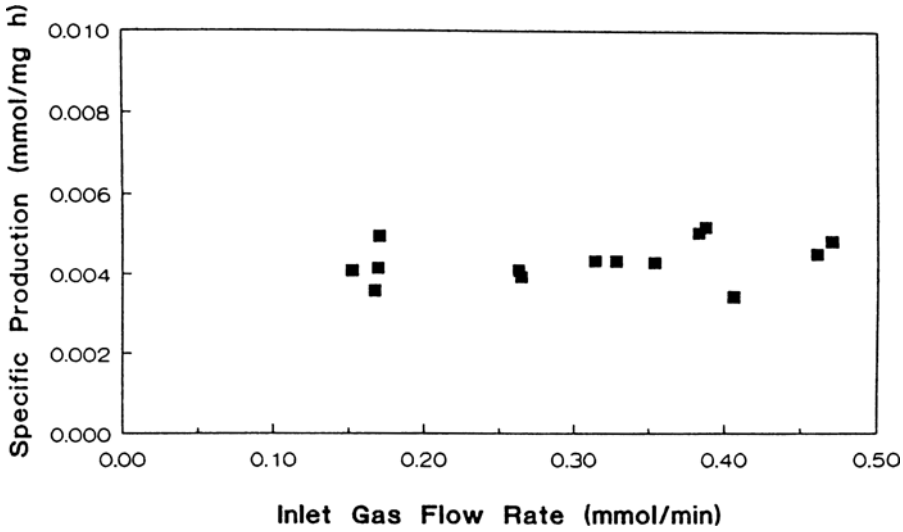

Fig. 6. Specific production rates of *C. ljungdahlii* grown on the designed medium in the CSTR.

trations on a molar basis. The maximum ethanol concentration in basal medium was about 1.5 g/L compared to about 23 g/L in the designed medium. The product ratio was also dramatically shifted toward ethanol, showing much more ethanol than acetate with the designed medium. The specific production rates, calculated as the moles of products produced per gram of cells per hour, were essentially constant and identical

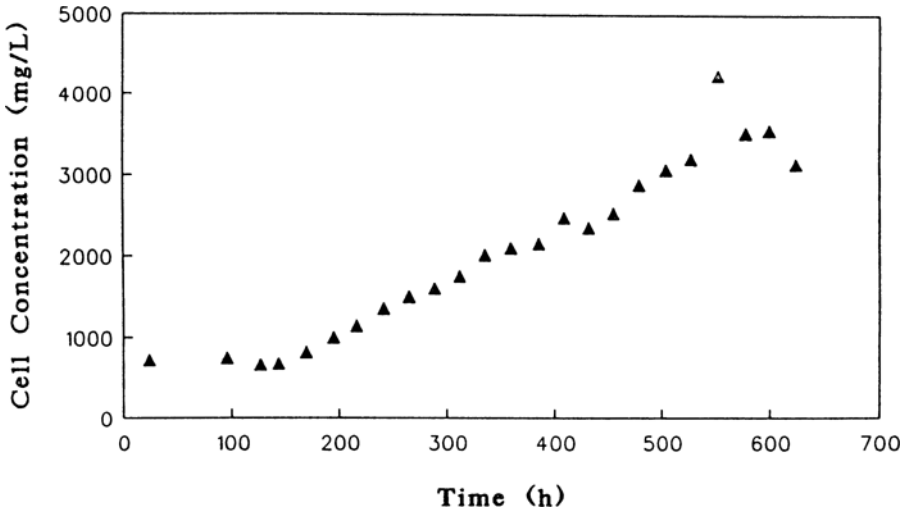

Fig. 7. Cell concentration measurements in the CSTR with cell recycle (designed medium).

at 0.004 for both media (*see* Figs. 3 and 6). This result is coincidental, because the nutrient supply and substrate gas composition can affect the culture performance. Typical specific production rates in batch culture with basal medium are near 0.01 mol/g·h with concomitant specific growth rates of 0.1 h^{-1}. The dilution rates and thus the specific growth rates in these experiments were 0.024 h^{-1} in basal medium and 0.014 h^{-1} in the designed medium (1.75 and 2.92 d liquid retention time, respectively). Growth in the defined basal medium is limited by the trace metals concentration. Ethanol has been found to be inhibitory at 20 g/L in initial growth experiments with *C. ljungdahlii;* such inhibition could be manifested as reduced performance in specific growth and production rates in the designed medium. The comparison of the media on a gross performance basis is nonetheless valid.

Studies were also carried out with *C. ljungdahlii* in the designed medium in a CSTR with cell recycle. In this study, the liquid vol was 1000 mL, the temperature was 36°C, and the pH was held constant at 4.5. The agitation rate was increased from 300 to 450 rpm, and the gas flow rate was increased from 10 to 30 mL/min during the study to accommodate cell growth in the reactor. The liquid flow rate to the cell recycle reactor was 3.5 to 12 mL/h, decreasing with time of operation.

