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ENERGY & ENVIRONMENT
DIVISION

BIOCONVERSION OF CELLULOSE

Charles R. Wilke and Harvey W. Blanch

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BIOCONVERSION OF CELLULOSE

DEPARTMENT OF ENERGY
DIVISION OF BASIC ENERGY SCIENCES

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Work Progress Report for FY 1980

TABLE OF CONTENT

I. STUDIES ON THE MECHANISM AND KINETICS OF CELLULOSE HYDROLYSIS

A. Kinetic and Mechanistic Studies on Cellulase Enzymes

1. Introduction

A scheme for the separation of the components of the cellulase complex of enzymes obtained from batch cultures of the fungus Trichoderma reesei, Rut-C-30 strain, has been scaled up to obtain partially purified endoglucanase (E.C.3.2.1.4), cellobiohydrolase (E.C.3.2.1.91), and β-glucosidase (E.C.3.2.1.21). While the latter is not a cellulase in the sense that it acts on a soluble substrate, cellobiose, rather than insoluble cellulose and higher cellooligomers, it is a necessary component of the complex of enzymes involved in the ultimate degradation of cellulose to glucose.

2. Current Research

The scheme, which is shown in Fig. 1, results in the preparation of these enzymes in amounts on the order of grams rather than the previously obtained milligram quantities from analytic separations, and will be sufficient for kinetic studies of the separate activities. Description of the reactions catalyzed by the separated enzymes is essential to the development of a general mechanism for the overall rate of cellulose hedrolysis in the production of glucose from agricultural wastes.

In the first step of the separation scheme, an ultrafiltration cell removes salts and other non-essential low-molecular weight species, including the chromatophore, from the raw culture filtrate. This enhances the separation obtained by elution with a salt gradient in the subsequent ion exchange, which is shown in Fig. 2. This results in the preparation of eight fractions when the pooled fractions are lyophilized and resuspended. Activities of these pooled fractions, and of the raw enzyme, with respect to cellobiose, carboxymethylcellulose (soluble cellulose), and cotton (crystalline cellulose) are

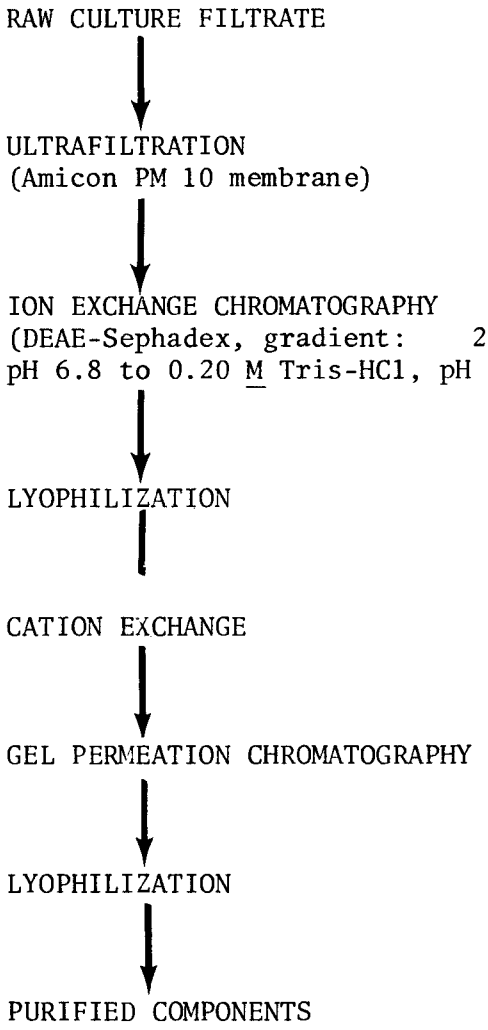


Fig. 1. Scheme for the Preparative Separation of Cellulase Components

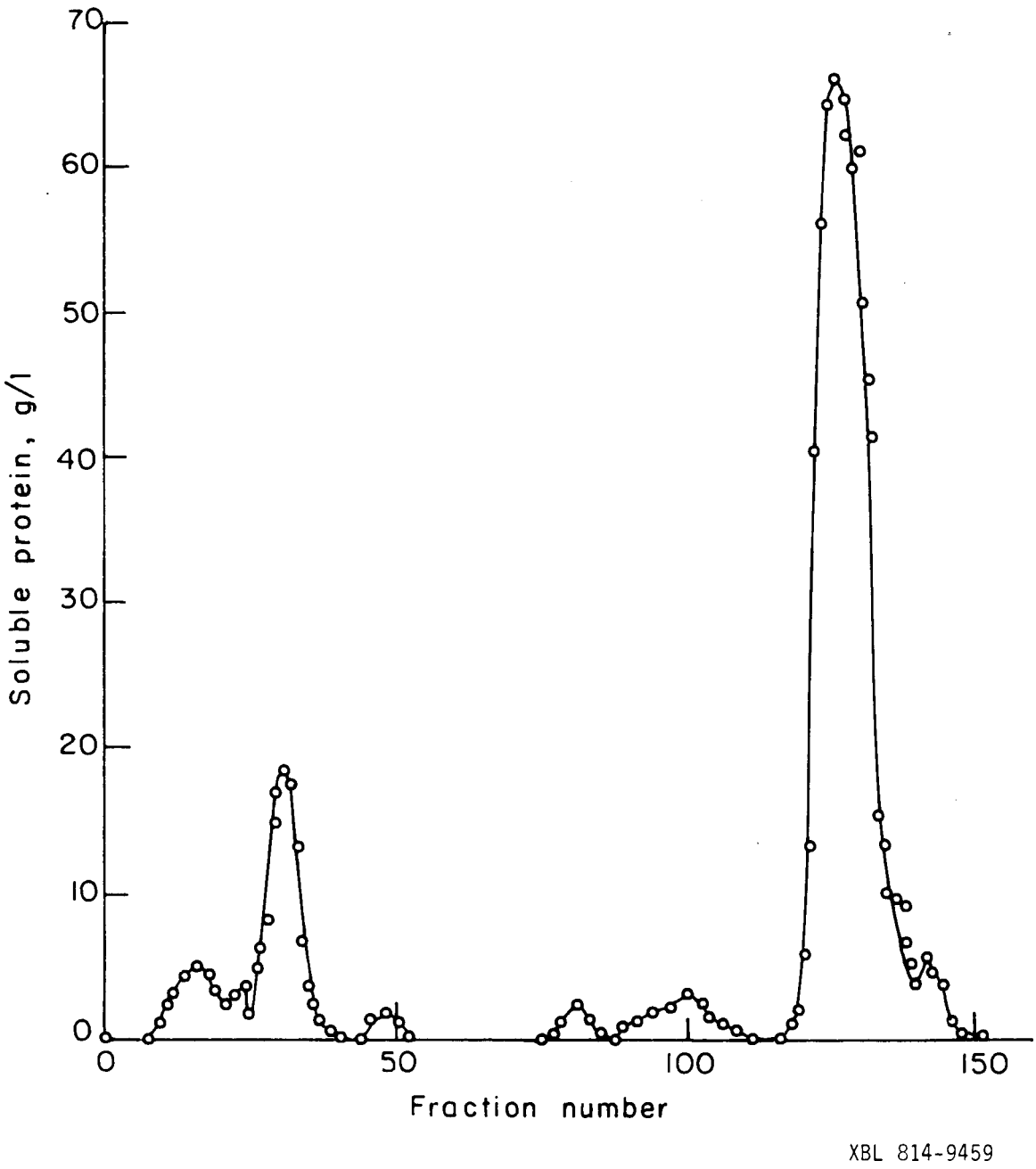


Fig. 2. Ion Exchange Chromatography of Cellulase Components.

Presented in Table 1. This demonstrates the synergistic nature of the components, particularly in the hydrolysis of highly crystalline cellulose.

The material obtained is further purified by gel permeation chromatography. Homogeneity is examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which indicates the requirement for an additional separation by cationic exchange of the proteins eluted before the salt gradient in the anionic exchange step.

The preparative scheme results in the isolation of three major components--the principal cellobiohydrolase, endoglucanase, and β-glucosidase (listed in order of their prevalence in the cellulase complex)--as well as minor components showing each of these types of activities. Xylanase activity is presumably an ancillary activity of a principal component, and the protease irreversibly bound during the anion exchange step.

3. Future Research

The described separation scheme will be instrumental in the development of a kinetic model to be used in optimization of the hydrolysis of cellulosic materials. Such a model will incorporate the effects of multiple kinetic constants and adsorption characteristics found from the purification components, the model will be useful in the resolution of a basic problem that has previously not been studied very much--enzymatic action on an insoluble substrate.

Table I

Specific Activities of Pooled Fractions
From Ion Exchange Chromatography

	β-Glucosidase (Cellobiose activity)	C_x (CMC activity)	C_1 (Cotton activity)
Raw Enzyme	1.14 IU/mg	12.5 IU/mg	0.056 IU/mg
I (fractions 10-20)	2.15	8.12	0.0014
II (21-25)	0.71	14.3	0.0014
III (26-37)	0.04	16.5	0.0033
IV (54-65)	0.17	11.8	0.0019
V (75-85)	-	7.06	0.0033
VI (93-105)	0.0013	6.56	0.0041
VII (119-139)	0.030	0.20	0.0037
VIII (140-145)	-	0.50	0.0033

II. FUNDAMENTAL STUDIES OF HIGH PRODUCTIVITY FERMENTATIONS

A. Basic Studies on Yeast Nutrition

1. Introduction

A basic study of the nutrient requirements for yeast fermentation of sugar to ethanol is important because of the high cost contribution of the nutrients to the ethanol production cost and because of the major effects of media composition on ethanol and yeast productivities. In all known ethanol producing processes, the growth factors and many of the minerals are provided from complex sources such as molasses or cornsteep liquor. Up to now there has been little effort devoted to defining the active components of these complex sources in yeast fermentation and to defining their optimal ratios for a balance medium. A balanced medium is important to eliminate the cost of excess components and because of their inhibitory effects on build up in recycle processes. Therefore, batch and continuous fermentations are being carried out with synthetic and semi-synthetic media to quantatively define the usage of all the major media components for cell growth and ethanol production.

2. Current Research

a) Approach

After the identification of the important growth factors in batch fermentation, continuous cultures were started to determine the optimum levels of all the medium components in ethanol fermentation. A novel procedure extending developments by Mateles and Battat (1) was employed. Each medium component is made the limiting substrate in terms of cell and ethanol yields. The limiting nutrient is first determined by observing which component when injected as a concentrated shot directly into the fermenter produces a transient increase in cell mass or ethanol. The yields of cell

mass and ethanol with respect to the limiting nutrient can then be determined from the known steady state feed rate of the limiting nutrient and known productivity rates of cell mass and ethanol prior to the injection. When this limiting nutrient is found, its concentration in the feed reservoir, which is feeding at a steady state dilution rate is increased such that it is no longer yield limiting up to a given level of cell mass and ethanol. This component can then be eliminated from further testing as the other components in turn are each made yield limiting.

b) Results

Previously it had been shown that for low (10 g/l) glucose concentrations, 88% of the complex component of the medium, yeast extract, could be replaced by synthetic vitamins with a resulting increase in both specific and total ethanol productivities and with almost total glucose utilization. The same relative concentrations of nutrient components were then tried with 100 g/l glucose, using the minimal medium given in Table 2. The results are given in Table 3. The initial steady state with a minimal medium (Medium 27), at a dilution rate of 0.19 hr^{-1} resulted in 53.2% glucose utilization. Raising the dilution rate to 0.28 hr^{-1}, which was the dilution rate used for the 10 g/l glucose case, decreased glucose utilization to 22.3%. Supplementing the minimal medium with additional biotin (Medium 28) in the steady state feed or with pulses of pyridoxine, pantothenate, and biotin (Medium 29) did not significantly change the residual sugar level. Although these three vitamins were shown to be essential in the lower glucose level cases, they were apparently in sufficient quantity in the minimal medium. It was then shown that the minimal medium was deficient in some combination of p-aminobenzoic acid, thiamine, nicotinic acid, and inositol since supplementing the mininal medium with this combination (Medium 30)

Table 2

Minimal Medium Concentration

Glucose	100 g/l
$(NH_4)_2 SO_4$	3.54 g/l
$CaCl_2 \cdot H_2O$	0.059 g/l
$MgSO_4 . 7H_2O$	0.248 g/l
K_2HPO_4	0.730 g/l
Yeast extract	1.0 g/l
Trace elements	2.5 ml/l
EDTA	0.25 g/l
Biotin	26.9 µg/l
Pantothenate	6.92 mg/l
Pyridoxine	6.66 mg/l

Table 3

Continuous Culture with 100 g/l Glucose Feed

Feed	Medium Number	Medium	Dilution rate (hr^{-1})	Dry Cell Mass (g/l)	Ethanol (g/l)	Residual Sugar (g/l)
Steady State	(27)	Minimal	0.19	3.54	18.0	46.8
Steady State	(27	Minimal	0.28	1.86	8.92	77.7
Steady State	(28)	Minimal & Biotin	0.28	2.30*	8.91	75.8
Pulse	(29)	Pyridoxine Pantothenate Biotin	0.28	2.24*	8.92	76.0
Steady State	(30)	Minimal + Biotin + p-amino benzoic acid thiamine nicotinic acid Inositol	0.28	2.77*	11.5	69.2
Pulse	(30)	$(NH_4)_2SO_4$	0.28	2.87*	12.3	64.8
Steady State	(31)	minimal + minerals	0.28	2.64*	12.3	66.0
Steady State	(32)	minimal + minerals + vitamins	0.28	3.86	16.7	52.2

Notes:

*Determined by Beckman spectrophotometer, unstarred dry cell mass determined by Bausch and Lomb spectrophotometer

significantly increased cell mass, ethanol, and sugar utilization. Supplementing the minimal medium with additional minerals (Media 30,31) also improved sugar utilization significantly. The best medium combined supplemental vitamins and minerals (Medium 32) to produce 47.8% sugar utilization. The vitamin content of this best medium was higher than that for the vitamins supplemented Medium 30, so the extent of synergism is not clear.

Even with the best medium above, the sugar utilization is poor relative to that obtained with high yeast extract concentration. Possible reasons are ethanol inhibition, substrate inhibition, carbon dioxide inhibition, non-optimal oxygen tension, and changes in the organism. Investigations into these areas are discussed in the next section on modeling of yeast growth.

3. Future Research

After the source of inhibition of cell growth and ethanol production for 100 g/l glucose feed has been eliminated, a balanced synthetic medium will be derermined for this glucose level.

