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BY-PRODUCT INHIBITION EFFECTS ON ETHANOLIC FERMENTATION BY SACCHAROMYCES CEREVISIAE

Brian Maiorella, Harvey W. Blanch, and Charles R. Wilke

August 1981

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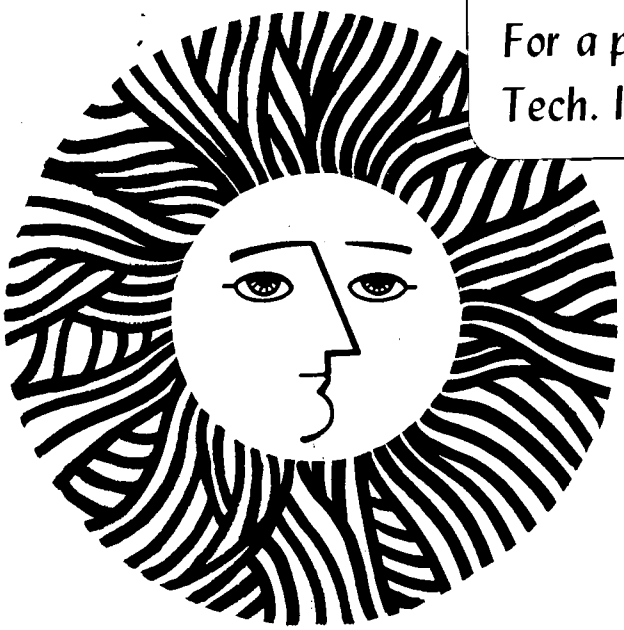

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BY-PRODUCT INHIBITION EFFECTS ON ETHANOLIC FERMENTATION BY *SACCHAROMYCES CEREVISIAE*

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This work was supported by the Director for Energy Research, Office of Basic Energy Sciences of the U.S. Department of Energy under Contract W-7405-ENG-48.

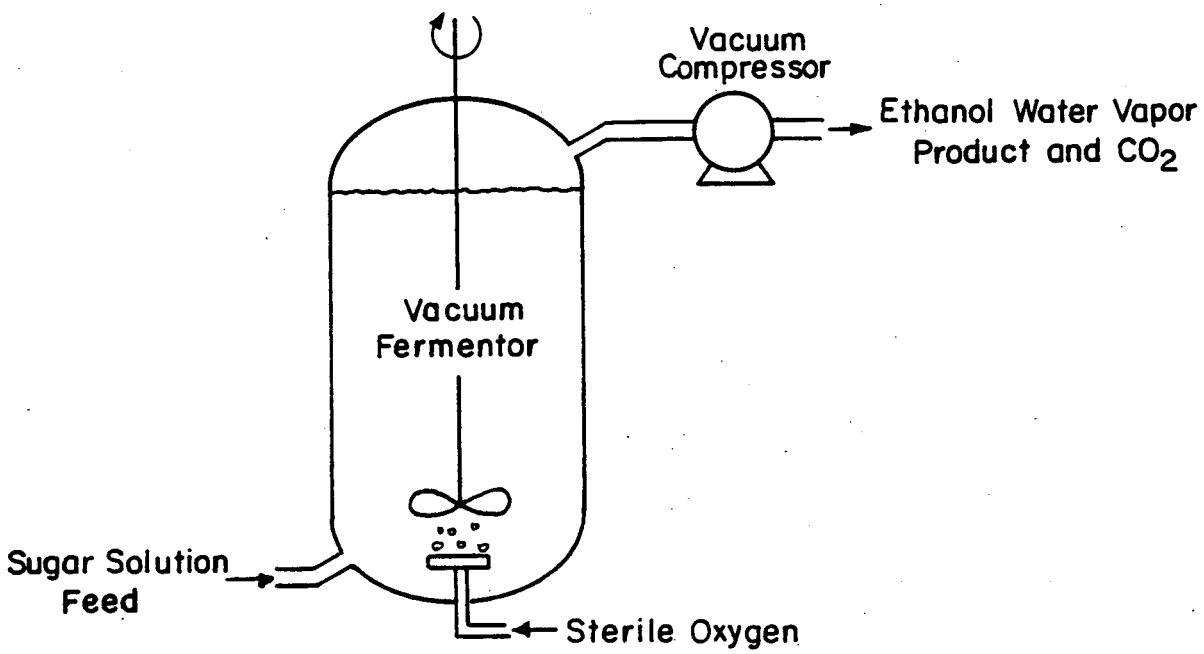
ABSTRACT

Inhibition by secondary fermentation products may limit the ultimate productivity of new glucose to ethanol fermentation processes. New processes are under development whereby ethanol is selectively removed from the fermenting broth to eliminate ethanol inhibition effects. These processes can concentrate minor secondary products to the point where they become toxic to the yeast. Vacuum fermentation selectively concentrates nonvolatile products in the fermentation broth. Membrane fermentation systems may concentrate large molecules which are sterically blocked from membrane transport. Extractive fermentation systems, employing nonpolar solvents, may concentrate small organic acids. By-product production rates and inhibition levels in continuous fermentation with *Saccharomyces cerevisiae* have been determined for acetaldehyde; glycerol; formic, lactic and acetic acids; 1-propanol; 2-methyl-1-butanol; and 2,3-butanediol; to assess the potential effects of these by-products on new fermentation processes. Mechanisms are proposed for the various inhibition effects observed.

INTRODUCTION

Cysewski found in operation of a laboratory vacuum fermentation (Fig. 1) that the buildup of some nonvolatile inhibitor limited the ultimate productivity of the fermentation (1). In the vacu-ferm process, the fermentation is conducted at reduced pressure (approximately 50 mm Hg). Ethanol is boiled away at 35°C as it is produced, maintaining the beer ethanol concentration at below 3.5 wt%. Ethanol end product inhibition is alleviated and the specific ethanol productivity (g ethanol/g cells-hr) is increased. A concentrated glucose feed can be fully converted. The product leaves as a concentrated vapor stream (thus reducing distillation costs). Cells grow during fermentation but cannot escape the fermentor in the vapor product stream and so the yeast density increases, further increasing the fermentor productivity.

Figure 2 shows the results of a continuous vacuum fermentation of a 334 g/L glucose feed solution. Cell density and fermentor productivity increase for the first 40 hours of operation with a maximum ethanol productivity of 44 g/L-hr (ten times the average for a conventional batch fermentation)(1). After 48 hours, however, the density of viable cells and hence the productivity sharply declined. The sharp decline in viable cells after two days is indicative of the buildup of some nonvolatile component to a level toxic to the yeast.



Continuous Vacuum Fermentation

XBL803-4903

Figure 1.

