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XYLOSE FERMENTATION WITH
Clostridium Thermohydrosulfuricum

Anthony Mancuso*, Charles R. Wilke,
and Harvey W. Blanch

(*M.S. Thesis)

December 1982

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Xylose Fermentation with *Clostridium Thermohydrosulfuricum*

Anthony Mancuso*
Charles R. Wilke and Harvey W. Blanch

*M.S. Thesis
December 1982

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ABSTRACT

In the biological conversion of cellulosic biomass to ethanol, a significant fraction of the available carbohydrates are not fermentable by common brewing yeast. Xylose, a five carbon sugar which is not metabolized by brewing yeasts, represents approximately 25% of the total carbohydrate content of cellulosic biomass.

In this study, the fermentation of xylose to ethanol with a thermophilic, strictly anaerobic bacterium, *Clostridium thermohydrosulfuricum*, was examined. The focus of this investigation was on the physiological parameters which most strongly affect the economic feasibility of using this bacterium for industrial ethanol production.

In rich medium (containing economically impractical concentrations of yeast extract) yields as high as 0.43 gm ethanol/gm xylose and growth rates of 0.4 to 0.5 hr^{-1} were observed. The predominant by-products of the fermentation were acetate and lactate. Nutritional studies indicated that the cost of the growth medium could be dramatically reduced by replacing most of the yeast extract used with nicotinic acid and vitamin B_{12}.

Ethanol was found to be very inhibitory to growth and ethanol formation. The low ethanol concentrations produced by this organism, typically less than 0.6%, do not allow economical recovery of the ethanol. To overcome the problem of inhibition, cells were gradually adapted to high concentrations (up to 4.2%) of ethanol. However, the ethanol yield of adapted cells was typically 30 to 40% less than the yield of non-adapted cells.

Environmental parameters such as pH and by-product concen-

trations had only a slight effect on the ethanol yield produced by tolerant cells. A mutant, selected from an adapted strain, was found to produce 60% less lactate than its parent. This low-lactate producing mutant had a slightly improved ethanol yield.

The results obtained with the tolerant, low-lactate producing mutant were used in the design of an industrial-scale fermentation process. An economic evaluation of the process indicates that ethanol production with this bacterium is currently uneconomical. Further increasing the yield and concentration of ethanol produced will significantly improve the economic feasibility of using this bacterium for ethanol production.

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TABLE OF CONTENTS

I. INTRODUCTION 1

II. GENERAL BACKGROUND 3

2.1 Description of the Proposed Process 3

2.2 Xylose Metabolism 6

2.2.1 Absence of Xylose Fermenting Capability in Common Yeasts 6
2.2.2 Possible Alternatives for Xylose Conversion with Common Yeast 8

2.3 Organisms Capable of Fermenting Xylose to Ethanol . . . 10

2.3.1 Desired Properties for Prospective Xylose Fermenters 10
2.3.2 Newly Identified Yeasts Capable of Semi-aerobic Xylose Fermentation 11
2.3.3 Conversion of Xylose to Ethanol with Fungi . . . 13
2.3.4 Xylose Fermenting Bacteria 14

III. PREVIOUS WORK WITH CLOSTRIDIUM THERMOHYDROSULFURICUM 21

3.1 Species History 21

3.2 General Physiology 22

3.3 Properties of Strain 39E 23

3.3.1 Growth Characteristics 23
3.3.2 Metabolic Pathway of *C. thermohydrosulfuricum* 39E 26
3.3.3 Metabolic Control and End-Product Formation . . 29

IV. MATERIALS AND METHODS 33

4.1 Growth Procedures 33

4.1.1 Organism and Culture Media 33
4.1.2 Medium Composition 35
4.1.3 Medium Reduction 39
4.1.4 Preparation of Inocula 42
4.1.5 Incubation 42
4.1.6 pH Controlled Experiments 42

4.2 Analytical Procedures 45

4.2.1 Growth Rate and Dry Weight 45
4.2.2 Sample Preparation for Xylose and End-Product Assays 45
4.2.3 Ethanol 45
4.2.4 Xylose 48
4.2.5 Acetate 48
4.2.6 Lactate 49

V. RESULTS AND DISCUSSION 51

5.1 Batch Fermentation Profile 51

5.2 Nutritional Requirements 56

5.2.1 Effect of Yeast Extract Concentration on Growth and Yields 56
5.2.2 Corn Steep Liquor as a Source of Nutrients . . . 61
5.2.3 Growth in Defined Media 67

5.3 Ethanol Inhibition and Tolerance 87

5.3.1 Ethanol Inhibition 87
5.3.2 Adaptation 92
5.3.3 De-adaptatin and Maintenance of Tolerance . . . 93
5.3.4 Comparison of Parent and Adapted Strains 93
5.3.5 Further Adaptation 98

5.4 Effect of pH on Strain UC-42 98

5.5 Effect of Acetate, Lactate and Xylose on Growth Rate and Yields 108

5.6 Improving Ethanol Yield by Mutation 115

5.6.1 Selection of Mutants 115
5.6.2 Evaluation of Strain UC-42-L2 117
5.6.3 Recommendations 123

VI. PROCESS DESIGN AND ECONOMIC EVALUATION 124

6.1 Plant Design 124

6.1.1 Raw Material 124
6.1.2 Process Description 126
6.1.3 Material Balance and Design Basis 129

6.2 Economic Evaluation 130

6.2.1 Purchased Equipment Costs 130
6.2.2 Operating Costs 134
6.2.3 Waste Treatment 134
6.2.4 The Advantage of Using Thermophilic Organisms for Ethanol Production 136
6.2.5 Further Reductions in Production Costs 136

VII. CONCLUSIONS AND RECOMMENDATIONS 138

APPENDIX I: Isolation of Low Acid Producing Mutants 139

APPENDIX II: Determination of Carbon Recovery 157

REFERENCES 159

I. INTRODUCTION

An ever-increasing awareness that petroleum reserves are not limitless has led to a recent surge in interest in alternative feedstocks for liquid fuel production. Cellulosic biomass is an attractive alternative feedstock since it is abundant and renewable, and can be converted by microorganisms to clean burning fuels, such as ethanol (1).

Cellulosic biomass is primarily composed of cellulose (a glucose polymer), hemicellulose (a heteropolymer containing mostly xylose), and lignin (a non-carbohydrate). Many processes for the biological conversion of biomass carbohydrates to ethanol have been proposed. Because glucose is the most abundant carbohydrate in cellulosic biomass, most schemes have focused on the hydrolysis of cellulose to glucose (2). The subsequent fermentation of glucose to ethanol can be readily accomplished with common brewing yeasts. However, efficient conversion of cellulosic biomass will necessarily include the fermentation of xylose which represents approximately 25% of the available carbohydrates (3). Unfortunately, common yeasts do not have the metabolic capability to ferment xylose (4).

In this study, the fermentation of xylose to ethanol with a thermophilic, obligate anaerobe, *Clostridium thermohydrosulfuricum*, was investigated. Important physiological characteristics, such as nutrient requirements and end-product tolerance, were examined since they may limit the economic feasibility of using this organism in an industrial biomass conversion process. Various alternative approaches to improving the yield of ethanol from xylose were also studied.

The experimental results obtained were used to design a xylose

fermentation process, intended to extend a biomass conversion scheme developed at the University of California, Berkeley (3). Previously, the xylose fraction of cellulosic biomass was left unused in this process.

A preliminary economic evaluation for the xylose fermentation process is presented at the end of this work. Based on the evaluation, suggestions for further research are given which may improve the economic feasibility of converting xylose to ethanol.

II. GENERAL BACKGROUND

2.1. Description of the Proposed Process

Sources of cellulosic biomass include agricultural wastes (e.g. corn stover, wheat straw, and sugar cane bagasse), forest residues, and municipal wastes (e.g. newsprint). Corn stover was selected as a representative source of cellulosic biomass (3). The composition of corn stover is presented in Table 2.1. Most of the glucose present in untreated corn stover exists as cellulose, which is a β, 1-4 linked D-glucose polymer. The other sugars, D-xylose, L-arabinose, D-mannose, and D-galactose, along with some D-glucose, are linked together in a branched heteropolymer: hemicellulose. The other organic compounds listed, lignin and extractives, are generally non-fermentable (5).

A flow diagram for the process is given in Figure 2.1. Initially, corn stover is hammer milled and pretreated with dilute sulfuric acid to remove most of the hemicellulose. The pretreatment is necessary to make the cellulose fraction more accessible for the subsequent hydrolysis. Cellulose is hydrolyzed by enzymes called cellulases in a series of stirred tank reactors. Cellulase enzymes used for the hydrolysis are produced by the fungus *Trichoderma reesei* Rut C-30. The enzyme is produced continuously in a two stage fermentation process.

After hydrolysis, the enzyme-containing sugar solution is contacted countercurrently with fresh incoming solids. Solids contacting is a useful means for recovering much of the cellulase leaving hydrolysis. Cellulase adsorbs onto the solids in the adsorption vessels and returns to the hydrolysis tanks with the fresh solids. The

Table 2.1

Corn Stover Composition*

% Carbohydrate		% Sugar Equivalent	
Hexosans:			
Glucan	35.1	Glucose	39.0
Mannan	0.25	Mannose	0.28
Galactan	0.75	Galactose	0.83
Pentosans			
Xylan	13.0	Xylose	14.8
Arabinan	2.98	Arabinose	3.39
Total Carbohydrate	52.1	Total Sugar Equivalent	58.3

15.1%	Lignin
4.3%	Ash
5.5%	Azeotropic Benzene/Ethanol extractives
1(±1)%	Acid insoluble material
4.0%	Protein

Note that total is less than 100% since not all components were assayed.

*Adapted from Wilke, et al. (3).

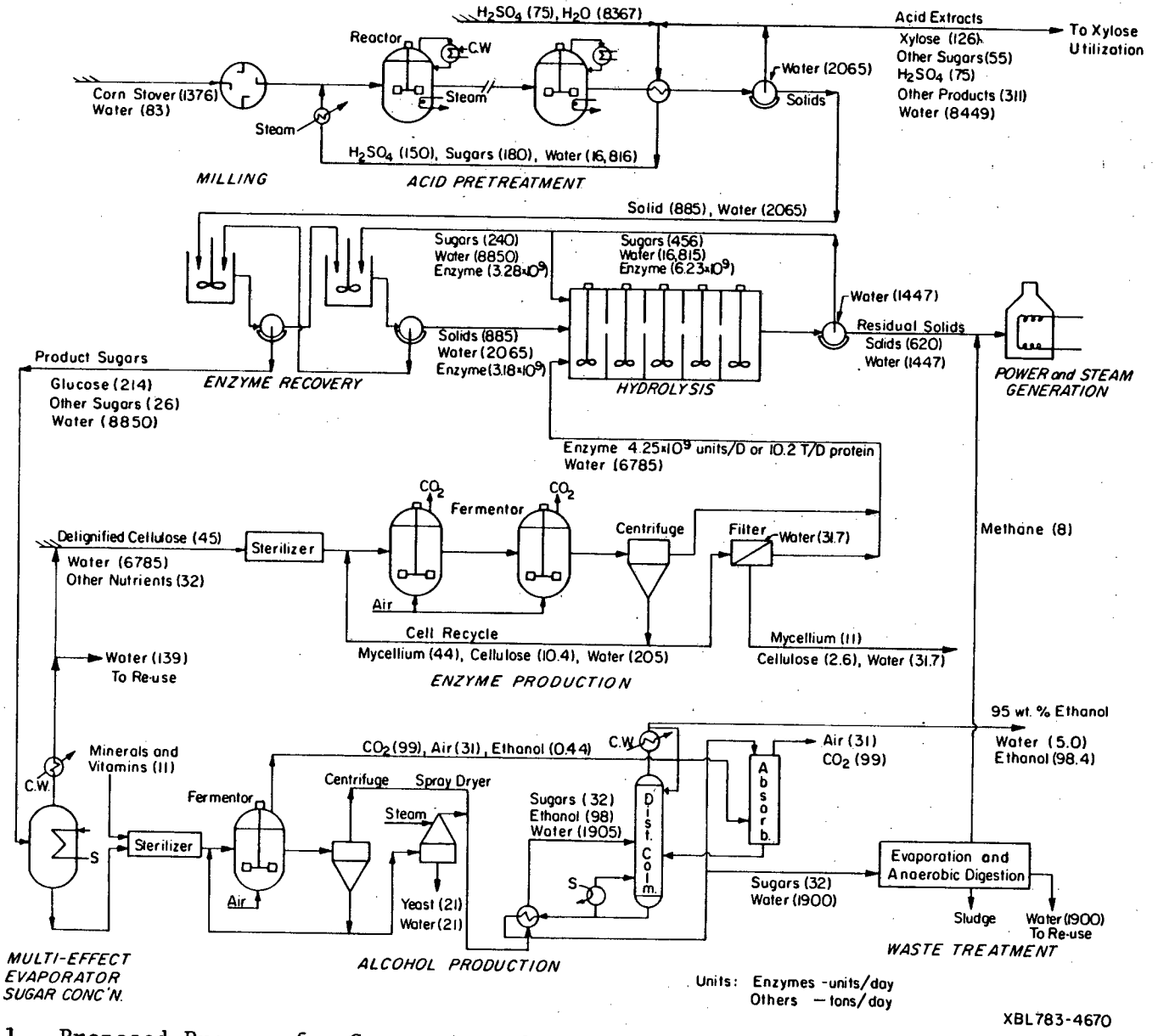

Figure 2.1. Proposed Process for Conversion of Corn Stover to Ethanol. From Wilke et al. (3).

dilute glucose solution is concentrated in a multiple effect evaporator and fermented by the yeast Saccharomyces cerevisiae in a continuous, cell recycle fermentor. Beer leaving the fermentation section contains 4.9% ethanol (by weight). Ethanol is recovered by distillation while by-product yeast cells are recovered by drying to be sold as a protein supplement. The overall process ethanol yield, based on the total carbohydrates initially present in corn stover, is 12%.

One possible way to increase the yield of ethanol from corn stover would be to ferment sugars formed during acid pretreatment. Dilute sulfuric acid used in the pretreatment hydrolyzes hemicellulose to form a solution containing 1.5% xylose and small amounts of other sugars. Efficient conversion of xylose to ethanol could increase the overall yield of ethanol by 70% (3). Hence xylose fermentation may have a very significant effect on the economic feasibility of converting cellulosic biomass to ethanol.

2.2. Xylose Metabolism

2.2.1. Absence of Xylose Fermenting Capability in Common Yeasts

Yeasts commonly used for industrial ethanol production such as Saccharomyces cerevisiae, Saccharomyces carlsbergensis, and Schizosaccharomyces pombe are unable to metabolize D-xylose (6). Other yeasts including Candida albicans, Candida utilis, Kluyveromyces lactis, and Saccharomyces amurcae can utilize xylose but only for aerobic growth (4,6). The pathway for aerobic xylose metabolism is shown in Figure 2.2.

Wang, et al. (6) have attempted to identify why common yeasts can not ferment xylose. They showed that D-xylulose, the first intermediate in aerobic xylose metabolism, can be converted to ethanol

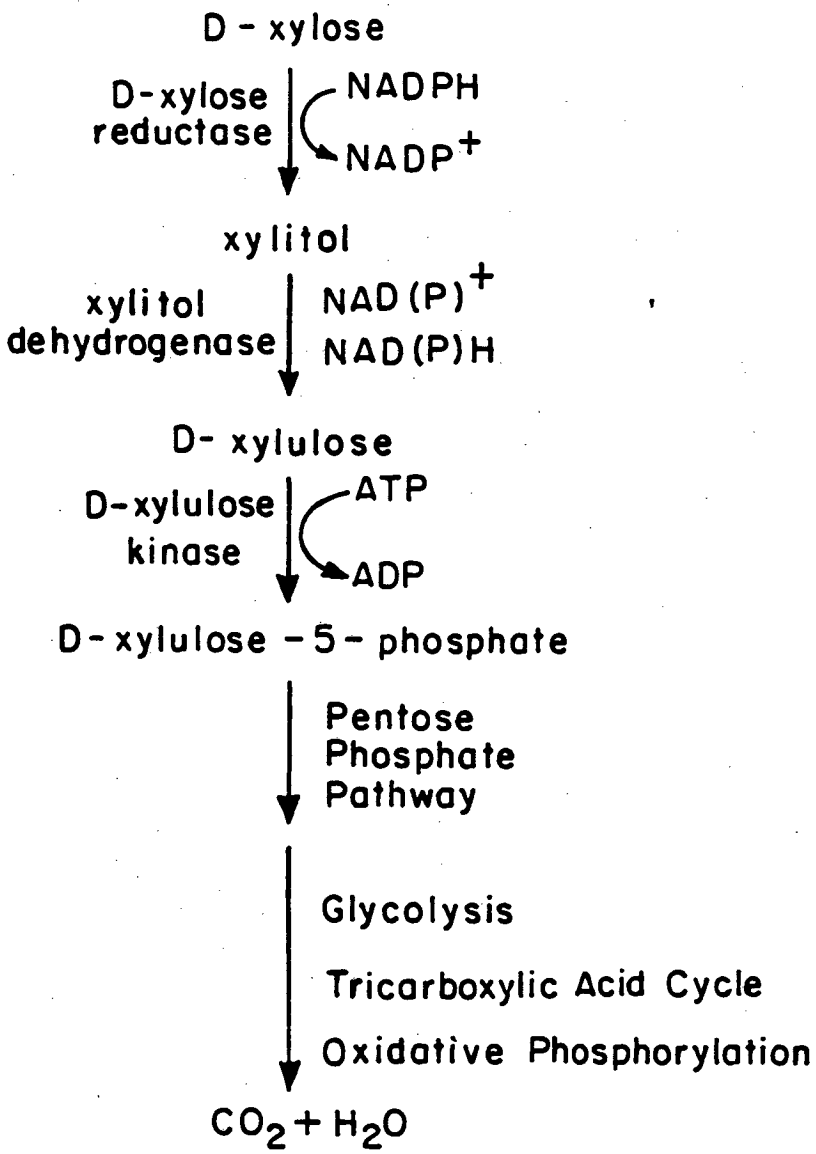

Figure 2.2. Pathway for Aerobic Xylose Metabolism in Yeast. Adapted from Flickinger (4) and Barnett (7).

by S. cervisiae, S. carlsbergensis, S. pombe, C. utilis, K. lactis and S. amurcae. This result suggests that all of the above yeasts possess both a xylulose kinase and the pentose phosphate pathway under anaerobic conditions. In addition, S. cerevisiae and S. pombe have some xylose permease activity; however, the rate of xylose uptake by these yeasts is extremely slow (7,8). Hence, the lack of xylose fermenting ability in S. cerevisiae, S. carlsbergensis and S. pombe is due to either the absence of enzymes necessary to isomerize xylose to xylulose or inadequate xylose transport.

For C. utilis, K. lactis, and S. amurcae, which grow aerobically on xylose and anaerobically on xylulose, some intracellular regulatory mechanism must prevent the conversion of xylose to xylulose in the absence of oxygen. The level of regulation for this phenomenon is not known (6).

2.2.2. Possible Alternatives for Xylose Conversion with Common Yeast

Several alternatives have been proposed for converting xylose to ethanol with the above mentioned yeasts.

Isomerization

Some researchers have suggested converting xylose to xylulose by some chemical or biological means and then fermenting the xylulose with yeast (9,10,11).

D-xylose isomerase (glucose isomerase) has been used for the conversion of xylose to xylulose. The enzyme is usually used in heat-treated, whole-cell form. Species of Bacillus, Streptomyces, and Actinoplanes have all been used as sources for this enzyme. Xylose is usually isomerized in columns packed with immobilized enzyme. Unfortunately, the conversion to xylulose is limited by the equilibrium

xylulose/xylose ratio which is only 28/72 at 70°C. The equilibrium can be shifted to favor xylulose formation by using sodium tetraborate in the sugar solution. With 0.1 M sodium tetraborate and 1M xylose initially, the equilibrium xylulose/xylose ratio is 70/30. However, 0.1 M tetraborate decreases enzyme activity by 40% and also causes a 60% reduction in the rate of the subsequent yeast xylulose fermentation (9).

Alternatively, xylose isomerase has been added directly to the yeast fermentation (10). In this way, xylulose is continuously converted to ethanol, hence equilibrium limitations are no longer a problem.

Another difficulty with this scheme is that xylulose fermentation by yeast is relatively slow (10,12). For *Saccharomyces cerevisiae*, the rate of ethanol formation from xylulose is one-third the rate from glucose. In addition, the overall yield of ethanol from xylose via xylulose is 0.3 gm/gm, which is 30% lower than yields normally obtained from glucose (9,13). Other important factors such as the stability and cost of xylose isomerase have not been investigated. A realistic assessment of the economic feasibility of this proposed process will not be possible until more information is available on the enzyme.

Genetic Manipulations

The direct conversion of xylose to ethanol may be possible with genetically altered yeasts. For yeasts that are able to metabolize xylose aerobically, it may be possible to mutate these organisms so as to eliminate regulatory mechanisms which prevent the anoxic conversion of xylose to xylulose (6). This would eliminate the need for an extracellular xylose isomerase.

It may also be possible to splice the genes required for the xylose to xylulose conversion into yeasts using recombinant DNA techniques. Research in this area is currently underway at the Solar Energy Research Institute in Golden, Colorado (14).

2.3. Organisms Capable of Fermenting Xylose to Ethanol

2.3.1. Desired Properties for Prospective Xylose Fermenters

Recently, many microorganisms capable of producing ethanol directly from xylose have been described (4,15,16,17). However, most of these organisms are not well suited for industrial ethanol production for a number of reasons. Some xylose fermenters produce by-products along with ethanol. Ideally, xylose would be fermented to only ethanol and carbon dioxide:

$$C_5H_{10}O_5 \xrightarrow{\text{cells}} 5/3\ C_2H_5OH + 5/3\ CO_2$$

Given the above stoichiometry, the maximum theoretical yield of ethanol from xylose is 0.51 gm/gm. Since some carbohydrate will necessarily be consumed in the production of microbial cells, actual yields will be slightly less.

Other proposed xylose fermenters have low tolerances of ethanol or require expensive nutrients (e.g., amino acids, nucleic acids, and vitamins). High ethanol tolerance is desirable since it allows the production of high alcohol content beer, hence reducing subsequent product recovery costs. Costly nutrients are clearly undesirable, since they are essentially raw materials for ethanol production and will have a very significant effect on production costs. Finally, some organisms capable of fermenting xylose have very slow growth rates, and

consequently, low specific ethanol productivities (i.e., low productivity per cell). Capital costs for fermentation are inversely related to specific productivity making the use of slow growing organisms undesirable.

The advantages and disadvantages of using some of the recently described xylose fermenters are discussed below.

2.3.2. Newly Identified Yeasts Capable of Semi-aerobic Xylose Fermentation

Recently, several yeasts capable of converting xylose to ethanol in the presence of oxygen have been described.

Pachysolen tannophilus can produce ethanol from xylose at a yield of 0.30 gm/gm (18,19). Batch fermentation of 50 gm/L xylose produces 15 gm/L ethanol in the presence of oxygen; much less ethanol is formed in the absence of oxygen. Neither the by-products formed nor the metabolic role of oxygen have been identified. The formation of ethanol is slow and not strongly growth associated. Average volumetric productivities of 0.16 to 0.19 gm/L-hr have been measured in batch culture (19). In comparison, brewing yeasts grown on glucose have average batch productivities of 1.8-2.5 gm/L-hr (11). One promising characteristic of *P. tannophilus* is that it is fairly ethanol tolerant. Concentrations as high as 40 gm/L have been produced (19). (Common yeast are able to produce up to 110 gm/L ethanol (13).)

To overcome the problem of low fermentation rates, high densities of *P. tannophilus* cells have been used to ferment xylose (20,21). The use of high cell densities has been shown to increase fermentation rates dramatically (13). *P. tannophilus*, immobilized in calcium alginate beads, was used at a concentration of 43 gm of cells/L

in a constant-flow stirred-tank fermentor. From a feed concentration of 50 gm/L xylose, 14 gm/L of ethanol was produced at a productivity of 1.5 gm/L-hr. This represents approximately an eight-fold improvement over simple batch fermentation.

Other advantages of using immobilized P. tannophilus are that the cells require no nutrients (other than salts) or aeration to produce ethanol. Nutrients and aeration normally used in batch fermentation are only necessary for cell reproduction (20). A major disadvantage in using immobilized cells is that they are not extremely stable. Slininger et al. (20) observed that after 6 days of continuous fermentation, the ethanol productivity began to decline. Further research is needed to improve immobilized cell stability.

Another yeast capable of producing ethanol from xylose is Candida sp. (22). A high ethanol yielding mutant of this organism, Candida sp. XF 217, has recently been isolated by Gong and co-workers (22). Oxygen has been found to enhance growth and possibly ethanol production for XF 217. Aerobic-batch fermentation of 50 gm/L xylose produces 20 gm/L of ethanol (yield = 0.40 gm/gm) at an average volumetric productivity of 0.33 gm/L hr. Both the yield and productivity are higher than the corresponding values for P. tannophilus. However, the results for XF 217 were obtained with very rich medium so an accurate comparison of the two yeasts will require additional data.

Within the last year, many other yeasts capable of converting xylose to ethanol have been described (16,23,24). However, none of these appear to be nearly as promising as P. tannophilus or Candida sp. XF 217.

2.3.3. Conversion of Xylose to Ethanol with Fungi

Many species of the genera *Fusarium* and *Mucor* are able to ferment xylose to ethanol (11,17,25,26). *Fusarium oxysporum* f.sp. *lini* has been studied extensively (11,26). This organism is capable of producing ethanol at a yield of 0.41 gm/gm from an initial xylose concentration of 10 gm/L. Ethanol concentrations as high as 15 gm/L have been produced by *F. lini*. Small amounts of complex nutrients (e.g., yeast extract) are required for growth. Like most fungi, the growth rate of this organism is very slow and cell mass increases linearly, rather than exponentially with time. The batch-volumetric productivity of ethanol is also very low; a value of 0.05 gm/L-hr has been measured (26).

Other xylose fermenting species of *Fusaria* with higher ethanol tolerances and productivities have recently been isolated (17,25). The best of these appears to be *F. oxysporum* VTT-D-80134 (17). This organism has simple nutritional requirements and can tolerate up to 6% (w/v) ethanol. A yield of 0.50 gm/gm and a batch productivity of 0.17 gm/L-hr result when of 50 gm/L xylose is fermented. The high yield and ethanol tolerance are promising characteristics of this fungus. The problem of low productivity may be at least partially overcome by the use of high cell densities; however, this awaits further investigation.

Mucor sp. 105 has properties similar to many *Fusaria*. It is slow growing, produces ethanol at a yield of 0.3 gm/gm and can produce concentrations of ethanol as high as 20 gm/L (11,25). Unlike *Fusaria*, it requires no nutrients other than mineral salts. Again, research using high cell densities is necessary before this fungus can be

realistically evaluated for use in an industrial process.

2.3.4. Xylose Fermenting Bacteria

Many bacteria are able to catabolize xylose to ethanol. Xylose fermenting species of *Aerobacter*, *Aeromonas*, *Bacillus*, *Clostridia*, *Escherichia*, *Ruminococcus*, *Thermoanaerobacter*, and others have been described (4,15,27,28,29,30). However, bacterial ethanologens generally produce significant quantities of by-products such as organic acids and higher alcohols. Hence, economical xylose conversion with bacteria will require either suppression of by-product formation via mutation (or some other means) or by-product recovery (assuming the by-products have significant value).

Aerobacter indologens, *Aeromonas hydrophilia*, and *Bacillus polymyxa* all produce 2,3-butanediol along with ethanol (4,15). Total alcohol yields (ethanol + butanediol) are aproximately 0.40 gm/gm of xylose for these bacteria. Butanediol can be used as a liquid fuel along with ethanol, however it is difficult to separate from the fermentation broth because it boils at 180°C (15). Recovery by distillation would not be practical due to the tremendous amount of energy required to remove all the water from a dilute butanediol solution. Hence, these bacteria will have little, if any, industrial applications in fuel production until an acceptable butanediol recovery scheme is developed.

Xylose conversion with *Bacillus macerans* has been studied extensively (31,32). In batch fermentation, ethanol is produced at a yield of 0.27 gm/gm along with acetate 0.22 gm/gm and acetone 0.01 gm/gm (31). Unfortunately, *B. macerans* is strongly inhibited by ethanol; the rate of growth is 50% inhibited at only 3 gm/L. In addition, continuous

culture studies have revealed that growth on xylose by B. macerans is unstable, probably due to poor metabolic regulation (32). Low ethanol tolerance and unstable behavior make this bacterium industrially impractical.

Researchers at the University of Georgia at Athens have isolated an ethanol producing bacterium, Thermoanaerobacter ethanolicus, from hot springs in Yellowstone National Park (28). T. ethanolicus, an obligate anaerobe and an extreme thermophile, has an optimum growth temperature of 69°C. Thermophilic bacteria are particularly well suited for continuous ethanol production in vacuum fermentation processes. In vacuum fermentation, ethanol is removed from the fermentation broth continuously by boiling under reduced pressure, at metabolically tolerable temperatures. Since ethanol is generally a growth inhibitor, continuous, selective ethanol removal by boiling allows increased rates of fermentation. Pressures as low as 50 mmHg are used in vacuum fermentation with yeast such as S. cerevisiae which is grown at 35°C (13). At 69°C, the vapor pressure of the growth medium is much higher (approximately 220 mmHg) than at 35°C; hence, capital and energy costs for subsequent product vapor recompression are greatly reduced (33). Vacuum fermentation will be discussed further in Chapter VI.

When grown on glucose, the specific growth rate (0.3 hr^{-1}) and ethanol yield (0.45 gm/gm) for T. ethanolicus are comparable to the corresponding values for brewing yeasts (28,34). Xylose is fermented by T. ethanolicus; however, quantitative results obtained with this substrate have not been published.

A major limitation in using this organism is that it does not produce ethanol at concentrations greater than 4 gm/L, despite the fact

that it can tolerate up to 60 gm/L ethanol. Apparently, some form of feedback regulation exists in T. ethanolicus that prevents the formation of high alcohol concentrations. Attempts to overcome this regulation via mutation have been successful. Mutants capable of producing 18 gm/L ethanol have been isolated. Further improvements may be possible (35). Data for xylose metabolism are obviously necessary before the potential for this bacterium in practical applications can be determined.

Clostridium thermosaccharolyticum is another thermophilic anaerobe capable of converting xylose to ethanol. Extensive research with C. thermosaccharolyticum has been conducted at the Massachusetts Institute of Technology (29,36). A typical profile for batch gowth of strain HG-2 is shown in Figure 2.3. The maximum specific growth rate and yield of ethanol from xylose are 0.25 hr^{-1} and 0.18 gm/gm, respectively. Lactate (not shown) and acetate are also produced as by-products. These results were obtained with medium containing 15 gm/L xylose and 5 gm/L yeast extract.

Ethanol inhibition studies have demonstrated that cell growth is slightly inhibited by 10 gm/L ethanol and 50% inhibited by 20 gm/L. (29). Clearly, both the ethanol yield and tolerance of strain HG-2 are unacceptably low.

To overcome these problems, Wang et al. (29) adapted C. thermosaccharolyticum HG-2 to high ethanol concentrations, which resulted in ethanol tolerant strain HG-3. A low acid producing mutant, HG-4, was isolated from a nitrosoguanidine-treated culture of HG-3. HG-4 was further adapted to higher alcohol concentrations until it grew in up to 48 gm/L ethanol. Ethanol tolerant HG-4 (renamed HG-6) is able to produce 39 gm/L of ethanol at a yield of 0.40 gm/gm, in a fed-batch

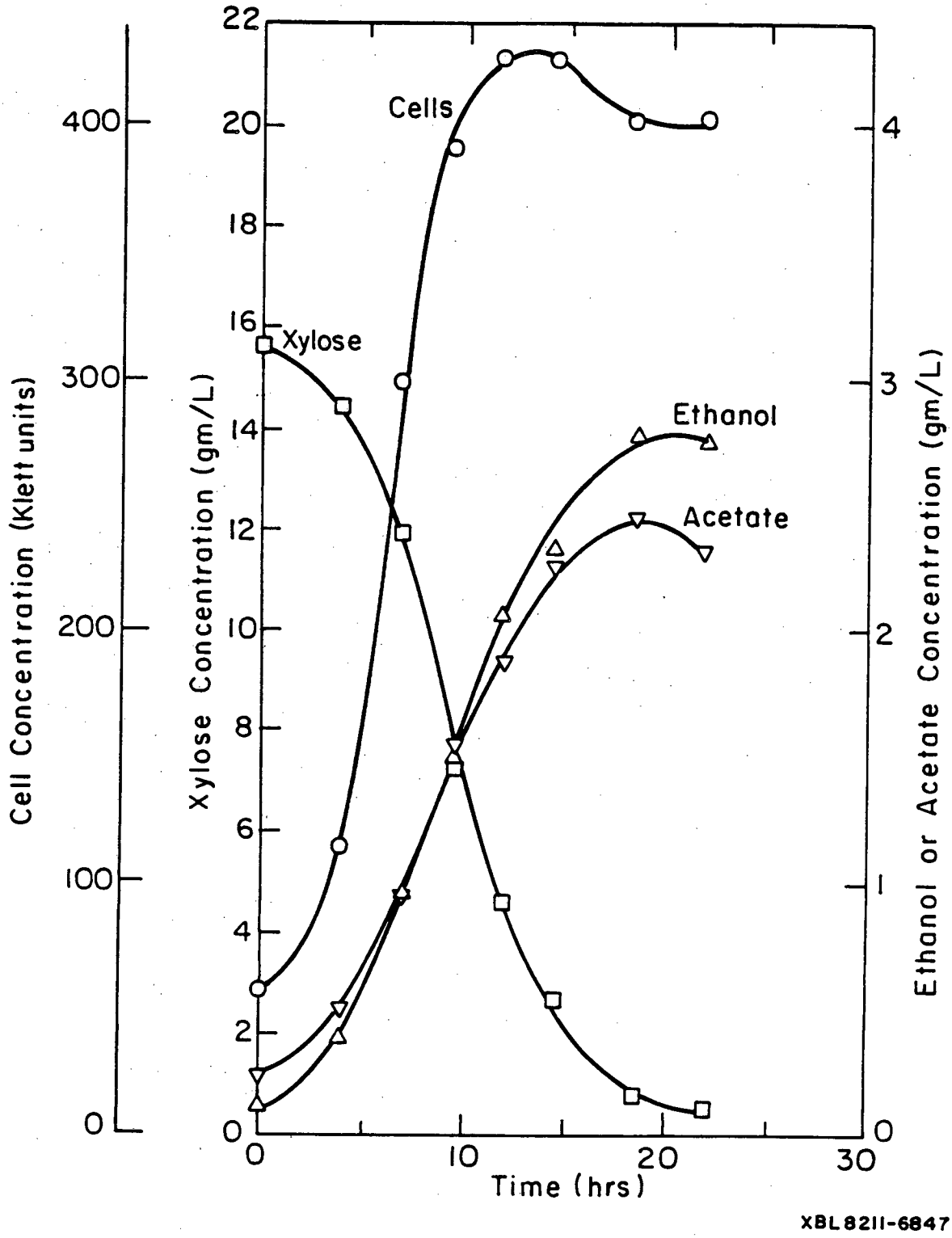

Figure 2.3. Batch Fermentation Profile for Clostridium thermosaccharolyticum strain HG-2. Adapted from Wang et al. (29).

xylose fermentation (see Figure 2.4). The average volumetric productivity for HG-6 in batch culture is 0.78 gm/L-hr. Further mutagenesis and adaptation has produced strain HG-8, which can produce 65 gm/L ethanol at a yield of 0.37 gm/gm (37).