Reference

1. Mateles, R.J., Battat, E., Applied Microbiology, 28(6), 901-905 (1974).

B. Models of Yeast Growth and Ethanol Production

1. Introduction

The productivities of cell mass and ethanol from fermentation are in general functions of the environmental conditions of fermentation for a given organism. The major independent variables which comprise these conditions are listed in Table 4. The temperature and pH optima were previously determined by Cysewski and Wilke. The medium related variables in this group consisting of the carbon, mineral, and growth factor sources and concentration, have been discussed in the above section on yeast nutrition. In this section the effect of oxygen concentration, carbon dioxide concentration, and ethanol concentration will be discussed. In the next section on high cell density recycle operations, the effects of glucose concentration, fermentation by-products concentration, and ethanol concentration again will be discussed.

Although the discussion has been segmented, all the variables potentially interact. In particular, the effects of oxygen, carbon dioxide, and ethanol concentrations are suspected to be important in explaining the relatively lower yields with higher sugar substrate concentrations noted above. Therefore, before the medium can be optimized, at least a conceptual understanding of the effects of these nonmedium variable is needed.

2. Current Research

a) Oxygen effect

The effect of dissolved oxygen was studied because previous reports showed important but contradictory results in the very low ranges of 0 to 10 parts per billion dissolved oxygen. Cysewski and Wilke reported a minimum specific ethanol productivity with respect to dissolved oxygen in this range. Nishizawa, Dunn, and Bourne reported a maximum

Table 4

Key Variables in Yeast Growth and Ethanol Production

Carbon source and concentration

Minerals and concentrations

Growth factors and concentrations

Oxygen concentration

Carbon dioxide concentration

Ethanol concentration

By-products concentrations

Temperature

pH

specific ethanol productivity in the same range of dissolved oxygen.

Unfortunately, many attempts at measuring this low level of oxygen did not succeed. Dissolved oxygen probes, chemical tests, and gas chromatography (with thermal conductivity detector) all lacked the sensitivity to measure this level of dissolved oxygen.

Attention was then turned to determining the effects of higher measurable concentrations of dissolved oxygen, which would be in the range of practical importance. Control of the reported low optimal levels would be impractical because of its narrow range. Preliminary results show that above a minimum oxygen level (less than 2% of air saturation) there is no clear effect of oxygen on cell mass and ethanol, up to about 50% of air saturation. However, the oxygen effect very likely was obscured by varying carbon dioxide concentrations. Subsequently, when carbon dioxide was controlled over a fairly narrow range, the oxygen concentrations in the outlet gas, reflecting varying dissolved oxygen was varied from about 5 to 10% air saturation. There was essentially no effect on ethanol production and no discernible trend on dry cell mass, in this range, as shown in Table 5.

Also, an interesting change, which may be an adaptation, appeared to take place in the organism when an essentially anaerobic continuous culture was aerated vigorously. Both the cell and ethanol yields, 18 g/l and 32 g/l, respectively, increased considerably over previously obtained yields with 100 g/l glucose. The cell yield was two to three times that expected from fermentative yeast growth and indicated substantial aerobic or oxidative growth. Aerobic growth in the presence of 1 to 2 g/l glucose residue in the medium implied that catabolite repression (the glucose effect) was at least partially lifted. Measurement of oxygen uptake and carbon dioxide evolution are in progress to determine the relative extents.

Table 5

Effect of O_2 Concentration in Fermentor Outlet Gas

Outlet Gas Mole Fractions		Cell Dry wgt. (g/l)	Ethanol (g/l)	Specific productivity of Ethanol (hr^{-1})
Y_{O_2}	Y_{CO_2}			
0.010	0.513	5.99	32.8	1.10
0.0121	0.508	5.98	31.9	1.08
0.0136	0.502	6.48	32.2	0.995
0.0157	0.498	6.71	31.5	0.939
0.0226	0.465	6.14	33.1	1.08

of fermentative and oxidative growth as a function of aeration conditions. If this function can be determined, the ratio of cell mass to ethanol production can be controlled. A complicating factor to this study has been the stability of the possible adaptations.

b) Carbon dioxide effect

Brandt reported that under anaerobic conditions carbon dioxide did not affect the metabolism or cell constituents of Baker's yeast. From Table 6, the carbon dioxide concentration from 5.7% to 51.3% in the gas outlet is seen to have practically no effect on ethanol production, but there is very significant inhibition of cell growth by carbon dioxide. From the second and third points in this table, it was also established that Yo_2 from 0.011 to 0.020 had no effect on cell mass or ethanol production. Therefore the effects in Table 6 should be due solely to carbon dioxide. The carbon dioxide inhibition appears to begin at about 10% of the gas phase.

c) Ethanol inhibition

It has been determined that de Bazua's ethanol inhibition model underestimates the degree of inhibition in systems with 100 g/l glucose feed. de Bazua's model was fitted from data obtained using 10 g/l glucose and should be expanded to include 100 g/l glucose feed. There is a wide discrepancy in the degree of inhibition proposed by various models. Part of the difference may come from carbon dioxide inhibition or insufficient oxygen.

3. Future Research

The effect of dissolved oxygen over a wide range with constant carbon dioxide will be determined.

The carbon dioxide concentration will be increased by adding

Table 6

Effect of CO_2 Concentration in Fermentor Outlet Gas

Outlet Gas Mole Fractions		Cell Dry wgt(g/l	Ethanol (g/l)	Specific productivity of Ethanol (hr^{-1})
Y_{O_2}	Y_{CO_2}			
0.010	0.513	5.99	32.8	1.10
0.020	0.202	8.01	35.3	0.882
0.011	0.195	8.16	34.5	0.844
0.015	0.103	9.15	34.3	0.752
0.019	0.057	8.84	33.6	0.760

CO_2 to the fermenter to extend the CO_2 inhibition model.

The ethanol inhibition model will be extended by increasing the glucose feed concentration to 100 g/l. Because the temperature optimum was previously established in batch culture, it will be checked in continuous culture.

References

1. Cysewski, G., and Wilke, C.R., Ph.D. Thesis in Chemical Engineering University of California, Berkeley, March 1976

2. Nishzawa, Y., Dunn, I.J., and Bourne, J.R., "The Influence of Oxygen and Glucose on Anaerobic Ethanol Production," Abstracts from the European Congress of Biotechnology held at Interlaken, Switzerland, September 1978.

3. Brandt, K.M., Acta Physiologica Scandinavica, Vol 10, Supplementum XXX, 1945.

4. de Bazua, M.C.D., M.S. Thesis in Chemical Engineering, University of California, Berkeley.

C. By-Product Inhibition Effects in Cell Recyle and Vacuum Fermentation

1. Introduction

Inhibition by secondary fermentation products may limit the ultimate productivity of new fermentation processes. New processes (including flash fermentation) are under development whereby ethanol is selectively removed from a fermenting broth to eliminate ethanol inhibition effects. These processes can concentrate minor secondary fermentation products to the point where they become toxic to the yeast. Flash fermentation selectively concentrates non-volatile products in the fermentor broth. Membrane fermentation systems may concentrate large molecules which are sterically blocked from membrane transport. Extractive fermentation systems, employing nonpolar organic solvents, may concentrate organic acids. By-product production rates and inhibition levels must be determined to assess the potential for fermentation by-product inhibition.

2. Current Research

a) Approach

The products of alcoholic fermentation of glucose by Saccharomyces cerevisiae are given by Rainbow and Rose (1) (Table 7). In addition to those by-products shown, volatile acetaldehyde is known to be produced in about equimolar proportion to glycerol. When sugar from natural sources containing amino acids (such as corn) are used, fusel oils will also be produced, with up to 5 grams of these propyl and butyl alcohols produced per liter of alcohol.

To test the effects of these by-products, continuous fermentations were conducted with increasing amounts of each individual by-product added to the feed until cell growth and ethanol productivity inhibition was observed.

Table 7

Products of the alcoholic fermentation of glucose by *Saccharomyces cerevisiae*

	mM product/100 mM glucose fermented
Ethanol	177.0
Carbon dioxide	180.8
2,3-Butanediol	0.48
Acetoin	-
Glycerol	6.60
Acetic acid	0.69
Butyric acid	0.32
Formic acid	0.42
Lactic acid	0.38
Succinic acid	0.26
Glucose assimilated	16.1
Fermentation time (h.)	14.5

Conditions for the experiments are given in Table 8. The feed glucose concentration has been chosen low to limit ethanol production and prevent the masking of by-product inhibition effects by ethanol inhibition.

b. Results

Results to date are summarized in Table 9 which lists the by-products studied and the by-product concentration at the onset of inhibition effects. Results for ethanol and glucose inhibition studies are included for comparison. All of the inhibition effects studied can be explained by three basic inhibition mechanisms.

i. Inhibition by direct interference with the ethanol production or cell growth pathways.

Ethanol inhibition has been shown by Bazua (2) to be by direct noncompetitive inhibition of the glucose to ethanol pathway. Inhibition begins at about 25 g ethanol/l beer and is total at 95 g/l. The ethanol metabolic pathway generates ATP for cell maintenance and growth. Typical of this direct inhibition of the metabolic pathway is a constant proportional decrease in cell growth rate (μ) as ethanol productivity (ν), and hence available ATP, decreases with increased inhibition (see Fig. 7).

Direct inhibition of the cell growth pathway has not been observed in these experiments but has been induced by nitrogen starving the yeast. Cell growth rate (μ) is decreased but ethanol productivity (ν) is maintained as ATP is shunted away for production of by-products such as glycerol and acetadelhyde

Direct inhibition of the metabolic (ethanol) or cell growth pathways was not observed for any of the by-products tested.

ii. Inhibition by chemical interference with cell maintenance functions

Inhibition by chemical interference with cell maintenance functions is well illustrated in the case of acetic acid (which inhibits in

Table 8

Conditions for Continuous By-product Inhibition Studies

Base medium composition:

Glucose	20 g/l
Yeast extract	1.7 g/l
NH_4Cl	.26 g/l
$(NH_4)_2SO_4$	.65 g/l
$MgSO_4 \cdot 7H_2O$	.022 g/l
$CaCl_2$	.012 g/l
Antifoam	.040 ml/l

Fermentation conditions:

pH	4.0
Temperature	35°C
Dissolved O_2	5% of air saturation
Dilution rate	acetaldehyde and glycerol expt. .11 hr^{-1}
	other experiments .16 hr^{-1}

Table 9

Inhibition effects for Saccharomyces cerevisiae

Inhibition	Concentration at Onset of Inhibition
Ethanol	25 g/l
Glucose	150 g/l
Acetadelhyde	2 g/l
Gylcerol	100 g/l
2-3 Butanediol	50 g/l
Acetic Acid	.5 g/l
n-propanol	4 g/l

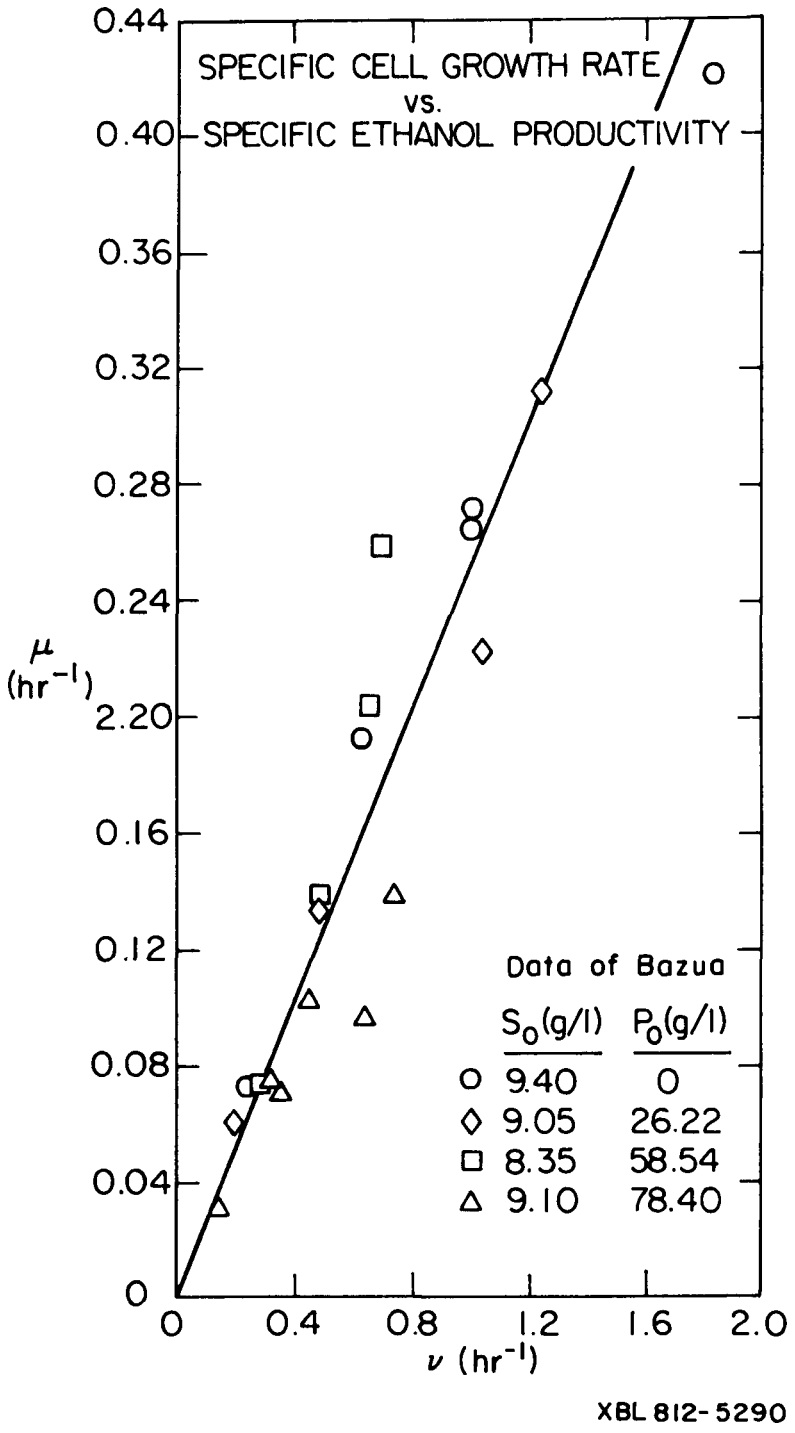

SPECIFIC CELL GROWTH RATE
vs.
SPECIFIC ETHANOL PRODUCTIVITY
μ (hr⁻¹)
ν (hr⁻¹)
0.44
0.40
0.36
0.32
0.28
0.24
2.20
0.16
0.12
0.08
0.04
0
0
0.4
0.8
1.2
1.6
2.0
Data of Bazua
S0(g/l) P0(g/l)
9.40 0
9.05 26.22
8.35 58.54
9.10 78.40
XBL 812-5290

Figure 7.

the range from 1 to 8 g/l, see Fig. 8). Samson (3) has shown that acetic acid (or sodium acetate) inhibits by chemically interfering with transport of phosphate. Phosphate transport through the cell membrane is an activated transport process requiring the expenditure of ATP. Acetic acid interference results in an increase in the ATP requirement for this maintenance function. Typical of this type of inhibition, cell production decreases while ethanol production increases to make available sufficient ATP for cell maintenance. The ratio of μ/ν decreases as inhibitor concentration increases. Chemical interference effects can typically occur at very low inhibitor concentrations and where membrane disruption is involved (as in acetic acid attack) cell morphology is altered with cells becoming irregular and elongated.

