16:38:33 OCA PAD AMENDMENT - PROJECT HEADER INFORMATION 09/20/90

Active

Project #: E-19-630 Cost share #: E-19-348 Rev #: 2
Center # : R6507-0A0 Center shr #: F6507-0A0 OCA file #:
Work type : RES
Contract#: TV-74261A Mod #: 2 Document : CONT
Prime #: Contract entity: GTRC

Subprojects ? : N
Main project #:

Project unit: CHEM ENGR Unit code: 02.010.114
Project director(s):
ROBERTS R S CHEM ENGR (404)894-2889

Sponsor/division names: TENN VALLEY AUTHORITY, TN /
Sponsor/division codes: 119 / 000

Award period: 880601 to 910930 (performance) 911030 (reports)

Sponsor amount	New this change	Total to date
Contract value	15,000.00	36,000.00
Funded	15,000.00	36,000.00
Cost sharing amount		34,952.00

Does subcontracting plan apply ?: N

Title: ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC

PROJECT ADMINISTRATION DATA

OCA contact: Brian J. Lindberg 894-4820

Sponsor technical contact

MR. M. R. MOORE
(205)386-2601

TENNESSEE VALLEY AUT
435 CEB
MUSCLE SHOALS, ALABA

Sponsor issuing office

MR. OWEN P. WATKINS
(205)386-2917

TENNESSEE VALLEY AUT
2T 218I NFDC
MUSCLE SHOALS, ALABA

Security class (U,C,S,TS) : U ONR resident rep. is ACO (Y/N): N
Defense priority rating : N/A N/A supplemental sheet
Equipment title vests with: Sponsor GIT
NONE PROPOSED OR ANTICIPATED.
Administrative comments -
SUPPLEMENT NO. 2 ADDS $15,000 IN NEW FUNDS AND EXTENDS PERIOD OF PERFORMANCE THROUGH SEPTEMBER 30, 1991. TOTAL FUNDING NOW: $36,000 C/S = $34,952.

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Closeout Notice Date 03/03/92

Project No. E-19-630_____ Center No. R6507-0A0_____

Project Director ROBERTS R S_____ School/Lab CHEM ENGR_____

Sponsor TENN VALLEY AUTHORITY, TN/_____

Contract/Grant No. TV-74261A_____ Contract Entity GTRC

Prime Contract No. _____

Title ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC_

Effective Completion Date 910930 (Performance) 911030 (Reports)

Closeout Actions Required:	Y/N	Date Submitted
Final Invoice or Copy of Final Invoice	Y	_____
Final Report of Inventions and/or Subcontracts	Y	_____
Government Property Inventory & Related Certificate	N	_____
Classified Material Certificate	N	_____
Release and Assignment	N	_____
Other _____	N	_____

Comments_____

_____

Subproject Under Main Project No. _____

Continues Project No. _____

Distribution Required:

Project Director	Y
Administrative Network Representative	Y
GTRI Accounting/Grants and Contracts	Y
Procurement/Supply Services	Y
Research Property Managment	Y
Research Security Services	N
Reports Coordinator (OCA)	Y
GTRC	Y
Project File	Y
Other _____	N
_____	N

NOTE: Final Patent Questionnaire sent to PDPI.

QUARTERLY PROGRESS REPORT #1
July 1, 1988 - September 30, 1988
Contract # TV-74261A

ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC RESIDUES

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

J. R. Kastner and Professor R. S. Roberts

School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

December 31, 1988

ABSTRACT

The yeast, *Candida shehatae*, was used to perform mixed sugar and pure glucose fermentations under semi-anaerobic and anaerobic conditions (at low starting cell densities) and pure d-xylose fermentations under anaerobic conditions (at high starting cell densities). In the mixed sugar fermentation under anaerobic conditions, 8 g/L of ethanol was formed from a total of 20 g/L of carbohydrates (50% d-xylose and 50% glucose). Also, a diauxy of 20 hours was seen in the biomass formation and about 10 hours in ethanol formation.

Under semi-anaerobic conditions 6 g/L of ethanol was formed from 20 g/L of carbohydrates. A diauxy of 30 hours was seen in ethanol formation but was not observed in biomass formation. Semi-anaerobic conditions increased the uptake rate of d-xylose and glucose, increased the amount of biomass formed, decreased the quantity of xylitol formed and resulted in the consumption of ethanol.

The pure glucose fermentations under the same conditions did not outperform the mixed sugar fermentations. Under anaerobic conditions, 7 g/L of ethanol was formed from 20 g/L of glucose. If semi-anaerobic conditions were imposed, 5 g/L of ethanol were formed from 20 g/L of glucose.

The most important results were obtained in the pure d-xylose fermentations under anaerobic conditions. In these fermentations the medium composition was changed and the fermentation parameters correlated with cell viability. If the medium lacked a nitrogen source (substrate only), cell viability decreased rapidly; at a fermentation time of 20 hours the viability index was 0.95 compared to 0.7 at 80 hours. The viability index for all other medium compositions (Yeast Extract, Malt Extract and Peptone, Yeast Extract only, Peptone only) ranged between 0.95 and 0.98 at the end of the fermentation (80 hours).

With the onset of cell death in the fermentation containing substrate only, the d-xylose uptake rate decreased and ethanol was consumed. This resulted in residual d-xylose and a lowered ethanol yield ($Y_{p/s}$; 0.22); compared to complete d-xylose utilization and higher ethanol yields (Yeast Extract, Malt Extract, and Peptone; 0.33, Yeast Extract; 0.33, and Peptone; 0.46) in the other fermentations.

It was determined that the ability to monitor cell viability via methylene blue staining is a vital analytical technique. Future experimental work will correlate cell viability with ethanol concentration, mixed sugar ratios (meaning diauxy effects), type of nitrogen source, addition of TCA cycle intermediates and qualitative oxygen levels.

INTRODUCTION

In an attempt to utilize the hemicellulosic constituent of biomass for the formation of ethanol, research in recent years has centered on microorganisms capable of fermenting d-xylose. One such

microorganism capable of fermenting both pure d-xylose and mixed sugars (mainly composed of glucose and d-xylose) is *Candida shehatae*.

Several researchers have investigated d-xylose and mixed sugar fermentations via *Candida shehatae* (Wayman and Parekh, 1985; Wayman and Tsuyuki, 1985; du Preez, et al., 1983; du Preez, et al., 1984; du Preez, et al., 1986; Screenath, et al., 1986). Based on data from this research a single stage fermentation of mixed sugars was proposed (Roberts and Kastner; Research Proposal: Ethanol Fermentation of Mixed Sugars Derived from Lignocellulosic Residues, 1988).

To determine the feasibility of a single staged mixed sugar fermentation utilizing *Candida shehatae*, the effect of process parameters on both pure d-xylose and mixed sugar fermentations were studied. Parameters studied in this phase of the research include initial cell densities, initial sugar concentrations, d-xylose to glucose ratio and medium composition.

MATERIALS and METHODS

Microorganism:

A strain of *Candida shehatae* (ATCC 22984) was obtained from the American Type Culture Collection. It was maintained on YM agar slants (YM broth in 2% agar). Long-term stock cultures were maintained at 4°C, and subcultured at one to two month intervals.

Medium:

The medium of Wayman and Tsuyuki (1985) [yeast extract (3g/L, spray dried and autolyzed), malt extract (3g/L), peptone (5g/L, Type II; enzymatic hydrolysate from meat), and 20g/L of either d-xylose (Grade II; 99% pure), d-glucose or a mixture of d-xylose and d-glucose] was used in low cell density fermentations. All components of the medium were purchased from Sigma. The xylose and nutrient solution (yeast extract, malt extract and peptone) were autoclaved separately. After the two solutions were autoclaved and combined the pH was initially between 5.5 and 6.0; the pH was not controlled in these set of experiments. In high cell density experiments, media were formulated with systematic variations of yeast extract, malt extract, and peptone.

Seed Cultures:

In the **low cell density fermentations** inoculations were made from 48 hour slants of *Candida shehatae* into spinner flasks (250 ml working volume). For inoculations into the benchtop fermentor a 48 hour slant of *Candida shehatae* was rinsed with 5 ml of the fermentation medium and aseptically transferred to the 2L NewBrunswick Benchtop fermentor.

In the **high cell density fermentations**, seed cultures were grown in the medium of Wayman and Tsuyuki (1985). The seed cultures were grown in 500 ml erlenmeyer flasks with working volumes of 250ml (the same volume as the spinner

flasks). The seed cultures were inoculated via loop transfer from a 48 hour slant of *Candida shehatae* and incubated at 30°C under mild agitation (magnetic stirrer with a stirring bar) for about 48 hours. After the 48 hour incubation period, the cells were centrifuged at 5000 rpm for 15 minutes. The supernatant was decanted and the cells resuspended with 10-15 ml of the fermentation medium (from the spinner flask). The cells were then transferred to the spinner flasks.

Fermentors:

Fermentations performed in the Bellco Spinner Flasks were conducted at 30°C. The spinner flask contained two side ports and a domed top. Each reactor had a working volume of 250 ml. Agitation, approximately 250 revolutions per minute (rpm), was provided by a magnetic stirrer (the rpm was estimated by a digital stroboscope).

The benchtop fermentor consisted of a NewBrunswick Multigen 2L Benchtop Fermentor (F-2000). Temperature was controlled between 25°C and 32°C. A working volume of 500 ml was used and agitation was maintained at 350 rpm. The oxygen in the dead space above the liquid level was not removed with nitrogen nor was the oxygen in the medium removed before inoculation. The dissolved oxygen level was monitored via a Dissolved Oxygen Probe (900 Series, with a replaceable membrane) and Dissolved Oxygen Analyzer (Model DO-40). It should be noted, that in the benchtop fermentations air was initially sparged through the system to set the dissolved oxygen concentration at 100% of saturation. Once 100% saturation was reached the air was turned off. For this reason, and the fact that the benchtop contained 3 internal baffles and 2 turbine impellers, the fermentations in the benchtop were considered semi-anaerobic relative to those carried out in the spinner flasks (anaerobic).

Analytical Methods:

Two to five ml samples were taken at periodic intervals and placed in ice. An aliquot of the sample was mixed 1:1 with 0.1% methylene blue and a hemacytometer was used to determine viable cell concentration (Kim, et. al, 1987). After viable cell counts were made, the remaining sample was centrifuged at 5000 rpm for 15 minutes. The supernatant was decanted and stored in glass vials at 0°C. Xylose, Glucose, Xylitol and Ethanol concentration were determined by HPLC analysis using cellobiose (0.01 g/ml) as the internal standard. A Waters GPC-I, equipped with a Bio-Rad Aminex Ion Exclusion HPX-87H column and a differential refractometer, was used. The differential refractometer was set at 16x, and the mobile phase was 0.01N H_2SO_4 at a temperature of 60°C and a flowrate of 0.6 ml/min.

RESULTS

Carbohydrate Utilization Pattern and Rates:

In the initial four experiments, relatively low concentrations of *Candida shehatae* were inoculated into spinner flasks or the two liter benchtop fermentor. The experimental protocol resulted in essentially anaerobic fermentation conditions in the spinner flasks, while conditions in the benchtop fermentor were semi-anaerobic. As shown in Figures 1 and 2, fermentations were conducted with media containing both 10g/L xylose and 10g/L glucose. The rate of glucose consumption was considerably faster than xylose consumption for both the benchtop fermentor and spinner flask.