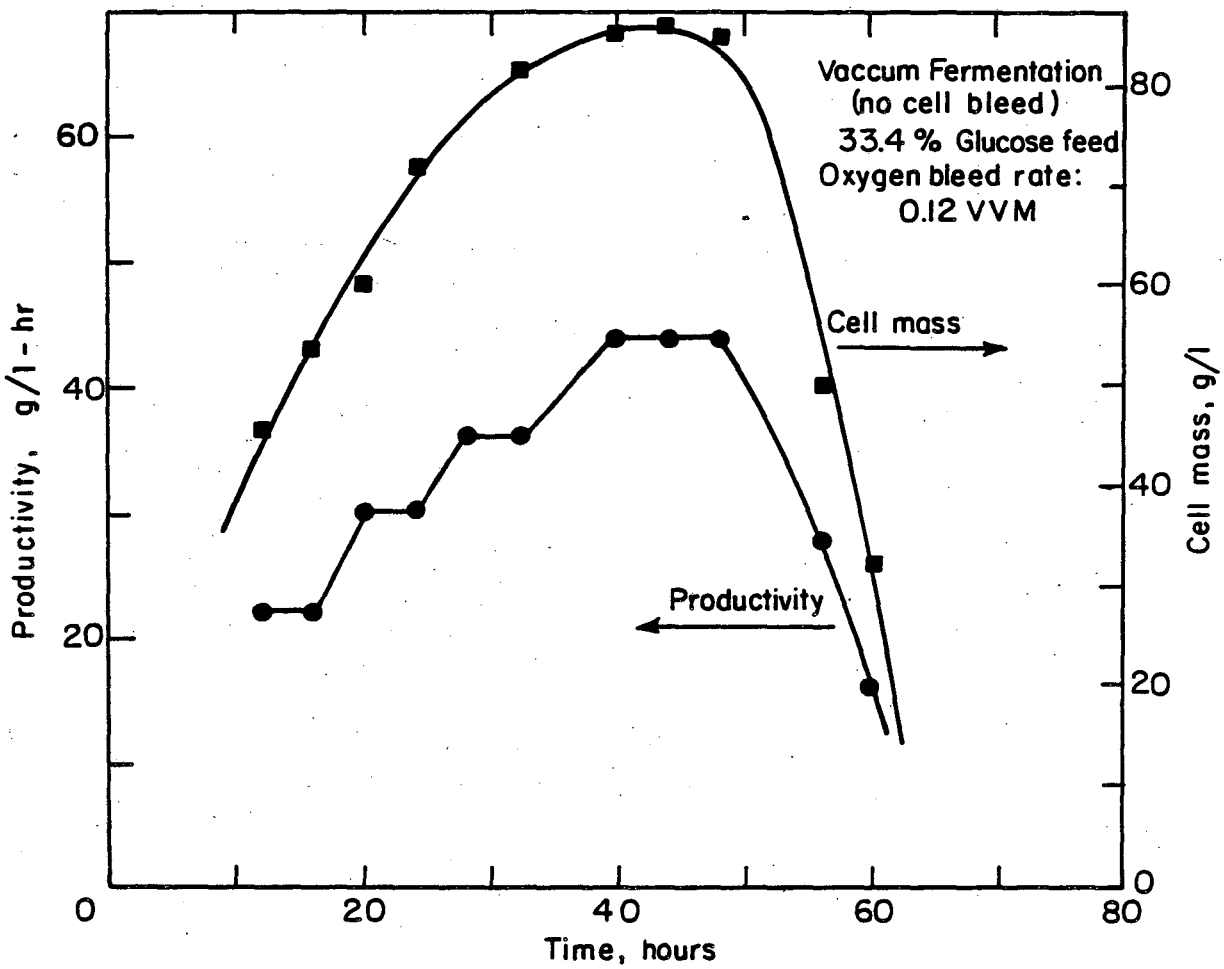


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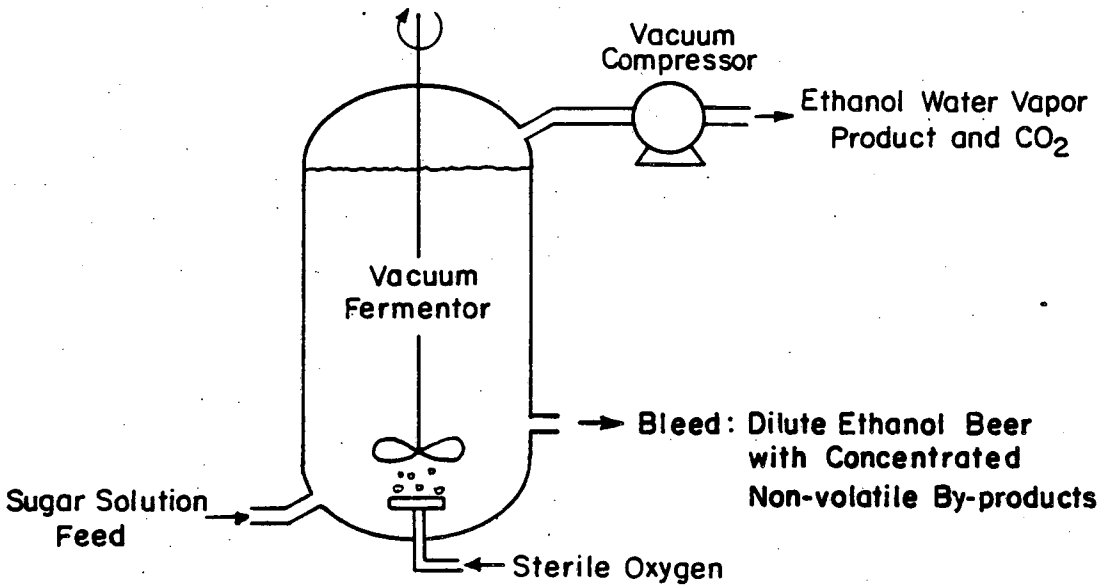
Figure 2

continuously withdrawing a bleed stream of fermentor beer (Fig. 3) to maintain the concentration of nonvolatiles below the level of toxicity. The results of such a continuous vacuum fermentation with bleed are shown in Fig. 4 where cell mass and cell yield are plotted versus a concentration factor (the ratio of the feed rate to the bleed rate)(1). The fermentation was begun with a large bleed. A decrease in bleed rate, while holding the feed rate constant, then increased the concentration factor. As the concentration factor was first increased from 1 to 2.5, cell growth continued and the cell mass concentration increased with fewer cells being washed out in the bleed. Above a concentration factor of 2.5, however, the cell yield decreased and cell concentration dropped. At this concentration factor, the bleed was insufficient to maintain the concentration of the nonvolatile inhibitor below toxic levels.

Use of a large bleed limits the productivity of the fermentation process as cell density is diminished. Further, ethanol product removed in the bleed is very dilute and costly to distill. It is therefore desirable to identify and, if possible, control limiting inhibitors to decrease the bleed and increase productivity.

The problem of toxin buildup may be commmon to many of the new fermentation processes which remove ethanol as a concentrated product from the beer (Fig 5)(2). Nonvolatiles are concentrated and may be inhibitory in vacuum fermentation. Selective membrane fermentations (3) may concentrate larger molecules which are sterically blocked from membrane transport. Extractive fermentation



Continuous Vacuum Fermentation

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Figure 3.

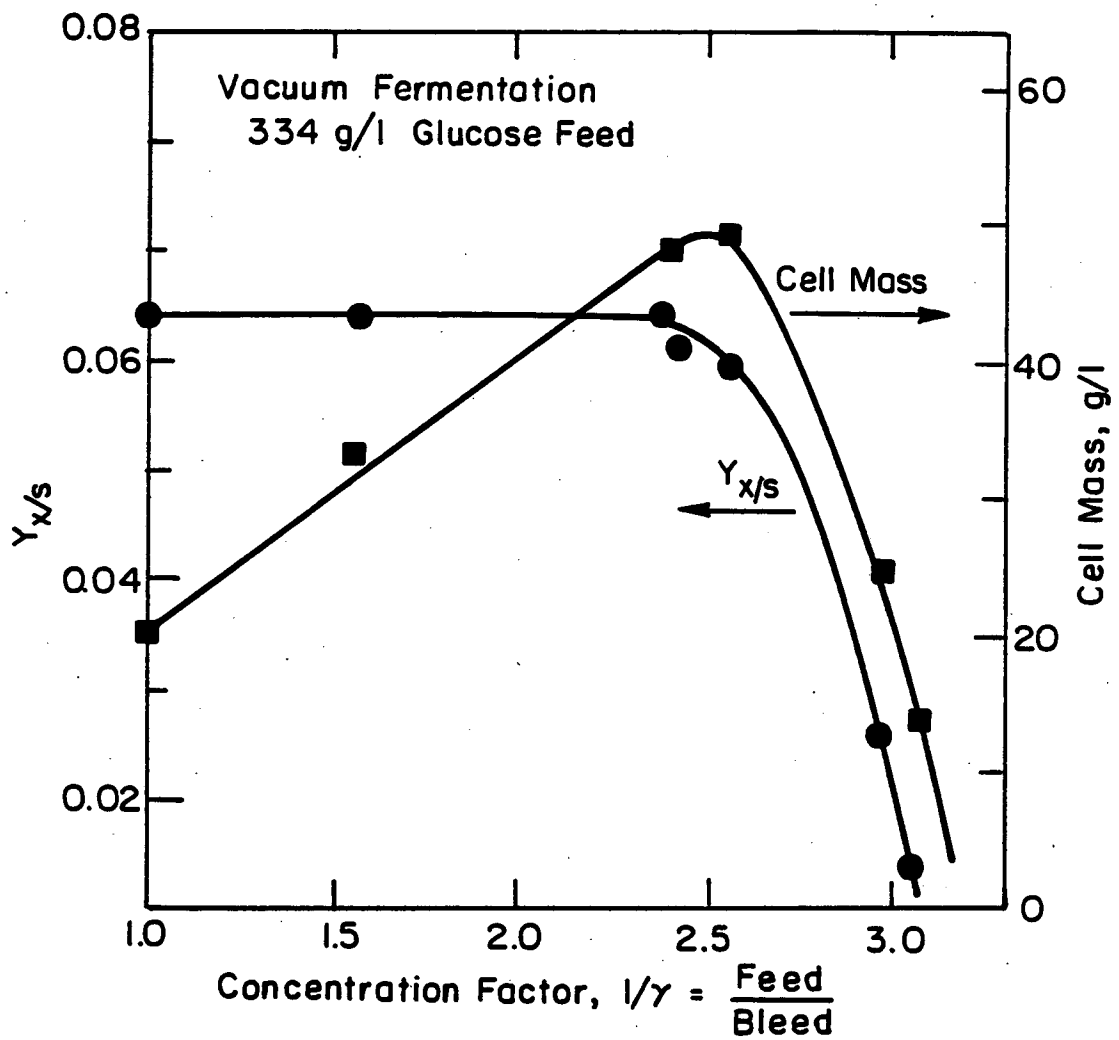

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Figure 4.

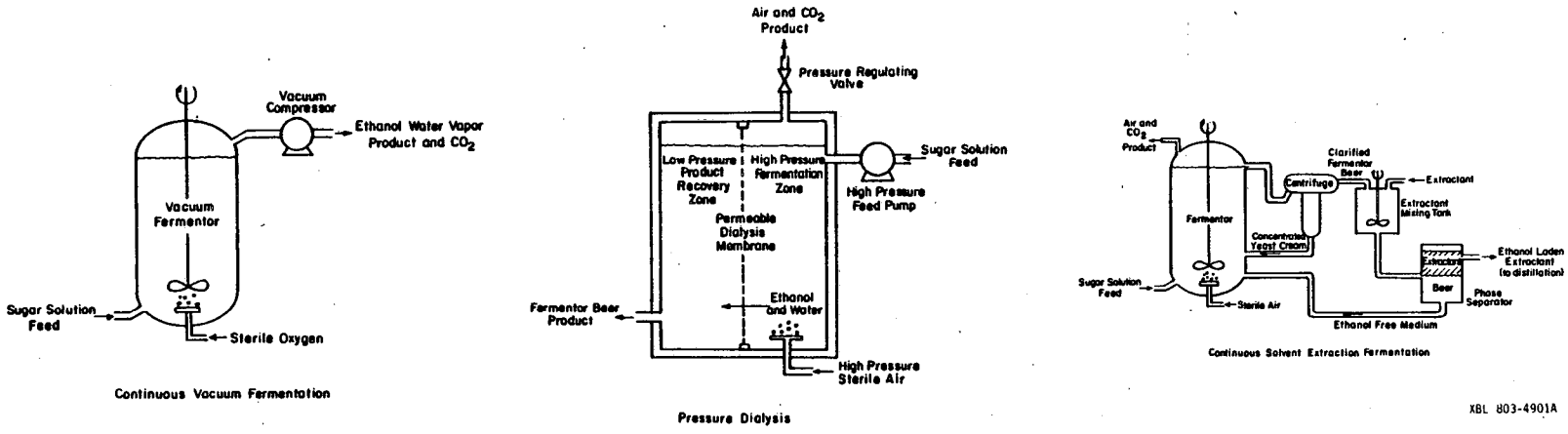

Vacuum Compressor
Ethanol Water Vapor Product and CO_2
Vacuum Fermentor
Sugar Solution Feed
Sterile Oxygen
Continuous Vacuum Fermentation
Air and CO_2 Product
Pressure Regulating Valve
Low Pressure Product Recovery Zone
High Pressure Fermentation Zone
Sugar Solution Feed
High Pressure Feed Pump
Permeable Dialysis Membrane
Fermentor Beer Product
Ethanol and Water
High Pressure Sterile Air
Pressure Dialysis
Air and CO_2 Product
Clarified Fermentor Beer
Centrifuge
Extractant
Extractant Mixing Tank
Fermentor
Concentrated Yeast Cream
Ethanol Laden Extractant (to distillation)
Sugar Solution Feed
Beer
Phase Separator
Sterile Air
Ethanol Free Medium
Continuous Solvent Extraction Fermentation
XBL 803-4901A

Figure 5.

systems (4), employing nonpolar solvents to remove ethanol, may concentrate organic acids.

The source of inhibitors may be feed components which are not fully metabolized and which concentrate in the fermentor, or they may be fermentation by-products. In this paper, we consider the effect of by-products as these may be hard to eliminate from the fermentation system and may thus set an ultimate limit on fermentation productivity with a given organism. In a further paper, the effects of common feed components, when concentrated to high levels, are presented (15).