In most laboratory studies done with C. thermosaccharolyticum, high concentrations of yeast extract, which would be economically impractical for industrial ethanol production, have been used. This prompted the researchers at M.I.T. to quantify the nutritional requirements of this bacterium. Biotin, nicotinic acid, riboflavin, methionine, and histidine have been determined to be essential for rapid growth and a high ethanol yield (38). However, their nutritional studies were done exclusively with an initial xylose concentration of only 15 gm/L. At higher substrate concentrations and consequently higher alcohol concentrations, the inhibitory effects of ethanol may alter the nutritional requirements of C. thermosaccharolyticum (39). Further work is needed to determine the requirements of C. thermosaccharolyticum in the presence of high ethanol concentrations and to find an economical source of the required nutrients.

In general, because of its high tolerance, high yield, and high productivity, C. thermosaccharolyticum HG-8 appears to be the best organism presently available for fermenting xylose.

Finally, Clostridium thermohydrosulfuricum is also a thermophilic, strictly anaerobic, xylose fermenting ethanologen (40). Strain 39E, isolated by J.G. Zeikus and co-workers, has the highest yield of ethanol from glucose (0.48 gm/gm) of any thermophile yet described (41). Growth rates as high as 0.55 hr^{-1} on glucose have been reported. This organism ferments xylose, however, very few results for growth on this

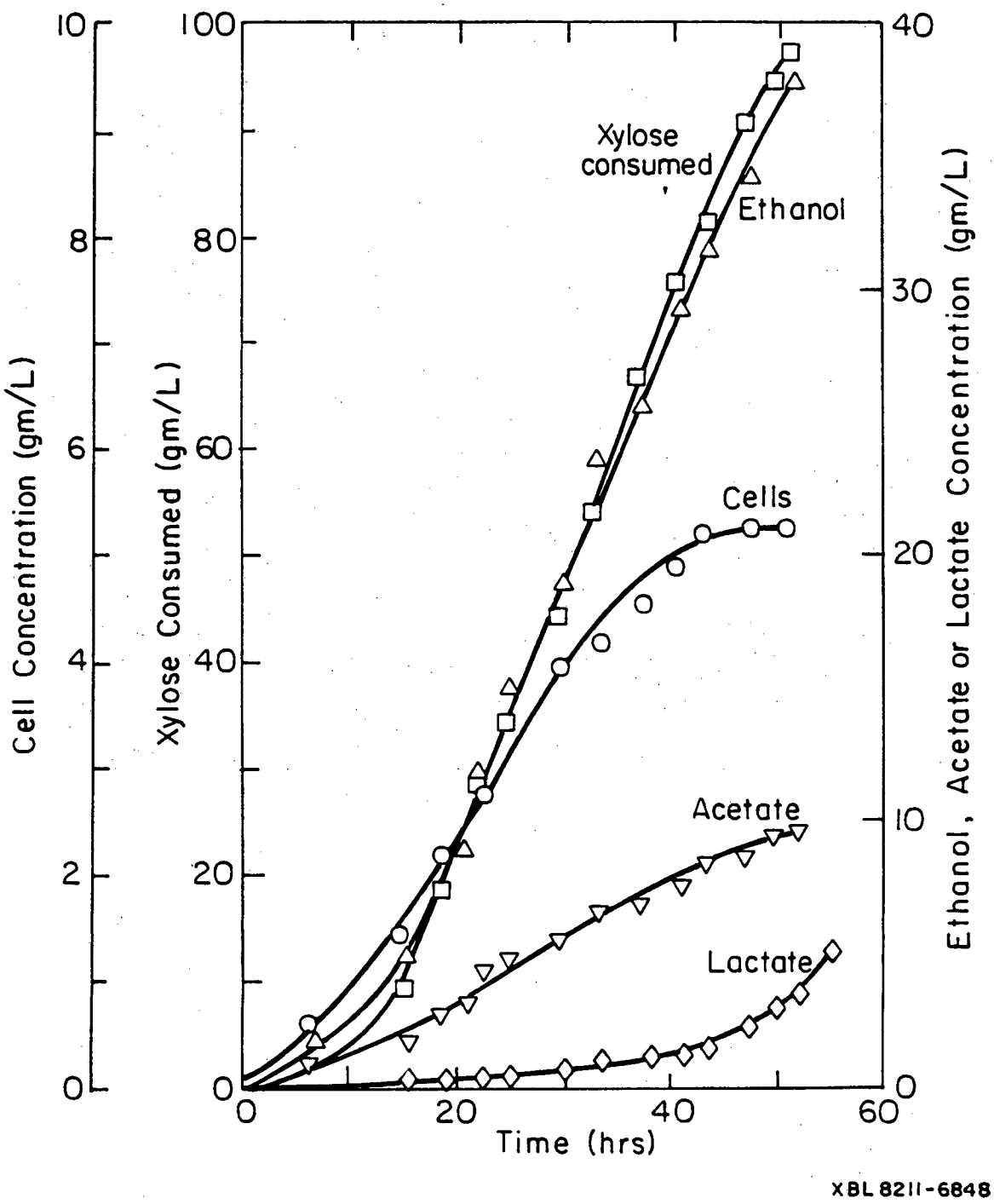

Figure 2.4. Fed-Batch Fermentation Profile for Clostridium thermosaccharolyticum strain HG-4. Adapted from Wang et al. (29).

substrate have been published. The yield of ethanol from xylose is 0.4 gm/gm (42).

Zeikus et al. (42) have stated that ethanol concentrations in excess of 1% are not produced. However, they did not extensively study the inhibitory effects of ethanol on this organism.

In general, little work has been done to determine the economic feasibility of fermenting xylose to ethanol with C. thermohydrosulfuricum strain 39E. In addition, because 39E has a higher growth rate and yield than C. thermosaccharolyticum HG-2 (the parent of high ethanol yielding and tolerant HG-8), the potential for improved strains from 39E should be very high. For these reasons C. thermohydrosulfuricum 39E was chosen for this study.

III. PREVIOUS WORK WITH *CLOSTRIDIUM THERMOHYDROSULFURICUM*

3.1. Species History

Clostridium thermohydrosulfuricum was originally isolated from extraction juices in an Austrian beet factory by Parrkinen and Klausshofer in 1964 (40). They described the organism as a strict anaerobe capable of: degrading saccharides, producing hydrogen sulfide gas, and growing at temperatures in excess of 75°C. They subsequently designated the organism as a new species and proposed the name *C. thermohydrosulfuricum* (43). However, they did not provide a complete description for the new species nor did they maintain the original culture (40).

In 1972, Hollaus and Sleytr examined 13 new strains of *C. thermohydrosulfuricum* and provided a full taxonomic description for this species (40). Their description, along with a later report on its DNA composition (44), clearly distinguished *C. themohydrosulfuricum* from similar bacteria, *C. thermosaccharolyticum* and *C. tartarivorum*.

Most of the characteristics of *C. thermohydrosulfuricum* listed by Hollaus and Sleytr were morphological, although some of the more important physiological properties were also included. Cells are peritrichously-flagellated, gram-variable rods, capable of forming endospores. A double outer cell wall, revealed by electron microscopy, accounts for the cells gram-variable nature. *C. thermohydrosulfuricum* grows at temperatures ranging from 37 to 76°C and can metabolize a wide variety of carbohydrates including xylose. This bacterium produces hydrogen sulfide gas in small amounts from peptone, tryptone, and yeast extract and in large amounts from sulfite or thiosulfate. Hydrogen

sulfide formation will be discussed in more detail in section 3.3.3.

3.2. General Physiology

Ljungdahl et al. (45) have studied the physiological characteristics of thirteen strains of *C. thermohydrosulfuricum* isolated from thermophilic (e.g., hot springs) and mesophilic environments. Strain JW102, which was studied extensively, has an optimum growth temperature of 69°C. At 80°C vegetative cells are killed rapidly. Spores are much more heat resistant; they can survive in boiling water for 20 hours. Spore formation can be induced by slow cooling of growing cells.

The optimum pH for growth ranges from 6.9 to 7.5 for strain JW102; however, cells can be grown at pH's ranging from 5.0 to 9.2. The maximum specific growth rate in medium containing 5 gm/L glucose and 2 gm/L yeast extract at optimal pH and temperature is 0.6 hr^{-1}. Reducing the initial yeast exract concentration from 2.0 to 0.5 gm/L has no significant effect on the initial growth rate but it does decrease the final cell yield from glucose.

Many hexoses and pentoses, including xylose, support the growth of all strains tested by Ljungdahl and co-workers (45). Fermentation products from glucose include ethanol, L-lactate, acetate, hydrogen gas and carbon dioxide. Typical ethanol yields are approximately 0.25 gm/gm glucose, although yields as high as 0.33 gm/gm have been reported. Growth at a low (controlled) pH does not favor ethanol production over acid production. The ethanol yield is highest (0.33 gm/gm) when the starting pH is 7.5 and is allowed to fall (due to acid formation) to 6.8.

Total inhibition of growth occurs in the presence of any oxygen in the growth medium. However, cells exposed to oxygen resume growing

when they are returned to a strictly anaerobic environment. The effects of oxygen on cell viability have not been quantified.

3.3. Properties of Strain 39E

3.3.1. Growth Characteristics

C. thermohydrosulfuricum strain 39E, which was isolated from a hot spring in Yellowstone National Park by J.G. Zeikus (46) (University of Wisconsin) was chosen for this work (see section 2.3.4). This bacterium grows at temperatures ranging from 40 to 70°C. The optimum temperature for growth is 65°C. The maximum specific growth rate of strain 39E is 0.55 hr^{-1} in medium containing 5 gm/L glucose, 3 gm/L yeast extract, and 10 gm/L tryptone (46). A typical batch-growth curve is shown in Figure 3.1. Note that the growth rate drops slightly (from 0.55 to 0.34 hr^{-1}) after 3.5 hours of growth. Zeikus and co-workers gave no explanation for the decrease in the growth rate but it may have been due to the depletion of some limiting nutrient in the growth medium.

Strain 39E produces ethanol, L-lactate, acetate, hydrogen and carbon dioxide from various carbohydrates including: cellobiose, glucose, pyruvate, sucrose, starch, xylobiose, and xylose (42,47). Hence in addition to being used as a xylose fermenter, this organism potentially has a wide range of applications. Carbon sources which do not support growth are: cellodextrins, cellulose, xylan, and mannan. The distribution of fermentation products formed from cellobiose, glucose, and xylose (as growth limiting substrates) is shown in Table 3.1. As can be seen from the table, the principal product formed is ethanol. From an initial concentration of 5 gm/L xylose, 1.95 gm/L ethanol is produced (yield = 0.4 gm/gm).

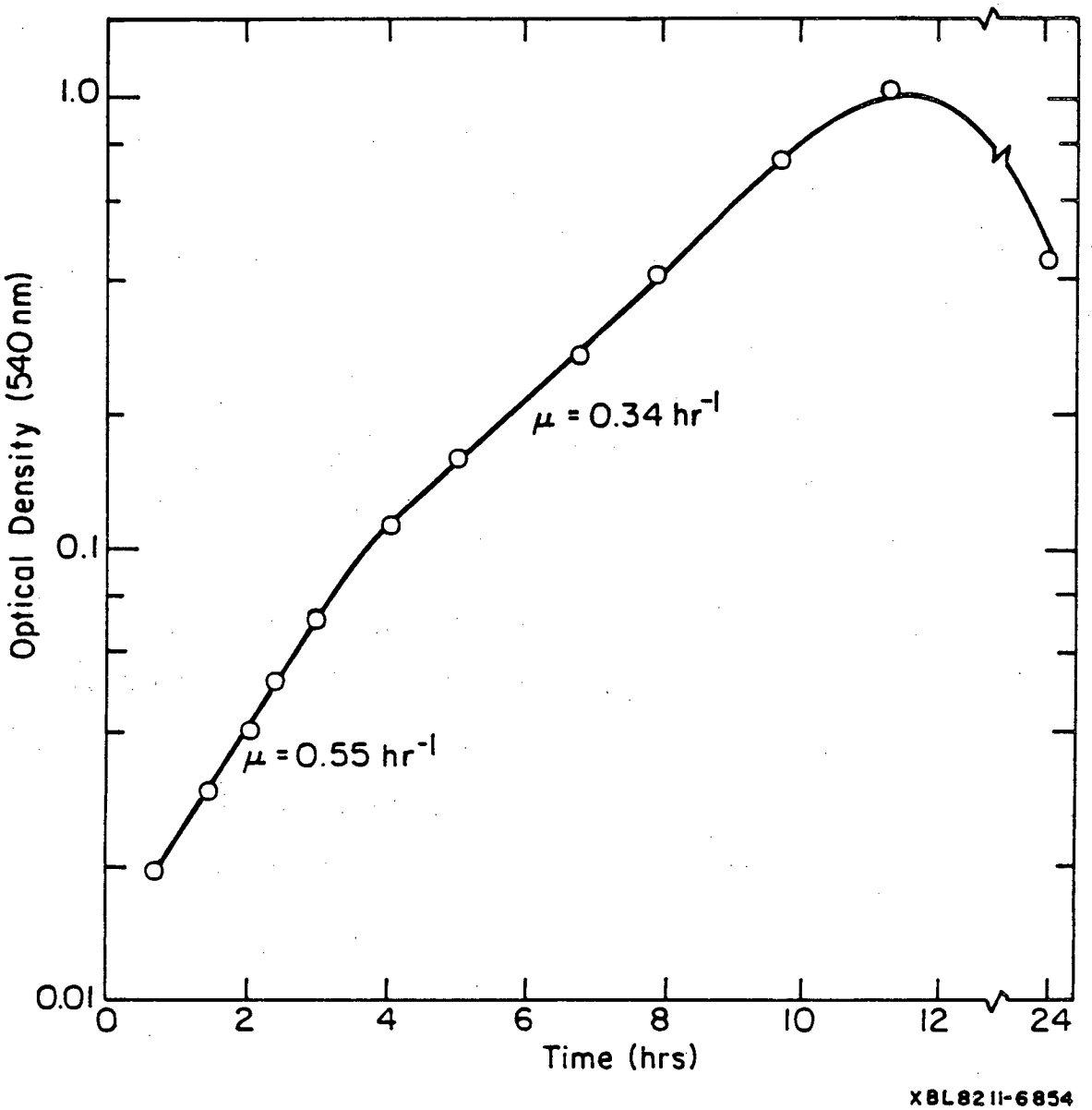

Figure 3.1. Batch Growth Curve for Clostridium thermohydrosulfuricum strain 39E. Cells were grown on glucose without agitation.
Adapted from Zeikus et al. (46).

Table 3.1

Fermentation Products from Cellobiose, Glucose, and Xylose for *C. thermohydrosulfuricum*

	Concentrations (mM)		
	Cellobiose	Glucose	Xylose
Ethanol	48.2	54.9	42.4
Acetate	6.7	3.1	6.0
L-lactate	5.1	5.0	4.4
CO_2	55.2	58.0	47.0
H_2	6.4	3.1	5.0

Results were obtained with medium containing 0.5% substrate, 0.3% yeast extract, 1.0% tryptone. Cultures were grown at 65°C without pH control in phosphate-buffered medium. Adapted from Zeikus, et al. (42).

Zeikus et al. (42) also noted that C. thermohydrosulfuricum is unable to produce high concentrations of ethanol. Ethanol concentrations in fermentation broths of strain 39E do not exceed 1%. The exact reason for this limitation is not known (47).

3.3.2. Metabolic Pathway of C. thermohydrosulfuricum 39E

Researchers at the University of Wisconsin have elucidated most of the catabolic pathways in strain 39E (41,47,48). Key intermediates and enzymes present in the xylose fermentation pathways are shown in Figure 3.2.

Xylose is converted to xylulose which is phosphorylated to xylulose-5-phosphate. The enzymes transaldolase and transketolase of the pentose phosphate pathway convert xylulose-5-phosphate to fructose-1,6-diphosphate and glyceraldehyde-3-phosphate. These two intermediates feed into the Embden-Meyerhof-Parnas pathway and are converted to pyruvate. 1.2 moles of xylose are consumed for every 2 moles of pyruvate produced. Along with each mole of pyruvate, a net of one mole of adenosine triphosphate (ATP) and one mole of reduced nicotinamide adenine dinucleotide (NADH) are formed.

Pyruvate can be reduced by NADH to lactate via lactate dehydrogenase. Alternatively, pyruvate can be oxidatively decarboxylated to acetyl-CoA (via pyruvate dehydrogenase) with the concomitant formation of reduced ferredoxin. Reduced ferredoxin is used to form either hydrogen gas from hydronium ions or additional reduced nucleotides (NADH and NADPH).

Acetyl-CoA can be phosphorylated to acetyl-phosphate and subsequently converted to acetate with acetate kinase. The formation of acetate from acetyl-CoA is advantageous for the cell since additional

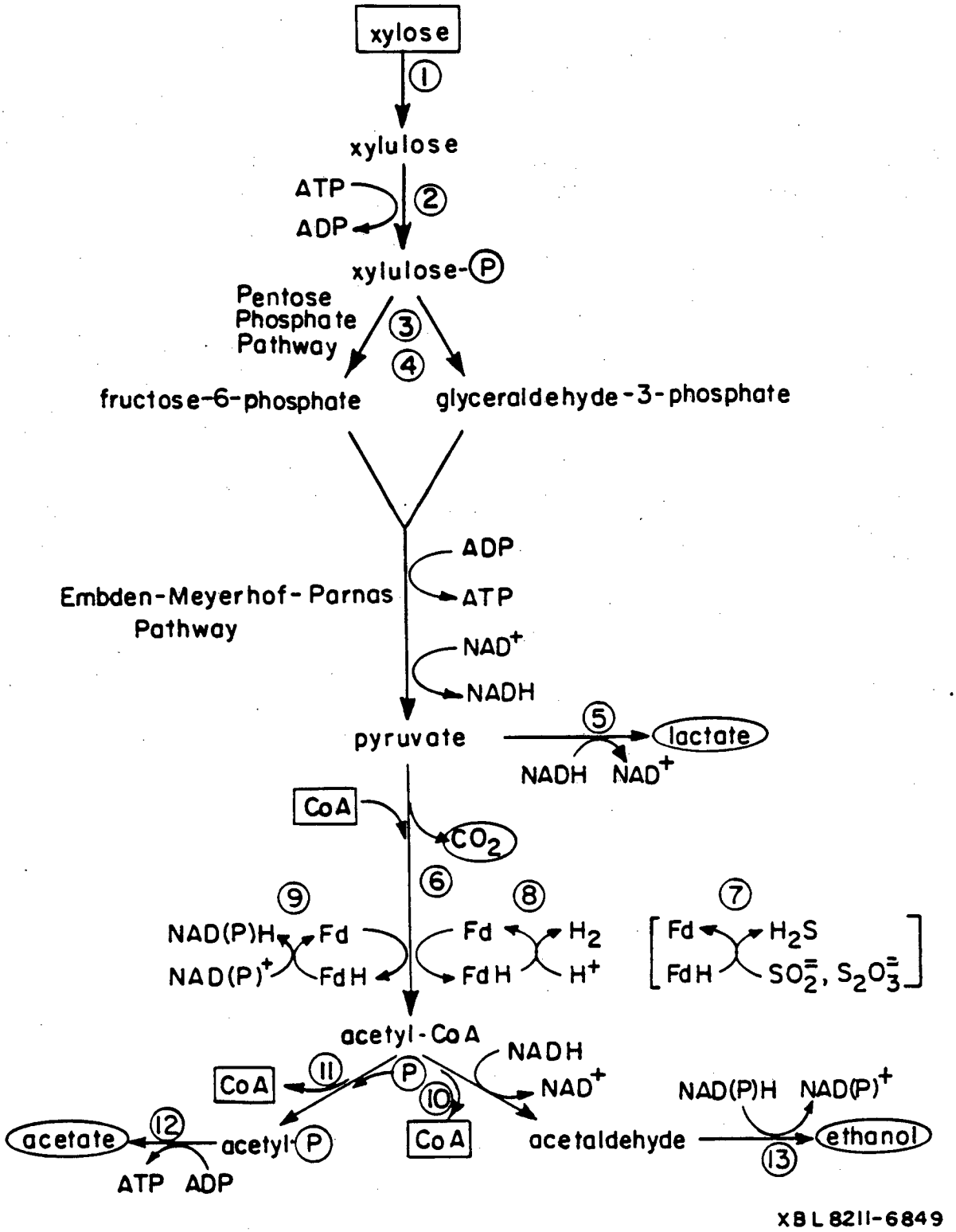

Figure 3.2. Xylose Fermentation Pathways in Clostridium thermohydrosulfuricum.
Enzymes in pathways are listed in Table 3.2.
Adapted from Zeikus et al. (41,47,48).

Table 3.2

Enzymes for Xylose Fermentation Pathways of

C. thermohydrosulfuricum strain 39E.

1. xylose isomerase
2. xylulose kinase
3. transketolase
4. transaldolase
5. lactate dehydrogenase
6. pyruvate dehydrogenase
7. bisulfate reductase, thiosulfate reductase
8. hydrogenase
9. ferredoxin NAD(P) oxidoreductase
10. acetaldehyde dehydrogenase
11. phosphotransacetylase
12. acetate kinase
13. alcohol dehydrogenase

ATP is produced; hence increasing the amount of energy available for biosynthesis. Alternatively, acetyl-CoA can be reduced with NADH to acetaldehyde and further reduced with NAD(P)H to ethanol. Both NADH and NADPH-linked alcohol dehydrogenases have been detected in cell extracts of C. thermohydrosulfuricum.

3.3.3. Metabolic Control and End-Product Formation

The distribution of end products formed by C. thermohydrosulfuricum is determined by intracellular regulatory mechanisms that are strongly affected by the growth environment.

Lactate dehydrogenase is an allosteric enzyme which requires fructose-1,6-diphosphate (FDP) as an activator (48). The rate of lactate formation by this organism depends on the intracellular FDP concentration. To illustrate the importance of FDP in regulating lactate production, Zeikus et al. (47) grew C. thermohydrosulfuricum in continuous culture under carbon and nitrogen-limited conditions. Figure 3.3 shows the relative amounts of lactate, acetate, and ethanol produced at various dilution rates. Under carbon-limited conditions, the lactate yield is low at all dilution rates and ethanol is the predominant end product. Because nitrogen sources (yeast extract and tryptone) are present in large excess, xylose is rapidly metabolized. The concentration of intracellular FDP is low whereby little lactate is produced. When nitrogen is limiting, excess xylose is available which leads to a build-up of FDP. This effect is pronounced at high dilution rates (greater than 0.1 hr^{-1}) where very high levels of intracellular FDP result in high lactate yields. These results indicate that for rapid industrial ethanol production, which necessitates the use of high dilution rates, xylose must be maintained as the limiting nutrient.

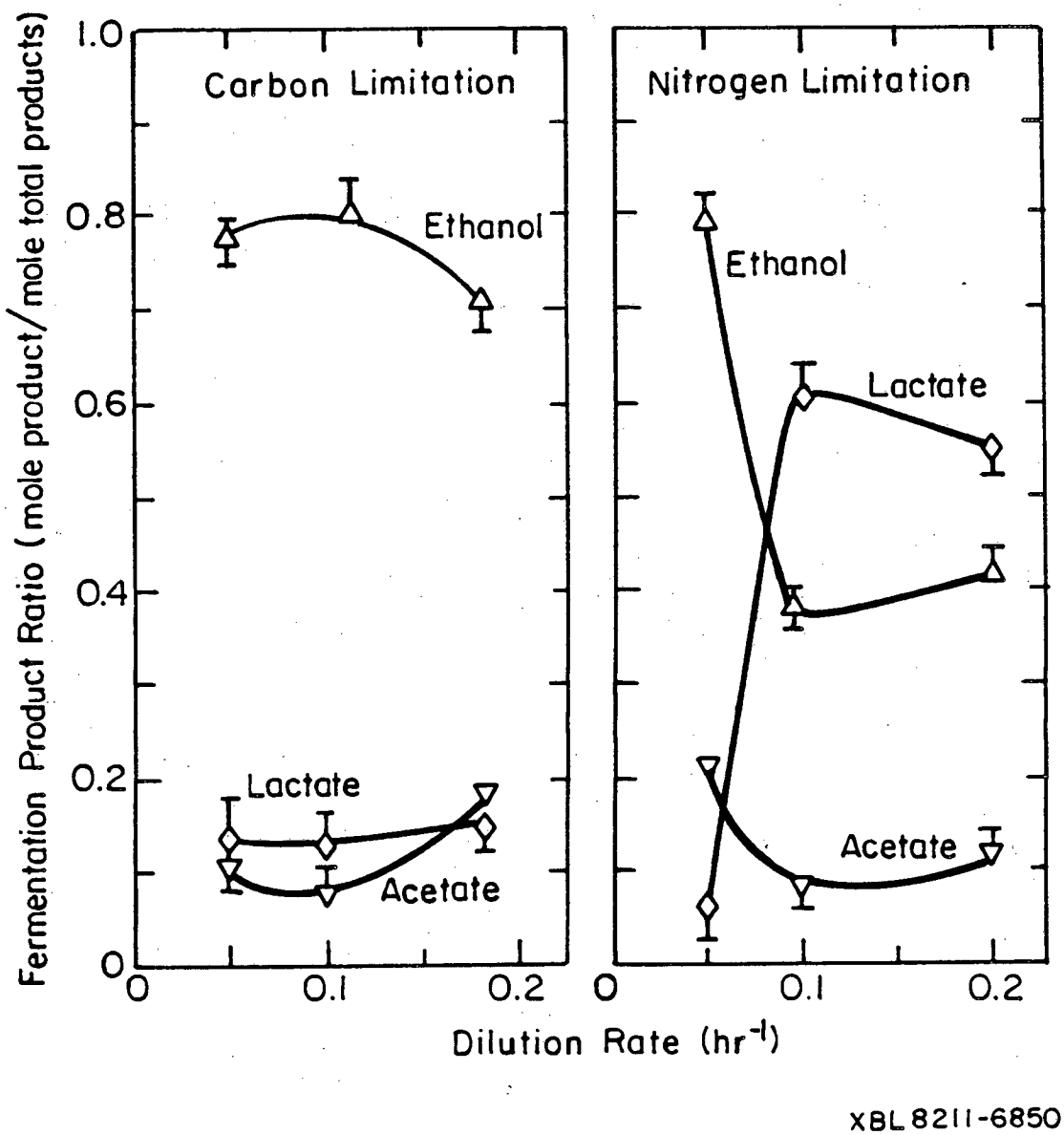

Figure 3.3. Growth of Clostridium thermohydrosulfuricum strain 39E Under Carbon and Nitrogen-Limiting Conditions. Adapted from Zeikus et al. (47).

As stated in section 3.3.2, C. thermohydrosulfuricum 39E possesses both NAD and NADP-linked alcohol dehydrogenases. The NADP-linked enzyme has been studied extensively by Zeikus et al. (49). The activity of this enzyme is reversible whereby ethanol can be metabolized by 39E (50). A wide range of substrates can be reduced by this enzyme including many aldehydes and ketones. The enzyme is also very solvent stable; it is active at 2-propanol concentrations as high as 45% v/v. In addition, at alcohol concentrations of 100 to 150 mM no inhibition of activity was observed. 150 mM ethanol corresponds to 0.8% v/v ethanol. Given these properties for alcohol dehydrogenase and the fact that growth is inhibited by less than 1% ethanol, it appears that ethanol inhibition in this bacterium is not the result of inactivation of this enzyme. A proposed mechanism for ethanol inhibition will be given in Chapter V.

Zeikus et al. (48) have also studied the regulation of reducing-electron flow in C. thermohydrosulfuricum. Electron donors and acceptors added to the growth medium have been found to alter the distribution of end products formed. Both sulfite and thiosulfate can act as electron acceptors in the medium. They are reduced to hydrogen sulfide gas via bisulfate reductase and thiosulfate reductase (refer to Figure 3.2). A corresponding decrease in ethanol, hydrogen, and lactate yields are observed when electron acceptors are added to the growth medium. Apparently, electrons are diverted from NAD(P)H (which would have been used in the formation of ethanol, hydrogen and lactate) and are used to reduce sulfite and thiosulfate.

Electron donors (e.g. hydrogen) have the opposite effect. High partial pressures of hydrogen (0.5 atm) enhance the formation of reduced

end products (ethanol and lactate). Electrons from exogenous hydrogen are transferred to ferredoxin via reversible hydrogenase. These electrons are transferred from ferredoxin to NAD(P) and are used to reduce acetaldehyde and pyruvate to ethanol and lactate, respectively. However, high partial pressures of hydrogen also inhibit growth since nucleotides which would normally be used in glycolysis are tied up in the formation of excess reduced end products. Conversely, the rate of growth is increased by the addition of electron acceptors, thiosulfate and sulfite, which rapidly regenerate reduced nucleotiedes.

These findings may be useful in the design of an industrial ethanol process. Electron acceptors should be excluded from the fermentation broth to maintain a high yield of ethanol. The use of sulfur dioxide, which has been proposed for pretreating various forms of biomass, probably would not be well suited for processes using C. thermohydrosulfuricum. Dissolved SO_2 ($SO_3^{=}$) would be reduced to H_2S thus lowering the ethanol yield (48). In addition, the use of slow fermentor agitation rates may be advantageous. With slow agitation, the fermentation broth becomes super saturated with hydrogen gas produced by the bacterium (51). The high dissolved-hydrogen concentration should supress H_2 formation in favor of ethanol production (48).

In summary, maintaining xylose as the limiting substrate, excluding electron acceptors from the fermentation broth, and using slow agitation rates should enhance the yield of ethanol.

IV. MATERIALS AND METHODS

4.1. Growth Procedures

4.1.1. Organism and Culture Media

Clostridium thermohydrosulfuricum strain 39E was obtained from the American Type Culture Collection (ATCC 33223). Cultures were maintained in soft agar as suggested by Hollaus and Sleytr (40). The composition of the agar medium is given in Table 4.1. All medium components (except xylose) were combined and sterilized at 121°C for 30 minutes. After the sterile medium was cooled to 65°C, 2 ml of a filter sterilized xylose syrup (500 gm xylose/L) were added to the medium. Xylose was not autoclaved with the rest of the medium components to prevent caramelization. The molten agar medium was dispensed in 8 ml portions to sterile 125 x 16 mm screw-cap culture tubes (VWR Scientific; Honolulu, HI). A few drops of the liquid suspension culture from ATCC were used to inoculate the molten agar. The tubes were sealed with plastic screw caps, inverted to disperse the inoculum, and cooled to allow the agar to solidify. No special precautions to exclude oxygen from the growth environment were necessary. The reducing environment created by the tryptone in the hardened agar was adequate for growth. Cultures were incubated for 2 to 3 days at 65°C. Growth was apparent when the medium blackened due to the reaction of H_2S produced by the organism with $FeSO_4$ included in the medium (which formed FeS). After most of the agar had turned black, the cultures were stored at 4°C. Stock cultures were subcultured monthly; a core of soft agar, obtained by inserting a sterile Pasteur pipette into a stock culture, was used to inoculate fresh molten agar.

Alternatively, cells could be stored for up to one year at -80°C

Table 4.1

Composition of Soft Agar Medium

Component	Amount Per Liter of Water
Tryptone	10 gm
Xylose	1 gm
$FeSO_4 \cdot 7H_2O$	200 mg
$Na_2S_2O_3 \cdot 5H_2O$	80 mg
Na_2SO_3	200 mg
Agar	7 gm

in suspension culture. Cells grown to mid-exponential phase in pre-reduced liquid medium were mixed aerobically with sterile glycerol (50% medium with cells, 50% glycerol). Glycerol treated cells were frozen immediately at -80°C.

4.1.2. Medium Composition

The growth medium used in most experiments was similar to that suggested by Ljungdahl (45,52). The composition of the medium is given in Tables 4.2A to 4.2C. Any changes in composition made for specific experiments are clearly indicated in the discussion of results (see chapter 5).

The medium was prepared by adding yeast extract, phosphate buffer salts, ammonium chloride, and resazurin (a redox indicator) to 950 ml. This solution was sterilized at 121°C for 30 minutes.

The high phosphate concentration, 100 m*M*, was necessary to prevent the pH of the growth medium from dropping significantly during batch growth experiments conducted without pH control. In most experiments, this level of buffering was sufficient to keep the pH above 6.0. With lower concentrations of buffer, acid produced by the organism reduced the pH to less than 5, resulting in the cessation of growth. In experiments where pH was controlled by base addition, 11 m*M* phosphate was used.

Trace mineral and vitamin solutions were added after the medium had cooled to room temperature. Xylose was also added after autoclaving as a concentrated (500 gm/L) syrup. Trace mineral solution was prepared by mixing 1.5 gm of nitrilotriacetic acid (NTA) in a liter of water. The pH of the solution was adjusted to 6.5 with 14 *N* potassium hydroxide to allow the NTA to dissolve. The use of NTA (a chelating agent) was

Table 4.2A

YEX1 Medium

Component	Amount Per Liter
Xylose	10 gm
Yeast Extract	2 gm
$Na_2HPO_4 \cdot 7H_2O$	20 gm
KH_2PO_4	4 gm
NH_4Cl	500 mg
Resazurin	2 mg
Vitamin Solution*	5 ml
Mineral Solution**	10 ml
Cysteine·HCl · H_2O	400 mg

Initial pH = 7.2. All medium components were reagent grade. Yeast extract was obtained from Difco Laboratories, Detroit, Michigan.

*see Table 4.2B

**see Table 4.2C

Table 4.2B

Vitamin Solution (53)

Component	Milligrams Per Liter
Biotin	2
Folic Acid	2
Pyridoxine Hydrochloride	10
Riboflavin	5
Thiamine	5
Nicotinic Acid	5
Pantothenic Acid	5
Vitamin B_{12}	1
P-aminobenzoic Acid	5
Lipoic Acid	5

Table 4.2C

Mineral Solution (52)

Component	Amount Per Liter
Nitrilotriacetic Acid	1.5 gm
$MgSO_4 \cdot 7H_2O$	3.0 gm
NaCl	1.0 gm
$FeSO_4 \cdot 7H_2O$	100 mg
$Co(NO_3)_2 \cdot 6H_2O$	100 mg
$ZnSO_4 \cdot 7H_2O$	100 mg
$CuSO_4 \cdot 5H_2O$	10 mg
$Al_2(SO_4)_3$	10 mg
H_3BO_4	10 mg
$Na_2MoO_4 \cdot 2H_2O$	10 mg
Na_2SeO_3	10 mg

necessary to prevent precipitation of the mineral solution. Each salt was added in the order given in Table 4.2C and was allowed to dissolve before the next salt was added. Careless preparation of the mineral solution lead to precipitation of some salts.