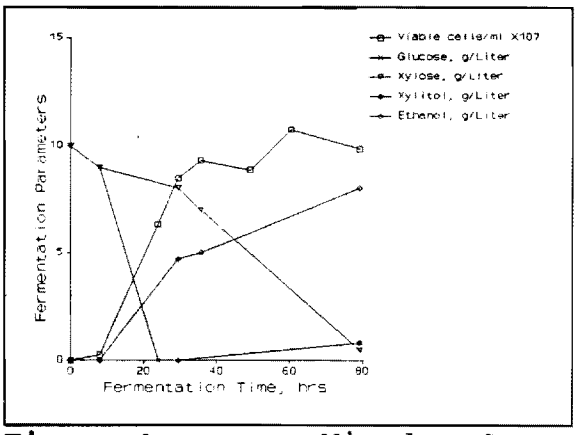

Figure 1 -- Mixed Sugar Fermentation in a Spinner Flask.

As can be seen from Figures 1 through 4, a much longer time was required to consume essentially all the carbohydrates in the mixed sugar fermentations (compared to the pure glucose fermentations; this was apparent for fermentations in both the spinner flasks and the benchtop fermentor). Less than 38 hours was required to consume 20 g/L of glucose in the spinner flask compared to 80 hours for a mixture of 50% glucose and 50% xylose at the same concentration. This same trend was observed in the benchtop fermentor; it required about 25 hours to consume 20 g/L of glucose compared to 70 hours for a mixture of 50% glucose and 50% d-xylose.

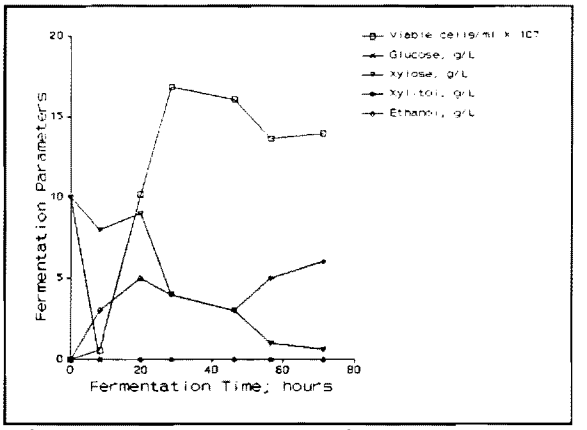

Figure 2 -- Mixed Sugar Fermentation in a Benchtop Fermentor.

Biomass Formation:

Figure 1 shows the biomass formation as a function of time under anaerobic conditions. After a lag in biomass formation of 10 hours, an exponential phase of growth was seen. When the microorganisms shifted metabolism from glucose to d-xylose, a lag in biomass formation (diauxic lag) of about 20 hours was observed. After this lag a slight increase in viable cell density occurred.

The increased uptake rate and more efficient utilization of glucose resulted in higher biomass levels for pure glucose fermentation. This trend in biomass formation can be seen in Figures 5 and 6. The final cell density for pure glucose

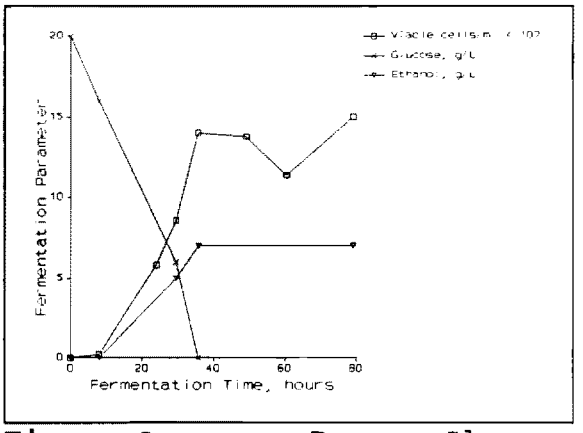

Figure 3 -- Pure Glucose Fermentation in Spinner Flask.

fermentation in the spinner flask was 15 X 10^7 cells/ml compared to 10 X 10^7 cells/ml (**all cell densities reported are the number of viable cells/ml**) in the mixed sugar fermentation. A similar trend was seen in the fermentations performed in the benchtop (see Table 1). In addition, fermentation in the benchtop resulted in higher biomass levels when compared to fermentations in the spinner flask (see Table 1).

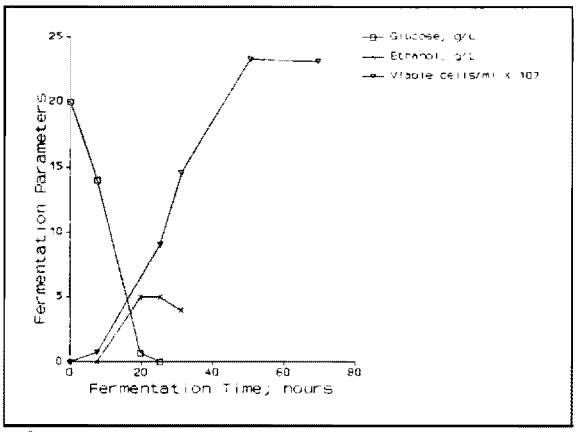

Figure 4 -- Pure Glucose Fermentation in Benchtop Fermentor.

Table 1.0 -- Comparison of Final Cell Densities for Mixed Sugar and Glucose Fermentation

Conditions	Cell Density, X 107 cells/ml
BT, Glucose only	25
SF, Glucose only	15
BT, Mixed Sugars	15
SF, Mixed Sugars	10

Ethanol Production:

Figures 1 and 2 show ethanol concentration as a function of time for both the benchtop fermentor (semi-anaerobic) and the spinner flasks (anaerobic). A lag period of about 10 hours was seen in the spinner flask (Figure 1) before ethanol was produced. However, no lag in ethanol production was seen in the benchtop fermentor (Figure 2). Later in the fermentations (both under anaerobic and semi-anaerobic conditions) a second lag in ethanol formation was seen.

Under anaerobic conditions (spinner flask) the lag occurred 30 hours into the fermentation and lasted about 10 hours. The lag in ethanol formation appeared to result after the glucose had been consumed and carbohydrate metabolism had shifted to d-xylose catabolism. Under semi-anaerobic conditions (benchtop fermentor) ethanol reached a peak but was then slowly consumed for about 30 hours. After 30 hours the ethanol concentration increased to a final level of 6 g/L at 80 hours.

One can also compare ethanol levels between the

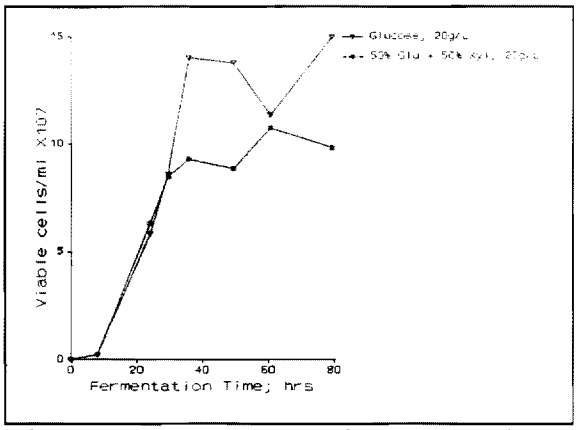

Figure 5 -- Comparison of Mixed Sugar and Pure Glucose Fermentation in a Spinner Flask. Legend -- Glu=Glucose and Xyl=d-Xylose; 50%(w/w)

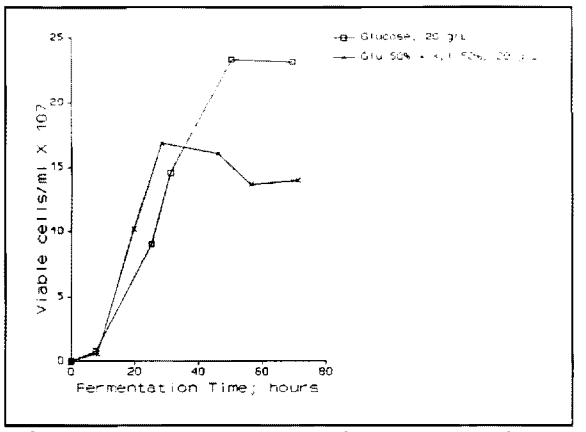

Figure 6 -- Comparison of Mixed Sugar and Pure Glucose Fermentation in Benchtop Fermentor. Legend -- Glu=Glucose and Xyl=d-Xylose; 50% (w/w)

mixed sugar fermentation and pure glucose fermentation. If one contrasts Figures 1 through 4, it can be seen that a lag in ethanol production occurred in all cases except for mixed sugar fermentation in the spinner flask. Ethanol reached a maximum at about 20 hours and was then consumed (see Figure 4).

In a more specific sense one can compare mixed sugar fermentation and pure glucose fermentation under different conditions; benchtop (**semi-anaerobic**) versus spinner flask (**anaerobic**). As indicated by Figures, 1 and 3, and 2 and 4, the final ethanol levels for mixed sugar fermentation and pure glucose fermentation were about the same; 7 to 8,g/L in the spinner flask and 5 to 6 g/L in the benchtop fermentor.

However, under anaerobic conditions, the pure glucose fermentation did result in a shorter time period required to produce the maximum ethanol concentration; about 38 hours compared to 80 hours for the mixed sugar (see Figures 1 and 3). Under semi-anaerobic conditions the maximum ethanol concentration was achieved in 20 hours compared to 80 hours in the mixed sugar fermentation (compare Figures 2 and 4).

Ethanol Yields:

As expected fermentation under semi-anaerobic conditions (in the benchtop) resulted in reduced ethanol yields ($Y_{p/s}$). The data in Table 2.0 show that under semi-anaerobic conditions the ethanol yield for **pure glucose** fermentation was 0.25 compared to 0.35 under anaerobic conditions. Also, the data indicate a similar

trend for mixed sugar fermentations; the ethanol yield in the benchtop was 0.30 contrasted to 0.40 in the spinner flask.

Table 2.0 -- Comparison of Ethanol Yields; Yp/s

Conditions	$Y_{p/s}$
SF, Pure Glucose	0.35
Mixed Sugars	0.40
BT, Pure Glucose	0.25
Mixed Sugars	0.30

By-Product Formation:

Xylitol, a by-product of d-xylose fermentation, was formed in the mixed sugar fermentations performed in the spinner flasks. As can be seen from Figure 1, 0.8 g/L xylitol was formed late in fermentation (about 80 hours into the fermentation). However, as indicated by Figure 3, if mixed sugars were fermented in the benchtop fermentor, xylitol was not detected in the HPLC analysis.

In addition to xylitol several unknown peaks were detected in the HPLC analysis; Table 3.0 indicates the residence times and conditions under which these intermediates were formed.

Table 3.0 -- Unknown By-Products Formed in Ethanol Fermentation via *Candida shehatae* (HPLC Analysis *)

Conditions	Unknown Retention Times, min
SF, Pure Glucose	14
Mixed Sugar	14
BT, Pure Glucose	9.70
	13.66
Tested Components	
Glycerol	13.81
α-Keto Glutaric Acid	8.56
Citric Acid	8.04
Pyruvic Acid	10.17

* see Materials and Methods Section for conditions of HPLC Analysis

Cell Viability:

Although a viability index (percentage of total cells which are viable) was not calculated, each cell count was a viable cell count. As indicated in Figures 1 through 6, under each fermentation condition the cell counts reached a plateau which was maintained throughout the fermentation (approximately 80 hours). Declines in viable cell counts were not observed.