EXPERIMENTAL APPROACH

The products of alcoholic fermentation of a synthetic glucose media by Saccharoyces cerevisiae as given by Neish and Blackwood (5) and as determined in our own laboratory (6) are presented in Table 1. When sugar from natural sources containing amino acids (such as corn hydrolyzate or molasses) is used, fusel oils will also be produced (7), with up to 5 grams of these propyl and butyl alcohols produced per liter of ethanol (8).

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 were inhibited. Those by-products marked by an asterisk in Table 1 were tested. The six major synthetic media

TABLE 1

PRODUCTS OF THE ALCOHOLIC FERMENTATION OF GLUCOSE BY *SACCHAROMYCES CEREVISIAE*

FROM SYNTHETIC MEDIA:

PRODUCT	mM PRODUCT/100 mM GLUCOSE
* 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
* ACETALDEHYDE	5.0

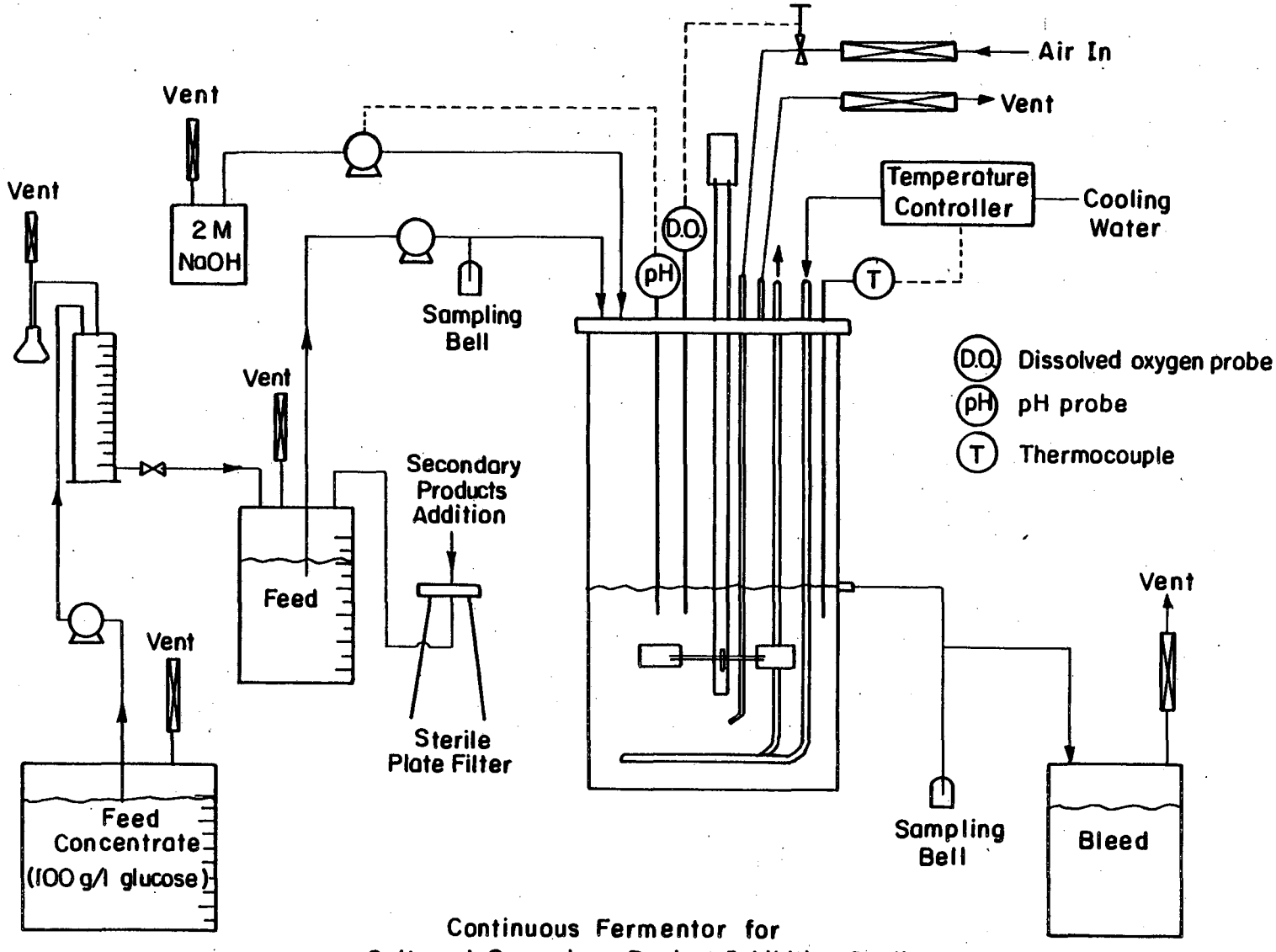
ADDITIONAL FROM MOLASSES MEDIA

FUSEL OIL: *1-PROPANOL	0.34
*2-METHYL-1-BUTANOL	0.11

by-products and a representative straight chained and a branched fusel oil component were used.

The inhibition studies were carried out in 5 liter (2.4 liter working volume) New Brunswick fermentors, arranged as shown in Fig. 6. Conditions for these studies are summarized in Table 2. Temperature and pH were controlled at established optima for the yeast strain (Sac. cer. var anamensis ATCC 4226). A feed glucose concentration of only 20 g/L was chosen to limit ethanol production and prevent the masking of by-product inhibition effects by ethanol inhibition. While the basic fermentation reaction to produce ethanol is anaerobic, oxygen is required for the biosynthesis of unsaturated fatty acids and sterols. A dissolved oxygen concentration of 5% of air saturation was maintained in the fermentor. The dilution rates were chosen to insure a substantial residual glucose level as according to Moss, et al. (9), the matabolism will be fermentative and independent of oxygen concentration as long as the glucose level is above 3 g/L. Unfortunately, this was not the case. Initial ethanol yields (before the addition of any by-products) were only approximately .37 g ethanol/g glucose consumed as compared to the anticipated .47. A complete mass balance (including carbon dioxide offgas analysis) confirmed a high fraction of aerobic metabolism. An interpretation of the meaning of changes in ethanol and cell yield factors is thus complicated.

A stock solution of feed concentrate made to five times the



Continuous Fermentor for Salt and Secondary Product Inhibition Studies

XBL 818-6254

Figure 6

Table 2

Conditions for Continuous By-Product Inhibition Studies

Base medium composition:

Glucose	20	g/L
Yeast extract	1.7	g/L
NH_4Cl	.25	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}

A stock solution of feed concentrate made to five times the final feed concentration as given in Table 2 (i.e. 100 g/L glucose) was prepared and sterilized by autoclaving. The final feed with added by-products was prepared by measuring four liters of the sterile feed concentrate into the feed reservoir and then adding the by-products and water though a sterilizing filter to make up to a final twenty liters volume (20 g/L glucose concentration).

The fermentations were begun in by-product free media, inoculating the fermentors with 100 cc of a dense (approximately 10 g cells/L) actively growing yeast culture. The fementor was kept in batch growth until actively fermenting (about 12 hours) and then switched to continuous flow. A benchmark steady state was achieved without by-product addition. By-products were then added and steady states achieved at successively higher by-product concentrations.

Samples from the feed and fermentor overflows were taken at regular intervals and a steady state was noted when three successive fermentor samples separated by at least six hours each gave the same cell density, ethanol concentration and residual glucose concentration. Steady state was normally achieved within six fermentor volume flows.

Cell densities were determined by optical density measurement at 600 nm and confirmed by actual dry weight measurements of filtered samples. Glucose concentrations were measured using the dinitrosalicyclic acid method (10). Ethanol concentrations and the concentrations of the volatile by-products

were determined by gas chromatography. The concentrations of lactic and formic acid in the fermentor could not be determined by gas chromatography and thus, were not measured. It was assumed that like acetic acid (and all of the by-products except acetaldehyde) the fermentor lactic and formic acid concentrations were the concentrations in the prepared feed.

RESULTS

Results are summarized in Table 3 which lists the by-products studied and by-product feed concentrations at high inhibition (where the cell density is reduced by 80%). Results for earlier ethanol and glucose inhibition studies (11) are included for comparison.

The yeast cell is a complex system with many transport, energy and biosynthetic pathways. Each inhibitor may have many individual points of metabolic effect. An exact knowledge of the modes of effect of each inhibitor would be of tremendous value as specifically "engineered" organisms resistant to each mode of attack, might then possibly be developed.

For the present purpose of assessing the limitations placed upon new selective ethanol removal processes by by-product inhibition effects, a generalization of modes of inhibitor effect is most desirable. Recognizing that these are not necessarily specific descriptions of the mode of action of the inhibitory by-products, all of the inhibition effects observed can nonetheless be classified

Table 3

By-Product Inhibition Summary

By-Product	Concentration at High Inhibition* (g/L)	Inhibition Mechanism
Ethanol	70	Direct inhibition of the ethanol production pathway
Formic Acid	2.7	Chemical interference with cell maintenance functions
Acetic Acid	7.5	"
Lactic Acid	38	"
1-Propanol	12	"
2-Methyl-1-Butanol	3.5	"
2,3-Butanediol	90	"
Acetaldehyde	5.0	" (Largely reconsumed)
Glycerol	450	Osmotic Pressure Effects
Glucose	380	"

*80% reduction in cell mass

into three basic mechanistic schemes, each with particular characteristics. The postulated modes of inhibition of each by-product are given in Table 3.

A. Inhibition by Direct Interference with the Ethanol Production or Cell Growth Pathways

Ethanol inhibition has been shown by Bazua (11) 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) and ethanol productivity (ν) as ethanol productivity and hence available ATP for biosynthesis decreases with increased inhibition (see Fig. 7). There is no apparent change in cell morphology associated with ethanol inhibition.

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 while ethanol producivity (ν) decreases or may be partially maintained as ATP is shunted away for production of by-products such as glycerol and acetaldehyde.