The vitamin solution was prepared by adding the components listed in Table 4.2B to a liter of water. Lipoic acid, the last component added, required up to two hours to dissolve. Both the mineral and the vitamin solutions were filter sterilized.

4.1.3. Medium Reduction

Growth of strictly anaerobic bacteria is possible only in the absence of oxygen and all other oxidizing compounds (54). Hence, before the growth medium can be inoculated, it must be reduced to a low redox potential. Many techniques for culturing strictly anaerobic bacteria have been described (54-62). The technique used in this study is a modification of that suggested by Hungate (54).

Growth in Culture Tubes

For all experiments where the time course of substrate utilizaton and end-product formation were not followed, the organism was grown in 125 x 16 mm screw-cap culture tubes. These tubes were used since they could be directly inserted into a spectrophotometer to allow optical density measurements without sampling (see section 4.2.1). All experiments employing culture tubes were performed in duplicate.

Culture tubes were aseptically filled with 8 ml of medium and sealed with #14 serum caps (Bittner Corporation, Norcross, GA.). Oxygen was removed from the air space by flushing the tubes with a minimum of 20 volumes of oxygen-free (less than 1.5 ppm O_2) nitrogen (Pacific Oxygen Co., Oakland, CA). Nitrogen entered and exited the tubes through

20-gauge needles that easily pierced the serum caps. The nitrogen line was butyl rubber tubing, which is less permeable to oxygen than any other autoclavable, flexible tubing. A 0.5-ml glass syringe barrel, with the flange removed, was used to connect the rubber nitrogen line to the needle. To remove potential contaminants from the nitrogen, it was passed through a glass wool filter before it entered the culture tubes.

After nitrogen gasing, the tubes were heated in a boiling water bath to remove dissolved oxygen from the liquid and to reduce oxidized compounds present in yeast extract. After the color of the redox indicator changed from pink to colorless (approximately 5 minutes) the tubes were removed from the boiling water bath and flushed with nitrogen as they cooled to room temperature.

After boiling, the redox potential of the medium may be sufficiently low to allow the growth of strictly anaerobic microorganisms (63). However, introduction of traces of oxygen, perhaps during inoculation, would dramatically increase the potential of the medium. Hence, it is necessary to "poise" the medium with a strongly reducing substance (e.g. cysteine or sodium sulfide) that will scavenge traces of oxygen entering the system (64).

Using a gastight syringe fitted with a 20-gauge needle, 0.1 ml of an anoxically prepared reducing agent (either 32 gm/L cysteine hydrochloride monohydrate or 8 gm/L sodium sulfide) were added to each culture tube. A 1-ml glass syringe sealed with silicon grease was used as a gastight syringe. The syringe was thoroughly flushed with sterile nitrogen immediately before use. Any exposure of the reducing agent to oxygen decreased its reductive capacity. In addition, oxidized reducing agents (either cystine or sodium sulfite) have adverse effects; high

concentrations of cystine are inhibitory to growth for most microorganisms (62) and sulfite tends to lower ethanol yields (see section 3.3.3).

Reducing agent solutions were prepared by adding the reducing compound to boiling water. (Boiling water contains essentially no dissolved oxygen.) The hot solution was immediately filter sterilized into a culture tube which was subsequently sealed with a serum cap. The air space in the tube was flushed with 20 volumes of nitrogen while the solution cooled.

In experiments where ethanol was included in the medium, oxygen free ethanol was added to tubes containing pre-reduced medium. Oxygen was removed from ethanol by boiling under vigorous nitrogen gasing.

All anoxic solutions not used immediately after preparation were stored in an anaerobic glove box (see Appendix I).

Growth in Shake Flasks

In experiments where multiple samples were withdrawn to follow the time course of end-product formation (without pH control), cells were grown in 250-ml side arm flasks (Bellco Glass Inc., Vineland, NJ). Side arm flasks were useful because they could be directly inserted into a spectrophotometer to permit simple and rapid optical density determinations. Glue was used to seal the large screw cap at the top of the flasks to prevent air from leaking in. The side arm opening was sealed with a serum cap through which nitrogen flushing and sample withdrawal were performed. Flasks were filled with 50 ml of medium which was reduced in a manner analogous to that used for culture tubes. Samples were aseptically withdrawn with an airtight syringe through the serum cap which was ethanol sterilized before sampling. Experiments

conducted in shake flasks were duplicated.

4.1.4. Preparation of Inocula

In all experiments, cells taken from stock deep-agar cultures were first grown in liquid medium before being used as an inoculum. A core of cell-containing agar was removed from a stock tube with a nitrogen flushed pasteur pipette. The cap of a tube containing pre-reduced medium was removed and the agar core was dispensed from the pipette into the liquid. During this procedure, the tube air space was flushed vigorously with nitrogen to prevent oxygen from entering the tube. Nitrogen was introduced just above the gas-liquid interface with a 2 1/2-inch 20-gauge needle. The tube was resealed by sliding the serum cap on while simultaneously removing the needle.

The inoculated tube was incubated in a shake bath at 65°C overnight. After approximately 24 hours, the late exponential phase culture was used as an inoculun. Cells were transferred to tubes to be inoculated with a nitrogen-flushed, gas-tight syringe. Unless otherwise stated a 1% inoculum was used in all experiments.

4.1.5. Incubation

Both culture tubes and shake flasks were incubated in a covered water bath at 65°C. Unless otherwise specified, cultures were agitated in the bath at a rate of 160 rpm.

4.1.6. pH Controlled Experiments

Apparatus

All pH cntrolled experiments were conducted in a 5-liter New Brunswick Micro-Ferm^R fermentor. The fermentor was sparged with a 95:5 nitrogen/hydrogen mixture at a rate of 10 cc/min and was maintained at a slightly positive pressure to prevent inward air leaks. Hydrogen was

used with the nitrogen to facilitate the removal of trace oxygen from the sparging gas. The nitrogen/hydrogen mixture was passed through a catalytic oxygen remover (Engelhard Industries, Union, New Jersey) where traces of oxygen reacted with hydrogen to form water. Sparging lines carrying nitrogen to the fermentor were either stainless steel or butyl rubber vacuum tubing (3/16-inch wall) both being essentially impermeable to oxygen. The fermentor agitation rate was 140 rpm. The temperature of the fermentor was controlled to 65°C (± 0.5°C).

The off-gas condenser was cooled with a refrigerated (5°C) 50:50 ethylene glycol/water mixture. Ethanol loss from the fermentor was measured and found to be negligible (less than 1% per day). pH was controlled (± 0.1 pH units) by the automatic addition (PEC fermenter pH controller, Chemapec, Woodbury, NY) of oxygen-free 2N potassium hydroxide. Anaerobisis in the base feed jar was maintained by constant nitrogen sparging.

Most types of flexible rubber tubing are somewhat permeable to oxygen. Tygon^R has the lowest oxygen permeability of any rubber tubing (65). However in tests where pre-reduced medium was pumped through nitrogen-washed Tygon, the resazurin changed from colorless to pink indicating that significant amounts of oxygen diffused through the tubing wall. Hence, stainless-steel tubing was used for all base feed lines except for a small section of Tygon that fit into the head of a peristaltic pump. The oxygen permeable tubing in the pump head (as well as the pump itself) were enclosed in a plexiglass nitrogen-filled container. In this way, oxygen was completely excluded from base added to the fermentor for pH control. All fittings and connectors used were airtight stainless-steel Swage-loks^R.

Samples were withdrawn through a siphon-flow 1/8-inch stainless steel sampling line.

Procedure for pH Controlled Experiments

The 5-liter fermentor jar was initially filled with two liters of medium (less xylose, vitamins, minerals and reducing agent) and autoclaved for 40 minutes at 125°C. After the medium cooled, heat labile components were aseptically added through a port at the top of the fermentor. The fermentor jar was then connected to the rest of the Mini-Ferm^R apparatus and heated to 75°C (highest temperature possible with Mini-Ferm^R). Nitrogen was sparged through the fermentor at a rate of approximately 2 liters per minute. After the medium changed color, indicating anaerobisis, 25 ml of sterile cysteine hydrochloride solution (32 gm/L) were added through an ethanol sterilized septum at the top of the fermentor. A 50-ml glass syringe was used to add the reducing agent.

The temperature of the fermentor was adjusted to 65°C before inoculation. 8 ml of a culture grown to mid-exponential phase were used to inoculate the fermentor. The culture tube containing the inoculum was connected to the fermentor by a two-pointed 20-gauge needle which pierced the septa of both vessels. Because the culture tube was at a higher pressure than the fermentor (due to a build-up of CO_2 during growth) the inoculum flowed from the tube to the fermentor. Samples were withdrawn periodically from the fermentor for the 48 hours immediately following inoculation.

4.2. Analytical Procedures

4.2.1. Growth Rate and Dry Weight

Growth rate was determined by following the change in culture optical density at 650 nm with a Fisher Electrophotometer II. Culture tubes and side arm flasks could be directly inserted into the electrophotometer, whereby the growth vessel served as the cuvette. With this technique, optical densities could be rapidly and conveniently measured. For cells grown in a fermentor, samples were drawn into culture tubes which were used as cuvettes in the electrophotometer.

Dry weight determinations were made by centrifuging a known volume of a culture at 10,000 rpm for 15 minutes. The pellet was washed, resuspended in 0.05 M saline, and recentrifuged. Cell pellets were dried at 65°C for 24 hours, allowed to cool in a desiccator, and weighed on a Mettler^R balance which was accurate to ± 0.1 mg. For cells grown in medium containing 2 gm/L yeast extract and 10 gm/L xylose, a standard dry weight curve was prepared (Figure 4.1). With this curve optical densities could be easily converted into dry weights. Optical density was a linear function of dry weight up to 0.17 gm/L cells.

4.2.2. Sample Preparation for Xylose and End-Product Assays

Culture samples were clarified by centrifugation at 10,000 rpm for 15 minutes. Samples were stored at 4°C and assayed within 2 weeks of sampling. Samples were not frozen since the stability of lactic acid on freezing is not known (66). All measurements of xylose and metabolic product concentrations were duplicated.

4.2.3. Ethanol

Ethanol was determined with either gas chromatography (for high

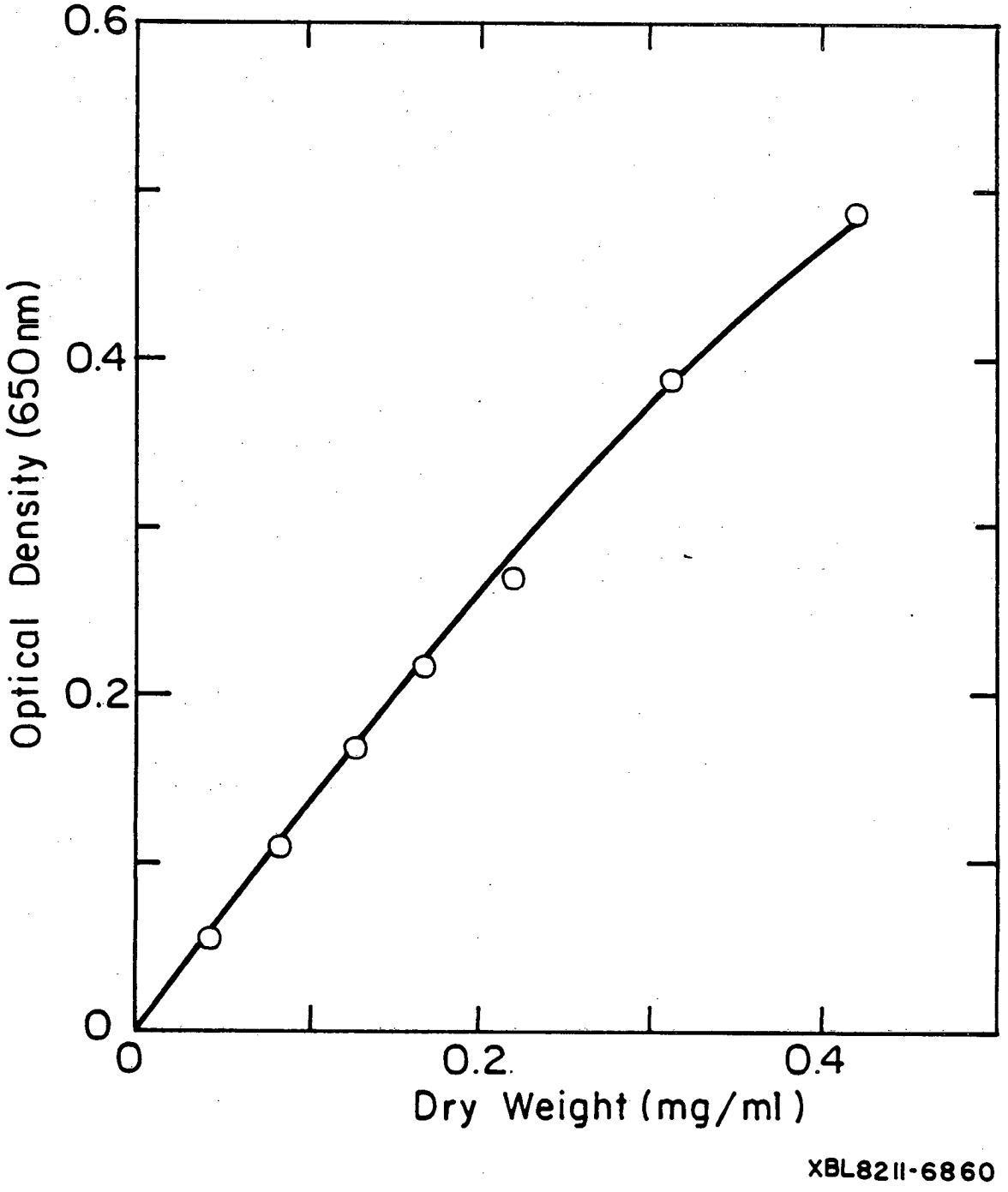

Figure 4.1. Dry Weight Calibration Curve.

concentrations, i.e. greater than 1 gm/L) or an enzymatic technique (for low concentrations).

Gas Chromatography

Samples were analyzed with an Aerograph 1520 gas chromatograph equipped with a flame ionization detector. The column was a 6-foot by 1/4-inch stainless steel tube packed with 100/120 mesh Chromosorb^R 101. Either helium or argon was used as the carrier gas and the column was maintained at 170°C. With this method, ethanol concentrations greater than 1 gm/L could be determined $\pm$ 3%.

Enzymatic Determination

Low concentrations of ethanol were determined as described in Sigma^R Technical Bulletin No. 332-UV (Sigma Chemical Co., St. Louis MO). Alcohol dehydrogenase (ADH) is used to convert ethanol and NAD to acetaldehyde and NADH. The reaction is driven by continuous removal of acetaldehyde by a trapping agent (which was not disclosed by Sigma). The change in NADH concentration can be read directly since it absorbs light very strongly at 340 nm.

The assaying reagent contained 0.2 mg ADH (Sigma No. A-7011) and 0.44 mg NADH (Sigma No. N-7381) per ml of glycine buffer solution (Sigma No. 332-9). Any protein left undissolved after the above three components were mixed was removed by filtration with Whatman #1 filter paper.

2.9 ml of the enzyme solution were mixed with 0.1 ml of diluted sample which contained less than 0.3 gm/L ethanol. After the mixture was incubated for 20 minutes at 25°C, the absorbance was measured at 340 nm with a Bausch and Lamb Spectronic System 400 (also used for all other colorimetric asays). Ethanol concentrations were read from a standard

curve of absorbance versus concentration. A calibration curve was determined for each batch of freshly prepared enzyme solution. Concentrations as low as 0.05 gm/L could be determined accurately (± 3%).

4.2.4. Xylose

Xylose was usually determined with a standard dinitrosalicylic acid technique (67). However when other reducing compounds were present (e.g. caramelized xylose) which interferred with the assay, high pressure liquid chromatography was used.

For HPLC analysis, a Partisil-10 PAC column (Whatman, Inc.) operated at room temperature was used. The detector was a Waters Associates refractometer and the carrier solvent was an azeotropic acetonitrile/water 84:16 mixture. The solvent flow rate was 1 ml/minute.

4.2.5. Acetate

A modification of the method developed by Rose (68) was used to determine the concentration of acetate. In this procedure, acetate and ATP are converted to acetyl-phosphate and ADP by acetyl kinase in the presence of magnesium.

$$CH_3COOH + ATP \xrightarrow{\text{acetyl kinase}} CH_3COO\text{-}\textcircled{P} + ADP$$

Hydroxylamine is used to convert acetyl-phosphate to its hydroxamic acid which is subsequently converted to a colored ferric-hydroxamate complex by ferric chloride.

$$CH_3COO\text{-}\textcircled{P} + NH_2OH \longrightarrow CH_3CONOH + H_2PO_4^-$$

$$CH_3CONOH + FeCl_3 \longrightarrow \text{colored complex}$$

The assay solution contained per ml: 2.0 mg $MgCl_2 \cdot 6H_2O$, 6.0 mg Tris, 4.6 mg $NH_2OH \cdot HCl$, 6.23 mg ATP (Sigma No. A-2383), and 0.012 ml acetyl kinase solution (Sigma No. A-2384). 0.1 ml of sample containing less than 1.5 gm/L acetate were mixed with 0.9 ml of the above reagent and incubated at 38°C. After one hour, the color was developed by the addition of 3 ml of 1.5% $FeCl_3$ in 1 N HCl. The absorbance at 540 nm was directly porportional to the acetate concentration; however, xylose was found to interfere with this assay (see Figure 4.2). Hence the acetate concentration measured with this technique had to be corrected for xylose interference. Acetate concentrations as low as 0.3 gm/L could be determined accurately (± 3%).

4.2.6. Lactate

Lactate was determined enzymatically with lactate dehydrogenase (Sigma Technical Bulletin No. 826-44). Lactate and NAD react via lactate dehydrogenase (LDH) to form pyruvate and NADH. Hydrazine, which reacts with pyruvate, is used in the assaying reagent to shift the equilibrium to favor NADH formation.

The reagent contained per ml of solution: 0.33 ml glycine buffer (Sigma No. 826-3), 0.67 ml water, 1.7 mg NAD (Sigma No. 7381), and 0.017 ml LDH aqueous suspension (Sigma No. 826-6).

A 2.8 ml portion of assay reagent was mixed with 0.2 ml of sample (containing less than 0.2 gm/L lactate). The mixture was incubated at 37°C for 45 minutes. The absorbance at 340 nm was measured and the lactate concentration was read from a standard curve. Accurate measurements (± 2%) could be made on lactate concentrations as low as 0.05 gm/L.

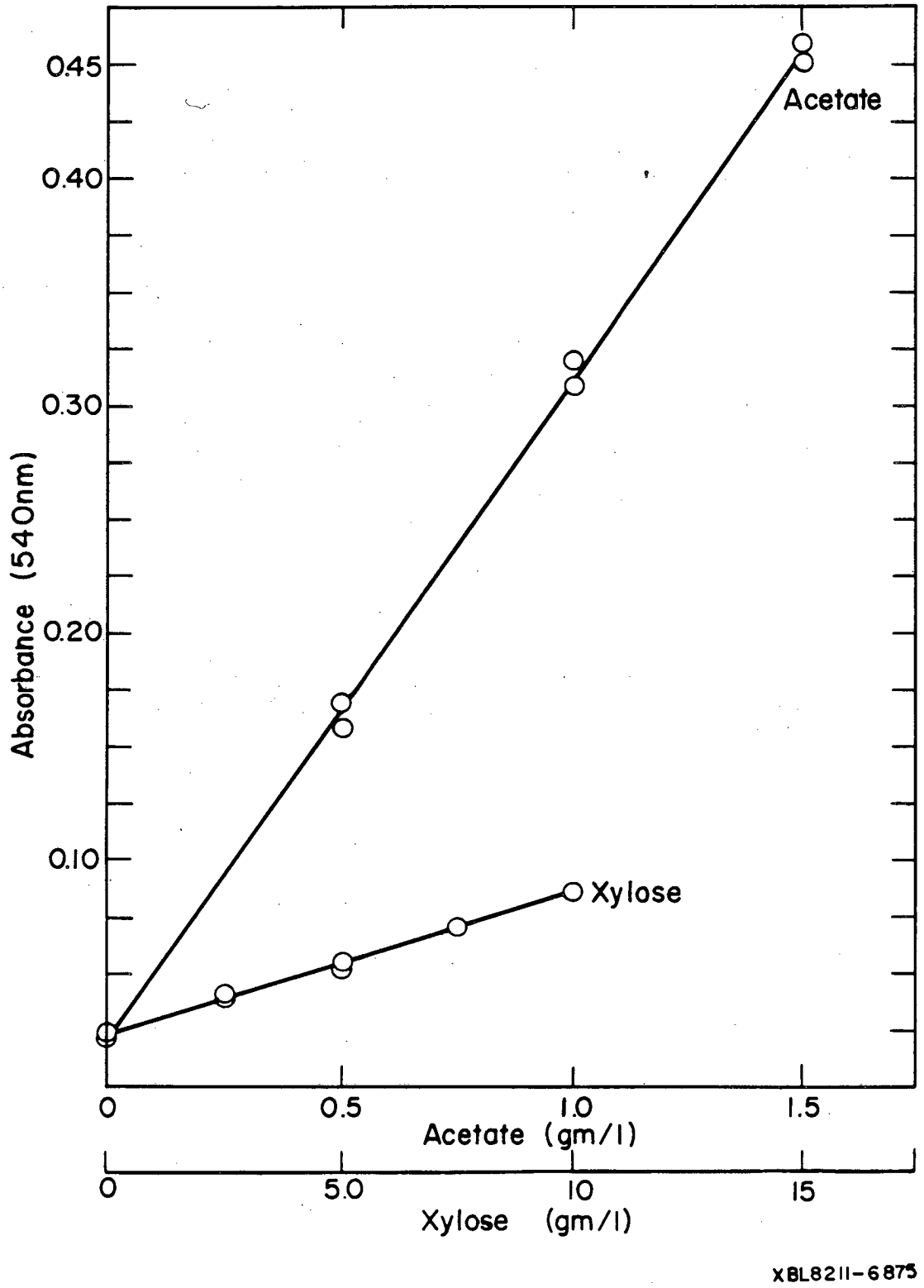

Figure 4.2. Interference by Xylose in Enzymatic Determination of Acetate.

V. RESULTS AND DISCUSSION

5.1. Batch Fermentation Profile

A typical batch fermentation profile for *C. thermohydrosulfuricum* strain 39E is shown in Figure 5.1. Ethanol was produced as the primary end product; from an initial concentration of 11.7 gm/L xylose, 3.5 gm/L ethanol was formed (overall yield = 0.33 gm/gm). The yields of acetate and lactate from xylose were 0.098 and 0.036 gm/gm, respectively. In calculating these yields, xylose consumption had to be adjusted to account for caramelization of the growth medium which occured during incubation at the high growth temperature (see Appendix II).

Cell growth was not truly exponential; the specific growth rate dropped from 0.8 hr^{-1} at 7 hours to 0.10 hr^{-1} at 13 hours. The drop in growth rate was probably due to depletion of nutrients in the medium (e.g. vitamins, amino acids, or nucleic acids). Sustained exponential growth occurs only when *all* nutrients are present in excess. The specific ethanol productivity also dropped as the fermentation proceeded. At 10 hours the specific ethanol productivity was 0.6 gm ethanol/gm cell-hr while at 20 hours it was 0.2 gm/gm-hr.

The total carbon recovery (moles of carbon appearing as fermentation products and cells, per mole of carbon consumed as xylose) for this experiment was 94% (see Appendix II).

These results differ somewhat from those obtained by Zeikus and co-workers (see section 3.3.1). They did not observe as large a drop in specific growth rate and also reported a yield of 0.40 gm ethanol/gm xylose. However, they grew the organism in richer medium and without agitation.

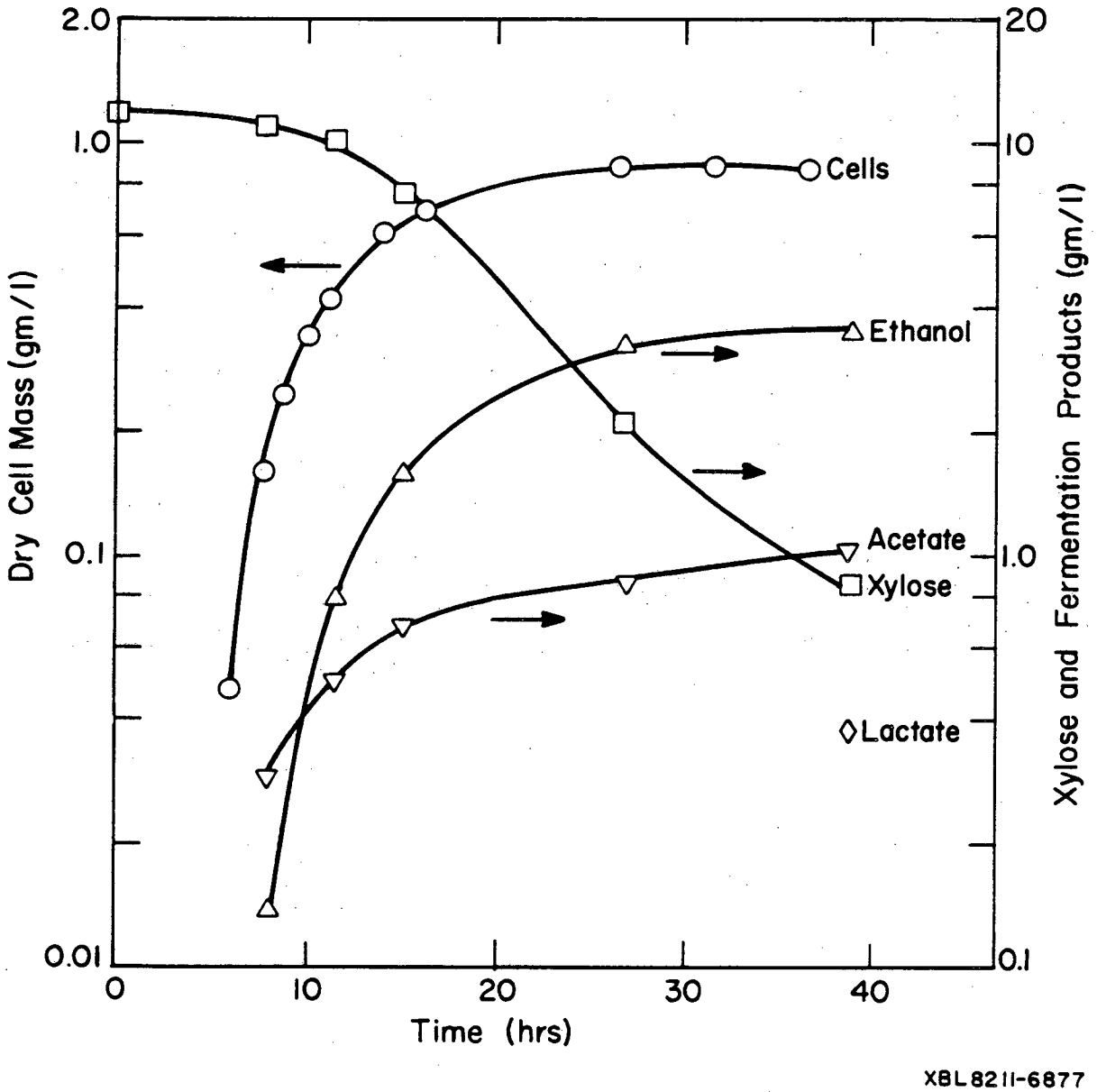

Figure 5.1. Batch Fermentation Profile for Clostridium thermohydrosulfuricum strain 39E. Cells were grown in YEX1 medium with sodium sulfide as the reducing agent. Carbon Recovered = 94%.

To see if their results could be duplicated, C. thermohydrosulfuricum was grown in unshaken flasks on enriched YEX1 medium. (The yeast extract concentration was doubled.) The results, which are shown in Figure 5.2 and Table 5.1, are in fairly good agreement with those obtained by Zeikus. The specific growth rate was constant for the first eight hours at 0.35 hr^{-1}; Zeikus observed a sustained rate of 0.34 hr^{-1}. A yield of 0.43 gm ethanol/gm xylose was measured after 4.9 gm/L of xylose had been fermented. Zeikus reported a yield of 0.40 gm/gm for the fermentation of 5 gm/L.

Table 5.1 also indicates that the yield dropped significantly as xylose utilization continued. A corresponding increase in lactate yield is also evident from Figure 5.2. There are two possible explanations for the shift in end-product yields. Cell growth ceased after approximately 20 hours while end-product accumulation continued for an additional 13 hours. This suggests that yeast extract was growth limiting and as indicated in section 3.3.3, under "nitrogen" limiting conditions lactate is the primary end product. Alternatively, ethanol formation may be strongly feed back inhibited; whereby, in the presence of ethanol, lactate formation is enhanced. These possibilities are further examined in sections 5.2 and 5.3.

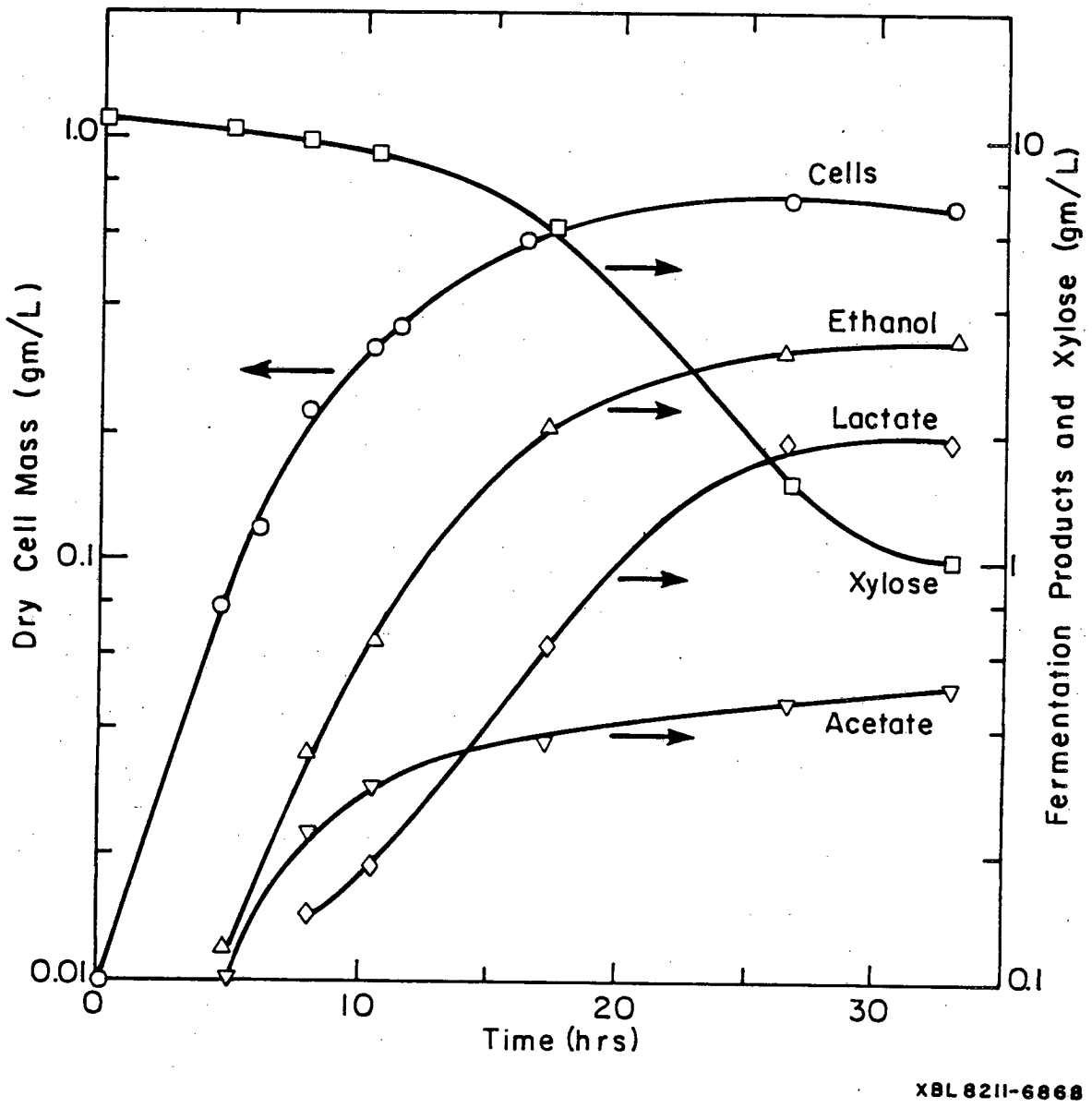

Figure 5.2. Batch Fermentation Profile for Clostridium thermohydrosulfuricum strain 39E. Cells were grown in medium containing 4 gm/L yeast extract with cysteine hydrochloride as the reducing agent. The culture was not agitated. Carbon recovered = 98%.

Table 5.1

Ethanol Yield During Batch Growth

Xylose Fermented (gm/L)	Overall Yield (gm/gm)
4.9	0.43
9.1	0.34
9.6	0.34

5.2. Nutritional Requirements

Laboratory yeast extract is a complex source of nutrients made from autolysed yeast cells (69). In an industrial ethanol fermentation process, the cost of nutrients must be kept at a minimum. The current (1982) market price for industrial autolysed yeast is $1.78/Kg (70). Using 2 gm of yeast extract to produce 3.5 gm of ethanol (Figure 5.1) contributes approximately 79 cents to the cost of producing a liter of ethanol. Since the current market price of ethanol is 48 cents/liter, decreasing or completely eliminating the use of yeast extract is clearly desirable (71).

5.2.1. Effect of Yeast Extract Concentration on Growth and Yields

The effect of yeast extract concentration on the growth of *C. thermohydrosulfuricum* is shown in Figure 5.3. In this experiment, sodium sulfide was used as the reducing agent since cysteine may have served as a nutrient, especially at low yeast extract concentrations. The initial growth rate is rapid in all cases; however, as growth proceeds and nutrients are depleted the rate slows appreciably. The decrease in growth rate with time is most marked at low yeast extract concentrations.

