2

DOE/PC/89876--T17

DEC 04 1992

DOE/PC/89876--T17

DE93 008947

BIOLOGICAL PRODUCTION OF ETHANOL FROM COAL

Task 4 Report: Continuous Reactor Studies

Published: October 1992

Prepared by

University of Arkansas
Department of Chemical Engineering
Fayetteville, Arkansas 72701

Prepared for

The United States Department of Energy
Pittsburgh Energy Technology Center
Contract No. DE-AC22-89PC89876

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

ACKNOWLEDGEMENT

Financial support for this work was provided by the United States Department of Energy, Pittsburgh Energy Technology Center, on Contract No. DE-AC22-89PC89876.

1.0 INTRODUCTION

A significant research effort has occurred over the past 15 years concerning the conversion of coal to liquid fuels. Researchers at the University of Arkansas have concentrated on a biological approach to coal liquefaction, starting with coal-derived synthesis gas as the raw material. Synthesis gas, a mixture of CO, H_2, CO_2, CH_4 and sulfur gases, is first produced using traditional gasification techniques. The CO, CO_2 and H_2 are then converted to ethanol using a bacterial culture of *Clostridium ljungdahlii*.

C. ljungdahlii converts CO, CO_2 and H_2 to ethanol and acetate by the equations:

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \quad (1.1)$$

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \quad (1.2)$$

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ H_2O \quad (1.3)$$

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (1.4)$$

In these equations, ethanol is the desired product if the resultant product stream is to be used as a liquid fuel. However, under normal operating conditions, acetate is produced in favor of ethanol in conjunction with growth in a 20:1 molar ratio. Thus, research needs to be conducted to determine the conditions necessary to maximize not only the ratio of ethanol to acetate, but also to maximize the concentration of ethanol resulting in the product stream.

Significant progress has been made toward reaching this goal.[1] Several factors have been identified that cause the culture to produce ethanol in favor of acetate. These factors include manipulation of the nutrients composition and concentration, the addition of reducing agents or sporulating agents to liquid medium and reduction of the medium pH. Perhaps the most

variable in maximizing both the product ratio of ethanol to acetate and the ethanol concentration is the use of a minimal nutrient medium in conjunction with a low operating pH. Ethanol to acetate product ratios of over 100 (essentially no acetate) have been obtained with this technique. At the same time, ethanol concentrations as high as 47 g/L have been obtained.

The purpose of this report is to present results from continuous reactors used in producing ethanol from synthesis gas. The continuous stirred tank reactor (CSTR), the CSTR with cell recycle and the trickle bed reactor have been used for this purpose. In this report, comparisons are made between the results obtained from the three reactors, along with recommendations for reactor design with this bacterium.

2.0 BACKGROUND ON CLOSTRIDIUM LJUNGDAHLII

In 1987, researchers at the University of Arkansas reported the isolation of a strictly anaerobic mesophilic bacterium from animal waste that was capable of converting CO, H_2 and CO_2 to a mixture of acetate and ethanol.[2] This discovery was the result of extensive screening studies utilizing natural inocula, such as sewage sludge, animal waste, coal pile runoff and soils. The method of successive transfer was applied, while simultaneously blocking methanogenesis.

Early batch fermentation experiments with the newly isolated microorganism, tentatively identified as a member of the *Clostridium* genera, did not show a high degree of reproducibility. Although product ratios of 1 mole ethanol to 10 moles of acetate (1:10) were sometimes obtained, the standard product ratio in these early studies was 1:20. Ethanol concentrations were also quite low, although the concentration could be increased in batch culture through multiple gas additions. Clearly, an

experimental program was needed to increase the product ratio and concentration of ethanol while, at the same time, carrying out parallel studies on culture identification and process stoichiometry.

An experimental program was developed early to identify and characterize the bacterium, evaluate overall culture stoichiometry, improve the ethanol/acetate ratio and optimize culture operating conditions. Highlights of this work include improving the product ratio from 1:20 (ethanol to acetate) to 3:1, and improving the specific ethanol productivity of the culture by 800 percent. These and other results are summarized in the paragraphs below.

2.1 Culture Isolation and Characterization

Bacterial isolation and characterization studies showed that the culture is a new clostridial species, named *Clostridium ljungdahlii*, Strain PETC, in honor of Dr. Lars G. Ljungdahl. *C. ljundahlii* is a gram-positive, motile, rod-shaped anaerobic bacterium which sporulates infrequently. The bacterium only produces ethanol at pH levels below 6.0. At higher pH levels, acetate is the only liquid phase product.

A list of substrates tested for growth as the sole carbon and energy source is shown in Table 2.1. In these studies, 1 g/L of yeast extract was added to the medium along with the substrate, and all fermentations were carried out at an initial pH of 6.0. As noted in Table 2.1, growth was obtained when H_2/CO_2, ethanol, pyruvate, xylose, arabinose and fructose were used as substrates. *C. ljungdahlii* grew weakly in the presence of fumarate, ribose, glucose and casamino acids.

Table 2.1

Growth of C. ljungdahlii on Substrates as the Sole Carbon/Energy Source

$H_2:CO_2$	+	ribose	w
CO	+	xylose	+
formate	-	arabinose	+
methanol	-	fructose	+
ethanol	+	glucose	w
pyruvate	+	galactose	-
lactate	-	mannose	-
glycerol	-	sorbitol	-
succinate	-	sucrose	-
fumarate	w	maltose	-
citrate	-	lactose	-
		starch	-
		casamino acids	w

+ positive growth
- no growth
w weak growth

C. ljungdahlii is distinguishable from related organisms by its substrate use, pH requirements, end-products of fermentation and physical characteristics. It can be distinguished from *Clostridium aceticum* by its pH optimum, and the utilization of glucose, formate, ribose and xylose as substrates. The bacterium can be distinguished from *Clostridium barkeri* by the end-products of fermentation, motility, and the utilization of glucose, lactate and xylose as substrates. *C. ljungdahlii* is distinguishable from *Clostridium formicoaceticum* by the utilization of glucose, lactate, ribose and H_2/CO_2 as substrates, and is distinguished from *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* by thermophilicity and the utilization of glucose as a substrate.

The bacterium can also be differentiated from other acetate forming species. It is distinguished from *Ace bacterium* sp. by spore formation and the utilization of formate, lactate and pentoses as substrates. It is distinguished from *Eubacterium limosum* by motility, spore formation, and the utilization of glucose, lactate, methanol, ethanol and arabinose as substrates.

Figure 2.1 shows an electron micrograph of *C. ljungdahlii*, Strain PETC. As is seen in the micrograph, the cells are petrichously flagellated. Also, there is good evidence for an internal membrane structure, which is visible both in fixed and unfixed preparations and in fixed and unfixed cells stained with uranyl acetate or phosphotungstic acid. The membranes are also seen in different culture preparations. These internal structures, which have not been observed in clostridial species before and which usually require thin sectioning to visualize in any eubacteria, may explain how the organism can metabolize in the presence of acetic acid at pH 4.

DNA composition studies have shown that *C. ljungdahlii* contains 22 mole percent guanine plus cytosine. This composition is at the lowest end of bacteria, with only one other clostridia having a composition this low. This result further substantiates the fact that *C. ljundahlii*, Strain PETC is a new clostridial species.

2.2 Evaluation of Reaction Stoichiometry

The stoichiometry for the formation of acetate from carbon monoxide, as well as hydrogen and carbon dioxide, has been well-established for many acetogenic bacteria.[3] These reactions are:

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (1.1)$$

$$\Delta G^\circ = -37.8\ \text{kcal/gmole}\ CH_3COOH$$

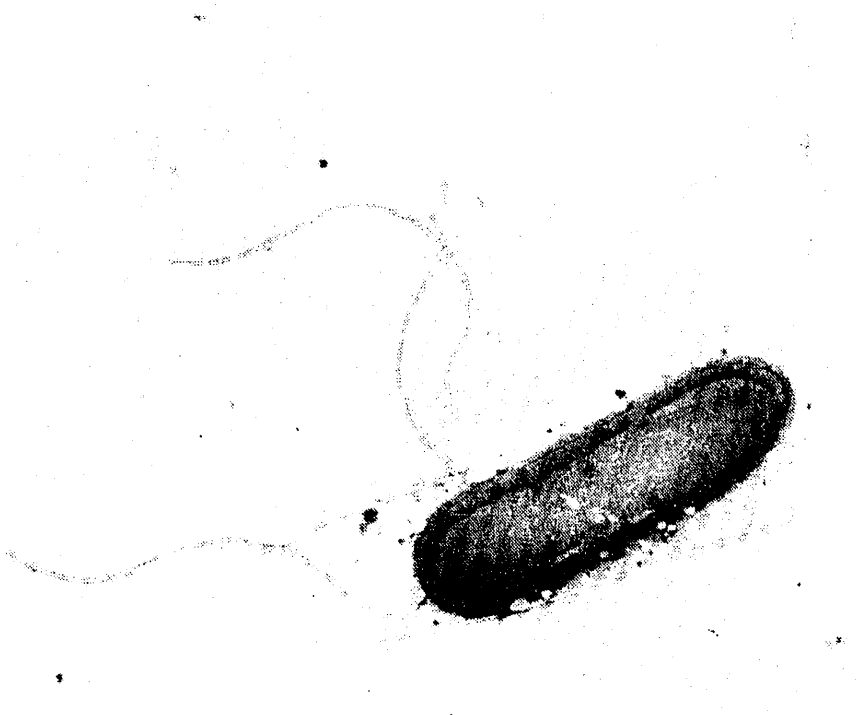


Figure 2.1. Electron micrograph of *Clostridium ljungdahlii*, strain PETC.

and

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (1.2)$$

$$\Delta G^\circ = -18.6 \text{ kcal/gmole } CH_3COOH$$

Similar equations may be written for the formation of ethanol from CO, CO_2 and H_2:

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \qquad (1.3)$$

$$\Delta G^\circ = -59.9 \text{ kcal/gmole } C_2H_5OH$$

and

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \qquad (1.4)$$

$$\Delta G^\circ = -23.2 \text{ kcal/gmole } C_2H_5OH$$

Experiments were carried out in the University of Arkansas laboratories to verify the stoichiometries of Equations (1.1-1.4) for *C. ljungdahlii*.[4] Both single substrate (CO or CO_2/H_2) studies and mixed substrate (CO, CO_2 and H_2) studies were conducted. The results of these studies indicated that stoichiometric quantities of acetate and ethanol were produced from CO, CO_2 and H_2 as indicated in Equations (1.1-1.4), thus verifying the stoichiometric relationships.

3.0 ELIMINATION OF ACETATE AS A BY-PRODUCT IN CLOSTRIDIAL FERMENTATION

3.1 Biochemistry and Energetics of Clostridia

As mentioned previously, the production of ethanol by *C. ljungdahlii* is accompanied by the production of acetate, an undesirable by-product if a fuel is sought. Although *C. ljungdahlii* is the first organism to show the production of ethanol from CO, H_2 and CO_2, several other clostridial species are known to manufacture ethanol from sugars.[5] *C. thermocellum*, *C. thermohydrosulfuricum* and *C. saccharolyticum* have been shown to produce ethanol from complex carbohydrates such as cellulose and starch, and from

simple sugars such as glucose. In all three cases, however, acetic acid and sometimes lactic acid are always produced as by-products of the fermentation. It is very likely that similar biochemical pathways exist for these bacteria and *C. ljungdahlii*, thus providing information on the methanisms that lead to the production of ethanol and acetate.

The ethanologenic clostridia convert sugars to pyruvate via the fructose-biphosphate pathway producing two moles of ATP and two moles of NADH per mole of hexose, as shown in Figure 3.1. The majority of the pyruvate is converted to acetyl-CoA with small amounts going to lactate or CO_2 and H_2. Acetyl-CoA can then be reduced to acetaldehyde and then to ethanol, or it can be converted into acetate with stoichiometric production of ATP.

The pathway utilized by clostridial species and other acetogenic bacteria to autotrophically grow on H_2/CO_2 and CO has been recently established by Wood *et al.*[6] in studies conducted with *C. thermoaceticum*, and has been termed "the acetyl-CoA pathway". Evidence is accumulating that this pathway is utilized by other bacteria that grow with CO_2 and H_2 as the sources of carbon and energy. This group includes all of the acetogenic bacteria, the methane producing bacteria and the sulfate-reducing bacteria. The mechanism involves the reduction of one molecule of CO_2 to a methyl group and then its combination with a second molecule of CO_2 (or a molecule of CO) and CoA to form acetyl-CoA. The reduction of CO_2 to a methyl group in the tetrahydrofolate pathway requires one molecule of ATP and one molecule of NADH per molecule of CO_2 reduced. Also, it is important to note that the conversion of acetyl-CoA to acetate is the only source of substrate level phosphorylation in the acetogenic clostridia during unicarbonotrophic growth.[7]

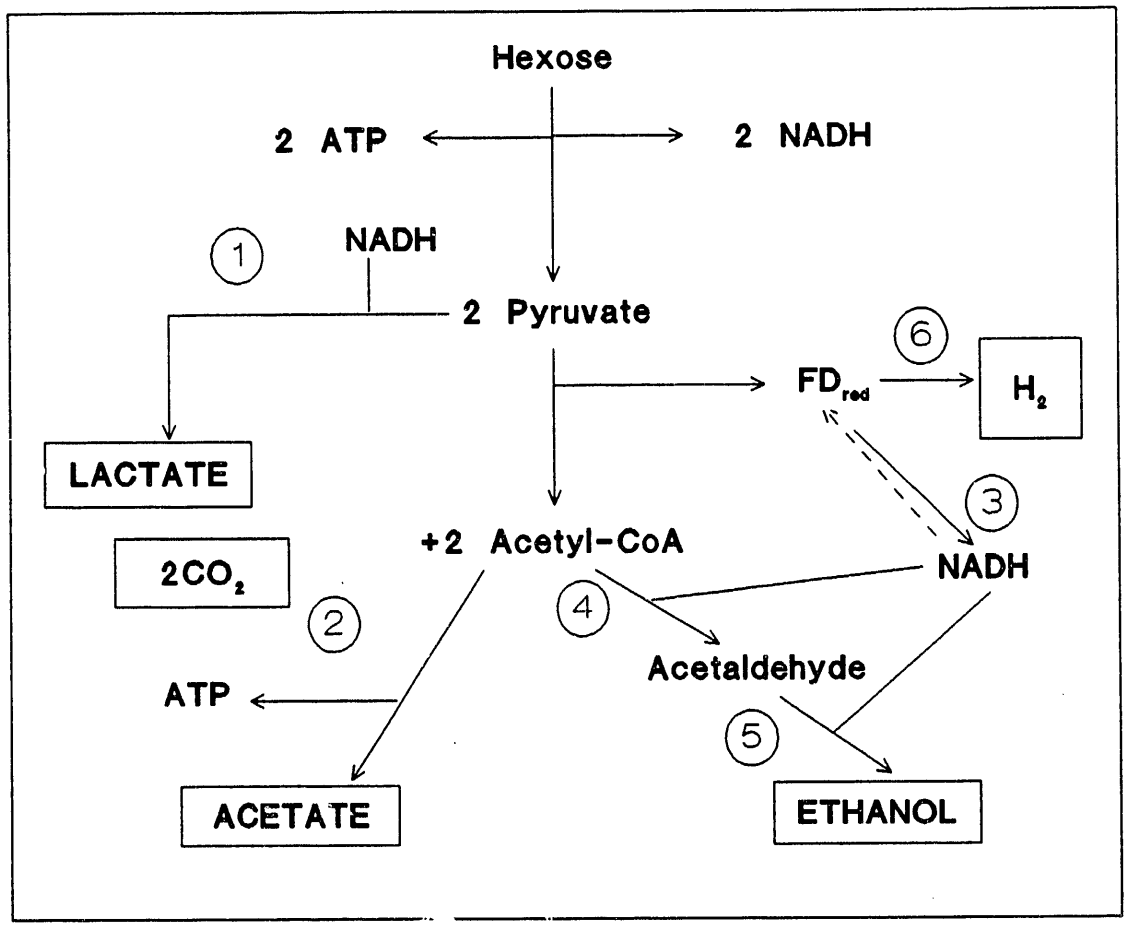


Figure 3.1. Clostridial ethanolic fermentations. Numbers indicate enzymes: (1) Lactate dehydrogenase; (2) Phosphotransacetylase + acetate kinase; (3) NADH ferredosin oxidoreductase; (4) Acetaldehyde dehydrogenase; (5) Ethanol dehydrogenase; (6) Hydrogenase.

When terminating in acetate, the pathway is balanced in ATP and the production of ethanol or other end-products would result in a net consumption of ATP which would not support growth of the bacteria.

The high growth yields of homoacetogenic bacteria on sugars and the discovery of a large variety of electron transport proteins have provided the first clues to indicate that in clostridial species, ATP may be generated by a proton gradient mechanism and electron coupled phosphorylation. It has been suggested that the generation of the proton gradient may be caused by the free flow of H_2 through the cyloplasmic membrane and the presence of membrane bound hydrogenase.[7] In view of these findings, it is possible to expect that end products other than acetate (such as ethanol) may be manufactured in microcarbonotrophic growth of clostridial species. The understanding of the basic energetics supporting growth and metabolism is fundamental to the development of products other than acetate in clostridial fermentations.

3.2 Elimination of Acetate as a Product

Clostridial fermentations yield a wide variety of end-products which include two to five major acids and/or solvents as well as gaseous products such as H_2 and CO_2. The amount of reduced versus neutral and oxidized products is always balanced with the amount of H_2 and also ATP produced and has the potential of a great deal of natural variation. 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. Some of these strategies have been investigated with *C. ljungdahlii* and the results are summarized below.

3.2.1 Control of Growth Rate Parameters

Studies in the acetone-butanol fermentation have shown that high solvent yields depend upon adjusting growth-limiting factors such as phosphate, nitrogen, etc. in a range which allows some growth but not under optimum conditions.[8,9,10] Solventogenesis, then, is apparently a metabolic response to a condition of unbalanced growth where the utilizable energy source remains in excess but growth is restricted by other limiting factors or growth inhibitors. Still, the molecular changes that trigger the formation of solvents due to growth restriction are unknown.

Early in the studies conducted with *C. ljungdahlii*, the importance of the yeast extract concentration employed in the medium on the ratio of ethanol to acetate obtained during a fermentation was recognized. In a typical batch fermentation, the production of ethanol occurs simultaneously and a rather constant ratio of products is maintained along the fermentation. The ratio of ethanol to acetate, however, is highly dependent upon the yeast extract concentration as is shown in Figure 3.2 for higher levels of yeast extract. Unfortunately, the bacteria have adapted to lower levels of yeast extract and the effect observed in batch culture has not proven useful in continuous experiments.

While growth control in continuous culture has not been successful through nutrient limitation strategies, the rate of growth of bacteria in chemostats can be easily controlled by adjusting the dilution rate. At steady-state, in a continuous stirred-tank reactor that is fed a cell-free solution, it is necessary that the specific growth rate equals the dilution rate.[11] Therefore, low growth rates can be achieved by using low dilution rates. The molar product ratio of ethanol to acetate achieved at various

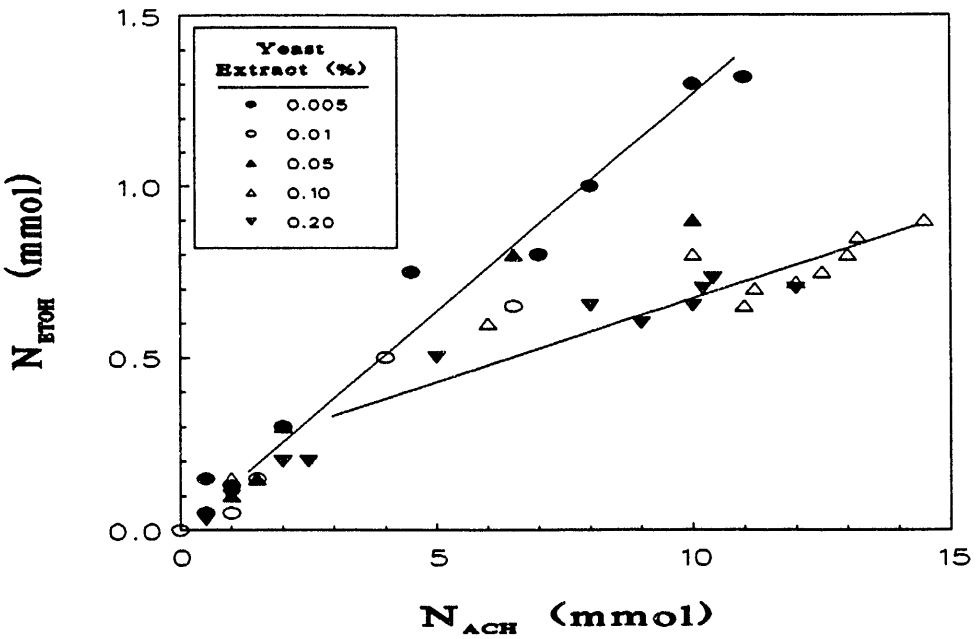


Figure 3.2. Effect of yeast extract concentratoin on the product ratio for *C. ljungdahlii*.

liquid flow rates in a New Brunswick Bioflo C.30 chemostat specially adapted for anaerobic operation is shown in Figure 3.3. As is observed, when all other variables are held constant, the lower dilution rates yielded the higher ratios. Values of 0.6-0.7 mol of ethanol/mol of acetate were maintained for over 20 days.

Better product ratios have been obtained in other continuous experimental studies where a combination of factors that are known to improve solventogenesis in other clostridial fermentations are applied. The product ratios obtained in two other runs are shown in Figures 3.4 and 3.5. A combination of sudden decreases in yeast extract concentration with decreases in dilution rates led to oscillatory behavior with ratios as high as 1.2 mol ethanol/mol acetate. Even higher ratios (as much as 2.8 mol ethanol/mol acetate) were achieved by employing a sudden decrease in the operating pH from 5.0 to 4.0, followed by a decrease in the dilution rate. The sudden change in operating pH, however, may have brought about an induction of sporogenesis in the culture and is discussed below.

This same pH shift technique was repeated while using a lower yeast extract concentration in the liquid medium (0.02%). These experiments were carried out after a long period of culture acclimation, so that liquid flow rates over 600 mL/day were possible as opposed to flow rates of 200-400 mL/day in previous studies. The major significance of the latter results is in the increase in specific productivity of the culture with acclimation. The specific productivity may be defined as the quantity of ethanol produced per unit of cells per unit time, typically in units of mmol ethanol/gcell•day. This parameter sets the quantity of ethanol that can be produced from a culture (per cell) in a given reactor volume. Previously a peak ethanol

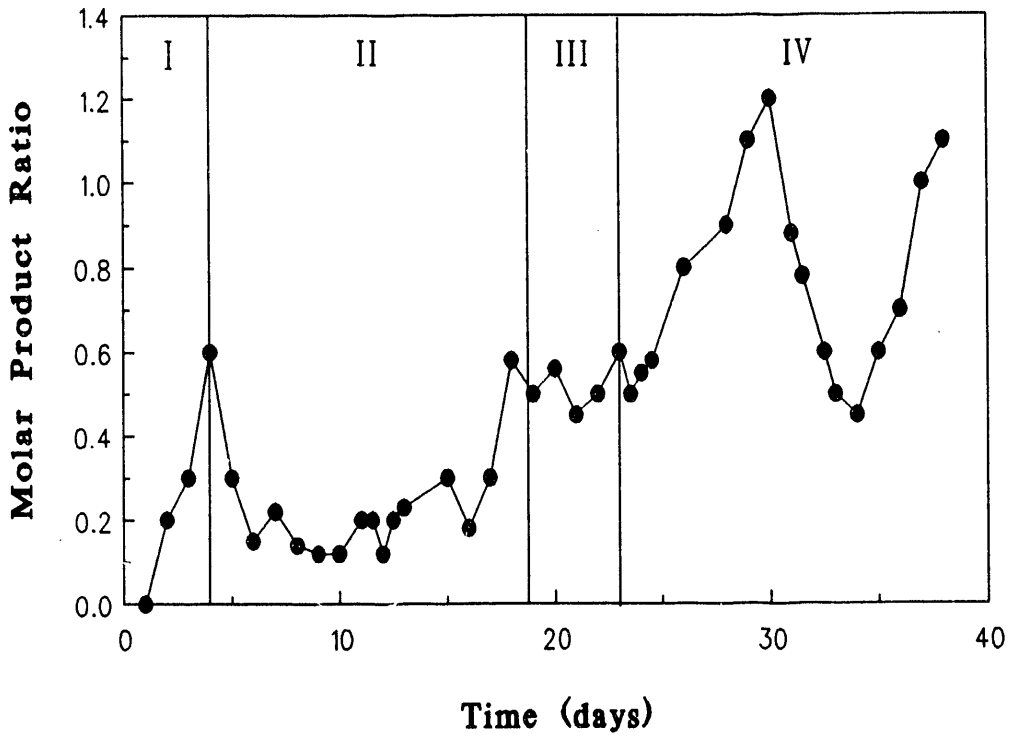


Figure 3.3. Molar product ratio for *C. ljungdahlii* in a CSTR.

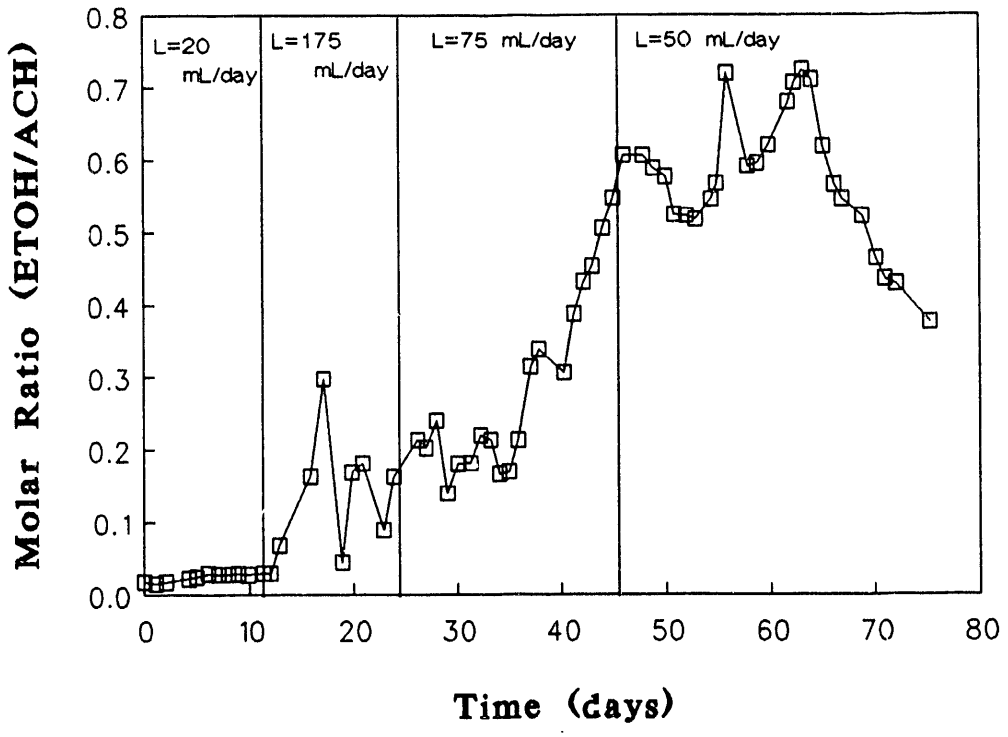


Figure 3.4. Molar product ratio versus liquid flow rate in a CSTR.

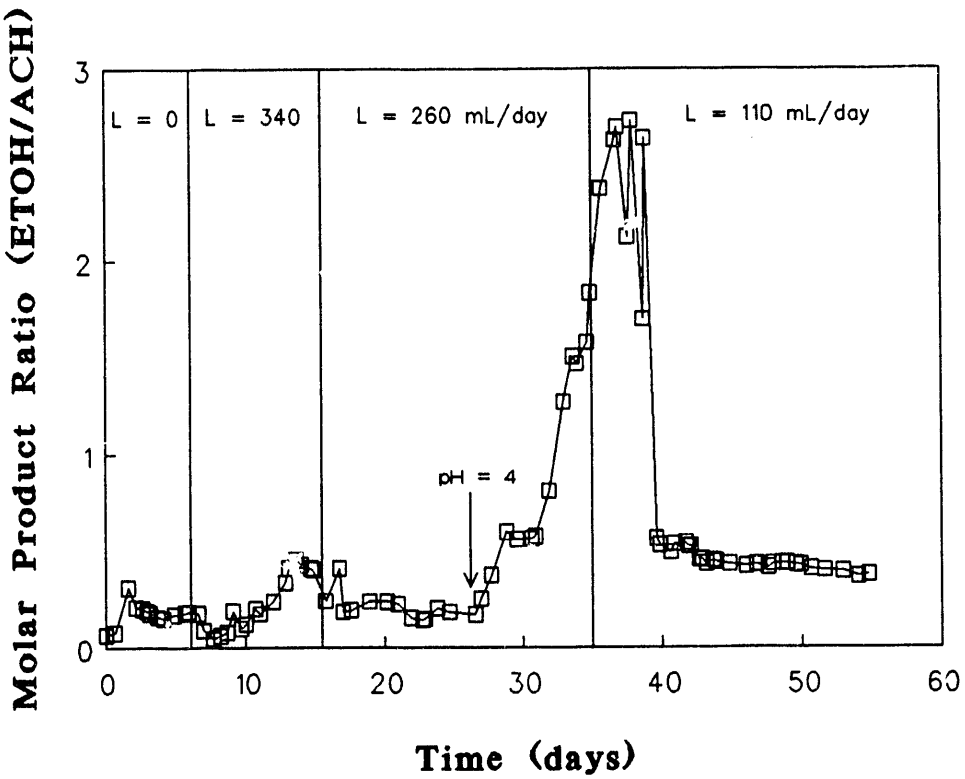


Figure 3.5. Molar product ratio in a CSTR with yeast extract depletion.

concentration of 1.6 g/L was achieved at a liquid flow rate of 60 mL/day. In the latter work, a peak ethanol concentration of 0.7 g/L was reached at a liquid flow rate of 530 mL/day. The productivity in the latter work was 178 mmol EtOH/gcell•day, which is an 800 percent improvement in the quantity of ethanol produced per cell. Improvements such as this will significantly enhance process economics in a commercial production scheme.

3.2.2 The Use of Reducing Agents as a Method to Improve the Ethanol/Acetate Product Ratio

Several researchers have shown that the presence of reducing agents in the liquid media of clostridial fermentations has brought about an increase in solvent formation in the product stream at the expense of acid formation. Rao and Mutharasan,[12] for example, showed a five-fold increase in acetone yields from glucose at pH 5 when adding methyl viologen to a culture of *C. acetobutylicum*. In a similar study, the addition of benzyl viologen to the media in a *C. acetobutylicum* fermentation was responsible for increasing the quantity of butanol in the product stream to over 90% of the total solvents formed. Acid formation decreased significantly in favor of butanol formation. Other reducing agents produced similar results on *Clostridium* strains, including sodium sulfide, cysteine hydrochloride, sodium thioglycolate and electrochemical energy.[13-16] Reducing agents apparently cause altered electron flow, which direct carbon flow and acid to alcohol production. Reducing equivalents are directed to the formation of NADH which, in turn, resulted in increased alcohol production.

Since the bacteria isolated at the University of Arkansas for the conversion of CO, H_2 and CO_2 to ethanol and acetate is also a *Clostridium*, it was felt that the addition of reducing agents to the medium might also result

in increased solvent (ethanol) production at the expense of acid formation. Batch experiments were thus carried out with small quantities of reducing agents (30, 50 and 100 ppm) added to the feed in order to assess the feasibility of increasing the ethanol to acetate ratio through reducing agent addition. The experiment carried out with 100 ppm of reducing agents resulted in very limited growth in all cases. On the other hand, 50 ppm and 30 ppm concentrations were successful in improving the ethanol to acetate ratio in some cases, as is shown in Table 3.1. The flask containing benzyl viologen at a concentration of 30 ppm produced 3.7 mmol of ethanol with a product ratio of 1.1, the highest ratio observed in batch experiments so far. It is interesting to mention that those reducing agents that improved the product ratio always resulted in slower growth rates of the bacteria, as could be expected from decreased ATP formation.

Table 3.1

Peak Levels for Ethanol Production and the Molar Ratio (ETOH/ACH) at 30 and 50 ppm Reducing Agent Concentrations

	(50 ppm)		(30 ppm)	
Reducing agent	ETOH(mmol)	ETOH/ACH	ETOH(mmol)	ETOH/ACH
Control	0.60	0.12	1.40	0.24
Sodium thioglycolate	1.30	0.20	1.30	0.25
Ascorbic acid	1.50	0.24	1.50	0.25
Methyl Viologen	1.90	0.20	2.50	0.40
Benzyl Viologen	1.25	0.21	3.70	1.10

3.2.3 Induction of Sporulation

Recently, the connection between sporulation and solventogenesis has been the subject of extensive studies, both in batch cultures[17-19] and chemostat cultures.[10,20] Under certain conditions, which are strain-dependent, a shift of the bacteria into a sporulation phase occurs which is accompanied by

morphological changes (elongation of the cells) and the production of solvents rather than acids. In some cases, such as *C. thermosaccharolyticum*, an uptake of previously produced acetate takes place during sporulation.[21] The selection of two classes of mutants, the *cls* and the *spo* mutants of *C. acetobutylicum*, has confirmed the relationship between sporulation and solventogenesis. The *cls* mutants are unable to form a clostridial stage and cannot switch to solventogenesis. The *spo* mutants, however, blocked at a later stage in sporogenesis, can all produce solvents.[17]

The conditions that cause the onset of sporulation and 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.[22] In the case of *C. acetobutylicum*, switching to the solventogenic phase and differentiation to the clostridial stage requires the presence of glucose and nitrogen, low pH and a minimum acetate and butyrate concentration. It has also been observed that, in general, good sporulation can be induced in batch culture using carbon sources that tend to reduce the bacterial growth rate.[23]

Experiments have been conducted with *C. ljungdahlii* grown in basal media at pH 5.0 with a 0.01% concentration of various carbohydrates (starch, galactose, rhamnose and cellobiose) in the presence of synthesis gas. Table 3.2 summarizes the results obtained for each of the nutrient studies, along with the maximum values obtained for cell concentration, ethanol concentration and molar product ratios. As noted, the highest product ratios were obtained

for cellobiose with product ratios over 3 times the ratio obtained in the presence of yeast extract. Ethanol and cell concentrations were highest in the presence of cellobiose and galactose, where the ethanol concentrations were over 4 times the value obtained in the presence of yeast extract.

Table 3.2

Summary of Results with Nutrient Sources Bringing About Sporulation

Nutrient	Cell Conc (mg/L)	ETOH (mmol)	ETOH/ACH molar ratio
Yeast Extract	140	0.13	0.13
Cellobiose	170	0.56	0.45
Rhamnose	135	0.31	0.31
Galactose	168	0.53	0.36
Starch	130	0.27	0.36

3.2.4 Nutrient Limitation at Reduced pH

As was mentioned previously, the most important technique found to date in increasing ethanol concentration and product ratio is to employ a minimal medium at reduced pH. The medium was developed using *Escherichia coli* as a microbial model and will be shown in Section 4. A pH level of 4.0 to 5.0 must be used in conjunction with this medium. Results using this medium in continuous reactors are presented later in this report. Ethanol concentrations as high as 47 g/L have been obtained with only small amounts of acetate produced.

4.0 MATERIALS AND METHODS

4.1 Organism and Medium

Clostridium ljungdahlii, Strain PETC, was originally isolated from chicken waste in the University of Arkansas laboratories, and later identified and characterized by Dr. R. S. Tanner, University of Oklahoma,

Department of Botany and Microbiology. The culture was stored in a non-shaking incubator (Precision Scientific, Chicago, IL) at pH 5 and 37°C on basal medium (see Table 4.1) and synthesis gas (65% CO, 24% H_2, and 11% CO_2), and transferred every two weeks. The medium of Table 4.1 was also used in most of the early experimental studies. The specific components of the basal medium (without yeast extract) is shown in Table 4.2. In several studies the yeast extract concentration was varied or eliminated. In other studies, a minimal medium modified from the undefined medium of Savage and Drake[26] without amino acids was utilized. Finally, a medium designed especially for *C. ljungdahlii* was used in later studies. The composition of this medium is shown in Table 4.3.