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

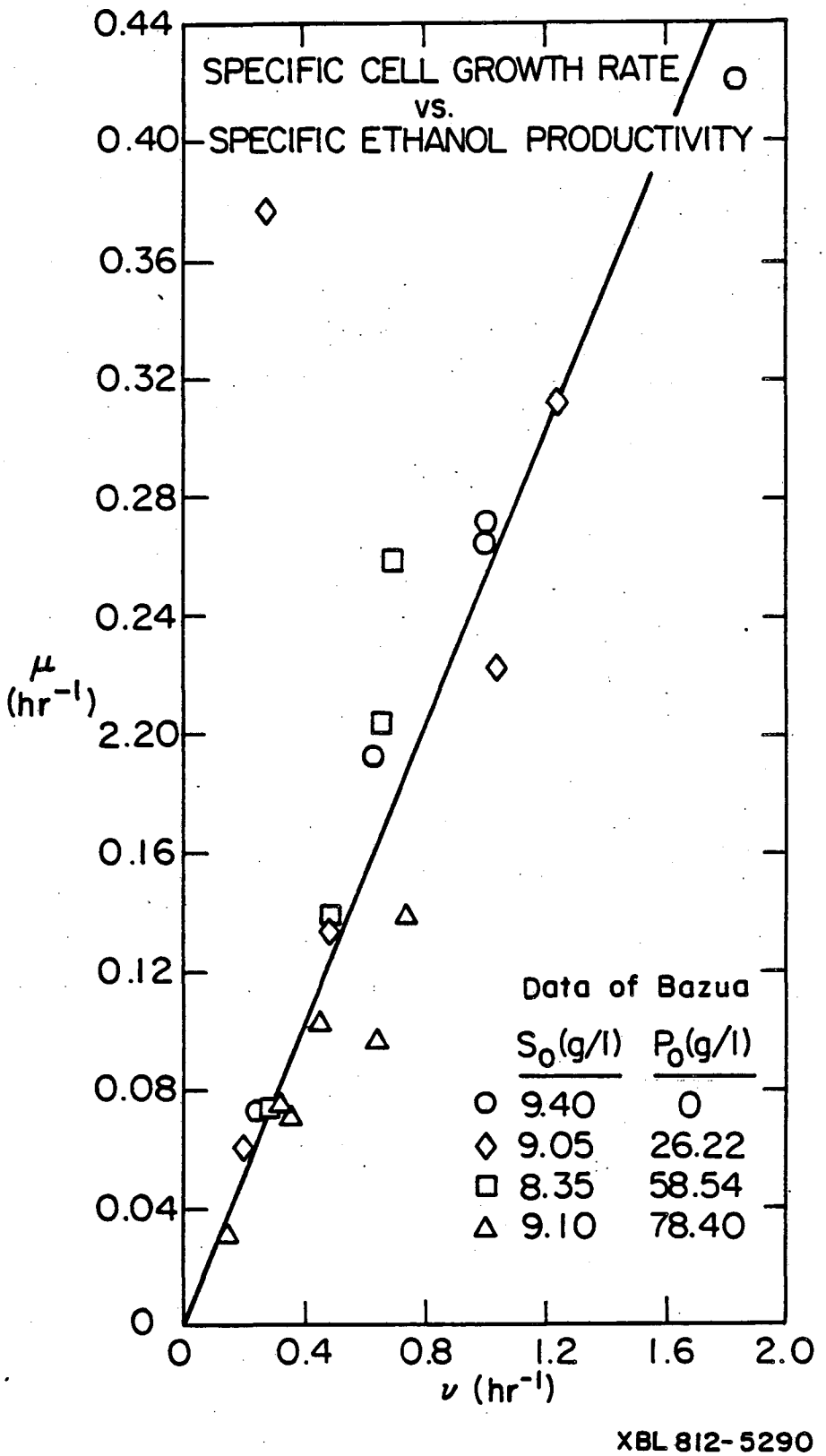

SPECIFIC CELL GROWTH RATE
vs.
SPECIFIC ETHANOL PRODUCTIVITY
0.44
0.40
0.36
0.32
0.28
0.24
2.20
0.16
0.12
0.08
0.04
0
μ (hr⁻¹)
0
0.4
0.8
1.2
1.6
2.0
ν (hr⁻¹)
Data of Bazua
S₀(g/l) P₀(g/l)
9.40 0
9.05 26.22
8.35 58.54
9.10 78.40
XBL 812-5290

Figure 7

B. 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 the range from .5 to 9 g/L, see Fig. 8). Acetate is soluble in the lipids of the cell membrane (12). Samson (13) has shown that acetic acid (or sodium acetate) inhibits by chemically interfering with membrane 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 <u>increases</u> 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 mophology is altered with cells becoming irregular and elongated (Fig. 9).

Formic acid is very similar in lipid solubility to acetic acid (12). Both acids inhibit at similar concentrations (Fig 10) and for both, while the cell density is decreased, specific ethanol productivity increases to a maximum of 1.5 hr^{-1} as the by-product concentation is increased. It is probable that the mechanisms of

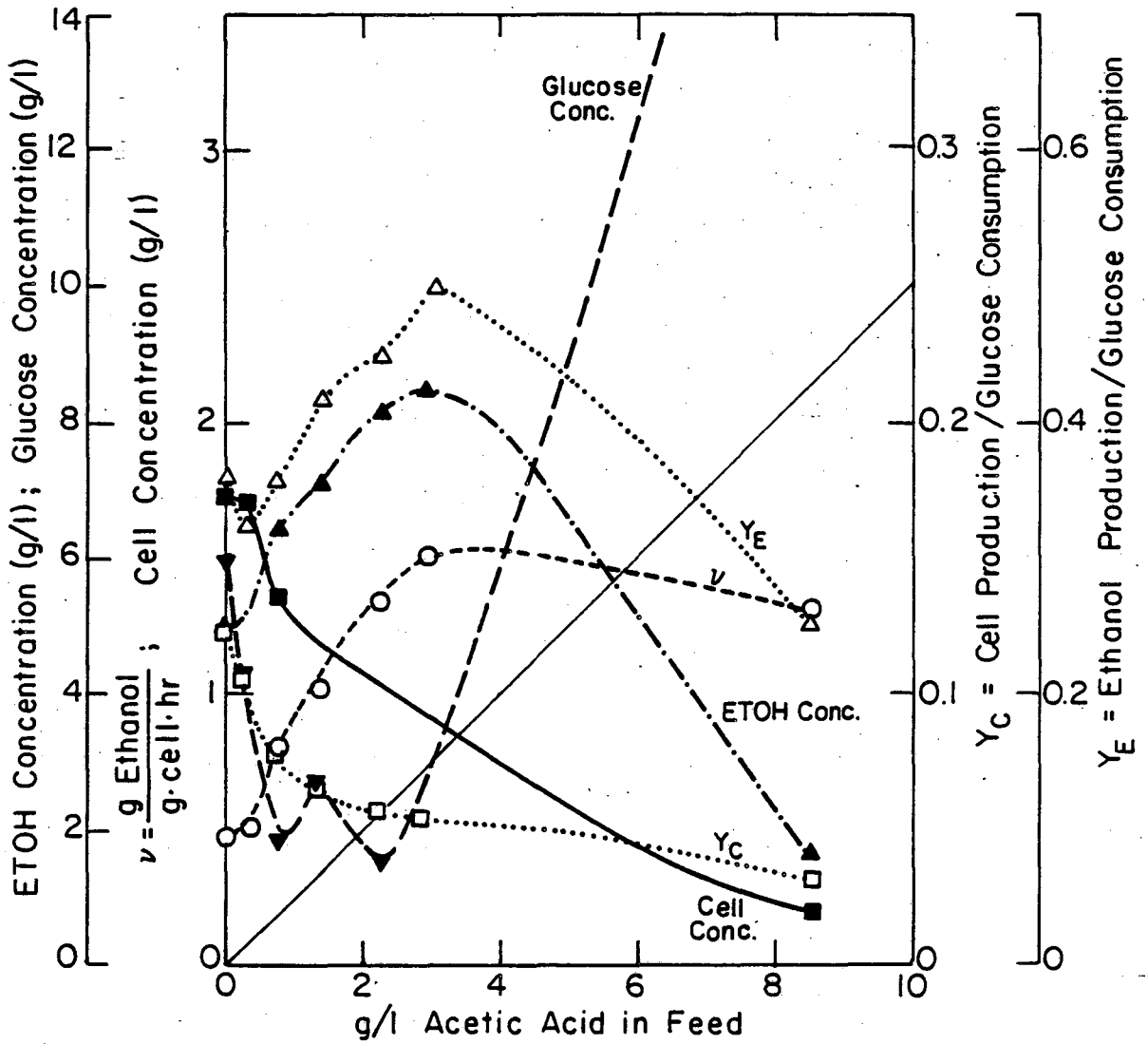


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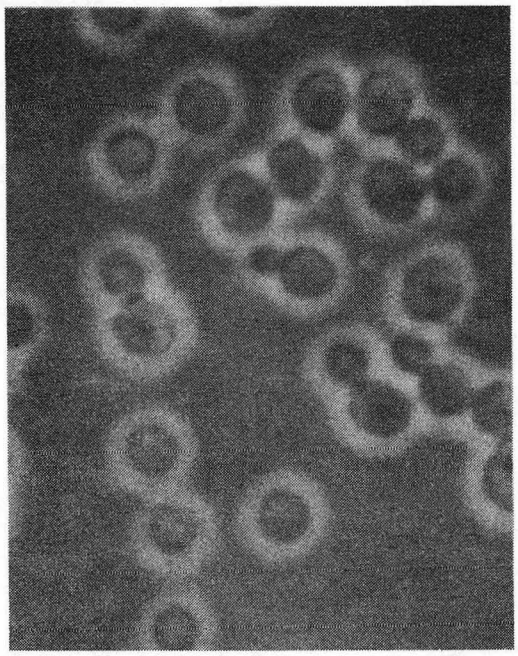
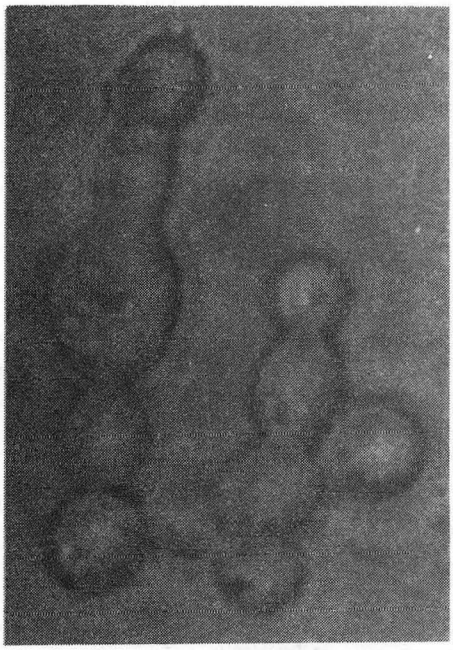
Figure 8.