Table 5.2 shows the average specific growth rate (from optical density 0.1 to 0.2) obtained with the 5 different yeast extract concentrations. Decreasing the yeast extract concentration reduced the rate of growth significantly. Note that because optical density measurments were obtained by direct insertion of culture tubes into a spectrophotometer, they are not directly proportional to cell mass for optical densities greater than 0.22 (see section 4.2.1). Hence, the

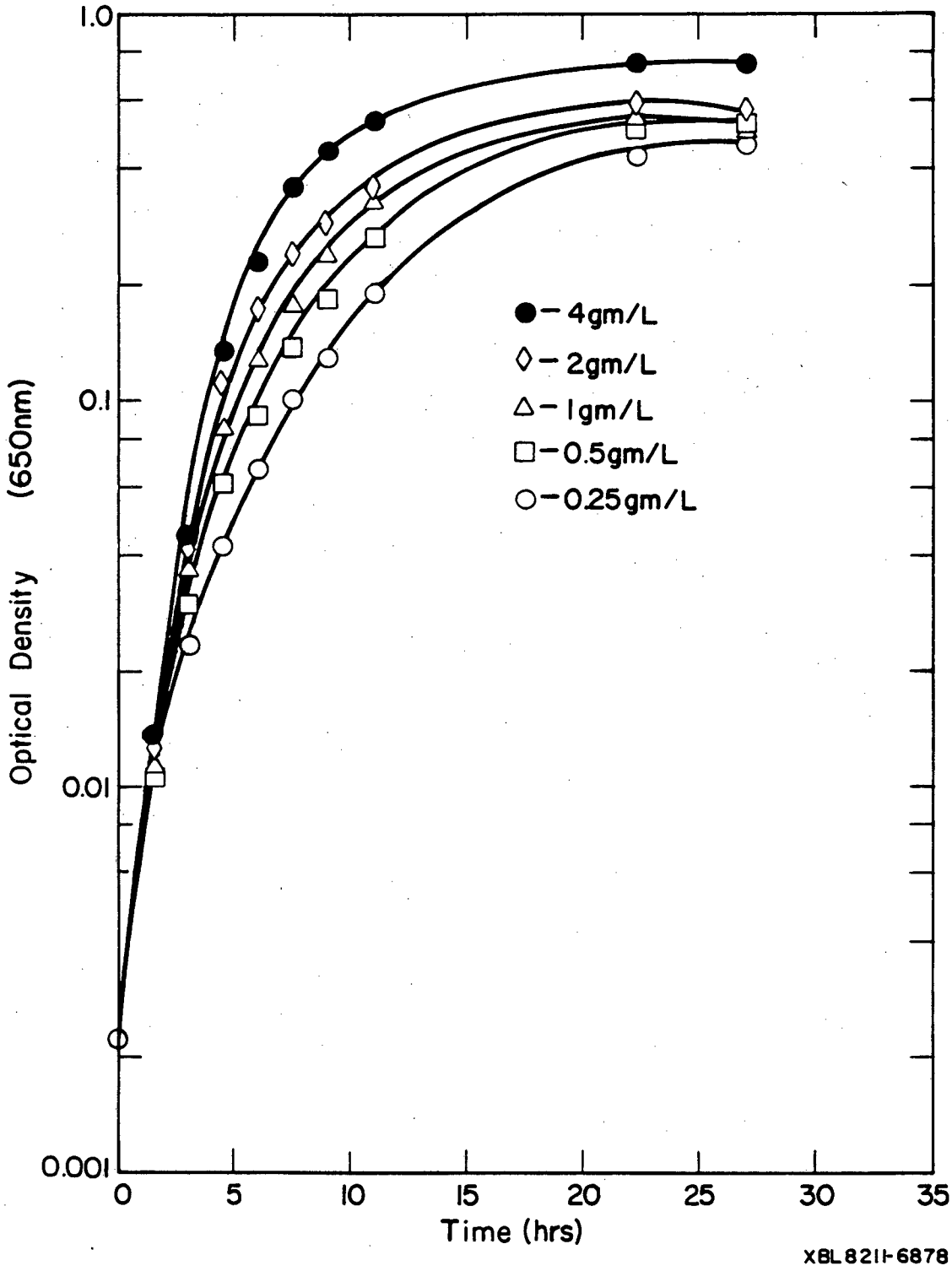

Figure 5.3. Effect of Yeast Extract Concentration on Growth.

Table 5.2

Effect of Yeast Extract Concentration

on Growth Rate

Yeast Extract (gm/L)	Growth Rate (hr^{-1})*
0.25	0.18
0.50	0.18
1.0	0.22
2.0	0.28
4.0	0.58

*Rate of growth observed between optical density 0.1 and 0.2.

non-linearity of the growth curves at optical densities above 0.22 is due to both a slowing of growth and slightly erroneous optical density measurements.

Figure 5.4 shows the dependence of end-product and cell yields, and extent of xylose utilization on yeast extract concentration. The results in this figure were determined from samples withdrawn at the end of stationary phase (approximately 30 hours after inoculation). Over the range of yeast extract concentration examined, the yield of ethanol increased from 0.17 to 0.28 gm/gm, while the yield of lactate decreased from 0.51 to 0.35 gm/gm. This is consistent with the findings of Zeikus and co-workers (section 3.3.1) who demonstrated that under nitrogen-limited conditions (low yeast extract concentrations) lactate formation is greatly enhanced. The yield of acetate was relatively unaffected by changes in the yeast extract concentration. The cell yield increased with yeast extract concentration up to 2 gm/L after which it gradually leveled off. Hence, at 4 gm/L the limiting substrate for growth was not yeast extract. The slope of the linear portion of this curve (0.25 to 2.0 gm/L) gives an approximate value for the yield of cells from yeast extract (0.15 gm cells/gm yeast extract). This value is used in Appendix 2 to calculate carbon recovery.

Because both the ethanol yield and the extent of xylose utilization are adversely affected, decreasing the yeast extract concentration is not an effective way to reduce the cost of the growth medium.

During the course of this work, it was observed that the yield of ethanol from xylose was also dependent on the lot of yeast extract supplied by Difco. Apparently some component of yeast extract, which is

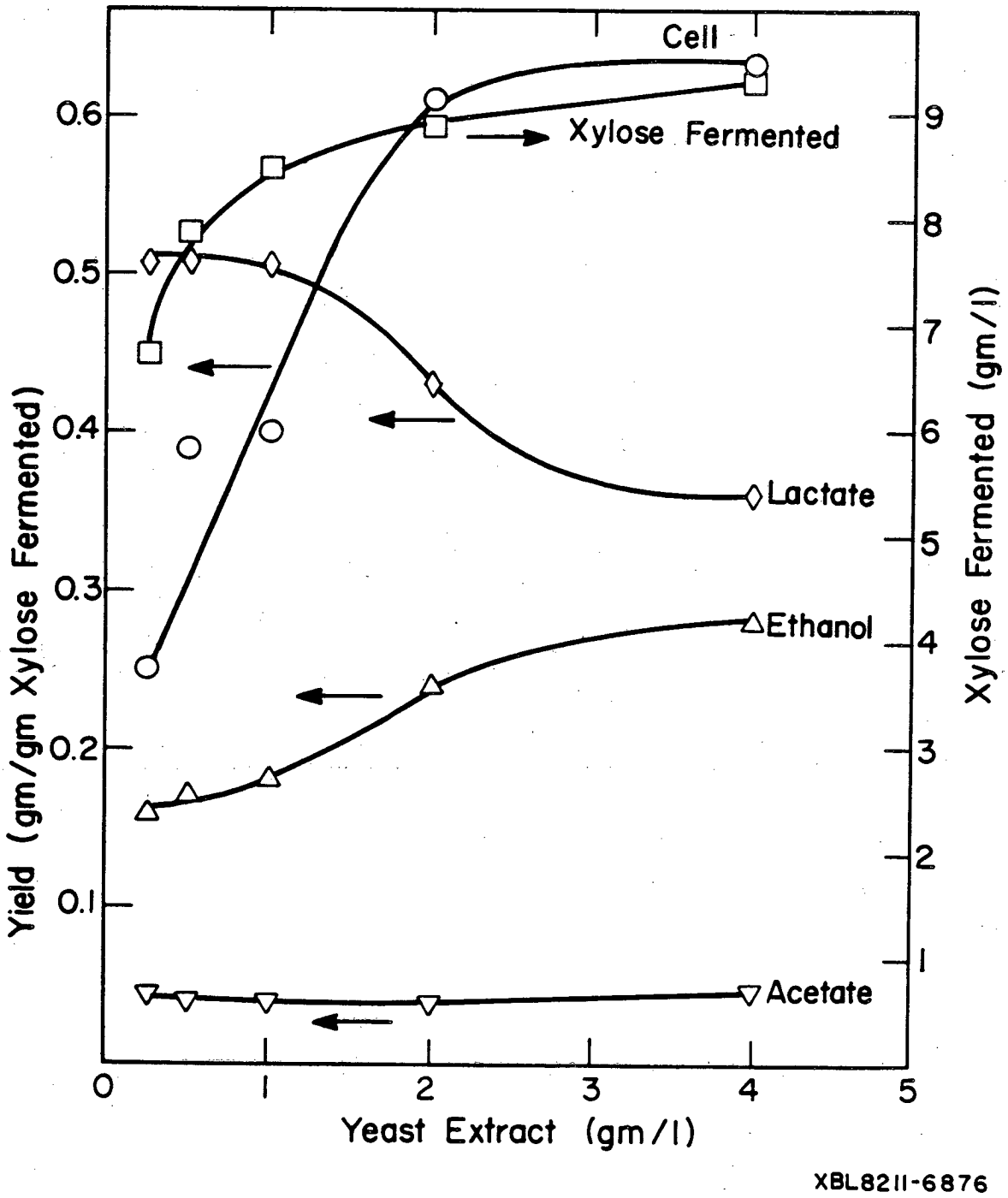

Figure 5.4. Dependence of Cell and End-Product Yields and Extent of Xylose Utilization on Yeast Extract Concentration.
In all cases carbon recovery was greater than 90%.

not carefully controlled at Difco, strongly affects the end-product distribution of C. thermohydrosulfuricum. For example, in Figure 5.4 the yield of ethanol with 2 gm/L yeast extract was only 0.24 gm/gm, while the yield in Figure 5.1 (which was obtained with a different batch of yeast extract but at the same concentration), was 0.33 gm/gm. In all experiments where low ethanol yields were measured due to poor quality yeast extract, the acetate yield was also reduced while the yield of lactate was increased. Wood (72) has reported that a drop in ethanol and acetate formation with a corresponding increase in lactate production was observed with Clostridium perfringens (a mixed acid and ethanol producing bacterium) grown in iron deficient medium. Examination of the metabolic pathways shown in Figure 3.2 indicates that ferredoxin, an iron containing electron carrier (73), is important for acetate and ethanol formation. In addition, in studies on the nutritional requirements of C. thermohydrosulfuricum (see section 5.2.3) growth appeared to be enhanced by the addition of iron and magnesium.

Given the above information, it was hypothesized that low ethanol yields observed with some batches of yeast extract were due to a lack of iron. To examine this idea, cells were grown in YEX1 with and without 2 mg/L $FeSO_4 \cdot 7H_2O$ added. The results, shown in Table 5.3, clearly demonstrate that iron is important for ethanol and acetate formation. In future work with C. thermohydrosulfuricum it is recommended that ferrous sulfate (2 mg/L) be included in the medium.

5.2.2. Corn Steep Liquor as a Source of Nutrients

A potentially economical alternative to yeast extract is corn steep liquor (CSL). CSL is a by-product of corn wet milling and is used frequently in the fermentation industry as a source of nutrients (74).

Table 5.3

Effect of Adding Iron to YEX1 Medium

$FeSO_4 \cdot 7H_2O$ (mg/L)	0	2
Extent of Xylose Utilization (gm/L)	8.9	9.1
Final Cell Mass (gm/L)	0.74	0.74
Growth Rate (hr^{-1})	0.49	0.53
Yields (gm/gm)		
Ethanol	0.28	0.31
Acetate	0.054	0.071
Lactate	0.34	0.18
% Carbon Recovered	105	98

It is a thick black slurry containing approximately 50% water by weight and has a crude protein content (non-dialyzable proteins, polypeptides, and amino acids) of 47% (dry basis). In addition, small amounts of vitamins, minerals, and nucleic acids are also present in CSL (74).

The composition of the medium used to examine the growth characteristics of C. thermohydrosulfuricum on corn steep liquor is shown in Table 5.4. When CSL was combined with the other medium components, it did not dissolve completely. The insolubles, which appeared to be mostly cellulosic materials, were removed from the medium by filtration with Whatman #1 paper. Solids removal was necessary to facilitate cell mass optical density determinations. Subsequent tests revealed that including the insolubles in the growth medium did not significantly affect carbohydrate utilizaton or ethanol formation during batch growth. Even without the insolubles, obtaining accurate optical density measurements at low cell densities was difficult due to the deep-brown color of the medium. All heat-stable components of the medium were sterilized by heat; xylose and cysteine were added after the medium cooled.

Figure 5.5 shows the effect of CSL concentration on the growth of C. thermohydrosulfuricum. The initial rate of growth increased with increasing CSL concentration. This effect is shown more clearly in Figure 5.6 which also shows the dependence of the final ethanol yield and extent of xylose utilization on the CSL concentration after 55 hours of incubation. Both increase with the amount of CSL used in the medium. These trends are similar to those observed with yeast extract (Figure 5.4); thus some component of corn steep liquor was the limiting nutrient in the medium.

Table 5.4

Corn Steep Liquor Medium

Component	Amount per liter
$Na_2HPO_4 \cdot 7H_2O$	15.0 gm
KH_2PO_4	3.0 gm
$FeSO_4 \cdot 7H_2O$	2.0 mg
Resazurin	2.0 mg
Cysteine·HCl·H_2O	400 mg
*Corn Steep Liquor	1,2,5, or 10 gm

*Obtained from Corn Products (75).

CSL concentrations given are based on wet weight.

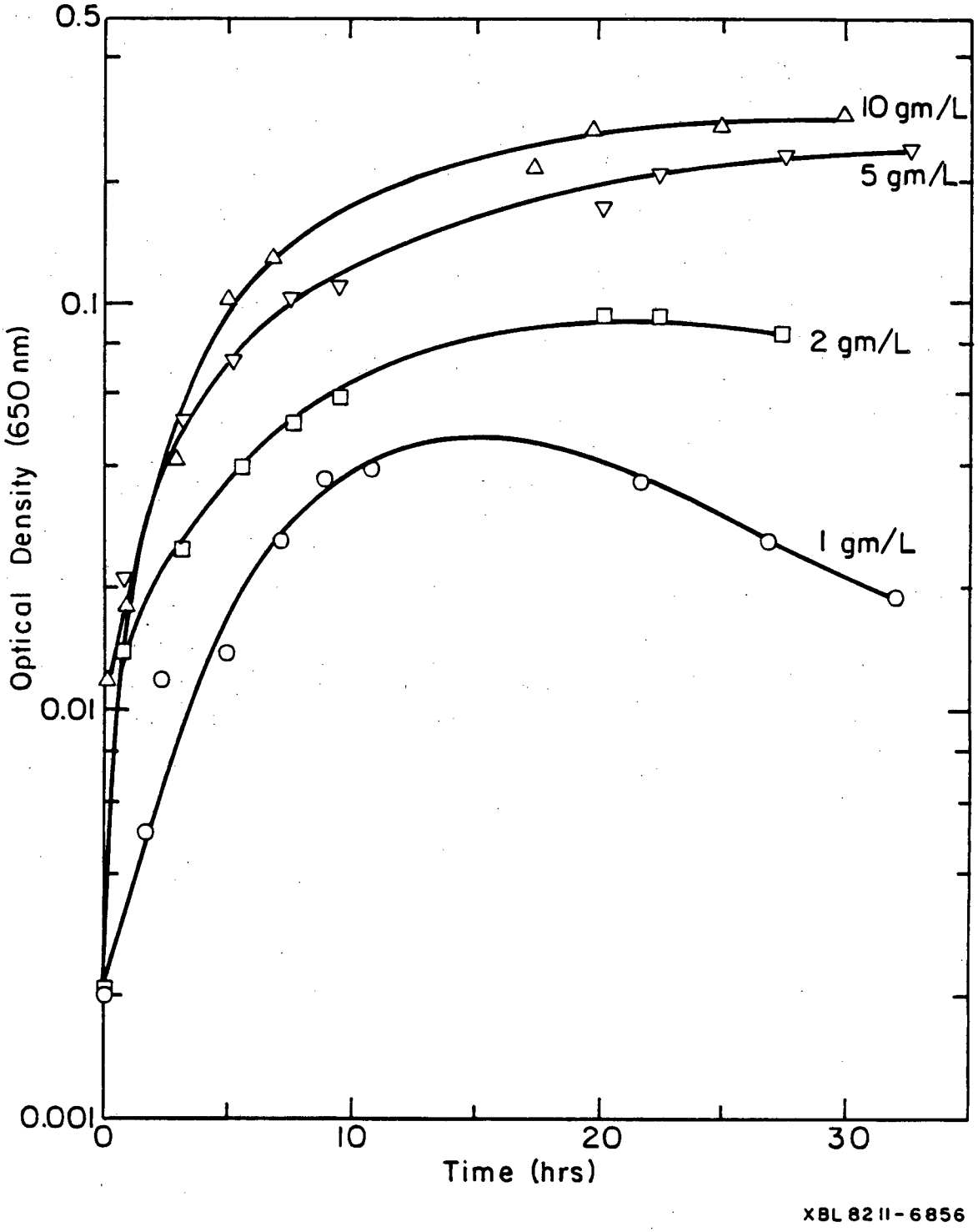

Figure 5.5. Effect of Corn Steep Liquor Concentration on Growth.

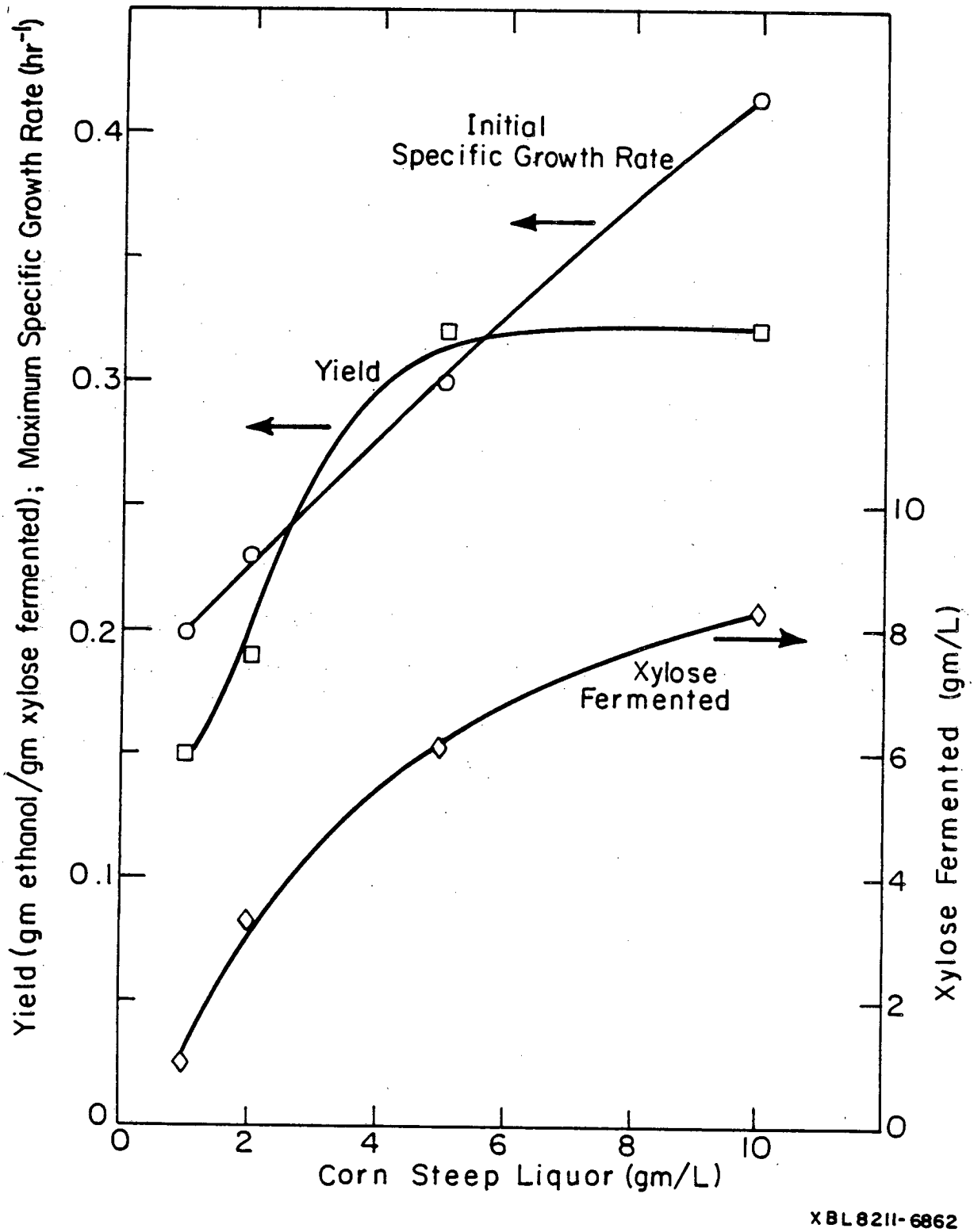

Figure 5.6. Effect of Corn Steep Liquor Concentration on Growth Rate, Extent of Xylose Utilization, and Ethanol Yield.

The present market price of CSL is a 12.1 cents/Kg (74). If 10 gm of CSL are required to produce 2.8 gm of ethanol, then the use of CSL will contribute 34 cents to the cost of each liter of ethanol produced. Clearly, this cost is unacceptably high.

If corn steep liquor is deficient in some inexpensive trace factor, it may be economically practical to use low concentrations of CSL with some supplemental nutrients. Alternatively, growth in CSL medium may have been poor due to a lack of ammonium ions.

To examine these possibilities, a base medium containing 1 gm/L CSL, 0.5 gm/L $(NH_4)_2SO_4$, and all the other components listed in Table 5.4 was prepared. Table 5.5 summarizes the results obtained after 48 hours of growth on the base medum with and without supplements. Both small amounts of yeast extract, and vitamins and minerals in the medium produced improvements over the base case. However, in neither case does the extent of xylose fermentation proceed nearly to completion (10 gm/L). Even when yeast extract, vitamins, and minerals are all used as supplements, only half the available xylose is fermented. Therefore, it appears that corn steep liquor is not a good source of of all required nutrients for C. thermohydrosulfuricum.

5.2.3. Growth in Defined Media

Another possible way to reduce the cost of the growth medium is to replace yeast extract with a synthetic combination of amino acids, vitamins, and minerals. To develop an economical, defined medium, it is necessary to determine which nutrients are essential for rapid growth and high ethanol yield.

The synthetic medium used for examining the nutritional requirements of C. thermohydrosulfuricum is shown in Table 5.6. It

Table 5.5

Supplemented Corn Steep Liquor*

Addition	Xylose Fermented (gm/L)	Ethanol Yield (gm/gm)
None	1.8	0.15
0.2 gm/L Yeast Extract	2.7	0.20
5 ml Vitamin Solution + 5 ml Mineral Solution	3.4	0.22
5 ml Vitamin Solution + 5 ml Mineral Solution + 0.2 gm/L Yeast Extract	5.2	0.26

*Base medium same as Table 5.4 with 1 gm/L CSL and 0.5 gm/L $(NH_4)_2SO_4$.

Vitamin and mineral solutions were the same as those used in YEX1.

Table 5.6

Defined Medium I (DMI)

Component	Amount per liter
Arginine	75 mg
Histidine	75 mg
Isoleucine	75 mg
Leucine	75 mg
Lysine	75 mg
Methionine	75 mg
Phenylalanine	75 mg
Threonine	75 mg
Tryptophane	75 mg
Valine	75 mg
Biotin	100 µgm
Folic Acid	100 µgm
Pyridoxine	100 µgm
Riboflavine	100 µgm
Thiamine	100 µgm
Nicotinic Acid	100 µgm
Pantothenic Acid	100 µgm
Para-aminobenzoic Acid	100 µgm
KH_2PO_4	2.0 gm
$Na_2HPO_4 \cdot 7H_2O$	10.0 gm
$(NH_4)_2SO_4$	0.5 gm
Na_2S	0.1 gm
Trace Mineral Solution	10 ml
Resazurin	2.0 mg

includes many of the amino acids and vitamins normally found in yeast extract (76,77).

Essential Amino Acids

To determine which amino acids are essential, C. thermohydrosulfuricum was cultured in 10 different media, each deficient in a single amino acid. (All solutions prepared for defined media studies were filtered sterilized since many amino acids and vitamins are heat labile (78).) The effect of omitting each amino acid from the growth medium is shown in Table 5.7. To ensure that any nutrients introduced with the inoculum were insignificant, these results were verified by subculturing. For example, cells grown in the "absence" of arginine were transferred to arginine deficient medium and re-grown. In all cases, the data shown in Table 5.7 were found to be accurate.

The results indicate that methionine is an important amino acid for C. thermohydrosulfuricum. In addition, the maximum optical density obtained with DM1 (all 10 amino acids present) was much less than that normally obtained with YEX1 medium. The maximum specific growth rate in the defined media was also slow (approximately 0.13 hr^{-1}); growth rates in YEX1 typically range from 0.4 to 0.5 hr^{-1}. The poor growth in DM1 may have been due to several factors. DM1 may have been deficient in some nutrients such as other amino acids or vitamins, or an unidentified trace factor present in yeast extract. Alternatively, some of the vitamin concentrations may have been too high. Low concentrations (approximately 100 μgm/L) of biotin, folic acid, and para-aminobenzoic acid (PABA) have all been found to be inhibitory in some species of bacteria (78).

To examine these possibilities, a new base medium (DM2) was

Table 5.7

Effect of Omitting Individual Amino Acids

From DM1 on Growth

Deficient Amino Acid	Maximum Optical Density
Arginine	0.16
Histidine	0.19
Isoleucine	0.18
Leucine	0.19
Lysine	0.18
Methionine	0.10*
Phenylalanine	0.19
Threonine	0.19
Tryptophane	0.18
Valine	0.18
Control (DM1)	0.20
YEX1 Medium	0.60

*Appears to be essential

prepared (see Table 5.8). The vitamin concentrations used were intended to be approximately equal to those in YEX1. These concentrations were calculated from an approximate compositional analysis of Difco yeast extract (77). Vitamin B_{12} and lipoic acid, which are not normally found in yeast extract but were used in the YEX1 vitamin solution, were added to DM2. In addition, BBL yeast extract (Becton, Dickinson, and Co.; Cockeysville, MD) typically contains significant amounts of iron and magnesium (76); hence, the concentrations of these two metals were also increased in DM2 (Mineral composition analyses of Difco yeast extract are not supplied by Difco).

C. thermohydrosulfuricum was grown in DM2 with and without supplements. The supplements tested were: additional biotin, PABA, and folic acid to see if high concentrations of these vitamins were inhibitory, 10 additional amino acids (other than those used in DM2) to determine whether or not they were important for growth, and 100 mg/L yeast extract which may supply important unidentified trace nutrients. The growth curves obtained with these four media are shown in Figure 5.7.

In all cases, growth was significantly better than with DM1. With all four media, there was an initial, short phase of rapid growth followed by sustained exponential growth lasting for approximately 10 hours. The growth rates during exponential phase are shown in Table 5.9 along with the maximum optical density, the total amount of xylose fermented, and the yield of ethanol. In DM2, 6.7 gm/L of xylose was consumed and ethanol was produced at a yield of 0.28 gm/gm. The final optical density, 0.46, was more than twice that observed with DM1.

Addition of the 10 amino acids to the medium improved growth and

Table 5.8

Vitamins and Minerals Used in DM2*

Component	Amount per liter
Biotin	10 μgm
Folic Acid	10 μgm
Pyridoxine	100 μgm
Riboflavin	100 μgm
Pantothenic Acid	100 μgm
Thiamine	30 μgm
Nicotinic Acid	500 μgm
Para-aminobenzoic Acid	25 μgm
Viatmin B_{12}	1.0 μgm
Lipoic Acid	25 μgm
$FeSO_4 \cdot 7H_2O$	2.0 mg
$MgSO_4 \cdot 7H_2O$	50 mg
All 10 amino acids in Table 5.5	100 mg

*Concentrations of phosphates, ammonium sulfate, sodium sulfide, and trace minerals were the same as in DM1.

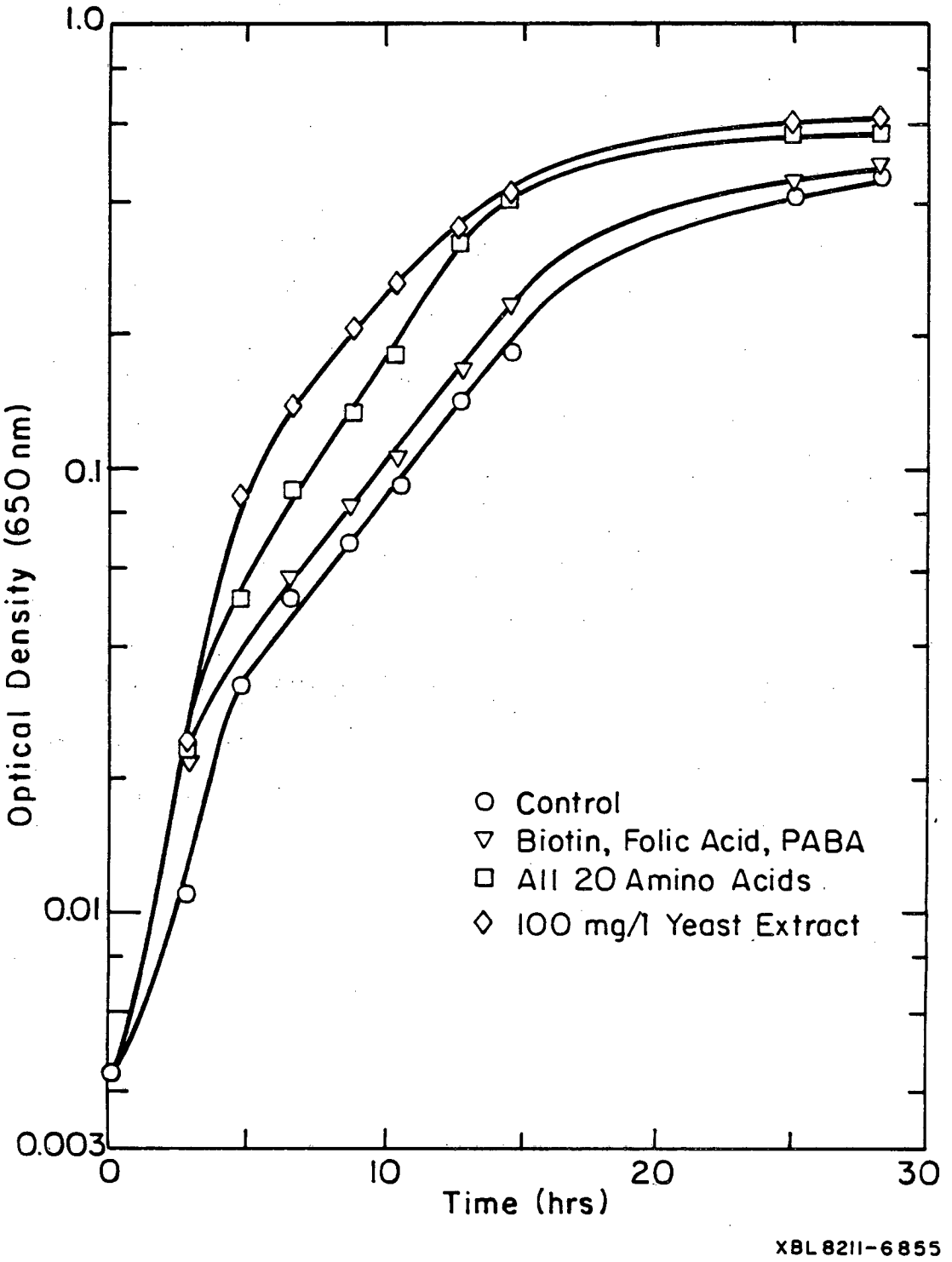

Figure 5.7. Growth in Defined Medium DM2 with supplements.

Table 5.9

Growth on Defined Medium II With Supplements

Addition	Max. O.D.*	Growth Rate (hr^{-1})	Xylose Fermented* (gm/L)	Yield (gm etoh/ gm xylose)
None	0.46	0.18	6.7	0.28
All Other Amino Acids (75 mg/L)	0.58	0.22	8.9	0.24
Biotin (90 µgm/L) Folic Acid (90 µgm/L) PABA (75 µgm/L)	0.48	0.18	7.0	0.29
Yeast Extract (100 mg/L)	0.63	0.17	8.9	0.29

*After 29 hours of fermentation

xylose utilization; however, the ethanol yield was significantly reduced. Hence, these amino acids do not appear to be necessary for ethanol production with C. thermohydrosulfuricum. Results with additional biotin, folic acid, and PABA were not significantly different form those obtained with DM2 alone. Thus, 100 μgm/L of all three vitamins does not inhibit the gowth of C. thermohydrosulfuricum. The above results indicate that DM1 was deficient in either nicotinic acid, vitamin B_{12}, lipoic acid, ferrous sulfate, or magnesium sulfate.

With 100 mg/L yeast extract in DM2, growth was rapid up to an optical density of 0.1 but then slowed to about the same rate observed with the other media. Xylose utilization was significantly improved with the addition of yeast extract while the yield was approximately the same as with DM2 alone. Thus, it appears that yeast extract supplies some nutrients, other than the amino acids and vitamins tested, that are beneficial for ethanol production. Since using 100 mg/L of yeast extract in DM2 contributes only 7.5 cents to the cost of producing a liter of ethanol, it was used in all subsequent nutritional studies.

Data shown in Table 5.7 indicate that, of the amino acids tested, only methionine was essential. To confirm that the other amino acids are non-essential, cells were grown in medium (DM3) containing vitamins, minerals, 100 mg/L yeast extract, and only methionine (see Table 5.10). The maximum optical density obtained during batch growth in this medium (shown in Table 5.11), was 0.41. This value is one-third less than the maximum optical density obtained with methionine and the nine "non-essential" amino acids. Although none of these amino acids appeared to be essential, omitting all of them from the medium adversely affected growth.

Table 5.10

Defined Medium III*

Component	Amount per liter
Methionine	75 mg
Biotin	25 μgm
Folic Acid	25 μgm
Pyridoxine	100 μgm
Riboflavin	100 μgm
Pantothenic Acid	100 μgm
Thiamine	30 μgm
Nicotinic Acid	500 μgm
PABA	25 μgm
Lipoic Acid	25 μgm
Vitamin B_{12}	1.0 μgm
$FeSO_4 \cdot 7H_2O$	2.0 mg
$MgSO_4 \cdot 7H_2O$	50 mg

*Buffer, trace minerals, ammonium sulfate, and reducing agent same as in DM1.

Table 5.11

Growth on Defined Medium III

Deficient Component	Max. O.D.*	Specific Growth Rate(hr^{-1})
None	0.41	0.23
Methionine	0.36	0.20
All Vitamins	0.23	0.23

*Obtained after 35 hours of growth.

Apparently, in the absence of a single amino acid, C. thermohydrosulfuricum can synthesize the deficient compound from other amino acids. Inter-substitution of amino acids is common in many microorganisms (79).

Also shown in Table 5.11 is the effect of deleting methionine from DM3. The results indicate that in this medium, methionine was not as important as it was in DM1. Hence, some of the nutrients included in DM3 that were not used in DM1 have decreased the methionine requirement.

Finally, cells grew poorly in DM3 containing no vitamins (Table 5.11). Thus, some of the vitamins listed in Table 5.10 are important for growth.

Vitamin Requirements

To determine which vitamins of DM3 are important growth factors for C. thermohydrosulfuricum, cells were grown in 10 different media, each deficient in a single vitamin. The composition of the base medium used was the same DM3. The effect of omitting each vitamin from DM3 on growth is shown in Table 5.12. The data shown were verified by repeated subculturing to ensure that nutrients introduced with the original inoculum were insignificant.