Table 4.1

Basal Medium for C. ljungdahlii

	per 100 mL
Pfennig's mineral solution[24]	5.0 mL
B-vitamins[25]	0.5 mL
Yeast extract	0.1 g
Pfennig's trace metal solution[25]	0.1 mL
Cysteine-HCl, 2.5% solution	2.0 mL
Resazurin, 0.1% solution	0.1 mL

4.2 General Laboratory Procedures

Detailed procedures for inoculation, gas addition and gas and liquid sampling are shown in Appendices A2-A4. These procedures were developed after several months of refinement and modification. The anaerobic techniques employed are essentially those of Hungate,[27] as modified by Bryant[28] and Balch and Wolfe.[29]

Table 4.2

Basal Medium Composition*

Constituent	Concentration (mg/L)
Pfennig Minerals	
KH_2PO_4	500
$MgCl_2 \cdot 6H_2O$	330
NaCl	400
NH_4Cl	400
$CaCl_2 \cdot 2H_2O$	50
Pfennig Trace Metals	
$ZnSO_4 \cdot 7H_2O$	0.100
$MnCl_2 \cdot 4H_2O$	0.030
H_3BO_3	0.300
$CoCl_2 \cdot 6H_2O$	0.200
$CuCl_2 \cdot H_2O$	0.010
$NiCl_2 \cdot 6H_2O$	0.020
$Na_2MoO_4 \cdot 2H_2O$	0.030
$FeCl_2 \cdot 4H_2O$	1.500
Na_2SeO_3	0.010
B Vitamins	
Biotin	0.100
Folic Acid	0.100
Pyridoxal HCl	0.050
Lipoic a.(Thiotic a.)	0.300
Riboflavin	0.250
Thiamine HCl	0.250
Ca-D-Pantothenate	0.250
Cyanocobalamin	0.250
P-aminobenzoic acid	0.250
Nicotinic acid	0.250
Cysteine HCl	490

*resazurin also added as indicator of anaerobicity

JRP's Medium		Fresh Medium		Inoculum		Overall
Volume (ml)		100	2000	100	0	2020
JRP Minerals I (ml)		0.5	10	1	0	10
JRP Minerals II (ml)		5	100	1	0	100
JRP Salts (ml)		0.5	10	0.1	0	10
PFN Tr. Metals(ml)		1	20	0.1	0	20
JRP's Vitamins (ml)		0.1	2	0.02	0	2
Resazurin (ml)		0	0	0.1	0	0
Cysteine HCl 2.5% (ml)		1	20	2	0	20
JRP Minerals I		In Sol'n (g/l)	In Media (g/l)	In Sol'n (g/l)	In Media (g/l)	In Media (g/l)
	NH4Cl	0	0	20	0.2	0.000
	MgCl2.6H2O	100	0.5	5	0.05	0.495
	CaCl2.2H2O	40	0.2	2	0.02	0.198
JRP Minerals II		In Sol'n (g/l)	In Media (g/l)	In Sol'n (g/l)	In Media (g/l)	In Media (g/l)
	(NH4)2HPO4	40	2	20	0.2	1.980
JRP Salts		In Sol'n (g/l)	In Media (g/l)	In Sol'n (g/l)	In Media (g/l)	In Media (g/l)
	NaCl	40	0.2	40	0.04	0.198
	KCl	30	0.15	30	0.03	0.149
PFN Trace Metals		In Sol'n (g/l)	In Media (mg/l)	In Sol'n (g/l)	In Media (mg/l)	In Media (mg/l)
	ZnSO4.7H2O	0.1	1	0.1	0.1	0.990
	MnCl2.4H2O	0.03	0.3	0.03	0.03	0.297
	H3BO3	0.3	3	0.3	0.3	2.970
	CoCl2.6H2O	0.2	2	0.2	0.2	1.980
	CuCl2.H2O	0.01	0.1	0.01	0.01	0.099
	NiCl2.6H2O	0.02	0.2	0.02	0.02	0.198
	Na2MoO4.2H2O	0.03	0.3	0.03	0.03	0.297
	FeCl2.4H2O	1.5	15	1.5	1.5	14.851
	Na2SeO3	0.01	0.1	0.01	0.01	0.099
B Vitamins		In Sol'n (mg/l)	In Media (mg/l)	In Sol'n (mg/l)	In Media (mg/l)	In Media (mg/l)
	Biotin	101	0.101	101	0.020	0.1000
	Thiamine HCl	254	0.254	254	0.051	0.2515
	Ca-D-Pantothenate	401	0.401	401	0.080	0.3970
		In Sol'n (g/l)	In Media (mg/l)	In Sol'n (g/l)	In Media (mg/l)	In Media (mg/l)
Cysteine HCl in 0.2 N NaOH		25	248	25	490	248
			Total Ion Concentration (M)			0.070
Supplements		In Sol'n (mg/l)	Vol. Added (ml)			In Media (mg/l)
	Biotin	20	0.50			0.0050
	Folic Acid	20	0.50			0.0050
	Pyridoxal HCl	10	0.50			0.0025
	Lipoic a. (Thiotic a.)	60	0.50			0.0149
	Riboflavin	50	0.50			0.0124
	Thiamine HCl	50	0.50			0.0124
	Ca-D-Pantothenate	50	0.50			0.0124
	Cyanocobalamin	50	0.50			0.0124
	P-aminobenzoic acid	50	0.50			0.0124
	Nicotinic acid	50	0.50			0.0124
	H3PO4	1453500	2.00			1439.1

Table 4.3 Designed Medium Composition

The development of procedures for quantitatively using gas substrates (CO, CO_2/H_2) with *Clostridium* sp. was necessary. An outline of the procedures for quantitative gas utilization is shown in Appendix B.

4.2.1 Chemostat Equipment and Procedures

A New Brunswick Bioflo C.30 chemostat was adapted for use under anaerobic conditions with continuous flow of gas. The chemostat was actually a semibatch reactor with continuous pH control. Figure 4.1 shows a schematic diagram of the experimental system. The reactor vessel was made of Pyrex glass, 750 mL nominal volume. Agitation was provided by magnetic coupling in the range of 200-1000 rpm. Verification of the agitation level indicator against a tachometer has shown no significant deviation. The system was equipped with temperature and pH control, and had gas flow meters.

The gas flowed from the pressurized tank through a fine metering valve and rotameter into the reactor. A large capacity oxygen trap, an oxygen indicator column and a filter were installed in the gas line to eliminate all traces of oxygen from the synthetic gas feed, and to avoid contamination. A 1 psig check valve was placed before the gas sparger to help stabilize the flow at the very low flow rates employed. The gas exited the system either through the liquid overflow and finally from the product reservoir or directly from the reactor. In both cases, 1/3 psig check valves were located in the exit gas line to help maintain the system slightly above atmospheric pressure. This arrangement prevented any air leaks into the reactor system. All tubing connections were made of butyl rubber or stainless steel.

The anaerobic techniques for the start-up of the chemostat system and the preparation of media used in the studies were slightly different from those used in traditional batch culture. The feed reservoir and reactor vessel,

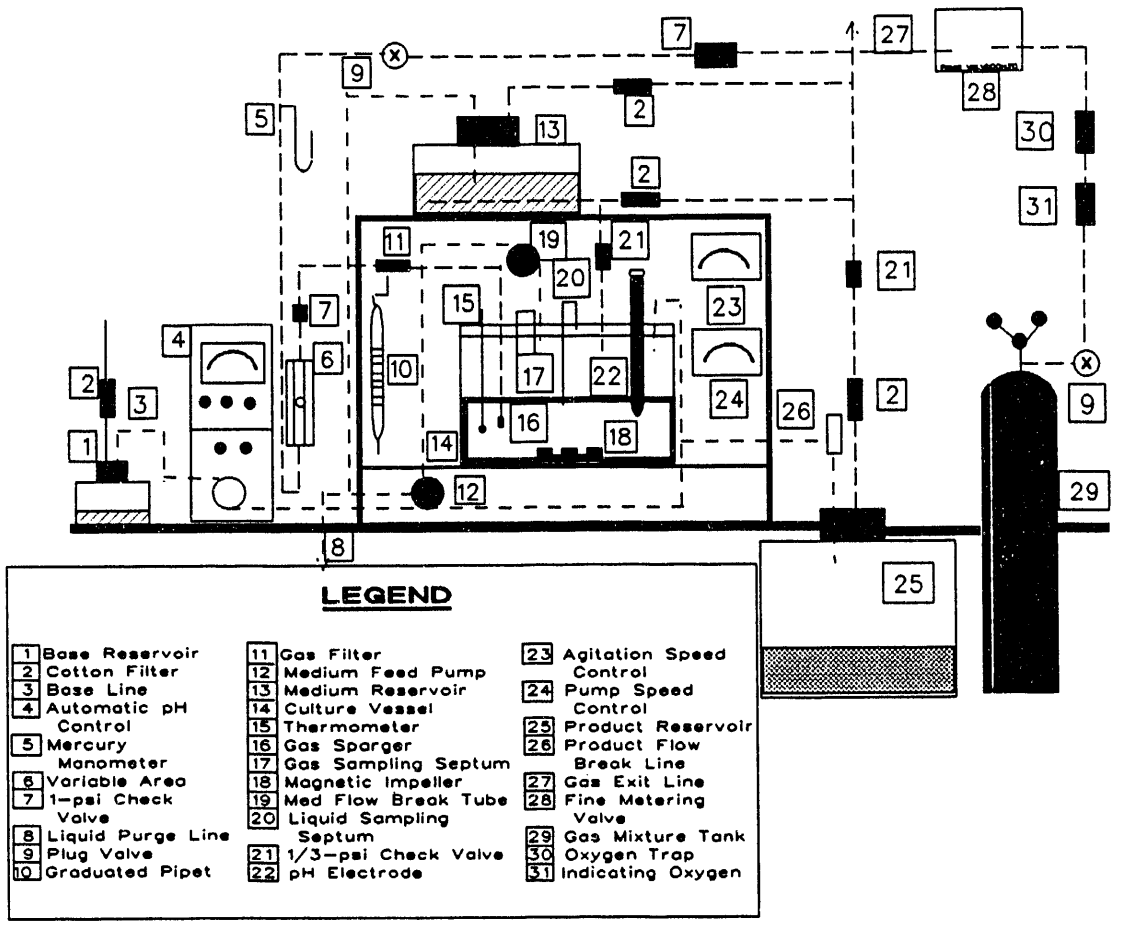


Figure 4.1. Schematic of the chemostat fermentation system.

filled with medium, as well as the product reservoir, empty, were autoclaved separately. The medium contained all of the required components except the reducing agent. A large cotton filter attached by a screw cap to the vessels allowed for pressure equilibrium inside the autoclave. After autoclaving, an oxygen free mixture of nitrogen and carbon dioxide was introduced into each vessel until they cooled. Enough carbon dioxide was bubbled into the media solutions to make up for that lost during autoclaving by disproportioning of biocarbonate into carbonate and carbon dioxide. When cool, the media solutions were clear; any color was due to the added resazurin, which is blue when organic reducing matter such as yeast extract is not present or light red otherwise. The cotton filter was then replaced by previously sterilized screw cap with a septum and all lines in and out of the vessel were clamped.

The reactor vessel was then connected to the gas lines of the system and allowed to flow for about 8 hours prior to inoculation. The gas composition used in the continuous studies consisted of 19.28 percent H_2, 15.29 percent Ar, 55.59 percent CO and 9.89 percent CO_2. Other compositions may be used if desired. The argon was used as an inert in determining conversion, as described previously. The medium in the reactor was then reduced by the addition of cysteine hydrochloride. Cysteine hydrochloride was chosen due to its adequacy in previous experiments. The standard redox potential achieved with cysteine (-210mV) is less negative than that of sodium sulfide (-571mV). In our system, cysteine hydrochloride has allowed growth and gas uptake to occur. Still, a careful study to determine the optimum reducing agent to use and its effects on the ratios of alcohol/acetate obtained will be required in the future. Five milliliters of stock culture was used as the inoculum. the reactor was then operated batchwise for the liquid and continuously for the

gas (about 4.2 mL/min of gas flow rate) for 138 hours. The agitation rate was 400 rpm during this experiment.

The externally controlled variables in the system were the agitation level, the pH level, and gas flow rate. These parameters can be controlled in all experiments. Other variables expected to affect the performance of the system are nutrient level and addition of antifoam. Antifoam is required to avoid excessive foaming, and is added directly into the reactor at regular intervals or when considered necessary. Three to five drops from a needle was usually enough to provide a foam free liquid culture.

Sampling of the reactor gas composition was routinely carried out with gas-tight syringe through a setpum placed on top of the reactor vessel. Liquid samples were withdrawn directly from the reactor through an immersed tube ending in a septum on top of the reactor vessel and analyzed for cell and acetate concentrations. To avoid the emergence of a pink color in the sample which interferes with optical density measurements, a sterile syringe and a spectronic tube sealed with parafilm were flushed with an oxygen free gas mixture prior to sampling. When dilutions are needed, a solution of approximately 0.05 weight percent $Na_2 \cdot 9H_2O$ was employed.

4.2.2 Cell Recycle System

In order to boost product concentrations in the existing CSTR, a cell recycle system has been constructed. This system should be able to maintain a higher cell concentration inside the reactor than in a comparable CSTR without cell recycle since part of the exiting broth is filtered with the cells and returned to the reactor. This corresponds to a CSTR system with different residence times for the cells and the liquid medium. Since filter clogging is one of the main obstacles in using cell recycle, a novel self-cleaning

filtration system has been constructed (See Figure 4.2) which consists of two cycles, a "feed cycle" and a "filtration cycle." During the feed cycle, fresh feed is used to clear the filter as it enters the reactor. During the filtration cycle, the exiting liquid stream is filtered. The cells deposited on the filter are then returned to the reactor during the next feed cycle. Each cycle is approximately 1-2 min long. In order to further extend the life of the filter, the feed cycle is slightly longer than the filtration cycle, allowing a longer cleaning time. To maintain a constant volume in the CSTR, an overflow control is also used to adjust for the slightly higher feed rate. Thus, the total effluent will consist of a filtered and an unfiltered part, the filtered part being larger. This will assure cell renewal and removal of old or deal cells, a problem with immobilized cell systems.

4.3 Analytical Techniques

Standard analytical techniques used on a routine basis included cell density measurements using optical density, gas analysis using gas-solid chromatography, liquid analysis using gas-solid chromatography, and reaction pH. Each of these techniques are briefly discussed in Appendix C. Other analytical techniques used in culture identification are standard microbiological techniques and are not discussed.

5.0 RESULTS AND DISCUSSION

5.1 Two-Stage CSTR System

A two-stage CSTR system was used in early studies with basal medium. The first reactor (volume of 350 mL) was used primarily for the growth of *C. ljungdahlii*. The liquid feed to this reactor consisted of 0.02 percent yeast extract in basal medium at pH 4.5, with a flow rate of 360 mL/day. In addition, synthesis gas was fed to the reactor to supply carbon and energy

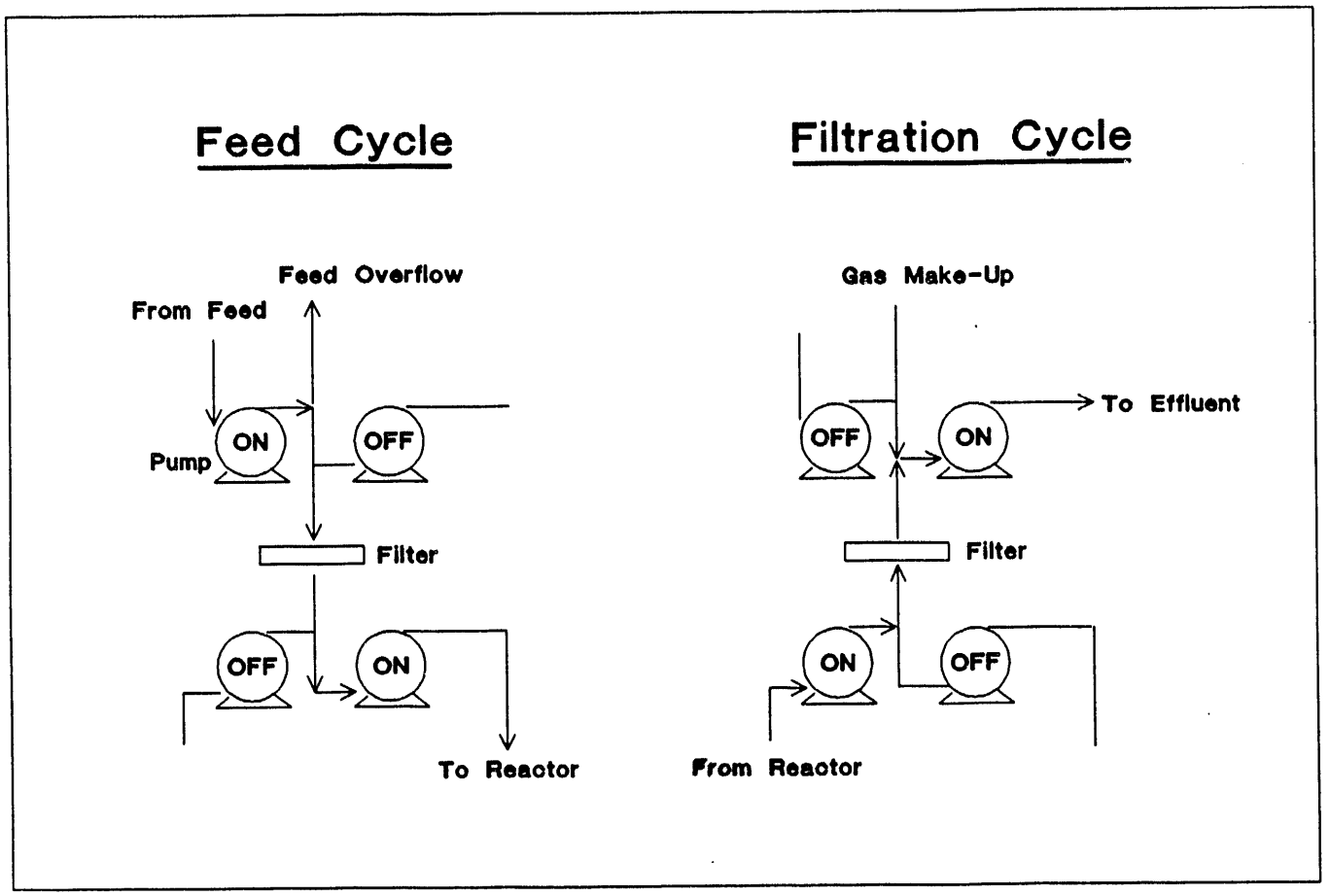


Figure 4.2. Pump and Filter Set-Up for Cell Recycle System.

sources for growth. Because this reactor was designed primarily for growth, significant acetate production was also expected. The second reactor (volume of 1.44 L) was used primarily for ethanol production by *C. ljungdahlii* cells formed in the first reactor. No additional liquid was fed to the second reactor so that nutrients were limiting. In addition, the pH was adjusted to pH 4.0 and the reducing agent benzyl viologen was added at a concentration of 10-20 ppm. Synthesis gas was bubbled into the second CSTR for conversion to ethanol. it was hoped that only ethanol would be produced from the cells in the second CSTR. However, because the conditions in the second reactor were not conducive for growth, the efficiency of ethanol production in the second CSTR was in doubt. An experiment was thus run with the above system to determine the overall performance of this two-stage system and to determine the suitability of the second stage in efficiently producing ethanol by utilizing the pH shift, nutrient limitation and benzyl viologen addition. The results are summarized below.

5.1.1 Operating Conditions

The reactor volumes and liquid flow rates were held constant at the values shown above throughout the experimental study. In addition, the feed to the first reactor always consisted of 0.02 percent yeast extract in basal medium. The pH varied a bit in each reactor, but was essentially 4.5 in Reactor A (the first reactor in series) and 4.0 in Reactor B.

The gas flow rate to Reactor A varied from 2.34 mL/min during start-up to a high of 3.40 mL/min on day 53. However, during most of the experimental study the gas flow rate was held constant at 2.76 mL/min. Similarly, the gas flow rate to Reactor B varied from a low of 1.79 mL/min during startup to 2.20 mL/min. Adjustments in these gas flow rate were made periodically in an attempt to improve reactor performance but, as noted, were not significant adjustments.

Benzyl viologen at a concentration of 20 ppm was added to Reactor B on day 13. The higher concentrations utilized in previously batch studies (30-50 ppm) were found to be excessive in continuous culture, resulting in culture death. The concentrations of benzyl viologen was lowered to 10 ppm on day 45. Initially, no benzyl viologen was added to Reactor B.

5.1.2 Results and Discussion

Cell concentration profiles for the two reactors are shown in Figures 5.1 and 5.2. As is noted in Figure 5.1, after a brief start-up period, the cell concentration varied about a cell concentration of approximately 300 mg/L from 270 to 317 mg/L until day 65. A temperature decrease in the constant temperature room is then thought to be responsible for the significant drop in cell concentration seen after day 60. The cell concentration in Reactor B was also seen to gradually increase during start-up until the addition of benzyl viologen on day 13. A cell concentration of 340 mg/L was seen at this point. The reducing agent benzyl viologen, while yielding reduced conditions necessary for ethanol production, also resulted in the death of cells particularly at high concentrations (a high concentration may be as little as 20 ppm, as is noted in Figure 5.2). The cell concentration decreased to only 250 mg/L throughout the addition of 20 ppm benzyl viologen. When 10 ppm benzyl viologen was used instead of 20 ppm, the cell concentration in Reactor B also decreased, but only to a 280 mg/L concentration. Again, the results after day 60 are due to the temperature upset.

The conversions of CO and H_2 in the synthesis gas feed to Reactors A and B are shown in Figure 5.3 and 5.4. As is noted in Figure 5.3, the conversions of both CO and H_2 remained constant at 90-95 percent throughout the whole experimental study (excluding start-up and the temperature upset). Both the

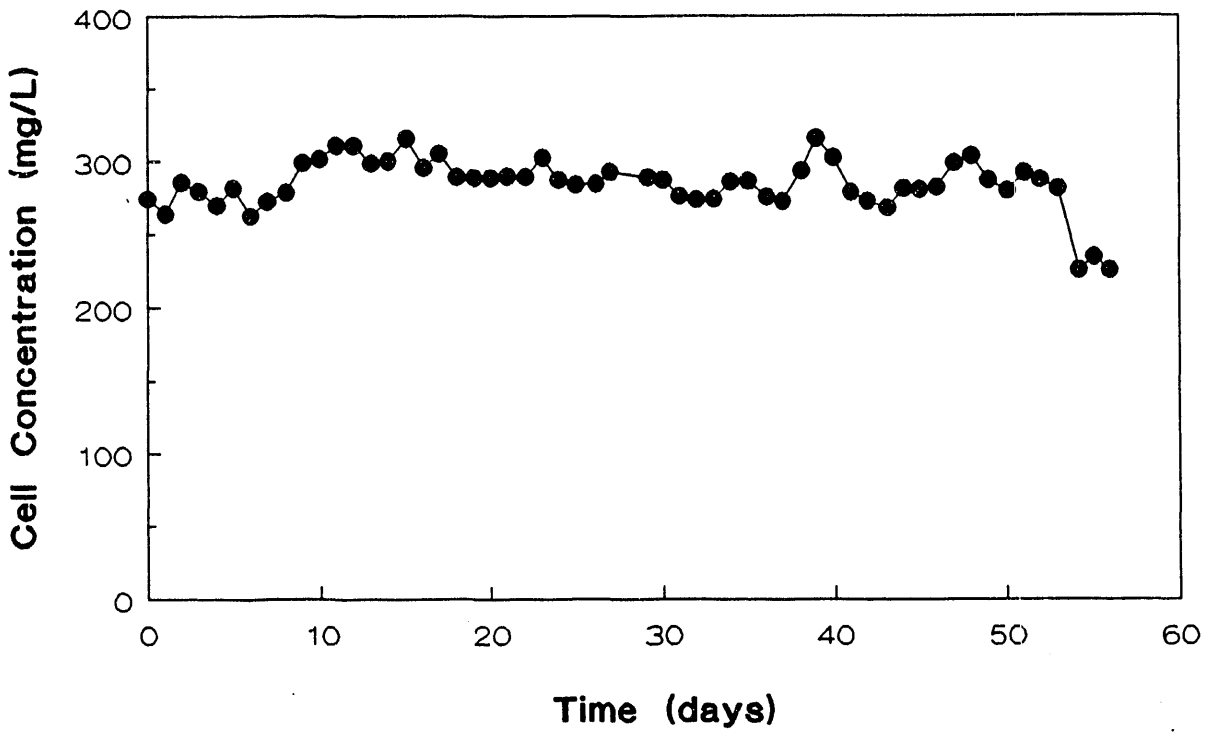


Figure 5.1. Cell Concentration Profile in Reactor A in a Two-Stage CSTR System Using *C. ljungdahlii*.

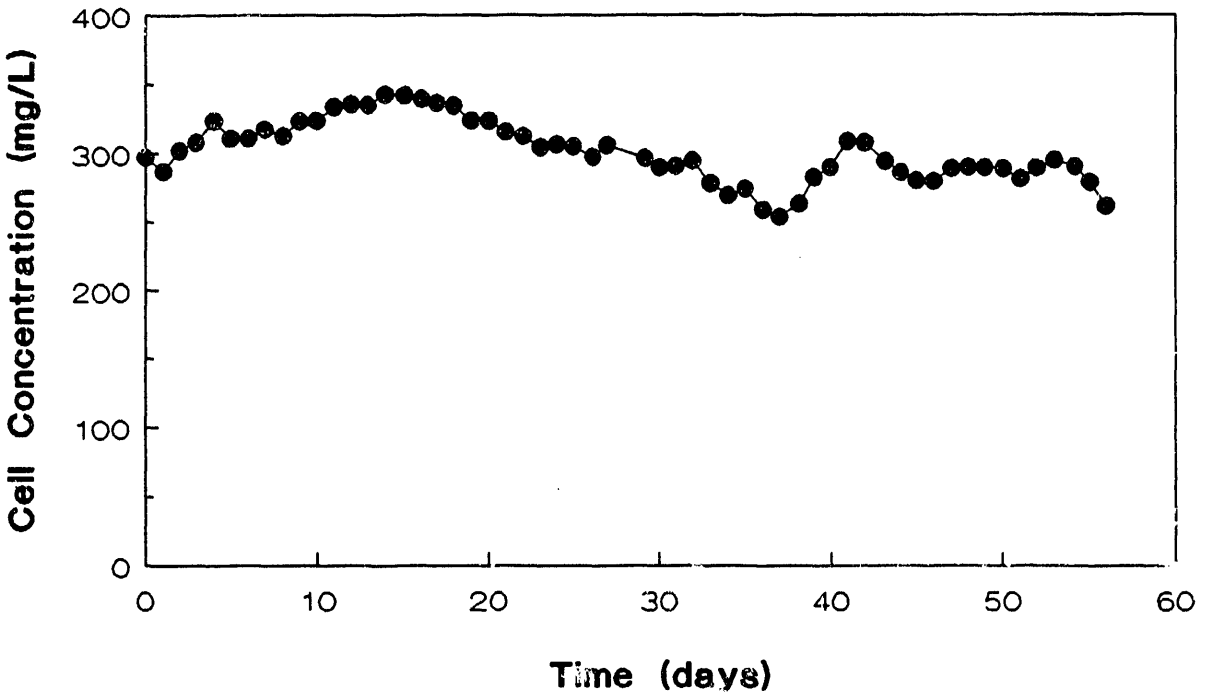


Figure 5.2. Cell Concentration Profile in Reactor B in a Two-Stage CSTR System Using *C. ljungdahlii*.

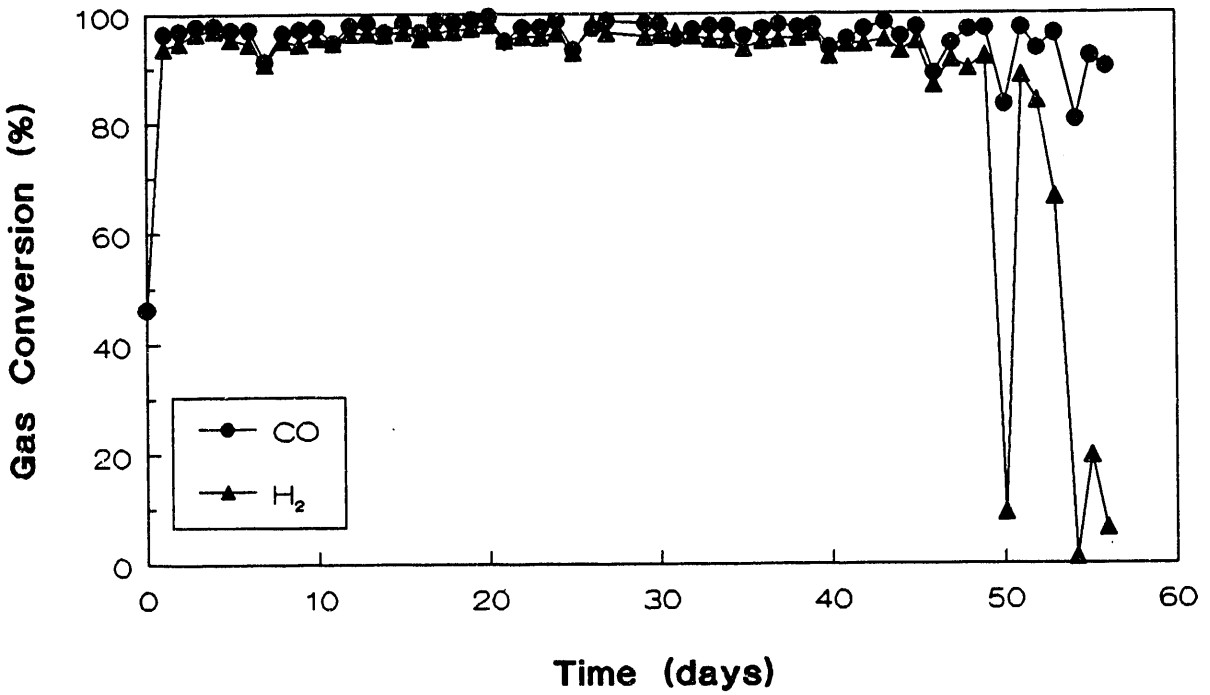


Figure 5.3. CO and H_2 Conversion in Reactor A in a Two-Stage CSTR System Using *C. ljungdahlii*.

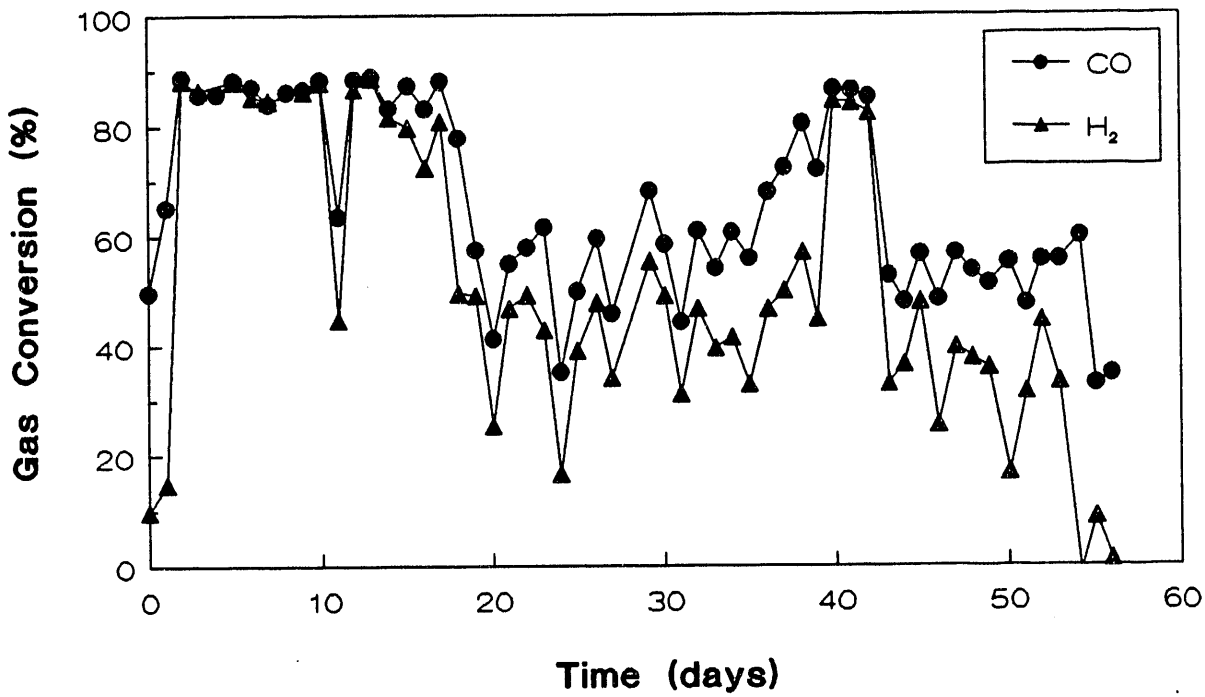


Figure 5.4. CO and H_2 Conversion in Reactor B in a Two-Stage CSTR System Using *C. ljungdahlii*.

CO and H_2 conversions decreased as a result of benzyl viologen addition in Reactor B (see Figure 5.4) regardless of whether 20 ppm or 10 ppm benzyl viologen was used. This phenomenon has been seen previously in the University of Arkansas laboratories, and may signal that the efficiency of the cells in utilizing substrate or forming product is reduced in the presence of benzyl viologen or other reducing agents.

Product formation in the reactors are shown in Figures 5.5-5.7. As expected, acetate formation was much greater in Reactor A than ethanol formation due to the growth-enhancing conditions employed. Concentrations of approximately 3.5 g/L acetate and 0.5 g/L ethanol were formed except during start-up and after the temperature upset. This translates into a molar ratio of 0.19 moles ethanol per mole acetate, or just over 5 moles acetate produced per mole of ethanol.

Figure 5.6 shows the overall product concentration profile from the two-stage system. As is noted, the concentration of ethanol stayed approximately constant at about 1 g/L, with peak concentrations of about 1.3 g/L. These concentration levels have been exceeded in the past. The concentration of acetate, however, fell with the addition of benzyl viologen, especially when using 20 ppm of the reducing agent. A better idea of what happened upon the addition of benzyl viologen can be seen in Figure 5.7, where the concentrations of ethanol and acetate produced in Reactor A are subtracted from the overall product concentrations. It can be clearly seen that only ethanol was produced in the second reactor. As a matter of fact, the negative acetate concentrations indicate that acetate was actually being consumed in the second reactor. The only negative aspect of this result is the hope that the ethanol concentrations would have been higher. This probably can be accomplished by varying the benzyl viologen concentration.

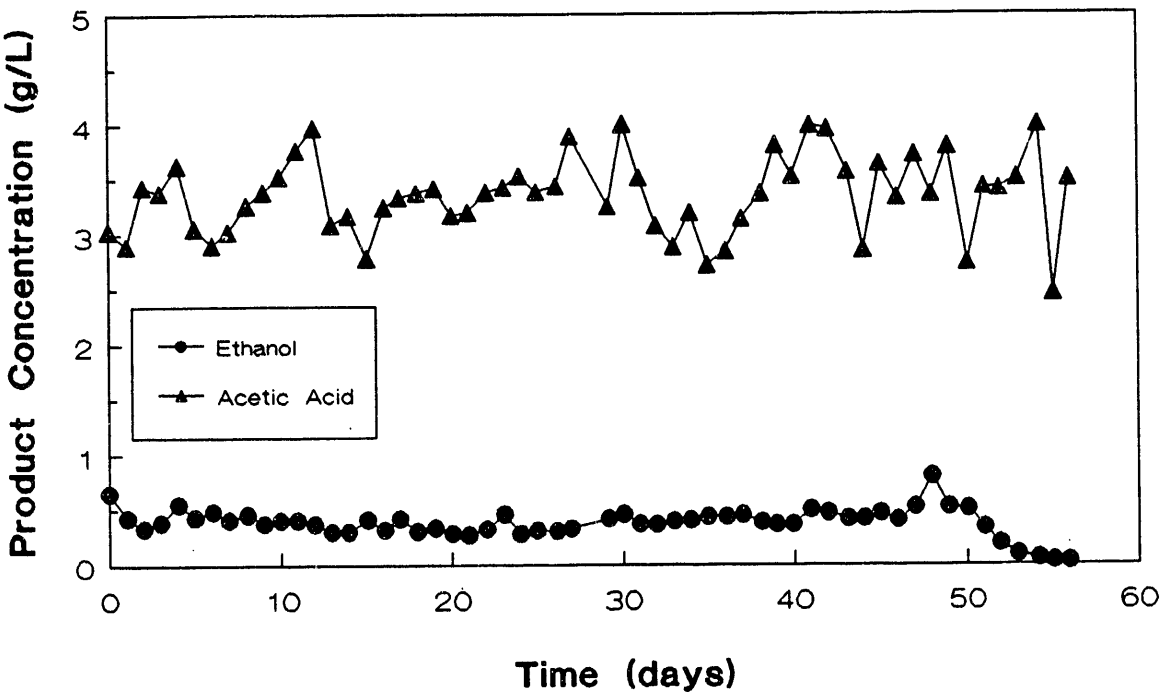


Figure 5.5. Production of Ethanol and Acetate by *C. ljungdahlii* in Reactor A of a Two-Stage CSTR System.