Nitrogen Source Effect(s) on Xylose Fermentation:

Various types of non-chemically defined additives are routinely added to *C. shehatae* fermentations to enhance growth and productivity of the micro-organisms. In the next set of experiments, the

effect(s) of yeast extract [3g/L], peptone [5g/L] and malt extract [3g/L] on *C. shehatae* fermentation of xylose was examined. These additives are sources of nitrogen and growth factors. Figures 7 through 10 indicate the presence of these additives had little effect on the initial xylose consumption rates (the initial slope of xylose concentration versus time plot for each condition was about the same). Even in the experiment in which the media consisted only of xylose and water the xylose concentration plot versus time is initially the same as for media containing the additives. However, after about 10 hours the slope is reduced (xylose consumption rate is reduced), and as time increases the slope is reduced to zero (which results in some residual xylose left after the fermentation).

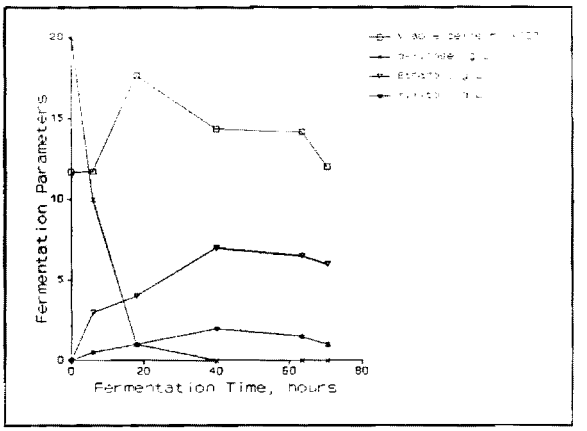

Figure 7 -- d-Xylose Fermentation in a Medium Containing Yeast Extract, Peptone and Malt Extract; the Fermentor was a Spinner Flask.

Biomass Formation:

As demonstrated in Figures 7 - 10, three or four phases of growth seemed apparent in the pure xylose fermentations; the number of phases depended on the medium. In the case of the control (Figure 7) and peptone only (Figure 10) a short lag phase was seen. A lag phase was not seen in the nitrogen deficient (Figure 8, yeast extract only) and non-growing (Figure 9, no components added) mediums. Next, a short burst of growth was seen for each condition. Once the exponential phase of growth reached a plateau a stationary phase was observed. **However, it should be noted that under non-growing conditions (Figure 9) a stationary phase was not observed (after the growth peaked a decline or death phase was immediately observed).** In the later stages of the fermentation a death phase was seen to occur under only one condition, the non-growing medium.

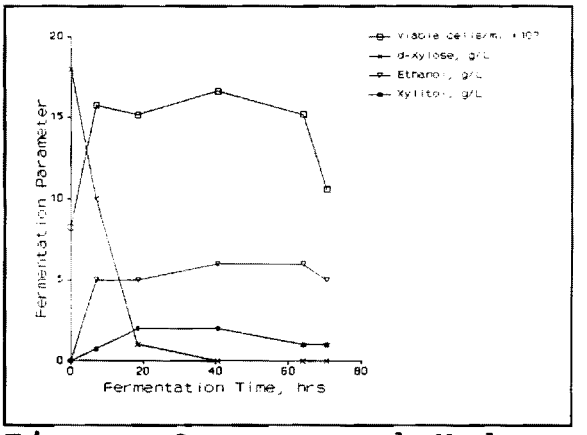

Figure 8 -- d-Xylose Fermentation in a Medium Containing Only Yeast Extract; the Fermentor was a Spinner Flask.

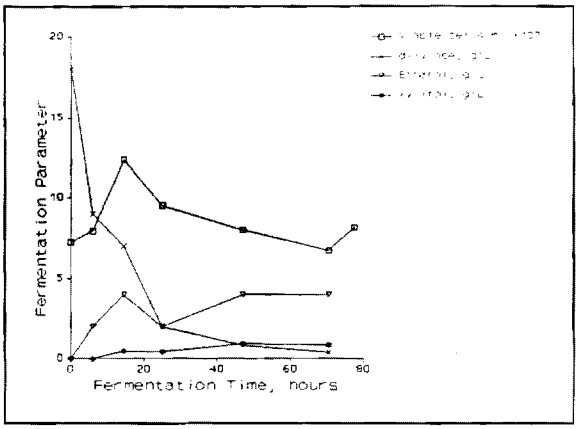

Figure 9 -- d-Xylose Fermentation in a Medium Containing Substrate Only; the Fermentor was a Spinner Flask.

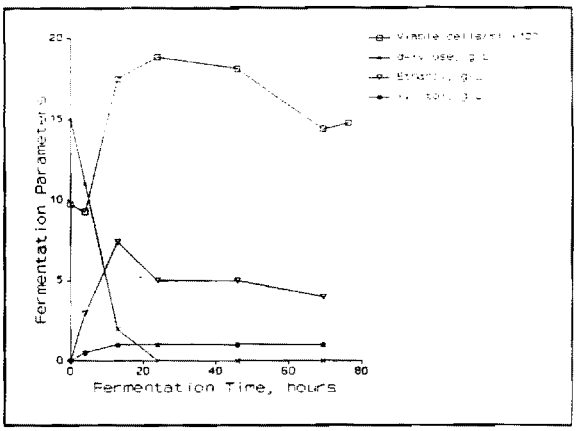

Figure 10 -- d-Xylose Fermentation in a Medium Containing Peptone Only; the Fermentor was a Spinner Flask.

Ethanol Production:

There was no significant difference in ethanol production rates under the different fermentation conditions; however, the ethanol yields did vary. The highest ethanol yield was achieved in the fermentation medium containing only peptone (see Table 4).

Table 4 -- Ethanol Yields of d-Xylose Fermentation via *Candida shehatae* under Varying Medium Components

Reactor System	$Y_{p/s}$, g etoh/g xylose
Control, YE,ME,Pep	0.33
YE only	0.33
---	0.22
Peptone only	0.46

By-Product Formation:

In every case, except under non-growing conditions, xylitol was formed very early in the fermentations; within the first 5 hours. A maximum of 2g/L of xylitol was formed under the control conditions (YE, ME and Peptone) and with the addition of yeast extract only. Much lower levels of xylitol were formed in the fermentations with substrate only and peptone only; about 1.0 g/L respectively. An unknown peak was detected under the non-growing conditions (substrate only).

Cell Viability:

A viability index (percentage of viable cells) was calculated in the four different fermentation conditions (methylene blue stain; 1% w/v). In all cases except under non-growing

conditions, the viability index initially ranged between 0.85 and 0.9, increased during the short burst of cell growth, and maintained a level between 0.97 and 0.98 throughout the fermentation (see Figure 11). Under non-growing conditions (Figure 9), after the exponential phase the viability index steadily decreased to a final value of 0.7 after 80 hours.

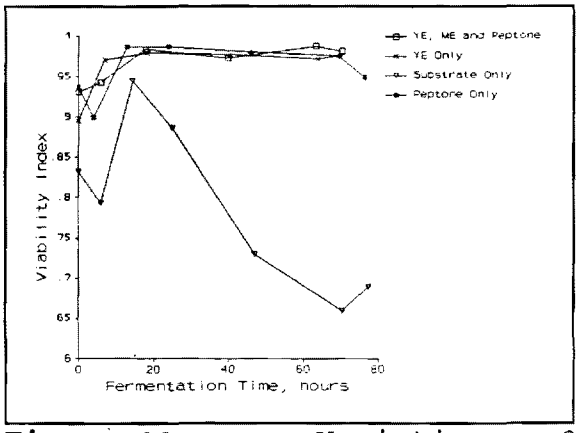

Figure 11 -- Variation of Viability Index with Medium Composition in d-Xylose Fermentation. Legend -- Viability Index = Viable Cells/(Viable Cells + Non-Viable Cells)

DISCUSSION OF RESULTS

Mixed and Pure Glucose Fermentation:

The results from this initial phase of research are in good agreement with data presented in the literature, except for data in Figures 1 and 2. Data for the mixed sugar fermentations suggest that d-xylose and glucose were consumed simultaneously. This observation does not agree with previous work in mixed sugar fermentations. du Preez (1986) reported that *Candida shehatae* preferentially consumed glucose first in a mixture of d-glucose, d-galactose, L-rhamnose, d-cellobiose and d-xylose (10 g/L each). Once the glucose had been consumed the other sugars were consumed simultaneously; except for cellobiose (*Candida shehatae* did not consume cellobiose). In order to confirm the pattern of carbohydrate utilization in mixed sugar fermentations two changes in the experimental procedure are needed: One, increase the number of samples taken in the first 20 hours of fermentation; and two, run each fermentation in duplicate (or at least replicate the experiments).

As reported with other d-xylose fermenting yeast (Slininger et al., 1985), *Candida shehatae* consumed glucose at a faster rate than d-xylose. When the microorganisms shifted carbohydrate metabolism from glucose to d-xylose a diauxic lag in ethanol and biomass formation was seen under anaerobic conditions (Figure 1). Enzymes necessary for d-xylose metabolism must be induced (contrary to glucose enzymes which are constitutive), this period of induction results in the diauxic lag. Once the enzymes are formed via protein synthesis, biomass and ethanol formation continue at a different rate.

Under semi-anaerobic conditions a diauxy in ethanol formation was observed but not in biomass formation. Apparently, when glucose was completely consumed the microorganisms were able to metabolize ethanol until the

proper enzymes for d-xylose metabolism had been formed. As a result, biomass formation continued while ethanol concentration decreased. Once the induction period was completed the ethanol concentration increased (see Figure 2).

Biomass levels were highest in the benchtop fermentor (compared to the spinner flasks). This was due to higher initial oxygen concentrations and longer extended oxygen concentrations (this is a qualitative assessment; the percent saturation of O_2 in the spinner flasks was not determined). At time zero the oxygen concentration was 90% saturation, but after 8 hours it had dropped to 0%. The longer extended oxygen concentrations in the benchtop fermentor were attributed to a larger head space (1500 ml, compared to 250 ml in the spinner flasks), four baffles and a better agitation system (two impellers). Contrary to the benchtop fermentor the spinner flask contained no baffles, one impeller and a smaller head space (approximately 250 ml).

Under anaerobic conditions many researchers report the formation of xylitol in d-xylose and mixed sugar fermentations (Screenath et al., 1986 and du Preez et al., 1986). Xylitol was produced late in the mixed sugar fermentation (at the 30 hour point in the spinner flask), thus coinciding with data in the literature. Under the semi-anaerobic conditions generated in the benchtop fermentor no xylitol was formed. This data supports the hypothesis that one of the limiting steps in d-xylose catabolism is the conversion (reduction/oxidation step) of xylose to xylulose via the intermediate xylitol (du Preez and Van der Walt, 1983). If mass transfer of oxygen becomes severely limited, the cofactor (NAD) required for conversion of xylitol to xylulose can not be regenerated. Without NAD, xylitol builds up internally in the cell and is excreted (Alexander, 1986).

Pure d-Xylose Fermentation with High Starting Cell Densities:

The concentration and type of nitrogen source has a dramatic effect on fermentation parameters. The xylose uptake rate was severely affected by a lack of nitrogen (see Figure 9). Lack of an external nitrogen source terminates the microorganism's capability of synthesizing new protein from a fresh nitrogen source. Thus, with no outside nitrogen source the microorganisms synthesized protein from nitrogen reserves for the first 10-18 hours in the fermentation. This accounts for the similar d-xylose uptake rates when compared to the other conditions (Figures 7,8 and 10) and an increase in the viability index from 0.8 to 0.95 (corresponds to an increase in viable cell density). After this point in the fermentation, the d-xylose uptake decreases, the viability index decreases and ethanol is consumed.