Nicotinic acid, pantothenic acid, and para-aminobenzoic acid (PABA) all appear to be important vitamins for C. thermohydrosulfuricum. In all media used in this experiment, the maximum specific growth rate was approximately 0.2 hr^{-1}, except in medium deficient in vitamin B_{12}. The growth curve obtained in the absence of vitamin B_{12} was atypical. When cells grown in the presence of B_{12} were transferred to B_{12} deficient DM3, growth slowed (for a period of approximately 3 hours) in the middle of the exponential phase. However, cells repeatedly

Table 5.12

Effect of Omitting Individual Vitamins

From DM3 on Growth

Deficient Vitamin	Maximum Optical Density
None	0.39
Biotin	0.38
Folic Acid	0.38
Lipoic Acid	0.40
Nicotinic Acid	0.17*
Pantothenic Acid	0.32*
PABA	0.32*
Pyridoxine	0.41
Riboflavin	0.44
Thiamine	0.40
Vitamin B_{12}	0.39**

*Important for growth

**Unusual growth curve

All cultures were incubated for 38 hours.

subcultured in the absence of vitamin B_{12} grew as well as cells with B_{12}. These findings suggest that vitamin B_{12} may be an important growth factor for C. thermohydrosulfuricum. Vitamin B_{12} is known to be a co-factor in the synthesis of methionine in many organisms (79). This fact supports the idea that B_{12} may be important, since methionine was found to enhance the growth of C. thermohydrosulfuricum.

If the requirement for methionine could be met with B_{12}, the cost of the growth medium may be substantially reduced. Unlike amino acids which are consumed in the synthesis of cellular materials, only minute quantities of vitamins are necessary for microbial metabolism since they normally have only catalytic roles.

The importance of nicotinic acid, pantothenic acid, PABA, vitamin B_{12} and methionine was further examined with the base medium shown in Table 5.13. This medium (DM4) contained no vitamins except nicotinic acid and those supplied by 100 mg/L yeast extract. The effects of various additions to and deletions from DM4 on growth, xylose utilization, and ethanol yield are shown in Table 5.14. The extent and rate of growth with DM4 was approximately the same as with DM3. Thus, vitamins other than nicotinic acid were not needed for growth with 100 mg/L yeast extract in the medium. Reducing the nicotinic acid concentration to 200 μgm/L did not affect growth or ethanol formation. Additional PABA, pantothenic acid, and vitamin B_{12} also had no significant effect.

When methionine was deleted from the medium, growth was very poor. However, with just 2.0 μgm/L vitamin B_{12}, the requirement for methionine is essentially eliminated. No benefit resulted by including PABA and pantothenic acid in the methionine deficient medium. These

Table 5.13

Defined Medium IV

Component	Amount per liter
Yeast Extract	100 mg
Nicotinic Acid	500 µgm
$FeSO_4 \cdot 7H_2O$	2.0 mg
$MgSO_4 \cdot 7H_2O$	50 mg
Methionine	75 mg
KH_2PO_4	2.0 gm
$Na_2HPO_4 \cdot 7H_2O$	10 gm
$(NH_4)_2SO_4$	0.5 gm
Na_2S	0.1 gm
Trace mineral solution	10 ml
Resazurin	2.0 mg

Table 5.14

Growth in Defined Medium IV

Changed Component	Max. O.D.	Xylose Fermented (gm/L)	Ethanol Yield (gm/gm)
None	0.44	6.2	0.28
Reduced: Nicotinic Acid 200 μgm/L	0.39	6.0	0.29
*Add: PABA, 25 μgm/L Pantothenic acid, 100 μgm/L Vitamin B_{12}, 2 μgm/L	0.42	6.1	0.27
*Delete: methionine	0.08	2.8	0.09
*Add: Vitamin B_{12}, 2 μgm/L Delete: Methionine	0.41	5.6	0.30
*Add: PABA, 25 μgm/L Pantothenic acid, 100 μgm/L Vitamin B_{12}, 2 μgm/L Delete: Methionine	0.40	5.5	0.28

*Contained 200 μgm/L nicotinic acid

All cultures were incubated for 35 hours. Maximum specific growth rates in all cases were approximately 0.20 hr^{-1}.

results clearly indicate that vitamin B_{12} is a co-factor in methionine synthesis in C. thermohydrosulfuricum.

To determine wether or not growth in DM4 (with B_{12} substituted for methionine) was stable, cells were subcultured in this medium 6 times. The growth characteristics measured after the last transfer are summarized in Table 5.15. The cells appeared to have adapted somewhat to this medium; the growth rate increased 50% over the rate initially measured with cells transferred directly from YEX1. The ethanol yield measured was slightly lower than the 0.30 gm/gm yield originally observed with this medium; however this may have been the result of inadequate compensation for caramelization that occured during incubation. This possibility is supported by the fact that the carbon recovery was only 84%.

The cost of nutrients used in the growth medium per liter of ethanol produced is summarized in Table 5.16. Using B_{12} instead of methionine results in a significant savings in production costs.

Recommendations

Attempts to further reduce nutrient costs should focus on decreasing the amount of yeast extract used. In addition, most of the results presented have been fairly qualitative. Improved economics will probably result if nutrient concentrations are optimized. Data on mineral requirements (e.g. ammonia and phosphate) may also be important for optimizing the growth medium. Finally, the results presented above were all obtained at low substrate and end-product concentrations. At higher, more inhibitory concentrations (especially of ethanol) additional nutrients may be necessary (39).

Table 5.15

Growth in DM4 with Vitamin B_{12}*

Maximum Optical Density	0.37
Maximum Specific Growth Rate	0.30 hr^{-1}
Total Xylose Fermented	7.2 gm/L
<u>Yields (gm/gm)</u>	
Ethanol	0.26
Acetate	0.068
Lactate	0.18
Cells	0.04
% Carbon Recovered	84

*Medium contained 2.0 μgm/L of vitamin B_{12} and no methionine.

Table 5.16

Cost of Nutrients in Growth Medium

Component	Cents/liter Ethanol
100 mg/L Yeast Extract ($1.78/Kg)	7.5
200 μgm/L Nicotinic Acid ($6.82/Kg)	0.05
2 μgm/L Vitamin B_{12} ($10.75/Kg)*	0.1
75 mg/L Methionine ($6.80/Kg)**	46.0

*Price is for crude extract containing 1% B_{12}.

**Price is for D,L racemic mixture.

All prices are for October, 1982 (71).

5.3. Ethanol Inhibition and Tolerance

5.3.1. Ethanol Inhibition

Another important economic parameter in the production of fermentative ethanol is the cost of recovering the ethanol from the fermentation broth. To minimize this cost, it is necessary to produce ethanol at a high concentration. Therefore, the inhibitive effect of ethanol on the growth of *C. thermohydrosulfuricum* 39E was examined.

Figure 5.8 shows the effect of including 6 gm/L ethanol in YEX1 medium on the growth of strain 39E. Initially, cells were not significantly affected by the ethanol. However, 3 hours after inoculation, growth ceased. After an 8 hour lag, growth resumed but at an inhibited rate. The final extent of growth was also decreased in the presence of ethanol.

Similar growth patterns were observed at initial ethanol concentrations ranging from approximately 2 to 15 gm/L. The rate of growth after the lag phase was found to be dependent on the amount of alcohol added to the medium (see Figure 5.9). Inhibition was severe at very low concentrations; the rate of growth was reduced by 80% in only 10 gm/L ethanol. At concentrations greater than 20 gm/L very little growth was observed. In fact, the culture optical density often decreased at greater than 20 gm/L ethanol, suggesting that high alcohol concentrations caused the cells to lyse.

In addition to inhibiting growth, exposing cells to ethanol also decreased the ethanol yield. The effect of ethanol on end-product distribution is shown in Table 5.17. At 6.2 gm/L, the ethanol yield was reduced by approximately 50% while the yields of lactate and acetate

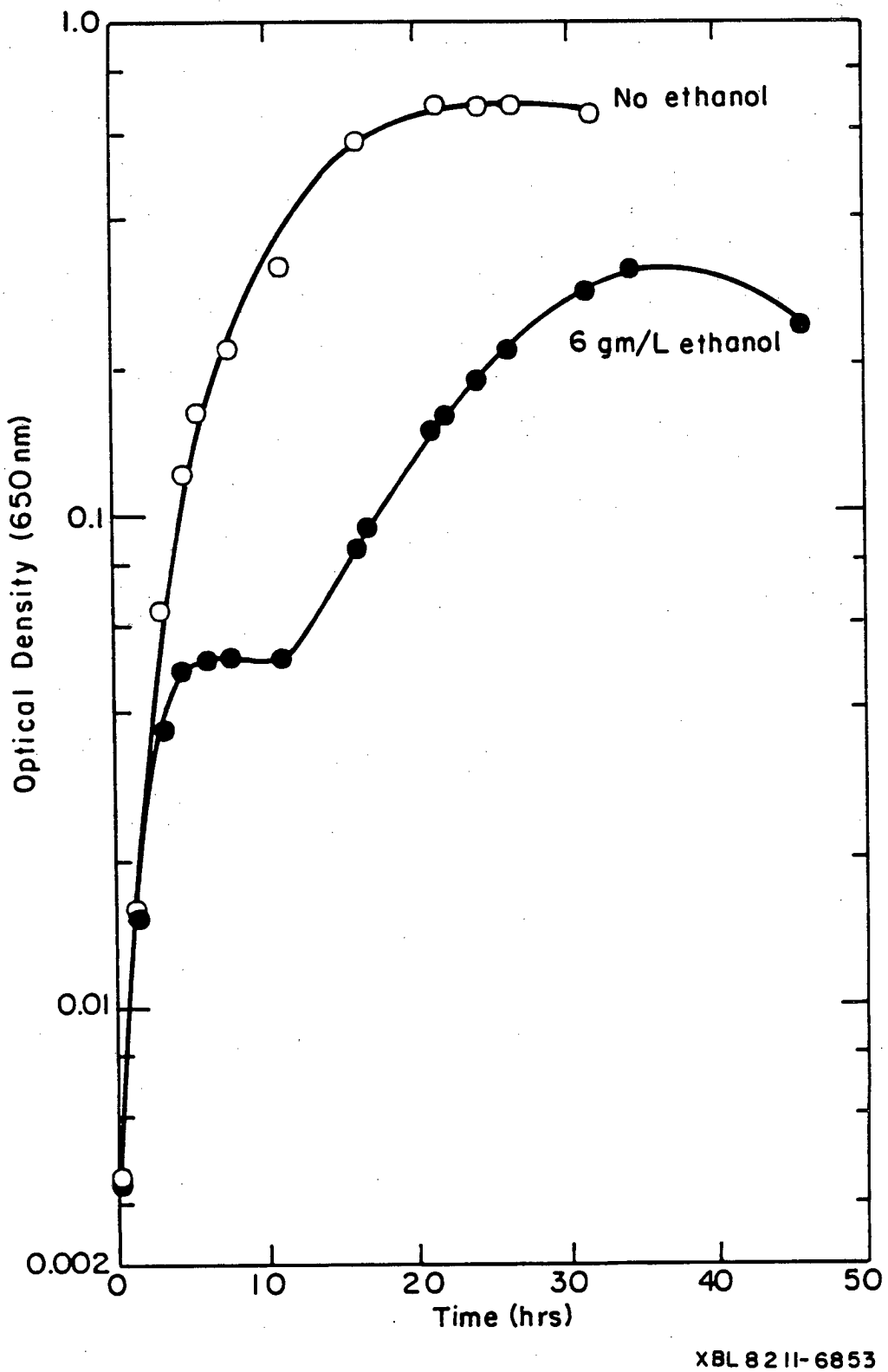

Figure 5.8. Effect of 6 gm/L Ethanol on the Growth of Strain 39E.
Ethanol was introduced to the culture at time zero.

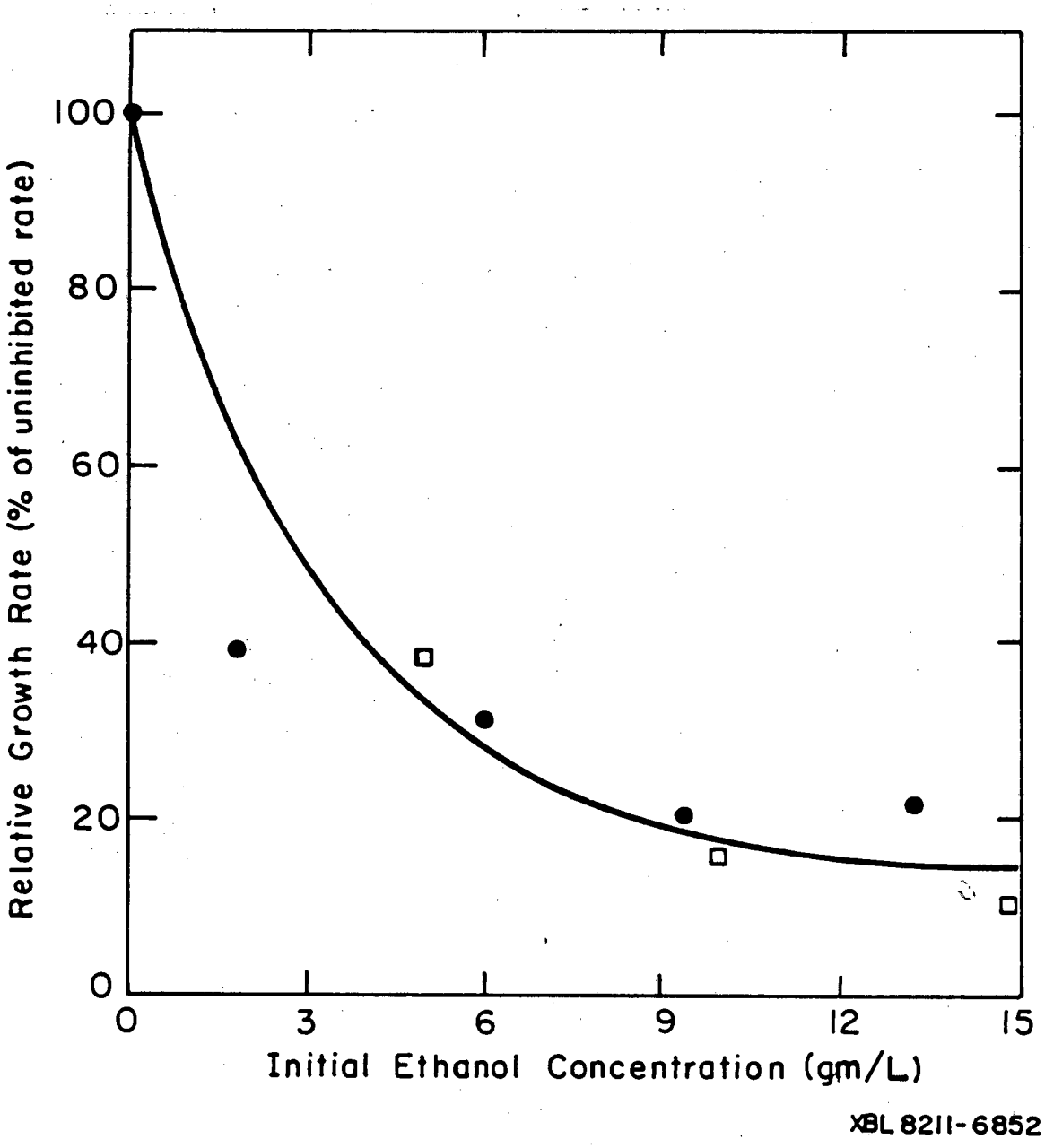

Figure 5.9. Effect of Ethanol Concentration on Growth Rate Following Lag Phase.
The different symbols used represent the results of independent experiments.

Table 5.17

Effect of Initial Ethanol Concentration on the Yield of Strain 39E

Initial Ethanol Concentration (gm/L)	0	6.2	10.0
Total Xylose Fermented (gm/L)	9.1	6.7	6.0
Length of Fermentation (hrs)	30	55	55
Yields (gm/gm)			
Ethanol	0.31	0.15	0.03
Acetate	0.071	0.22	0.25
Lactate	0.18	0.43	0.46
% Carbon Recovered	97	110	95

were increased. At 10 gm/L even less ethanol and more acetate and lactate were formed. Apparently, the metabolic pathway leading to ethanol formation is feedback inhibited. Zeikus has demonstrated that NADP-linked alcohol dehydrogenase (ADH) is not strongly inhibited by ethanol in strain 39E (section 3.3.3). Hence, the reduced yield may be the result of reduced NAD-linked ADH activity, inhibition of acetaldehyde reductase, or repression of formation of any of these enzymes.

Mechanism for Ethanol Inhibition

Ethanol inhibition has been shown to be related to changes in the cell membrane (80,81,82,83,84). During the initial phase of growth (Figure 5.8), the freshly inoculated cells were probably not in equilibrium with their environment. However, after 3 to 4 hours, the ethanol had sufficient time to diffuse into the membrane and caused a total cessation of growth.

Ethanol is an amphipathic molecule and can interact with polar and non-polar molecules. In the membrane, it disrupts the non-polar interactions between phospholipids which results in decreased membrane fluidity. Membrane-bound enzymes can not function properly in the "stiff" membrane; hence, growth is inhibited. (80,82).

During an ethanol induced lag phase, which has been observed with other bacteria (80), cells are believed to undergo an adaptation process where the structure of the membrane is altered to compensate for the ethanol. Unsaturated fatty acids are substituted for saturated fatty acids, whereby the fluidity of the membrane is increased and growth resumes (79,80,83). This phenomenon probably also occurs in *C. thermohydrosulfuricum*.

An inhibited rate of growth, which was observed with strain 39E following the adaptation period, has also been reported for other bacteria (80,82). This result is believed to be due to a direct inhibition of glycolysis, which reduces the rate of ATP generation and subsequently, the rate of growth (85). Recent evidence indicates that some of the enzymes of the glycolytic pathway are bound to the membrane (86,87). Hence, changes in membrane structure and inhibition of glycolysis may be closely related (84).

At high ethanol concentrations (20 gm/L), strain 39E grew only slightly and lysis was often observed. Lysis by ethanol is believed to be due to inhibition of peptidoglycan cross-linking in the cell wall (81). Enzymes on the outside of the cell membrane, necessary for cross-linking, can not function at high ethanol concentrations. The weak uncross-linked cell wall formed in the presence of ethanol eventually fails and the cells lyse.

5.3.2. Adaptation

To alleviate the problem of inhibition, strain 39E was slowly adapted to high concentrations of ethanol. Cells exposed to ethanol for extended periods of time eventually were able to grow rapidly at high alcohol concentrations.

Adaptation Procedure

Initially, cells were grown batch-wise in 5 gm/L ethanol for 48 hours. This late exponential-phase culture was used to inoculate (5% v/v inoculum) medium containing 10 gm/L ethanol. Subculturing in this manner was repeated with the ethanol concentration being increased by 5 gm/L between each transfer. Cells transferred from 25 gm/L ethanol were unable to grow at 30 gm/L, hence the change in ethanol concentration

between transfers had to be reduced to 2 gm/L. To continue adaptation above 35 gm/L, the change had to be further reduced to 0.5 gm/L. After more than one month of repeated subculturing, adapted cells were able to grow in 40 gm/L ethanol. The adapted culture was purified by isolation in deep agar containing 30 gm/L ethanol (see Appendix I for deep-agar isolation procedure).

5.3.3. De-adaptation and Maintenance of Tolerance

Adapted cells could be grown in the absence of ethanol for many generations without de-adapting significantly. For example, cells able to tolerate 40 gm/L ethanol were grown in YEX1 medium with no alcohol for 30 generations. When these cells were returned to medium containing ethanol, they were able to grow at concentrations as high as 35 gm/L. Apparently, regulatory mechanisms responsible for controlling tolerance are slow to respond when stress by ethanol is removed.

Some de-adaptation was apparent when cells were stored for long periods of time (approximately one month) in the absence of ethanol. Tolerance was best maintained by storing cultures in medium containing ethanol (35 to 40 gm/L) at room temperature. Stock cultures of tolerant cells were subcultured monthly.

5.3.4. Comparison of Parent and Adapted Strains

To assess the degree of tolerance of the new strain (designated UC-35), it was grown in YEX1 medium containing various concentrations of ethanol. The results summarized in Figures 5.10 and 5.11 compare the inhibitive effects of ethanol on UC-35 and 39E. UC-35 grew much faster and to a far greater extent than the parent strain (39E) in the presence of ethanol. The growth rate of UC-35 at 35 gm/L was 0.30 hr^{-1}. It is interesting to note that the adapted strain grew at an optimum

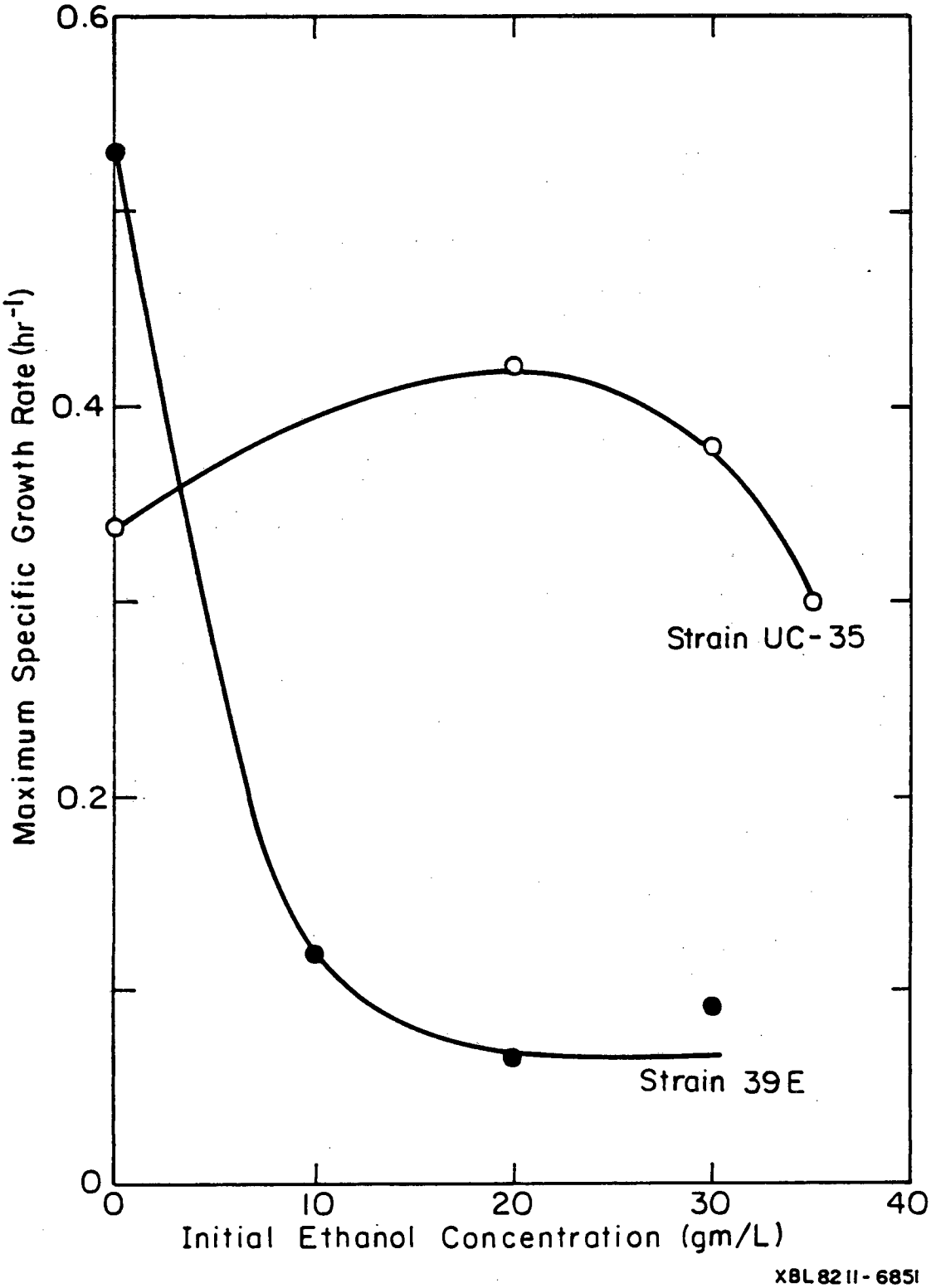

Figure 5.10. Growth Rates of 39E and UC-35 in the Presence of Ethanol.

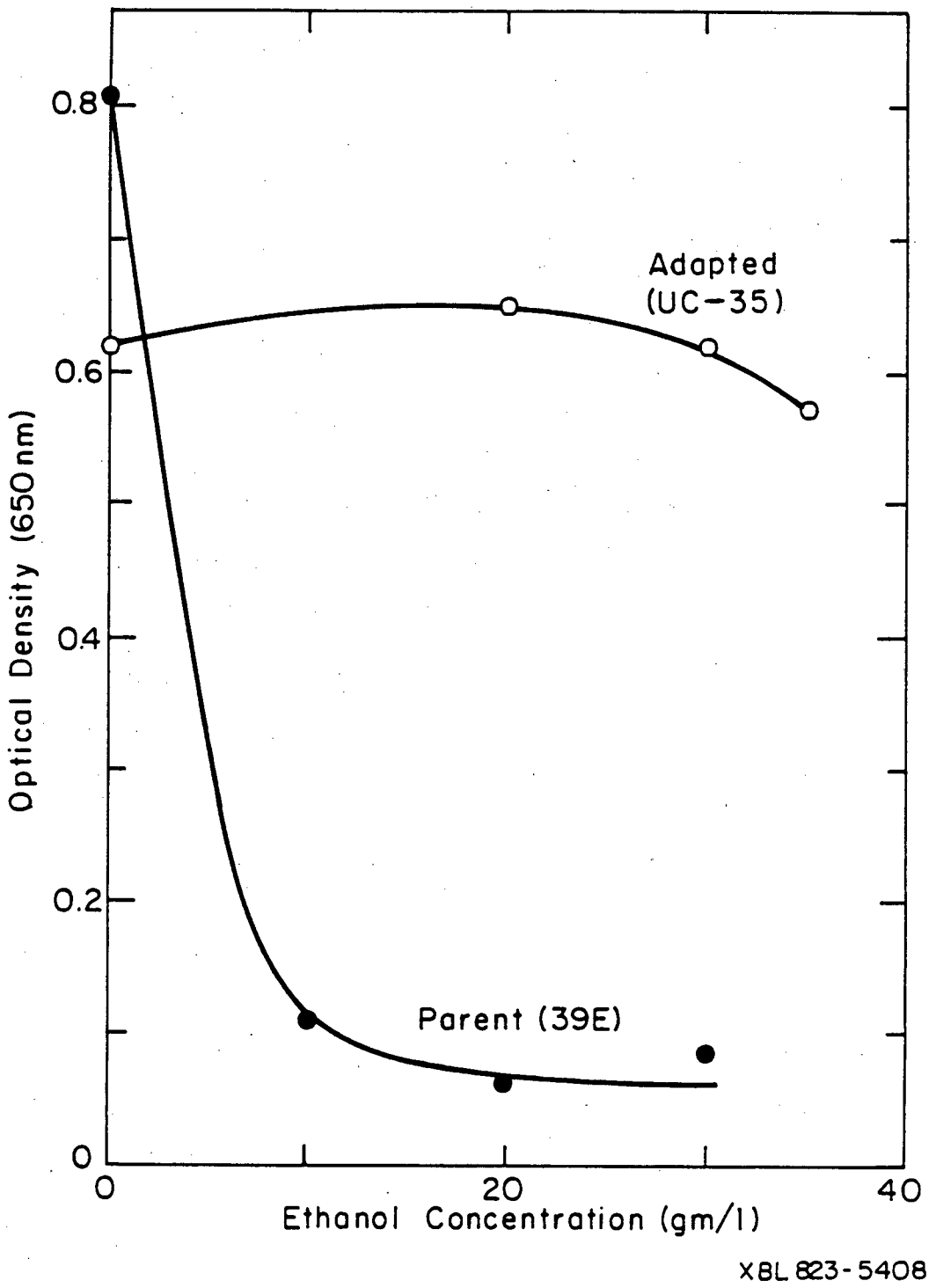

Figure 5.11. Maximum Extent of Growth of 39E and UC-35 in the Presence of Ethanol.

rate at 20 gm/L. Apparently, the cell membrane of UC-35, which probably contains a high degree of unsaturated fatty acids, is too fluid to function optimally in the absence of ethanol.

The end-product yields from xylose for strains 39E and UC-35 in medium containing 0 and 10 gm/L ethanol are compared in Table 5.18. The results shown were obtained with YEX2 medium (same as YEX1, with 20 gm/L xylose and 5 gm/L yeast extract). The purpose of increasing the initial xylose concentration was to see if ethanol tolerant UC-35 is able to produce a higher final ethanol concentration than 39E. With no ethanol initially, the yields of strain 39E and UC-35 were 0.36 and 0.23 gm/gm, respectively. Both acetate and lactate yields were higher with UC-35. These results are fairly surprising since it was expected that the ethanol tolerant strain would produce a higher yield of ethanol than the parent, which is strongly feedback inhibited by ethanol. At 10 gm/L ethanol initially, the yield of 39E was reduced significantly to 0.17 gm/gm while the yield of UC-35 dropped only slightly. Hence, although it has a lower yield than the parent, ethanol formation by UC-35 is not strongly feedback inhibited. Carbon utilization was incomplete with UC-35 because excessive acid formation decreased the pH to 5.0 which caused growth and end-product formation to stop.

A second adapted strain was developed independently of UC-35 to see if decreased ethanol yield with increased tolerance is truly characteristic of this bacterium. This strain also had a reduced yield of ethanol.

The shift in the distribution of fermentation products with adapted strains may be related to changes in membrane structure. Membrane-bound enzymes may be affected by the highly unsaturated

Table 5.18

Comparison of Yields for Strains 39E and UC-35*

Strain	39E		UC-35	
Initial Ethanol Concentration (gm/L)	0	10	0	10
Total Xylose Fermented (gm/L)	18.3	2.9	12.4	11.7
Final Culture pH	6.1	6.7	5.0	5.1
Yields (gm/gm)				
Ethanol	0.36	0.17	0.23	0.19
Acetate	0.048	0.34	0.15	0.11
Lactate	0.13	0.01	0.37	0.37
% Carbon Recovered	91	85	105	91

*Cells were grown in YEX2 which contained 20 gm/L xylose and 5 gm/L yeast extract. Cultures were incubated for 28 hours.

membrane in such a way that ethanol formation is decreased in favor of organic acid formation.

5.3.5. Further Adaptation

UC-35 was further adapted to produce a strain able to tolerant 42 gm/L ethanol. This strain (UC-42) had a maximum specific growth rate of 0.29 hr^{-1} in 40 gm/L ethanol. After re-isolation, the ethanol yield of UC-42 in YEX2 was 0.20 gm/gm, not significantly different from that of UC-35.

Further adaptation of this bacterium may be possible. However, the practicality of further adaptation is questionable given the low yield. Although product purification costs are significant, the low ethanol yield is economically very unattractive and it will have a much stronger bearing on the economic feasibility of using this bacterium in a large scale process than increased tolerance. Hence further experimentation was directed toward examining parameters which affect the yield of ethanol.

5.4. Effect of pH on Strain UC-42

The effect of pH on ethanol yield, growth rate, and specific ethanol productivity of UC-42 was examined in batch culture. Figures 5.12 through 5.16 show the fermentation profiles obtained at pH's ranging from 7.9 to 6.0 in YEX1 medium. The dependence of growth rate and specific ethanol productivity on pH is shown in Figure 5.17. Since the pH of the inoculum was approximately 6.7 in all cases, cells were allowed to adapt at each pH for 5 generations before growth rate and ethanol productivity were determined. The growth rate was highest at pH 7.2 but 6.8 appears to be the true optimum for growth since exponential growth was more sustained. At 6.0, growth stopped abruptly when the

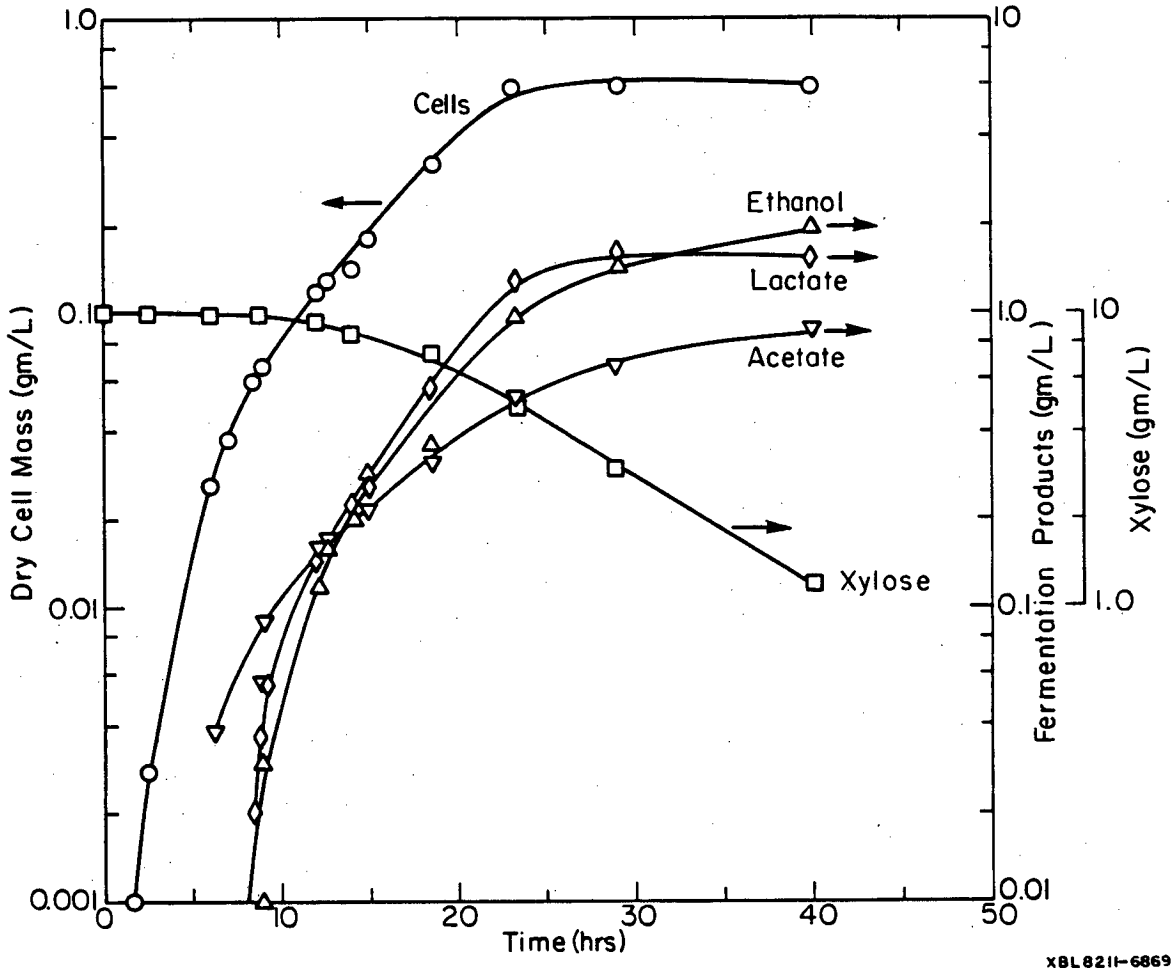

Figure 5.12. Effect of pH on the Fermentation Profile of Strain UC-42. pH controlled to 7.9.