Inhibition by 1-proponal (a major fusel oil component, Fig. 9) and by acetaldehyde (Fig. 10) like acetic acid inhibition appears to be by a mechanism of cell membrane attack resulting in increased maintenance energy requirements. Similar low by-product concentrations cause inhibition, cell production is depressed while specific ethanol productivity is increased and cell morphology is distorted.

A unique feature of acetaldehyde is consumption of the by-product by the yeast and conversion to alcohol. Thus, as the feed acetaldehyde concentration is increased from 0 to 4 g/l, the residual acetaldehyde concentration in the fermentor increases only from .25 to .8 g/l.

The mechanism of 2-3 butanediol inhibition (Fig. 11) is less certain. Inhibition by 2-3 butanediol begins at 50 g/l concentration--much higher than for the other chemical inhibitors. With two side chain alcohol groups, 2-3 butanediol is only modestly soluble in the cell membrane. Chemical disruption of the membrane at the high inhibition concentrations found may thus be the mechanism of inhibition.

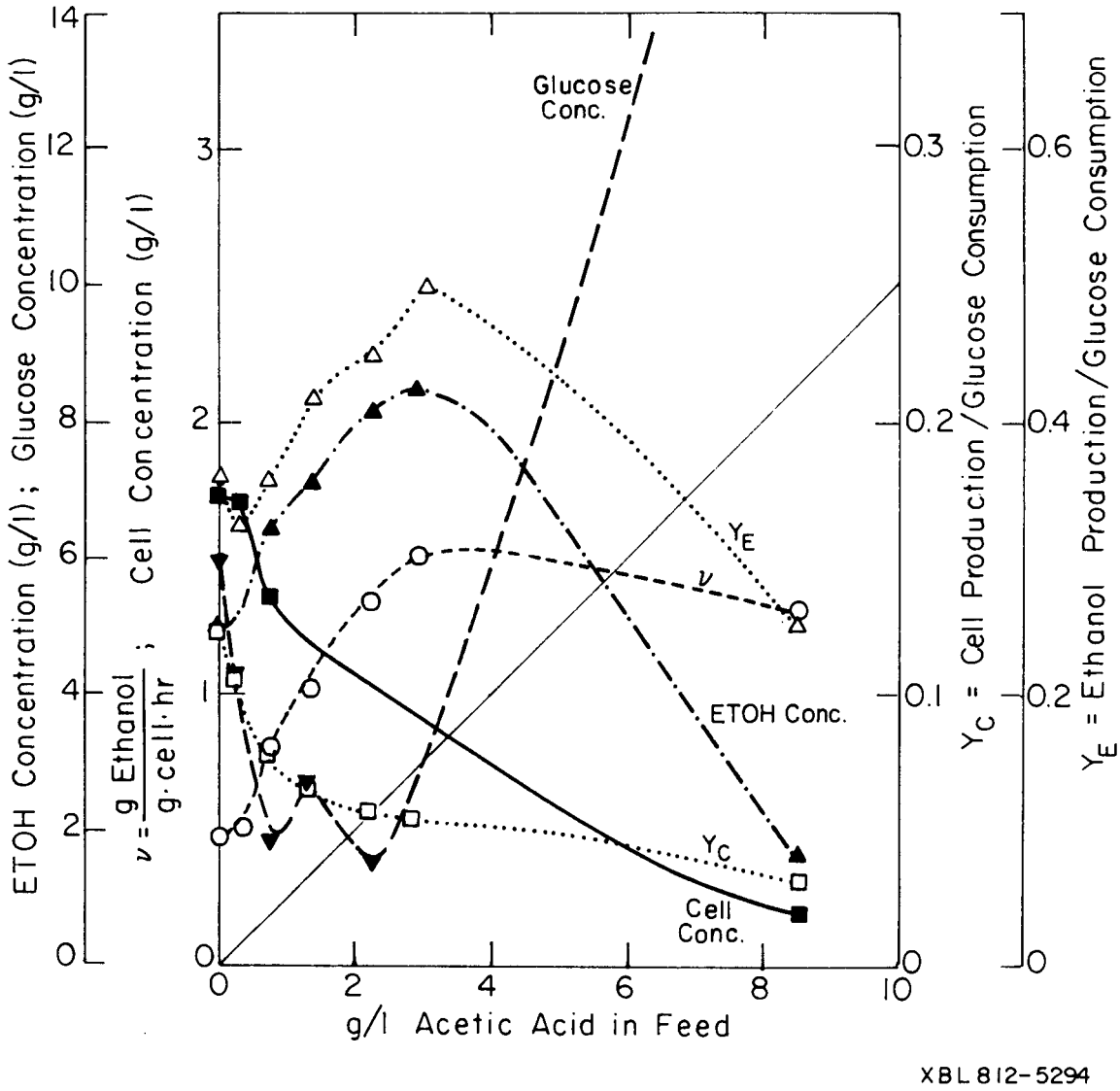

ETOH Concentration (g/l); Glucose Concentration (g/l)
$\nu = \frac{\text{g Ethanol}}{\text{g·cell·hr}}$; Cell Concentration (g/l)
Y_C = Cell Production/Glucose Consumption
Y_E = Ethanol Production/Glucose Consumption
Glucose Conc.
Y_E
ν
ETOH Conc.
Y_C
Cell Conc.
g/l Acetic Acid in Feed
XBL 812-5294

Figure 8.

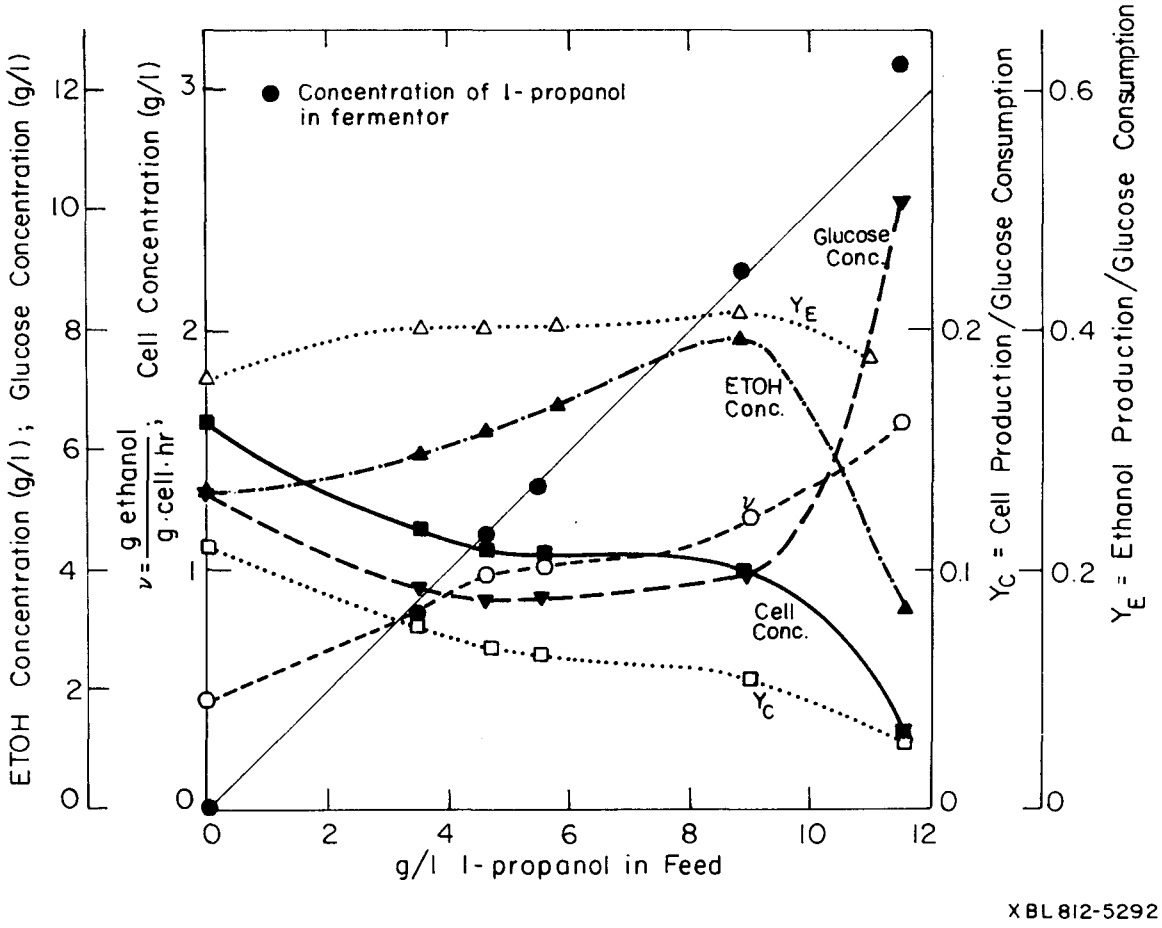

ETOH Concentration (g/l); Glucose Concentration (g/l)
Cell Concentration (g/l)
$\nu = \frac{\text{g ethanol}}{\text{g·cell·hr}}$
Y_C = Cell Production/Glucose Consumption
Y_E = Ethanol Production/Glucose Consumption
Concentration of 1-propanol in fermentor
Glucose Conc.
Y_E
ETOH Conc.
Cell Conc.
Y_C
g/l 1-propanol in Feed
XBL 812-5292

Figure 9

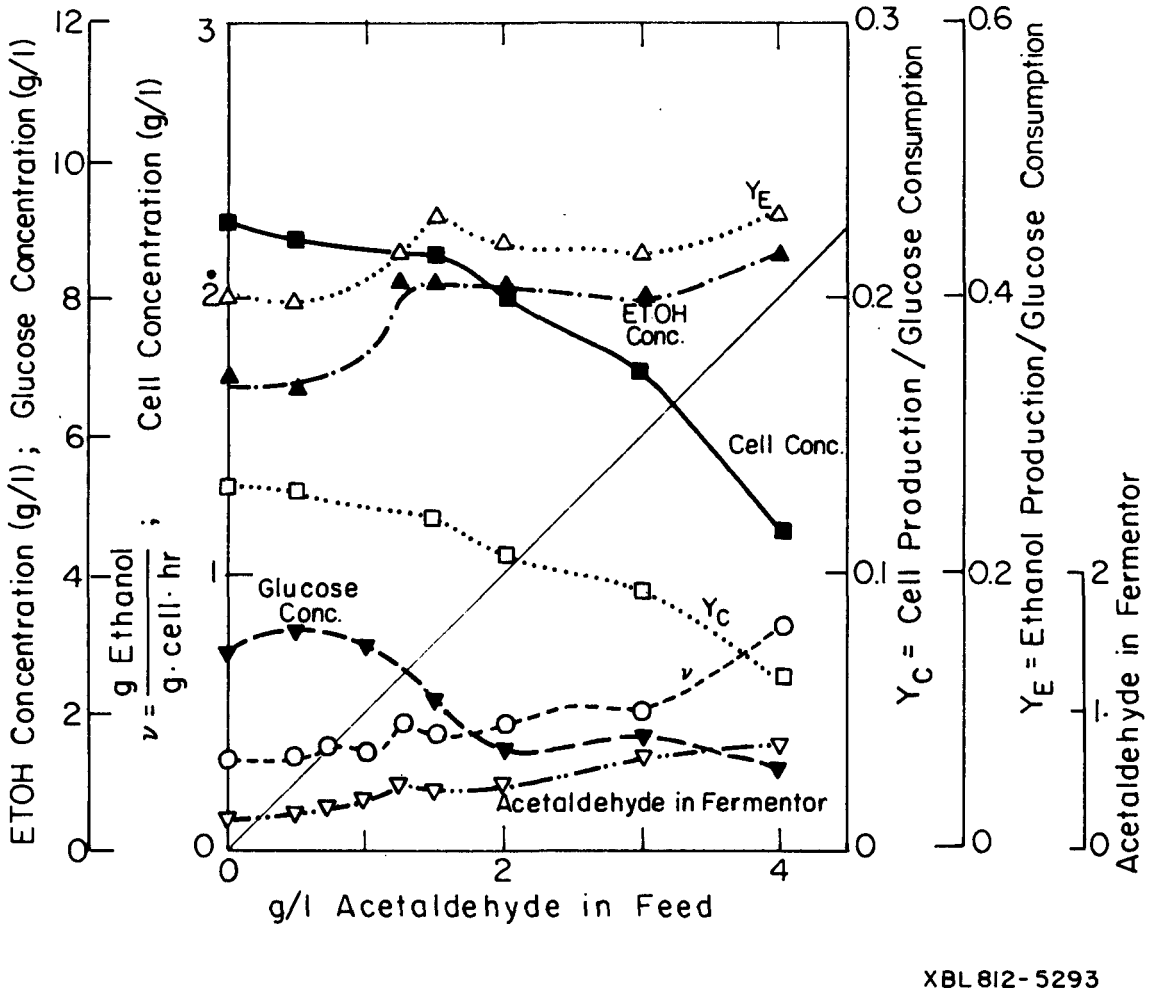
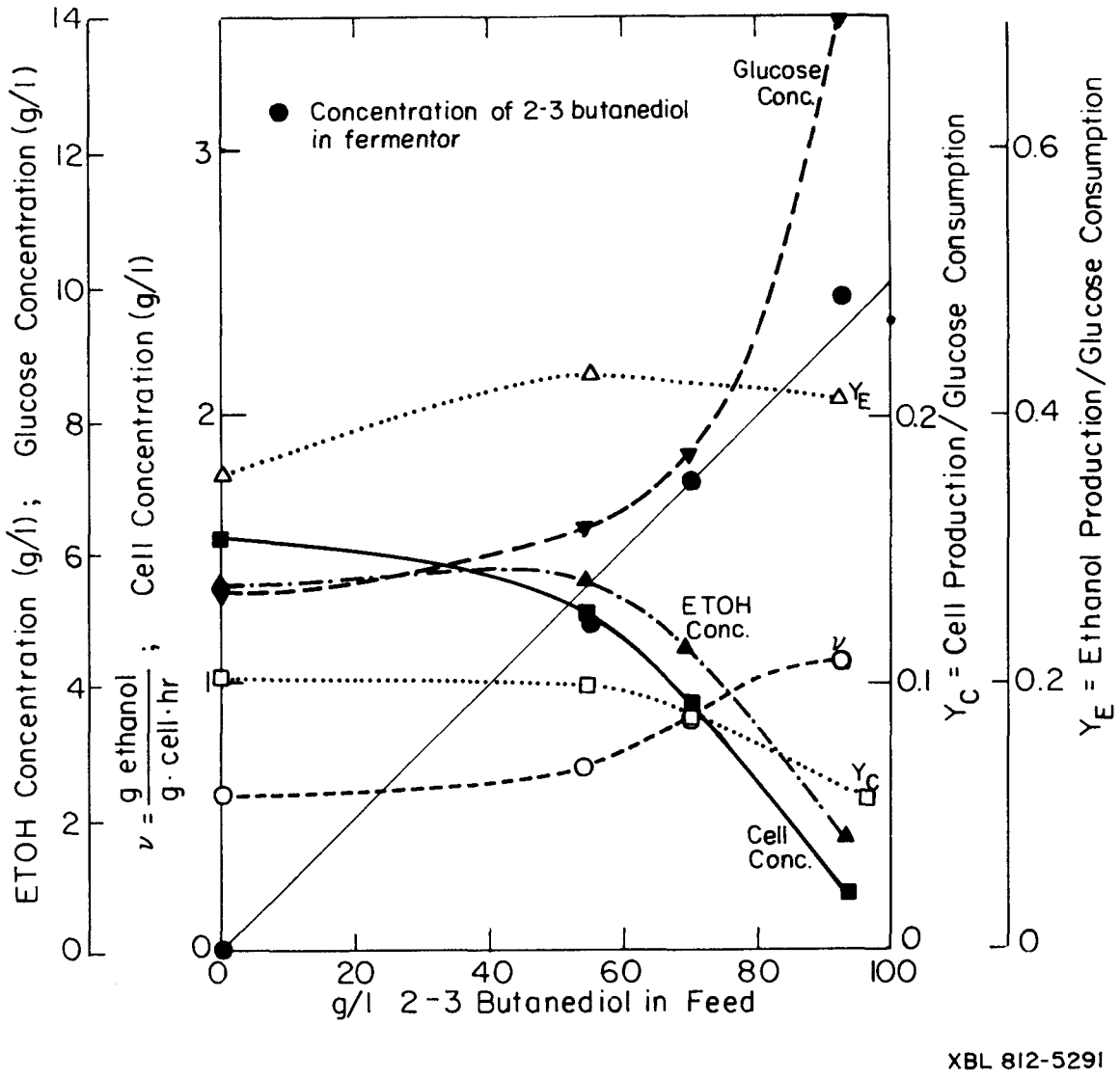