FUTURE PLANS

The initial phase of this research was designed to explore the response of the

yeast, *Candida shehatae*, to a limited number of experimental conditions. First, mixed sugar (50% d-xylose and 50% glucose) fermentations under anaerobic and semiaerobic conditions (and at low starting cell densities) were performed. Next, the same conditions were applied to pure glucose fermentation. Finally, pure d-xylose fermentations (at high starting cell densities) with a variation in medium composition were performed. From this experimental data the next phase of research can be formulated.

In the next phase of this research, additional mixed sugar fermentation experiments will be conducted. Data from these experiments are necessary to quantify carbohydrate utilization rates. Experiments will also be conducted to better understand the effects of anaerobic fermentation on the viability of *C. shehatae*.

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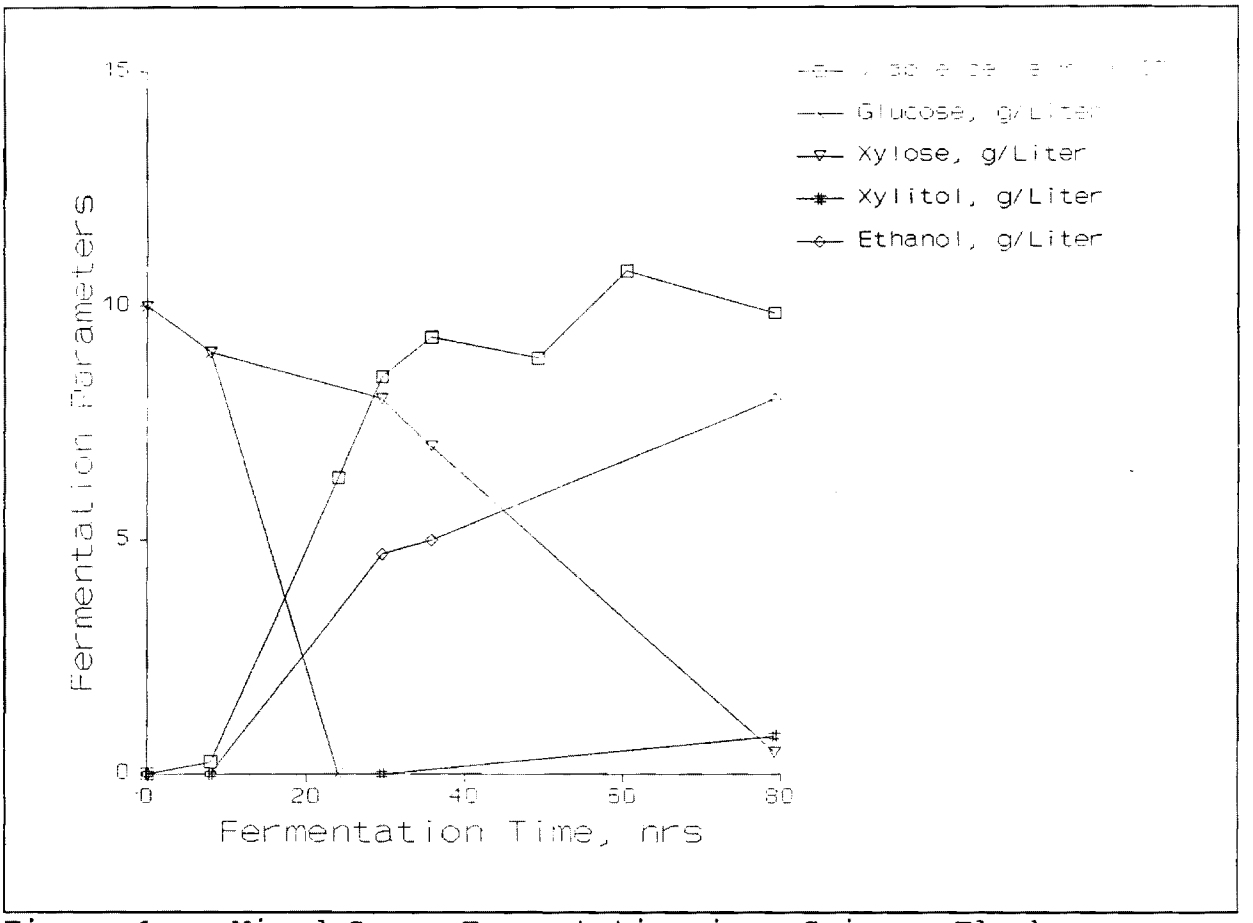

Figure 1 -- Mixed Sugar Fermentation in a Spinner Flask.

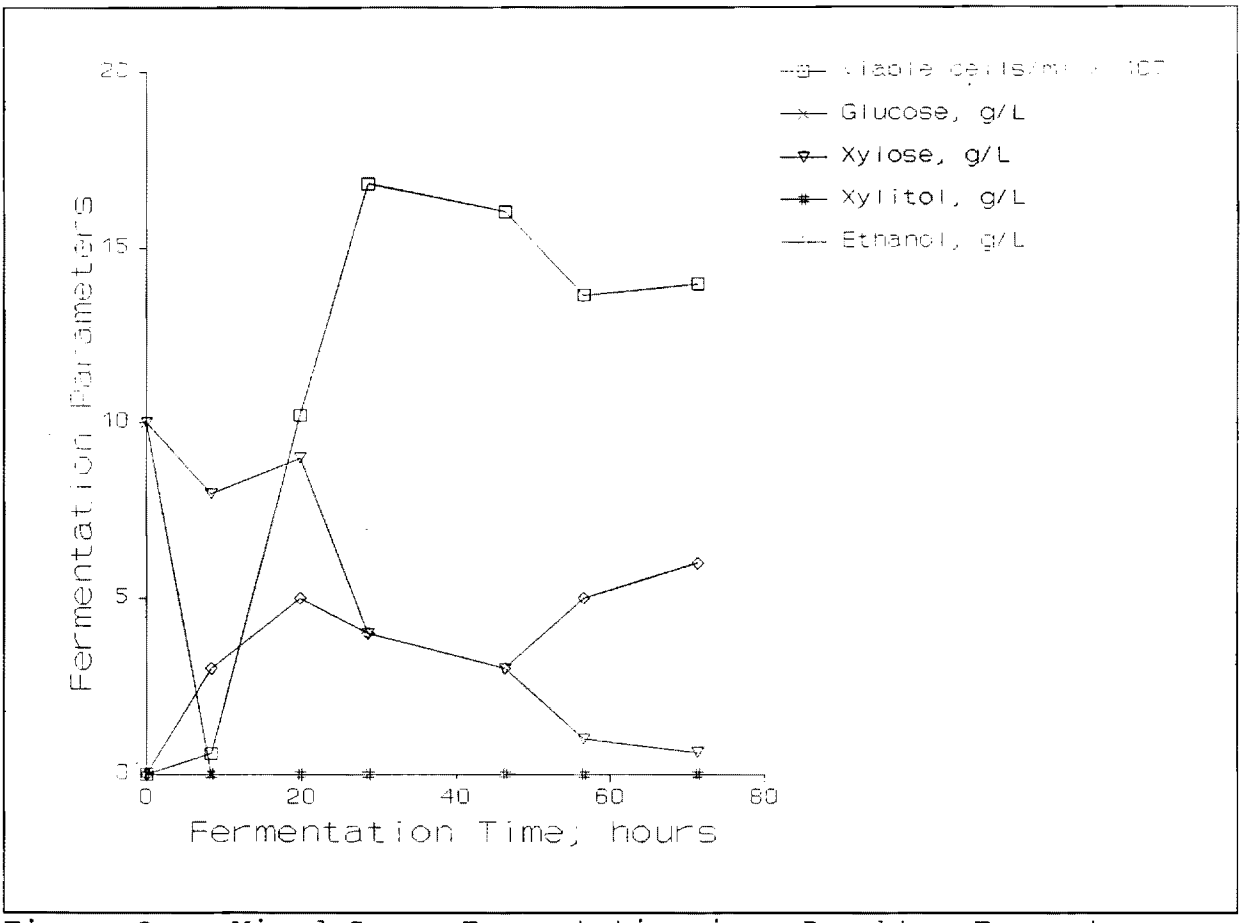

Figure 2 -- Mixed Sugar Fermentation in a Benchtop Fermentor.

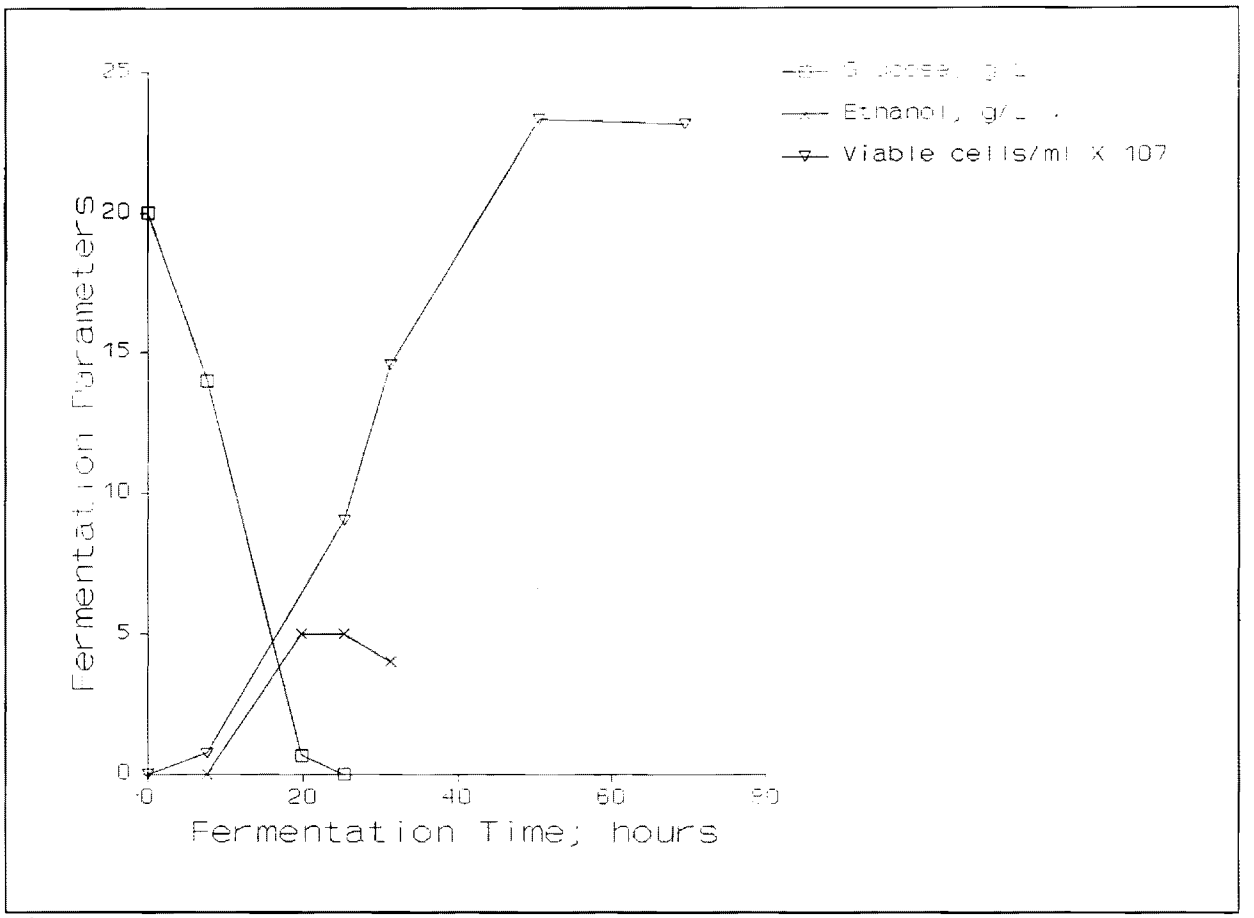

Figure 3 -- Pure Glucose Fermentation Benchtop Fermentor.