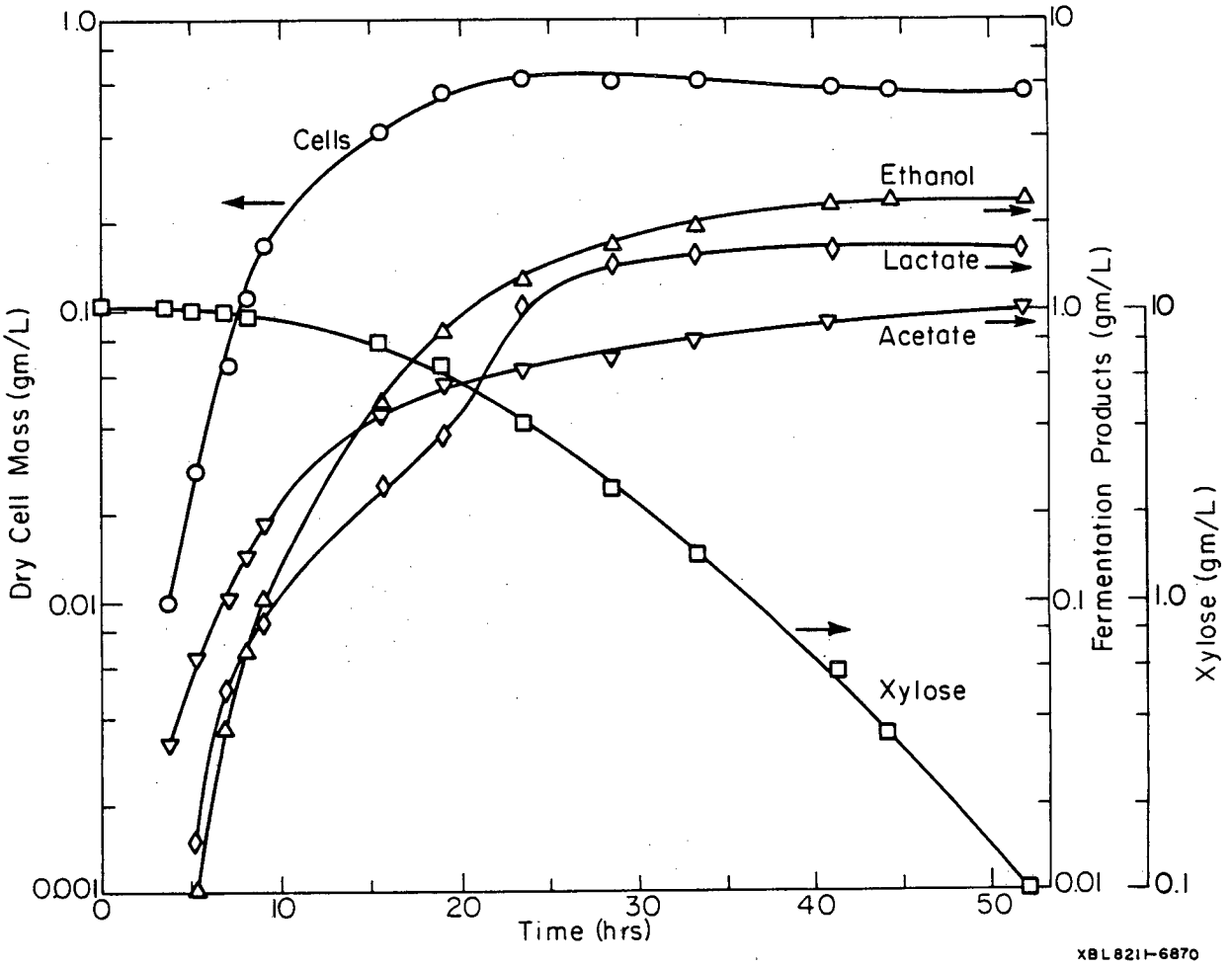

Figure 5.13. Effect of pH on the Fermentation Profile of Strain UC-42. pH controlled to 7.2.

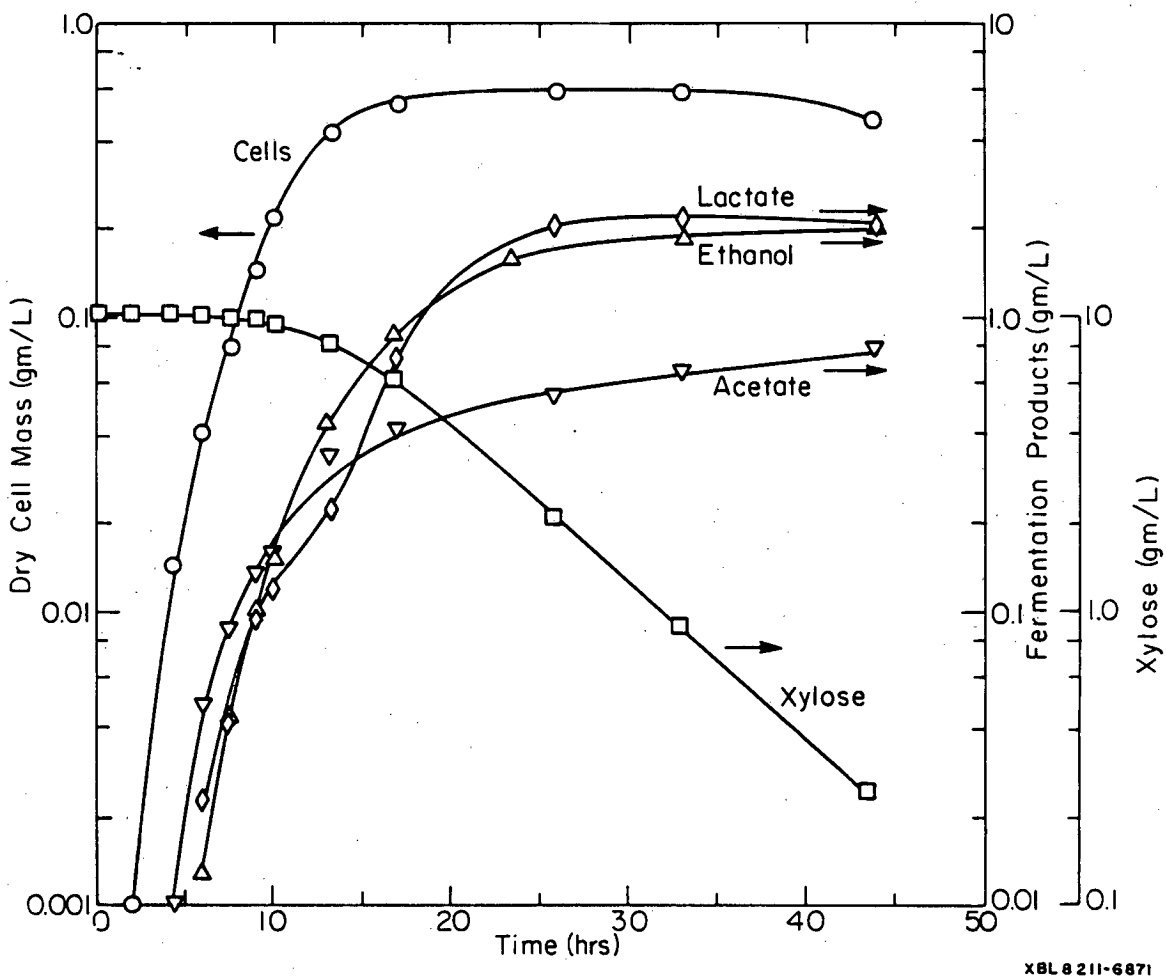

Figure 5.14. Effect of pH on the Fermentation Profile of Strain UC-42. pH controlled to 6.8.

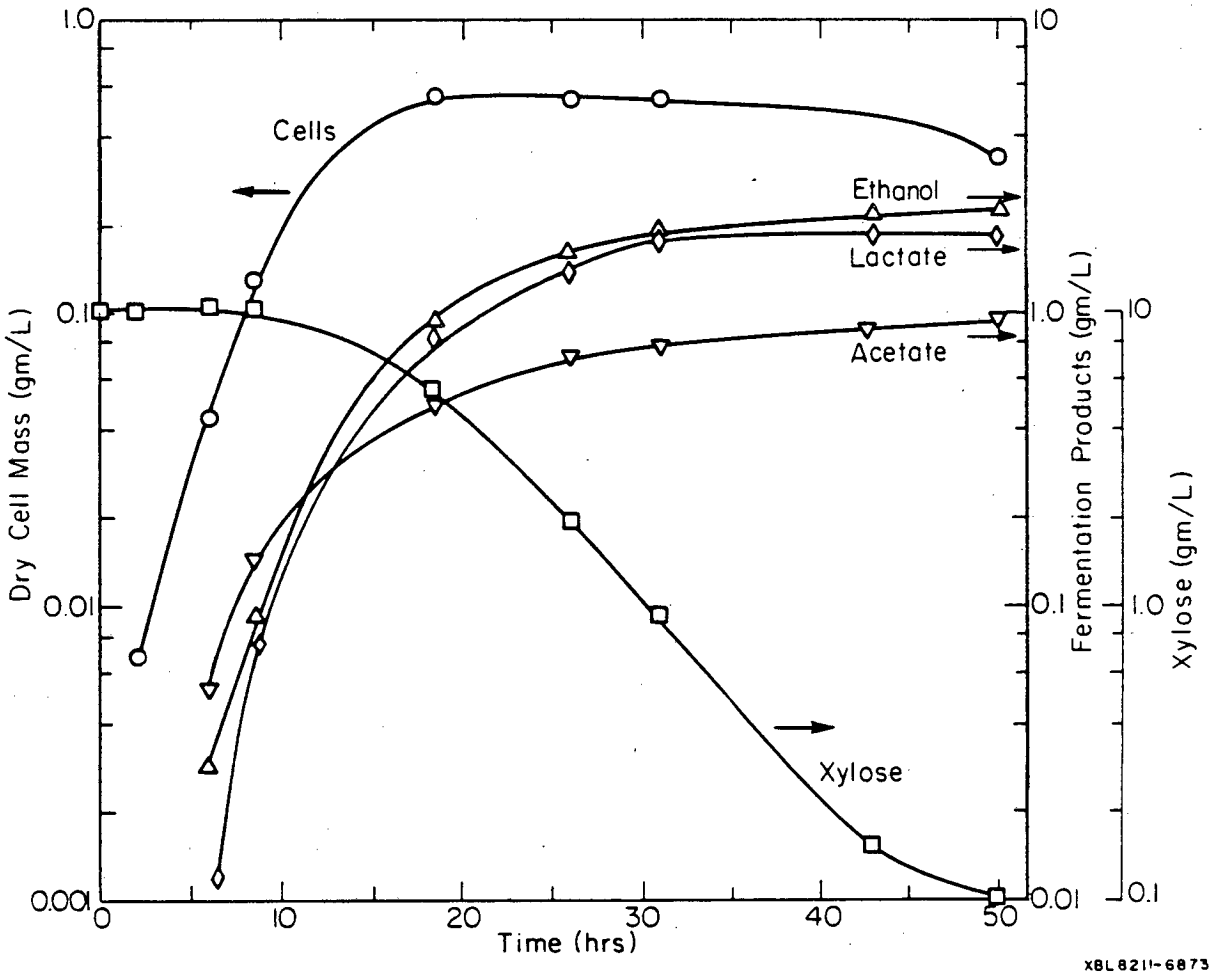

Figure 5.15. Effect of pH on the Fermentation Profile of Strain UC-42. pH controlled to 6.5.

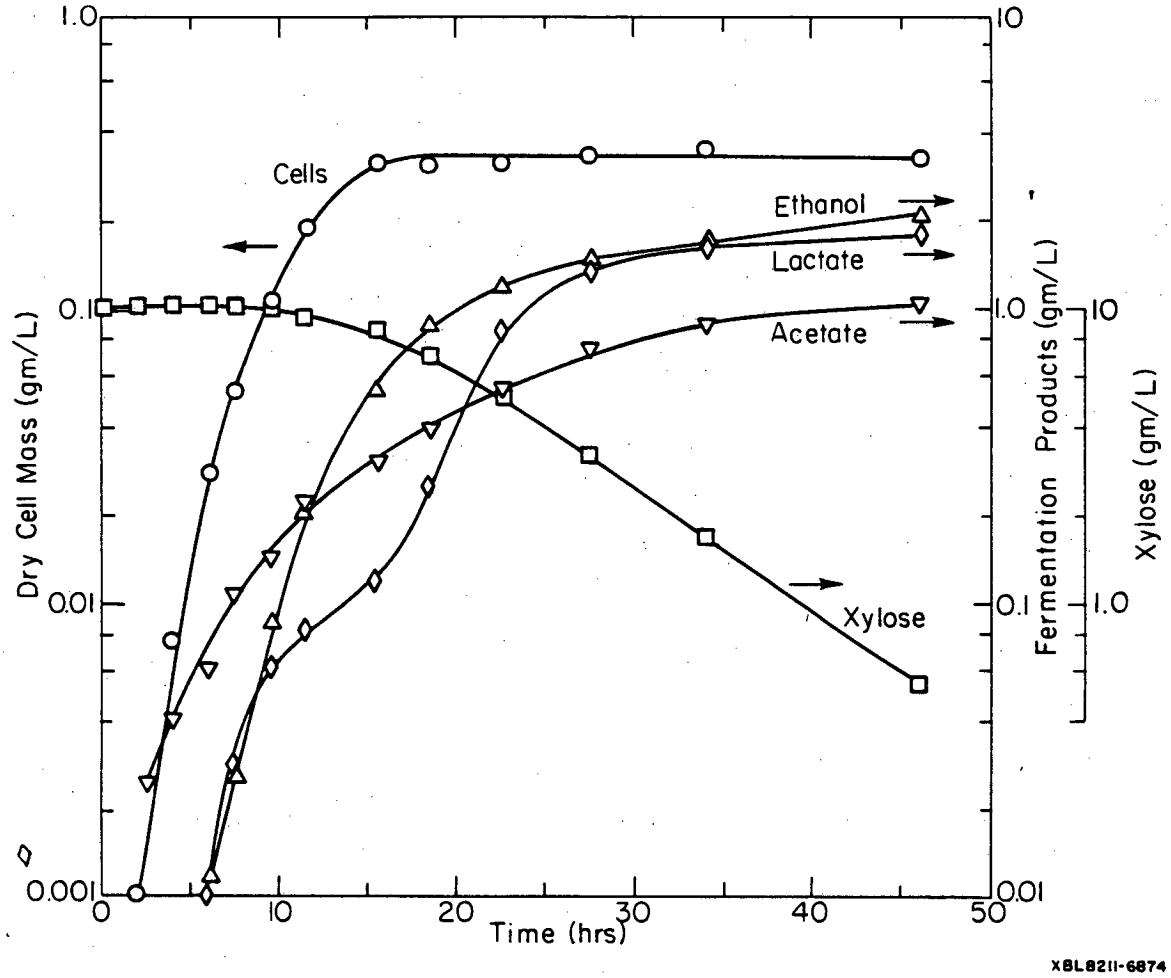

Figure 5.16. Effect of pH on the Fermentation Profile of Strain UC-42. pH controlled to 6.0.

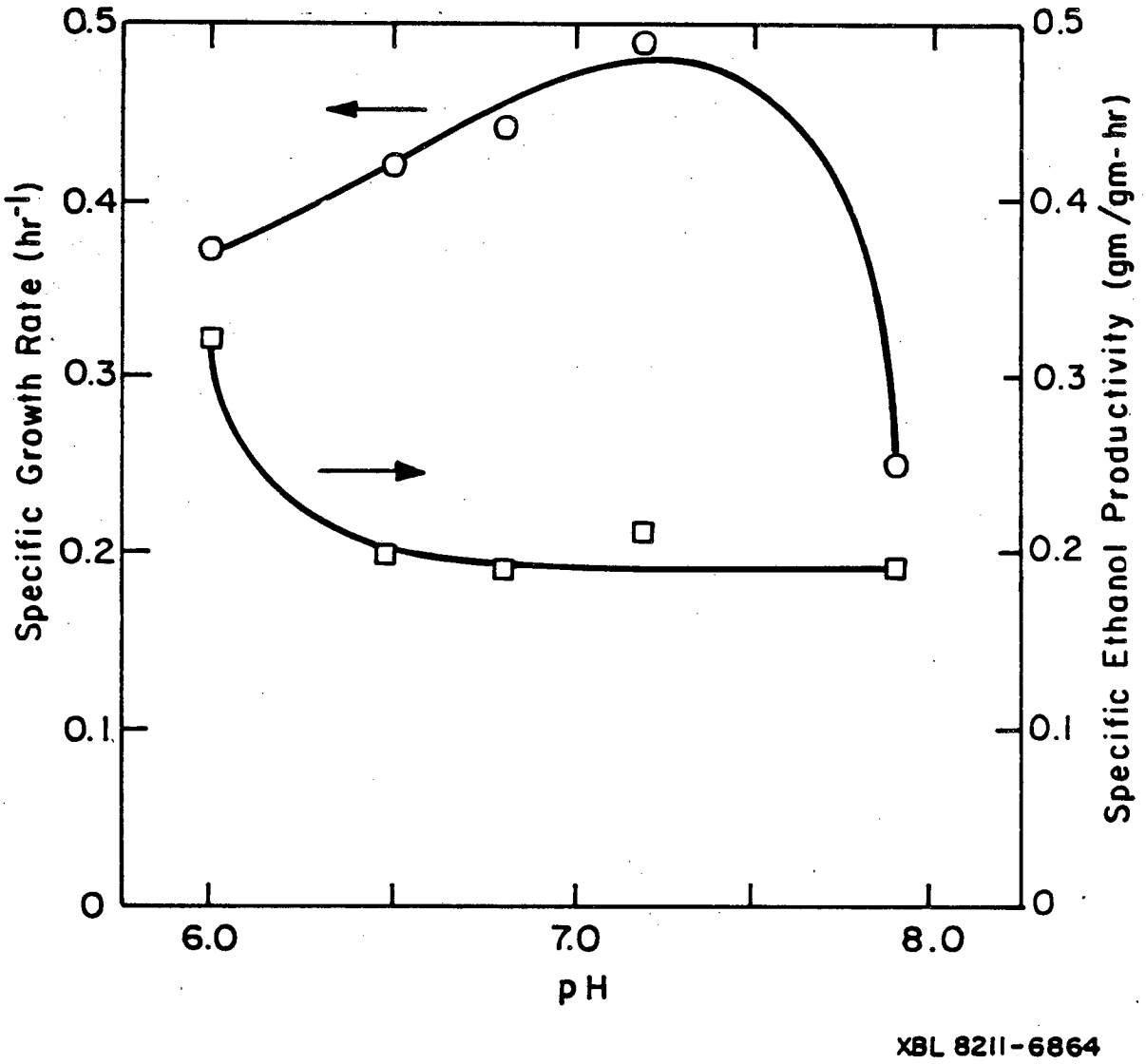

Figure 5.17. Dependence of Specific Growth Rate and Specific Ethanol Productivity of Strain UC-42 on pH.

cell mass concentration reached 0.33 gm/L; at all other pH's the final cell mass concentration was about 0.6 gm/L. In a duplicate experiment at pH 6.0, the abrupt stop was again observed. During the stationary phase at pH 6.0, foaming was apparent in the fermentor suggesting that cells were lysing. The reason for the cessation of growth and cellular lysis was not apparent. The high specific ethanol productivity at pH 6.0 reflects the poor cell growth; ethanol accumulated at about the same rate as at other pH's. In addition, lactate formation lagged significantly behind growth at pH 6.0. At pH's 6.8 and 7.2, similar lags were observed (although they were not as pronounced) while at pH 7.9, lactate formation was strongly growth associated. Data at pH 6.5 were insufficient to determine whether or not a similar lag occurred.

The yields of ethanol, acetate, and lactate at the end of the growth phase are summarized in Table 5.19. The ethanol yield was highest at pH 6.0, due to the lack of growth associated lactate formation. At pH 7.9, lactate was the predominant end product.

The overall yields for the entire batch fermentaton are listed in Table 5.20. In general, the overall ethanol yield did not change significantly with pH. Hence, the optimum pH for batch ethanol production is between 6.5 and 6.8 where growth was rapid and sustained.

For continuous culture, it appears that pH 6.0 would be optimal, since specific ethanol productivity and growth associated ethanol yield were highest at this pH. Whether or not sustained growth at pH 6.0 is possible will have to be determined in continuous culture.

Total carbon recoveries in pH controlled experiments were consistently low (see Tables 5.19 and 5.20). This may be related to the low yields of lactate (approximately 0.18 gm/gm) also observed in all pH

Table 5.19

Effect of pH on Growth Associated Yields

pH	6.0	6.5	6.8	7.2	7.9
Xylose fermented before stationary phase (gm/L)	3.3	5.0	4.1	6.1	5.25
Yields (gm/gm)					
Ethanol	0.27	0.20	0.21	0.21	0.18
Acetate	0.12	0.098	0.10	0.10	0.10
Lactate	0.078	0.17	0.18	0.17	0.24
%Carbon Recovered	84	81	87	83	85

Table 5.20

Effect of pH on Overall Batch Yields

pH	6.0	6.5	6.8	7.2	7.9
Yields (gm/gm)					
Ethanol	0.22	0.22	0.20	0.23	0.22
Acetate	0.11	0.095	0.074	0.10	0.10
Lactate	0.18	0.18	0.21	0.16	0.17
% Carbon Recovered	82	82	79	84	82

controlled fermentations. Lactate yields in non-pH controlled experiments with UC-42 were typically 0.4 gm/gm (see Tables 5.18 and 5.21). The exact reason for the low carbon recoveries and low lactate yields is not apparent.

5.5. Effect of Acetate, Lactate, and Xylose on Growth Rate and Yields

The inhibitive effects of high concentrations of acetate, lactate, and xylose on growth were examined. The results obtained may be useful in designing fermentation plants where high concentrations of these compounds are often necessary. The effect of acetate and lactate on the ethanol yield was also studied. If either by-product is feedback inhibited, an enhancement of ethanol yield may result at high concentrations of these organic acids.

The effect of xylose, acetate, and lactate on the growth of UC-42 is shown in Figures 5.18, 5.19, and 5.20. The maximum specific growth rates measured are summarized in Figure 5.21. For xylose, the rates of growth were approximately equal at 10 and 20 gm/L. However, the extent of growth at 20 gm/L was less than at 10 gm/L, possibly due to inhibitory caramelization products which formed during incubation at the high growth temperature (88). Inhibition was severe at 60 gm/L and extensive caramelization was observed during the fermentation. The reduced initial rate of growth was probably due to inhibition by xylose and not by caramelized xylose since growth was slow even before significant caramelization was observed. In addition, acetate and lactate yields were increased when caramelization was extensive.

These results indicate that this organism is not well suited for industrial batch fermentation where initial sugar concentrations are typically very high.

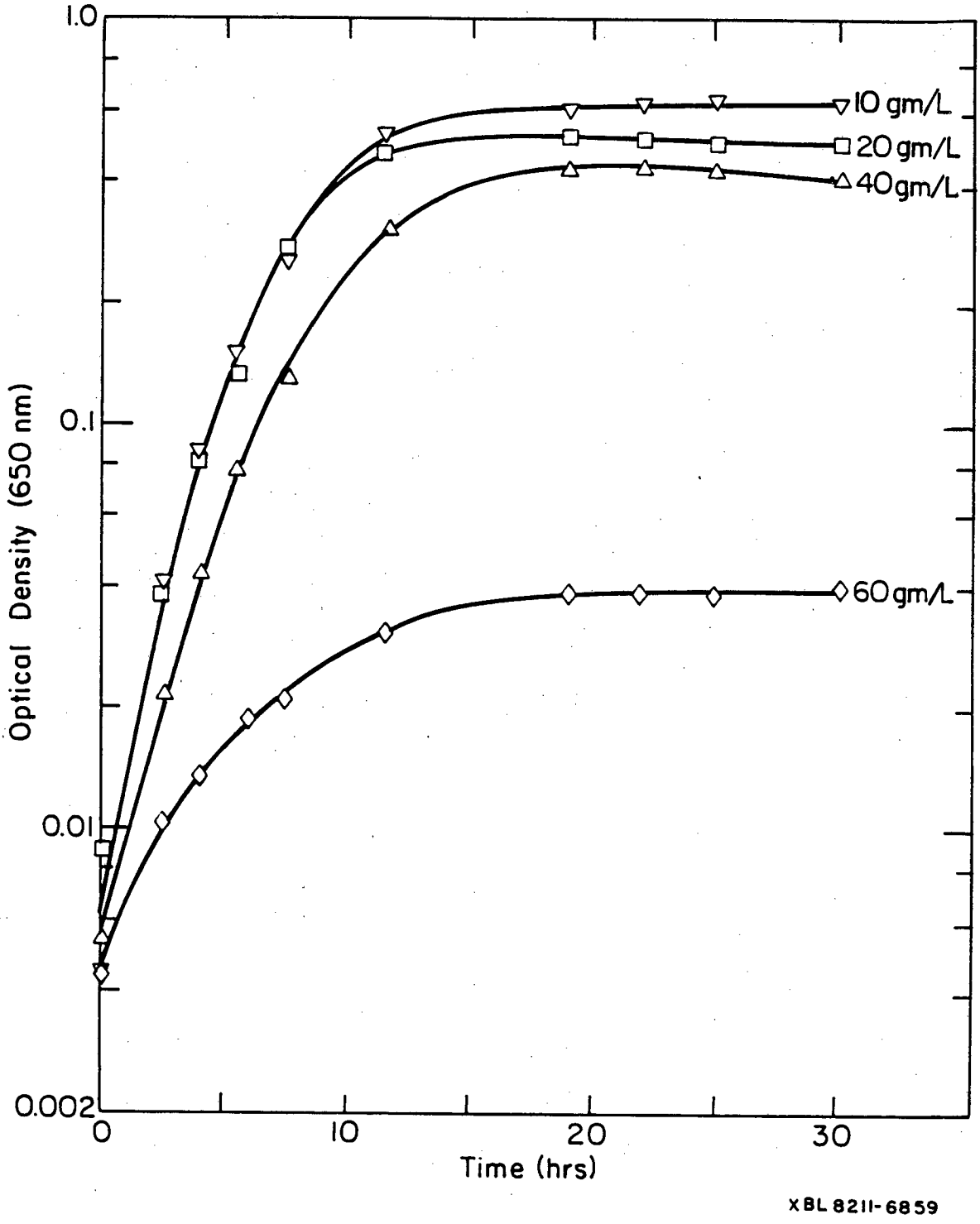

Figure 5.18. Inhibition of Growth of UC-42 by Xylose.

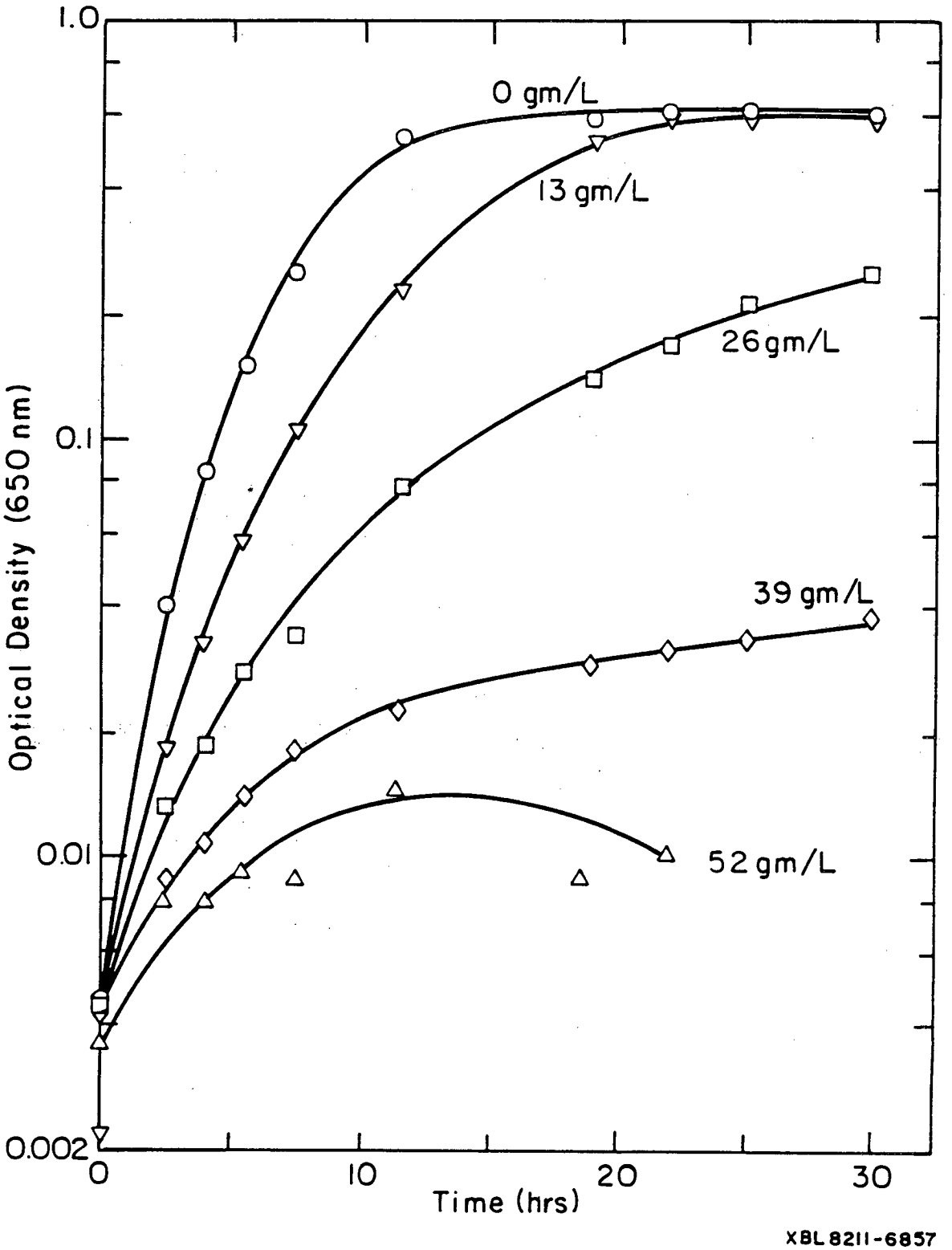

Figure 5.19. Inhibition of Growth of UC-42 by Acetate.

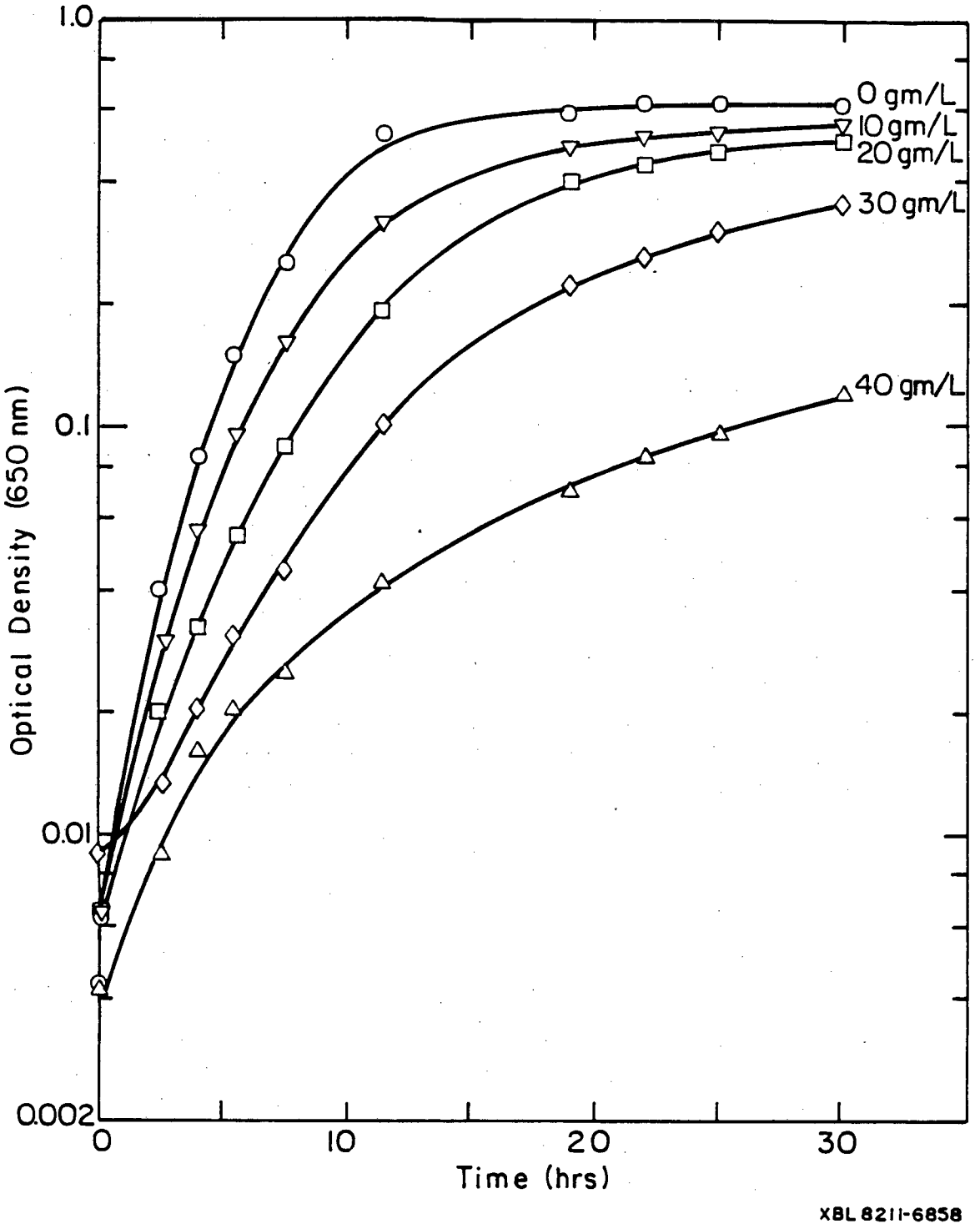

Figure 5.20. Inhibition of Growth of UC-42 by Lactate.