ETOH Concentration (g/l); Glucose Concentration (g/l)
Cell Concentration (g/l)
$\nu = \frac{g\ Ethanol}{g \cdot cell \cdot hr}$;
Y_C = Cell Production / Glucose Consumption
Y_E = Ethanol Production / Glucose Consumption
Acetaldehyde in Fermentor
g/l Acetaldehyde in Feed
ETOH Conc.
Cell Conc.
Glucose Conc.
Y_E
Y_C
ν
Acetaldehyde in Fermentor
XBL 812-5293

Figure 10



XBL 812-5291

Figure 11.

iii. Inhibtion by osmotic stress

Inhibition by osmotic stress occurs when the concentration of some by-product becomes so high that a large osmotic pressure gradient is established between the interior of the cell and the fermentor broth, and the cell must expend large amounts of energy to maintain homeostatic balance. There is no direct interference with any cell chemical process--no direct disruption of the cell membrane--and the inhibitor would normally be classed as non-toxic to the yeast. Like the mechanisms of inhibition by direct interference with cell maintenance functions, cell production is first reduced with an increase in specific ethanol productivity. Inhibition by osmotic stress occurs only at very high inhibitor concentration and osmotically stressed cells are rigid spheres.

Inhibition by osmotic stress is well illustrated by glycerol inhibition (Fig. 12). Glycerol has no effect at 100 g/l and significant cell growth continues at 400 g/l concentration.

It is instructive to compare glycerol and glucose inhibition effects. Total cell productivity was reduced by 25% at a glycerol concentration of 210 g/l with a corresponding osmolarity of 2.96. In batch experiments, cell productivity was reduced 25% at a glucose concentration of 270 g/l corresponding to an osmolarity of 2.26.

2-3 butanediol inhibition, occurring at the relatively high concentration of 50 g/l might be due in part to an osmotic pressure effect, however, the osmolarity of the inhibiting 2-3 butanediol solution is substantially lower than for glycerol or glucose inhibition.

iv. Conclusion

A generalized ethanol removal fermentation system is shown in Fig. 13. Feed glucose, nutrients, and water enters in stream 1. The

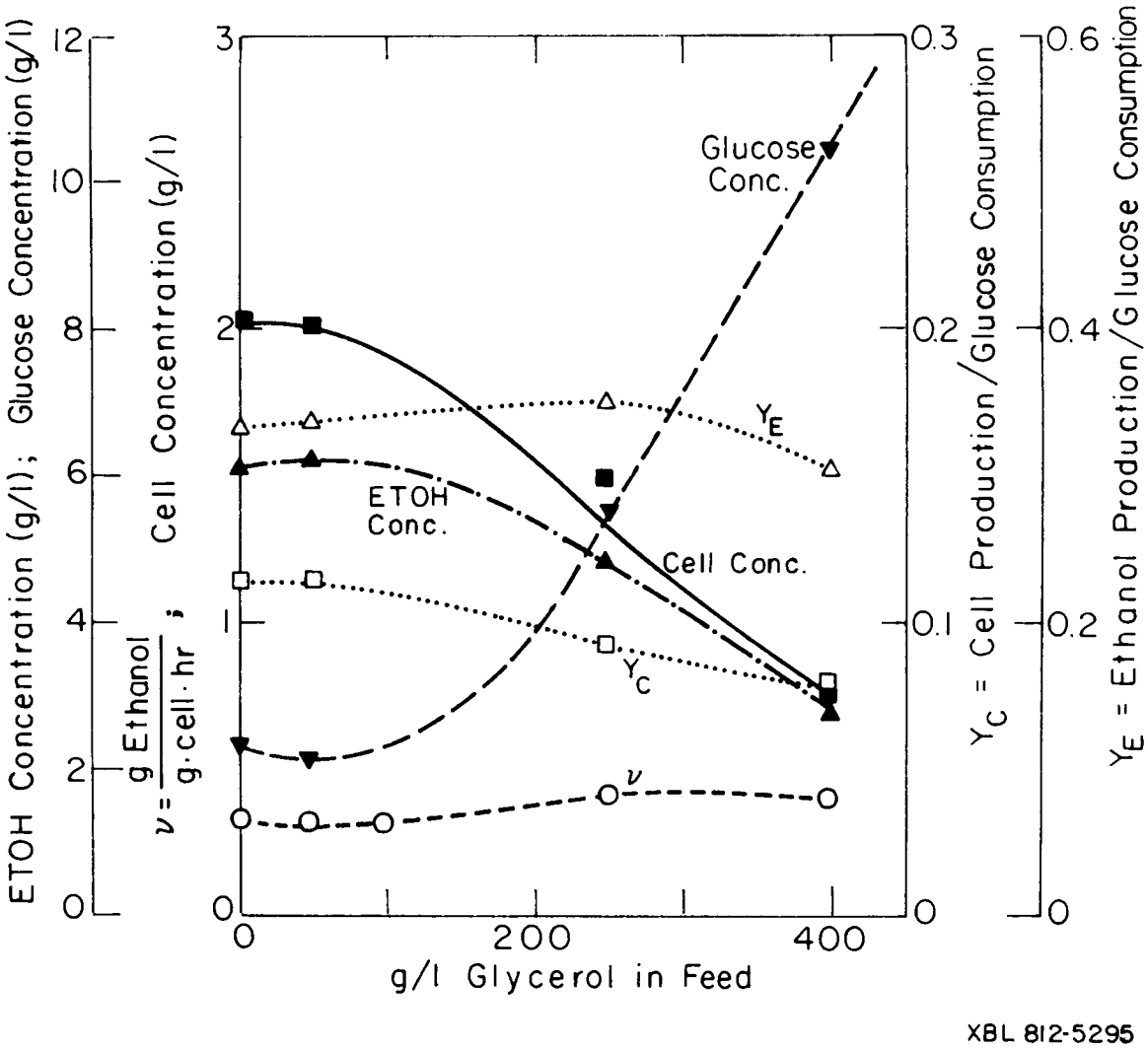

ETOH Concentration (g/l); Glucose Concentration (g/l)
Cell Concentration (g/l)
Y_C = Cell Production/Glucose Consumption
Y_E = Ethanol Production/Glucose Consumption
$\nu = \frac{g\ Ethanol}{g \cdot cell \cdot hr}$
Glucose Conc.
ETOH Conc.
Cell Conc.
Y_E
Y_C
ν
g/l Glycerol in Feed
0
200
400
XBL 812-5295

Figure 12

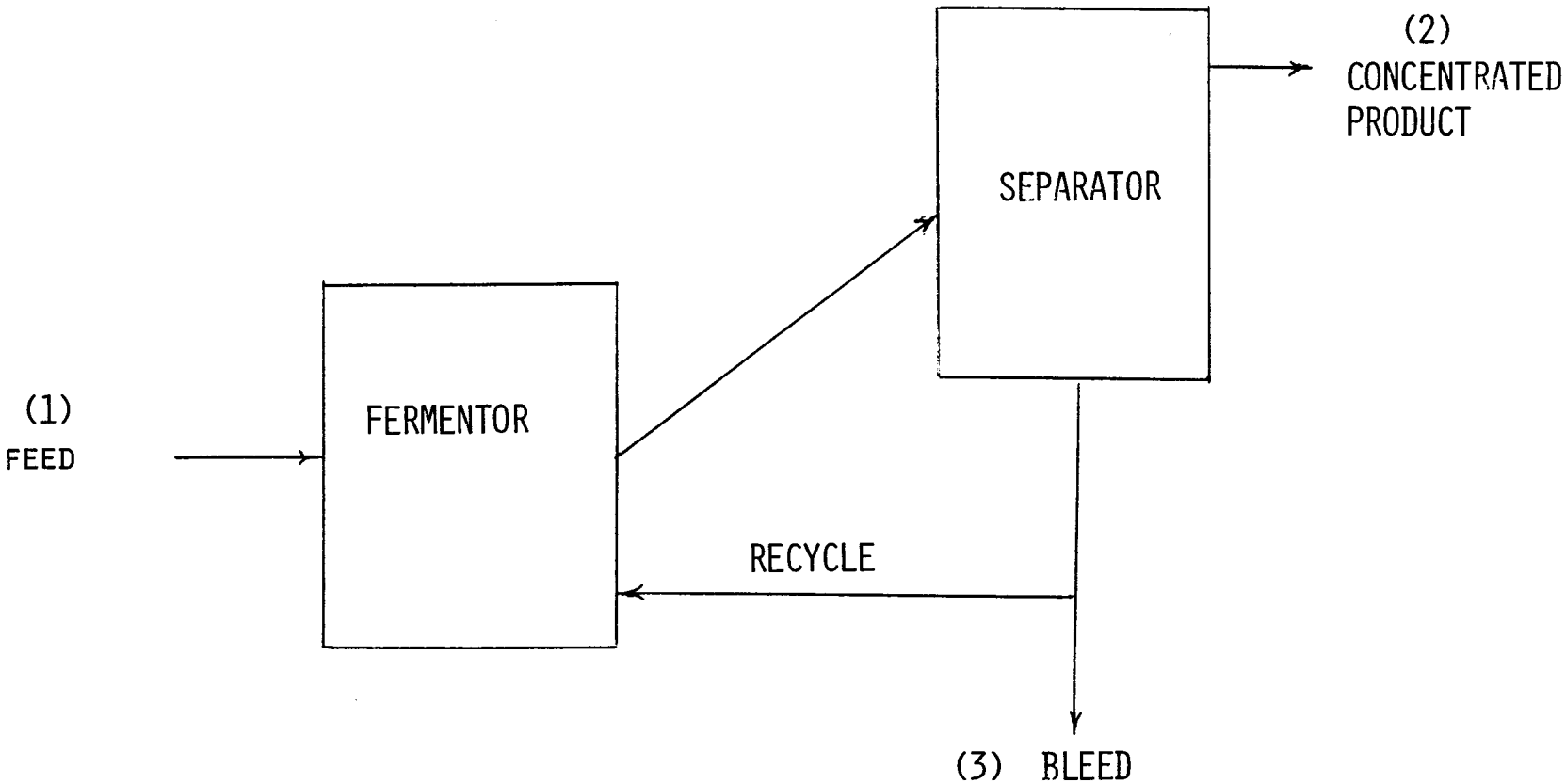


Fig. 13. Generalized Ethanol Removal Fermentation System.

concentrated ethanol product and some water leave as stream 2. A dilute bleed of water, residual product, unutilized nutrients, concentrated by-products and possibly cells, leaves as stream 3.. For the flash ferm process, stream 2 would be the flash vessel vapor product and stream 3 the centrifuged bleed stream.

To minimize costs, it is desirable to maintain the bleed stream as small as possible (thus removing most of the ethanol product as a purified concentrated stream). If the ratio of the size of the bleed stream 3 to the size of the feed stream 1 is γ, and if the ethanol recovery stream 2 contains no by-product contaminants and the by-products are not reconsumed by the yeast, then the concentration of any given by-product in the fermentor will be given by

$$C_{\text{by-product}} = \frac{\nu \cdot X \cdot Y_{\text{by-product}}}{\gamma \cdot D} \qquad (1)$$

where

$C_{\text{by-product}}$ = by-product concentration (g/l)

ν = specific ethanol productivity (1/hr)

X = cell density (g/l)

$Y_{\text{by-product}}$ = by-product production per ethanol production

$$\frac{\text{g by-product/hr}}{\text{g ethanol/hr}}$$

D = dilution rate

Thus the by-product concentration is increased by a factor of $1/\gamma$ over a conventional process--the smaller the bleed, the greater the by-product concentration effect.