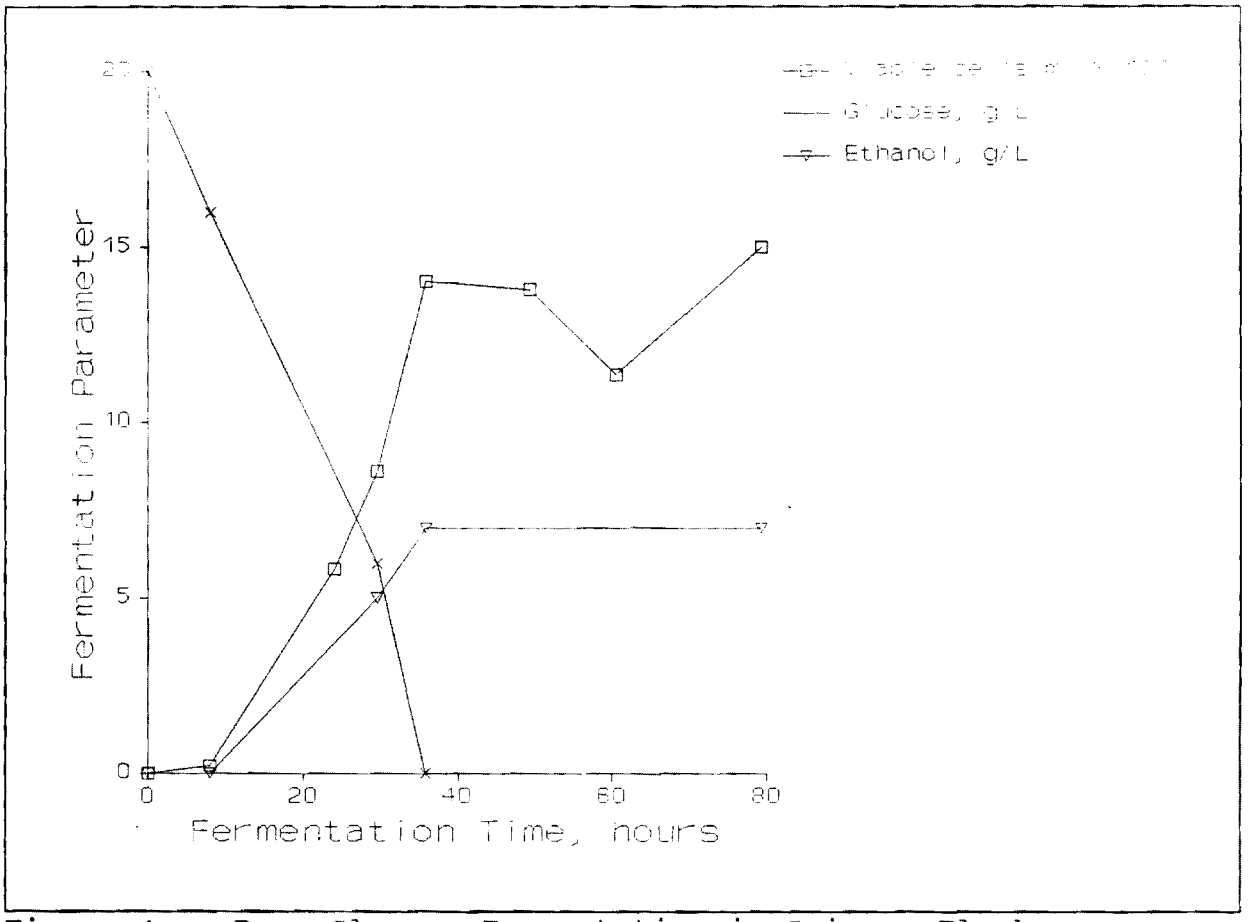

Figure 4 -- Pure Glucose Fermentation in Spinner Flask.

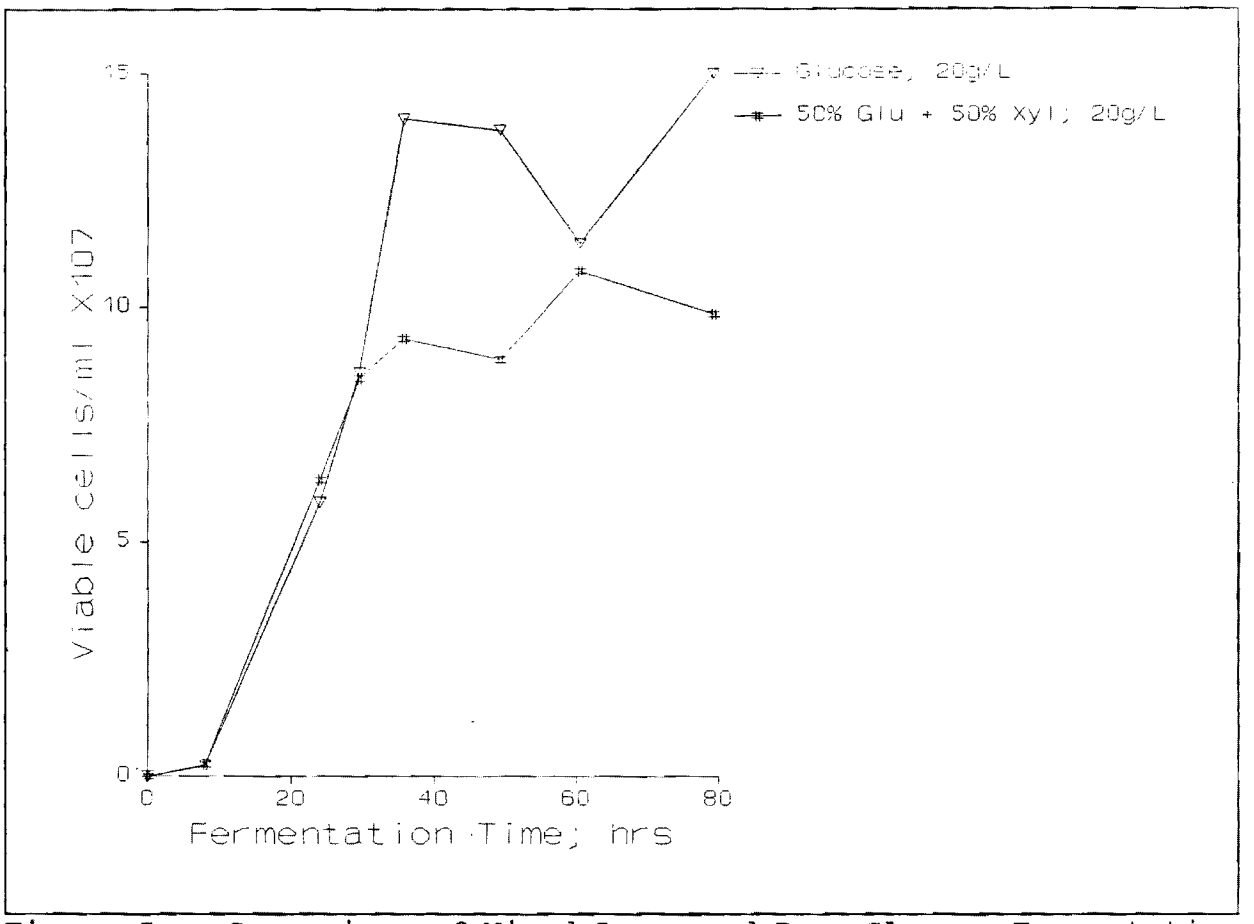

Figure 5 -- Comparison of Mixed Sugar and Pure Glucose Fermentation in a Spinner Flask. Legend -- Glu=Glucose and Xyl=d-Xylose; 50%(w/w)

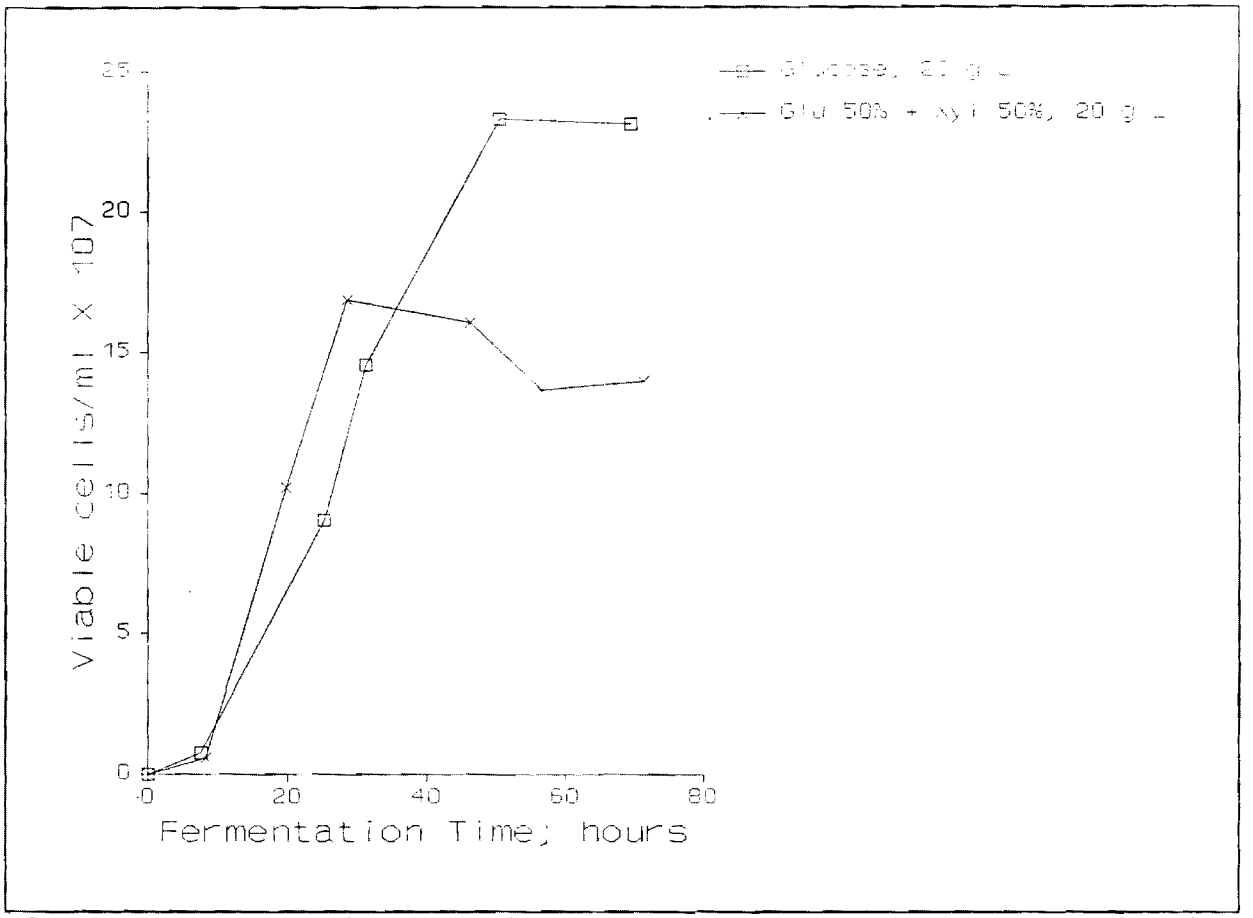

Figure 6 -- Comparison of Mixed Sugar and Pure Glucose Fermentation in Benchtop Fermentor. Legend -- Glu=Glucose and Xyl=d-Xylose; 50% (w/w)