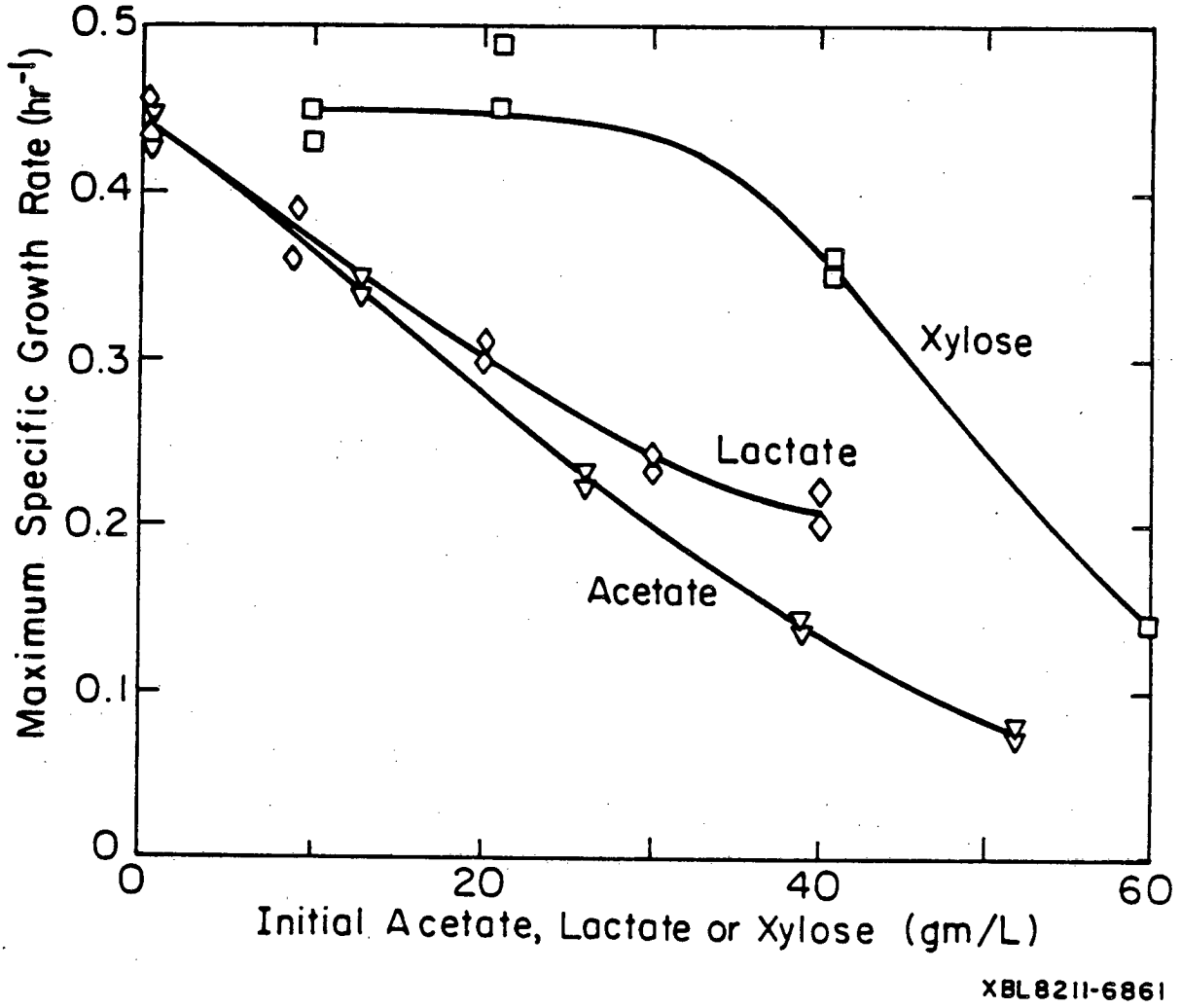

Figure 5.21. Dependence of Initial Specific Growth Rate of UC-42 on the Concentration of Acetate, Lactate, and Xylose.

Results in Figure 5.21 indicate that acetate was more inhibitory than lactate; 50% inhibition of growth occurred at 40 and 27 gm/L for lactate and acetate, respectively. In a continuous process where ethanol is removed selectively to increase the fermentation rate (e.g. vacuum fermentation), inhibition by acetate or lactate may limit the rate of ethanol production.

The effects of acetate and lactate on end-product yields are shown in Table 5.21. In the presence of 13 gm/L acetate, the yield of ethanol is increased by 17% while the yield of acetate is suppressed. At 26 gm/L, similar results were obtained. Hence, acetate formation appears to be feedback inhibited at fairly low concentrations. It should be noted that the yields of acetate shown are probably not extremely accurate. The amount of acetate produced at 13 gm/L was only 0.32 gm/L; hence, the change in acetate concentration was 3%. The accuracy of the acetate assay is only 3%, whereby precisely determining the small change was not possible.

Lactate formation did not appear to be strongly feedback inhibited. 10 gm/L lactate did not affect the yields of lactate and ethanol significantly but may have caused a slight increase in acetate yield. A corresponding decrease in cell yield (30%) was also observed. At 20 gm/L lactate, it was difficult to accurately measure the lactate yield, but the acetate yield was further enhanced. The poor carbon balance (80% carbon recovered) indicates that the lactate yield shown may be erroneously low. Thus, lactate formation may be slightly feedback inhibited; however, the inhibition is not advantageous since the ethanol yield is unaffected.

These results suggest that in processes employing selective

Table 5.21

Effect of Acetate and Lactate on End-Product Yields of UC-42.

	Control	Acetate		Lactate	
Initial Concentration (gm/L)		13	26	10	20
Xylose Consumed (gm/L)	8.5	7.9	5.0	7.9	7.2
Yields (gm/gm)					
Ethanol	0.18	0.21	0.21	0.18	0.19
Acetate	0.068	0.04*	0.0*	0.082	0.10
Lactate	0.43	0.46	0.40	0.45	0.23*
%Carbon Recovered	95	96	90	97	80

*Results were not accurately determined (see text).

ethanol removal, where high concentrations of by-product organic acids may develop, a slight increase in ethanol yield may result. However, this advantage will be offset, at least in part, by the reduced rate of growth caused by these compounds.

5.6. Improving Ethanol Yield by Mutation

5.6.1. Selection of Mutants

The yield of ethanol can be increased by developing mutants unable to produce acetate and lactate. This type of mutation was found to be very successful with *C. thermosaccharolyticum* (see section 2.3.4).

Ethyl methane sulfonate, a chemical mutagen, was used to induce mutations in *C. thermohydrosulfuricum* UC-42. The desired mutants were selected with plating techniques which employed colorometric methods to detect acid formation.

Detailed procedures for treating cultures with ethyl methane sulfonate and isolating mutants are given in Appendix I. Of the different stains examined, pH indicators were found to be the most useful for selecting low-acid producing mutants.

Using methyl red to detect acid formation, 2,500 colonies were screened. Eight of these, which appeared to produce little or no acid, were selected for further evaluation. These clones were grown in YEX3 medium (same as YEX1 but with 4 gm/L yeast extract) and the distribution of end products formed is given in Table 5.22. Isolate E produced more ethanol and much less lactate than any of the others selected. This isolate, designated UC-42-L1, did not de-adapt significantly during the selection process; the growth rate in 35 gm/L ethanol was found to be 0.25 hr^{-1}.

To compare the ethanol yields of UC-42 and UC-42-L1, both

Table 5.22

Fermentation Products for Methyl Red Isolates

Isolate	Ethanol	Acetate	Lactate
A	1.83	0.61	3.04
B	2.21	0.53	1.97
C	1.81	0.73	3.63
D	2.09	0.64	2.96
E	2.46	0.73	1.17
F	2.22	0.60	2.88
G	2.01	0.64	2.89
H	1.65	0.64	2.29

All concentrations are in gm/L. Results were determined after 28 hours of growth in YEX3 medium.

strains were grown in YEX1 medium. UC-42-L1 produced a 25% higher yield of ethanol than UC-42. Unfortunately, after a two week storage period and an additional 30 generations of growth, the yield of UC-42-L1 was identical to that of UC-42. Apparently, the mutation causing a reduction in lactate formation was unstable and UC-42-L1 reverted back to the original genotype.

Further screening (approximately 2,000 colonies) with another pH indicator, 2-(2,4-dinitrophenylazo)-1-napthal-3,6-disulfonic acid (DND), was conducted and 9 new isolates were selected. The fermentation products formed by these new clones in YEX1 medium are shown in Table 5.23. Note that in all cases (including the control) ethanol formation was low, probably due to a lack of iron in the medium (see section 5.2.1). Nevertheless, isolate J produced significantly more ethanol and isolate N produced significantly less lactate than UC-40. Isolates J and N were re-isolated and grown in YEX1 containing additional iron (2.0 mg/L $FeSO_4 \cdot 7H_2O$). The results are shown in Table 5.24. The yield of isolate J was the same as that of UC-42, indicating that the results shown in Table 5.23 for this clone were probably in error. The ethanol yield of isolate N (designated UC-42-L2) was 13% higher than UC-42. When transferred to the ethanol containing medium, UC-42-L2 was able to grow in concentrations up to 38 gm/L; the rate of growth at 35 gm/L was 0.26 hr^{-1}. Again, only slight de-adaptation occurred during the selection process.

5.6.2 Evaluation of Strain UC-42-L2

UC-42-L2 was grown in a fed-batch fermentation to determine the maximum concentration of ethanol that can be produced by this strain. Xylose was fed periodically to the fermentation broth to maintain the

Table 5.23

Fermentation Products for DND Isolates

Isolate	Ethanol	Acetate	Lactate
J	1.83	0.72	3.31
K	1.32	0.68	2.46
L	1.34	0.67	3.16
M	1.40	0.66	2.01
N	1.29	0.72	1.31
O	1.11	0.77	2.45
P	1.09	0.79	3.29
Q	1.06	0.79	3.21
R	1.29	0.69	3.00
UC-42	1.36	0.62	3.08

All concentrations are in gm/L.

Results were determined after 36 hours of growth in YEX1 medium.

Table 5.24

Comparison of Isolates J and N to UC-42

	UC-42	J	N
Ethanol (gm/L)	1.80	1.90	1.73
Lactate (gm/L)	2.5	2.8	1.5
Ethanol Yield (gm/gm)	0.23	0.22	0.26
Ethanol/Lactate (gm/gm)	0.72	0.69	1.15

sugar concentration between 4 and 10 gm/L. If all of the sugar had been added at the beginning of the fermentation, extensive caramelization would have resulted.

Xylose was fed to the fermentor as a concentrated syrup (500 gm/L). For every 5 grams of sugar added, 1 gram of yeast extract, 50 mg of ammonium chloride, 1 mg of ferrous sulfate and 1.25 ml of vitamin solution were also added. Initially the fermentor was filled with 2 liters of YEX1 (0.011 M phosphate) medium containing 4 gm/L yeast extract so that a high cell density would build up quickly. After inoculation, xylose and other nutrients were added when the cells reduced the residual sugar concentration to less than about 4 gm/L. The pH was controlled at 6.5.

The results for the fed-batch growth of strain UC-42-L2 are shown in Figure 5.22. Initially, growth was fairly rapid at 0.43 hr^{-1}. However, after 12 hours, the fermentor pH dropped to 6.4 and the cells began to lyse. Since lysis was generally observed at low pH, the pH was increased to 6.7, after which growth resumed.

The specific ethanol productivity reached a maximum of 0.25 gm/gm-hr 20 hours after inoculation and dropped steadily for the duration of the fermentation. The time courses of specific ethanol productivity and ethanol yield are shown in Figure 5.23. The ethanol yield remained fairly high at 0.25 gm/gm for 30 hours during the middle of the fermentation but dropped rapidly for the last 20 hours. As the ethanol yield dropped, a corresponding increase in lactate formation was observed. The final end-product concentrations were 8.8 gm/L ethanol, 4.6 gm/L acetate, and 13 gm/L lactate. The total carbon recovery dropped from 88% at 56 hours, to 82% at 82 hours possibly due to

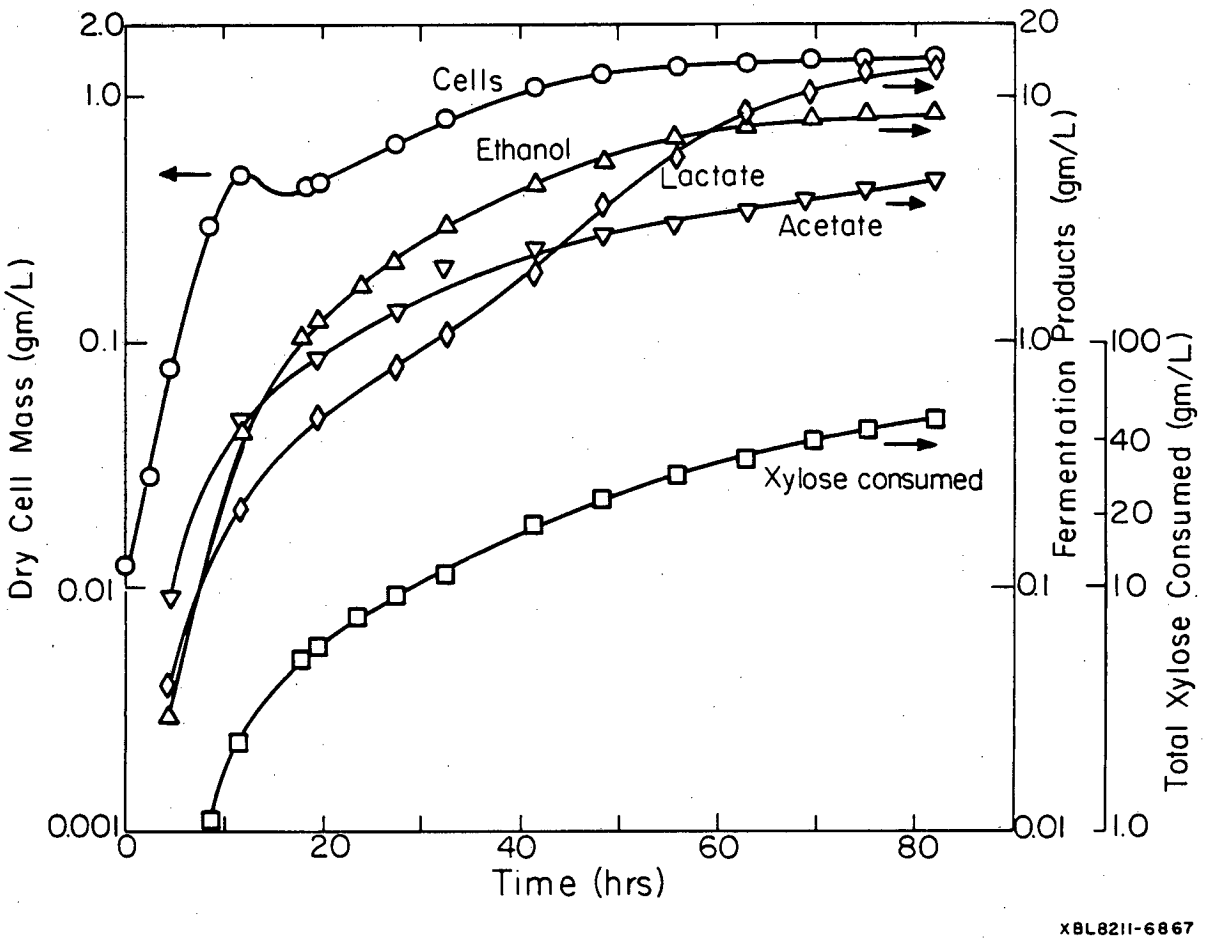

Figure 5.22. Fed-Batch Fermentation Profile for Strain UC-42-L2.

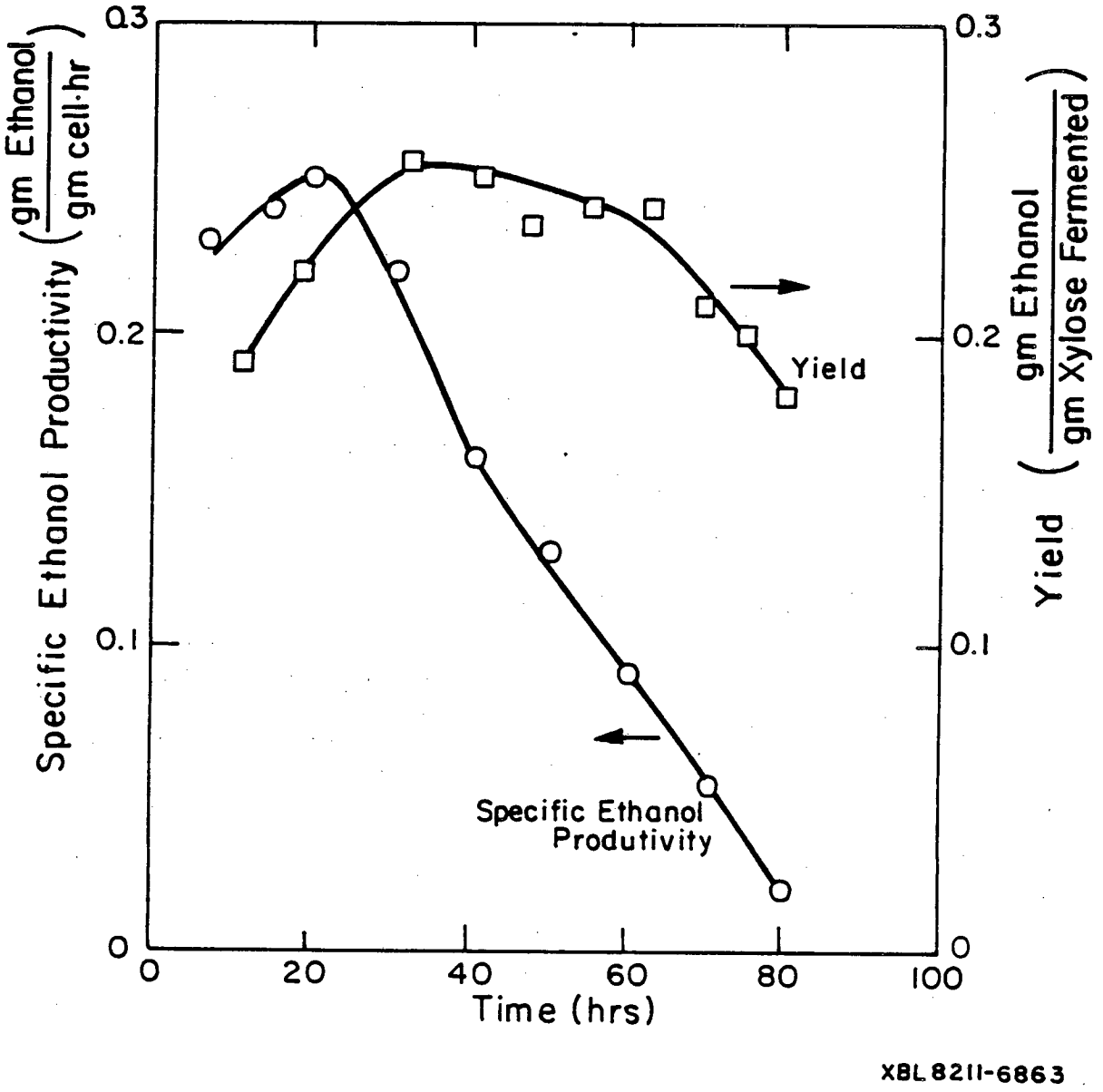

Figure 5.23. Ethanol Yield and Specific Ethanol Productivity During Fed-Batch Growth of Strain UC-42-L2.

caramelization of xylose.

The reason for the cessation of growth at 60 hours is not obvious. Neither ethanol, acetate, nor lactate were present at inhibitory concentrations. However, growth may have stopped because the three by-products are more inhibitory together than they are alone. Alternatively, growth may have ceased due to inhibition by caramelization products, which accumulated as the fermentation proceeded. This may also explain the drop in ethanol yield at the end of the fermentation since acetate and lactate yields are generally higher in the presence of caramelized sugar (see section 5.5). The full potential of tolerant mutants may be better demonstrated in continuous culture where residual sugar concentrations are low and hence, the rate of caramelization will be slow.

5.6.3 Recommendations

High ethanol yielding mutants appear to be selectable. Future work with this organism should focus on further decreasing acetate and lactate yields. More efficient mutant selection may be possible with optimized mutant induction and improved selection techniques.

VI. PROCESS DESIGN AND ECONOMIC EVALUATION

The results obtained for the fed-batch growth of *C. thermohydrosulfuricum* UC-42-L2 were used to construct a preliminary design for a xylose fermentation plant. Based on an economic evaluation of this plant, an ethanol production cost from xylose was calculated. In this evaluation, batch-culture data were used to design a complex, continuous fermentation plant; hence, the calculated ethanol cost is only approximate. Nevertheless, this evaluation should provide useful information for determining the emphasis of future research.

6.1. Plant Design

The design for the proposed xylose fermentation process is based on designs developed by Maiorella (89) and Wald (90). A flow diagram for the process is shown in Figure 6.1. The individual pieces of equipment are described in Table 6.2 (see section 6.2). The design basis for the material balance will be discussed later in this chapter.

6.1.1. Raw Material

The feed for the proposed plant is the dilute xylose stream generated during corn stover pretreatment in the process shown in Figure 2.1. This stream contains 1.5% xylose (by weight) as well as other carbohydrates, soluble lignin compounds, and sulfuric acid. The effects of carbohydrates other than xylose and soluble lignin on the growth of *C. thermohydrosulfuricum* were assumed to be negligible in this design. Further research is necessary to determine whether or not this assumption is realistic. In newer pretreatment processes, such as steam explosion of cellulosic materials (which appear to be superior to dilute acid pretreatment), sulfuric acid is not present in the xylose stream (2). Therefore, in this design, an acid neutralization step will not be

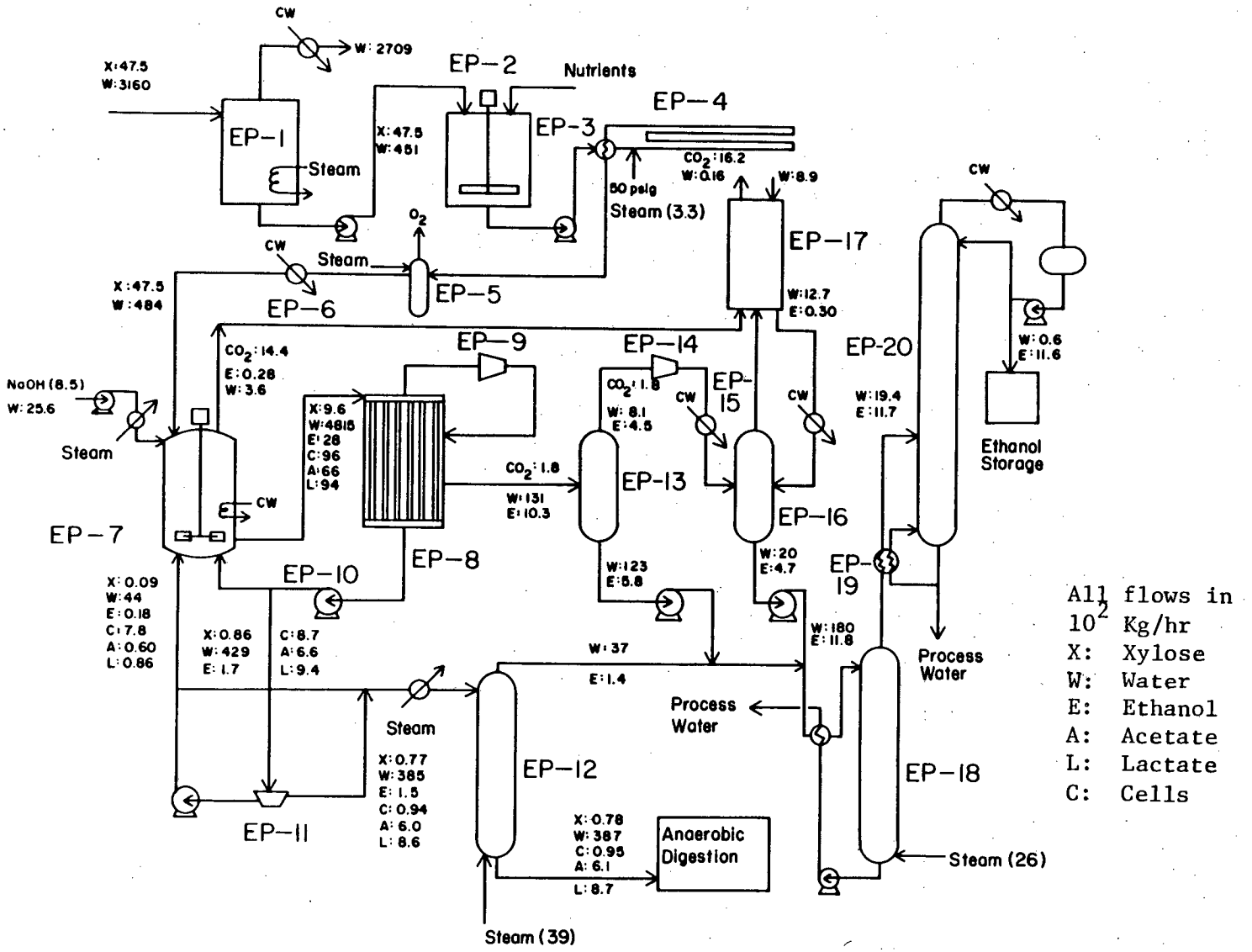

Figure 6.1. Flow Diagram for Xylose Fermentation Process

included.

The basis for the design shown in Figure 2.1 was 1,376 tons/day (52,120 Kg/hr) of dry corn stover. Results shown in the diagram indicate that a plant of this capacity will generate 126 tons/day (4,750 Kg/hr) of xylose. Hence, the design basis for the xylose fermentation plant is 4,750 Kg/hr.

6.1.2. Process Description

The dilute xylose feed is initially fed to a five-effect evaporator for concentration. 50 psig steam fed to the evaporator is used five times, which results in a significant energy savings. The concentrated sugar solution is mixed with nutrients and sterilized by direct steam injection. The super-heated medium is cooled by countercurrent heat exchange with the sterilizer feed to about 85°C. At this temperature, the equilibrium dissolved oxygen concentration in the medium is very small. Most of the oxygen originally dissolved in the feed is allowed to bubble out of the medium in a small degasser. Steam is used to sweep the head space of the degasser to prevent evolved oxygen from redissolving in the medium. The high xylose concentration of the feed (9.8%) should make the use of a reducing agent unnecessary (63). Before entering the fermentor, the feed is cooled to 65°C.

As mentioned in section 2.3.3, thermophilic ethanologens are particularly well suited for use in vacuum fermentation processes. Because the growth temperature is high (and consequently the vapor pressure of the medium is high) only a moderate vacuum has to be drawn on the fermentor to continuously boil the fermentation broth. Boiling the broth is an effective way to "selectively" remove ethanol since it is much more volatile than water (The relative volatility at 65°C is 2.3

for dilute ethanol solutions (91)). With selective ethanol removal, the beer alcohol content is kept at a low level; thus, the fermentation rate is not limited by the inhibitive effect of ethanol.

In the process shown in Figure 6.1, vacuum is not drawn on the fermentor itself but on a flash vessel, through which, the beer is continuously cycled. With the use of a flash vessel, carbon dioxide generated in the fermentor can be directly vented to the atmosphere without being compressed. Because compression costs are generally very high, minimizing the volume of vapor to be compressed is essential (89,91). To recover any ethanol leaving the fermentor with the carbon dioxide, the fermentor off-gas is scrubbed with water in a packed-column absorber.

The fermentor is a simple, stirred tank equipped with a small cooling coil for temperature control. Most of the metabolic heat produced by the cells is removed during medium vaporization in the flash vessel. The pH of the fermentor is controlled (at pH 6.7) by the addition of 10N sodium hydroxide. In this high ionic strength solution, the dissolved oxygen concentration is negligible and hence no provision is made for oxygen degassing of the added base(92). Lime (hydrated calcium oxide), a less expensive base, is normally used in industrial fermentations for pH control. Because the effect of high calcium concentrations on C. thermohydrosulfuricum is not known, lime was not used in this design.

The flash vessel is a vertical tube evaporator, designed so that the exiting vapors are in equilibrium with the incoming beer. Most of the spent beer is recycled to the fermenter while some is bled off and sent to a continuous centrifuge. The bleed is necesssary to prevent the

accumulation of toxic, non-volatile compounds in the fermentation broth, such as acetate and lactate. Approximately 88% of the cells in the bleed are recovered in the centrifuge and recycled to the fermentor as a concentrated (180 gm/L) paste. In this way, it is possible to maintain a high cell density in the fermentor, resulting in a high ethanol production rate per unit volume. The cell density in the fermentor is controlled with a small bleed from the concentrated cell recycle stream. Ethanol in the clarified bleed is recovered in a steam stripper and is sent to the distillation section. The stripper-bottoms (stillage) is sent to an anaerobic digestor where unused carbohydrate and cell protein are used to produced methane for steam generation.

Maintenance of anaerobisis in the entire recycle loop may present a significant engineering problem. Since information on large-scale, strictly anaerobic fermentations was not readily available, this problem was not addressed in this work.

Vapor removed in the flash vessel is recompressed in a steam driven compressor. By passing the compressed vapor through the shell-side of the flash vessel, heat generated during compression is used to provide the heat of vaporization required for flashing. Most of the compressed vapor is condensed on the shell side of the flash vessel. Uncondensed vapor and carbon dioxide (which enters the flash vessel as dissolved carbon dioxide) are removed from the condensed liquid in a gas-liquid separator. Liquid from the separator is pumped up to atmospheric pressure and sent to the distillation section.

The vapor phase from the separator is compressed and cooled a second time to remove ethanol and water from the carbon dioxide. Any traces of ethanol in the vapor phase of the second gas-liquid separator

are recovered in the off-gas absorber. The liquid phase from the second separator is also sent to the distillation section.

Final ethanol purification is accomplished with a dual distillation column system. Most of the water in the distillaton feed is removed in an atmospheric, steam stripping column. The hot bottoms product (which contains less than 0.15% ethanol) is used to preheat the stripper feed. The second column is operated under vacuum at a bottoms pressure of 170 mm Hg. Vacuum operation is advantageous because it shifts the ethanol/water equilibrium in such a way that the relative volatility (ethanol/water) is increased. Hence, the energy required for reduced pressure distillation is significantly less than for atmospheric distillation (93). The heat evolved during condensation of the stipping-column overhead product is used in the vacuum column reboiler. The final product, removed from the top of the vacuum column contains 95% ethanol and 5% water (by weight).

6.1.3. Material Balance and Design Basis

Most of the flow rates shown in Figure 6.1 were calculated with a computer package developed at the University of California, Berkeley by Maiorella and co-workers (94). The package was intended to be used for yeast fermentation of glucose, hence some modifications had to be made. Because cells of *C. thermohydrosulfuricum* have a much slower settling velocity than do yeast, the centrifuge residence time and velocity had to be increased. Assumptions made in the centrifuge design were based on the recommendations of an industrial centrifuge manufacturer (95). In addition, the amount of carbon dioxide formed per mole of ethanol produced is higher in *C. thermohydrosulfuricum* than in yeast since carbon dioxide is formed concomitantly with acetate.

(Equimolar amounts of carbon dioxide and ethanol are formed by both organisms.) Hence, the size of the off-gas absorber had to be increased.

The physiological parameters for C. thermohydrosulfuricum used in the design are listed in Table 6.1. These values were based on results shown in Figures 5.21, 5.22, and 5.23. The bleed to feed ratio (weight of beer bleed/weight of water in feed) was determined from results on the inhibitive effects of the non-volatile by-products, acetate and lactate. A thorough discussion of by-product inhibition in selective ethanol removal process is presented by Maiorella (96).

6.2. Economic Evaluation

6.2.1. Purchased Equipment Costs

The purchased costs of all major pieces of equipment are summarized in Table 6.2. These results were obtained from cost equations presented by Maiorella (94) and Perez (2). All prices listed are for the third quarter of 1982 (MSI = 750). The most costly pieces of equipment are the evaporator, the main compressor, and the centrifuges. The total purchased equipment cost, 7.2 million dollars, is very high for a fermentative ethanol plant of this capacity (89).

The evaporator cost may be unrealistically high since it is likely that the xylose concentration leaving corn stover pretreatment can be increased (97). If the feed contained 5% xylose rather than 1.5%, the purchased cost of the evaporator would be reduced from $2,400,000 to $600,000.

The high cost for the centrifuges reflects the difficulty in recovering slow-settling bacteria. Other techniques for cell recovery such as membrane separation may be very useful in this system; however,

Table 6.1

Design Basis

	Yields (gm/gm Xylose Consumed)
Ethanol	0.25
Acetate	0.13
Lactate	0.21
Cell	0.02
Xylose Lost to Caramelization (during fermentation)	0.05
Specific Growth Rate	0.011 hr^{-1}
Specific Ethanol Productivity	0.13 gm/gm-hr
Fermentor Ethanol Concentration	6 gm/L
Bleed to Feed Ratio (wt of water and ethanol in bleed/ wt of water in feed)	0.75 Kg/Kg*

*Based on data presented in section 5.5 on acetate and lactate inhibition.

Table 6.2

Purchased Equipment Summary
(All capacities are per unit)

	Equipment Description	Total Cost($)
EP-1	Xylose Feed Evaporator, 5 Effects CS, 37,600 ft^2, 2 units	2,239,000
EP-2	Feed Mixing Tank with Agitator CS, 58,600 L	8,200
EP-3	Sterilizer Pre-heat Exchanger SS, 720 ft^2	36,400
EP-4	Steam Injection Sterilizer SS, length=7.6 m, dia=0.31 m, temp=140°C	3,300
EP-5	Oxygen Degasser SS, 4,500 L	5,000
EP-6	Feed Cooler SS, 110 ft^2	12,400
EP-7	Fermentor with Agitator and Cooling Coil SS, 188,000 L, 150 Hp, 50 ft^2, 3 units	506,000
EP-8	Flash Expansion Vessel SS, 1,480 ft^2	140,000
EP-9	Primary Compressor (Steam Driven) SS, gas flow rate = 55,500 cf/hr, 861 Hp suction = 166 mm Hg, discharge = 320 mm Hg	1,130,000
EP-10	Beer Circulating Pump SS, 7,900 L/min, 14 Hp	6,300
EP-11	Centrifuge SS, 72 L/min, 392 Hp, 10 units	2,500,000
EP-12	Stillage Stripper CS, ht = 20 m, dia = 0.96 m, 29 trays	30,000
EP-13	Gas-Liquid Separator SS, 1,200 L	6,000
EP-14	Secondary Compressor (Steam Driven) SS, 2,290 cf/hr, 91 Hp suction = 320 mm Hg, discharge = 760 mm Hg	296,000

Table 6.2 (continued)

EP-15	Compressor Discharge Condenser/Cooler SS, 166 ft^2	15,500
EP-16	Gas-Liquid Separator SS, 110 L	1,600
EP-17	Fermentor Off-Gas Scrubber packed column, ht = 10.6 m, dia = 0.70 m	39,300
EP-18	Primary Stripper CS, ht = 11 m, dia = 0.77 m, 16 trays	24,100
EP-19	Vacuum Column Reboiler CS, 200 ft^2	8,160
EP-20	Vacuum Distillation Column CS, ht = 11 m, dia = 1.4 m 28 trays Column pressure (bottom) 170 mmHg	39,000
Storage (two weeks):	Ethanol (CS)	35,000
	Xylose Feed (CS)	124,000
Total Purchased Equipment Cost		7,205,000
Fixed Capital Investment (FCI) (4.13 x Purchased Equipment Cost)		29,758,000
Total Capital Investment (4.89 x Purchased Equipment Cost)		35,232,000

<u>Abbreviations Used</u>

CS - Carbon Steel

SS - Stainless Steel

Hp - Horse Power

these methods have not yet been demonstrated commercially. The compressor cost can be reduced by increasing the fermentor ethanol concentration. This will be discussed in section 6.2.5.