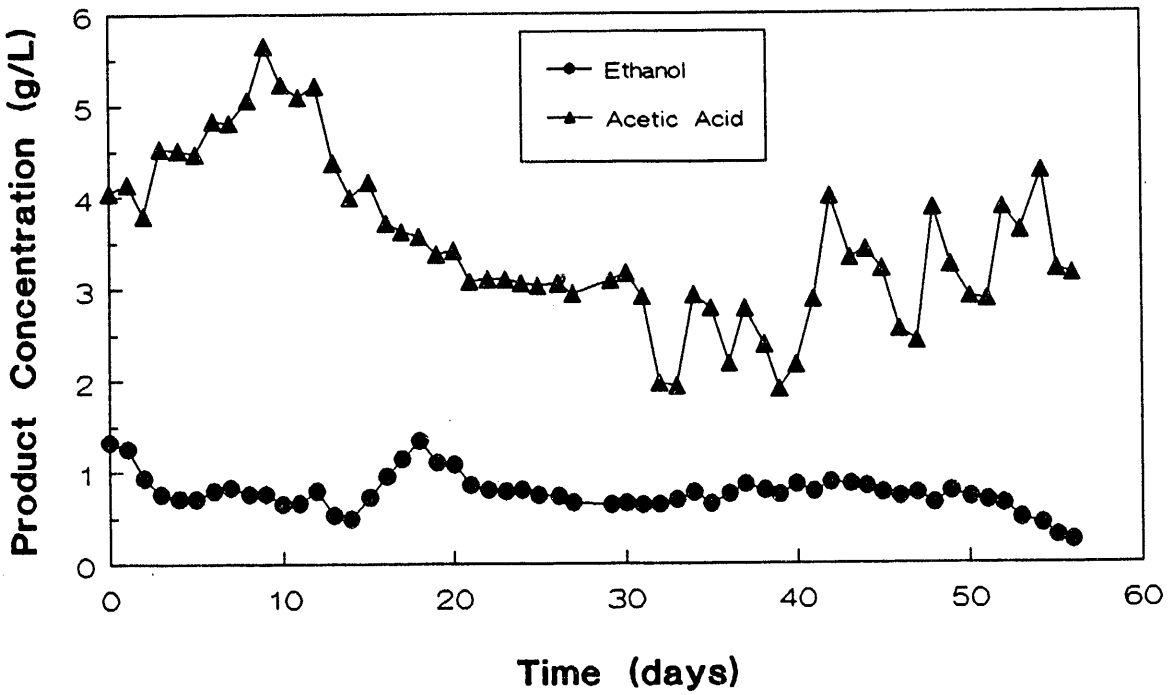


Figure 5.6. Product Concentration Profiles for the Overall Two-Stage Reactor System Using *C. ljungdahlii*.

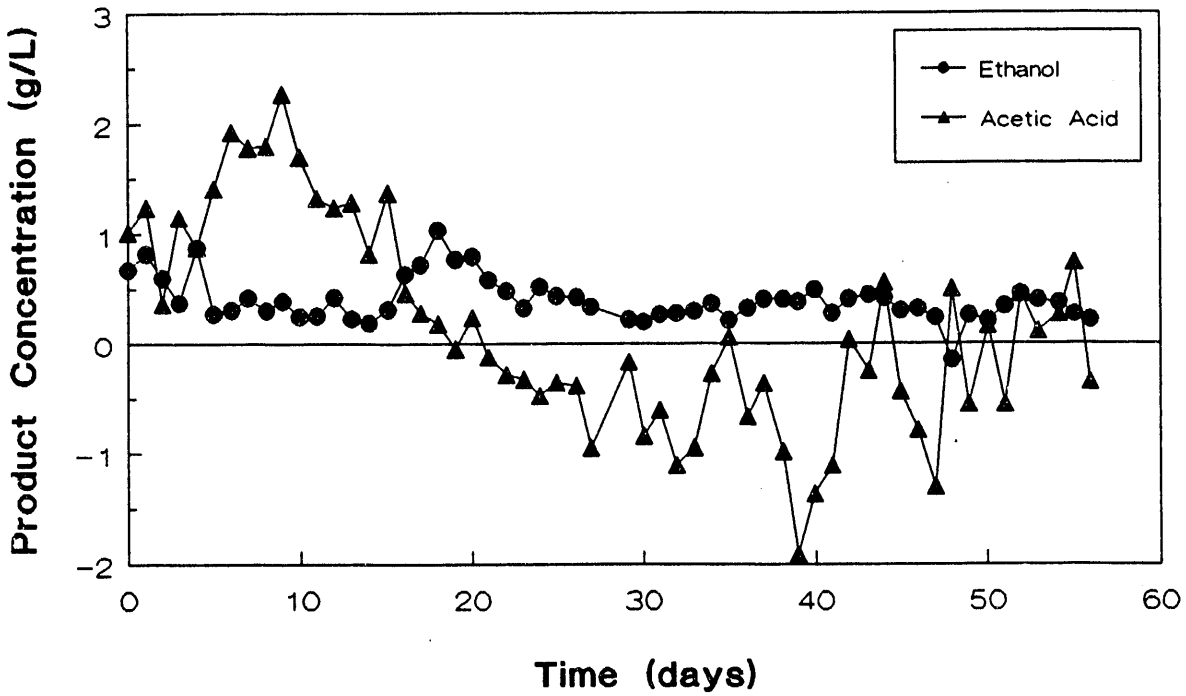


Figure 5.7. Production of Ethanol and Acetate by *C. ljungdahl* , Reactor B of a Two-Stage CSTR System.

5.2 Continuous Stirred-Tank Reactor Studies

A continuous stirred-tank reactor with both continuous liquid and gas feed was prepared in order 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 to 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.

5.2.1 Liquid Flow Rate Effects

Fermentation profiles of cell concentration, ethanol concentration and acetate concentration as a function of time were obtained in the LCSTR at each liquid flow rate. During these studies the agitation rate was held at 360-400 rpm, the gas flow rate was held at 0.145 mmol/min and the liquid flow rate was varied from 50 to 400 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.

Figures 5.8 and 5.9 show typical cell concentration and product concentration profiles in the CSTR at liquid flow rates of 200 and 100 mL/d. Time plotted on the abscissa ranges from 600 to 1200 h, since these data are part of a longer term study using various liquid flow rates in the CSTR. As

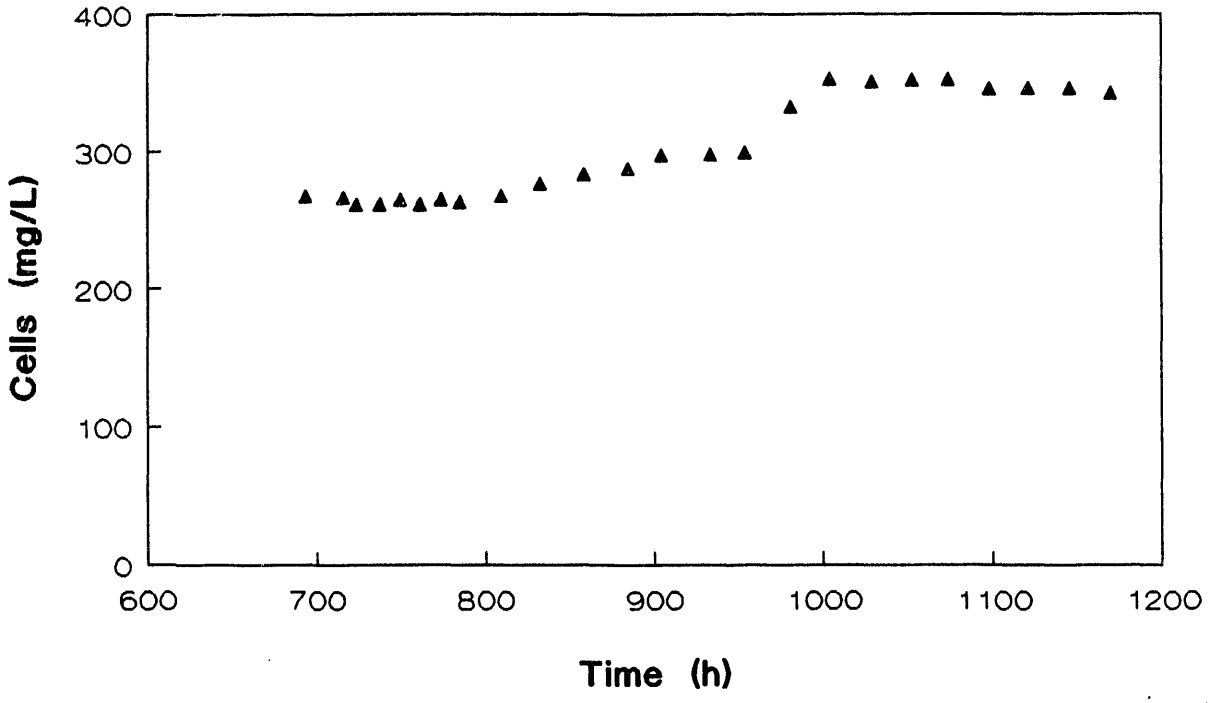


Figure 5.8. Cell Concentration Profile for *C. ljungdahlii* in the CSTR (400 rpm; Gas Flow Rate: 0.145 mmol/min; Liquid Flow Rates: 200, 100 mL/d)

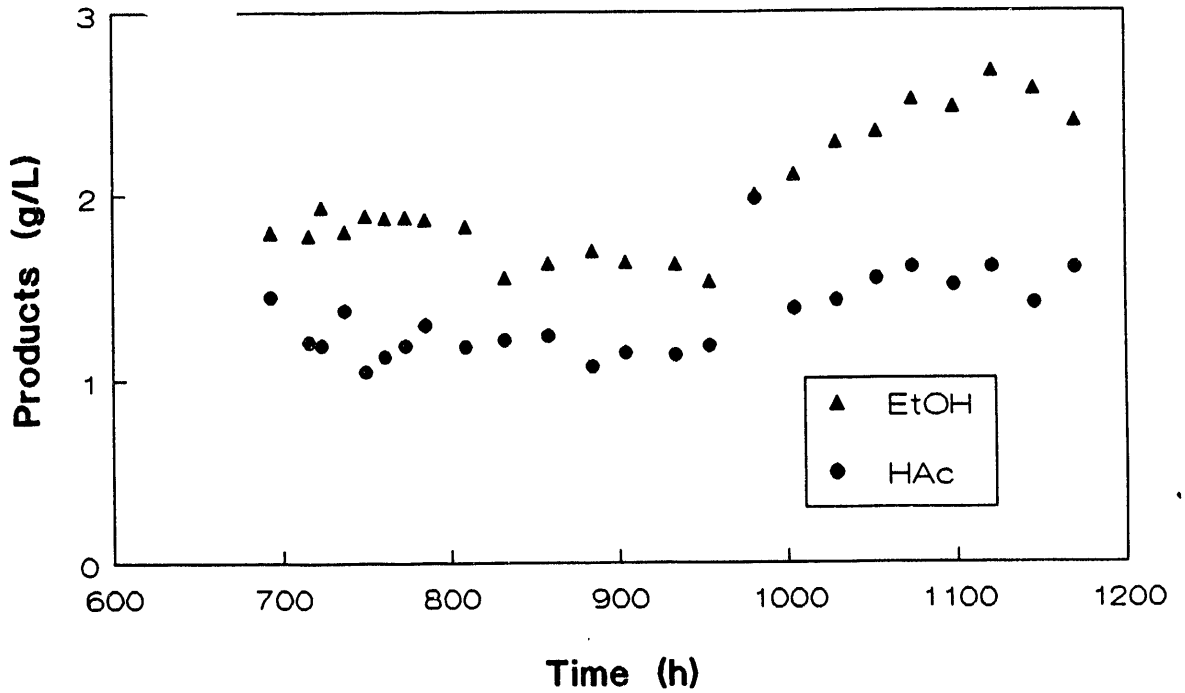


Figure 5.9. Product Concentration Profiles for *C. ljungdahlii* in the CSTR (400 rpm; Gas Flow Rate: 0.145 mmol/min; Liquid Flow Rates: 200, 100 mL/d)

is noted in Figure 5.8, a steady-state cell concentration of approximately 300 mg/L was obtained at a liquid flow rate of 200 mL/d, and a steady-state cell concentration of approximately 350 mg/L was obtained at a liquid flow rate of 100 mL/d. Thus, as expected, a decrease in the flow of liquid through the reactor brought about an increase in the steady-state cell concentration. Figure 5.9 presents product (ethanol and acetate) concentration profiles for the same flow rate study. The steady-state ethanol concentrations were approximately 1.8 g/L at 200 rpm and 2.5 g/L at 100 rpm. The corresponding steady-state acetate concentrations were approximately 1.2 and 1.6 g/L at 200 and 100 rpm, respectively.

The cell yield, $Y_{X/S}$, is shown as a function of time for the two liquid flow rates in Figure 5.10. As is noted, the yield remained essentially constant at about 0.6 mg cells/mol substrate. In fact, it was found that the cell yield was unaffected by liquid flow rate over the liquid flow rate range tested in the study. Figure 5.11 presents the product yield from substrate, $Y_{P/S}$, as a function of time for the two flow rates. As with the cell yield, the product yield was found to be essentially independent of liquid flow rate, having a value of approximately 0.3 mol of carbon in the products per mol of carbon in the substrate. It is worth noting that these steady-state values do not correspond well with the values presented earlier in this report during the initial stages of batch growth. The significance of the result needs investigation.

Finally, specific uptake rates in mmol CO/mg cells hr and specific productivities in mmol product/mg cells hr are shown in Figure 5.12 for the two liquid flow rates. The specific uptake rate was found to decrease from 0.04 to 0.02 mmol/mg h for a decrease in liquid flow rate of 200 to 100 mL/d.

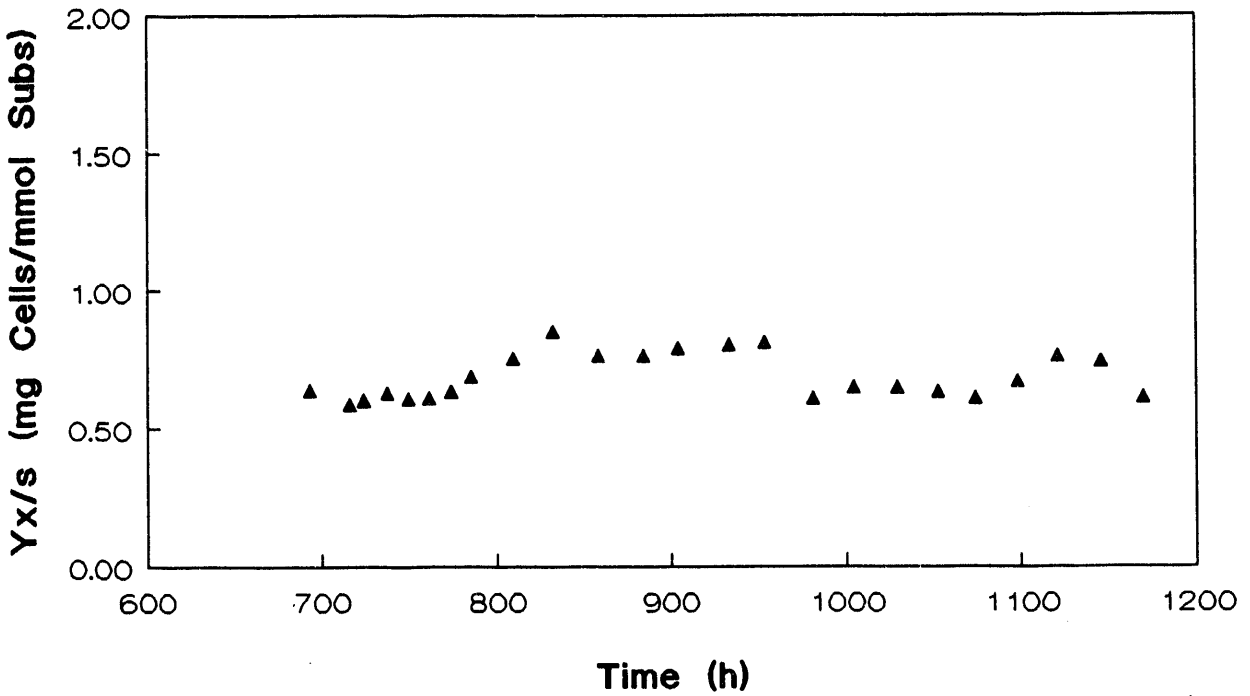


Figure 5.10. Cell Yield from Substrate for *C. ljungdahlii* in the CSTR (400 rpm; Gas Flow Rate: 0.145 mmol/min; Liquid Flow Rates: 200, 100 mL/d)

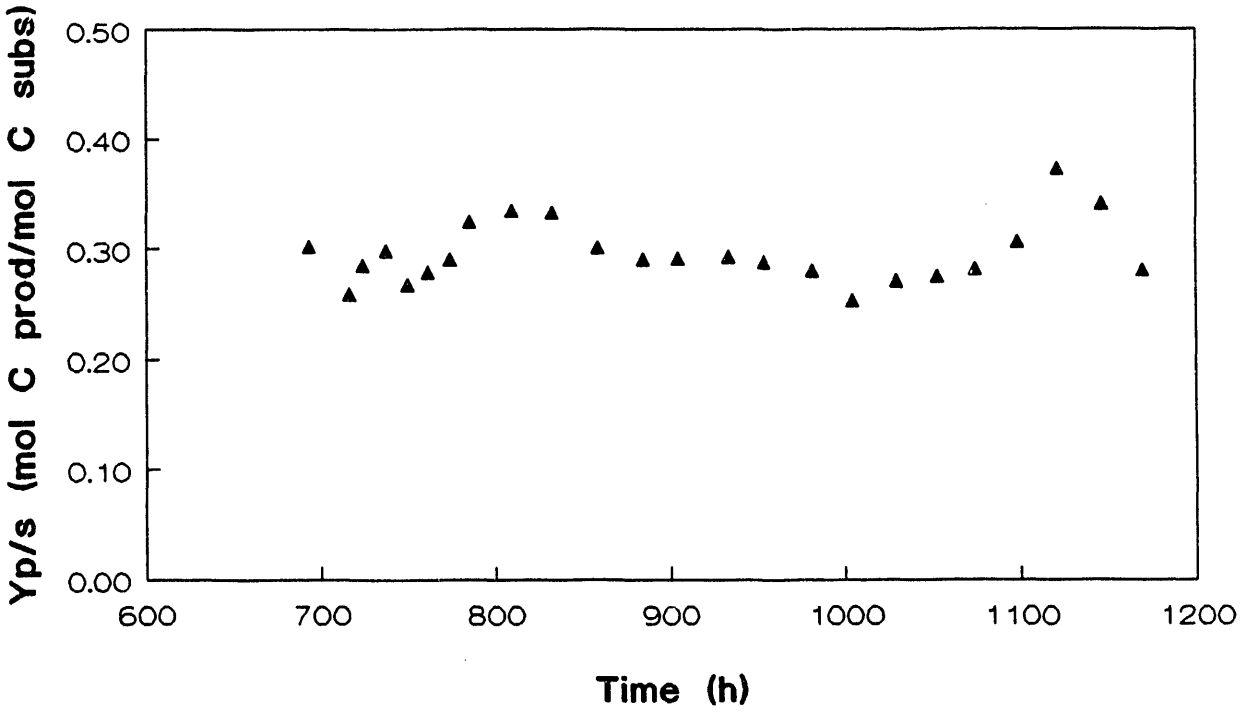


Figure 5.11. Product Yield from Substrate for *C. ljungdahlii* in the CSTR (400 rpm; Gas Flow Rate: 0.145 mmol/min; Liquid Flow Rates: 200, 100 mL/d)

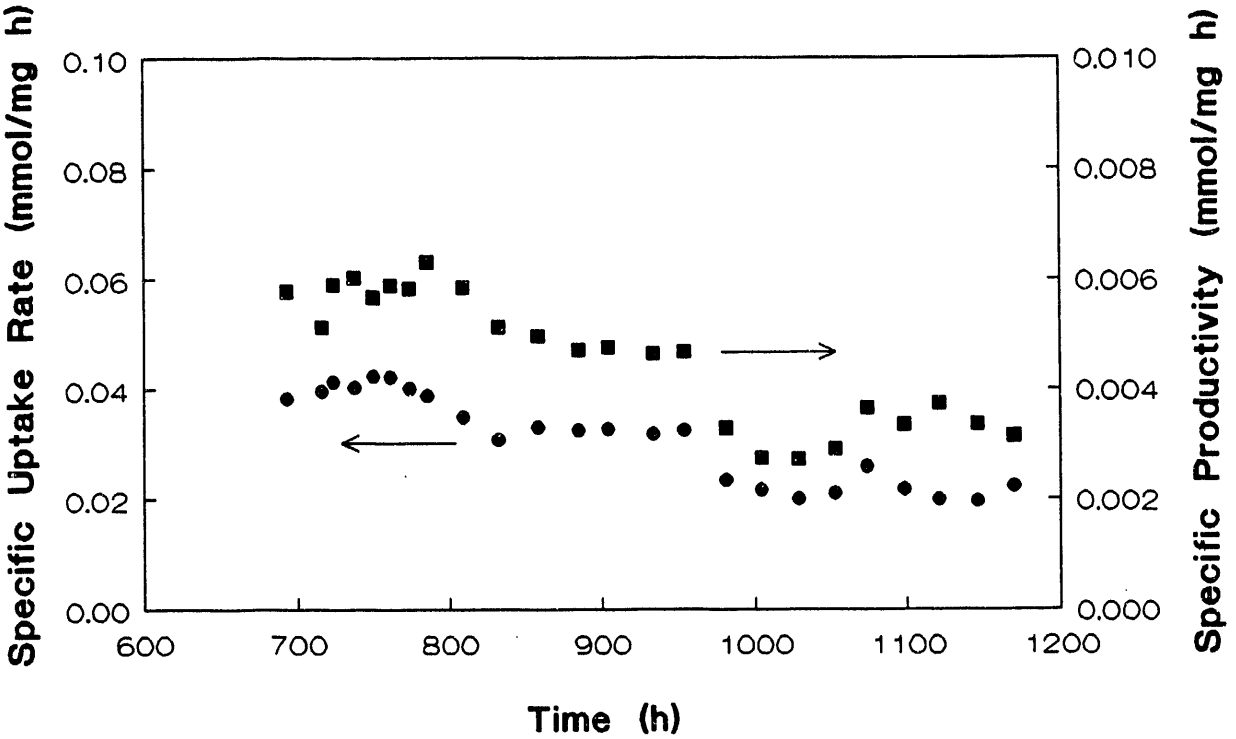


Figure 5.12. Specific Uptake Rate and Specific Productivity for *C. ljungdahlii* in the CSTR (400 rpm; Gas Flow Rate: 0.145 mmol/min; Liquid Flow Rates: 200, 100 mL/d)

Similarly, the specific productivity fell from 0.005 to 0.003 for the flow rate decrease. More will be said of these trends later.

A compilation of the CSTR data as a function of liquid flow rate is presented in Figures 5.13-5.17. Studies at two close but very different agitation rates are presented. This small difference in agitation rate is significant because the reaction system changes from gentle to vortex mixing at approximately 400 rpm. With vortex mexing, continuous breaking of the gas-liquid interface surface occurs, thereby significantly affecting gas-liquid mass transfer.

Figure 5.13 shows the effects of liquid flow rate and, to a lesser extent, agitation rate on cell density achieved in the CSTR. As is noted, the cell density decreased along a rather gentle curve, independent of agitation rate. The cell concentration was about 420 mg/L at a liquid flow rate of 50 mL/d, and was about 200 mg/L at a flow rate of 400 mL/d. The results in Figure 5.13 are expected since at lower flow rates, the liquid and cells spend more time in the reactor, thus allowing for the higher cell concentration.

Figure 5.14 shows the effects of liquid flow rate and the two agitation rates on the total products concentration, expressed as mmol of product per liter. As with cell concentration, the total product concentration fell from about 130 mmol/L at a flow rate of 50 mL/d to 30 mmol/L at a flow rate of 400 mL/d. Since it was earlier shown that product formation and cell concentration were directly linked, the results of Figure 5.14 are expected.

The effects of liquid flow rate and the two agitation rates on individual product (ethanol and acetate) concentrations are shown in Figure 5.15 and 5.16. In contrast to the total product concentration profile presented in Figure 5.14, the agitation rate was shown to affect both the ethanol and

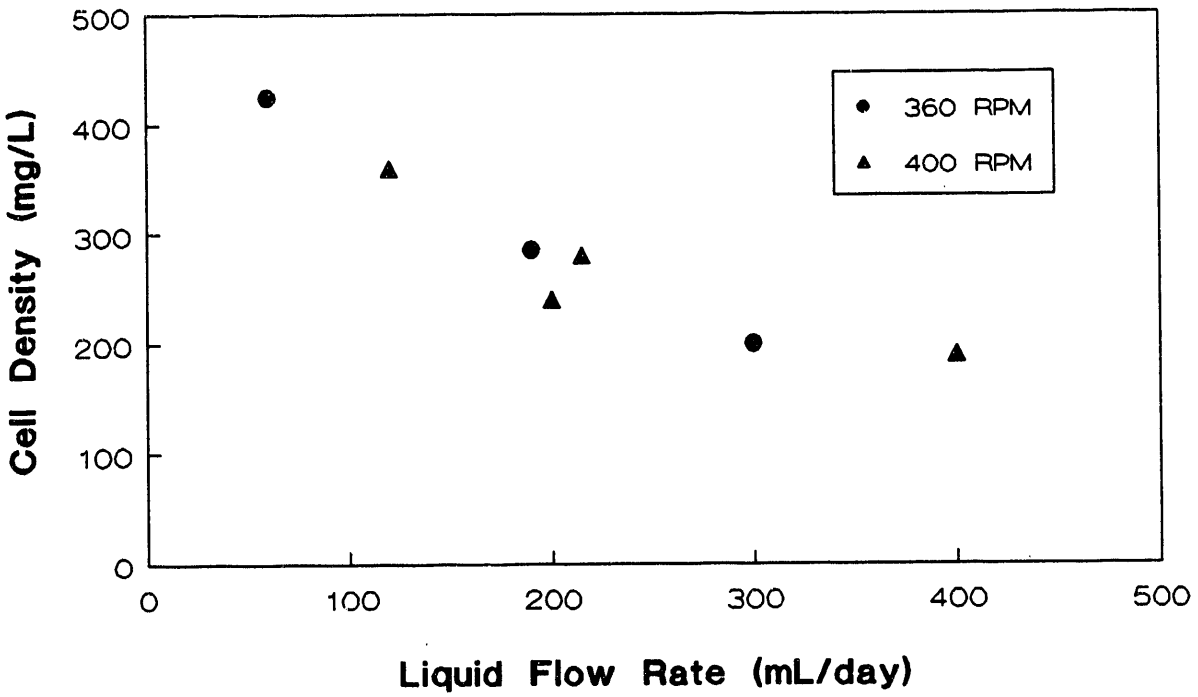


Figure 5.13. The Effects of Liquid Flow Rate On Cell Concentration Using *C. ljungdahlii* in the CSTR.

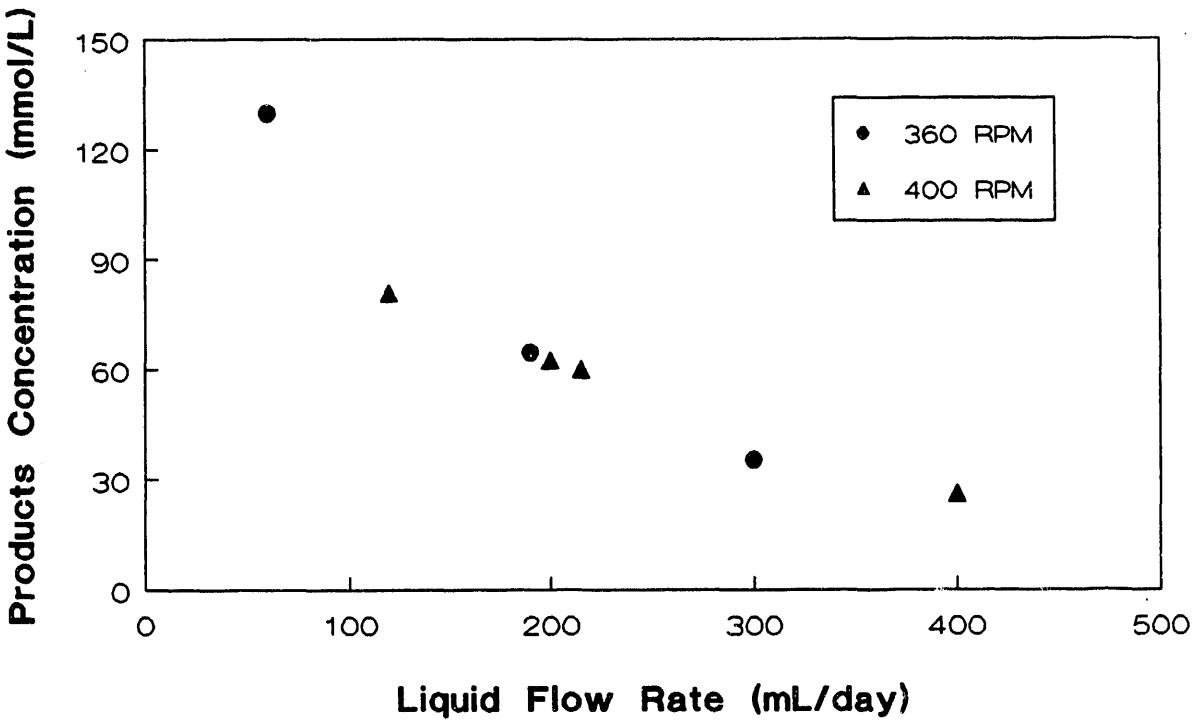


Figure 5.14. The Effects of Liquid Flow Rate on Total Product Concentration Using *C. ljungdahlii* in the CSTR.

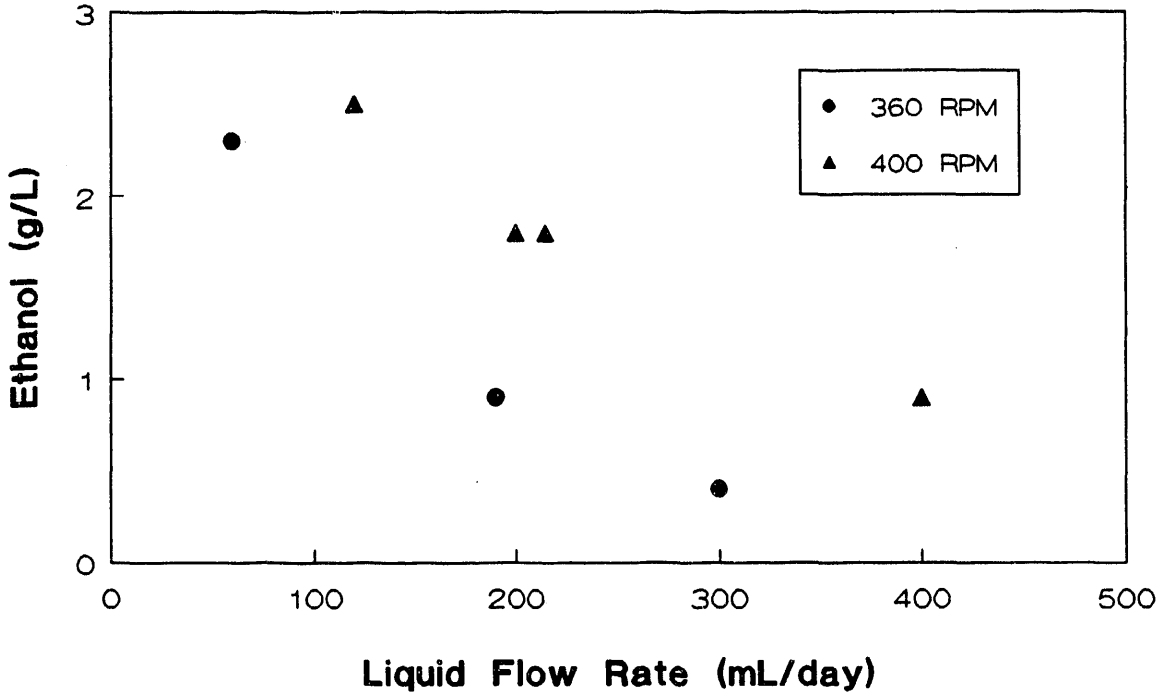


Figure 5.15. The Effects of Liquid Flow Rate on Ethanol Concentration Using *C. ljungdahlii* in the CSTR.

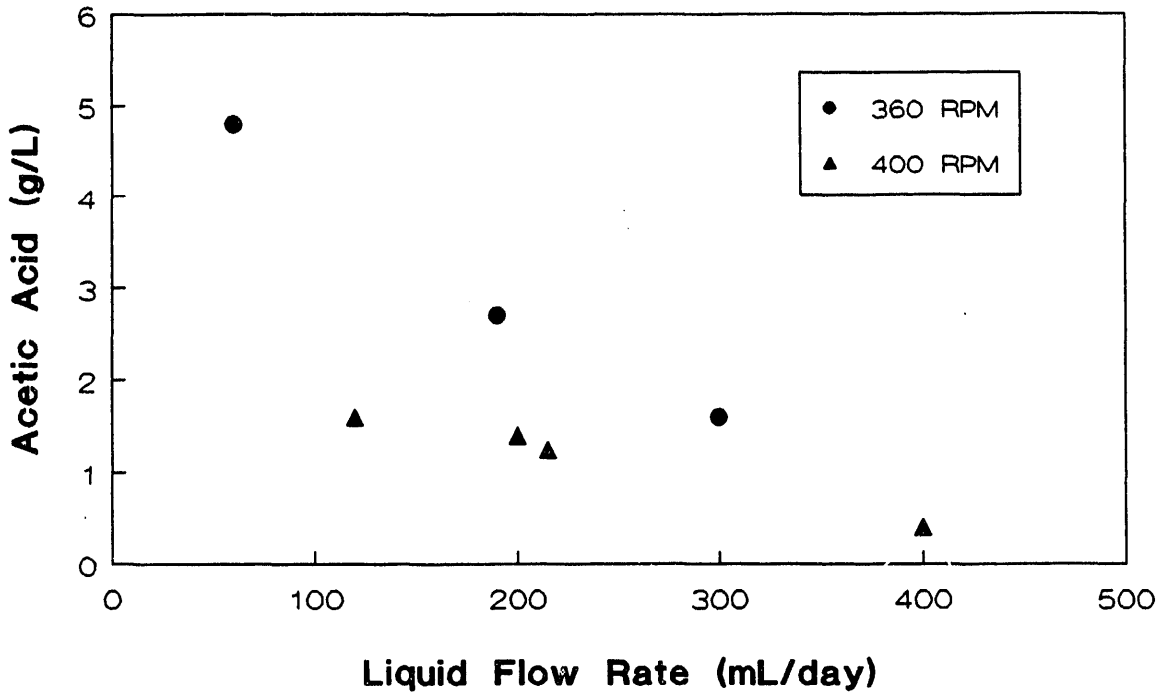


Figure 5.16. The Effects of Liquid Flow Rate on Acetic Acid Concentration Using *C. ljungdahlii* in the CSTR.

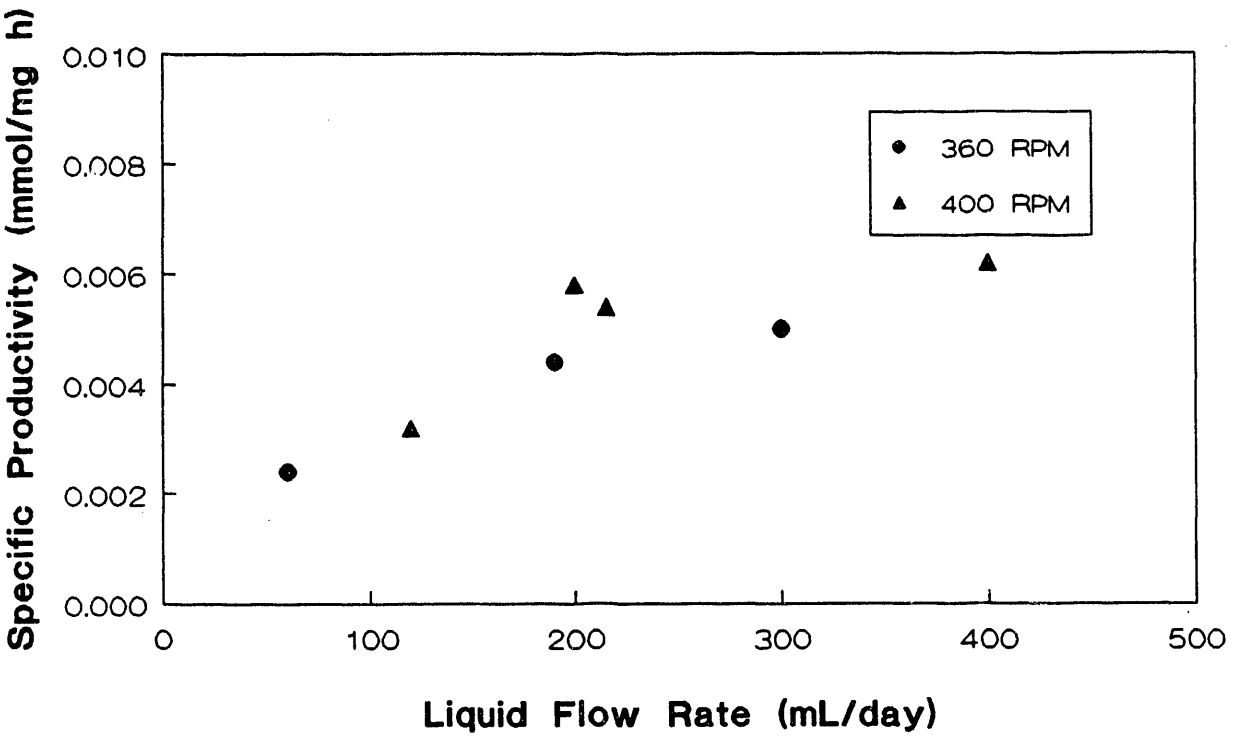


Figure 5.17. The Effects of Liquid Flow Rate on Specific Productivity Using *C. ljungdahlii* in the CSTR.

acetate concentrations. Higher ethanol concentrations were obtained at a given flow rate at 400 rpm (see Figure 5.15), and higher acetate concentrations were obtained at a given flow rate at 360 rpm (see Figure 5.16). In projecting continuous curves in the two figures, it is seen that at an agitation rate of 360 rpm and a liquid flow rate of 50 mL/d, the ethanol concentration was about 2.3 g/L and the acetate concentration was about 4,8 g/L. If the agitation rate is changed to 400 rpm at the same liquid flow rate of 50 mL/d, the projected ethanol concentration would be about 3.2 g/L and the acetate concentration would be about 2 g/L. Similar results are obtained at other liquid flow rates. Clearly, from the standpoint of attaining higher ethanol concentrations and higher product ratios, low liquid flow rates and higher agitation rates should be employed. Apparently the higher agitation rates cause more CO to be deposited into the liquid phase which stresses the culture. This stressful or inhibiting situation results in the production of ethanol in favor of acetate. The limits of this theory should be tested in agitation rate studies at a constant liquid flow rate.