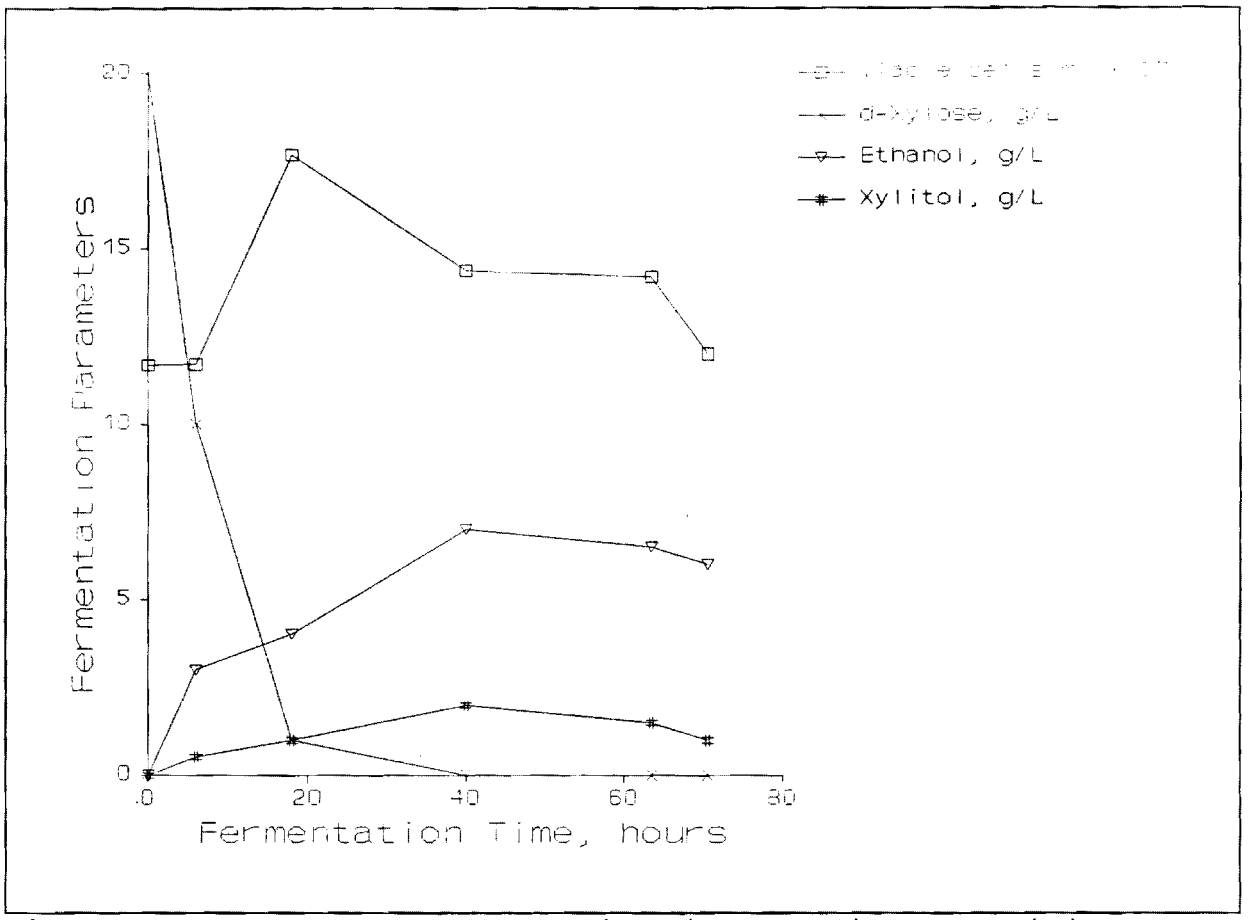

Figure 7 -- d-Xylose Fermentation in a Medium Containing Yeast Extract, Peptone and Malt Extract; the Fermentor was a Spinner Flask.

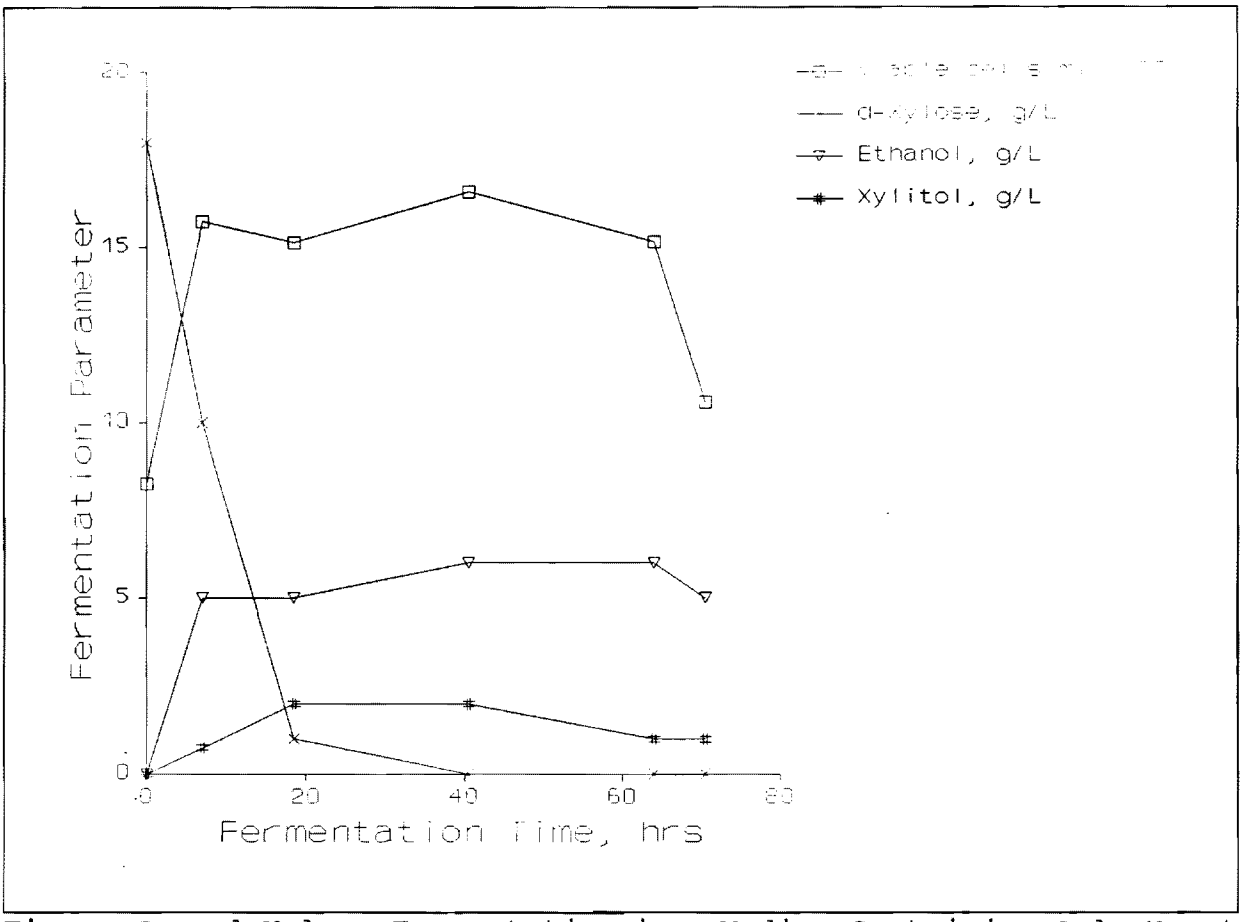

Figure 8 -- d-Xylose Fermentation in a Medium Containing Only Yeast Extract; the Fermentor was Spinner Flask.

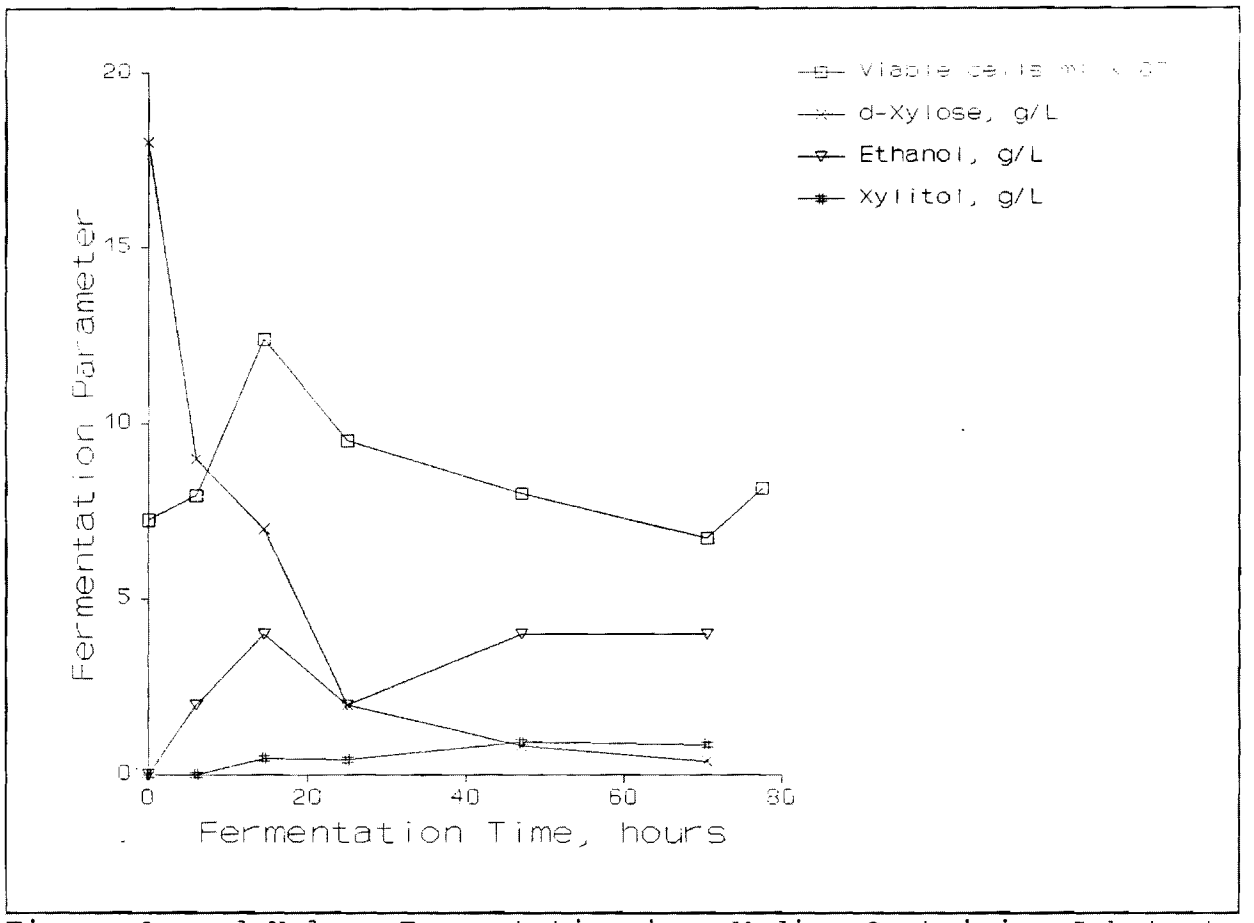

Figure 9 -- d-Xylose Fermentation in a Medium Containing Substrate Only; the Fermentor was a Spinner Flask.