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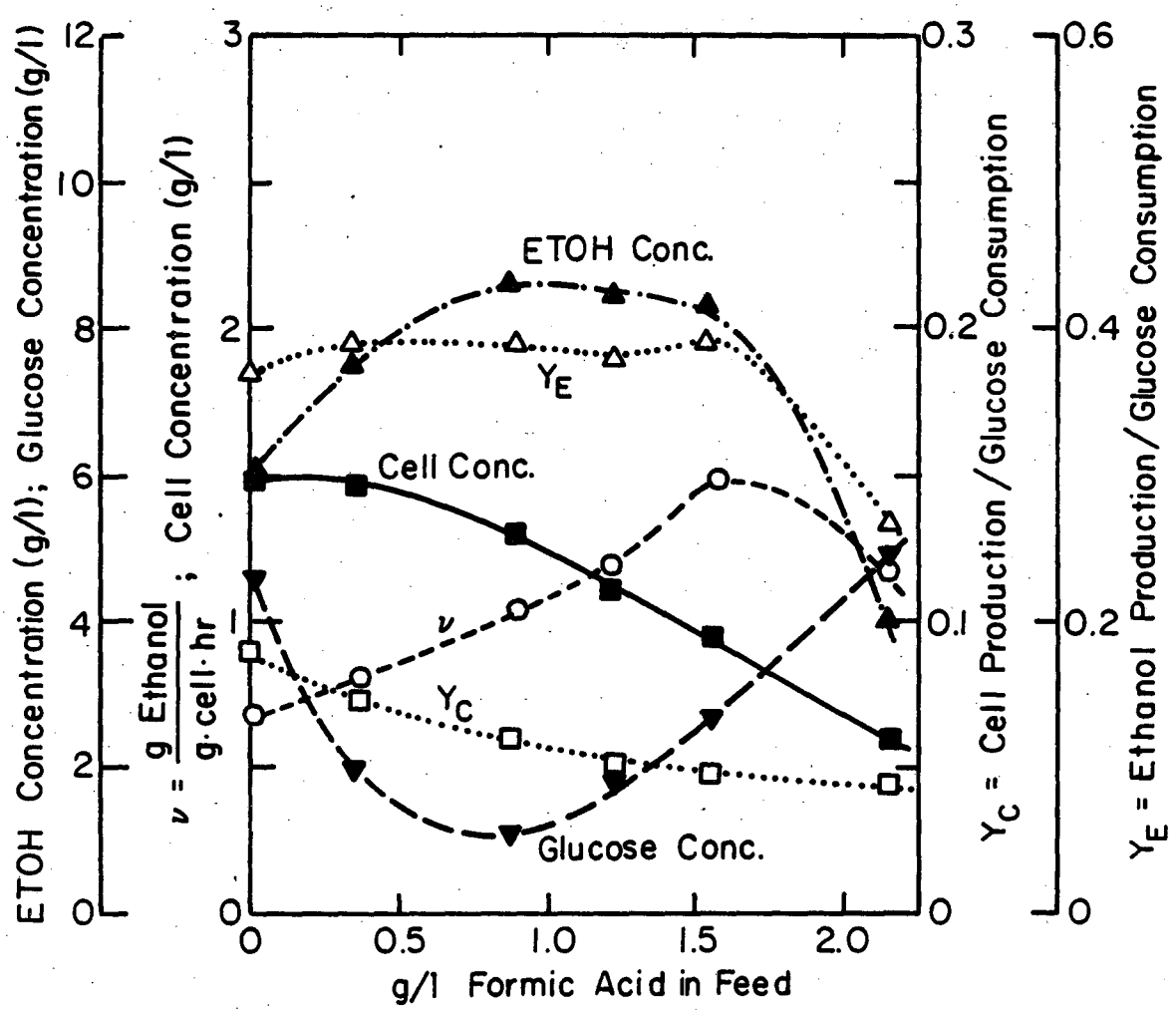
NORMAL

ACETIC ACID

Figure 9



XBL 818-6244

Figure 10.

inhibition are identical. Unlike acetic acid, no cell morphology change was seen with lactic acid. This may simply have been because the acetic acid experiment was carried up to higher inhibitor concentrations.

Lactic acid (Fig. 11) with its extra hydroxyl group is much less soluble in lipids than acetic or formic acids (12) and lactic acid inhibition occurs at a much higher concentration (10 to 40 g/L). Further, Samson reports that lactic acid does not inhibit phosphate transport (13). Thus, the exact mechanism of lactic acid inhibition is probably different than that for acetic acid. As cell density decreases, however, specific ethanol productivity does increase (from .55 hr^{-1} to .8 hr^{-1}) again indicative of some form of chemical interference with cell maintenance functions requiring increased ATP expenditure.

Both of the fusel oil components tested -- 1-propanol (Fig 12) and 2-methyl-1-butanol (Fig. 13)--inhibit at similar low by-product concentrations increasing the specific ethanol productivity from .45 hr^{-1} to 1.6 hr^{-1}. For both components, cell morphology is changed (Fig. 14). The inhibited yeast are pseudomycelial (long and rod shaped). It appears as if cells have repeatedly budded but that the buds have not pinched off into individual cells after reproduction.

Detergents and water immiscible solvents such as butanol are known lipid solvents and will cause the disintegration of the membrane. This is the probable mode of attack by the fusel oil components and may explain the observed morphology change. Under

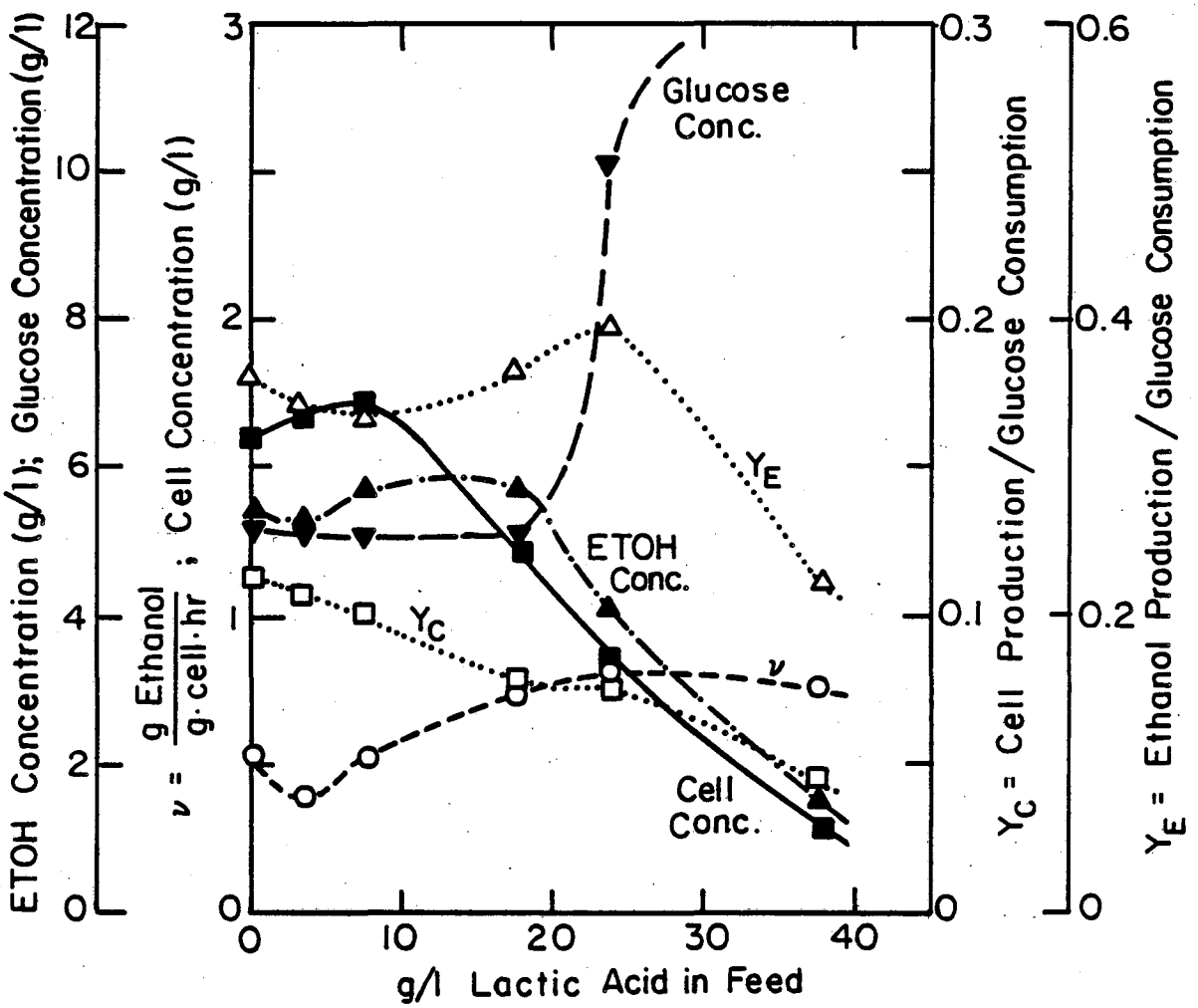


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Figure 11.