For all of the by-products studied, it is seen that specific ethanol productivity can be increased (from about $\nu = .7$) up to a uniform maximum of about $\nu = 1.5$ (where cell growth is arrested). Assuming a cell

recycle system so that a high value of cell growth rate (μ) is not necessary for high productivity, then by-product concentrations just below the total cell growth inhibition level are desirable. Using these values from our experiments, $Y_{by\text{-}product}$ values from the literature (Table 7) a high cell density of 100 g/l, a concentrated (300 g/l) glucose feed typical of flash fermentation, and formula (1), we find that a bleed ratio of 1:10 should be sufficient to prevent excessive toxic by-product build-up.

3. Future Research

Experiments are now underway testing the inhibition effects of lactic acid, formic acid and other fusel oil components.

The experimental portion of this work will be completed in April 1981. A paper summarizing this work is to be presented at the 1981 annual meeting of the American Chemical Society.

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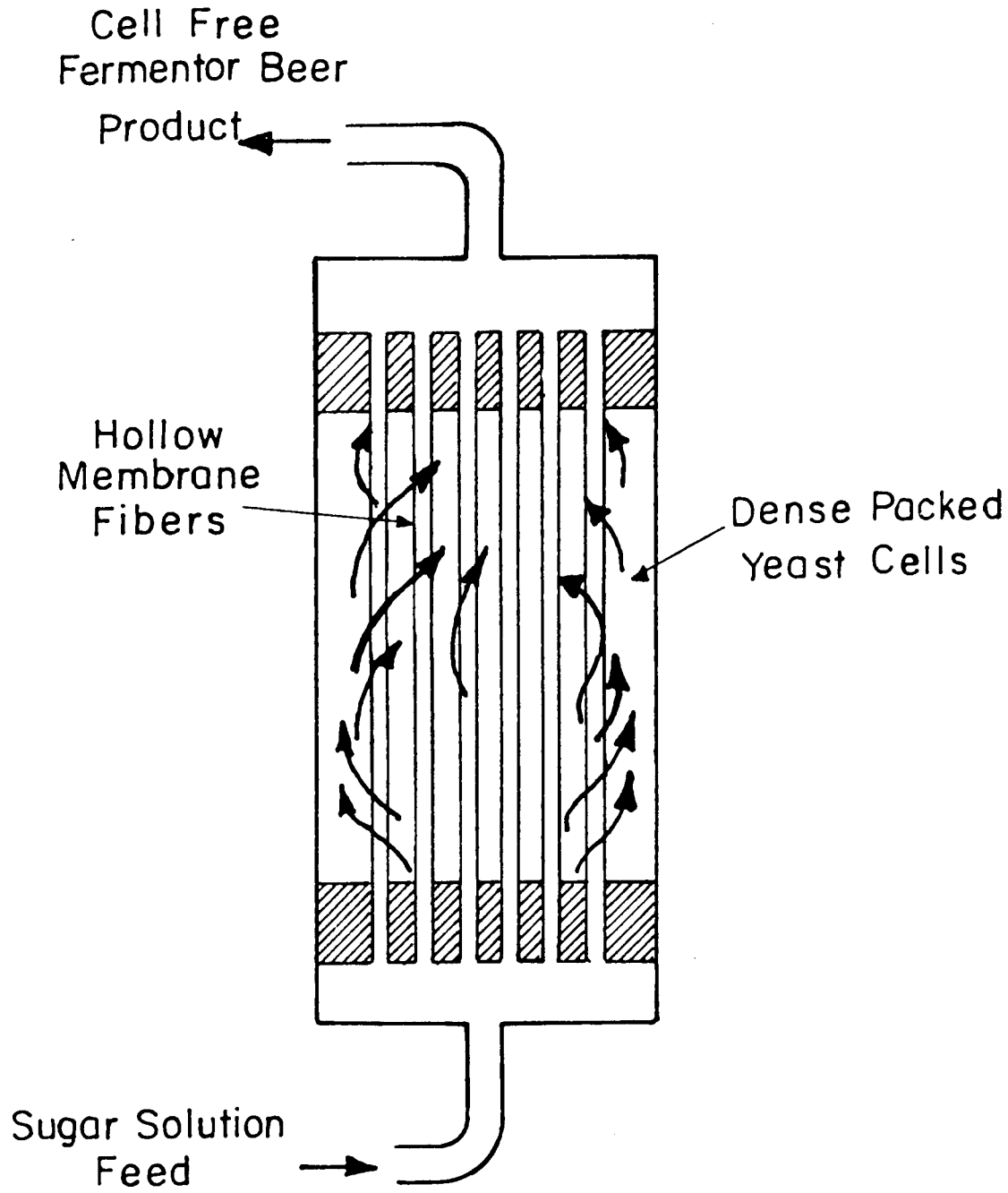
D. Hollow Fiber Reactor

1. Introduction

High density fermentations can, in principle, lead to higher productivities per unit fermentor volume. To achieve this goal, however, the specific productivity of the organism (productivity per gram of cells) must be maintained. At high cell densities, the broth becomes very viscous and mixing becomes inefficient in conventional fermentors. The growth rate is thus controlled by mass transfer limitations in the broth. The removal of metabolic waste products, which inhibit the growth rate, is also subject to the same mass transfer limitations. Thus, if it is desired to grow cells at very high densities (the extreme case is that of close packed cells), an alternative means of supplying nutrients and removing growth products must be provided. A hollow fiber fermentor, in principle, will be able to accomplish this by distributing the nutrients to the cells and withdrawing products by means of semipermeable hollow fiber ultrafiltration membranes. The cells are too large to pass through the membranes and therefore are immobilized on the shell side. In principle, the unit functions analogously to capillaries in the tissues of higher multicellular organisms. A diagram of a unit is shown in Fig. 14.

Similar work has been done previously using whole cell immobilized enzymes in a hollow fiber reactor by Schuler, et. al. (1,2,3). Their results show that the reactor can be mathematically described using effectiveness factors, and that the mass transfer limitation in their case is caused by diffusion on the shell side of the fermentor. The objective of this work is to utilize a modified version of this model to describe an active yeast fermentation. The resulting model will be used to optimize ethanol productivity with Saccharomyces cerevisiae anamensis (ATCC 4126).



Hollow Fiber Fermentor

XBL 803-4899

Fig. 14

2. Current Research

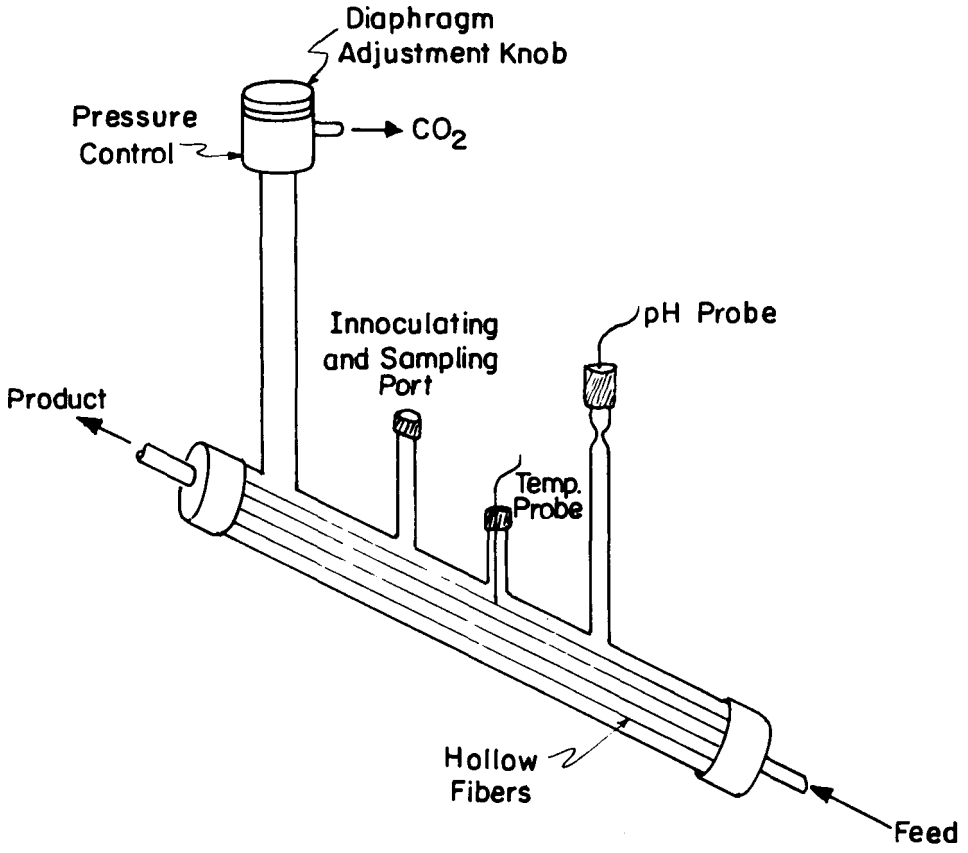
The first fermentation was performed using a commercial ultrafiltration unit. It showed that there are some additional complications that a model of the system must describe, and that equipment modifications must be made before a continuous high density fermentation is possible. Some of the most significant problems are:

1) The removal of gaseous CO_2 from the shell side.

2) Contamination of yeast on the tube side.

To remove the CO_2 gas from the fermentor, the shell side pressure is increased (which lowers the volumetric rate of gas evolution) and the spacing between fibers is increased which allows the bubbles to rise more easily. Increasing the fiber spacing, however, increases the diffusion length on the shell side, and may affect the rate of the supply of nutrients to the cells. An attempt was made to determine if the fiber spacing would cause large diffusional limitations at a sufficient spacing for adequate gas venting. Assuming a Newtonian fluid of a 'reasonable' viscosity (100-1000 cp.), it was found that the required spacing for gas venting is rather close to that at which diffusional limitations will occur. However, because the rheology of the thick fermentor broth is unknown and the wall effects due to the fibers on the rising bubbles are not readily estimated, the model is inconclusive.

It was not possible to determine if the contamination of the tube side by yeast cells was caused by growth through the membrane because there are some o-ring seals in the commercial units which are also suspect. Therefore, subsequent versions were constructed with glued, airtight seals. A unit with the modifications mentioned above was finally constructed after half a year and numerous attempts (Fig.15). The performance of this unit



HOLLOW FIBER FERMENTOR WITH MONITORING PROBES AND PRESSURE CONTROL

XBL 813-5317

Figure 15

was better, although not completely successful. The maximum fermentation rate was only about 5% of the theoretical maximum and yeast were found in the tube side and caused considerable contamination in the product line. Prior to the fermentation, the fibers were airtight at 10 psi of pressure. Afterwards three pinhole leaks were observed in the 1 to 4 psi range. The yeast may have grown through due to defective membranes. About half of the fibers were found to have surface abnormalities when viewed under the microscope. These same fibers stretched severely during the fermentation. The cause of the slow yeast growth is at present unclear. The ethanol concentration on the shell side was only about 13 gm/l and thus should not be responsible for the slow growth rate. The pH was maintained at 3.6 to 4.0 and the temperature was held at 35°C, both at the maximum for growth. The glucose level on the shell side was about 25 gm/l which is more than enough for maximum growth. Possible causes for the slow growth rate that need to be investigated are:

1) lack of O_2.

2) inhibition by CO_2 or other by-products.

The fermentation gases were easily vented, but the rate of evolution was only a few percent of the theoretical maximum, and the viscosity of the broth was far from that of a high density broth. Since air is not sparged through the fermentor, as it is in conventional fermentors the gas in the shell side is only CO_2. The effect of CO_2 on this strain is currently under investigation at LBL. A chemical method will be examined to check the O_2 level on the shell side.

3. Future Research

Some additional modifications are necessary before modeling can be attempted:

1) better pH regulation.

2) better O_2 supply.

3) removal of dead cells from the unit

4) better removal of waste products.

The longer range goals will be accomplished when a unit capable of continuous high cell density operation is constructed. The model presented by Schuler et al. (1,2,3) will be used in an appropriately modified form. Also to be explored is the effect of different types of fibers.

Another variation of the fermentor is in a unit where there are two sets of fibers, one to distribute the feed and one to remove the products. The advantage of this unit will be that the transport of the products will be by both convection and diffusion. A unit of this type has been constructed and will be tested in the near future.

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III. FUNDAMENTAL STUDIES ON PRODUCT RECOVERY

A. Thermodynamics of Ethanol/Water Systems

1. Introduction

A preliminary study has shown that vacuum distillation can be a highly efficient technique for ethanol recovery from fermentation beers (1). Energy savings of at least 50% are possible (2).

Design optimization of the vacuum distillation process requires ethanol/water vapor/liquid equilibrium data as a function of pressure. The goal of this project is to provide equilibrium data in a most usable form.

2. Current Research

1) 1600 ethanol/water equilibrium data points covering the pressure range from 35 to 850 mmHg have been collected from the literature.
2) The thermodynamic consistency test of Herrington (3) has been applied to all isothermal data sets and invalid data has been eliminated.
3) The vapor/liquid equilibrium data fitting program (VPLOFT) of Anderson (4) has been adapted for use in fitting the large ethanol/water data sets.
4) Isothermal (25°C) fits were developed to compare 2-parameter UNIQUAC, Wilson, and NRTL equations. The UNIQUAC equation best duplicates the data.
5) Fits of 10 data sets at six temperatures (from 35 to 110°C) were completed to determine the temperature dependence of the UNIQUAC parameters.
6) Equations were developed to specify the UNIQUAC parameters as functions of temperature. This allows direct computation of activity coefficients for the ethanol/water system at any composition and from 35 to 850 mmHg pressure.
7) An overall model for ethanol/water equilibrium was developed incorporating the new temperature dependent UNIQUAC equation

for activity coefficients along with the Hayden and O'Connell (5) equation for gas phase fugacity coefficients, a zero pressure reference fugacity equation of Prausnitz (4), and the modified Rackett equation of Spencer and Danner (5) for the Poynting correction.

a) Significance of Accomplishments to Date

The equilibrium data reduction makes available as a tool to the researcher or engineer as detailed a description of the complex ethanol/water equilibrium system as has been available for simple pure component systems in the past. Design and evaluation of new equilibrium based ethanol/water separation processes is greatly simplified.

b) Details of the Accomplishments

i. Data Collection and Evaluation

A summary of the ethanol/water equilibrium data sources and an evaluation of their consistency with the thermodynamic requirements of the Gibbs-Duhem relation is given in Table 8. Consistency with the Gibbs-Duhem equation is a necessary but not sufficient condition for determining the validity of equilibrium data. Data not meeting this requirement can be eliminated as invalid.

The criteria for consistency with the Gibbs-Duhem equation are shown in equations (1) and (2).