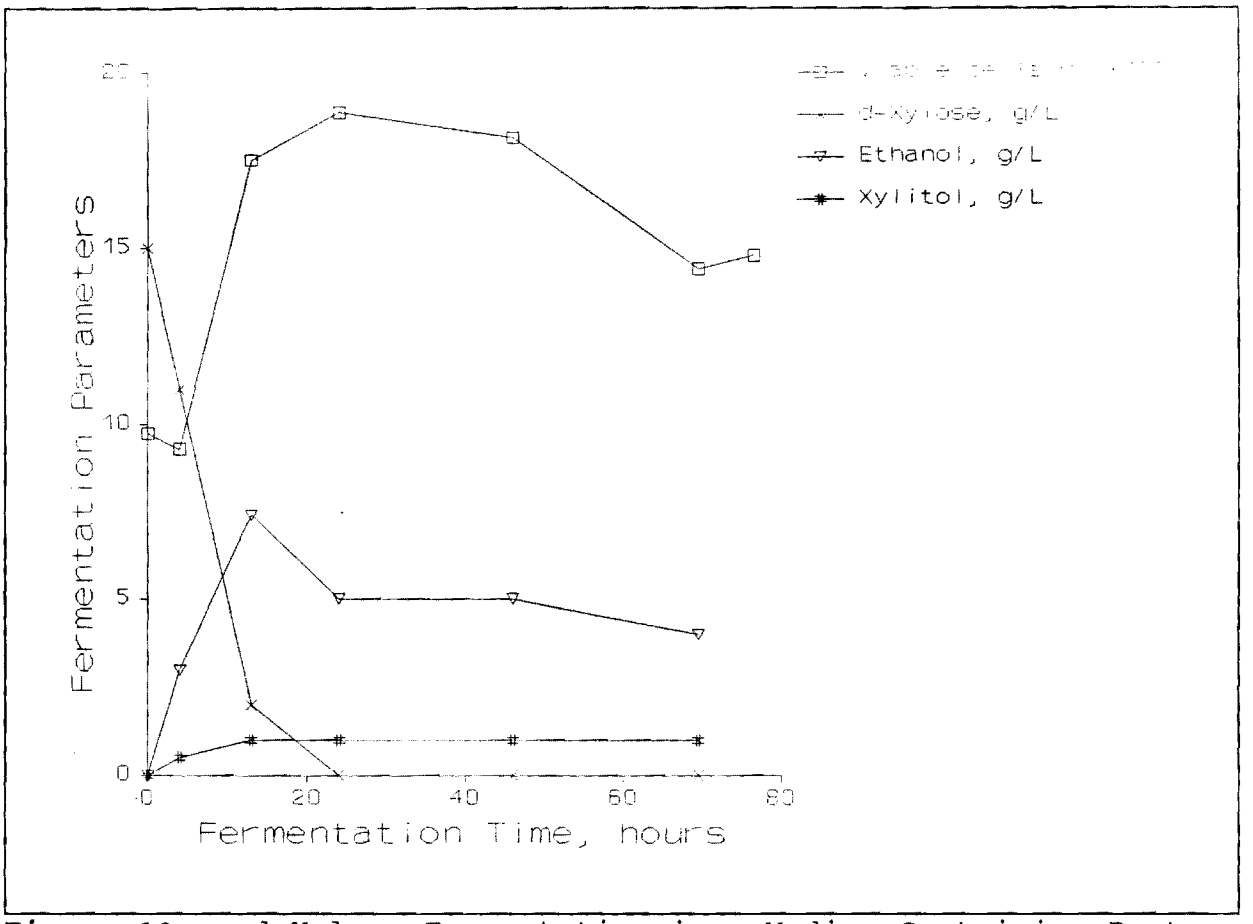

Figure 10 -- d-Xylose Fermentation in a Medium Containing Peptone Only; the Fermentor was Spinner Flask.

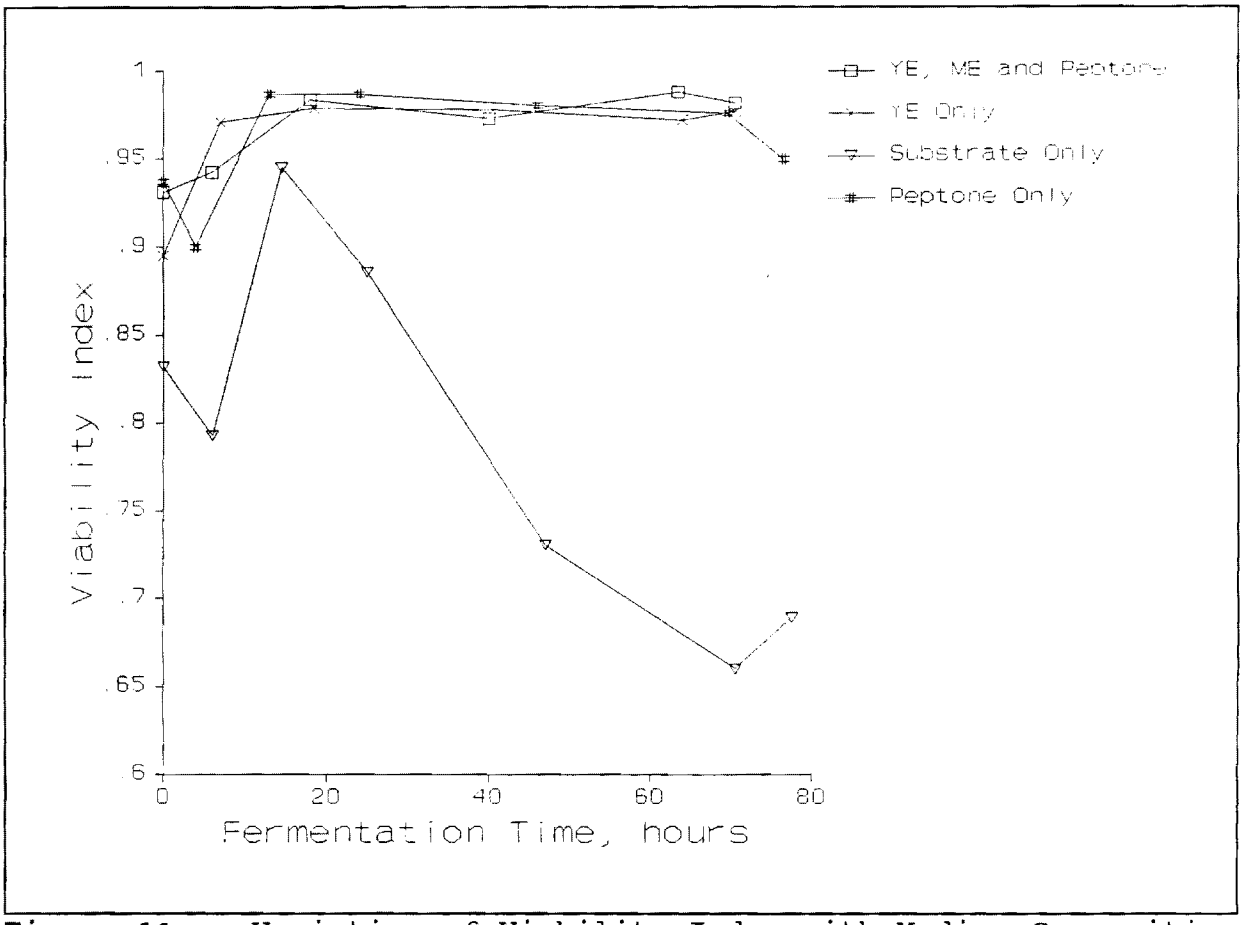

Figure 11 -- Variation of Viability Index with Medium Composition in d-Xylose Fermentation. Legend -- Viability Index = Viable Cells/(Viable Cells + Non-Viable Cells)

QUARTERLY PROGRESS REPORT #2
October 1, 1988 - December 31, 1988
Contract # TV-74261A

ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC RESIDUES

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

J. R. Kastner and Professor R. S. Roberts

School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

January 9, 1988

ABSTRACT

Mixed sugar and d-xylose fermentations (initial sugar concentrations were 100 g/L) were performed with the yeast *Candida shehatae* at a pH of 4.5, in an anaerobic mode and at a temperature of 30°C. The fermentations were performed at high cell densities by inoculating with cells aerobically grown in d-xylose.

In one set of pure d-xylose fermentations the nitrogen source was varied. It was found that the type of nitrogen source effected cell viability. Peptone, when compared to ammonium sulfate, maintained a higher percentage of viable cells (viability index) in the same time period; at 70 hours the viability index for peptone was 65% compared to 45% for ammonium sulfate.

In another set of experiments, pure d-xylose fermentations with and without TCA cycle intermediates were performed. Addition of the TCA cycle intermediates (citric acid 0.95 g/L, succinic acid 0.95 g/L, malic acid 0.95 g/L, alpha-keto-glutaric acid 0.95 g/L, and oxalacetic acid 0.477 g/L) maintained the viability index above 95% for a longer period of time and increased the final ethanol concentration. For example, without the TCA cycle intermediates the viability index was 38% at 110 hours, compared to 70% with the intermediates. Also, the final ethanol concentration was 30 g/L with the intermediates compared to about 22 g/L without.

Finally, it was found that aerobically growing the cells in the presence of d-xylose eliminated diauxy when transferred to a mixed sugar environment.

INTRODUCTION

The yeast *Candida shehatae*, is one of a few microorganisms capable of fermenting d-xylose and mixed sugars. Research with this organism has centered on evaluating the effect of process variables in terms of $Y_{p/s}$ (ethanol yield), u_{max} (maximum specific growth rate), q_p (ethanol production) and Q_p (maximum volumetric rate) (du Preez et al., 1984 and 1986; Screenath et al., 1986). It is felt that in addition to these parameters, **cell viability** would provide additional insight into the fermentation process (Jones, 1987 and Lee et al., 1981).

In previous fermentations with the yeast, *Candida shehatae* (Roberts and Kastner, Quarterly Report #1, 1988), cell viability was monitored via methylene blue staining. In pure d-xylose fermentations at high starting cell densities cell viability was correlated with medium composition. It was found that lack of a nitrogen source significantly reduced cell viability.

This paper reports the results for extension of this technique (correlation of cell viability with process variables) to other variables. The variables include the type of nitrogen source (peptone versus ammonium sulfate), initial sugar concentrations (about 100 g/L), mixed sugar

ratios (d-xylose to glucose ratio), seed culture technique and addition of intermediates to the fermentation broth.

MATERIALS AND METHODS

Microorganism and Culture:

See Procedures for first phase of study.

Medium:

The medium of Wayman and Tsuyuki (1985), excluding malt extract, was used for the seed cultures. The d-xylose concentration was 20 g/L in the seed culture and 90 g/L in the aerobic benchtop fermentations. Ammonium sulfate was substituted for peptone as the nitrogen source in the main fermentations (performed in spinner flasks). The pH of the nutrient solution (nitrogen source and yeast extract) was adjusted with 1N HCl to 4.5. After the pH adjustment a buffer, 10 g/L of KH_2PO_4, was added. The pH of the sugar solution was also adjusted to 4.5. The two solutions were autoclaved separately.