The specific productivity (total product) is plotted as a function of liquid flow rate for the two agitation rates in Figure 5.17. A single curve is shown for the two agitation rates which curves gently upward with increasing liquid flow rate. The highest productivity, 0.006 mml product/mg h, is attained at a liquid flow rate of 400 mL/d. It is obvious, as expected, that the more flow through the system, the higher the productivity of the reactor. The specific uptake rate similarly increases with increasing liquid flow rate (see Figure 5.12).

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.

5.2.2 Agitation Rate Effects

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 5.18 and 5.19 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 5.18, 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 5.19 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

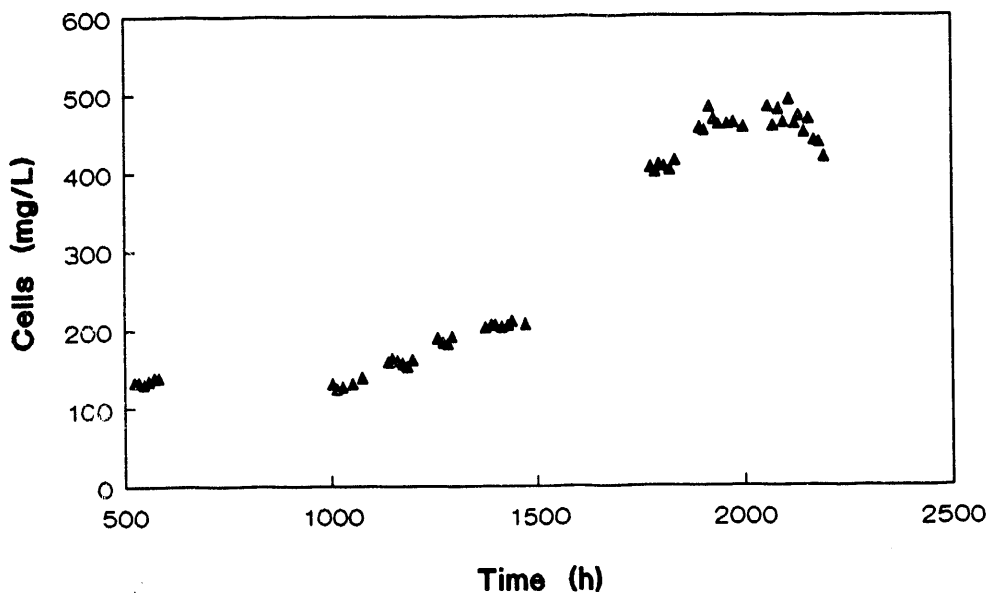


Figure 5.18. Cell concentration profile *C. ljungdahlii* in the CSTR (Gas Flow Rate: 0.02–00.15 mmol/mon; Liquid Flow Rate: 200 mL/d; Agitation Rate: 300–480 rpm).

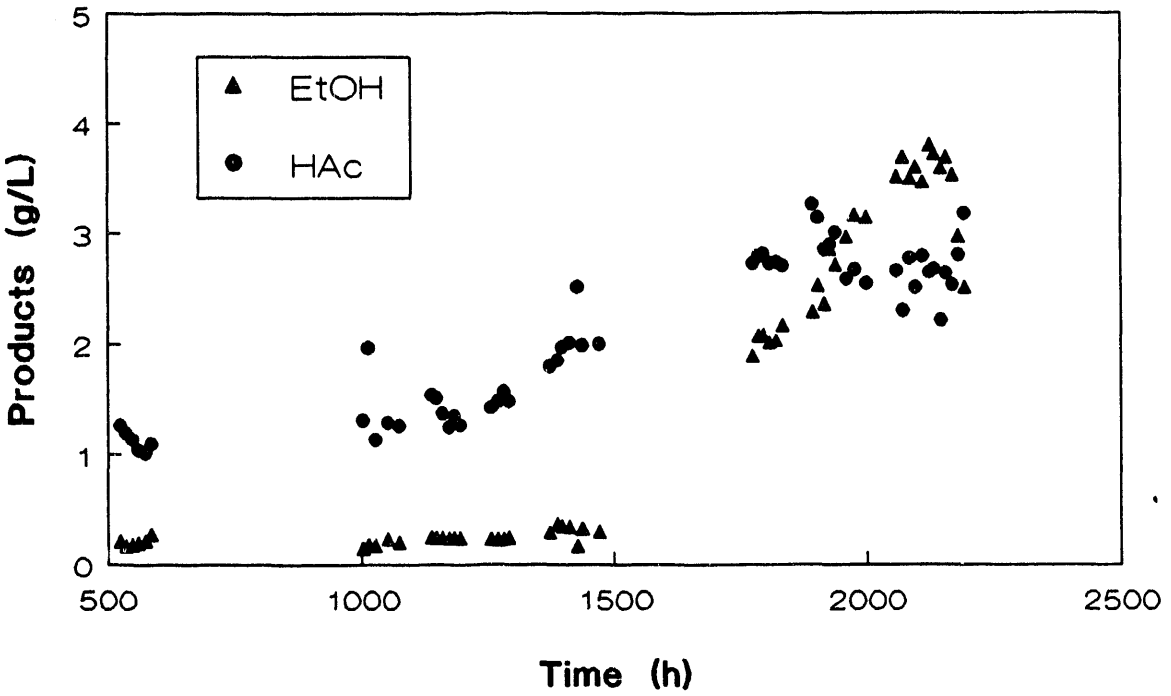


Figure 5.19 Product Concentration Profile for *C. ljungdahlii* in the CSTR; (Gas Flow Rate: 0.02 - 0.15 mmol/min; Liquid Flow Rate: 200 mL/d; Agitation Rate 300 - 480 rpm)

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 5.20. 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 5.21 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 5.22 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 5.23-5.25. Figure 5.23 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 would, at the same time, result in acetate production in favor of ethanol.

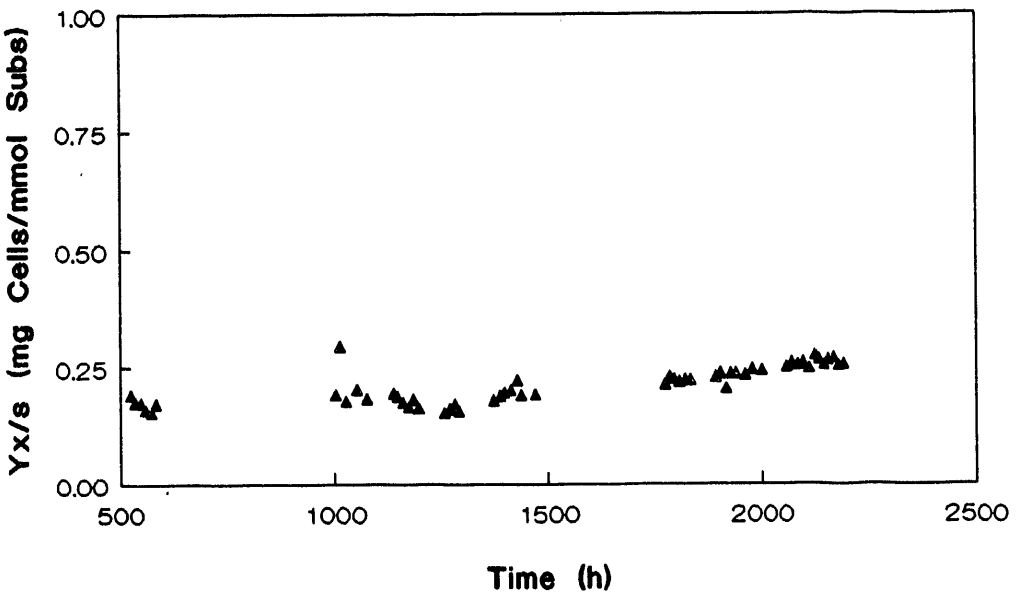


Figure 5.20. Cell yield from substrate for *C. ljungdahlii* in the CSTR (Gas Flow Rate: 0.02-00.15 mmol/mon; Liquid Flow Rate: 200 mL/d; Agitation Rate: 300-480 rpm).

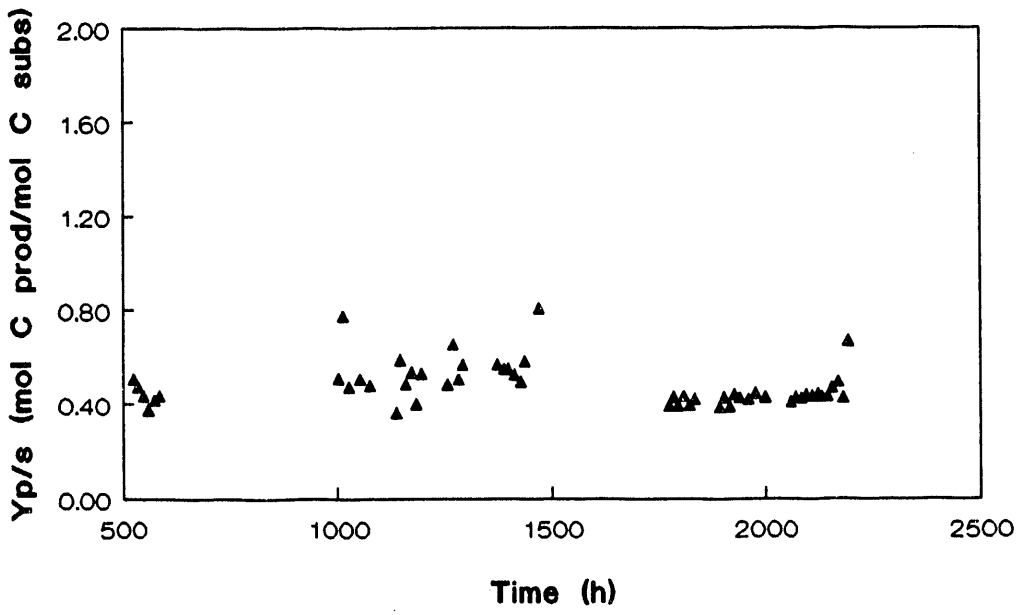


Figure 5.21. Product yield from substrate for *C. ljungdahlii* in the CSTR (Gas Flow Rate: 0.02–00.15 mmol/mon; Liquid Flow Rate: 200 mL/d; Agitation Rate: 300–480 rpm).

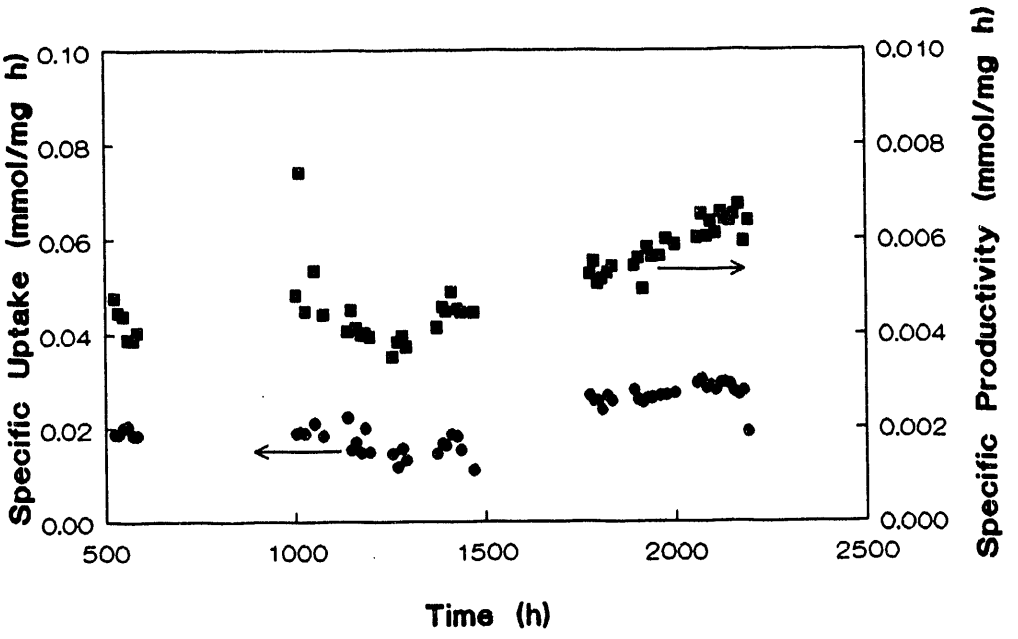


Figure 5.22. Specific uptake rate and specific productivity for *C. ljungdahlii* in the CSTR (Gas Flow Rate: 0.02–00.15 mmol/mon; Liquid Flow Rate: 200 mL/d; Agitation Rate: 300–480 rpm).

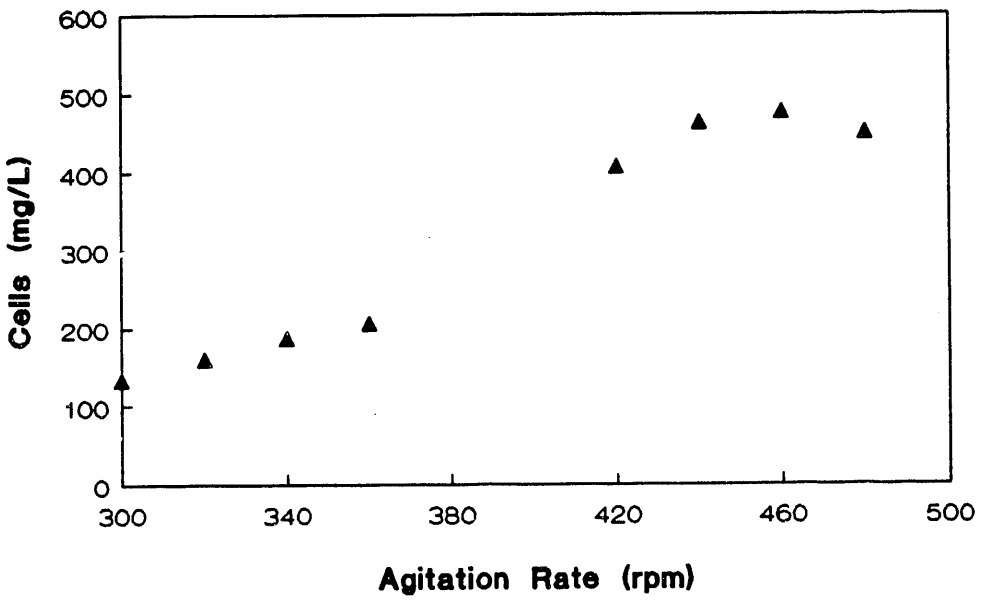


Figure 5.23. The effects of agitation rate on cell concentration using *C. ljungdahlii* in the CSTR.

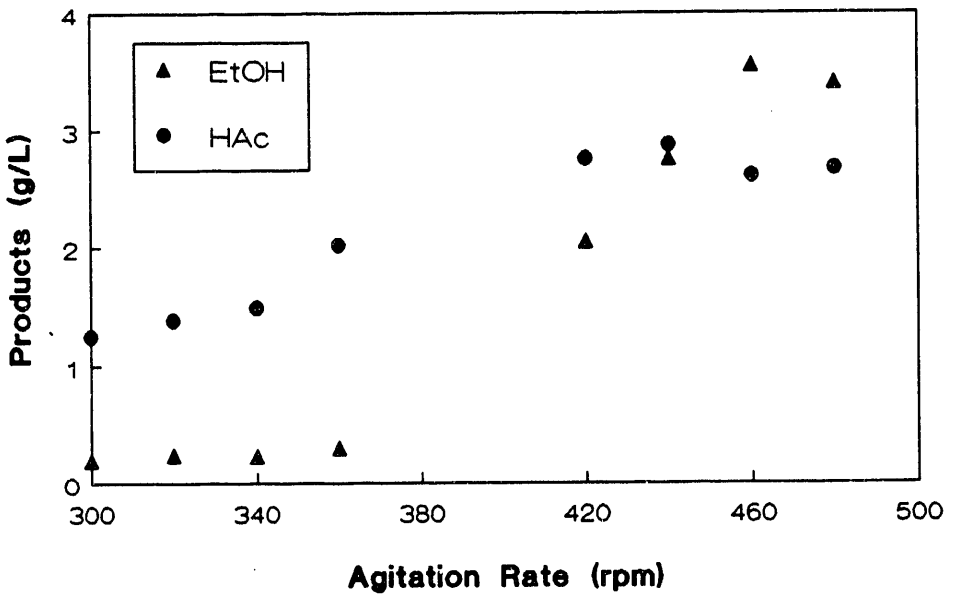


Figure 5.24. The effects of agitation rate on product concentration using *C. ljungdahlii* in the CSTR.

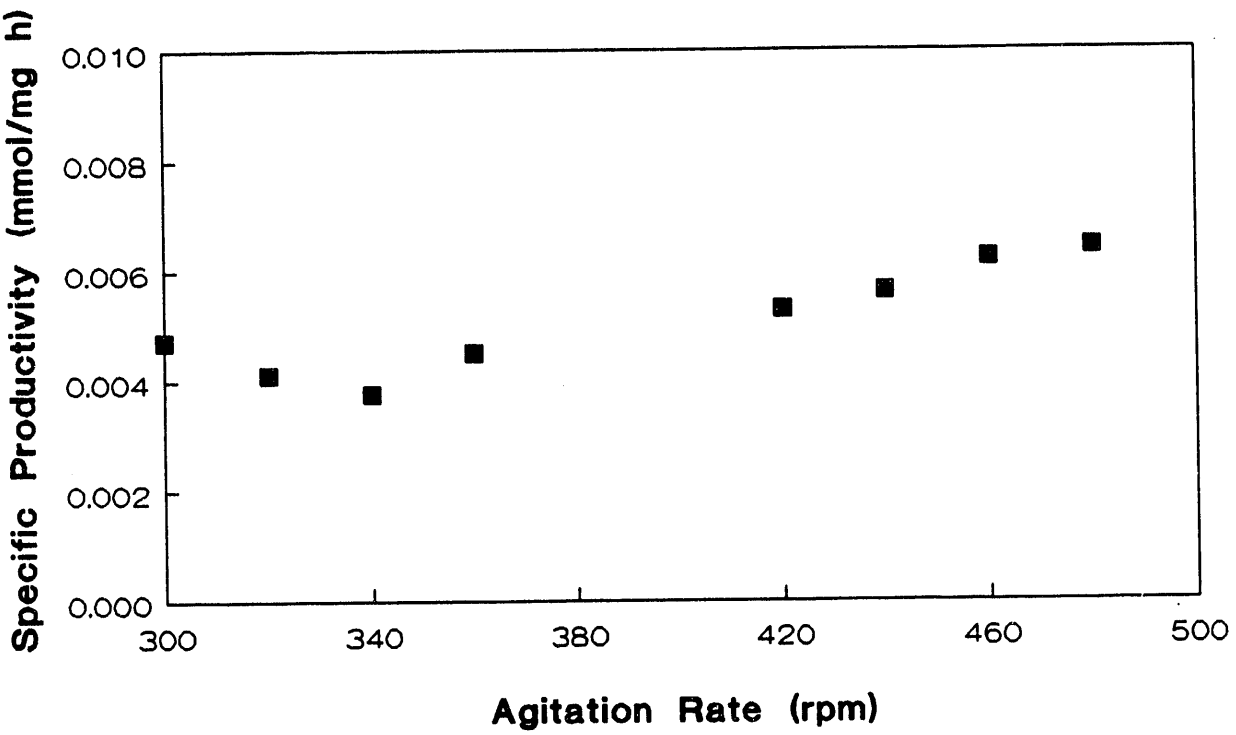


Figure 5.25 The Effects of Agitation Rate on Specific Productivity Using *C. ljungdahlii* in the CSTR.

Figure 5.24 shows the effects of agitation rate on the ethanol and acetate concentrations in g/L. A low agitation rates (≤ 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 5.23 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 5.23. 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 5.25. 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.

It was shown previously that higher agitation rates resulted in higher cell concentrations (up to 480 rpm), higher ethanol concentrations with essentially constant acetate concentrations and higher specific productivities. However, data were not available at agitation rates between 360 and 420 rpm. These data have now been obtained. The effects of agitation rate on cell concentration, products concentration, and specific productivity are shown, respectively, in Figures 5.26-5.28. These data are a compilation of data using procedures presented previously.

Figure 5.26 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 rpm and above. The cell density fell slightly at an agitation rate of 480 rpm indicating an increase in dissolved CO to inhibitory levels. 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 5.27 shows the effects of agitation rate on the ethanol and acetate concentrations in g/L. At 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.

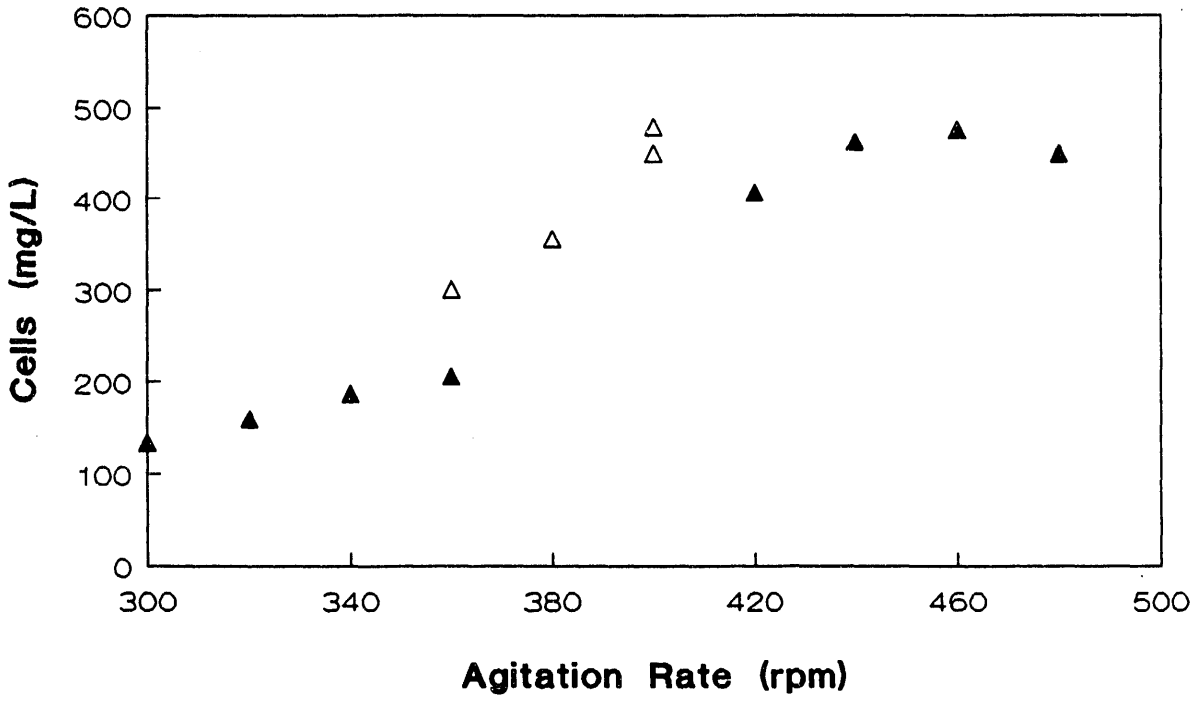


Figure 5.26 The Effects of Agitation Rate on Cell Concentration Using *C. ljungdahlii* in the CSTR.

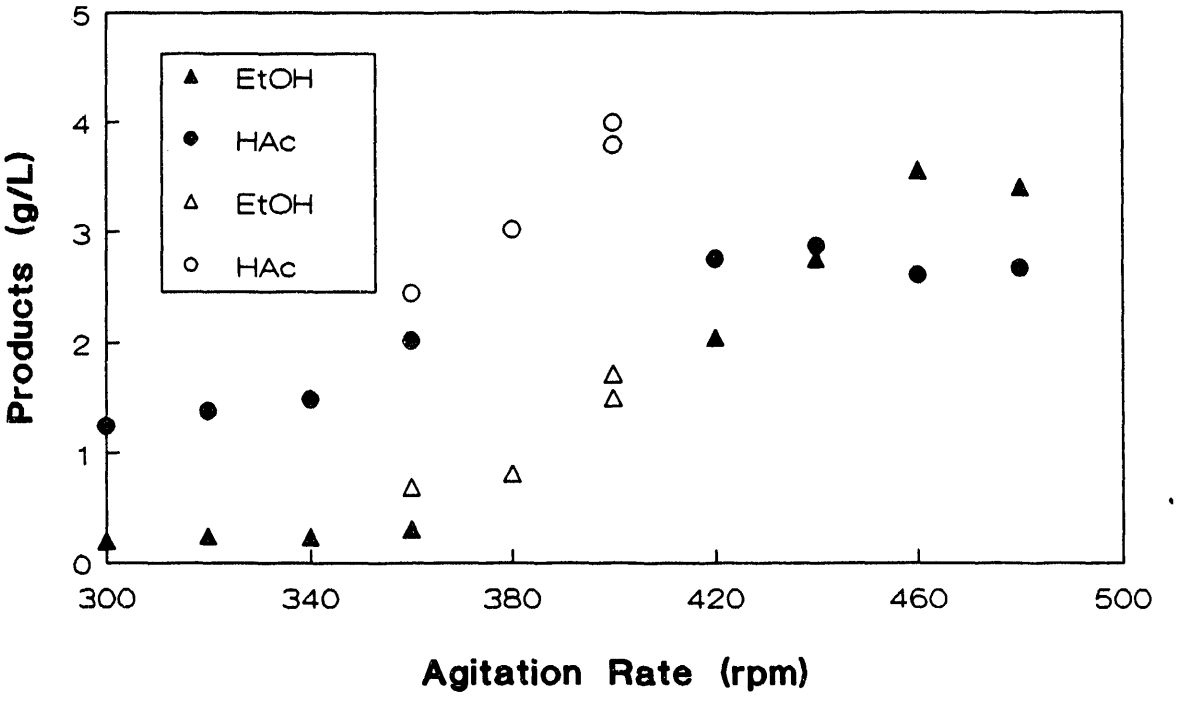


Figure 5.27 The Effects of Agitation Rate on Product Concentrations Using *C. ljungdahlii* in the CSTR.

A 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 5.26, 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 and substrate mass transfer exceeded that required for growth as indicated by the constant cell concentration in Figure 5.26. 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 5.28. 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.

5.2.3 Gas Flow Rate Effects

The effects of inlet gas flow rate on the performance of *C. ljungdahlii* in the CSTR were also studied. In this study, the agitation rate was held constant at 400 rpm, the liquid flow rate was 190 mL/d and the temperature was held constant at 35°C. Figures 5.29-5.31 show the effects of gas flow rate on

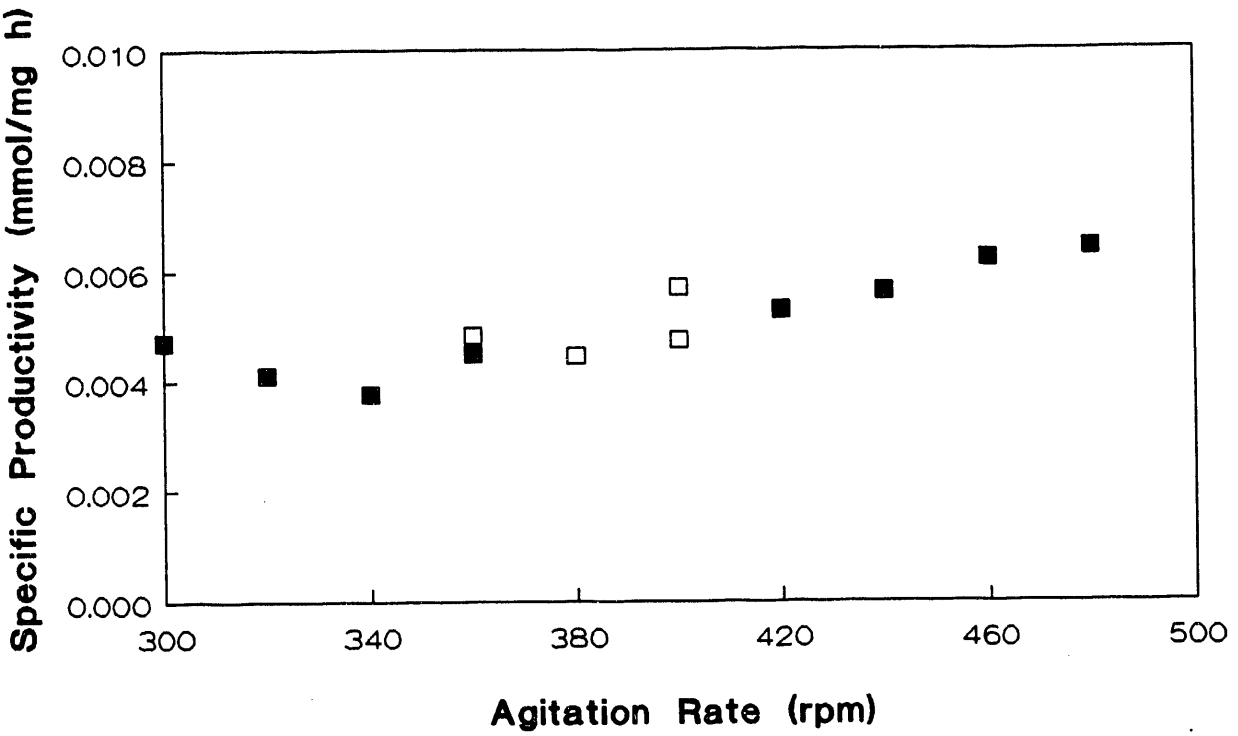


Figure 5.28 The Effects of Agitation Rate on Specific Productivity Using *C. ljungdahlii* in the CSTR.

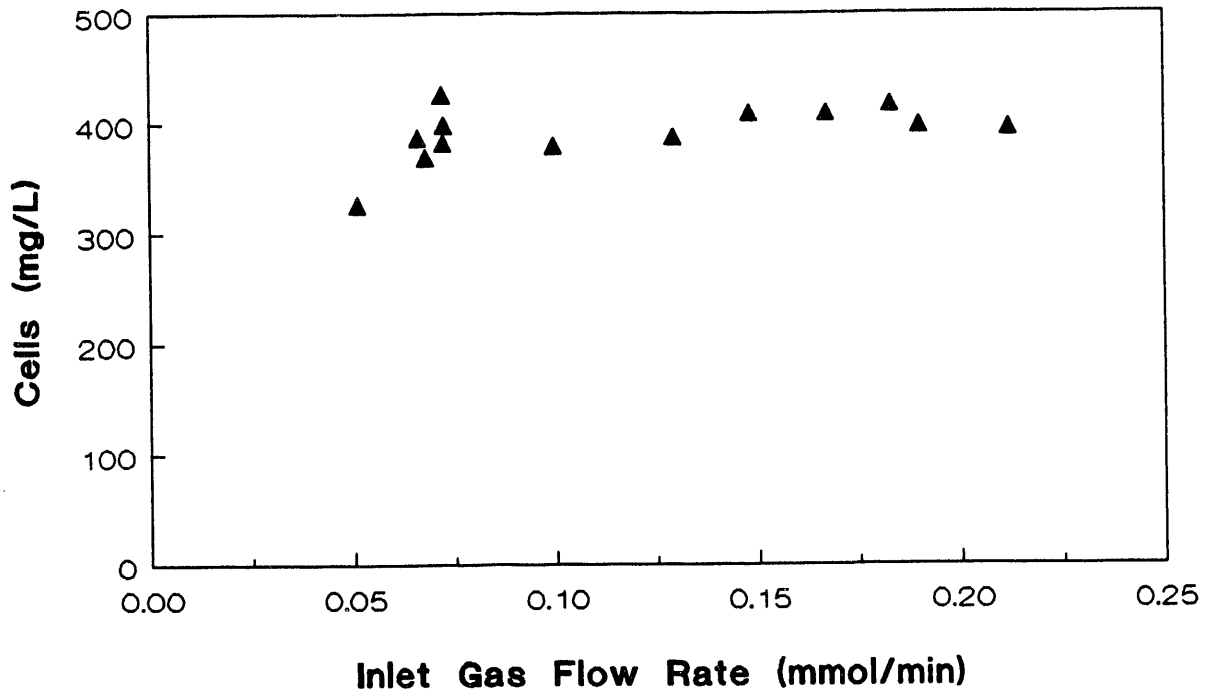


Figure 5.29 The Effects of Gas Flow Rate on Cell Concentration Using *C. ljungdahlii* in the CSTR.

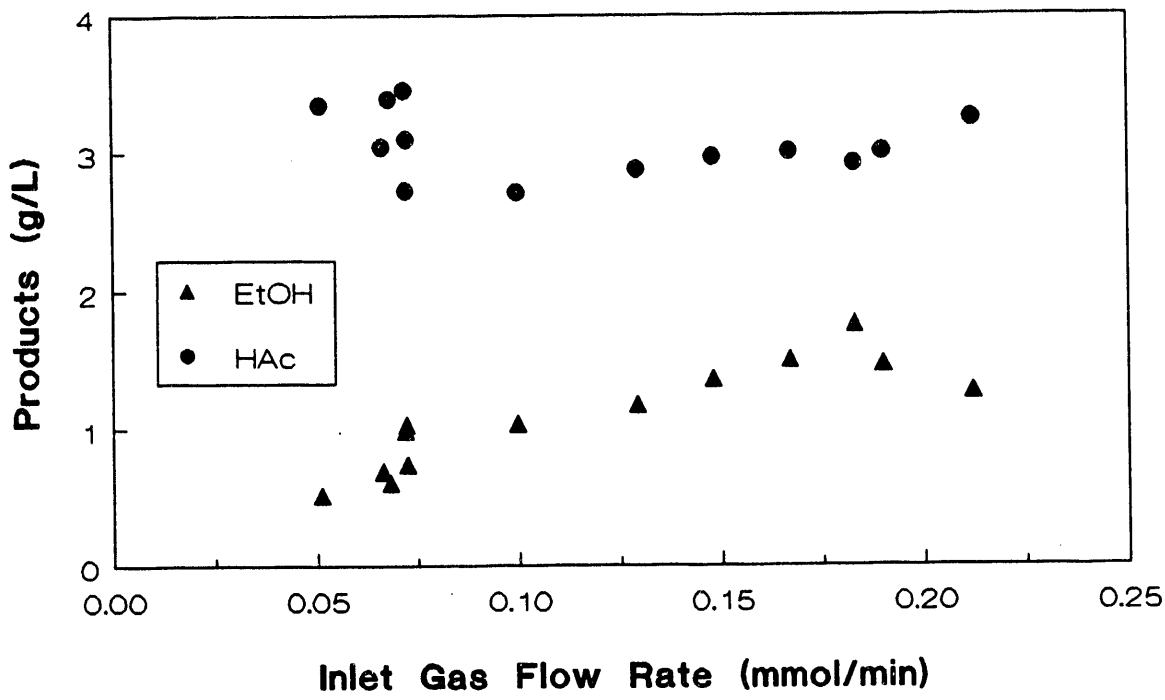


Figure 5.30 The Effects of Gas Flow Rate on Product Concentrations Using *C. ljungdahlii* in the CSTR.