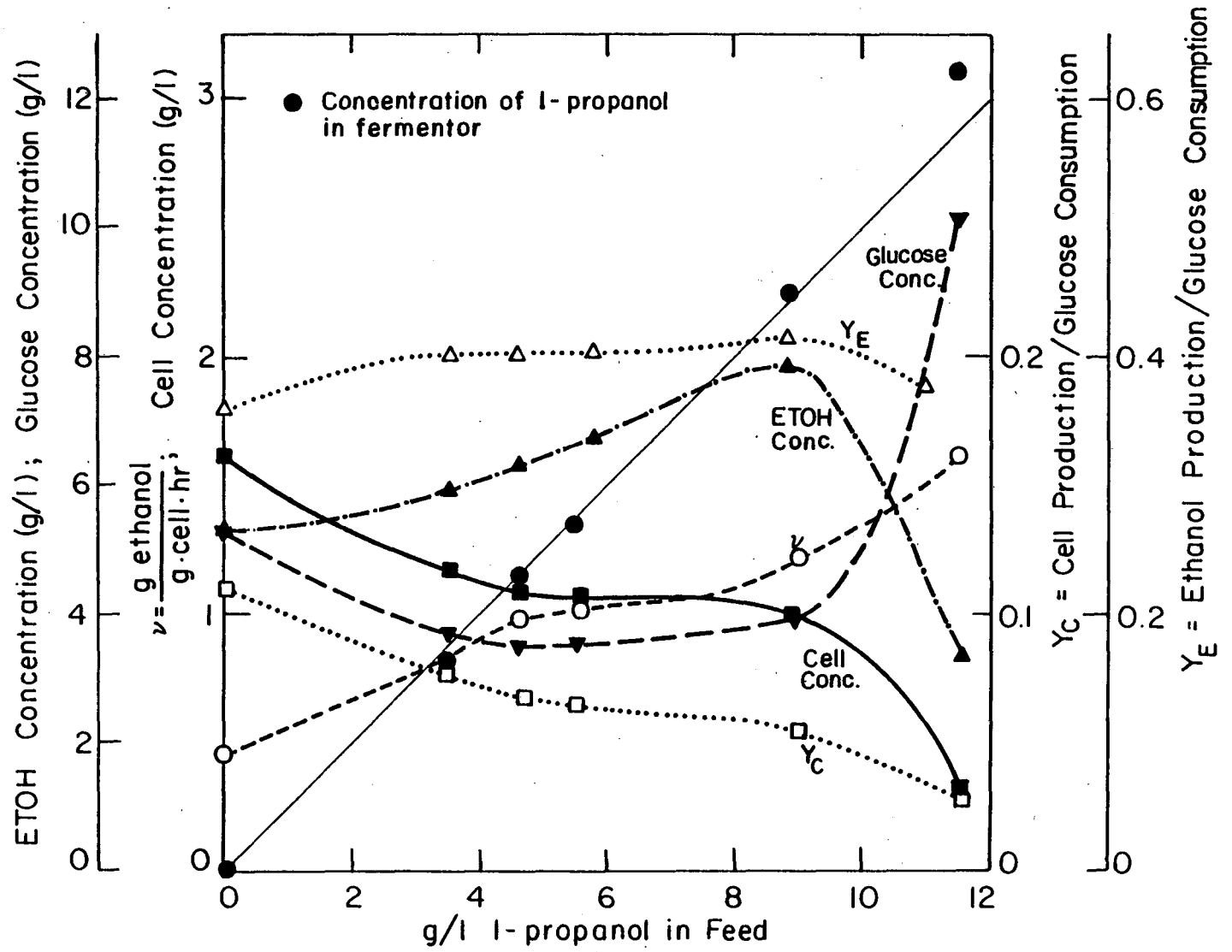

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Figure 12.

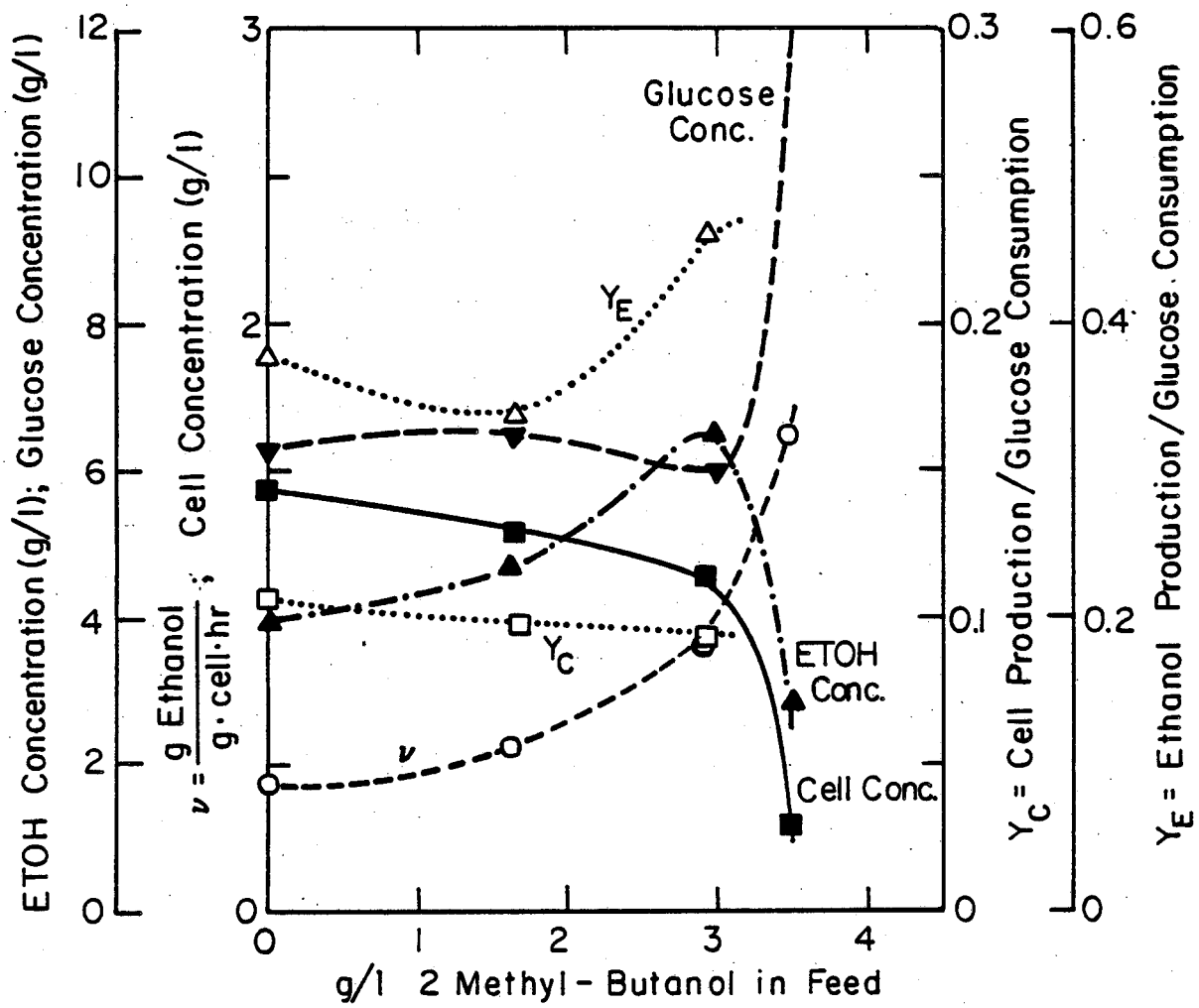

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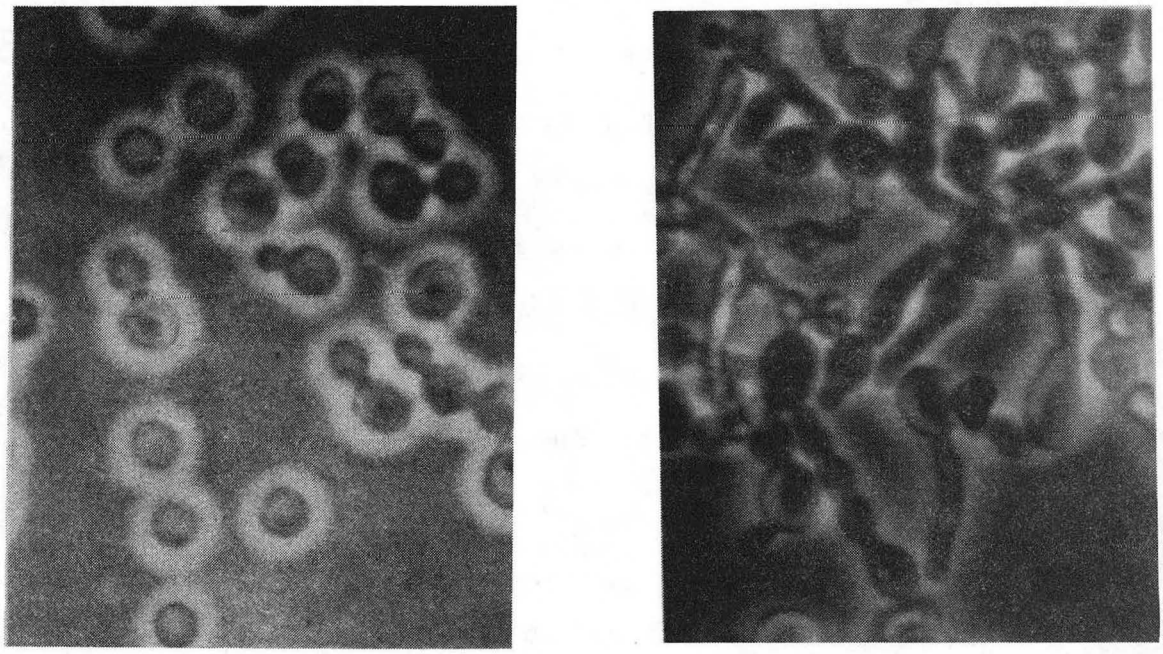
Figure 13