Figure 7 represents the cell concentration measurements for the CSTR with cell recycle. As is noted, the cell concentration increased (with agitation rate and gas flow rate increases) from approx 800 mg/L to over 4000 mg/L. The maximum in the previous CSTR study without cell recycle (*see* Fig. 4) was 1500 g/L. The CO conversion, shown in Fig. 8, hovered around the 90% level after 150 h of operation. The corresponding H_2 conversion, on the other hand, averaged 70% up to a time of 500 h. At this time, the H_2 conversion fell, probably because of an accumulation of CO in the

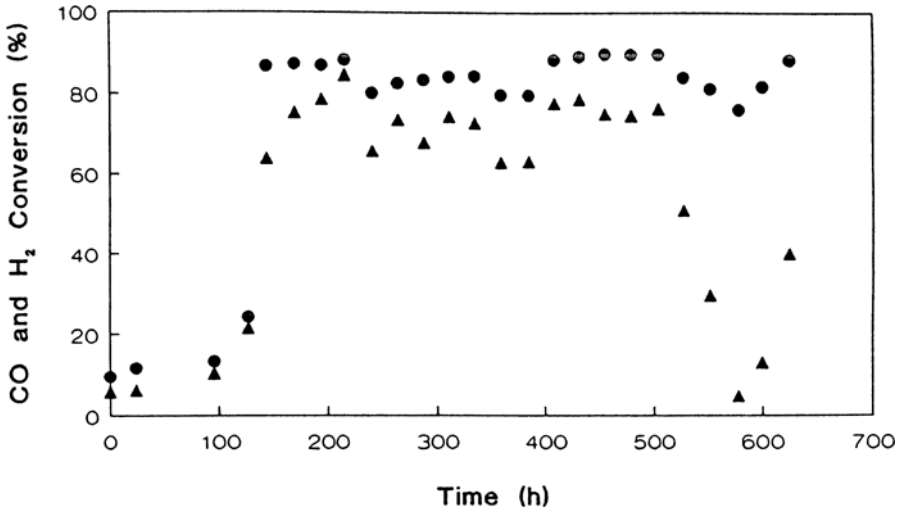

Fig. 8. ● CO and ▲ H_2 conversions in the CSTR with cell recycle (designed medium).

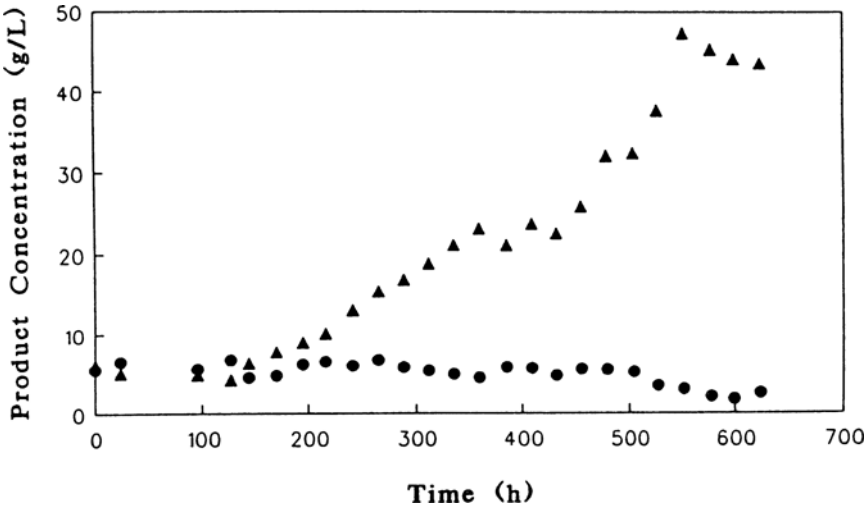

Fig. 9. Product concentration measurements in the CSTR with cell recycle (designed medium)—▲ EtOH; ● HAC.

liquid phase. It is likely that a growth limitation by a liquid constituent prompted a drop in CO conversion, resulting in CO buildup in the liquid phase and CO inhibition.

Product concentration mesurements during the study are shown in Fig. 9. The ethanol concentration ranged from 6 g/L at the beginning of the study to 48 g/L after 560 h of operation. The corresponding acetate concentrations at these times were 5 and 3 g/L, respectively. The ratio of ethanol to acetate ranged from 1.6 to 21 mol/mol. Thus, very high ethanol concentrations are possible with favorable product ratios.

CONCLUSIONS

The design of a medium based on *E. coli* was very successful despite the differences in the model organism in comparison to *C. ljungdahlii*. Cell and product concentrations in the CSTR improved from 400 mg/L cells and 5 g/L product (combined ethanol and acetate), respectively, on basal medium, to 1500 mg/L cells and 25 g/L products on the designed medium. The cell and product concentrations increased to 4000 mg/L and 50 g/L, respectively, on the designed medium with cell recycle in the CSTR. In addition, ethanol became by far the dominant product in the designed medium. The application of a model, even a poor one, can yield significant improvement in fermentation processes limited by mineral nutrients.

Clostridium ljungdahlii was shown to grow on synthesis gas with the production of high concentrations of ethanol and acetic acid. The relative amounts of ethanol and acetate can be controlled by nutritional factors, substrate gas supply, and pH. Low pH (4.0–4.5), high mass transfer of an adequate supply of substrate gas, and a minimal medium all favor ethanol production. Nutritional studies now under way still define an approximate stoichiometry for growth of *C. ljungdahlii*, and identify growth factors and inhibitors. The results of these studies will be used to review and adjust the design of the production medium further.

ACKNOWLEDGMENT

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