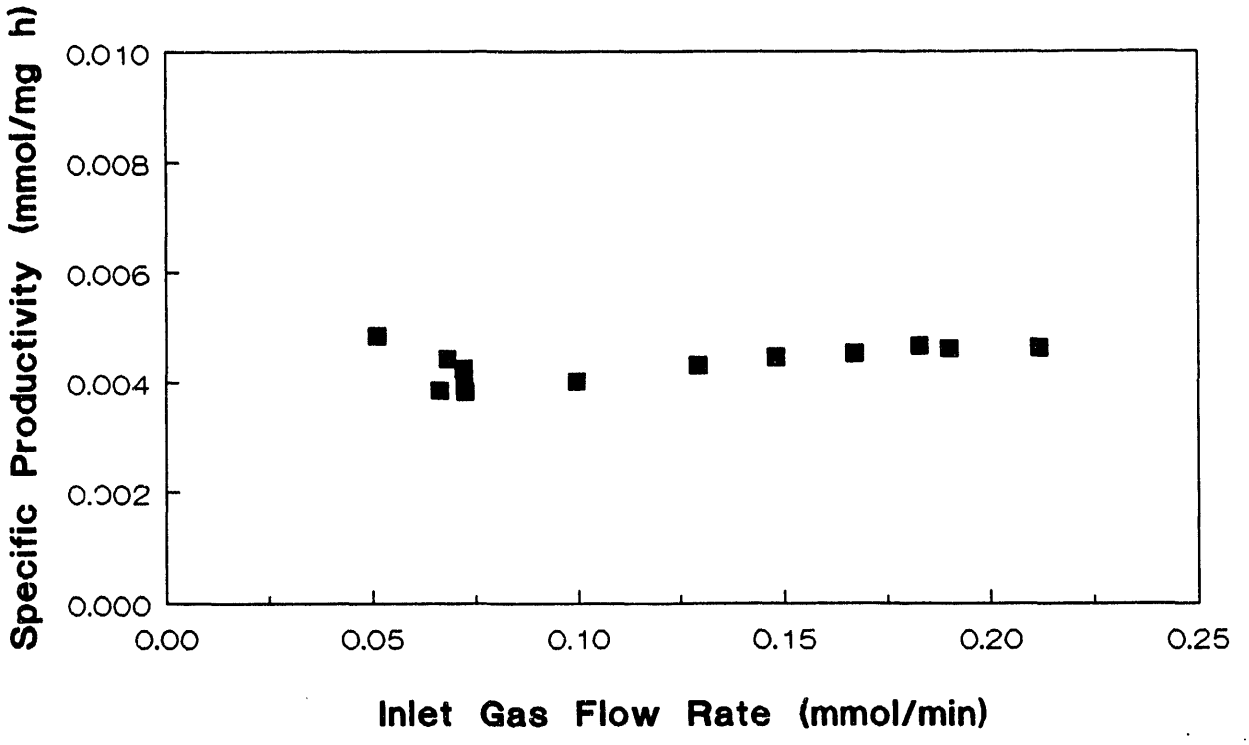


Figure 5.31 The Effects of Gas Flow Rate on Specific Productivity Using *C. ljungdahlii* in the CSTR.

cell concentration, product (ethanol and acetate) concentrations and the total specific productivity. As is noted in Figure 5.29, the cell concentration increased by almost 30 percent as the flow rate was increased from 0.05 to 0.075 mmol/min. At higher flow rates, the cell concentration leveled off at about 400 mg/L. This latter result is not expected since the cell concentrations should have increased with gas flow rate unless the system was mass transfer limited or the culture was nutrient limited. It is believed that the culture was probably nutrient limited since the conversion of CO was not complete and the nutrient medium employed was poor.

Figure 5.30 shows the effects of gas flow rate on ethanol and acetate concentration. At low flow rates (0.05-0.10 mmol/min), high acetate concentrations and low ethanol concentrations were observed. As the gas flow rate was increased, the acetate concentration nearly leveled off at about 3 g/L while the ethanol concentration increased from 1 to 1.5 g/L. This increase in ethanol concentration was probably due to nutrient limitations coupled with increased gas flow rate. As is noted in Figure 5.31, the specific productivity remained essentially constant at 0.004 mmol/mg h.

5.2.4 Performance Studies in the CSTR with Designed Medium

Growth, production and the specific production rate in the CSTR are shown for basal medium (Figures 5.32-5.34) and for the designed medium (Figures 5.35-5.37). Growth on the designed medium, while falling well short of the 3.0 grams of cells expected for nitrogen limitation, was 4 to 5 times the level achieved in basal medium (See Figures 5.32 and 5.35). The maximum cell concentration is basal medium was 400 mg/L, and the maximum in the designed medium was 1500 mg/L. The product concentrations in the CSTR (see Figures

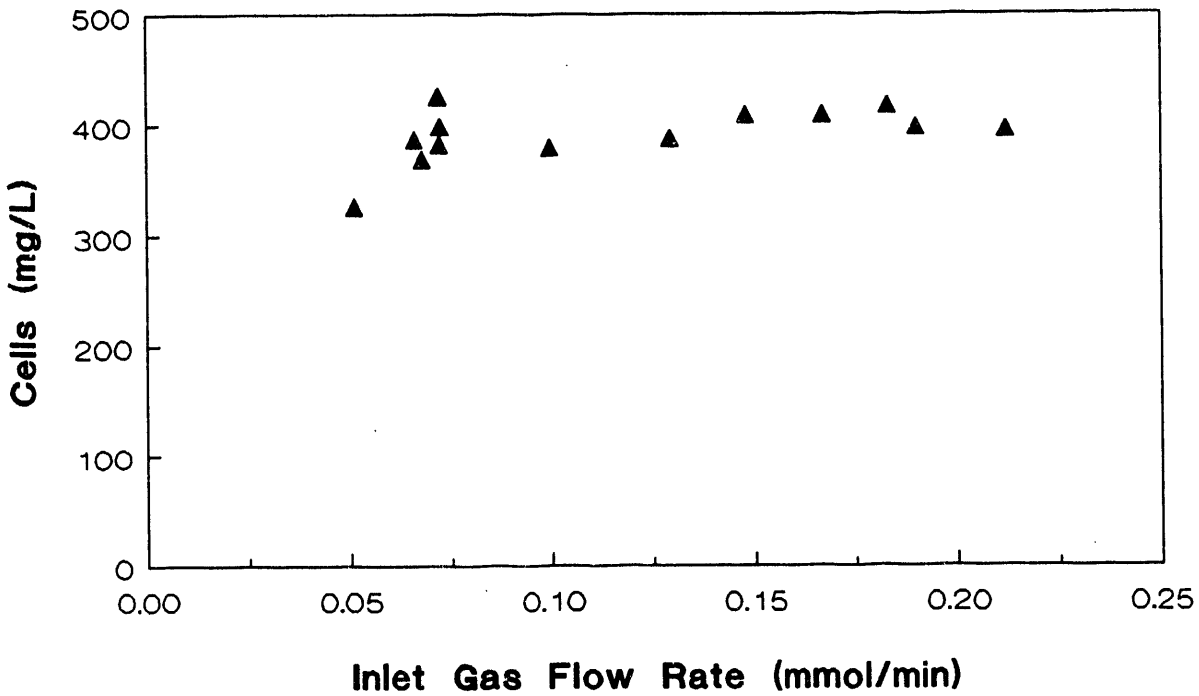


Figure 5.32. Cell Concentration of *C. ljungdahlii* Grown in Basal medium in the CSTR.

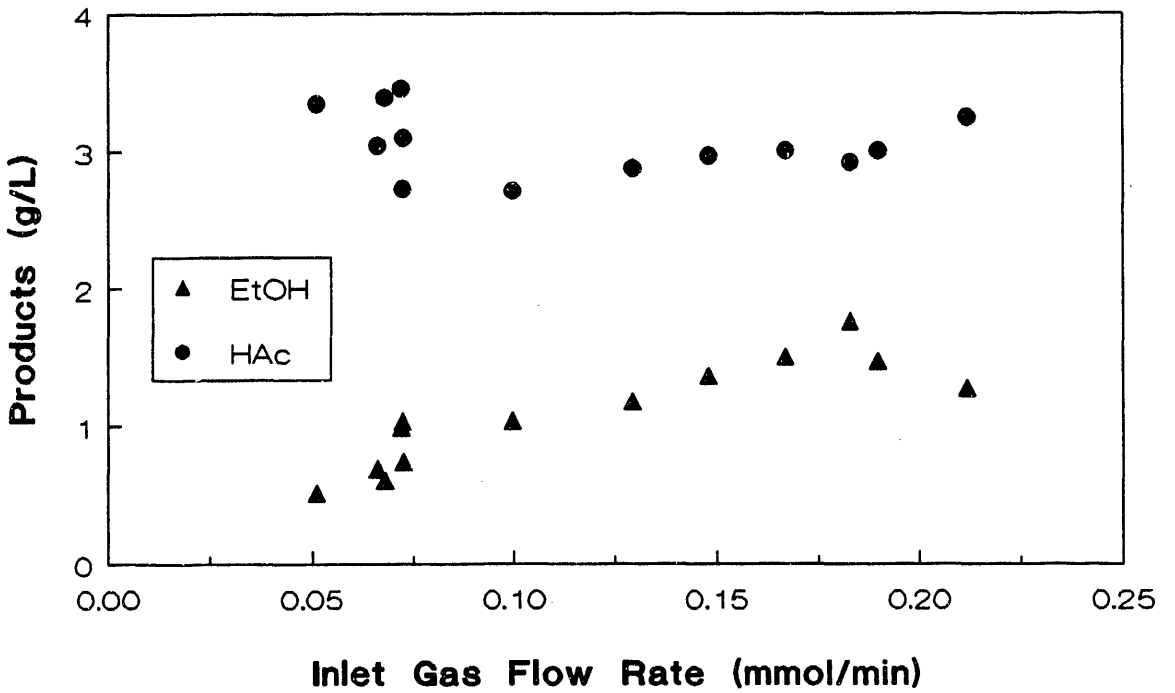


Figure 5.33. Product Concentrations from growth of *C. ljungdahlii* in Basal Medium in the CSTR.

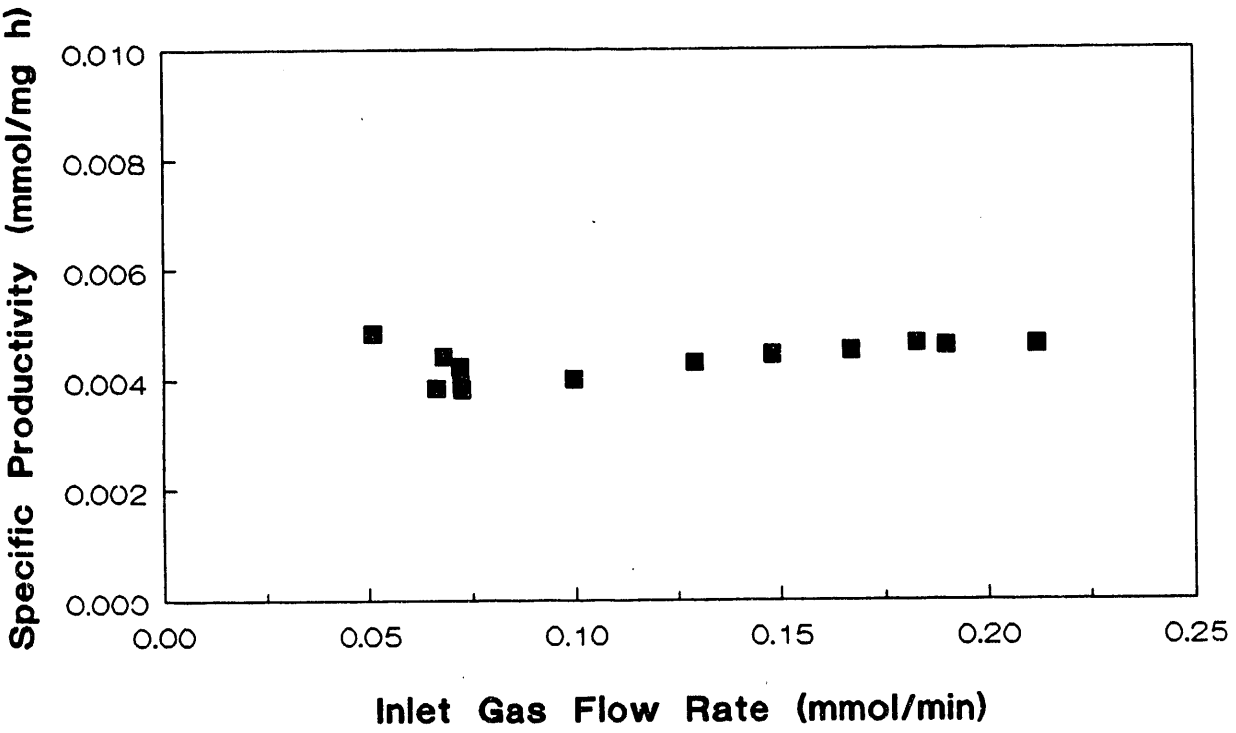


Figure 5.34. Specific Productivity of *C. ljungdahlii* Grown in Basal Medium in the CSTR.

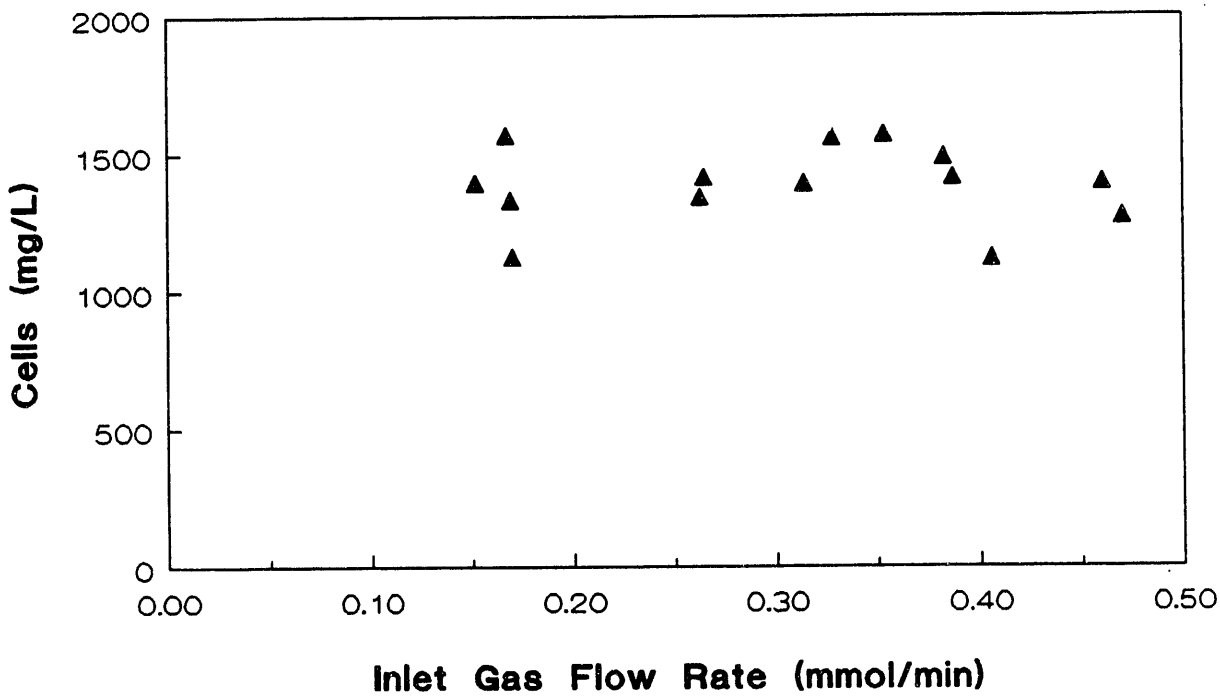


Figure 4.35. Cell Concentration of *C. ljungdahlii* Grown in Designed mediu in the CSTR.

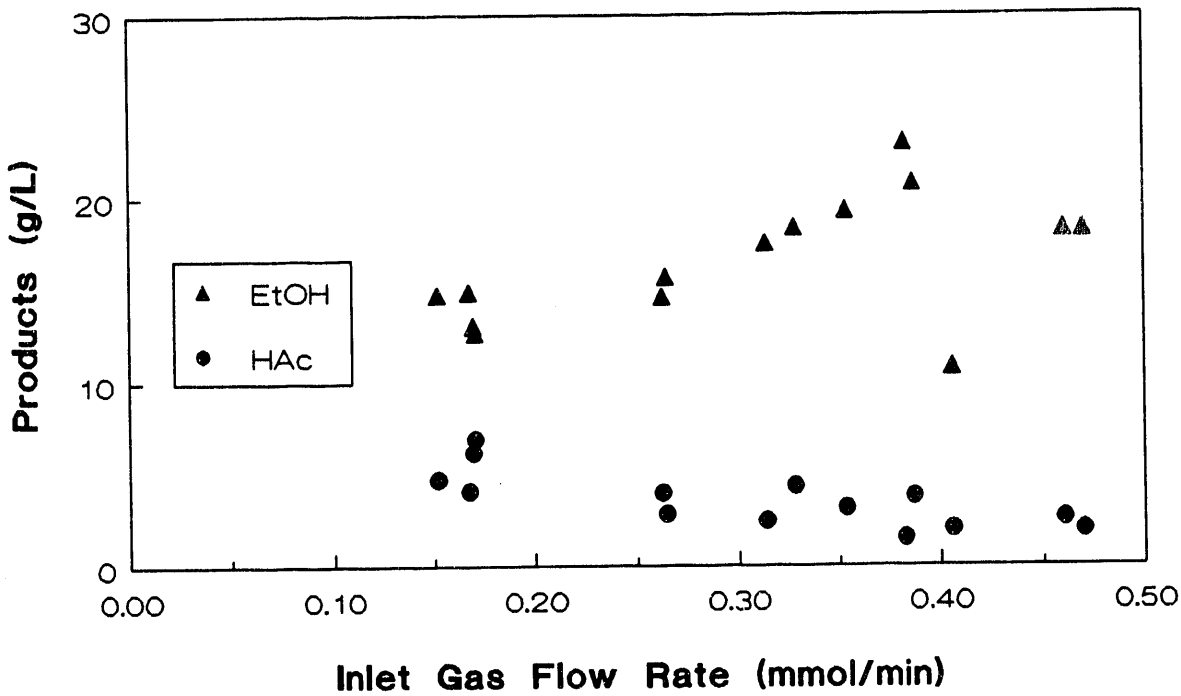


Figure 5.36. Product Concentrations from growth of *C. ljungdahlii* in Designed Medium in the CSTR.

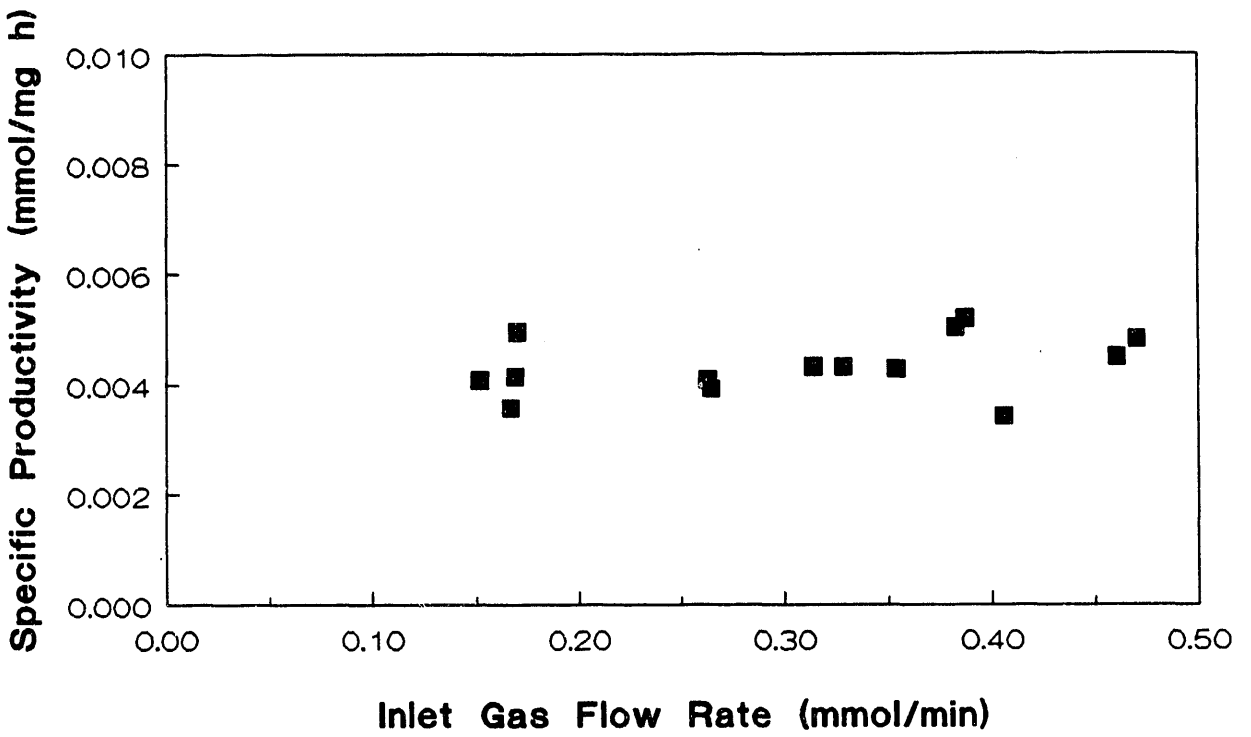


Figure 5.37. Specific Productivity of *C. ljungdahlii* Grown in Designed Medium in the CSTR.

5.33 and 5.36) for the designed medium were 5.5 times the concentrations in basal medium on a mass basis and 6.5 times the concentrations on a molar basis. The maximum ethanol concentration in basal medium was about 1.5 g/L and the maximum concentration in the designed medium was about 22 g/L. The product ratio was also dramatically shifted toward ethanol, showing much more ethanol than acetate with the designed medium. The specific production rates calculated for both media were identical (see Figures 5.34 and 5.37). However, if the cell density given for the designed medium is low as suspected, the specific production rate in the designed medium is about 20 percent lower than in basal medium. The dilution rates and thus the specific growth rates in these experiments were 0.024 h^{-} in basal medium and 0.014 in the designed medium (1.75 and 2.92 days retention time, respectively). The comparison of the media on a gross performance basis is nonetheless valid. 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 design of the medium based on *E. coli* was very successful despite the difference in the model organism in comparison to *C. ljungdahlii*. Culture performance should improve further if the medium design can be based on an analysis of *C. ljungdahlii*. Nutrient limitation studies, using an approximation of the basal medium with a specific nutrient concentration reduced, were used to determine minimum requirements of each element in the *E. coli* analysis.

As an example, a summary of the results of the potassium study is shown in Figures 5.38-5.41. Figure 5.38 shows growth limitation from potassium

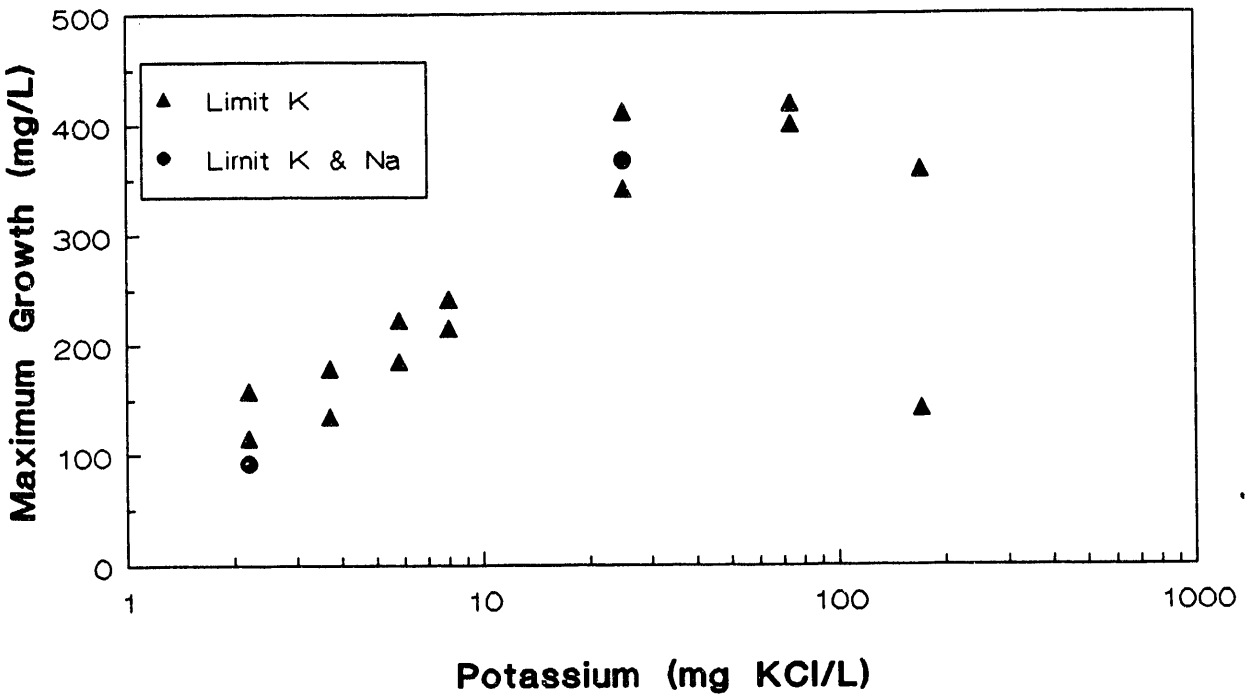


Figure 5.38. Maximum Cell Concentration of *C. ljungdahlii* Grown Under Potassium Limitation in Batch Culture.

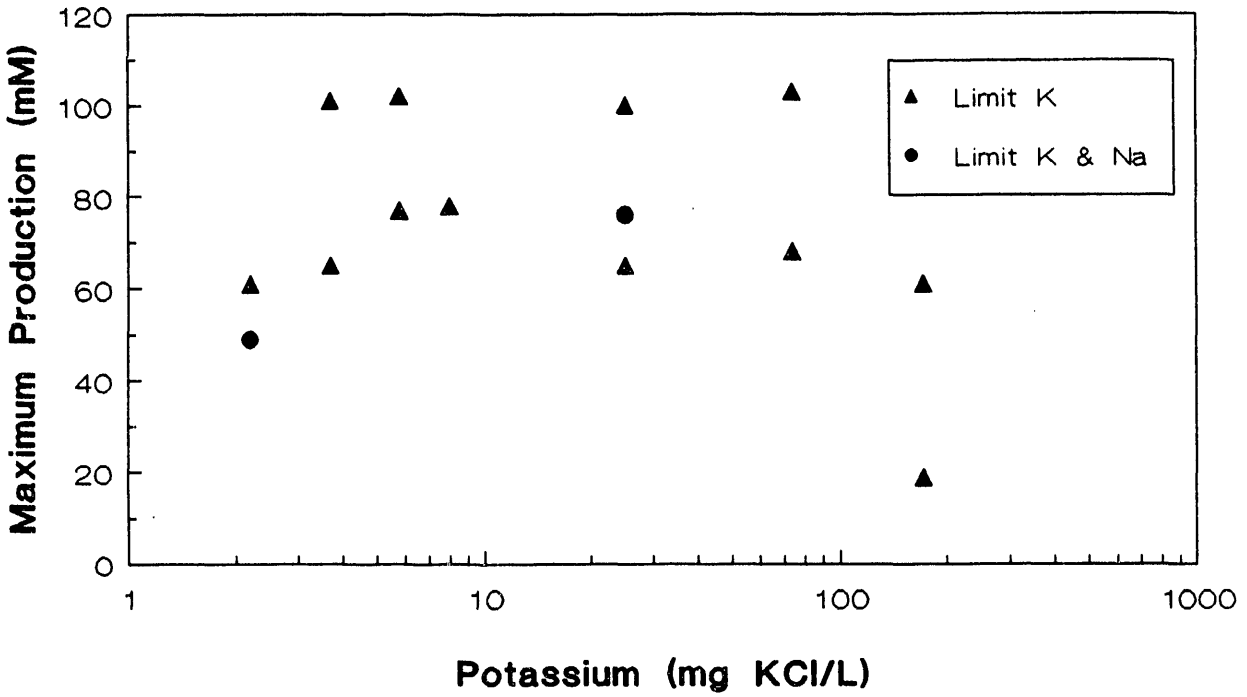


Figure 5.39. Maximum Product Concentration from *C. ljungdahlii* Grown Under Potassium Limitation in Batch Culture.

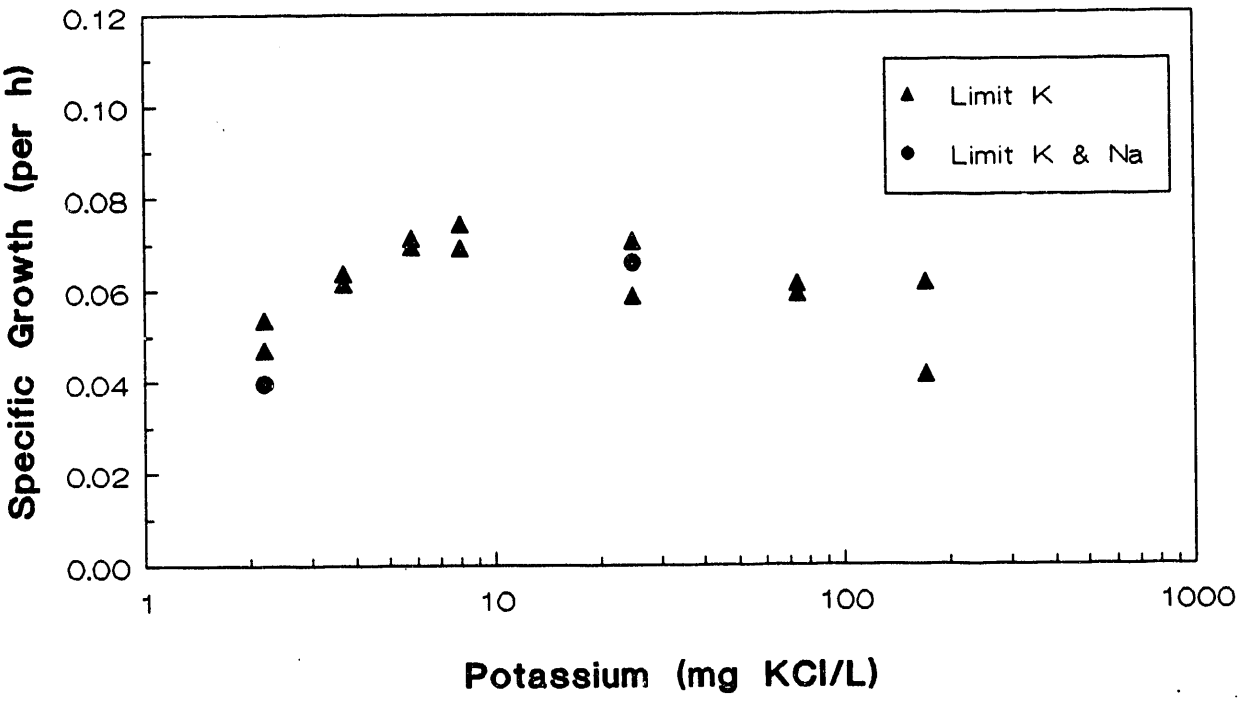


Figure 5.40. Specific Growth Rate of *C. ljungdahlii* Grown Under Potassium Limitation in Batch Culture.

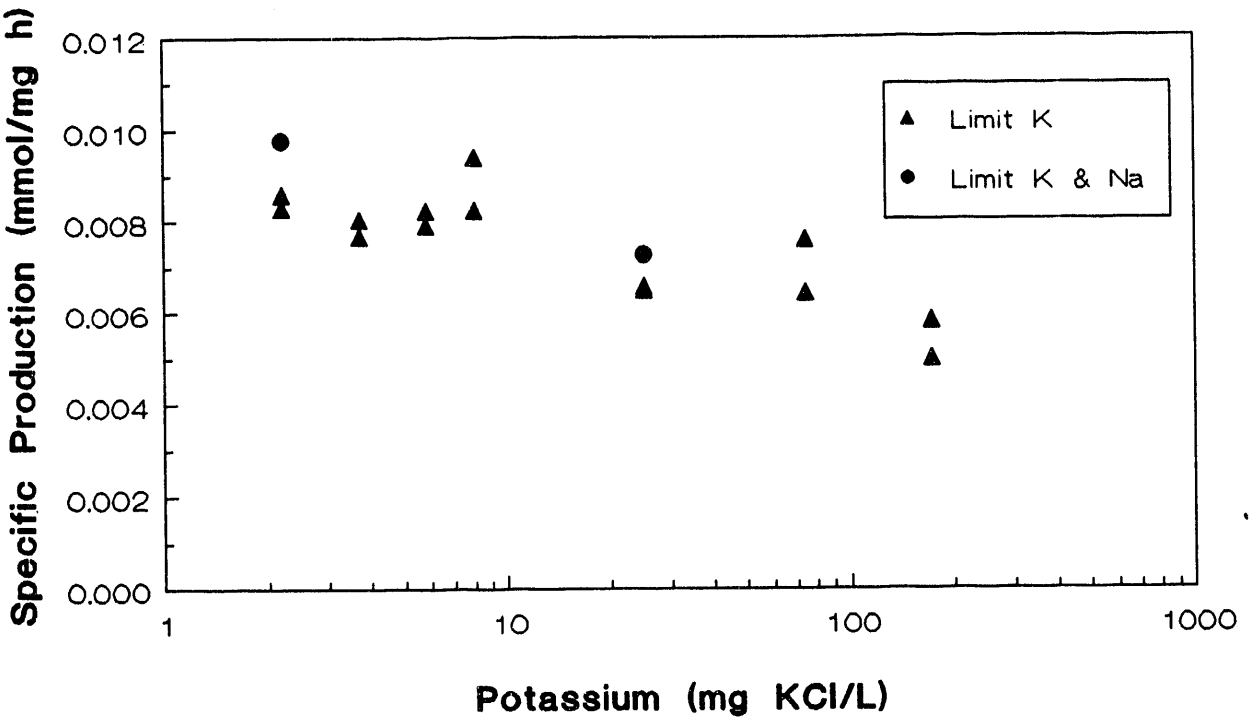


Figure 5.41. Specific Production Rate for *C. ljungdahlii* Grown Under Potassium Limitation in Batch Culture.

concentration, in basal medium, below 20 mg/L KCl. The maximum production accompanying this growth (Figure 5.39) appears to be relatively insensitive to potassium concentration. The specific growth and production rates (Figures 5.40 and 5.41) increase slightly as the potassium concentration is decreased to 10 mg/L KCl. This study shows a maximum of 71.8 g of cells per g KCl and an optimum concentration of potassium near 10 mg/L KCl. This implies that potassium is about 0.7 percent of the dry weight of *C. ljungdahlii* cells.

A similar study was performed for each of the major elements in the defined basal medium. The results of these studies are summarized in Table 5.1 as an approximate elemental analysis of *C. ljungdahlii*. This analysis will now be used to adjust the production medium for further study.

5.2.5 Arginine Addition to the Medium to Decrease Start-Up Time

Doyle[30] showed that *C. ljungdahlii* utilizes arginine from the peptone/amino acid pool as its primary carbon and energy source. It was thus felt that arginine could possibly stimulate the growth of *C. ljungdahlii*, thereby shortening the time necessary for the start-up of a CSTR fermentation system. The possible negative effects of utilizing aginine such as decreased ability to utilize CO and H_2, increased lag phases upon the addition of CO and H_2, etc. also need to be investigated.

A small quantity of arginine (10 mg/L) was added to the fermentation medium and fed to a CSTR. The pH of the system was 5.0 and the temperature was 35-37°C. The medium flow rate was held constant at about 180 mL/day, and the gas flow rate was fed at a very low level.

Figures 5.42-5.44 show the results of this experiment as cell concentration measurements, CO and H_2 conversions and product concentration

Elemental Composition of Bacteria

Percent of Dry Weight

	E. coli	*C. ljungdahlii*
Nitrogen	14.0	14.0
Phosphorus	3.0	1.2
Sulfur	1.0	1.2
Potassium	1.0	0.7
Sodium	1.0	<0.37
Calcium	0.5	<0.03
Magnesium	0.5	<0.086
Chlorine	0.5	*
Iron	0.2	0.097

Analysis of E. coli from J.E. Bailey and D.F. Ollis, "Biochemical Engineering Fundamentals" 2nd ed., McGraw Hill, 1986, Table 2.1 on p.28.

* Chlorine requirements were not determined; Chloride was used to balance ionic charge.

Table 5.1 Bacterial Composition

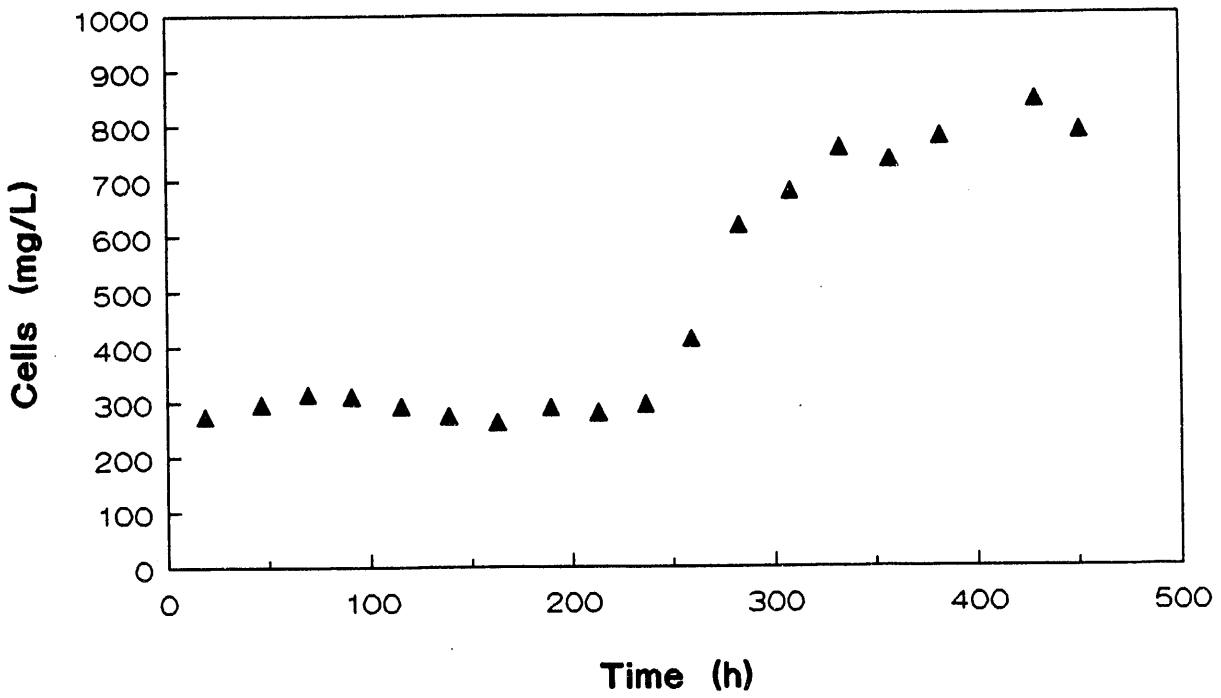


Figure 5.42 Cell Concentration Measurements in the CSTR for *C. ljungdahlii* with Arginine Addition.