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NORMAL 1-PROPANOL

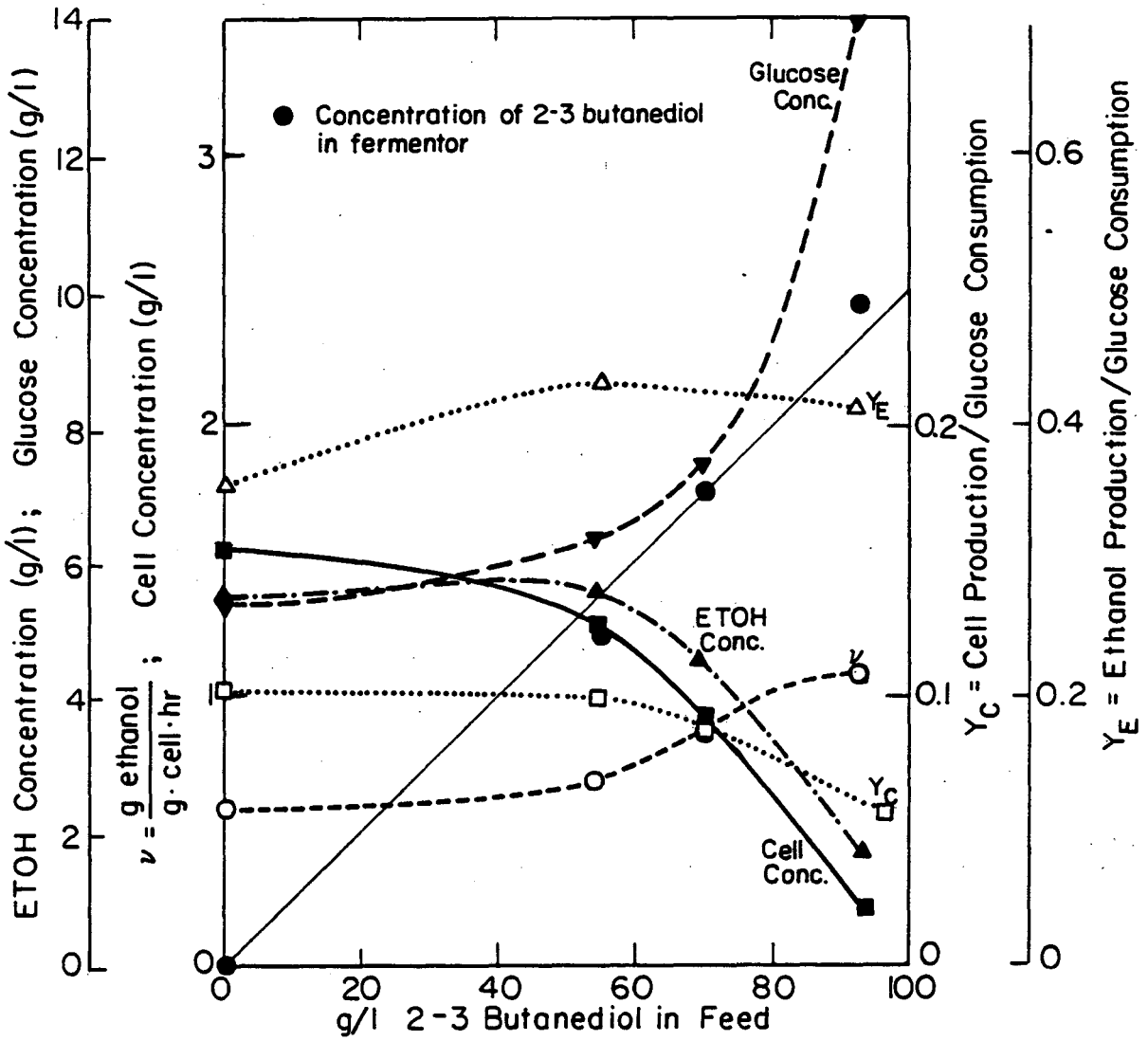
Figure 14

permeable to monovalent cations such as K^+ and NH_4^+ (14). More energy must be expended for maintenance, pumping these ions (by active transport) to maintain proper internal levels. The ATP demand for maintenance is increased, cell production decreases and the specific ethanol productivity is increased as was observed.

2,3-butanediol, with its two hydroxyl groups, is less lipid soluble. Inhibition by 2,3-butanediol occurs at a much higher by-product concentration (40-90 g/L) Fig.15). Inhibition by 2,3-butanediol may be due to cell membrane disruption as with propanol.

Being only slightly lipid soluble there may exist an active transport mechanism for the removal of internally produced 2,3-butanediol out through the lipid membrane. At high external butanediol concentrations, the required pumping energy might be increased, and this could also explain the increase in specific ethanol productivity (from .6 to 1.1 $hr,^{-1}$) as the butanediol concentration was increased in the fermentor.

Acetaldehyde is an immediate precursor to ethanol in the yeast metabolic pathway. A unique feature of the acetaldehyde inhibition studies was consumption of the by-product by the yeast and conversion to alcohol. Thus, as the feed acealdehyde concentration increased from 0 to 4 g/L, the residual acealdehyde concentration in the fermentor increased only from .25 to 8 g/L. Figure 16 plots the results of the acetaldehyde inhibition experiment, with the specific ethanol productivity (ν) based only on ethanol derived from glucose,



XBL 812-5291

Figure 15.

i.e. with acetaldehyde derived ethanol subtracted from the total. Acetaldehyde inhibits at about the same fermentor concentration and is structually similar to formic and acetic acids. Acetaldehyde inhibition is accompanied by a cell morphology change with the cells increasing to over twice their normal diameter and appearing "mushy" (Fig. 17). Acetaldehyde inhibition may be by a mechanism of interference with active transport similar to that for formic and acetic acids.

C. Inhibition 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. Uptake of nutrients will require additional energy. 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 nontoxic 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 small ridgid spheres.

Inhibition by osmotic stress is well illustrated by glycerol inhibition (Fig. 18). Glycerol has no affect at 100 g/L and significant cell growth continues at 400 g/L concentration.

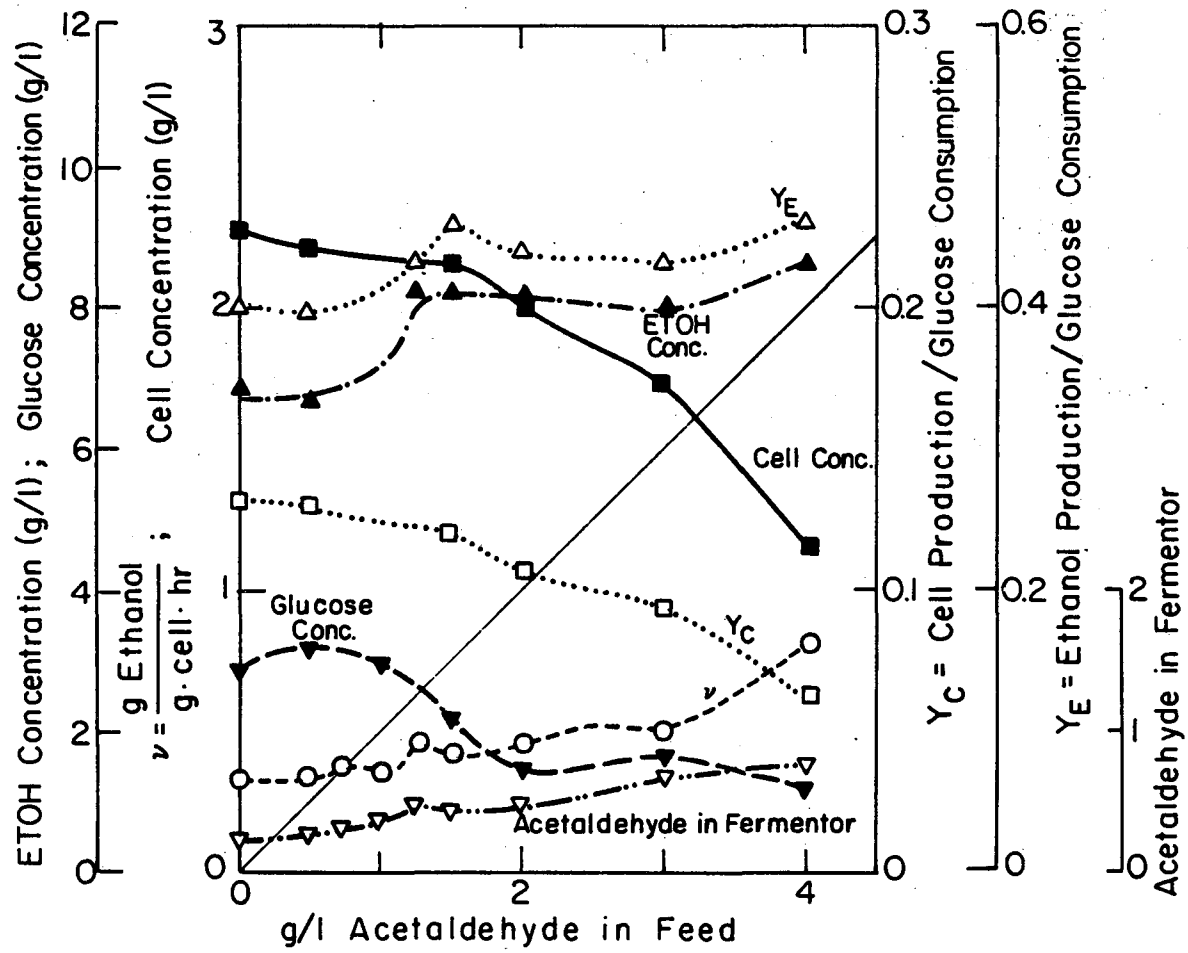


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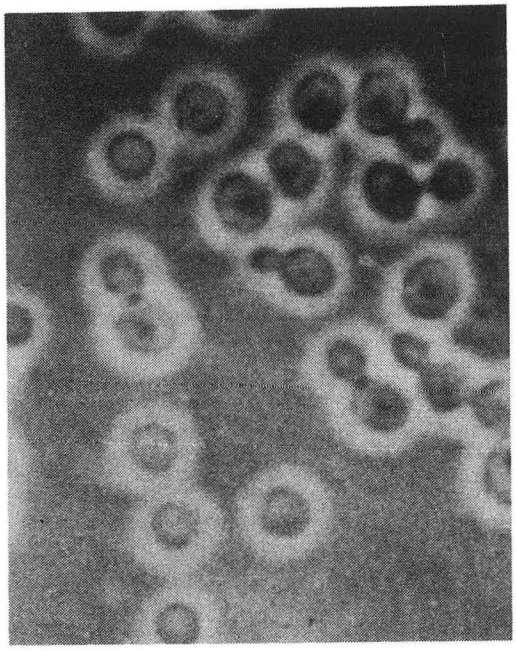
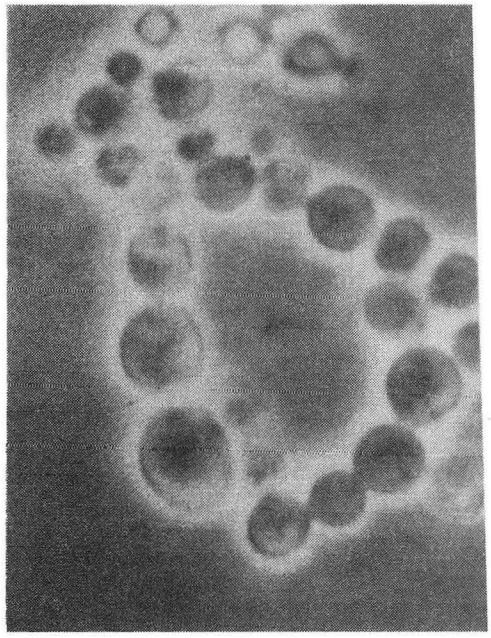
Figure 16.





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NORMAL

ACETALDEHYDE

Figure 17.

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 osolarity of 2.96. In batch experiments, cell productivity was reduced 25% by a glucose concentration of 270 g/L corresponding to an osmolarity of 2.26.

CONCLUSION

A generalized ethanol removal fermentation system is shown in Fig. 19. Feed glucose, nutrients, and water enter in stream 1. The concentrated ethanol product, and some water leaves as stream 2. A dilute bleed of water, residual product, unutilized nutrients, concentrated by-products and possibly cells, leaves as stream 3. For the vacu-ferm process, stream 2 would be the concentrated vapor product and stream 3, the centrifuged bleed stream.