For isothermal data:
$$\int_0^1 \ln \frac{\gamma_1}{\gamma_2}\, dx_1 = -\int_{x_1=0}^{x_1=1} \frac{V^E}{RT}\, dp \qquad (1)$$

For isobaric Data:
$$\int_0^1 \ln \frac{\gamma_1}{\gamma_2}\, dx_1 = \int_{x_1=0}^{x_1=1} \frac{h^E}{RT^2}\, dt \qquad (2)$$

h^E is not apriori known as a function of composition for the ethanol/water system, hence, for the isobaric equation no simplifications can be made and isobaric data

Table 8 : Results of the Thermodynamic Consistency Tests

T(°C)	Source	A	Judgement
25	Dornte	0.2888	Bad
25	Pemberton/Larkin	0.0027	Good
30	Mash/Pemberton	0.0017	Good
	Pemberton.Larkin	0.0023	Good
39.76	Wrewski	0.0558	Poor
40	Udovenko/Fatkulina	-0.0974	Bad
50	Dulitskaya	0.0084	Good
50	Jones/Shoenborn/Colburn	-0.0419	Poor
50	Mash/Pemberton	0.0026	Good
50	Pemberton/Larkin	0.0027	Good
50	Udovenko/Fatkulina	-0.0907	Bad
54.81	Wrewski	0.0275	Marginal
60	Jones/Shoenborn/Colburn	-0.1616	Gad
60	Udovenko/Fatkulina	-0.1320	Bad
70	Mash/Pemberton	0.0066	Good
70	Pemberton/Larkin	0.0058	Good
74.79	Wrewski	0.0355	Marginal
90	Mash/Pemberton	0.0139	Good
90	Pemberton/Larkin	0.0125	Good
110	Pemberton/Larkin	0.0204	Good

could not be tested for thermodynamic consistency. For isothermal data sets, however, the second integral can be approximated to zero with great confidence as V^E is extremely small. Graphical integration of equation (1) in the simplified form of equation (3) was used to test the thermodynamic consistency of the isothermal data. The sum should ideally equal zero and Prausnitz (8) suggests $|A| \leq 0.02$ for the consistency criterion.

$$\sum_{i \text{ at } x_1 = 0}^{i \text{ at } x_1 = 1} \left[\ln \frac{\gamma_1}{\gamma_2}\right]\left[x_i - x_{i-1}\right] = A \qquad (3)$$

Isobaric data sets taken by researchers with good isothermal data sets were assumed good.

The data of Pemberton and Larkin (7) and Mash and Pemberton (8) was chosen for use in developing the new equilibrium model, based both on the excellent consistency of this data with the Gibbs-Duhem equation and on the confidence in the data suggested by the careful experimental techniques of these researchers (as described in their papers).

ii. UNIQUAC Parameters as a Function of Temperature

The vapor/liquid equilibrium data fitting program of Anderson (4) (after some modification to allow handling of larger data sets) was used to fit the isothermal Pemberton-Larkin and Mash-Pemberton activity data at 25, 30, 50, 70, 90, and 110°C (corresponding to the pressure range from 35 to 850 mmHg). The resulting parameters (ΔU_{12} and ΔU_{21}) are plotted vs. temperature in Fig. 16. The parameters vary smoothly over the range studied and were easily fit to a polynomial expansion in T.

$$\begin{aligned}\Delta U_{12} = {} & -10845.010853 + 144.454638358T \\ & -.692588910466T^2 + .00143459625212T^3 \\ & -1.096233611 \times 10^{-6}\, T^4\end{aligned} \qquad (4a)$$

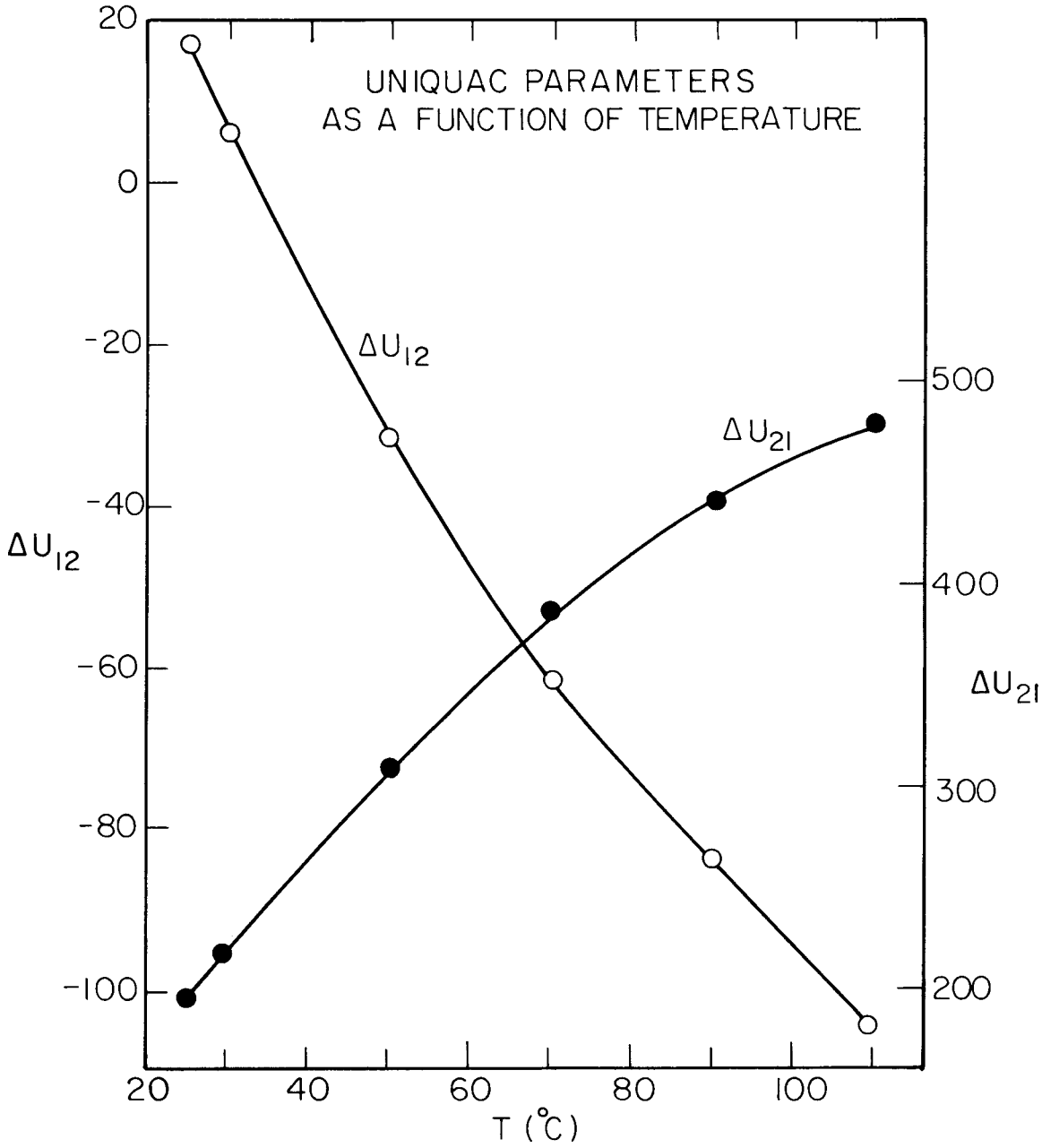
UNIQUAC PARAMETERS
AS A FUNCTION OF TEMPERATURE
ΔU12
ΔU21
ΔU12
ΔU21
20
0
-20
-40
-60
-80
-100
500
400
300
200
20
40
60
80
100
T (°C)
XBL 812-5289

Figure 16.

$$\Delta U_{21} = 38765.541772 - 476.052110708T + 2.14108540923T^2 - .00420316593674T^3 + 3.054583612 \times 10^{-6} T^4 \quad (4b)$$

(where the extravagant number of figures typical of the equilibrium literature has been maintained).

iii. The Overall Ethanol/Water Equilibrium Model

The overall ethanol/water equilibrium model begins with the general vapor/liquid equilibrium relation.

$$y_i \Phi_i p = x_i \gamma_i f_i^{OL} \exp(pV_i^L/RT) \quad (5)$$

where y_1 and y_2 are the mole fractions of ethanol and water respectively in the vapor phase, x_1 and x_2 are the mole fractions in the liquid and p and T are the system pressure and temperature.

The activity coefficients for ethanol and water (γ_1 and γ_2) are calculated from the new temperature dependent 2-parameter UNIQUAC equation:

Modified UNIQUAC Equation

$$\ln \gamma_1 = \ln \frac{\Phi_1}{x_1} + \frac{z}{2} q_2 \ln \frac{\Theta_1}{\Phi_1} + \Phi_2 \left(\ell_1 - \frac{r_1}{r_2} \ell_2\right) + C\, q_1' \left[- \ln(\Theta_1' + \Theta_2' \tau_{21}) + \frac{\Theta_2' \tau_{21}}{\Theta_1' + \Theta_2' \tau_{21}} - \frac{\Theta_2' \tau_{12}}{\Theta_2' + \Theta_1' \tau_{12}} \right]$$

$$\ln \gamma_2 = \ln \frac{\Phi_2}{x_2} + \frac{z}{2} q_2 \ln \frac{\Theta_2}{\Phi_2} + \Phi_1 \left(\ell_2 - \frac{r_2}{r_1} \ell_1\right) + C\, q_2' \left[- \ln(\Theta_2' + \Theta_1' \tau_{12}) + \frac{\Theta_1' \tau_{12}}{\Theta_2' + \Theta_1' \tau_{12}} - \frac{\Theta_1' \tau_{21}}{\Theta_1' + \Theta_2' \tau_{21}} \right]$$

where

$$\ell_1 = \frac{z}{2}(r_1 - q_1) - (r_1 - 1)$$

$$\ell_2 = \frac{z}{2}(r_2 - q_2) - (r_2 - 1)$$

$$\Phi_1 = \frac{x_1 r_1}{x_1 r_1 + x_2 r_2} \qquad \Phi_2 = \frac{x_2 r_2}{x_1 r_1 + x_2 r_2}$$

$$\Theta_1 = \frac{x_1 q_1}{x_1 q_1 + x_2 q_2} \qquad \Theta_2 = \frac{x_2 q_2}{x_1 q_1 + x_2 q_2}$$

$$\Theta_1' = \frac{x_1 q_1'}{x_1' q_1' + x_2' q_2'} \qquad \Theta_2' = \frac{x_2 q_2'}{x_1' q_1' + x_2' q_2'}$$

where: Z (coordination number) = 10

r_1 (ethanol structural size parameter) = 2.11

r_2 (water structural size parameter) = 0.92

q_1 (ethanol structural area parameter) = 1.97

q_2 (water structural area parameter) = 1.40

q_1' (ethanol modified area parameter) = 0.92

q_2' (water modified area parameter) = 1.00

are all physical parameters calculated from a knowledge of the molecular structure of ethanol and water.

τ_{12} and τ_{21} are the UNIQUAC parameters. The major temperature dependence of these parameters can be removed if they are expressed as

$$\tau_{12} = \exp\left(\frac{-\Delta U_{12}}{RT}\right)$$

$$\tau_{21} = \exp\left(\frac{-\Delta U_{21}}{RT}\right) \qquad (7)$$

ΔU_{12} and ΔU_{21} are then the final parameters which were found by fitting the actual ethanol/water equilibrium data and expressed as polynominal functions of

temperature (equation 4).

The fugacity coefficients for ethanol and water (Φ_1 and Φ_2, respectively) are given by the Hayden and O'Connell correlation for gas phase fugacities (5).

$$\Phi_1 = F(y_1, B_1, B_{12})$$
$$\Phi_2 = F(y_2, B_2, B_{12}) \qquad (8)$$

where B_1, B_2, and B_{12} are the temperature dependent virial coefficients.

The zero point reference fugacities for ethanol and water (f_1^{OL} and f_2^{OL}) are given by the expansion of Prausnitz (4).

zero point reference fugacities:

$$f_i^{OL} = \exp\ (A_i + B_1/T + C_iT + D_i \ln T + E_iT^2) \qquad (9)$$

with $A_1 = -.9091$

$B_1 = -.3466$

$C_1 = -.6230$

$D_1 = .2049$

$E_1 = .2066$

and $A_2 = .5704$

$B_2 = -.7005$

$C_2 = .3589$

$D_2 = -.6669$

$E_2 = -.8505$

The ethanol and water liquid specific molar volumes (V_1^L and V_2^L) used in the Poynting correction are given by the modified Rackett equation of Spencer and Danner as:

Modified Rackett Correlation

$$V_i^L = \frac{R\, T_{Ci}\, Z_{ri}^{\tau}}{P_{Ci}}$$

where $\tau = 1 + (1 - T_r)^{.286}$ for $T_r \leq .75$

$\tau = 1.6 + .00693/(T_r - .655)$ for $T_r > .75$

with $T_r = T/T_C$

and where T_{C_1} = (ethanol critical temperature) = 516.26°K

T_{C_2} = (water critical temperature) = 647.37°K

P_{C_1} = (ethanol critical pressure) = 63.89 bar

P_{C_2} = (water critical pressure) = 221.20 bar

Z_1 (ethanol modified Rackett parameter) = .2520

Z_2 (water modified Rackett parameter) = .2380

Of course, at low pressure the vapor phase closely approximates an ideal gas and Φ_1 and Φ_2 can be very closely approximated as equal to one. Also, the system pressure is close to the pure component saturation pressures so that the Poynting correction could be neglected and the term $f_i^{OL} \exp(PV_i^L/RT)$ of equation (5) could be replaced with a polynominal representation of the pure component vapor pressures as a function of temperature to give

$$y_i\, p = x_i\, \gamma_i\, p_i^o \tag{11}$$

The more complete form of equation (5) has been maintained to allow ready expansion of the model to high pressure cases should this be desired.

The excellent agreement between the model predictions and experimental data is clearly shown in Figure 17 which shows a McCabe-Thiele plot of the model results and actual data at 25°C with an RMS deviation of 0.06.

3. Future Research

a) 3-parameter Activity Models

3-parameter activity models are being studied to further increase the accuracy of the ethanol/water pure system vapor/liquid model already developed. This is important if sensitive properties such as the exact variation in the azeotropic composition are to be accurately predicted as a function of pressure.

b) Salt Effects on Ethanol/Water Vapor/Liquid Equilibrium

The ethanol/water separation factor $K = \frac{Y_{EtOH}/Y_{H_2O}}{X_{EtOH}/X_{H_2O}}$ is enhanced by the presence of certain salts (9). A surprisingly large increase has been observed for the combination of salts in ordinary fermentor beer. This enhanced separation factor (if substantiated) should allow a large reduction in required compressor capacity and cost for the flashferm process as compared to earlier design estimates.