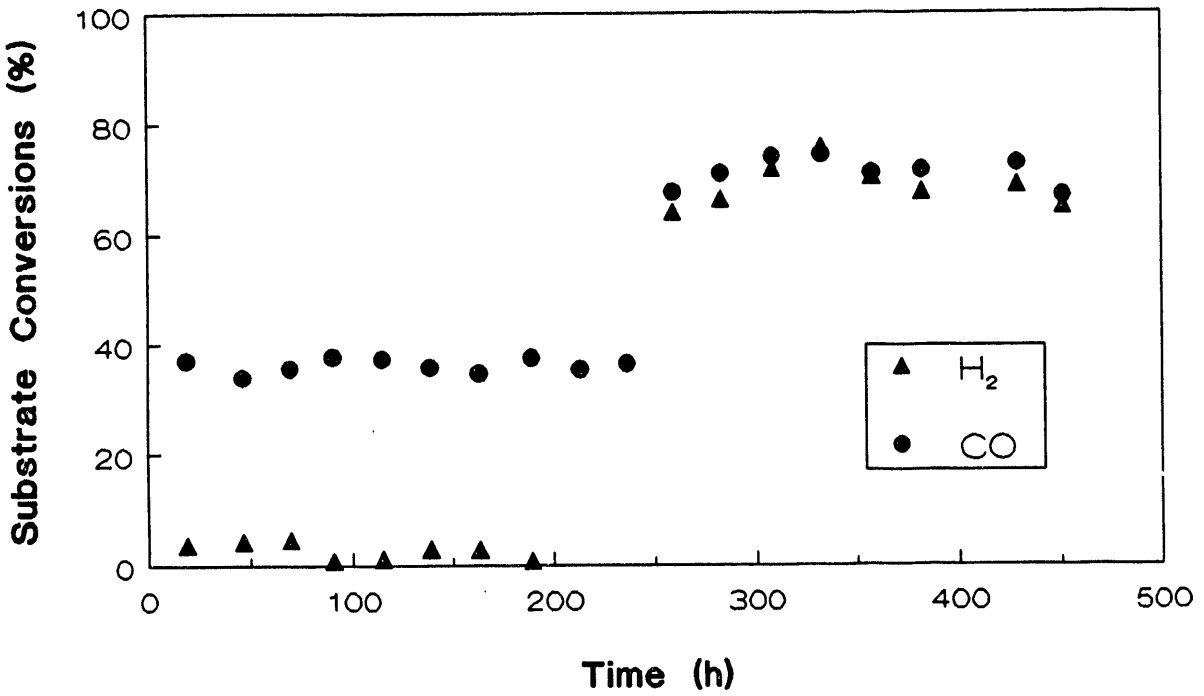


Figure 5.43 Substrate Conversion in the CSTR for *C. ljungdahlii* with Arginine Addition.

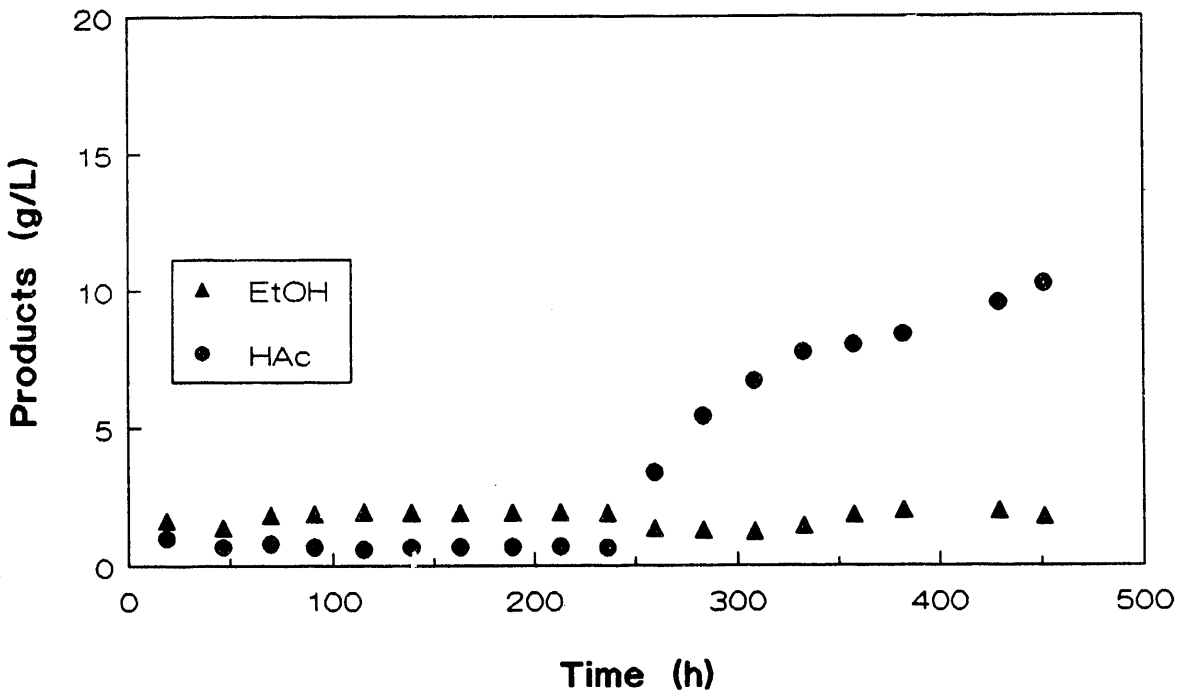


Figure 5.44 Product Concentrations Measurements in the CSTR for *C. ljungdahlii* with Arginine Addition.

measurements with time. As is noted in Figure 5.42, the cell concentration remained essentially constant at 300 mg/L during arginine addition. The quantity of arginine fed was not enough to support good growth but, as will be shown later, was enough to inhibit CO and H_2 uptake. At a time of 236 hr, the arginine flow was stopped. The cell concentration immediately increased to a concentration exceeding 800 mg/L. The phenomenon shown in Figure 5.42 is the phenomenon of diauxic growth, whereby the preferred substrate (arginine) is utilized entirely prior to secondary substrate (CO and H_2) utilization. One very interesting and important observation related to growth but not shown in Figure 5.42 is the rapid start-up achieved with arginine addition. CSTR start-up with arginine was immediate; start-up with CO, H_2 and basal medium alone often takes up to a week.

Figure 5.43 shows the substrate (CO and H_2) utilization in the CSTR with time. During arginine addition, the CO conversion was about 40 percent and the H_2 conversion was 0-5 percent. After arginine addition, both the CO and H_2 conversions increased to 70-80 percent. Again, this further illustrates the concept of diauxic growth, although a small amount of CO was used during arginine addition.

Figure 5.44 shows the product (ethanol and acetate) concentrations in the CSTR with time. During arginine addition, both product concentrations were very low (1-2 g/L). After arginine addition stopped, acetate production dominated ethanol production, with up to 10 g/L acetate and 2 g/L ethanol. It is expected that increased operation time will reverse the product concentrations trend. However, arginine addition may negatively affect product formation (more acetate than ethanol), which may be its major negative feature.

Figures 5.45 and 5.46 show the product yields and specific productivity of the system, respectively, with time. The yield of product from cells, Yp/x, increased only slightly (and probably not significantly) after arginine addition had stopped. The product yield was essentially constant at 0.20 mmol total product/mg cells, a level obtained in earlier CSTR experiments. The specific productivity was also nearly constant (a slightly increased trend) at 0.004-0.005 mmol/mg h, also a level attained previously. Thus, arginine addition did not affect product yield or specific productivity.

5.2.6 Analysis of the Results of CSTR Studies Employing *C. ljungdahlii*

A large number of CSTR studies have been performed with *C. ljungdahlii* in studying the effects of agitation rate, gas flow rate, liquid rate and other variables on culture performance. Steady-state results from these studies, as well as calculated yields, productivities and rates, are presented in Table 5.2. As is noted in the table, these experiments yielded results which gave both high and low ethanol concentrations, high and low cell densities and high and low ethanol to acetate product ratios (E/A).

CO and H_2 uptake rates in these studies ranged from 0.001 - 0.157 mmol/min. In general, the specific uptake rates for CO were approximately 3 times the values for H_2. The specific production rates ranged from 0.0014 - 0.0081 mol/g hr and the specific uptake rates ranged from 0.0075 - 0.0330 mol/g hr. The ethanol to acetate ratio (E/A) ranged from 0.28-20 mol/mol.

A material balance on Equations (1.1-1.4) indicates that 6 moles of either CO or H_2 are required to produce 1 mole of ethanol and 4 moles of either CO or H_2 are required to produce 1 mole of acetate. Thus, the sum of 6 times the moles of acetate formed, divided by the moles of substrate (CO and H_2) consumed should equal 1. This ratio, shown as (6E + 4A)/S in Table 5.2,

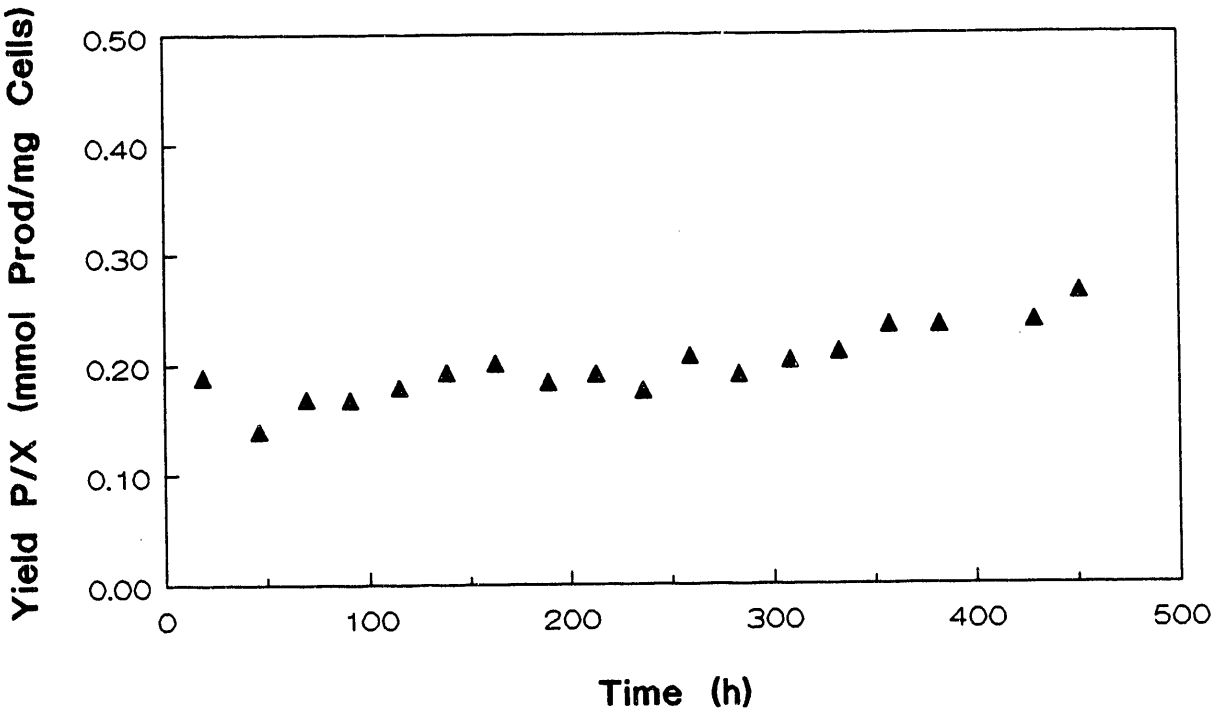


Figure 5.45 Product Yield from Cells for *C. ljungdahlii* in the CSTR with Arginine Addition.

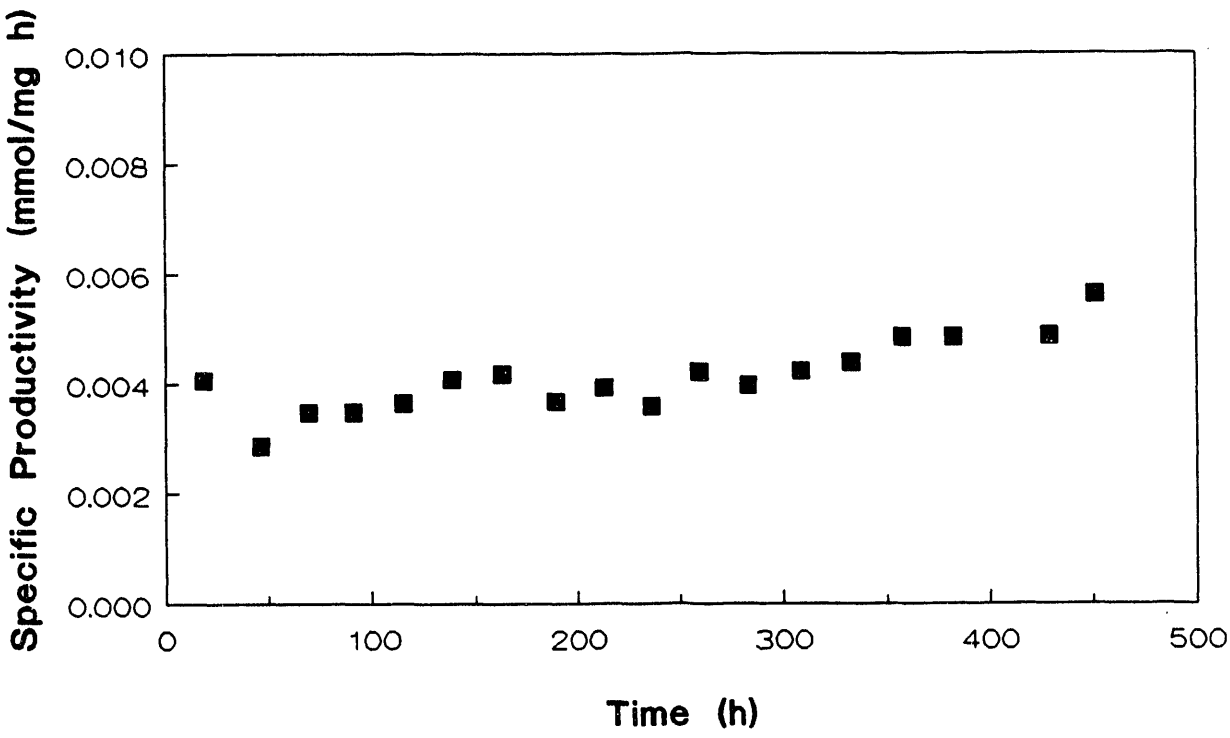


Figure 5.46 Specific Productivity for *C. ljungdahlii* in the CSTR with Arginine Addition.

Table 5.2 Analysis of the Results of CSTR Studies Employing *C. ljungdahlii*

Expt.	Date	LFR (mL/d)	X (mg/L)	EtOH (g/L)	HAc (g/L)	q CO (mmol/min)	q H2 (mmol/min)	Agitation (rpm)
F0	10/10/91	45	1196	13.460	0.880	0.041	0.011	340
	10/19/91	120	626	6.491	2.648	0.058	0.020	360
	10/28/91	120	849	6.271	6.974	0.076	0.028	380
	11/09/91	120	420	2.503	4.095	0.051	0.007	350
	11/19/91	120	741	3.755	7.249	0.068	0.025	370
	11/27/91	120	912	5.093	7.907	0.074	0.027	370
	12/05/91	120	1017	7.644	6.859	0.084	0.031	380
	12/11/91	120	1191	9.892	7.117	0.081	0.029	380
	12/15/91	120	1329	13.010	5.183	0.082	0.030	380
	12/27/91	120	1135	15.796	3.398	0.090	0.031	390
	01/07/92	120	1341	12.542	5.552	0.078	0.029	400
	01/15/92	120	1502	15.585	3.730	0.096	0.033	400
	01/26/92	120	1406	15.996	3.285	0.119	0.040	400
	02/11/92	121	1575	19.073	3.910	0.155	0.047	420
	02/27/92	120	1386	18.320	2.610	0.152	0.001	420
	03/06/92	120	1157	16.347	2.859	0.141	0.006	450
FR	05/05/92	190	314	1.824	4.335	0.045	0.016	420
	05/12/92	182	451	2.742	6.055	0.062	0.021	420
	06/24/92	196	353	0.037	6.706	0.044	0.002	400
FS	04/25/92	179	240	1.491	0.631	0.030	0.004	350
	05/03/92	178	323	2.631	0.580	0.047	0.004	380
	05/10/92	176	473	2.574	0.612	0.046	0.000	420
	05/27/92	172	280	1.938	0.651	0.030	0.001	400
	06/06/92	174	787	1.909	9.040	0.071	0.026	420
	08/21/92	169	1130	5.459	16.064	0.130	0.043	460
	09/08/92	170	1292	7.703	19.405	0.157	0.047	460

Table 5.2 Continued

D (1/h)	Sp.Prod. (mol/g h)	Sp.Upt. (mol/g h)	E/A (mol/mol)	(6E+4A)/S	Yx/s (g/mol)	Yp/s (mol/mol)	Yp/x (mol/g)	Yp/s (calc'd)
0.0054	0.0014	0.0075	19.96	1.079	0.711	0.183	0.257	0.171
0.0143	0.0042	0.0212	3.20	1.104	0.675	0.200	0.296	0.187
0.0143	0.0043	0.0209	1.17	1.034	0.684	0.204	0.298	0.205
0.0143	0.0042	0.0238	0.80	0.856	0.599	0.175	0.292	0.213
0.0143	0.0039	0.0217	0.68	0.866	0.660	0.180	0.273	0.216
0.0143	0.0038	0.0191	0.84	0.976	0.747	0.199	0.266	0.212
0.0143	0.0039	0.0193	1.45	1.056	0.739	0.204	0.276	0.201
0.0143	0.0040	0.0158	1.81	1.335	0.901	0.252	0.280	0.196
0.0143	0.0040	0.0145	3.27	1.519	0.989	0.275	0.278	0.186
0.0143	0.0050	0.0183	6.06	1.571	0.780	0.275	0.352	0.178
0.0143	0.0039	0.0138	2.95	1.551	1.037	0.282	0.272	0.188
0.0143	0.0038	0.0148	5.45	1.471	0.968	0.258	0.267	0.180
0.0143	0.0041	0.0193	6.35	1.212	0.739	0.212	0.286	0.178
0.0144	0.0044	0.0220	6.36	1.140	0.653	0.199	0.305	0.178
0.0143	0.0046	0.0190	9.16	1.392	0.753	0.240	0.319	0.175
0.0143	0.0050	0.0218	7.46	1.314	0.654	0.228	0.348	0.177
0.0226	0.0081	0.0330	0.55	1.150	0.686	0.244	0.356	0.220
0.0217	0.0077	0.0314	0.59	1.166	0.691	0.246	0.356	0.219
0.0233	0.0074	0.0224	0.01	1.332	1.040	0.332	0.319	0.249
0.0213	0.0038	0.0246	3.08	0.853	0.867	0.155	0.179	0.187
0.0212	0.0044	0.0268	5.91	0.933	0.789	0.163	0.207	0.179
0.0209	0.0029	0.0170	5.49	0.980	1.232	0.172	0.140	0.180
0.0205	0.0039	0.0191	3.88	1.136	1.073	0.203	0.189	0.184
0.0207	0.0050	0.0213	0.28	1.050	0.971	0.237	0.244	0.232
0.0201	0.0069	0.0264	0.44	1.205	0.764	0.261	0.342	0.224
0.0203	0.0077	0.0271	0.52	1.332	0.749	0.285	0.380	0.222

actually ranged from 0.85 - 1.5, indicating that a steady-state balance was not always attained. The yield of cells from substate ranged from 0.6-1.2 g/mol and the yield of combined product (ethanol and acetate) from substrate ranged from 0.15 - 0.33, with most of the values in the 0.2 - 0.25 range. The theoretical yield of product from substrate by Equations (1.1-1.4) is 0.17 - 0.25, depending upon whether ethanol or acetate is produced.

5.3 CSTR STudies with Cell Recycle

A cell recycle apparatus was used in conjunction with the standard CSTR 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 5.47 and 5.48 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 5.47 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 5.48, 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,

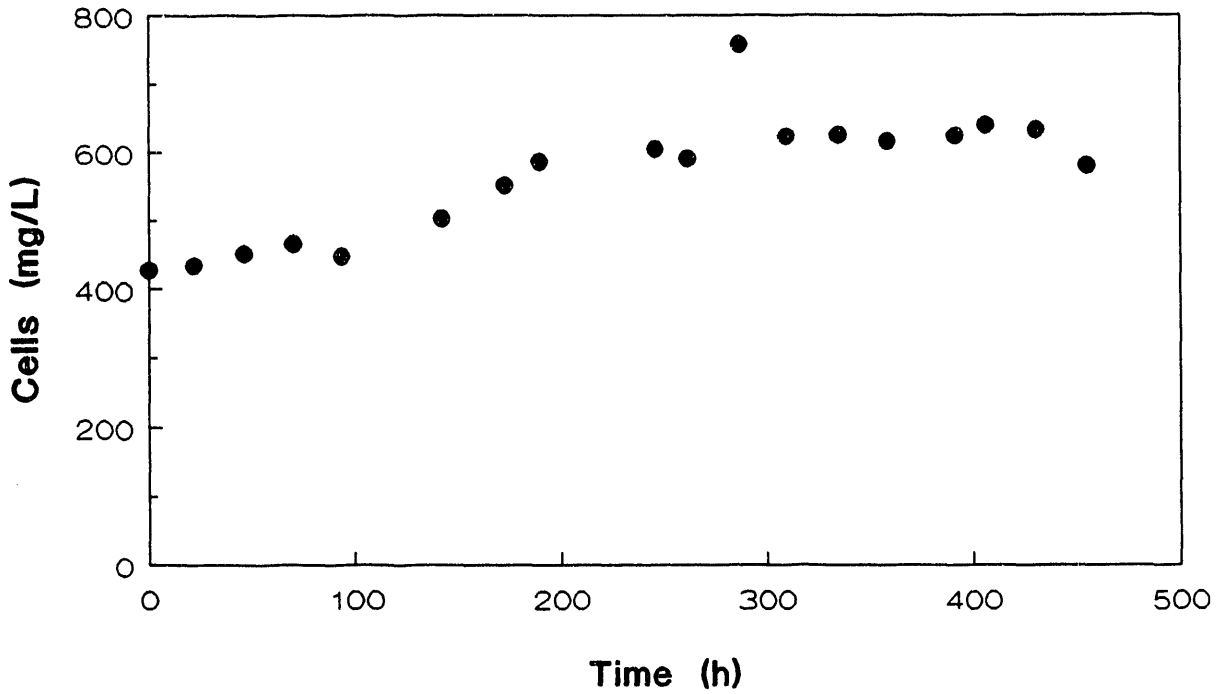


Figure 5.47 Cell Concentration Profile for *C. ljungdahlii* in the CSTR with Cell Recycle. (Basal Medium Contained no Yeast Extract, one half B-vitamins.)

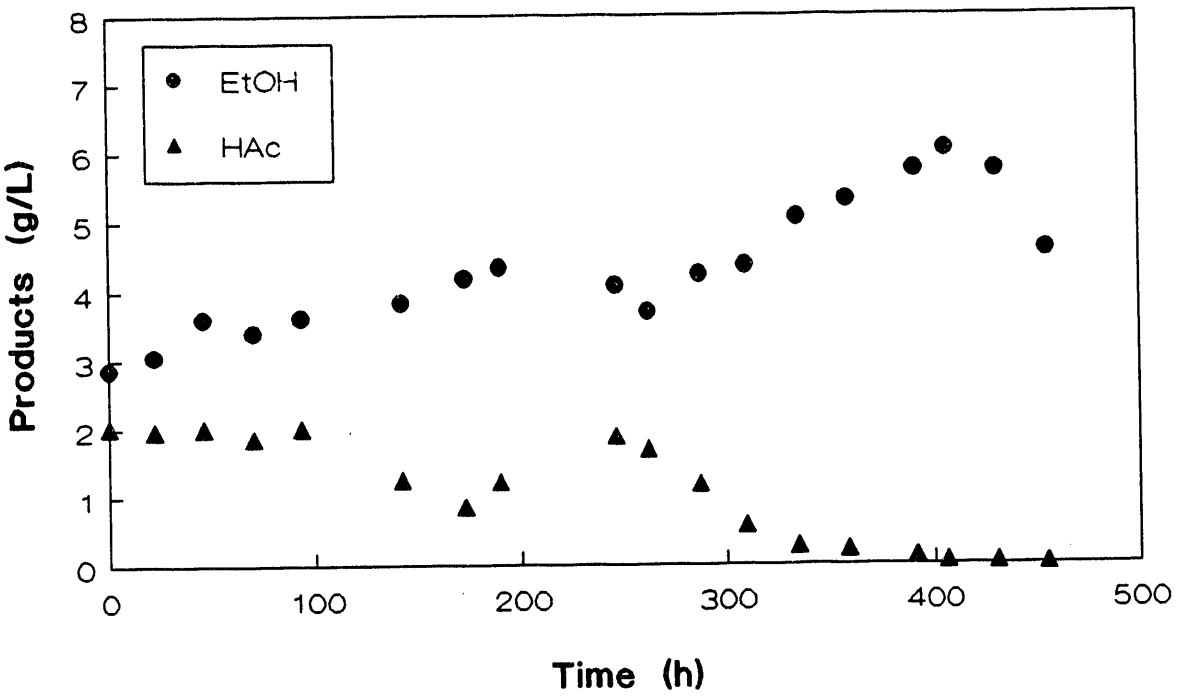


Figure 5.48 Product Profile for *C. ljungdahlii* in the CSTR with Cell Recycle. (Basal Medium Contained no Yeast Extract, one half B-vitamins.)

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.

To help alleviate the problem of nutrient limitation the cell recycle apparatus was operated in a second experimental run using medium supplemented with $(NH_4)_2\ HPO_4$. The ammonium phosphate dibasic was first added daily as spikes in small quantities (0.5 g/L), followed by continuous addition as a component in the medium.

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 5.49 and 5.50 show cell concentration and product concentration profiles for the CSTR with cell recycle. In these experiments, the CO conversion was rather low as had been in previous studies. As is shown in Figure 5.49, the maximum cell concentration reached was over 1300 mg/L, increasing from 200 mg/L with ammonium phosphate addition. This cell concentration was more than double the concentration obtained in the previous studies. The ethanol and acetate product concentrations are shown in Figure 5.50. At a time of about 300 h, the ethanol concentration was about 4 g/L and

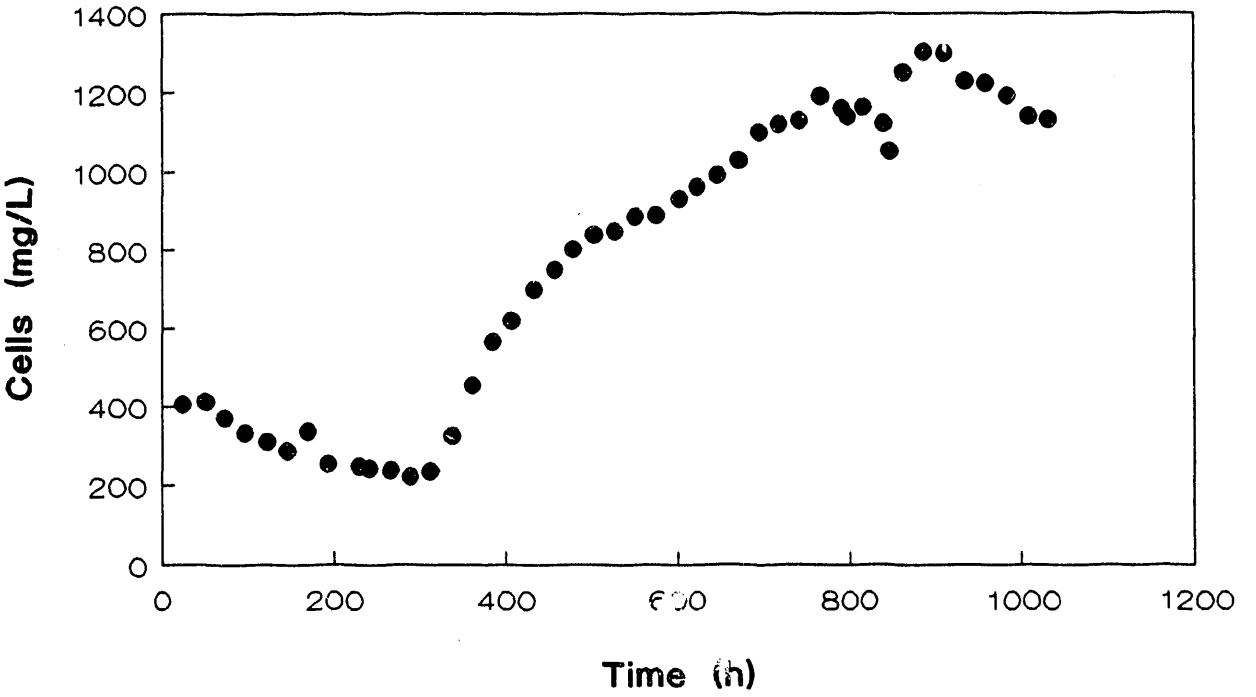


Figure 5.49. Cell Concentration Profile for *C. ljungdahlii* in the CSTR with Cell Recycle (With $(NH_4)_2HPO_4$ Stimulation)

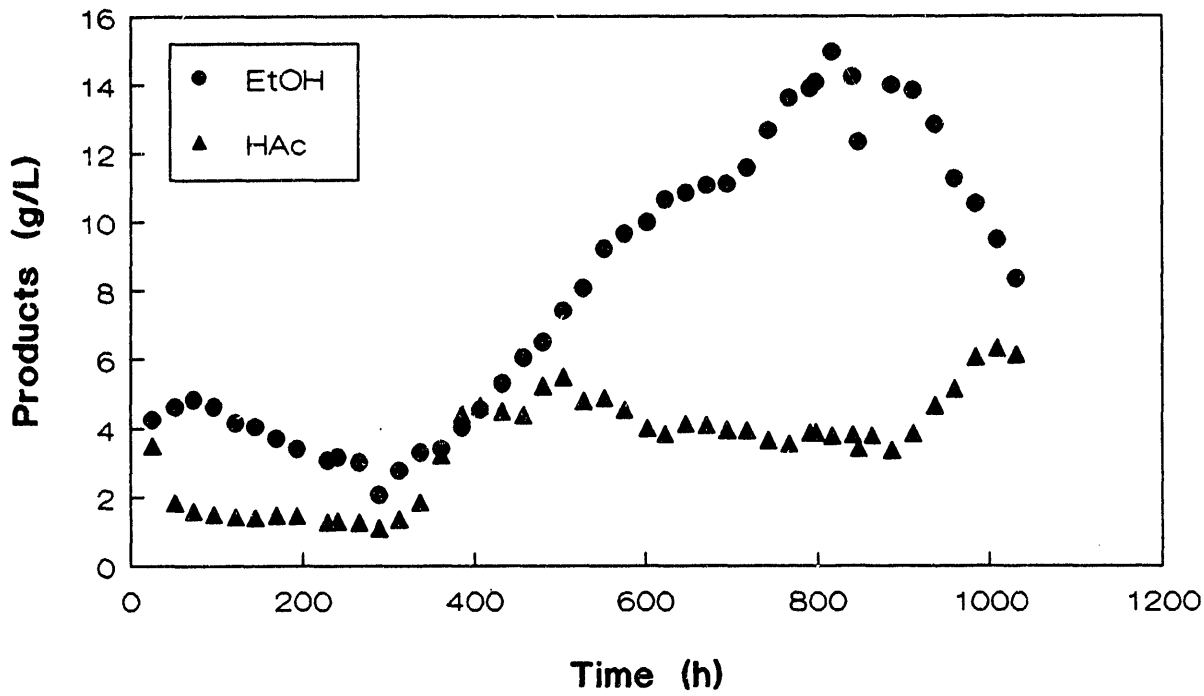


Figure 5.50. Products Concentration Profiles for *C. ljungdahlii* in the CSTR with Cell Recycle (With $(NH_4)_2HPO_4$ Stimulation)

the acetate concentration was nearly 2 g/L. This corresponds to the data at the same time in the previous work without ammonium phosphate. At a later fermentation time of 800 h, however, the ethanol concentration reached 15 g/L with a corresponding 4 g/L acetate concentration. The product ratio did not become infinite in favor of ethanol as it did in the previous study, yet the ethanol concentration reached levels that were 2.5 times greater. Increasing the all concentration is thus shown to be paramount in obtaining high product (ethanol) concentrations.

Studies were also carried out with *C. ljungdahlii* in the designed medium in a CSTR with cell recycle. In this study, the liquid volume was 1000 mL, the temperature was 35.5°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 accomodate 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. Thus, the reactor was effectively a CSTR without cell recycle using a very low liquid flow rate.

Figure 5.51 presents 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 approximately 800 mg/L to over 4000 mg/L. The increase is due to an increased mass transfer of CO and H_2 to the liquid phase with the increase in agitation rate and gas flow rate. The maximum in the previous CSTR study without cell recycle was 1500 g/L. The CO conversion, shown in Figure 5.52, hovered around the 90 percent level after 150 h of operation. The corresponding H_2 conversion, on the other hand, averaged 70 percent up to a time of 500 h. At this time, the H_2 conversion fell, probably due to an accumulation of CO in the liquid phase.

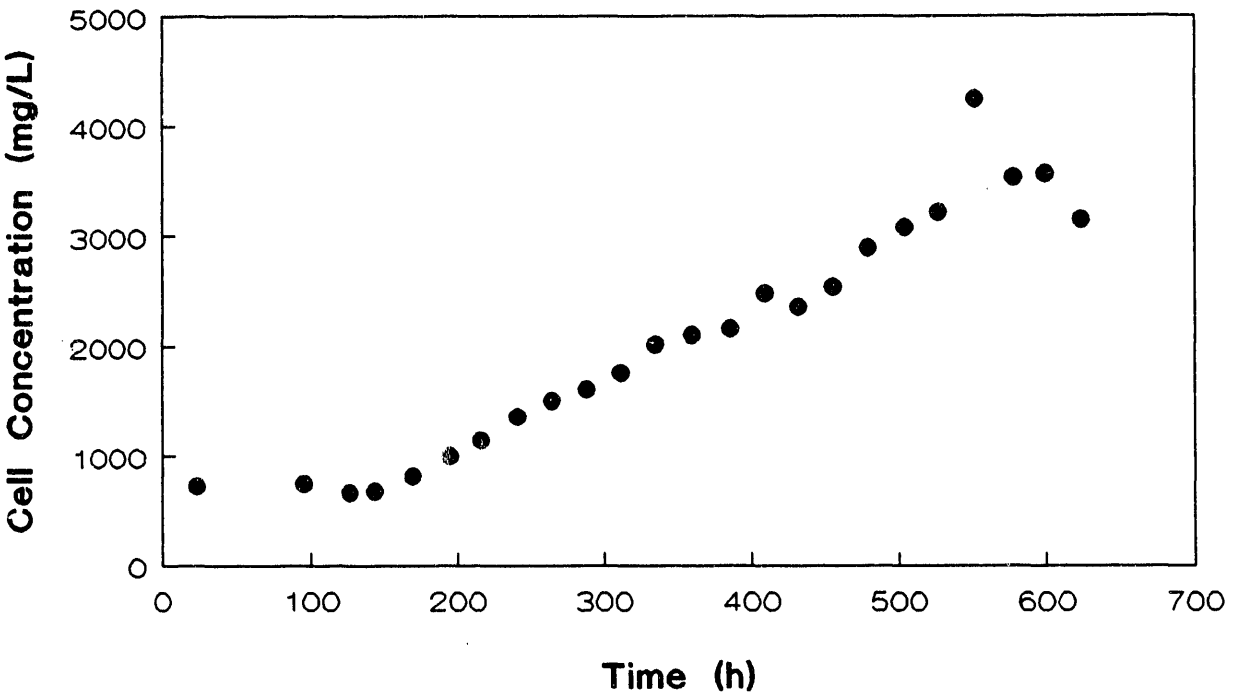


Figure 5.51. Cell Concentration Measurements for *C. ljungdalii* in the CSTR with Cell Recycle

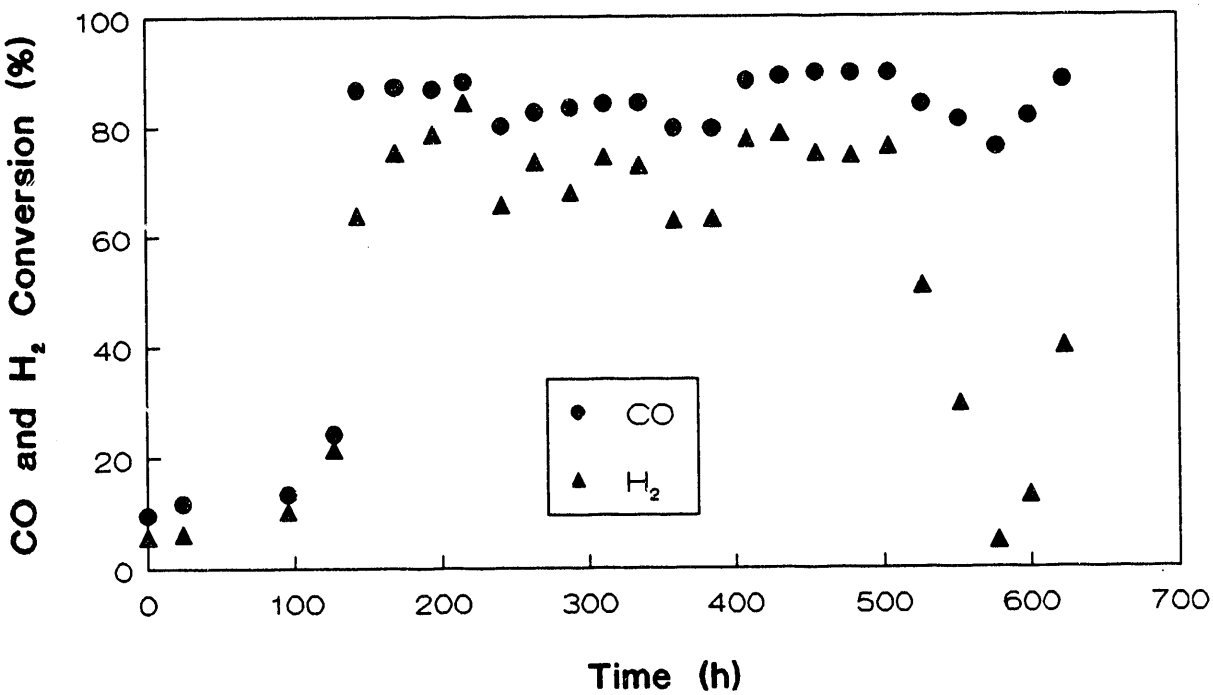


Figure 5.52. CO and H_2 Conversion for *C. ljungdalii* in the CSTR with Cell Recycle

Product concentration measurements during the study are shown in Figure 5.53. 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 g/L and 3 g/L, respectively. The ratio of ethanol to acetate ranged from 1.2 g/g to 16 g/g. Thus, very high ethanol concentrations are possible with favorable product ratios.