6.2.2. Operating Costs

The operating costs for the xylose fermentation process are listed in Table 6.3. Sodium hydroxide for neutralization and steam for evaporation comprise more than 40% of the total product cost. The high costs for maintenance and all indirect charges reflect the large capital investment required for the plant. The total cost of producing 95 wt% ethanol is $1.35/L, much higher than the current market price of 45 cents/L.

If the feed to the process contained 5.0% xylose instead of 1.5%, steam costs would be reduced by 20 cents/L. The cost of producing ethanol from a 5% xylose feed (including the effect of reduced capital costs) would be $1.01/L. Further reductions in costs would result with a higher fermentor ethanol concentration and a higher yield of ethanol (see section 6.2.5).

6.2.3. Waste Treatment

In most ethanol fermentation processes, the yeast cells produced are dried and sold as a protein supplement for cattle. Since the nutritional value of dried cells of *C. thermohydrosulfuricum* is not known, it is assumed that they will be fed, along with acetate, lactate and unused xylose, to an anaerobic digestor for conversion to methane. Based on estimates by Maiorella (98) and Wilke (99), the anaerobic digestor will increase the fixed capital investment for the xylose fermentation plant by 1.6 million dollars. If the methane produced in the digestor is used to generate some of the steam required in the

Table 6.3

Operating Costs

Raw Materials	Cents/L*
**Nutrients	10.00
Water	0.20
Sodium hydroxide ($0.53/Kg (71))	29.49
Utilities	
Steam: Evaporation	24.45
Distillation	2.44
Compressors	3.11
Electricity	3.78
Cooling Water	0.52
Operating Labor and Supervision	5.46
Maintenance, Operating Supplies, Lab Charges (7.9% of FCI)	18.51
DIRECT PRODUCTIONS COSTS	97.76
Depreciation (Linear, 18 year life)	13.65
Property Taxes (3% of FCI)	7.03
Insurance (0.7% of FCI)	1.64
Plant Overhead (60% op. labor, super, maint.)	14.38
Administration Expenses (15% op. labor)	0.87
TOTAL PRODUCTION COSTS	135.33

*Costs are per liter of 95 wt% ethanol.

**Based on results in section 5.2 and estimates for mineral costs for yeast ethanol fermentation as determined by Wong (39).

process, the ethanol cost per liter is reduced by 2.0 cents. Hence, the net effect of waste treatment on the ethanol cost is only a 1.3 cent/L increase.

6.2.4. The Advantage of Using Thermophilic Organisms for Ethanol Production

In sections 2.3.2 and 6.1.2, it was stated that thermophilic microorganisms are well suited for ethanol production in vacuum fermentation processes. To demonstrate the advantage of a high growth temperature, an ethanol production cost from xylose was calculated assuming that the fermentor was operated at 35°C rather than 65° (with all other parameters left unchanged). Because the vapor pressure of the fermentation broth is reduced at the lower temperature, the capital and energy costs for recompression are both increased. The net effect is an 11.4 cent/L increase in production cost.

6.2.5. Further Reductions in Production Costs

This work has focused on decreasing the cost of producing ethanol from xylose by decreasing nutrient costs, and increasing ethanol tolerance and yield. As stated in section 5.2, further reductions in nutrient costs may be possible by decreasing the yeast extract concentration used.

If the concentration of ethanol in the fermentor could be doubled to 12 gm/L, a significant savings would result. A higher beer alcohol content would decrease the amount of water processed in the compressors and all separation equipment, whereby, both capital and energy costs would be decreased. The net effect of doubling the ethanol concentration in the fermentor would be a 7.5 cent/L reduction in production cost.

Increasing the ethanol yield has a dramatic effect on production costs. Because less acids are formed, neutralizaton costs are reduced. Decreased acid formation also allows a smaller bleed stream since inhibitory by-products (acetate and lactate) accumulate much more slowly. If the ethanol yield was increased to 0.43 gm/gm (highest yield observed in this work), with acetate and lactate yields reduced to 0.04 and 0.08, respectively, a bleed to feed ratio of 0.4 is possible. With this low ratio (i.e., a smaller bleed), costs for centrifugation would be reduced by more than 50%. In addition, with a high yield, less xylose is wasted, thus the plant capital is used more efficiently. The net effect of increasing the yield of ethanol from 0.25 gm/gm to 0.43 gm/gm would be a 46 cent/L savings in production cost. Clearly, further improving the ethanol yield of UC-42-L2 will substantially improve the economics of fermenting xylose to ethanol with this bacterium.

VII. CONCLUSIONS AND RECOMMENDATIONS

In this study, the economic feasibility of converting xylose to ethanol with *Clostridium thermohydrosulfuricum* was examined. In rich medium (containing a high concentration of yeast extract) and in the absence of ethanol inhibition, yields as high as 0.43 gm ethanol/gm xylose were measured.

The cost of the growth medium could be significantly reduced by replacing some of the complex nutrients used with nicotinic acid and vitamin B_{12}. Low concentrations of ethanol were found to be very inhibitory for growth and ethanol formation. Inhibition could be substantially reduced by gradual adaptation; however, the ethanol yield of adapted cells was only 0.20 gm/gm. Low acid yielding mutants (developed from adapted cultures), were found to be selectable; however only slight improvements were obtained.

The economic evaluation indicates that ethanol production with this organism is currently uneconomical. To decrease the cost of producing ethanol from xylose, further research with this bacterium should focus on increasing the ethanol yield and the concentration of ethanol produced in the fermentation broth. Process development studies aimed at increasing the xylose concentration formed during pretreatment and finding economical alternatives to centrifugation for cell recycle should also significantly reduce production costs.

APPENDIX I

ISOLATING LOW ACID PRODUCING MUTANTS

AI.1. Background

One possible way of increasing the ethanol yield of *C. thermohydrosulfuricum* strain UC-42 is to develop mutants which are unable to produce acetate and lactate. The pathways for producing these by-products are shown in Figure 3.2 in Chapter 3. To eliminate organic acid formation, the genes which code for the enzymes of these pathways must be altered.

There are many different ways to induce mutations in bacteria. The advantages and disadvantages of the different techniques are discussed by Hopwood (100) and Miller (101). For this work, a chemical mutagen, ethyl methane sulfonate (EMS) was chosen because it produces a high yield of mutants in a culture without extensive killing and it is relatively easy to handle (100).

The mechanism for mutation by ethyl methane sulfonate has been described by Auerbach (102). Experimental evidence indicates that EMS usually alkylates ring nitrogens of guanine, although it may also react with adenine or cytosine. The ethyl group attached to a nucleic acid by EMS causes it to be misread during replication. For example, guanine normally pairs with cytosine; however, ethylated guanine pairs with thymine. Hence, the progeny of the altered cell will possess an adenine: thymine (A:T) pair instead of the original guanine: cytosine (G:C) pair of the parent. This type of mutation is called a point mutation since only one pair is affected.

Individual amino acids in proteins are specified by triplets of nucleotides. Hence changing a G:C pair to an A:T pair will affect one

amino acid in a single enzyme. There are several possible outcomes that may result when a base pair is changed. If the new triplet does not code for any amino acid (a nonsense mutation) the affected protein will be only partially synthesized. The incomplete protein will most likely be a totally inactive enzyme, whereby a blocked metabolic pathway results. Unfortunately, nonsense mutations frequently cause undesirable, adverse effects in adjacent genes (100).

Alternatively, if a base pair change results in the specification of a new amino acid (a missense mutation) a new enzyme is synthesized. The activity of the new enzyme will depend on how closely related the new amino acid is to the original. If the new and original amino acids are similar in structure (e.g. glycine and alanine) the enzyme is probably not significantly affected. However, if a totally different amino acid is substituted into the enzyme (e.g. substituting phenylalanine for serine) then its activity may be substantially decreased. Hence, missense mutations which change an amino acid to a very different one, are the most desirable for destroying enzymatic activity.

Mutagens react with DNA randomly, whereby, the probability of affecting a specific gene is very small (79,103). The probability of inducing two <u>desired</u> mutations in the same cell would be <u>extremely</u> small (approximately the product of the two individual probabilites). Hence in selecting low acid producers, it is impractical to try to block both acid forming pathways simultaneously. Therefore, cells will be treated with a dose of EMS which is designed to produce a large number of cells containing only single mutations.

Death Curve

Most mutations in bacteria are lethal. Therefore, in order to produce a significant number of viable mutants in a culture, it is necessary to kill a large fraction of the population (100). However, if the kill is too high, a significant fraction of the surviving clones will contain multiple mutations. No information on mutagenic treatments for *C. thermohydrosulfuricum* has been published. Hence, the optimal level of killing must be inferred from results with other organisms. Sherman, et al. (104) recommended a 99% kill for mutating *S. cerevisiae* with EMS. Van Tassell and Wilkins (105) induced mutations in *Bacteriodes fragilis* with EMS at a death rate of 90-95%. As a conservative estimate for the optimal level of killing, a 90% death rate was chosen.

AI.2. Procedure for Obtaining a Death Curve

The dose of EMS required to give a 90% death rate in *C. thermohydrosulfuricum* was determined by exposing resting cells to several concentrations of EMS (for a fixed amount of time) and counting the number of viable cells remaining after the treatment. Resting cells were used because some researchers have found that variable death rates result when replicating cells are used (100).

EMS Treatment

Cells were grown to mid-exponential phase (optical density = 0.30) in a culture tube containing 8 ml of YEX1 medium. They were centrifuged in the sealed culture tube (whereby exposure to oxygen was avoided) and the pellet was transferred anoxically to 8 ml of pre-reduced buffer solution (25 gm/L xylose and 0.6 gm/L cysteine

hydrochloride in 0.2 M phosphate buffer, pH = 7.4). In this buffer solution, cells remained viable without reproducing. 1.0 ml of the resuspended culture was added to several tubes containing 9 ml of buffer solution. The final optical density of the diluted suspensions was 0.036. A 0.5-ml syringe was used to add the EMS (Sigma Chem. Co., St. Louis, MO.), which is a liquid, to the cell suspensions at concentrations ranging from 0 to 1.0% (v/v). Safety procedures for handling EMS are described by Ehrenberg and Wachmeister (106,107). The tubes were vortexed to dissolve the EMS and incubated in a shake bath at 65°C for 20 minutes. (No significant kill resulted when cultures were treated at room temperature.)

Determination of Survival

To determine the number of cells that survived the EMS treatment, viable counts of each tube were made. Viability was determined using a deep-agar technique as suggested by Postgate (108). The agar medium used (AM1) had the same composition as YEX1 medium except: the phosphate concentration was reduced to 30 mM, 10 gm/L agar was added, and resazurin was not used.

Heat-stable components of the agar medium were combined and autoclaved. After the medium cooled to 65°C, xylose, vitamins, and minerals were added. Culture tubes were filled with 9.9 ml of molten AM1 and maintained at 65°C until they were inoculated. Cells were withdrawn from EMS treated cultures and diluted by 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} with pre-reduced medium. Molten agar tubes were inoculated with 0.1 ml of the diluted cultures and inverted several times to disperse the cells. After allowing the agar to solidify, the tubes were incubated at 65°C. Removal of oxygen from the head space

above the cysteine-containing agar was not necessary for growth to occur. After two days of incubation, distinct round colonies, approximately 2-3 mm in diameter, were visible. The total number of viable cells in the original EMS treated culture was calculated based on the assumption that each colony arose from a single cell. The results on the effect of EMS on culture viability are shown in Figure AI.1. After an initial lag where little killing was produced, the number of survivors decreased exponentially with increasing EMS concentration. The scatter in this graph reflects the difficulty in obtaining accurate viable cell counts with strict anaerobes. A 90% kill resulted with an EMS concentration of approximately 0.5% (v/v). Hence, this concentration was chosen for inducing mutations in C. thermohydrosulfuricum.

AI.3. Procedure for Mutagenesis

The procedure for producing mutants was similar to the procedure used for determining death rates. 0.05 ml of EMS was added to 10 mls of a suspension of cells (O.D. = 0.036) in phosphate buffer and incubated at 65°C for 20 minutes. After EMS treatment, the culture was centrifuged and resuspended in pre-reduced medium twice to remove the EMS. The treated cells were allowed to grow overnight in YEX1 medium to an optical density of approximately 0.20. This allowed expression of newly induced mutations, which may have been recessive immediately following mutagenesis (101). The recovered cells were isolated with plating techniques described in the next section.

AI.4. Plating Procedures.

Because the fraction of desired low acid producing clones in the recovered culture was very small, it was necessary to screen a large

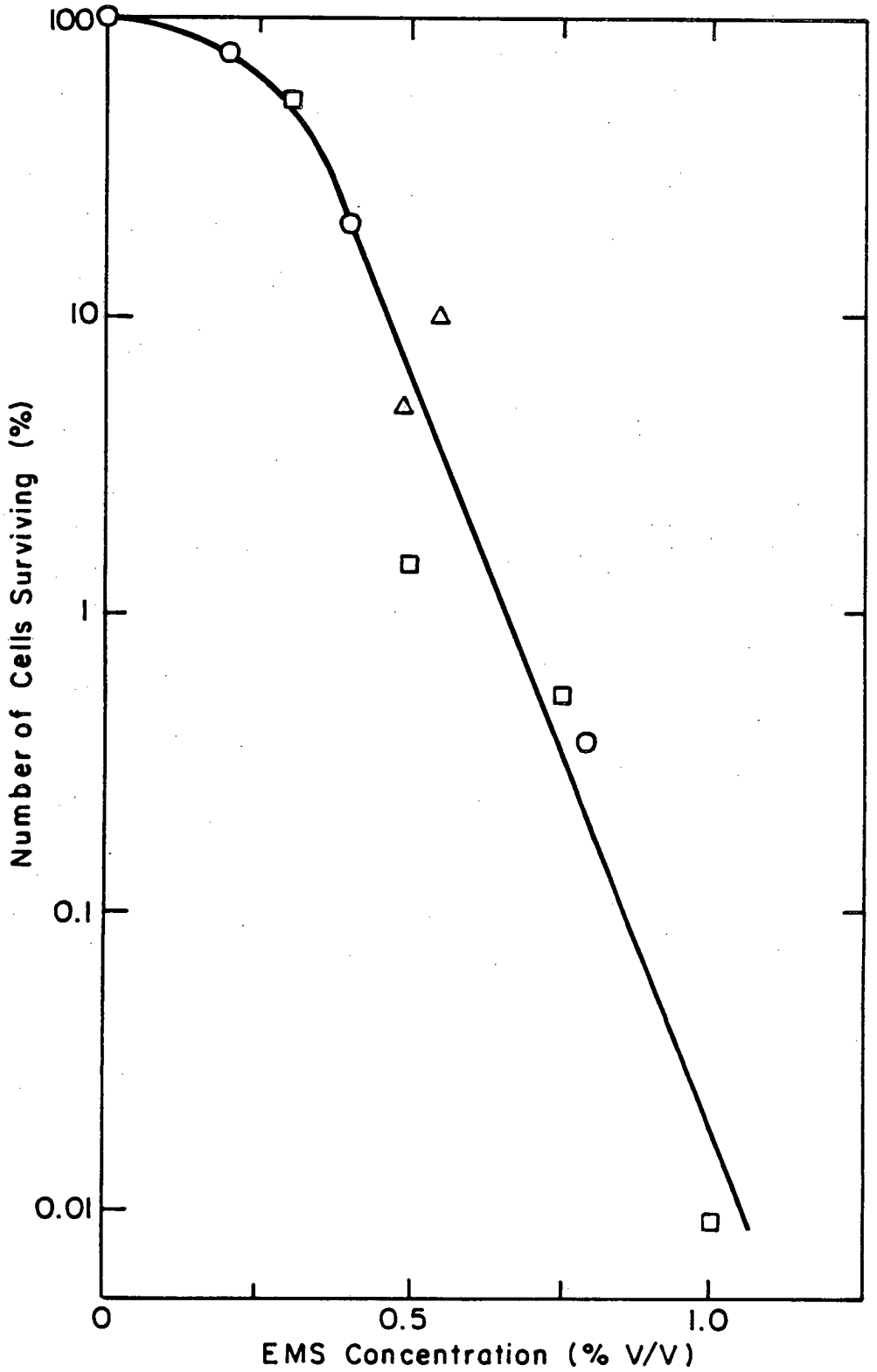

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Figure AI.1. Death Curve for Ethyl Methane Sulfonate Mutagenesis.
The different symbols represent the results of independent experiments.

number of prospective mutants for acid formation. The most efficient way to screen large numbers of cells is to plate them on agar-containing petri dishes. Colonies arising from individual cells are of a single genotype; hence, a low acid producing mutant will result in a low acid producing colony. Visual screening of prospective mutants is possible if an appropriate acid indicator is sprayed onto the colonies or included in the agar. Indicators are discussed in section AI.5.

Anaerobic Glove Box

C. thermohydrosulfuricum cells plated in air were frequently found to be non-viable. Hence, all plating was performed in an anaerobic glove box. A well-sealed glove box, filled with a 95:5 (volume ratio) nitrogen/hydrogen mixture, was maintained at a pressure slightly higher than the surrounding air. With positive pressure inside the box, the only possible way for oxygen to enter was by diffusion through the gloves (58). Oxygen in the glove box was scavenged by reaction with hydrogen on palladium catalyst pellets. (Becton, Dickinson and Co., Cockeysville, MD). The pellets were packed in a mono-layer between two wire-mesh screens which were secured to the face of a 4-inch pancake fan. The fan circulated the atmosphere within the box so that any traces of oxygen diffusing in were eventually converted to water on the pellets. A methylene-blue indicator strip (Becton, Dickinson and Co., Cockeysville, MD) was placed in the box to monitor the oxygen content. Small amounts of oxygen in the box turned the indicator blue. In the absence of oxygen the strip remained white.

Plating Techniques

Agar medium used for plating (AM2) was the same as AM1 except for the agar concentration, which was increased to 2.5%. Plates were

poured in a laminar flow hood, cooled, and stored in the anaerobic glove box where medium reduction occurred within approximately 30 hours.

In initial plate tests, C. thermohydrosulfuricum cells, which are flagellated, rapidly spread over the surface of the plates and did not produce distinct colonies. One way to prevent the cells from moving across the surface of the agar was to dry it by leaving the plates outside the glove box for 2 days. (The inside of the glove box was usually very humid.) When 0.1 ml of diluted cells were spread onto the surface during inoculation, the water introduced with the inoculum was quickly absorbed by the agar. Distinct and well-formed colonies were consistently obtained with pre-dryed plates.

Alternatively, cells could be plated in a layer of soft agar on top of the hard agar plates. Cells were mixed with molten AM1 agar and 3 to 4 ml of the mixture were poured onto the hard agar surface. To prevent colonies which grew to the top of the soft agar layer from spreading, a second layer of soft agar containing no cells was applied to the plates. With this technique, flagella were immobilized and distinct colonies resulted.

Incubation of Plates

Inoculated plates were incubated inside sealed vessels containing a nitrogen, hydrogen, and carbon dioxide atmosphere (an anaerobic jar). 2-liter reaction kettles (VWR Scientific, San Francisco, CA.) sealed with butyl-rubber stoppers were used as anaerobic jars. These kettles were chosen because they were small enought to fit inside the glove box air lock. Plates could be transferred to the anaerobic jars inside the glove box, whereby the cells were never exposed to oxygen. A hydrogen/carbon dioxide generator packet (Becton,

Dickinson and Co., Cockeysville, MD) was included in each jar during incubation. The hydrogen was necessary for establishing an anaerobic environment. It reacted with traces of oxygen in the jars on palladium catalyst pellets (contained in a wire mesh enclosure). The carbon dioxide produced, although not essential, helped to stimulate growth (62). Methylene blue strips were used in the jars to indicate anaerobisis. Plates were generally incubated for 3 days at 65°C.

AI.5. Indicators Used for Selection

Several techniques were examined for use in selecting low acid producing colonies.

AI.5.1. pH Indicators

The pH of an acid forming colony is lower than the pH of the surrounding agar. Colony pH is a function of the agar pH and buffer concentration. pH indicators, with color changes between 5 and 8 were examined for their possible use in the agar to permit rapid visual observation of acid formation. The indicators shown in Table AI.1 were all tested in AM2.

Phenol Red

Phenol red has been used successfully at the Massachusetts Institute of Technology in the selection of low acid producing mutants (109).

Phenol red agar was prepared by adding 25 mg of the indicator to a liter of AM2. (Because it is heat-stable, the phenol red was added to the medium before autoclaving.) Both soft-agar and surface plating techniques were used with phenol red. Colonies which formed acid on the orange-red colored agar were stained yellow.

A total of 3,500 prospective mutants were screened on phenol red

Table AI.1

pH Indicators

Indicator	Color Change		Concentration Used in Agar
Phenol Red	pH=6.5, yellow	pH=7.5, red	25 mg/L
Bromothymol Blue	pH=6.0, yellow	pH=7.6, blue	100 mg/L
*DND	pH=5.6, yellow	pH=7.0, blue	20 mg/L
Methyl Red	pH=4.9, red	pH=6.5, yellow	40 mg/L

*2-(2,4-Dinitrophenylazo)-1- Napthol-3,6-Disulfonic acid, disodium salt

plates (approximately 30 to 100 colonies per plate). Unfortunately this indicator was generally too sensitive for examining relative acid formation. Frequently, large areas on the plates turned yellow due to diffusion of acids away from the colonies. Increasing the initial agar pH to 7.5 (effectively increasing the buffering capacity of the surrounding agar) helped limit the size of yellow zones somewhat but selection was still difficult. With higher concentrations of phosphate buffer (100 m*M*) and high pH (7.5), the plates turned brown-orange due to caramelization of xylose which drowned out the color of the indicator (see Appendix II). In general, this method was not well suited for isolating low acid yielding mutants of strain UC-42.

Bromothymol Blue

Screening with bromothymol blue was not successful because the color change from blue to yellow was very gradual. Both the colonies and the agar turned from blue to green with little, if any, color differential between the two.

Methyl Red and DND

Both methyl red and DND turned colorless in AM1 medium after oxygen removal. Apparently, both indicators were inactivated by the strongly reducing conditions.

However, these indicators could be used by applying them to the surface of the plates after they were removed from the anaerobic jars. 1.0 ml of a methyl red solution (prepared by disolving 20 mg of methyl red in 20 ml ethanol and adding 80 ml of 0.01 *M* potassium hydroxide) was used to flood the surface of soft-agar plates containing approximately 100 colonies each. Surface-plated colonies were not used because they were frequently washed off during indicator application. The indicator

solution was allowed to soak into the agar for 10 minutes before any excess was poured off. Colonies producing acid were stained pink by methyl red while the color of the agar background was yellow.

Approximately, 2,500 clones were screened with this technique. Of these, 8 formed large (2-3 mm diameter), non-pink colonies which were picked and cultured in liquid medium. Large colonies were chosen since they are usually formed by faster growing clones. The distribution of end products formed from xylose for these 11 isolates is summarized and discussed in section 5.5.

A solution of DND (1 gm/L DND in 0.01 N potassium hydroxide) was also used to flood plates of soft-agar colonies. DND changes from blue-green to yellow at a pH of approximately 5.6. With 30 m$\underline{M}$ $PO_4^{=}$, the average agar pH was typically less than 5.9 making differential coloration with DND difficult. Hence, when DND was used, the phosphate concentration was increased to 60 m$\underline{M}$ to increase the average pH of the agar. With this technique, a yellow-green halo formed around acid producing colonies while the colonies themselves remained blue-green. From approximately 2,000 prospective mutants screened, 9 clones which formed large colonies without halos were isolated. The colonies were picked and characterized in YEX1 medium. The end-product yields of these isolates are summarized in section 5.5.

AI.5.2. Triphenyltetrazolium Chloride

Triphenyltetrazolium chloride (TTC) is a pH sensitive redox indicator (101). It has been used by Lederberg (110) to select non-acid forming colonies of *E. coli*. Dehydrogenase-active cells reduce TTC to a deep-red insoluble formazan provided that the colony is not too acidic; low pH colonies remain unstained.

One problem in using TTC with C. thermohydrosulfuricum is that it is converted to formazan by reaction with reducing compounds normally used in the agar (109). To prevent non-biological formazan formation, the concentration of reducing compounds in the medium had to be decreased. Both cysteine and yeast extract can reduce TTC at 65°C. Therefore, it was necessary to use agar containing no cysteine and not more than 0.3 gm/L yeast extract. Because 0.3 gm/L yeast extract may be insufficient for rapid colony growth, the agar was supplemented with 10 amino acids. 50 mg/L of each of the following were used: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine. Growth in amino acid supplemented agar (AASA) was fairly rapid; 2 mm colonies formed in less than 3 days. In working with AASA, which was very poorly poised, exposure to oxygen had to be carefully avoided (see section 4.1.3).

Despite the fact that AASA did not contain significant amounts of strongly reducing compounds, plates containing 25 mg/L TTC turned slightly red after three days of incubation at 65°C. Some formazan formation was also observed in agar containing only xylose (which is a reducing sugar) and TTC. To overcome this problem, xylose was spacially separated from TTC in the agar with multiple-layer plating. Cells were plated in soft agar as described in section AI.4. AASA medium was used for all three layers. Xylose (10 gm/L) was included only in the bottom hard agar layer. The top soft agar layer contained 50 mg/L TTC while xylose and TTC were both omitted from the middle cell-containing layer. Layered plates incubated at 65°C for three days, showed little, if any, formazan formation.

To determine if C. thermohydrosulfuricum colonies could be stained by TTC, colonies were grown on soft-agar plates containing no xylose in any of the three layers. Small colonies were able to form from the yeast extract and the amino acids. However, in the absence of a fermentable carbohydrate, little acid was formed. The resulting colonies were stained a very deep-red color, indicating that TTC was reducible by C. thermohydrosulfuricum and that it could diffuse from the top soft-agar layer to the colonies.

When xylose was included in the bottom agar layer, colonies were not stained, indicating that they had fermented some of the sugar and become acidic. Hence, xylose supplied in the bottom hard-agar layer diffused readily to colonies in the middle soft-agar layer.

To determine the critical pH for formazan formation, tubes containing complete AM1 (with reducing compounds) and 25 mg/L TTC were incubated at 65°C. The pH of the tubes ranged from 6.0 to 7.0. In tubes at pH 6.4 to 7.0, formazan formation was extensive. At pH 6.2 and 6.3, very little formazan was formed and at 6.1 the agar remained colorless. Hence, only colonies at pH 6.4 or greater would be definitively stained with TTC.

As with phenol red (pH change at approximately 6.6) this method is too sensitive for selecting low acid producing clones of UC-42 because the critical pH for differential staining is too high. Increasing the agar pH and buffer concentration to decrease the high sensitivity (to acid formation) was not possible since these changes produced excessive caramelization.

Another possible approach to decreasing the sensitivity is to reduce the xylose concentration. A low xylose concentration (less than one gram per liter) may decrease the rate of growth enough so that acid formation is slow. This would allow more time for buffer to diffuse into a colony, thus maintaining it at a higher pH. Determination of optimal xylose and phosphate concentration, and pH may make this technique useful in selecting the desired mutants.

AI.5.3. Selection of Low-Lactate Producing Clones

As an alternative to pH sensitive indicators which were non-specific, a reagent which reacted with lactate was used to screen colonies. Plates were sprayed with a solution normally used for the colorimetric determination of lactate (111). This method of mutant selection has been used successfully by other researchers (36).

The reagent contained lactate dehydrogenase and NAD which converted lactate to pyruvate and NADH. NADH subsequently reduced phenazine methosulfate (PMS) which in turn reduced tetranitro blue tetrazolium (TNBT) to an insoluble formazan. The reactions are summarized below.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{PMS(ox)} \longrightarrow \text{NAD}^{+} + \text{PMS (red)}$$

$$\text{PMS(red)} + \text{TNBT} \longrightarrow \text{PMS(ox)} + \text{Formazan}$$

The formazan, which formed as a deep-purple precipitate, stained lactate producing colonies. Table AI.2 shows the exact composition of the reagent.

To prepare the reagent, the components were mixed in the order given in the table. TNBT is hydrophobic and required a long time to

Table AI.2

Lactate Detection Reagent

Component	Amount per liter
$Na_2HPO_4 \cdot 7H_2O$	20 gm
*Tris (Sigma No. T 1378)	12 gm
*NAD (Sigma No. N 7381)	8 gm
*LDH (Sigma No. 826.6)	2.0 ml
*TNBT (Sigma No. T 4000)	0.5 gm
*PMS (Sigma No. P 9625)	10 mg

*All obtained from Sigma Chemical Co., St. Louis, MO.

dissolve. Any residual, insoluble lumps of TNBT were removed from the solution by filtration before the PMS was added.

The phosphate and tris buffers in the reagent were needed to neutralize the acidic colonies because the reagent was found to be inactive at low pH (less than 6.0). Higher buffer concentrations led to precipitation of the tetrazolium. In addition, the initial pH of the agar was increased to 7.5, and 60 mM phosphate was used so that final colony pH's would also be higher.

Soft-agar plates were sprayed with 1.0 ml of the reagent. Surface colonies were not used since they were often washed from the plate during spraying. 7,000 prospective mutants were examined using this method. From these, 20 unstained, apparently non-lactate forming colonies were selected. Subsequent characterization of these clones indicated that none produced significantly less lactic acid than strain UC-42. In addition to the twenty colonies picked for further examination, many others appeared to be non-lactate formers. Most of the unstained colonies were located deep below the soft-agar surface.

There was generally a faint formazan precipitate that formed throughout most of the soft-agar (probably due to reaction with lactate which diffused away from colonies). These unstained, deep colonies frequently were surrounded by clear zones where the faint precipitate was absent. The above observations suggest that the pH of the unstained colonies was too low for the reagent to work and they were falsely left unstained. As the reagent diffused into the agar, it was acidified by the acid above the deep colonies. Colonies very near or actually touching the surface were readily stained, since in diffusing only a short distance, the reagent was not appreciably acidified.

Successful screening with this technique may be possible with improved plating techniques that allow most colonies to be readily neutralized or by altering the reagent so that it remains active at low pH.

APPENDIX II

DETERMINATION OF CARBON RECOVERY

Carbon balances were performed to determine whether or not all of the xylose consumed could be accounted for in the fermentation products. In calculating the total carbon recovery, it was assumed that one mole of carbon dioxide was formed for each mole of ethanol or acetate produced (see figure 3.2). To calculate the amount of carbon from xylose converted to cells, the composition of the cells was approximated as $CH_{1.9}O_{0.3}N_{0.23}P_{0.023}$ (79), and the yield of cells from yeast extract was assumed to be 0.15 gm/gm (see section 5.2.1).

Caramelization

At the high growth temperature (65°C) some of the sugar caramelized during the course of fermentation. Figure AII.1 shows the time course of xylose loss due to carmelization. The rate of the reaction appears to slow with time in all cases. Phosphates increase the rate of the reaction; the initial rate in 0.1M phosphate is approximately 3 1/2 times faster than the rate in 0.011M. These results are consistent with observations made by Bridson and Brecker (88).

In addition, the loss of xylose measured with the dinitrosalysilic acid method (DNS) was much less than with liquid chromatography (LC). Hence, caramelized sugars appear to react, to some extent, with DNS. These results were used to correct calculated values for xylose consumption during fermentations. In general, correcting for caramelization improved carbon balances.

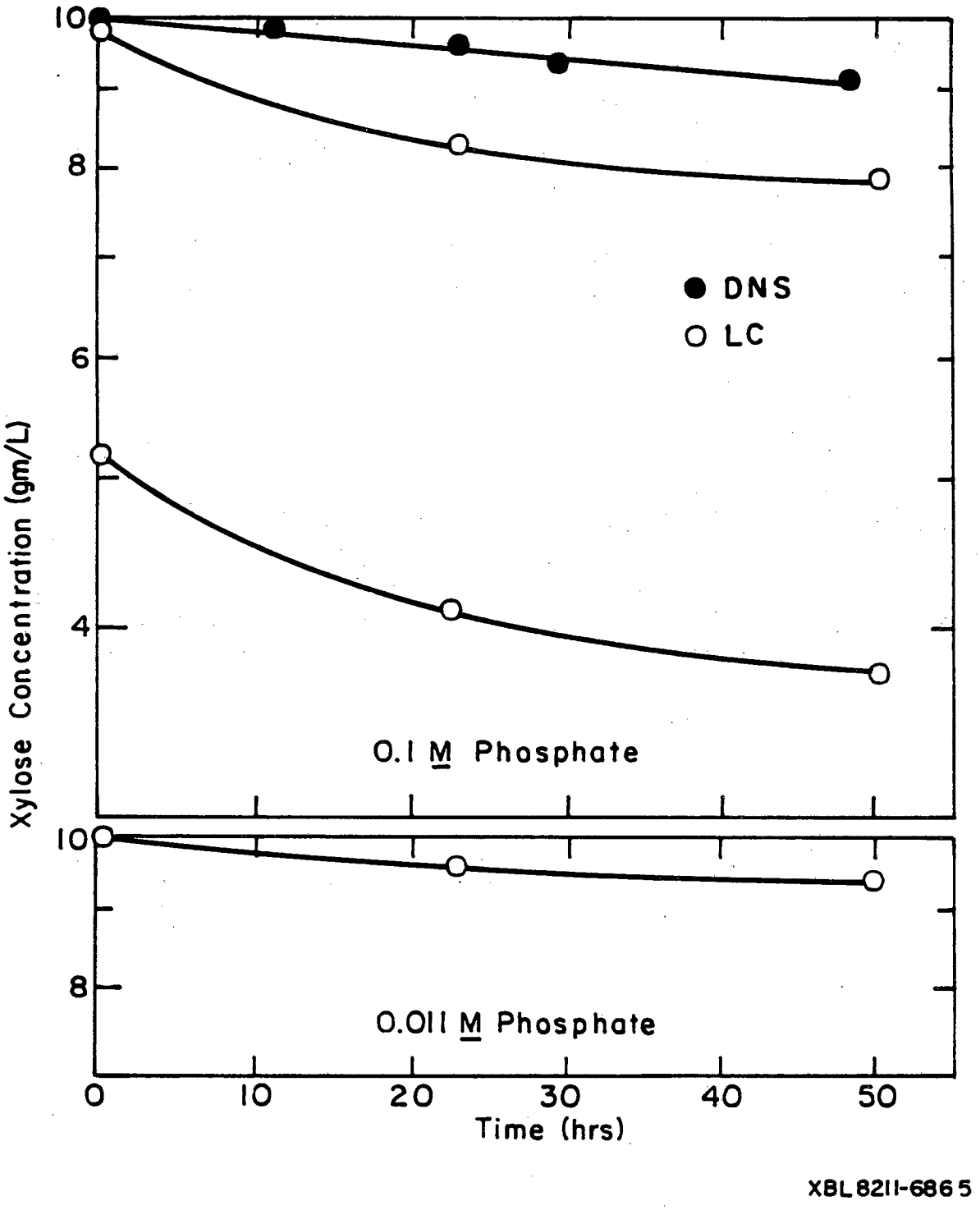

Figure AII.1. Caramelization of the Growth Medium at 65°C.

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