An Othmer equilibrium still has been ordered and will be used to measure ethanol/water vapor concentration over actual fermentor beers. This will allow an accurate assessment of the extent of relative volatility enhancement. Correlation of this enhancement factor will then allow extension of the pure system ethanol/water equilibrium model for use in modeling equilibrium behavior over actual fermentor beer.

c. Projected Timetable

Remaining experimental aspects of this project will be completed

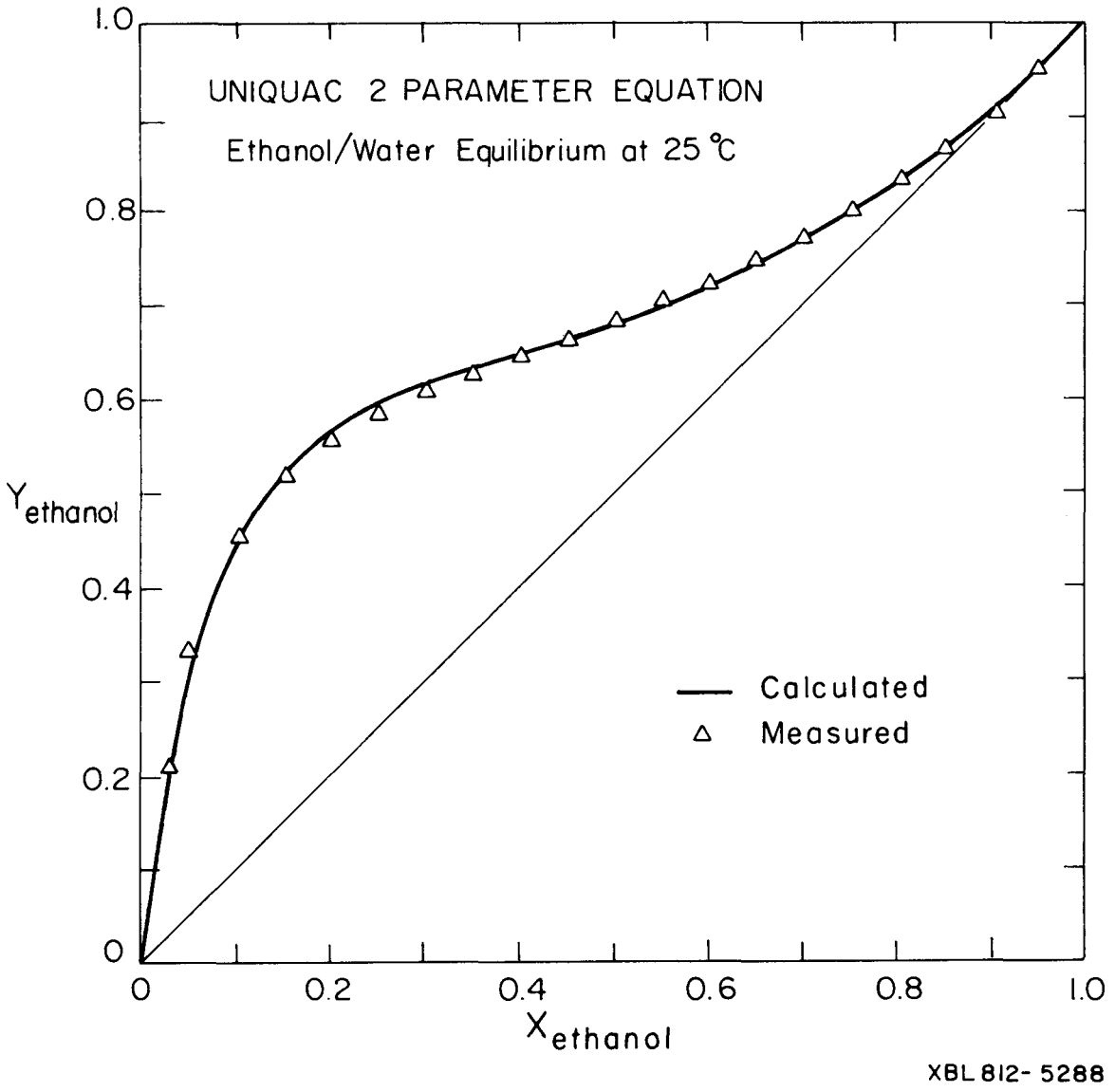
UNIQUAC 2 PARAMETER EQUATION
Ethanol/Water Equilibrium at 25 °C
Calculated
Measured
$Y_{ethanol}$
$X_{ethanol}$
0
0.2
0.4
0.6
0.8
1.0
XBL 812-5288

Figure 17.

by June 1981 and a final report detailing the full equilibrium model will be submitted by September 1981.

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B. Novel Ethanol-Water Separations

1. Introduction

The objectives of the project on novel ethanol-water separations are to investigate and develop the potential of alternatives to distillation for ethanol recovery. Earlier work has included assessment of fractional crystallization (freeze separation) and a type of field-flow fractionation. These approaches were determined to be technically or economically unfeasible, however, and all subsequent and continuing work has focused on various aspects of solvent extraction.

The main rationale for seeking alternatives to distillation as a product recovery method lies in the hope of reducing the considerable energy cost of distillation in the case of ethanol-water. As shown in Table 9, the energy required to product 95 weight % ethanol by distillation is over forty times the reversible thermodynamic work required to completely separate a 5 weight % ethanol solution into pure ethanol and water. This is due to the nature of the ethanol-water vapor-liquid equilibrium, particlularly to the existence of the ethanol-water azeotrope. Operation at reduced pressure can reduce the energy requirement by altering the vapor-liquid equilibrium favorably, but the savings in energy is offset somewhat by considerable capital expense for compressors and other equipment

Another reason for investigating extraction is its potential application to product recovery in other fermentations where, due to the nature of the product, distillation would not be a feasible technique. For example, the production of nonvolatile fatty acids by fermentation for conversion to hydrocarbon fuels, which has been proposed as an alternative way of recovering the energy in biomass, could benefit from improved understanding of extractive recovery of products from fermentation broths.

Table 9

Energy Requirements for Ethanol Recovery

	Energy Required (kilojoules/liter)
Theoretical Minimum (Complete separation of X_{ε} = .02)	147
Distillation, Conventional (to azeotrope only)	6216
Distillation, Vacuum (to azeotrope, calculated)	3568
Extraction (potential, calculated)	$\sim$ 1700

2. Current Research

a) Extraction Solvent Selection

The key to a solvent extraction process for fermentation product recovery lies in the discovery of a solvent which meets several important criteria. The most obvious of these is that the solvent have a high distribution coefficient, D_E, defined by $D_E = \frac{[EtOH]\ \text{solvent phase}}{[EtOH]\ \text{aqueous phase}}$ where [EtOH] is ethanol concentration in $\frac{\text{g.moles}}{\text{liter}}$ (or other appropriate units). An analagous coefficient, D_W, can be defined for water distribution. A further desirable property of an extraction solvent is that it have a high separation factor, S, defined by $S = \frac{D_E}{D_W}$. In addition, a solvent should be easily regenerable, inexpensive, and should have low water solubility.

All of these factors are interrelated and the quantitative requirements in each case are affected by the specific details of the process. For example, in the process schemes currently being considered, regeneration of solvent would be accomplished by vaporization of the ethanol at elevated temperature. This affects the requirement of high distribution coefficient because of the need to supply heat to the whole solvent stream. It also favors the selection of high-boiling solvents which separate easily from the volatile ethanol. The requirement of low water solubility is to prevent solvent loss and is thus associated with the solvent cost requirement. Furthermore, in processes under consideration which involve recycle of the fermentation broth after product extraction, this requirement is altered to one involving toxicity of the solvent to the fermentation. Finally, a variation on the conventional solvent extraction process scheme is being considered which changes the low water solubility requirement to a low volatility one. This involves replacing conventional liquid-liquid contacting with a stream of inert gas alternately stripping the fermentation broth of ethanol and is in turn depleted of ethanol by absorption of the product into the solvent. Such a process also has the advantage of avoiding the formation of emulsions

which can accompany attempts to apply liquid-liquid contacting to fermentation broths.

It is clear, then, that an extraction solvent and the process in which it is employed are mutually dependent. So it is appropriate to examine a range of solvents to determine the feasibility of ethanol extraction.

b) Measured Properties of Solvents

Distribution coefficient data and some separation factor data for a number of solvents are presented in Table 10. Distribution coefficients for ethanol were measured in this work by gas chromatographic analysis of samples from batch equilibration experiments using pure ethanol in water and reagent grade solvents. The work has been hampered to date by lack of a sensitive technique for determination of water in the solvent phase. Shown in Table 10 also are some results of Roddy (1981) who used ^{14}C labelled ethanol and tritiated water to determine D_W as well as D_E, and of Korenman and Chernorukovo who used a spectrophotometric technique based on reaction of alcohol with nitrous acid to determine alcohol concentrations. Most of the values in Table 2 are for aqueous alcohol concentrations at or near 1.0 M, but all investigators find that distribution coefficients vary little over a range of dilute ethanol concentration.

Several important points can be made about the data in Table 10. First, even the best value of S is rather low considering the fact that to produce an azeotropic (95 weight %) ethanol from a 1 M (5 weight %) aqueous solution in one step would require $S \geq 390$. Second, the only solvent with selectivities approaching this value (the alkanes are presumably all similar to cyclohexane) have quite low distribution coefficients. Also of interest is the difference between the oxygen-containing and chlorine-containing compounds. Both types of compounds are capable of participating in hydrogen-bonding with either water or ethanol (excepting carbon tetrachloride). However, the oxygen-containing

Table 10

Properties of Solvents, 20°-25°C

Solvent	D_E	S
alkanes		
n-alkanes (C_5-C_{10})	.008[c]	-
isoctane	.006[d]	-
cyclohexane	.007[b]	310
aromatics		
benzene	.046[b], .024[c]	110
toluene	.034[b], .020[c]	100
ethyl benzene	.029[b], .007[c]	97
diethyl benzene	.022[b]	120
triethyl benzene	.097[a]	
oxygenated		
2,6-dimethyl-4-heptanone	.143[a]	19
acetophenone	.257[a]	-
dibutyl phthalate	.123[a]	-
tributyl phosphate	.430[2], .430[c], .540[b]	8.3
chlorinated		
methylene chloride	.145	-
chloroform	.120[b], .140[c]	170
carbon tetrachloride	.056[a], .040[c]	-

Sources: a) this work
b) Roddy, 1981
c) Korenman & Chernorukova, 1974
(all S values from Roddy, 1981)

compounds can act only as nucleophilic hydrogen bonding partners while the partially chlorinated hydrocarbons act only as electrophilic hydrogen-bonding partners. The distribution coefficients for ethanol are of the same order of magnitude for both types of compounds, suggesting that ethanol interacts to roughly the same degree with either type of hydrogen-bonding. However, there is an order of magnitude difference in the separation factors (i.e., in D_W) for the two kinds of compounds, implying that water is much less able to interact with the electrophilic H-bonding sites than with the nucleophilic ones. Admittedly, this observation is based on little data as yet, but more evidence for it from the literature will be presented later.

A preliminary assessment of the toxicity of some solvents has also been done. This involved batch culture experiments with yeast growing in medium saturated with the solvent. The alkanes tested, cyclohexane and a mixture of high-boiling alkanes, had virtually no effect on growth or substrate utilization, possibly because of their low solubility. Aromatic compounds tested showed varying levels of toxicity, though all were more toxic than alkanes. Tributyl phosphate was toxic. Dibutyl phthalate, while showing no toxicity, formed a stable emulsion with the medium.

c. Improvement of Extraction Systems

It is apparent from the results in the previous section that anything which can be done to improve distribution coefficients, separation factors, or perferably both, will be very helpful, if not essential, to the realization of a solvent extraction recovery process for ethanol production.

Increasing the temperature can have a dramatic effect on distribution coefficients. Thus Roddy (1981) notes that increasing the temperature from 20° to 40°C can roughly double distribution coefficients. It seems likely that this is due to a decrease in hydrogen bonding in the aqueous phase since the hydrogen bond, like other dipole-dipole interactions, requires specific

orientation and thus have a strong inverse temperature dependence. This is just as true for water as it is for ethanol, though, with the result that temperature has very little effect on separation factors.

An approach which does improve both distribution coefficients and separation factors is the addition of an appropriate salt to the aqueous phase. This salting out effect has been studied for a great number of salts in ethanol-water solutions. Its potential application in distillation has been reviewed by Furter (1977) among others. It is equally applicable to extraction processes, however. The improvement of distribution coefficient is realted directly to the Setchenow parameter (or salt effect parameter), K_A, defined

$$\ln \frac{C_{A_s}}{C_A} = K_A Z_s$$

where C_A and C_{A_s} are the concentrations of solute (ethanol in this case) in the solvent in equilibrium with aqueous solutions without and with salt, respectively. Z_s is a measure of salt concentration, usually molality or mole fraction.

Some experiments have been done to confirm the salting out of ethanol from fermentation broths. Particularly effective salts include potassium carbonate, potassium phosphates and ammonium sulfate. An increase in the ethanol distribution coefficient by orders of magnitude is possible with sufficiently high salt concentrations. At saturation, for example, potassium carbonate salts out a separate phase of over 90 weight % ethanol from dilute solution without any solvent phase present. However, no salt is inexpensive enough to consider using it in this way without either recovery or recycle.

A third method for improving distribution coefficients is to employ a mixed solvent system which has desirable extraction properties. An example of this is the use of trioctyl phosphine oxide (TOPO in 2.6-dimethyl-4-heptanone. Results are shown in Table It is seen that D_E is improved at

Table 11

Mixed Solvent Extraction Using TOPO

(0.521 M TOPO in 2,6-dimethyl-4 heptanone)

Aqueous Ethanol Concentration, M	D_E	Extra Moles EtOH Per Mole TOPO
0.39	0.41	0.21
1.49	0.31	0.47
3.18	0.22	0.47

NOTE: D_E for range of aqueous ethanol concentrations 0.5 - 3.5 M with no TOPO added is constant at 0.14.

low concentration of ethanol with the improvement reduced as concentration is increased. When the results are expressed in terms of the additional ethanol brought into the organic phase by the TOPO, it is seen that a saturation appears to be reached at .47 moles of ethanol per mole of TOPO. The reason for this is not known, but appears to be related to the relative weakness of ethanol in competition with water for nucleophilic H-bonding sites mentioned previously. It is expected that separation factors in this case, already poor for the pure ketone, are not improved by TOPO addition.