5.4 Ethanol Production in a Trickle Bed Reactor

Many contacting schemes may be employed for gas-liquid biological reactors, including mechanically agitated reactors, bubble columns, packed columns, plate columns, spray columns and gas-lift reactors. A packed column or trickle bed reactor is particularly effective for the reactions because of the large mass transfer coefficients obtained in this contacting device without expensive agitation. Although packed beds are normally operated countercurrently; cocurrent operation is possible with irreversible biological reactors since the mean concentration driving force is the same for both modes of operation. Also, the capacity of cocurrent columns is not limited by flooding, and at any given gas and liquid flow rates, the pressure drop in the cocurrent column is less.[31] The principal purpose of this research work is to investigate the feasbility of employing a packed cocurrent column to produce ethanol using *Clostridium ljungdahlii*.

Experiments were carried out in a cocurrent packed column employing a cylindrical ceramic packing (height : 5mm, inner diameter : 2 mm and outer diameter : 5mm). The reactor consisted of a 5.2 cm inside diameter, 54 cm long plexiglass cylinder. The height occupied by the packing was 51.5 cm. The empty reactor volume was 1150 mL and the initial void volume after packing was 650 mL. The bottom of the reactor was provided with a perforated plate

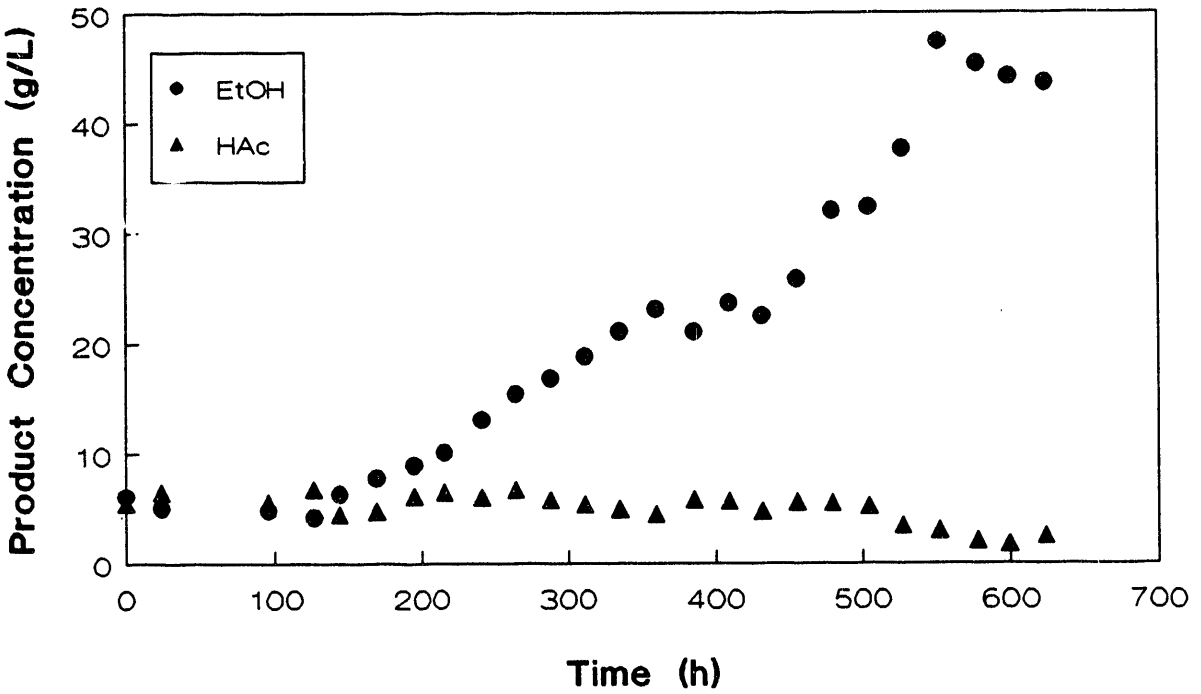


Figure 5.53. Product Concentration Measurements for *C. ljungdalii* in the CSTR with Cell Recycle

between the gas entrance and the packing in an effort to achieve better gas distribution in case the gas is introduced from the bottom with a countercurrent packed column. A sample port was thus installed at the bottom of the reactor to allow for liquid sampling.

The liquid and the gas flowed cocurrently from the top of the column. The liquid level inside the column was maintained with the aid of a level control positioned at the liquid outlet (this control also acted as a gas-liquid separator). The gas (9.9% carbon dioxide, 20.0% hydrogen, 54.9% carbon monoxide and 15.2% argon) flowed from a pressurized tank through a mass flow meter and filter into the reactor. The gas line out of the system was connected to the liquid headspace of the feed and the product reservoirs, as well as the level control device, in order to equilibrate pressures throughout the system while maintaining anaerobic conditions.

The liquid medium was pumped by means of a peristaltic pump from the feed reservoir into the liquid sample port situated at the bottom of the packed cocurrent column, and was kept constant at 42 mL/hr. The liquid from the outlet was recycled by means of a pump to the top of the column, and thereafter, the liquid flow was due to gravitational forces. The liquid recirculation rate in these experiments ranged from 85-415 mL/min. The packed cocurrent column in effect operated as a trickle bed reactor with the retention of the liquid in the column rather small. The gas flow rates for these experiments varied from 2 mL/min to 14 mL/min. Typically, each gas flow rate was maintained for 3 days during which gas samples were analyzed for CO conversion and liquid samples were analyzed for ethanol and acetate. The medium composition used for these experiments was the designed medium of Table 4.3.

The liquid porosity in the column is shown as a function of the liquid recirculation rate in Figure 5.54. As is noted, the porosity, defined as the ratio of the volume of voids to the total reactor volume, ranged from 0 to 15 percent depending upon the liquid recirculation rate. At recirculation rates of 415 mL/min and above, the liquid porosity was essentially 15 percent.

Figures 5.55-5.58 show CO and H_2 conversion profiles for *C. ljungdahlii* in the trickle bed reactor for liquid recirculation rates of 85, 232, 325 and 415 mL/min, respectively. As is noted in the figures, the conversion profiles at the three higher recirculation rates were quite similar. The profiles at the 85 mL/min recirculation rate were generally higher than at the other rates at a given gas flow rate. At a recirculation rate of 85 mL/min, the CO conversion was 100 percent at a 8 mL/min gas flow rate (1.35 hr gas retention time). The H_2 conversion under these conditions was greater than 90 percent. It is apparent from these results that increased liquid recirculation allowed less cell attachment in the packed column. Fewer attached cells, coupled with mass transfer, permitted better CO and H_2 conversions at the lower recirculation rate.

Product concentration profiles in the trickle bed reactor at the four recirculation rates are shown in Figures 5.59-5.62. These results showed the same trend as the conversion profiles, with a higher total product concentration at a given gas flow rate with the lowest recirculation rate. A maximum acetate concentration of 6.0 g/L and a maximum ethanol concentration of 0.8 g/L were attained at an 85 mL/min recirculation rate. These results again show the importance of mass transfer and cell attachment. Productivity profiles, shown in Figures 5.63-5.66, showed higher productivities at the lowest recirculation rate. The maximum productivity with an 55 mL/min

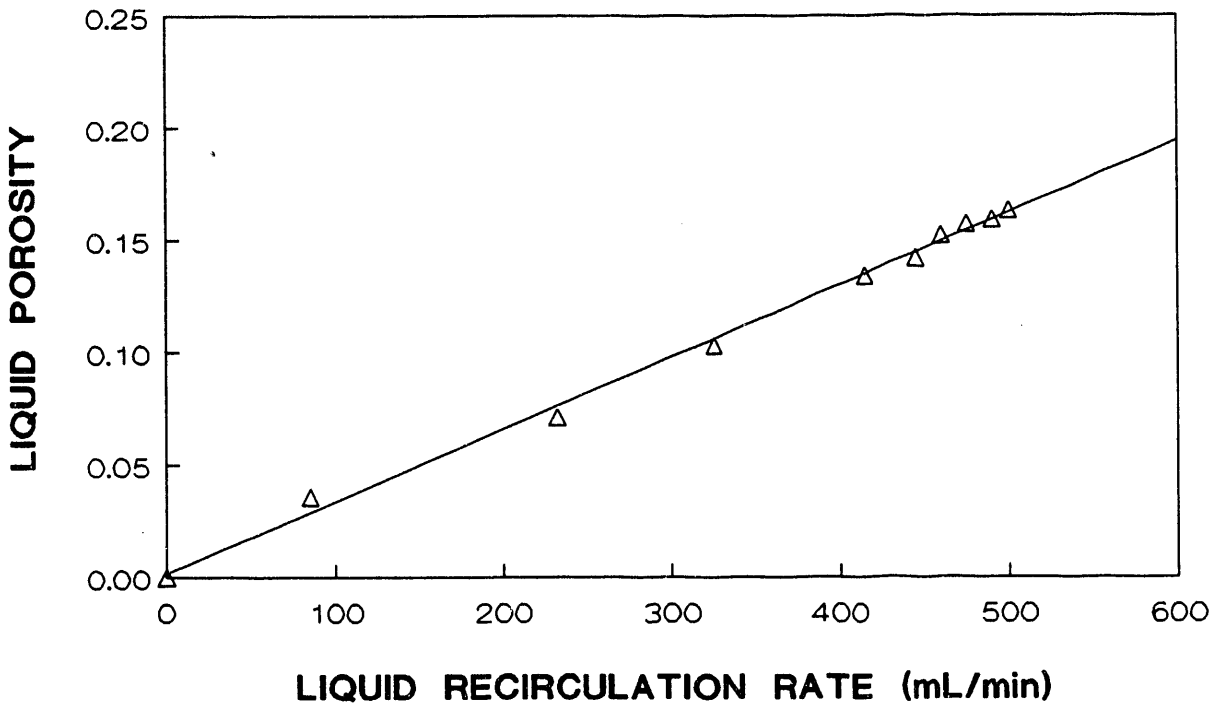


Figure 5.54. Liquid Porosity Measurements in the Packed Column as a Function of Recirculation Rate.

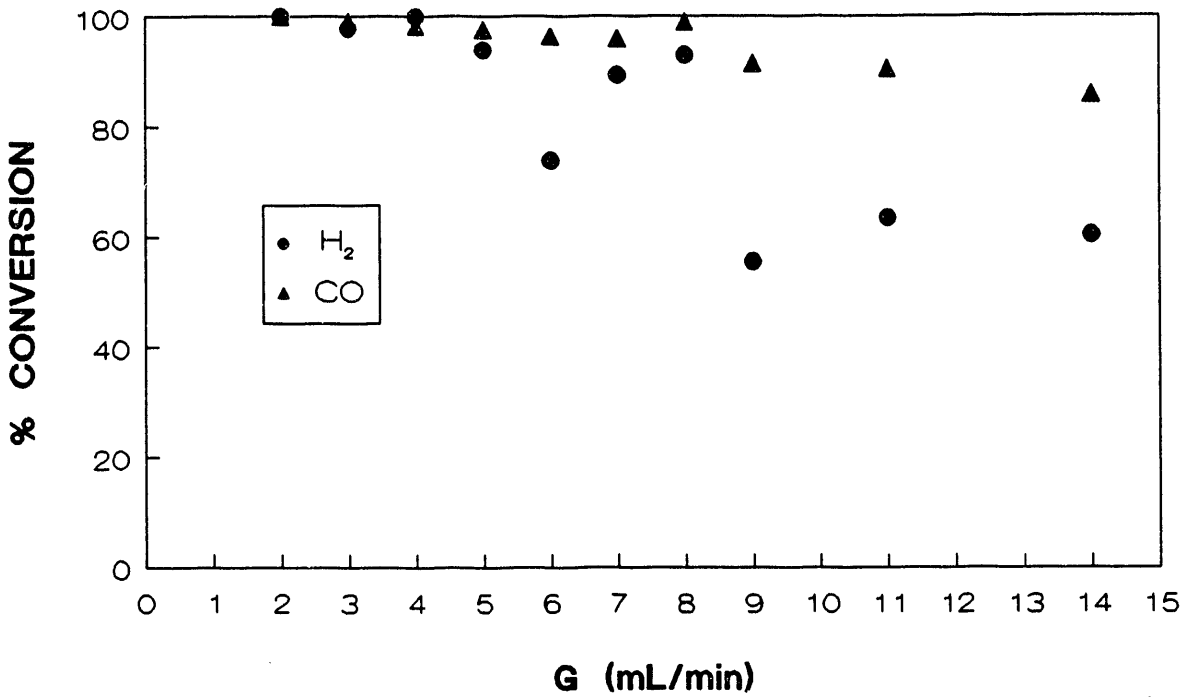


Figure 5.55. CO and H_2 Conversion Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 85 mL/min).

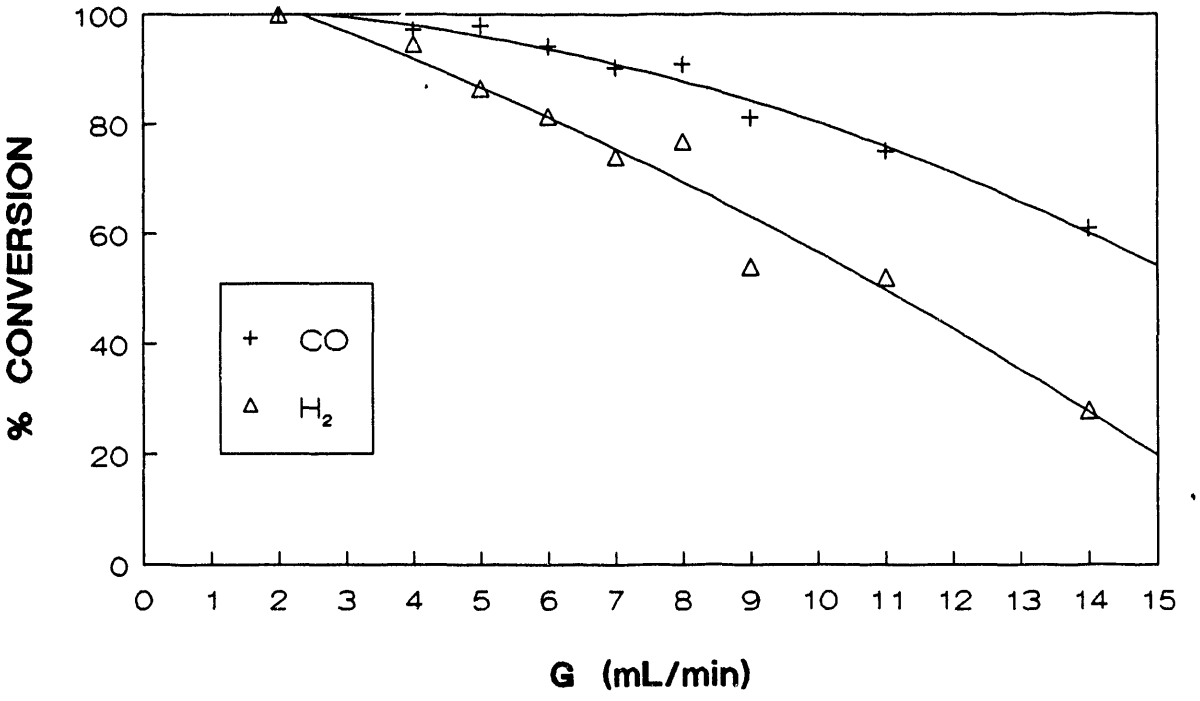


Figure 5.56. CO and H_2 Conversion Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 232 mL/min).

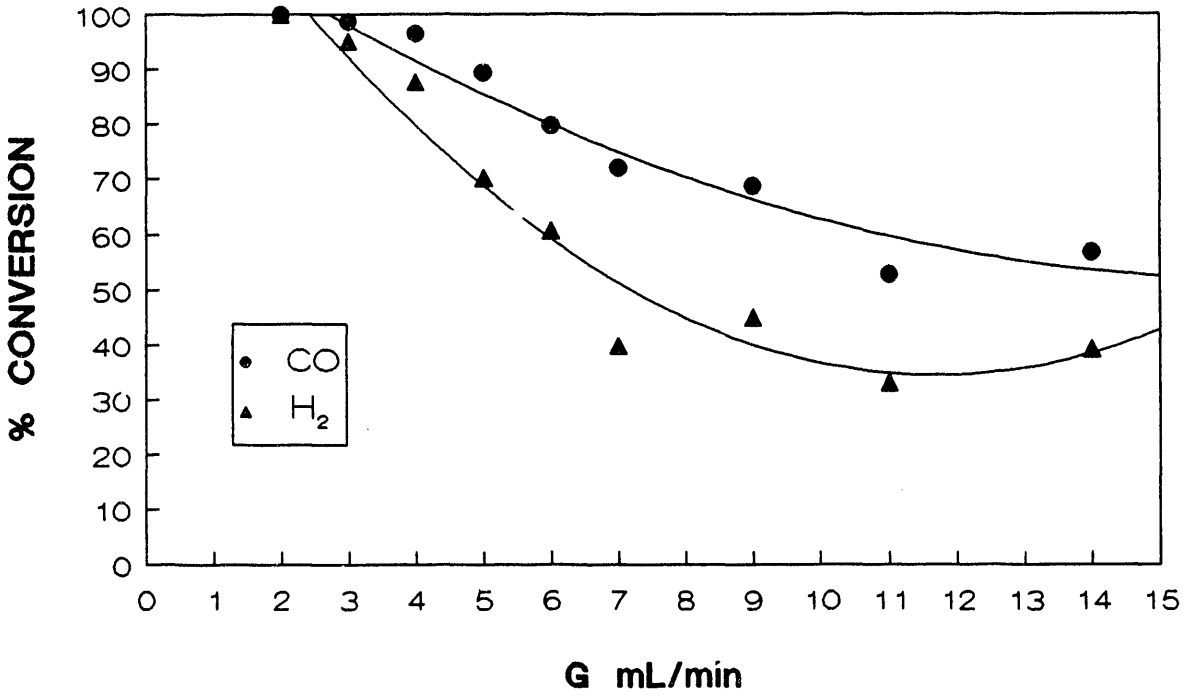


Figure 5.57. CO and H_2 Conversion Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 325 mL/min).

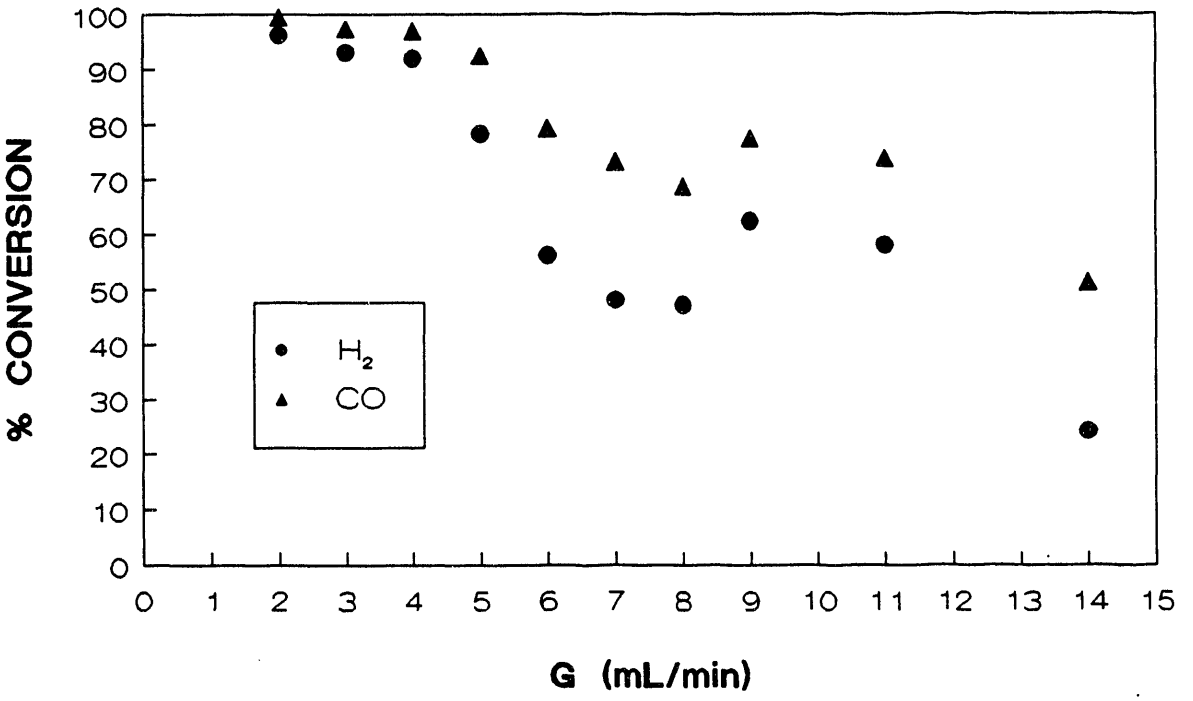


Figure 5.58. CO and H_2 Conversion Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 415 mL/min).

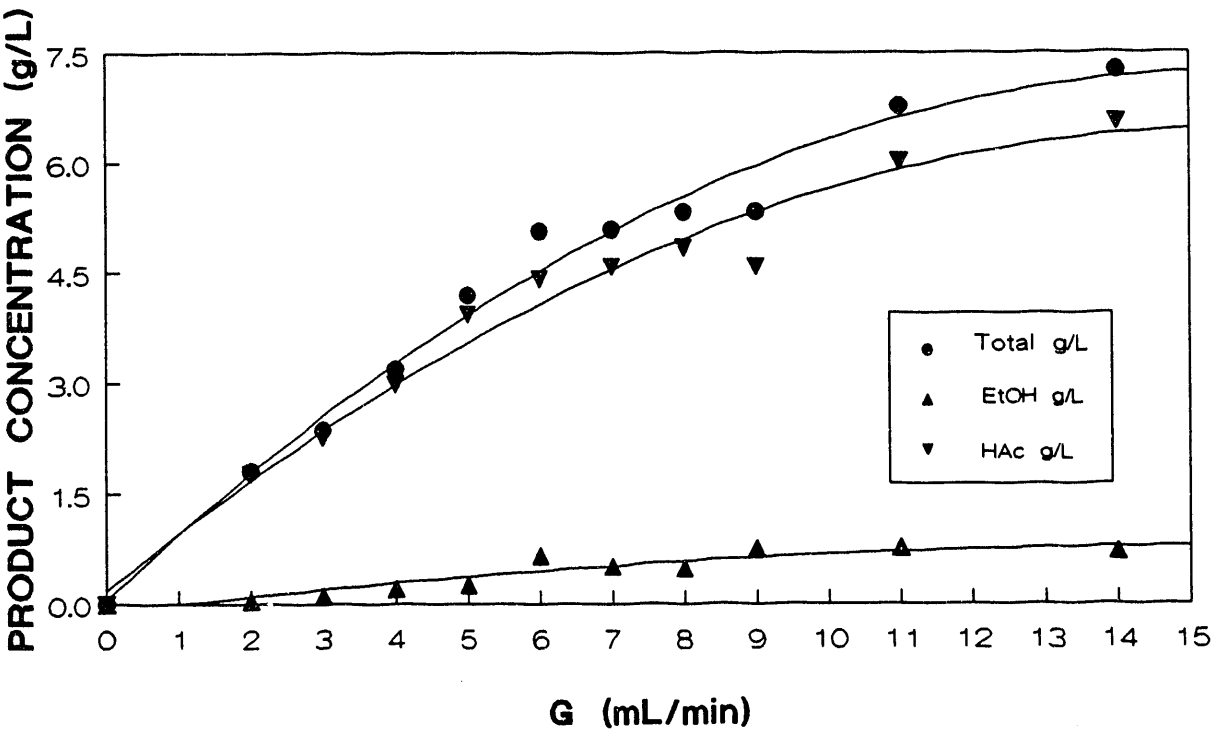


Figure 5.59. Product Concentration Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 85 mL/min).

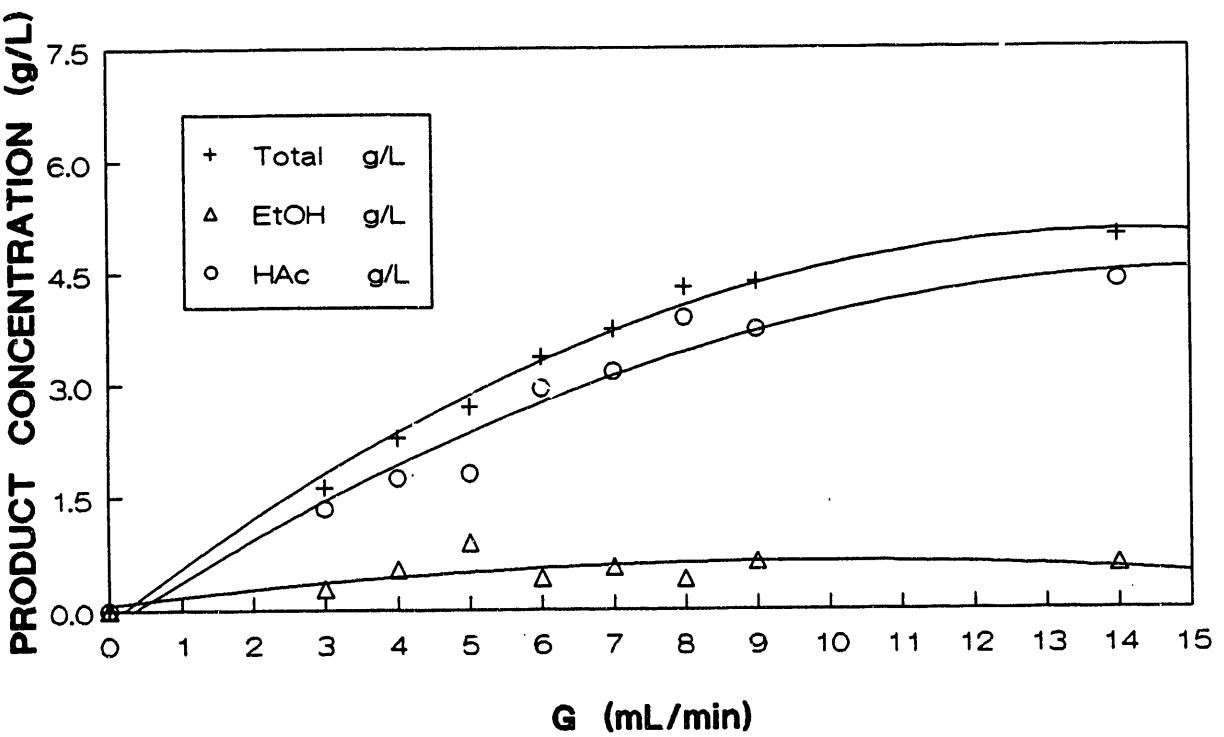


Figure 5.60 : Product Concentration Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 232 mL/min).

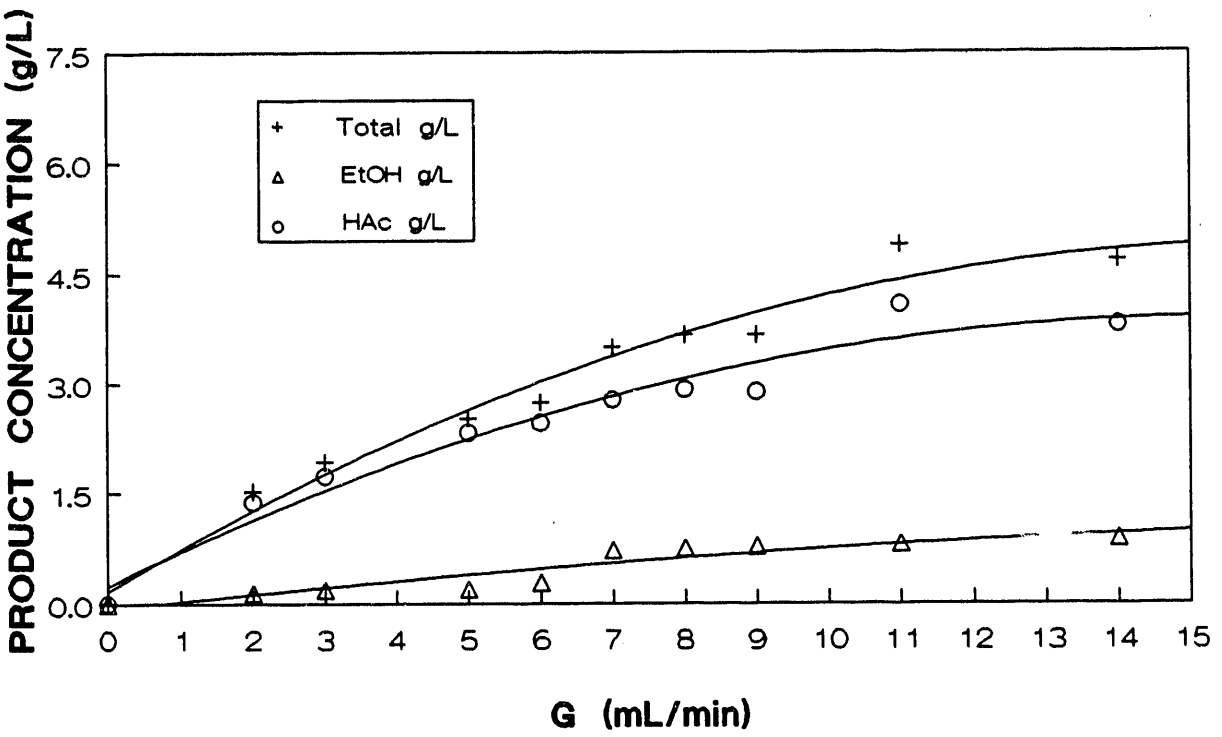


Figure 5.61. Product Concentration Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 325 mL/min).

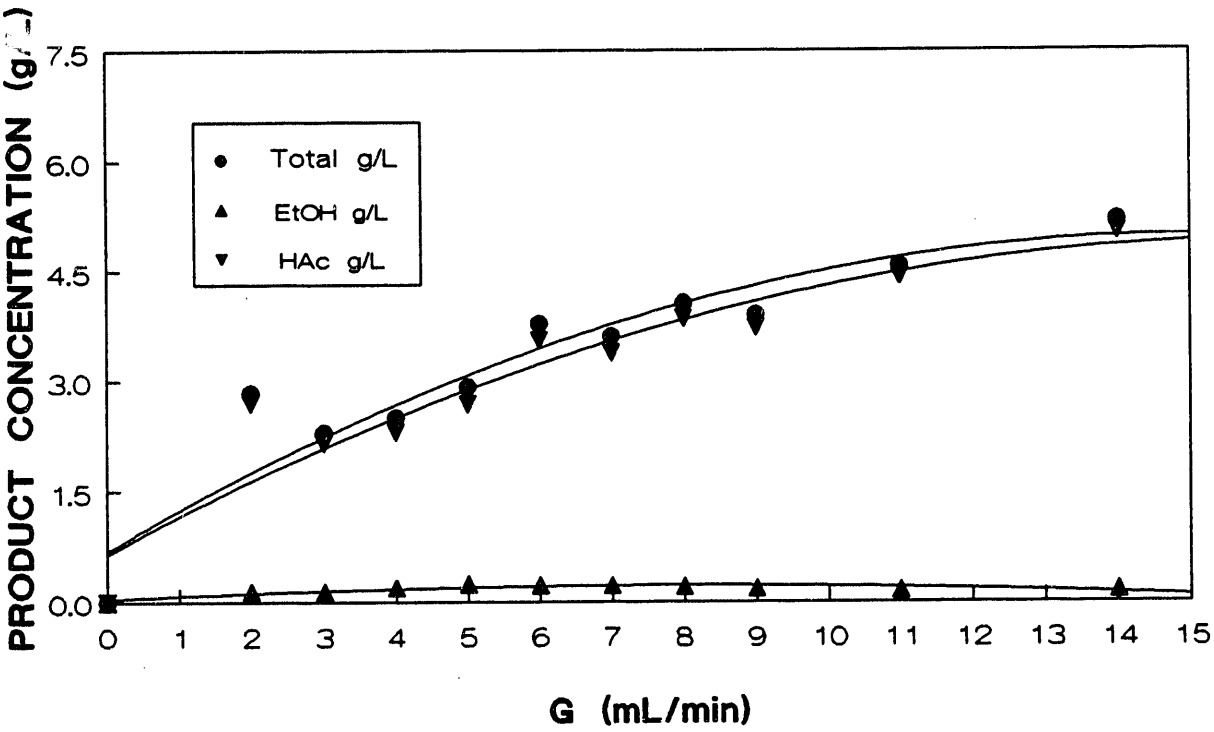


Figure 5.62. Product Concentration Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 415 mL/min).

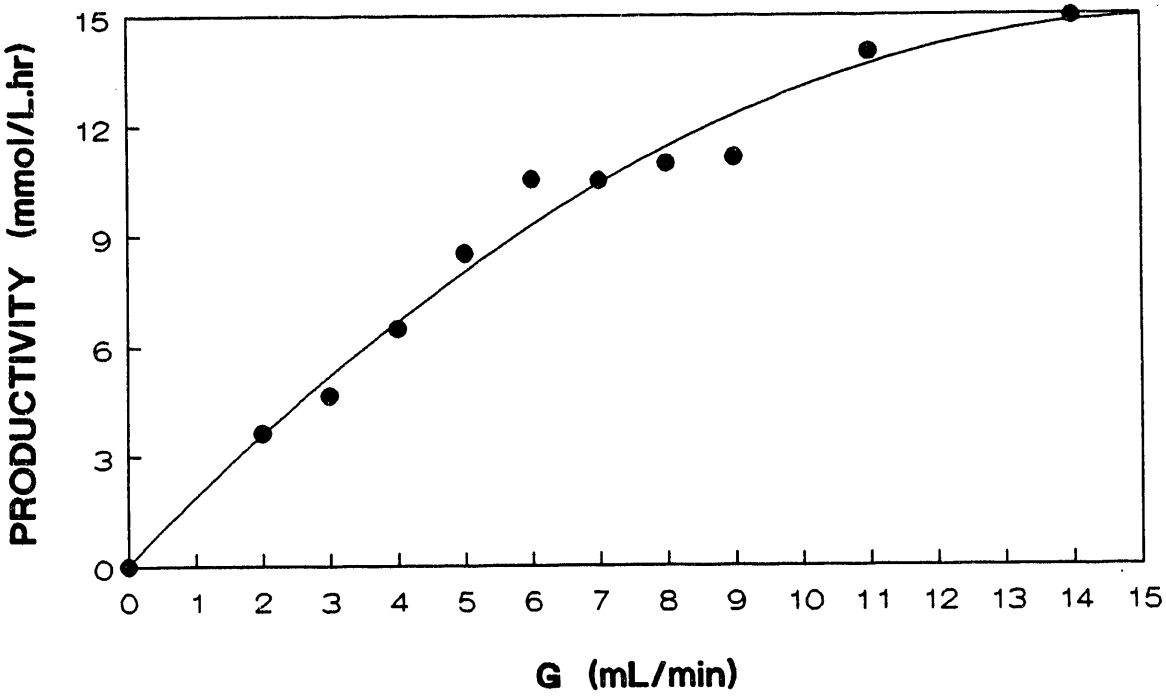


Figure 5.63. Productivity Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 85 mL/min)

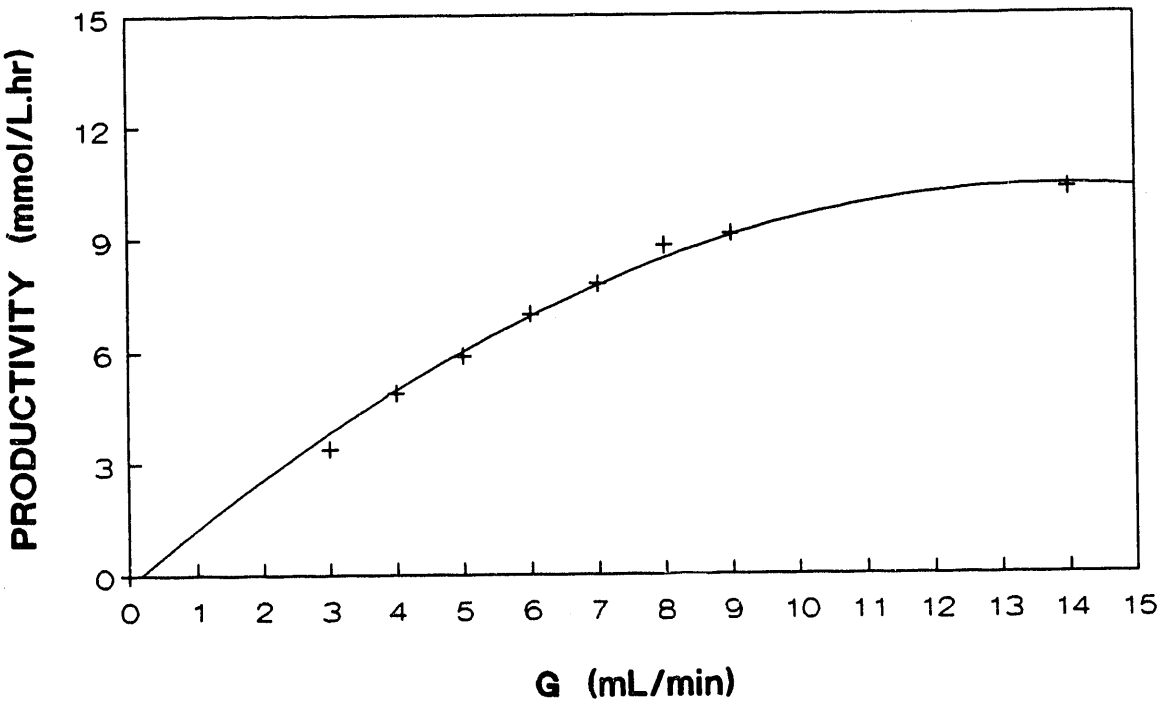


Figure 5.64 : Productivity Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 232 mL/min).