To minimize cost, 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 if the by-products are not reconsumed by the yeast, then the concentration of any given by-product in the fermentor will be given by

$$C_{\text{by-product}} = \frac{\nu X Y_{\text{by-product}}}{\gamma D}$$

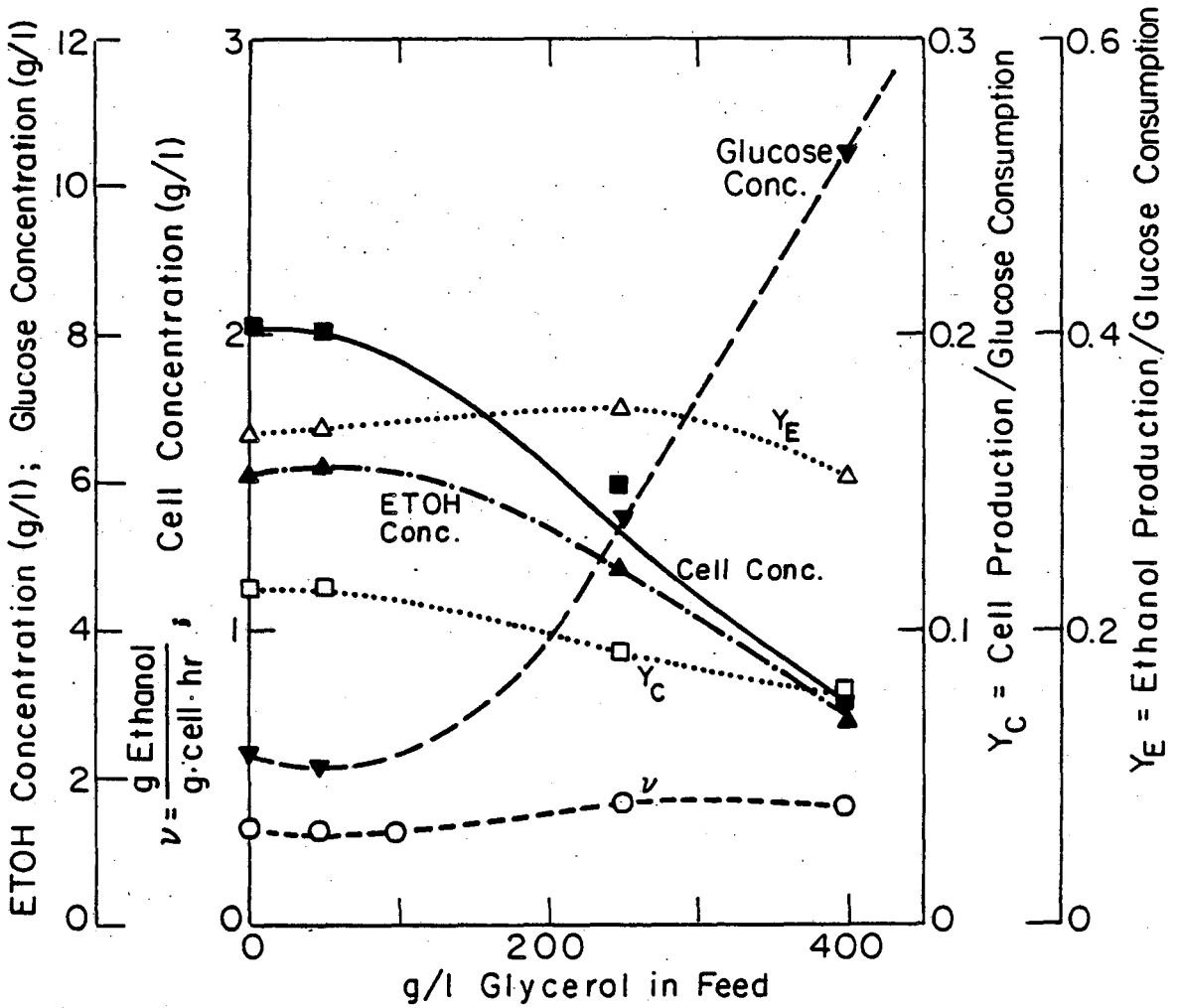
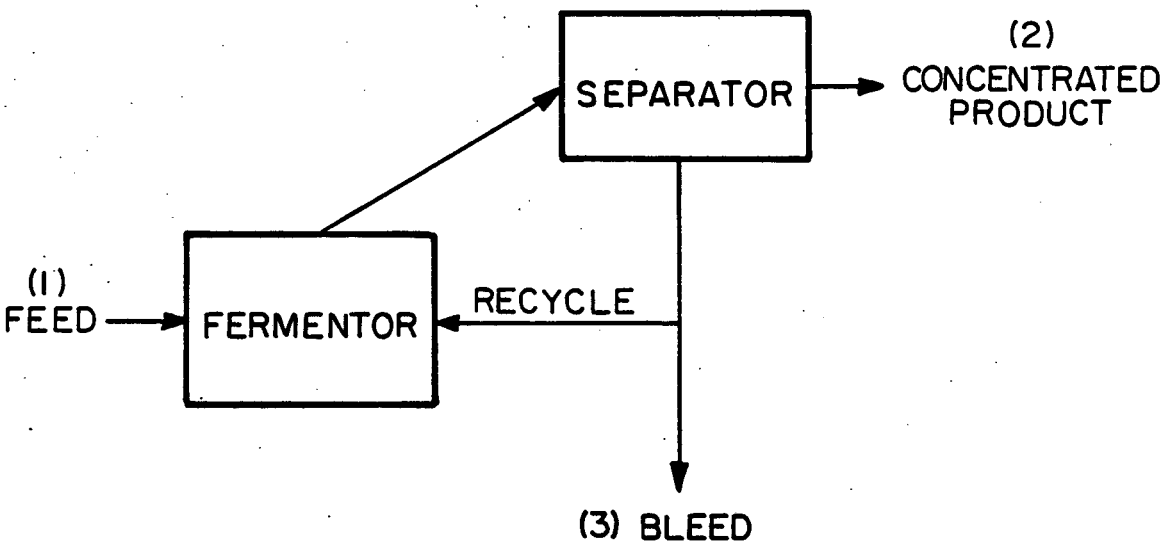

ETOH Concentration (g/l); Glucose Concentration (g/l)
Cell Concentration (g/l)
$\nu = \frac{g\ Ethanol}{g \cdot cell \cdot hr}$
Y_C = Cell Production/Glucose Consumption
Y_E = Ethanol Production/Glucose Consumption
Glucose Conc.
ETOH Conc.
Cell Conc.
Y_E
Y_C
ν
g/l Glycerol in Feed
0
200
400
XBL 812-5295

Figure 18.



Generalized Ethanol Removal Fermentation System.

XBL818-6281

Figure 19.

where

$C_{by\text{-}product}$	= by-product concentration (g/L)
ν	= specific ethanol productivity (1/hr)
X	= cell density (g/L)
$Y_{by\text{-}product}$	= by-product production ratio $\left(\frac{\text{g by-product/hr}}{\text{g ethanol/hr}}\right)$
D	= dilution rate (1/hr)
γ	= bleed to feed ratio
$\nu XY_{by\text{-}product}$	= by-product production rate (g by-product/L-hr)

Thus, the by-product concentration is increased by a factor of 1/ --the smaller the bleed, the greater the by-product concentration effect. This formula is not applicable in the case of acetaldehyde for which additional terms must be added to allow for acetaldehyde consumption. The concentration factor will also be less for by-products which "leek" out in the concentrated product stream (such as volatile acetaldehyde and formic acid, which will partially escape in the vacu-ferm concentrated vapor stream).

Formula 1 can now be used to determine if any of the by-products tested might have been responsible for the decline in cell growth in Cysewski's fermentation experiments. Using Cysewski's maximum overall fermentor productivity of 38 g ethanol/l-hr and a dilution rate of .27 hr^{-1} taking values of y by-product from Table 1 (converting to gram ratios) and recalling

that for Cysewski's synthetic media, fusel oil components were not produced, the limiting value of γ can be calculted. Cell productivity had declined 80% in Cysewski's experiment at a concentration factor of 3.0 corresponding to a γ of .333. Based on the inhibition experiments, formic acid would cause the earliest effect, and would not cause an 80% cell productivity decline until a γ of .128. Acetic acid would not cause an 80% decline until a γ of .098.

One may hypothesize that formic and acetic acids (with probably similar inhibition mechanisms) are working together to bring about the decline Cysewski observed. It must be remembered, however, that volatile formic acid would have been largely carried away in the vacu-ferm vapor product stream and should not have contributed strongly to inhibition effects. It must be concluded that a fermentation by-product probably was not the primary inhibitor affecting the vacu-ferm experiments. Buildup of nonmetabolized feed components is a more likely explanation. (Inhibition by concentrated feed components has been studied in our laboratory and is the topic of another paper.) Buildup of feed can be controlled. Inhibition by fermentation by-products may then still set an ultimate limit on fermentation processes.

For all of the by-products studied, it is seen that specific ethanol productivity can be increased (from about $\nu = .5$) up to a maximum of $\nu = 1.1$ to 1.6 where the cell reproduction rate drops below the fermentor dilution rate and washout occurs. Assuming a

cell recycle stystem so that a high value of cell growth rate (μ) and yield 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 Table 2, a high cell density of 100 g/L, a concentrated (300 g/L) glucose feed typical of vacuum fermentation, the overall fermentor productivity of 80 g/L found by Cysewski for vacu-ferm with cell recycle, and formula (1), we find that cell productivity will be reduced at a bleed to feed ratio 1:1.3, but that a bleed to feed ratio of only 1:7.8 should be sufficient to prevent excessive toxic by-product buildup, as long as cell recycle is employed to maintain the high cell density (Table 4).

TABLE 4

BY-PRODUCT	AT ONSET OF INHIBITION*1		AT HIGH INHIBITION*2	
	CONCENTRATION (G/L)	γ*3	CONCENTRATION (G/L)	γ
2,3-BUTANEDIOL	55	.014	90	.0086
GLYCEROL	200	.054	450	.025
ACETALDEHYDE	2.8	N.A.	5.0	N.A.
FORMIC ACID	1.2	.289	2.7	.128
ACETIC ACID	0.9	.794	7.5	.098
LACTIC ACID	17	.037	38	.0163
1-PROPANOL	2.0	.184	12	.030
2-METHYL-1-BUTANOL	2.8	.061	3.5	.049

*1 20% REDUCTION IN CELL MASS PRODUCTION

*2 80% REDUCTION IN CELL MASS PRODUCTION

*3 FOR VACUUM FERMENTATION WITH AN ETHANOL PRODUCTIVITY OF 80 G/L-HR

ACKNOWLEDGMENT

The assistance of Wai K. Lam and Jeff Vincent in conducting the continuous fermentation experiments is gratefully acknowledged.

This work was supported by the Director for Energy Research, Office of Basic Energy Sciences of the U.S. Department of Energy under Contract W-7405-ENG-48.

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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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