One further way of improving distribution coefficients is being investigated. In reviewing the literature on salting out, it was observed that certain salts have the opposite effect. That is they salt ethanol into the aqueous phase, reducing its volatility. Notable among these salts are those involving the large tetraalkyl ammonium ions. It was reasoned that, by using salts of these ions which would have very little water solubility but be soluble instead in an organic phase, an improved extraction solvent for ethanol might be devised. Table 12 shows some results of initial tests of this idea. While the effects in the cases of the ester solvents are small due to the small concentrations employed, at higher concentration in triethyl benzene, tetrabutyl ammonium benzoate clearly salts ethanol into the organic phase. The extra ethanol brought in is slightly more than 0.5 moles of ethanol per mole of salt. In the case of tetrabutyl ammonium iodide, however, salting out of ethanol is indicated, pointing to the importance of an anion in this interaction. This is confirmed by the work of Arnett et al. (1977) who found, in a study of the hydration of ions in nonaqueous media, that the influence of the tetraalkyl ammonium cation is small compared to the anion effect. The salting out by iodide is in keeping with other evidence that water is better able to compete with ethanol for nucleophilic sites in an organic medium than

Table 12

Salting Ethanol Into Organic Solvents

Solvent	Salt	$[EtOH]_{aq}$, M	D_E
dibutyl phthalate	none	1.0	.123
dibutyl phthalate	TBA benzoate, .04 M	1.0	.116
triethyl citrate	none	1.5	.072
triethyl citrate	TBA benzoate, .03 M	1.5	.091
triethyl benzene	none	1.0	.097
triethyl benzene	TBA iodide, sat.	1.0	.088
triethyl benzene	TBA benzoate, .18 M	1.0	.171

TBA ≡ tetrabutyl ammonium

electrophilic. In the case of the larger benzoate ion, the hydration forces are not as strong and presumably the cation dominates the overall effect.

3. Future Research

a) Experimental

Initial priority in planned experimental work will be given to development of techniques for water concentration measurement in organic solvents. Two approaches are being taken. One is based on the titrimetric Karl Fisher assay with potentiometric endpoint determination. The other is the use of a sensitive thermal conductivity detection system in gas chromatographic analysis of samples to which an internal standard such as propanol will be added. Both methods will be used to establish reliable data for D_W determinations.

Also planned is the testing of extraction properties of other electrophilic H-bonding compounds such as 1,1,2,3,4,4-hexachlorobutane. Such compounds are anticipated as having D_E and S values as good or better than those of the chlorinated methane derivatives, but with better solubility and volatility characteristics. The diluent solvent will be varied in these tests also to test the hypothesis that solvent should have little effect.

Investigation of the salting in of ethanol to extraction solvents will be continued with a study of salts of the $R_4\ B^-$ ions where R is phenyl or butyl. These ions should minimize the salting in of water (Arnett et al. found no hydration of tetraphenyl borate in their work) and allow the cation salting in of ethanol to exert its maximum effect on the distribution coefficient.

Fermentation studies will continue, and include further batch toxicity studies and will involve continuous culture studies to determine the possibility of adapting the organisms to the presence of solvent and to increase salt concentrations (osmophilic fermentation to utilize the salting

out effect.

b) Theoretical

It would be desirable to have predictive theories of the salting out and salting in effects to aid in the development of an extraction process. Accordingly, some theoretical work from the literature has been reviewed and its possible application to the problem will be assessed.

One approach to the calculation of Setchenow parameters is that of Tiepel and Gubbins (1973). These authors use a simplified version of second order statistical mechanical perturbation theory to relate the properties of real solvents in salt solutions to those of a model hard sphere fluid at the same temperature and pressure. The result of their treatment is the following expression for the chemical potential of the solute;

$$\mu_1 = \mu_1 = \mu_1^{hs} + \left(\frac{\partial \mu_1}{\partial \alpha}\right)_o - 11.608 \sum_j p_j \varepsilon_{1j} \alpha_{1j}^3$$

$$- \frac{2.962\ p_2\ \mu_2^{\ 2}\ \alpha}{\sigma_{12}^{\ 3}}$$

$$- \frac{1}{KT}\left[2.870 \sum_j p_j \varepsilon_{1j}^{\ 2} \sigma_{1j}^{\ 2} + \frac{1.3922\ p_2 \varepsilon_{12} \bar{\mu}_2^{\ 2} \alpha_1}{\sigma_{12}^{\ 3}} + \right.$$

$$\left. + \frac{0.2468\ p_2\ \bar{\mu}_2^{\ 4}\ \alpha_1}{\sigma_{12}^{\ 9}}\right]$$

where $$\left(\frac{\partial\ \mu_1}{\partial\ \alpha}\right)_0 == -\ 4\ kT \sum_{j \neq 1} pi\ d_{1j}^{\ 2}\left[d_{1j} - \delta_{1j}\right] g_{1j}^{hs}\ (d_{1j})$$

$$-2\pi VKT \sum_{i \neq j} \sum p_i p_j\ d_{1j}^{\ 2}\left[d_{ij} - \delta_{ij}\right]\left(\frac{\partial\ g_{ij}^{hs}(d_{ij})}{2N_1}\right)_{T_1 V_1 N_j \neq 1}$$

μ_1^{hs} is the chemical potential in the model hard sphere system

α_1 is the solute polarizability

ε_{ij} is the minimum potential energy of interacting ij

σ_{ij} is the Lennard-Jones collision diameter of the i-j pair

p_i is the number of i molecules per unit volume

g_{ij}^{hs} is the distribution function for the hard sphere model

and

d_{ij} and δ_{ij} are temperature dependent hard sphere diameters

Using this equation, the authors calculated salt effect parameters for a number of nonpolar solutes in a variety of salt solutions. Their results show better agreement with experimental data than older, electrostatic theories of salt effects in the case of small ion salts. In the case of tetralkyl ammonium salts, however, the theory predicts incorrect trends in the behavior of the salting coefficient with increasing size of the solute. A possible reason for this difficulty, and one which limits the theory to application to nonpolar solutes, is the neglect of ionic effects on the solute through any forces other than dispersion forces. The authors argue that, for a symmetrical distribution of ions about the solute, the net electric field at the solute will be zero. However, it seems questionable to assume a symmetrical distribution since the ion-induced dipole forces will be different for different ions and will tend to creat clusters of solute about the ions. Of course, in the case of polar solutes, the dipole-ion forces will have a significant contribution to the effect, also.

The other extreme is taken in the appraoch of Krishan and Friedmen (1974), who assume for their calculations of Setchenow constants, a potential of the form

$$u_{ij} = COR_{ij}(r) + \frac{e_i e_j}{\varepsilon r} + CAV_{ij}(r) + GUR_{ij}(r)$$

where the COR term is an exponential replusion term

$e_i e_j/\varepsilon r$ is coulombic potential with ε the solvent dielectric constant

CAV term accounts for dielectric effects, and

GUR is an adjustable parameter term which stems from considering the overlap of spherical solvation shells assumed to be present around the solute species.

Thus, no explicit consideration of dispersion forces is made and their effects are accounted for by fitting the GUR term to experimental data. Despite this disadvantage, however, the theory is attractive for its ability to treat polar solutes.

These theories, or modifications of them, will be used in the coming year to analyze the salting out and salting in effects. It will be especially interesting to apply them to calculation of the salting in of ethanol and water to the organic phase since the use of a nonpolar solvent simplifies the calculations by comparison with the salting out of water case.

A simplified correlations for the salt effects based on theoretical considerations will be developed. In this regard, the approach of de Visser et al (1978) may be useful. These workers extended the pairwise interaction concept of Savage and Wood (1975) to electrolyte solutions. In this scheme, enthalpies of interaction of solutes are estimated by breaking down the overall enthalpy into additive terms which are due to specific groups in the molecules. It may prove especially useful in the analysis of salting in data for ethanol by organic ions.

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IV. ORGANISM DEVELOPMENT

A. Simultaneous and Sequential Cellulose Fermenting Organisms

1. Introduction

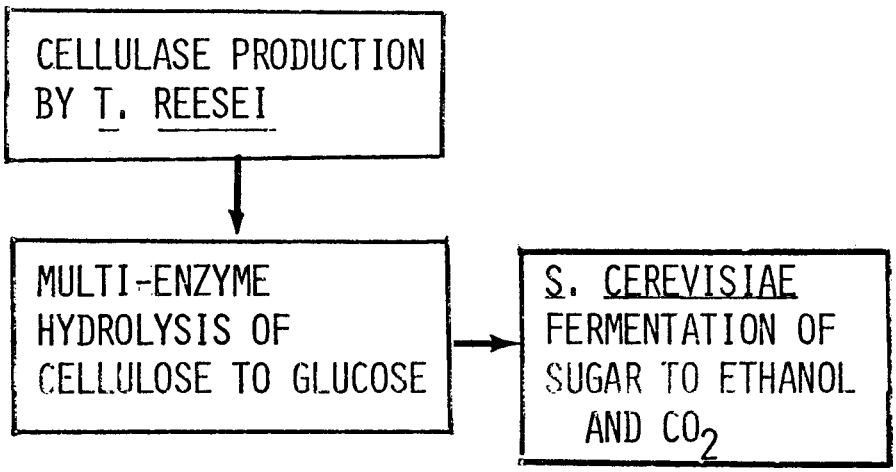
Work during this period was confined to selecting the most promising organisms for use in a direct cellulose to ethanol bioconversion scheme and studying their nutrition and physiology.

The current Berkeley process consists of three discrete steps: production of cellulase enzymes by the aerobic mold Trichoderma reesei, hydrolysis of cellulose to glucose using these enzymes, and fermentation of the glucose to ethanol and CO_2 by the yeast Saccharomyces cerevisiae.

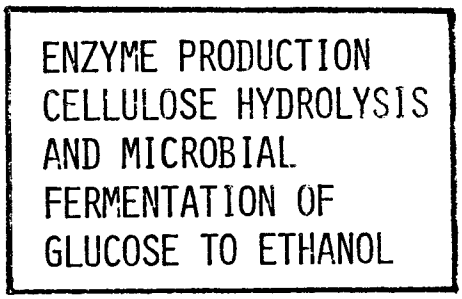
Although it has not been described in the literature, it is possible to conceive of an organism which ferments glucose like a yeast and produces cellulase enzymes. Such an organism would be able to ferment cellulose to ethanol in a single step. There appear to be no biochemical reasons why such an organism could not exist. Figure 18 illustrates the basic difference between our current process and one based on the organism we postulate. Because of the conceptual simplicity and possible economy of a direct fermentation process based on such an organism, we decided to make a careful search for it.

a) Background

In early 1979 an extensive literature survey was undertaken which disclosed that a number of fungi had been described by some workers as cellulolytic and by other (different) workers as able to ferment simple sugars, such as glucose, to ethyl alcohol and carbon dioxide. No one, apparently, had ever tested these organisms for the ability to ferment cellulose directly to ethanol and CO_2. With this goal in mind we assembled forty-seven promising strains from a number of private and government collections. To these we added thirteen of our own thermophilic, cellulolytic fungi which had not been tested



A) CURRENT PROCESS



B) PROPOSED PROCESS

Fig. 18. ORGANISM DEVELOPMENT FOR THE DIRECT FERMENTATION OF CELLULOSE TO ETHANOL AND CO_2.

previously for fermentative ability.

After constructing a medium upon which all strains would grow and defining approximate temperature optima for growth, we tested the organisms for the ability to ferment glucose, cellobiose and D-xylose. The tests were carried out in tubes containing our standard mineral medium, pH 6.0, plus 0.1% yeast extract and 2% sugar. The cultures were made anaerobic with vaspar seals which allow gas-producers to be identified. Gas production is usually correlated with ethanol production. Aerobic control cultures were included for each sugar. Both growth and gas production were scored. None of the thermophilic cellulose-degraders fermented any of the sugars. None of the cultures tested fermented D-xylose. Seventeen of the cultures fermented glucose and cellobiose.

The seventeen sugar-fermenting cultures were next grown both aerobically and anaerobically with phosphoric acid-swollen (Walseth) cellulose. Aerobic test tubes containing swollen cellulose in a mineral-agar medium were also surface-inoculated in order to indicate the relative abilities of these organisms to produce diffusible cellulases.

None of the cultures fermented the cellulose. That is, none produced obvious gas or gave evidence of significant cellulose degradation. Twelve cultures were able to grow on the cellulose aerobically, however. A comparison between those cultures which produce the most gas from glucose (associated with ethanol production) and those cultures which produce the greatest degree of clearing in the aerobic cellulose agar tubes led to the selection of six strains for further study.

2. Current Research

The six strains were compared with respect to maximum growth rate on glucose at optimum pH under anaerobic conditions, ethanol yield from glucose,

and relative rate and extent of degradation of avicell (crystalline cellulose), ball-milled avicell and acid swollen (amorphous) cellulose. The cultures were similar with respect to ethanol yield from glucose (40- 45%- g. ethanol/ g. glucose fermented) and amount of glucose fermented (> 98% in all cases), but one culture (A106) fermented glucose faster and degraded cellulose (both amorphous and crystalline) faster and more completely than the others.

We next proceeded with physiological and biochemical studies of A106. During anaerobic cultivation on glucose, it was noted that fermentation of the glucose was not always complete when culture growth, as measured by optical density, ceased. Inoculum size, type (spores versus vegetative mycelium), age and yeast extract concentration were all varied. In addition supplements of pure vitamins, rich nutrient media and culture extracts were used. None of these had a reproducible, positive effect on glucose utilization. It finally became apparent that two phenomena were involved. First, in our culturing system mycelium could be trapped in the rubber serum cap leading us to believe that growth (optical density increase) had ceased when in fact it hadn't. Second, even with this problem corrected, it was found that fermentations would continue after the true optical density increased had ceased. Current data suggest that growth may be inhibited by the rising carbon dioxide concentration in these un-vented cultures while glucose fermentation continues. Work is currently in progress to prove or disprove this thesis.

One possible explanation for the fact that significant cellulose degradation does not occur under anaerobic conditions is that one or more of the necessary enzymes are inactive in the absence of oxygen. Previous work suggested, but did not prove, that the cellulases were active under anaerobic conditions. This very important question is being examined now.

Even though our initial experiments indicated that neither this

organism nor any of the organisms screened could degrade cellulose anaerobically, our testing technique was rather crude and would indicate only the production of large quantities of CO_2 from cellulose. More recent experiments have indicated that this organism is able to grow very slightly on ball-milled cellulose under anaerobic conditions. Ethanol levels are extremely low, but measurable. This growth might be due to fermentation of some amorphous regions of the substrate, or it might be due to utilization of soluble sugar impurities created by the ball-milling. Experiments will be designed to distinguish between these two alternatives.

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