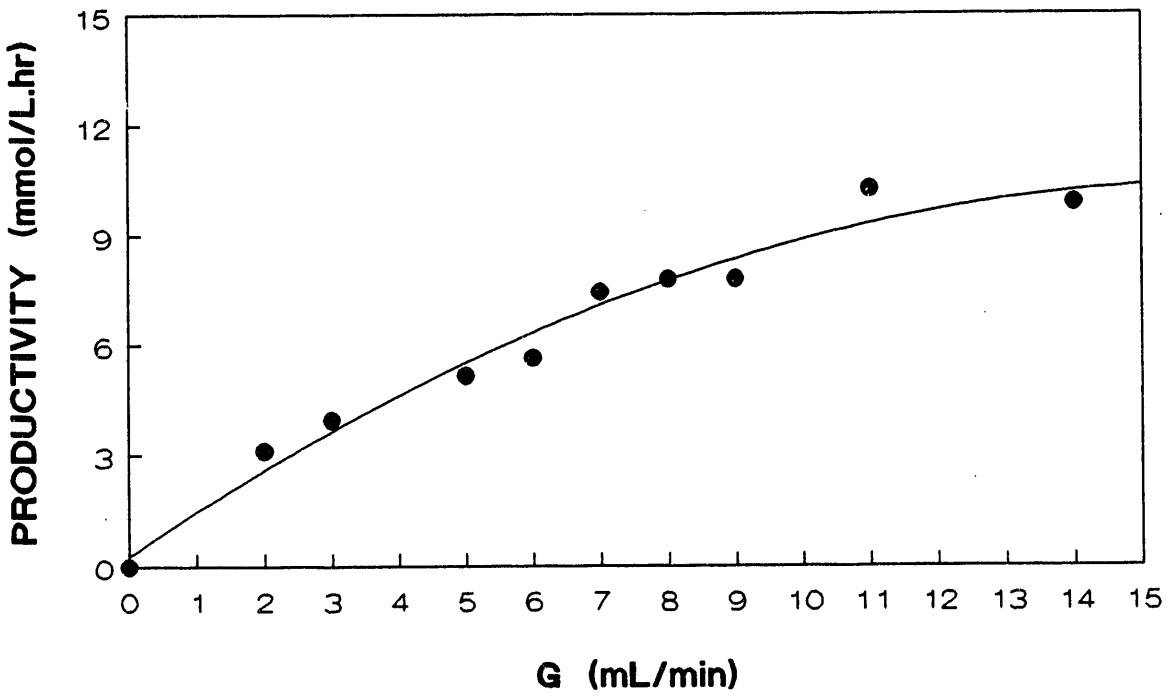


Figure 5.65. Productivity Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 325 mL/min).

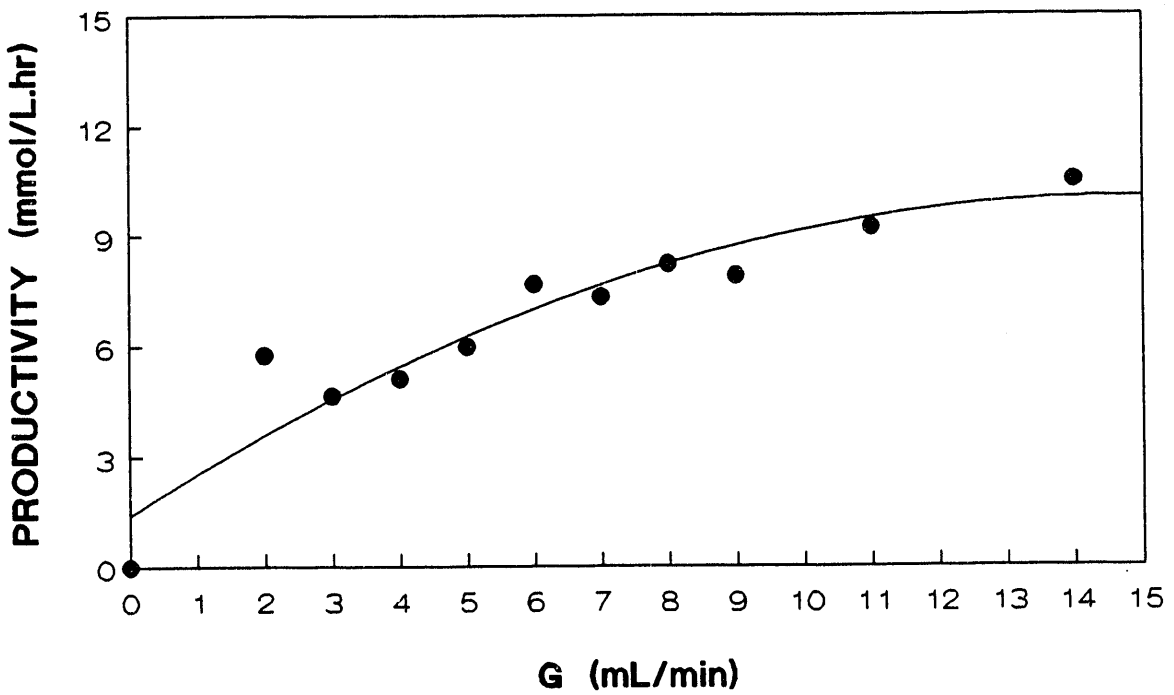


Figure 5.66. Productivity Profiles in the Packed Bed Reactor (Liquid Recirculation Rate : 415 mL/min).

recirculation rate was 15 mmol/L•hr. The maximum productivity at the other recirculation rates was 10 mmol/L•hr.

The transport of a sparingly water-soluble substrate from the gas phase to the liquid phase may be described by the equation:

$$\text{moles transported} = K_La/H(P^G - P^L) \qquad (5.1)$$

where P^G is the partial pressure of the substrate in the gas phase. In the same manner, P^L refers to the partial pressure of the substrate in equilibrium with the concentration of the substrate in the bulk liquid phase, K_La is the overall mass-transfer coefficient and H is the Henry's law constant. In the liquid phase, the substrate is consumed at a maximum rate by the cells according to the equation

$$\text{mole consumed} = q\,X \qquad (5.2)$$

where q is the specific uptake rate and X is the cell concentration. For a system not operating under mass-transfer limitation, the limiting step for substrate consumption is the microbial kinetics described by Equation (5.2). On the other hand, under mass-transfer limitation, the uptake rate is generated by the mass-transfer limitation of the reactor and Equation (5.1). It is important to realize that in both cases the moles of substrate transported from the gas phase to the liquid (Equation (5.1)) must be equal to the consumption rate by the cells (Equation (5.2)) at steady state.

In the column reactor used in this study, the outlet concentration of, for example, CO may be related to the gas flow rate and column parameters through the following equation[32]:

$$\ln\left[Y_{CO}^{i}/Y_{CO}^{o}\right] = \left[K_La/H\right]\epsilon_L\,[ShRT/G] \qquad (5.3)$$

where Y_{CO}^{i} and Y_{CO}^{o} are the molar fraction ratios of CO to an inert gas

component (Ar) in the inlet and outlet gases, respectively. Thus, by varying the gas flow rate, G, and measuring the compositions in the inlet and outlet gases, the mass transfer properties of the column reactors may be estimated. The main assumptions made in the derivation of Equation (5.3) are that mass-transfer limiting conditions apply and that the gas flow rate does not change significantly along the length of the column.

The relation of Equation (5.3) for finding the mass transfer coefficient is illustrated in Figure 5.67, where $\ln \left(Y_{CO}^{i} / Y_{CO}^{o} \right)$ is plotted as a function of hRTS/G. As is seen in the figure, the data fell on a straight line through the origin.

The experimental results from the trickle bed showed that acetate was the preferred product of CO, CO_2 and H_2 conversion over ethanol. Results in progress, however, indicate that ethanol is the preferred product over acetate at lower liquid flow rates. At an (inlet) liquid flow rate of 11 mL/hr, the product ratio (ethanol : acetate) was greater than one. Higher rates are possible at even lower liquid flow rates.

6.0 CONCLUSIONS

The production of ethanol from synthesis gas by the anaerobic bacterium *C. ljungdahlii* has been demonstrated in continuous stirred tank reactors (CSTRs), CSTRs with cell recycle and trickle bed reactors. Various liquid media were utilized in these studies including basal medium, basal media with 1/2 B-vitamins and no yeast extract and a medium specifically designed for the growth of *C. ljungdahlii* in the CSTR. Ethanol production was successful in each of the three reactor types, although trickle bed operation with *C. ljungdahlii* was not as good as with the stirred tank reactors. Operation in

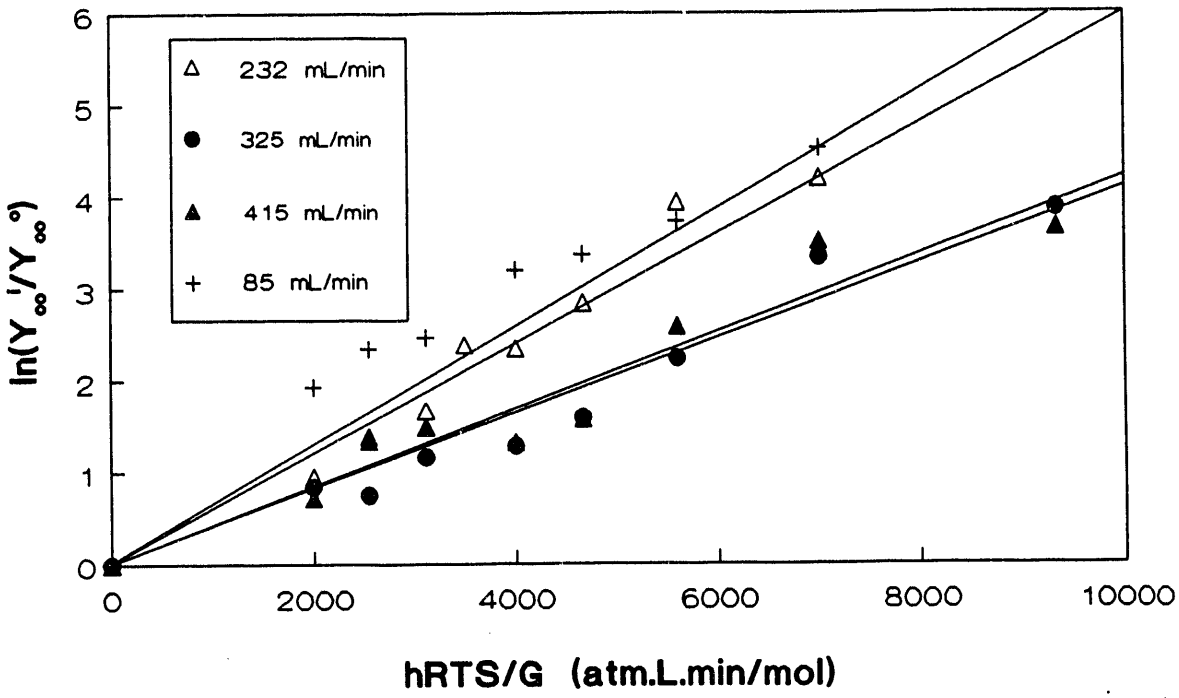


Figure 5.67. Determination of Mass Transfer Coefficient in a Packed Bed Reactor at Different Liquid Recirculation Rates.

the CSTR with cell recycle was particularly promising, producing 47 g/L ethanol with only minor concentrations of the by-product acetate.

7.0 LITERATURE CITED

1. Clausen, E.C., and J.L. Gaddy, "Advanced Studies of Biological Indirect Liquefaction of Coal: Final Report, "Prepared under DOE contract DE-AC22-88PC79813, Pittsburgh Energy Technology Center, (January 1990).

2. Barik, S., S. Prieto, S.B. Harrison, E C. Clausen and J.L. Gaddy "Biological Production of Alcohols from Coal Through Indirect Liquefaction," presented at the 9th Symposium on Biotechnology for Fuels and Chemicals, Boulder, CO, paper no 28 (May 1987).

3. Ljungdahl, L.G., *Ann. Rev. Microbiol.* 40, 415 (1986).

4. Vega, J.L., S. Prieto, B.B. Elmore, E.C. Clausen and J.L. Gaddy, "The Biological Production of Ethanol from Synthesis Gas," *Applied Biochem. Biotech.* 20/21, 781 (1989).

5. Rogers, P., *Ad. Appl. Microbiol.* 31, 1 (1986).

6. Wood, H.G., H.L. Drake and S.I. Hu, *Proc. Biochem. Symp.*, St. Louis, pp 29-56, Palto Alto, CA, Annual Reviews (1982).

7. Ljungdahl, L.G., In "Organic Chemicals from Biomass" (D. L. Wise, ed.), pp 219-248, Benjamin/Cummings, Menlo Park, California (1983).

8. Bahl, H. and G. Gottschalk, in Sixth Symposium on Biotechnology for Fuels and Chemicals, (D. I. C. Wang and C. D. Scott, eds.), 14, pp. 215-223, Wiley, New YOrk (1984).

9. Bahl, H., W. Andersch and G. Gottschalk, *Eur. J. Appl. Microbiol. Biotechnol.* 15, 201 (1982).

10. Gottschal, J.C. and J.G. Morris, *FEMS Mocrobiol. Lett.* 12, 385-389 (1981).

11. Bailey, J.E. and D.F. Ollis, "Biochemical Engineering Fundamentals, McGraw-Hill, New York (1977).

12. Rao, G. and R. Mutharasan, *Biotech. Letters*, 8, 893 (1986).

13. Rao, G., P.J. Ward and R. Mutharasan, *Ann. NY Acad. Sci.* 506, 76 (1987).

14. Rao, G. and R. Mutharasan, *Biotech. Letters*, 10, 129 (1988).

15. Rao, G. and R. Mutharasan, *Applied and Environ. Micro.* 1232, (June 1987).

16. Kim, T.S. and B.H. Kim, *Biotech. Letters*, 10, 123 (1988).

17. Jones, D.T., A. Van der Westhuizen, S. Long, E.R. Allcock, S.R. Reid and D.R. Woods, *Appl. Environ. Microbiol*, 43, 1434-1439 (1982).

18. Long, S.D., T. Jones and D.R. Woods, *Appl. Environ. Microbiol.* 20, 256-261.

19. Long, S.D., T. Jones and D.R. Woods, *Biotechnol. Lett.* 6, 529-535 (1984b).

20. Gottschal, J.C. and J.G. Morris, *Biotechnol. Lett.* 3, 525-530 (1981).

21. Pheil, C.G. and Z.G. Ordal, *Applied Microbiol.* 15, 893 (1967).

22. Landuyt, S.L., E.J. Hsu and M.Lu, *Ann. N. Y. Acad. Sci.* 413, 474-478 (1983).

23. Hsu, Edward and I. Ordal. *Journal of Bacteriology*, 97, 1511 (1969).

24. McInerney, M.J., M.P. Bryant and N. Pfennig, *Appl. Environ. Microbiol.* 122, 129.

25. Genthner, B.R.S., M.P. Davis and M.P. Bryant, *Appl. Environ. Microbiol.* 42, 12.

26. Savage, M.D. and H.L. Drake, *J. Bacteriol.* 165 (1), 315

27. Hungate, R.E., "Methods in Microbiology, 3B, 117 (1969).

28. Bryant, M.P., *Am. J. Clin. Nutr.* 25, p. 1324-1328 (1972).

29. Balch, W.E. and R.S. Wolfe, *Appl. Environ. Microbiol.* 32, 781 (1976).

30. Doyle, M., "Preferred Conditions for Growth and Product Formation by *Clostridium ljungdahlii* PETC in Complex Media," M.S. Thesis, University of Arkansas, (1992).

31. Charpentier, J.C., "Advances in Chemical Engineering," Vol. II, Academic, New York, (1981).

32. Klasson, K.T., B.B. Elmore, J.L. Vega, M.D. Ackerson, E.C. Clausen, and J.L. Gaddy,"Biological Production of Liquid and Gaseous Fuels from Synthesis Gas," *App. Biochem. Biotechnol.* 24/25, 857, (1990).

8.0 APPENDICES

Appendix A
Laboratory Procedures

A.1 Procedures for Media Preparation

1. Using sterilized or disposal pipettes, mix the media constituents.

2. Test the pH of the mixture, it should be a little above what is required, since the usually drops by 0.2 units in the autoclave

   If the pH is too low,; add dropwise sterilized 10% $NaCO_3$ (sodium bicarbonate)

   If the pH is too high, add dropwise 1.0% Acetic Acid.

3. Transfer the media into a 1000 or 500 ml round bottom flask.

4. An 80% N_2, 20% CO_2 gas mixture is bubbled through the media using a 1 ml cotton plugged pipe and attached to the gas hose.

5. Allow the solution to boil for 2 minutes

6. After the solution cools, check the pH
   If the pH is correct, anaerobically transfer the solution into serum bottles and seal with a septa. (This technique will be discussed later)

A.2 Inoculation of Media with Natural Inocula

Fresh natural inocula can be added to jars of media in the anaerobic chamber. Samples must be added to the media as soon as possible after collecting the inocula, trying not to let the sample dry out. After the gas has been added (CO or synthesis gas), put the sample in the incubator. Take gas samples weekly to check the progress of the organisms in the solution. Once gas consumption has begun, transfer a portion of the inoculated media to new media, always keeping the original sample. If the original sample doesn't consume gas after a period of a month to a month and a half, it must be transferred to ensure that the organisms have not depleted the nutrients. Samples have taken from 1 to 6 months to initiate gas consumption. Thus, try not to discard the cultures to soon.

The inoculation procedure must be anaerobic or the organisms may die. The procedures for inoculation are the same as in removing liquid samples:

1. Turn on the gas that is to be used (N_2 or CO or both) and turn the gas heater on.

2. When inoculating a sample, a substance must be added to tie up any dissolved oxygen in the solution. Sodium sulfide can be used. Add 0.2 ml of NaS_2 for every 10 ml of solution. Methane inhibitors should also be added prior to inoculation. BESA or monensin in 0.1 ml quantities for every 10 ml of solution were added.

3. To add something to the solution: Take a sterile or disposable needle and syringe and flush it twice with gas by placing it in the canula. Place methanol on the bottle of NaS_2 (example) and the serum bottle, and then flame the NaS_2 bottle and insert the needle into the septa. Withdraw the exact amount of solution needed. Flame the septa of the serum bottle and put the needle in the septa. After forcing the solution into the bottle, let the plunger come up on its own. This will relieve excess pressure from the serum bottle. Withdraw the needle and dispose of it in the proper container.

4. To remove liquid samples: Flush the needle with gas and put methanol on the top of the septa. Flame the septa and insert the needle into the sample and withdraw the amount needed. Withdraw the needle and place the sample in a sampling tube. Dispose of the needle and syringe. Don't use a syringe twice due to the possibility of cross contamination. Similar procedures are used for the pure culture.

A.3. Gas Addition to Serum Bottles

1. Place methanol on top of the septa. Let it evaporate before addition.

2. Place sterile needles on the gas lines that you will be using, making sure the tubing has been autoclaved.

3. Turn the gas on and turn on the regulator by the hood. Make sure the hood fan is on. Set the regulator for the pressure that is needed. If one atmosphere is required, fill the bottle to two atmospheres, then let the pressure out with a needle. Hold the needles up in the hood and flush the gas through them for a couple of seconds. Then place the needles in the septa. Fill the bottles to pressure, then pull a vacuum. Pressure again and vacuum again. Pressure and vacuum once more. Then fill the bottles with gas once more. Remove the needles and check for leaks in the septa. Turn the gas bottle off and vent any pressure in the lines.

4. If an inert gas is needed, place the gas in a gas bag. Then, using a sterile glass syringe, take out the amount that is needed and put in the bottle. Use caution not to over pressure the syringe since they have been known to break.

   NOTE: WHEN ADDING GAS, ESPECIALLY INERTS OR TRACERS, YOU NEED TO RECORD THE BAROMETRIC PRESSURE, VOLUME OF TRACE GAS AND ROOM TEMPERATURE. This enables a mole balance to be done.

A.4 Gas and Liquid Sampling from Serum Bottles

Gas and liquid samples are to be removed using the same procedures as outlined in Appendix A.2. Be sure to shake the bottles to insure

accurate readings. But make sure that liquid is not on the septa, otherwise the gas syringe may become clogged. The amount removed and the computer program used for calculation will depend on the current analysis.

A.5 Inoculum Preparation in the CSTR

The inoculum for the CSTR was prepared from two lines of stock cultures which are maintained in a shaker incubator and transferred weekly. These stock culture lines are designated "BIOB" and BH2S", respectively. The inoculum was grown as a seed culture in 1.2 L dilution jars adapted for anaerobic transfer and growth of bacteria. The medium was prepared which was very similar to that which was used in the CSTR. The medium was boiled and purged with oxygen free nitrogen. A bottle which had been similarly purged was then filled with 250 mL of the cooled medium, capped and autoclaved. The nitrogen in the sterile bottle was then replaced with synthesis gas and the bottle pressurized to about 7.5 psig. The medium was reduced with 5 mL of a 2.5 percent solution of cytsteine HCl (cysteine is not an essential nutrient for *C. ljungdahlii* but enhances growth rates), and inoculated with 10 mL of each line of stock culture. The seed culture was shaken at 37°C and transferred to the reactor during the late exponential growth phase.

A.6 CSTR Start-Up

REACTOR: The quick connect fitting near the inlet gas filter is disco nnected and the tubing between the filter and reactor is clamped to preclude the backup of liquid into the filter during autoclaving. The sodium hydroxide injectin needle is removed and the pH probe cable at the back of the pH control module is disconnected. The tubing connections to the feed and product reservoirs are then clamped, disconnected and plugged with glass wool filters to maintain sterility following autoclaving. The reactor and its stainless steel support plate is then removed and cleaned as needed. The reactor is filled with about 300 mL jof deionized water and one of the screw cap septa is replaced with a glass wool filter. The assembled reactor is autoclaved for 30 min at 120 C.

PURGING: The hot reactor is removed from the autoclave, the support plate is dried and the assembly is installed on the Bioflo. Purging with nitrogen immediately begins using a sterile glass wool filter and needle inserted in the gas sample port. The synthesis gas line is conncected to the reactor and the valves are opened before releasing the clamp on the tubing between the filter and the reactor. The flow rate is adjusted to the maximum that registers on the rotameter. Nitrogen flow is maintained for 10 to 15 min; the nitrogen is then shut off and the outlet tubing is connected to the product receiver and liquid feed and equalization tubing to the feed reservoir (allow gas to flow through each line to clear residual air prior to sealing). The pump tubing is replaced with new Masterflex tubing (Tygon 6409-13) and inserted into the pump head. The synthesis gas purge is maintained until the reactor gas analysis matches that for the feed (purge overnight at reduced rates).

INOCULUM: The deionized water is drained from the reactor and, using Vacutainer 21G1 blood sampling needle (double ended), the prepared 250 mL seed culture is transferred into the reactor. Agitation begins at low rates (200 rpm), along with fresh medium flow into the reactor. When the liquid covers the pH proble membranes, pH monitoring and control may begin. As the liquid level rises, the agitation rate should be increased to the level appropriate for the experimental work. The use of a seed culture in the exponential growth phase allows immediate initiation of continuous flow, yielding a short start-up time.

Appendix B
Gas Consumption Under Quantitative Conditions

Preparation of the media was carried out anaerobically in an atmosphere of 80 percent nitrogen (N_2), 20 percent CO_2 as described by Hungate and Ljungdahl and Wiegel. The medium was then transferred to serum bottles, 157.5 mL in volume. The bottles are sealed with butyl rubber stoppers and aluminum seals. The bottles were then autoclaved at 121°C for 21 minutes. A seed culture was started from a stock culture in a bottle similar to those described above about 48 hours before the experiment was to begin. This seed culture was grown in a shaker incubator kept at 37°C and 100 rpm. Once the seed culture was ready, sodium sulfide was added to the bottle and left in the shaker incubator for about 15 minutes to allow for complete oxygen removal and temperature acclimation. Then, 10 mL of seed culture were aseptically added to each bottle. The bottles were then flushed with the gas to be employed and pressurized to the desired level (up to 3 atm maximum) with the use of adjustable check valves. A measured amount of an inert gas was then introduced with a Leur-lock syringe. In these experiments 20 cc of methane will be as inert gas. The inert gas allows determination with high accuracy the changes in total pressure inside the bottle and thus the total amount of carbon monoxide present in the gas phase.

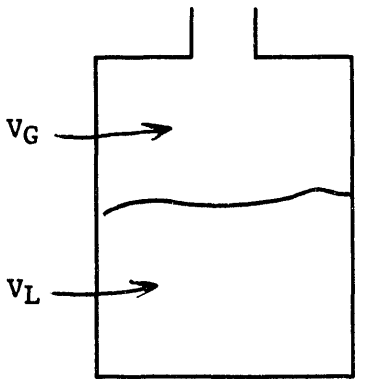
The bottles were left in the shaker incubator (100 rpm) at 37°C during the experiment. Sampling for gas composition, optical density, pH, acids and alcohols were carried out at adequate intervals. An outline of the experimental procedure is shown in Figure A.1.

The cell concentration (dry weight) may then be determined from an appropriate calibration curve of optical density as a function of dry cell weight. Acetate and ethanol concentration may be determined from a gas chromatograph calibration curve. Finally, the moles of carbon monoxide at any time may be found using the ideal gas law the gas chromatographic analysis, and the inert gas composition. The procedure is illustrated in Figure A.2.

Yields may then be determined by plotting cells or product produced as a function as the CO consumed. The yields are then found from the slopes of these plots.

Figure A.1

BATCH EXPERIMENTAL PROCEDURE FOR DETERMINING REACTION STOICHIOMETRY



V_T = 157.5 mL (total volume)

$V_L°$ = 86.5 mL (initial liquid volume)

$V_G°$ = 71.0 mL (initial gas volume)

Start-up

1. Anaerobically, fill the bottle with 75 mL of liquid medium.
2. Vent the gas phase with the desired gas mixture.
3. Sterilize the bottles in the autoclave.
4. Add 1.5 mL of Sodium Sulfide ($Na_2S.9H_2O$)(2.5%).
5. Shake for 20 minutes at 37°C.
6. Add 10 mL of seed culture.
7. Vent the gas phase with the desired gas mixture. Use variable cracking pressure check valves in the gas outlet.
8. Add 20 mL of CH_4 (inert gas) at room temperature and pressure.
9. Measure initial optical density and gas composition.
10. Place in the shaker incubator at 37°C.

Sampling

11. Sample the gas phase for gas composition (0.4 mL).
12. Sample the liquid phase for optical density, pH, ethanol and acetate (3 mL).

Figure A.2

DETERMINATION OF QUANTITATIVE INFORMATION DURING A GAS FERMENTATION

1. Cell concentration (dry weight) from OD (optical density) calibration curve.

2. Moles of carbon monoxide present in the gas phase from gas percent analysis:

   - At start-up:

$$N_{CH_4} = \frac{P_{room}\, V_G^o}{R \cdot T_{room}} \Rightarrow N_T^o = \frac{100}{(\%\ CH_4)}\, N_{CH_4}^o$$

$$N_{CO}^o = \frac{(\%CO)}{100}\, N_T^o$$

   - At each sampling time:

$$N_{CH_4}^i = N_{CH_4}^o - \sum_{j=1}^{i-1} \frac{V_{gas\ sample}}{V_G}\, N_{CH_4}^j$$

$$N_t^i = \frac{100}{(\%CH_4)}\, N_{CH_4} \Rightarrow N_{CO}^i = \frac{(\%CO)}{100}\, N_T^i$$

Thus, the moles of methane depend upon the initial amount of methane and the number of samples.

(3) Ethanol and acetate concentration from G.C. calibration curve.

Appendix C
Analytical Procedures

C.1 Bacterial Growth

A standard curve of absorbance as a function of cell density for the pure isolated culture has been developed. For this purpose, several different volumes of a ten-day old culture growing on synthesis gas were filtered through a 0.45 μm filter paper and dried in the oven until a constant weight was obtained. The dry cell weight density of the solution was found to be 0.167 g/L as from the slope of the line in Figure A3. The culture diluted to several levels and the absorbance at 580 nm measured to obtain the calibration curve shown in Figure A4. The result in Figure A4 can be expressed by the equation:

$$X \text{ (mg/L)} = 473.9 \ (OD)_{580}$$

where X = cell concentration (mg/L), and

$(OD)_{580}$ = optical density measurement at 580 nm.

C.2 Gas Analysis

The composition of the gas phase was determined by gas-solid chromatography in a Varian 3400 gas chromatograph. Samples (0.15 mL) were injected using a Pressure-Lok syringe into a 6 ft x 1/8 in. column packed with Carbosphere, 60/80 mesh, to allow the determination of H_2, Ar, CO, CH_4 and CO_2. A temperature program was required for the adequate separation of hydrogen and air components. The optimal program conditions were determined to be 30°C for 4 minutes, an increase in temperature to 125°C at 30°C/min for 5 minutes, and a final period at 200°C for 8 minutes, required to condition the column prior to analysis of the next sample and to remove any traces of water in the column. The detector and injector temperatures were both 175°C and the carrier gas used was helium at a flow rate of 40 mL/min. The total time for the analysis was approximately 20 minutes.

C.3 Liquid Analysis

Liquid analyses were also carried out by gas chromatography in the Varian 3400 gas chromatograph. Separate procedures for the analysis of alcohols and acids were required to avoid peak interference.

Alcohol analysis was performed using a 2 ft x 1/8 in column packed with Poropak QS, pretreated with NaOH. In a typical run, a 250 μl sample was mixed with 40 μl of NaOH to avoid acid peaks. The injection volume into the chromatograph was 1 μl. The oven temperature was 150°C. Both the detector and injector temperatures were 220°C and the carrier gas was helium at a flow rate of 40 μl/min. To separate methanol, ethanol, propanol and butanol, approximately 15 minutes were required.

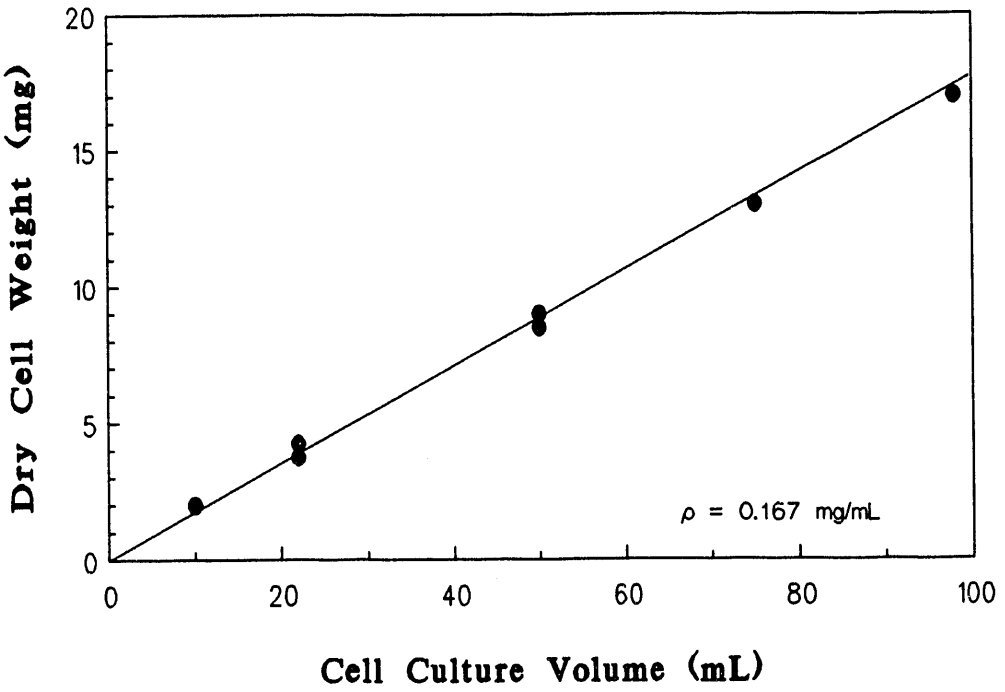


Figure A3. Determination of culture density.

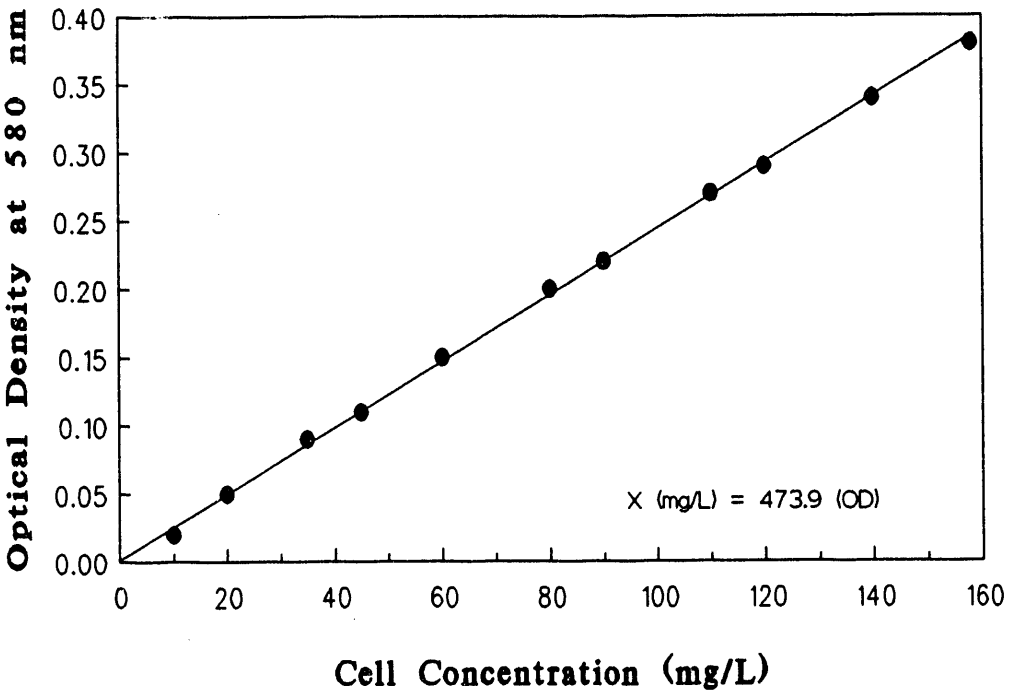


Figure A4. Optical density vs. cell dry weight concentration.

Acid analysis was performed using a 2 ft x 1/8 in column packed with Poropak QS, previously conditioned with H_3PO_4. A 250 μl sample was mixed with 40 μl of 50% H_3PO_4. A 1 μl sample of this mixture was injected into the chromatograph for analysis. The oven temperature was 170°C. The detector and injector were both maintained at 220°C and the carrier gas was helium at a flow rate of 40 ml/min. Approximately 15-20 minutes were necessary to separate acetic, propionic and butyric acid.

To switch from alcohol to acid analysis it was necessary to change the columns, followed by conditioning at 200°C overnight.

END

DATE FILMED

4/7/93