Experimental Design:

Table 1 -- Medium Compositions and Sugar Ratios Used in Fermentations

No.	Sugar Ratio xyl/glu	YE g/L	Pep g/L	AS g/L
1	10/90	3	5	-
2	20/80	3	5	-
3	50/50	3	5	-
4	100/0	3	5	-
5	100/0	3	-	5
6	100/0	3	5	-
7	100/0	3	-	5
8	100/0	3	-	5
9	75/25	3	-	5
10	50/50	3	-	5
11	25/75	3	-	5
12	0/100	3	-	5

- xyl stands for d-xylose
  glu for glucose;
  pep for peptone
  AS for $(NH_4)_2SO_4$

- **the seed culture preparation (aerobic fermentation) was different for the first six experiments.**

- **In experiment 6 the reactor system was spiked at time zero with 20 g/L of ethanol**

- **In experiment 7 the reactor system was spiked with TCA cycle intermediates 10-15 hours into the fermentation**

Seed Culture and Description of Fermentors:

Experiments 1-6:

The seed cultures for experiments 1 through 11 were prepared in the following manner. A YM Broth-2%Agar slant was inoculated with *Candida shehatae* and incubated at 30°C for 24 hours . A rinse transfer from the slant was then used to inoculate a 500 ml Erlenmeyer Flask with a 200 ml working volume; agitation was provided by a magnetic stirring bar. The flask was incubated at 30°C for approximately 24-36 hours. The seed culture was then aseptically transferred to the 2L NewBrunswick Multigen Benchtop Fermentor for aerobic batch fermentation.

The aerobic batch fermentation was performed under the following conditions; a temperature of 30°C, a pH between 4.5 and 5.0, an agitation rate of 600 revolutions per minute, and an air flow rate of 10 ml/min. After about 56 hours, 250 ml aliquots were withdrawn and centrifuged at 5000 rpm for 10-15 minutes. The centrifuge bottles were then decanted and used to inoculate the Bellco spinner flasks.

Experiments 7-12:

The seed culture for experiments 12 through 19 was developed in the following manner. A 24-48 hour slant of *Candida shehatae*, incubated at 30°C, was used for inoculation of the seed culture. A rinse transfer of 5ml was made from the slant to a 500 ml erlenmeyer flask (working volume of 300 ml). Upon aseptic inoculation the broth was incubated at 30°C with vigorous agitation (via a stirring bar) for about 24-36 hours. This culture was then used to inoculate an aerobic batch fermentation.

The aerobic batch fermentation was performed in an 8 liter NewBrunswick batch fermentor (Model FS-607 Fermentation system with a Model F7-100 batch reactor). The Model F7-100 had the following features: 1) height to diameter ratio of 4/1, , 2) an air sparger, four internal baffles and three bladed disk impellers, and 3) input/output ports consisting of a gas inlet (with filter), gas exhaust, inoculation port, thermocouple port, sampling line and two auxiliary ports. The fermentation was carried out under the following conditions; 1) working volume of 3 liters, 2) agitation at 400 rpm's, 3) aeration of 2 l/min and 4) a temperature of 30°C, 5) a d-xylose concentration of 90 g/L (it should be noted that a small amount of antifoam was added, 2-3 ml). Periodic samples were taken and microscopic observations performed to check for contamination.

After 56 hours, 250 ml volume aliquots (1 per spinner flask and 4 per NewBrunswick batch reactor) were withdrawn from the batch reactor (aseptic method). These aliquots were spun down at 5000 rpm for 10-15 minutes (the centrifuge bottles had been autoclaved before aliquots were withdrawn).

Fermentations:

Each 250 ml aliquot was decanted after centrifugation

(aseptic method) and used to inoculate either spinner flasks (Bellco) or a NewBrunswick Multigen batch reactor. All conditions imposed on the two reactor systems were the same as previously described (Quarterly Report #1), except the working volume for the Multigen Benchtop was **1 liter and agitation was maintained at 250 rpms**. Also, in the spinner flask fermentations, samples were taken through a rubber septum in one side port via a sterile syringe. The dissolved oxygen level was not monitored during the fermentations and no air was sparged through the reactor system.

TCA Cycle Intermediates:

In experiment 7 the following TCA cycle intermediates were added 11 hours into the fermentation; citric acid (0.952 g/L), succinic acid (0.952 g/L), l-malic acid (0.952 g/L), cis-oxalacetic acid (0.476 g/L), and alpha-keto-glutaric acid (0.95 g/L). The pH of the TCA cycle intermediate solution was not adjusted before aseptic addition. As a result the pH of the fermentor dropped from 4.5 to 3, and was then adjusted back to 4.5 by 10N NaOH.

Analytical Methods:

Cell densities were estimated with a hemacytometer. The method of Lee (Lee, et al., 1981) was used to obtain viable cell counts. Carbohydrates, ethanol and by-products were analyzed via HPLC analysis (see the previous analytical method section).

RESULTS and DISCUSSION

Variation of Nitrogen Source in d-Xylose Fermentation:

It should be noted that these fermentations were not run in duplicates. Thus, the data could not be represented with error bars. Therefore, any reference to exact values (such ethanol concentrations, xylitol concentrations, percent viability etc.) are only approximate values with no set confidence limits.

Two d-xylose fermentations with different nitrogen sources (peptone and ammonium sulfate) were performed. Each high cell density fermentation (inoculated from an aerobically grown seed culture) was carried out in a spinner flask. The conditions in the spinner flask were considered to be oxygen limited.

Figures 1 and 2 show results of d-xylose fermentations with the two different nitrogen sources; peptone and $(NH_4)_2SO_4$.

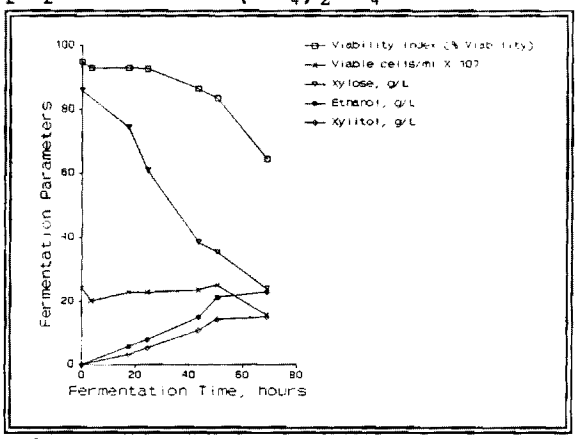

Figure 1 -- d-Xylose Fermentation in a Medium Containing Peptone; Fermentor was a Spinner Flask.

As portrayed by the two figures, the type of nitrogen source effected the d-xylose uptake rate, the xylitol level, and the viability index. There was approximately a 5 hour lag in d-xylose uptake if $(NH_4)_2SO_4$ was the nitrogen source, but no lag for peptone.

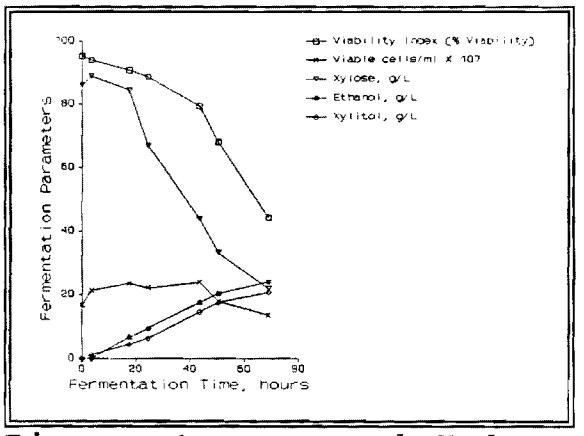

Figure 2 -- d-Xylose Fermentation in a Medium Containing $(NH_4)_2SO_4$; the Fermentor was a Spinner Flask.

However, the final d-xylose concentrations were about the same for the two fermentations; 20 g/L.

Another observed difference between the two fermentations was the final xylitol levels; 15.1 g/L if peptone was the nitrogen source and 21 g/L for $(NH_4)_2SO_4$.

The final and most noticeable difference was in the viability index. Figure 3 shows the change in percent viability versus time for the two fermentations. As can be seen from the graph, the viability index for the fermentation with $(NH_4)_2SO_4$ decreases at a faster rate than that for the fermentation in peptone.

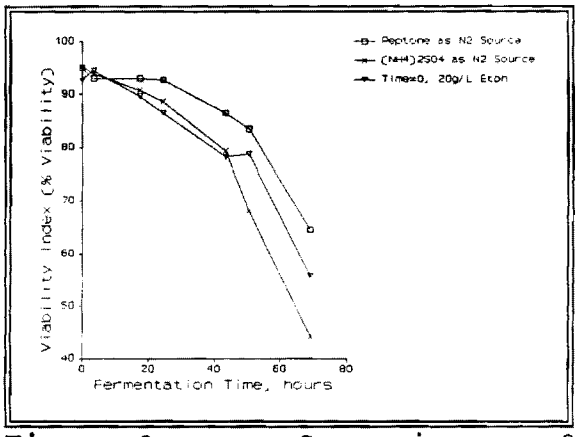

Figure 3 -- Comparison of Percent Viability for d-Xylose Fermentation in Two Different N_2 Sources and with an Initial Ethanol Concentration of 20 g/L; Fermentors were Spinner Flasks.

An amino nitrogen source (such as peptone) reduces the need for synthesis of amino acids from the carbohydrate source (Jones et al., 1981). If an amino nitrogen source is not present, the amino acids required for protein synthesis must be synthesized. Under oxygen limited conditions (anaerobic) two groups of amino acids can not be synthesized, the aspartic and glutamic acid family (Baily and Ollis, 1986). This is because precursors for amino acid synthesis are generated in the TCA cycle; a cycle which is shut down under anaerobic conditions.

Thus, the reduced viability and increase in xylitol concentration (in the fermentations with $(NH_4)_2SO_4$), may be a result of the microorganisms' inability to synthesize essential amino

acids required for d-xylose catabolism and cell maintenance (**under anaerobic conditions**).

Figures 1 and 2 also show a decline in cell viability with increasing ethanol concentration. In fact, the two figures indicate a limiting ethanol concentration of 20 g/L. To see if higher concentrations of ethanol could be achieved, a fermentation was performed in which ethanol (18 g/L) was added at time zero. The results are shown in Figure 4.

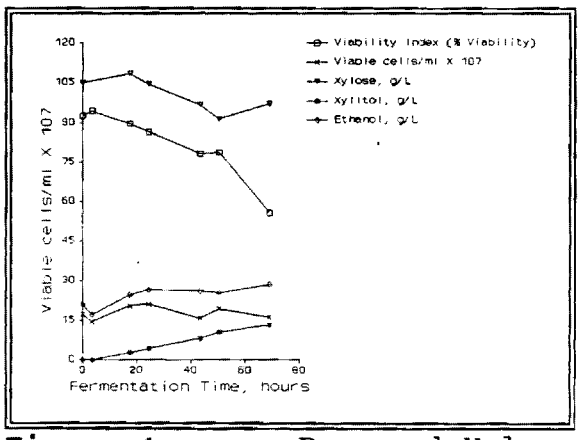

Figure 4 -- Pure d-Xylose Fermentation in a Medium Containing Peptone and an Ethanol Concentration of 20 g/L at Time Zero; Fermentor was a Spinner Flask.

The high initial ethanol concentration did cause a decline in the viability index, but not a sharp rapid decline. In fact, as indicated by Figure 3, the decline in percent viability was no more rapid than in the fermentation with $(NH_4)_2SO_4$ as the nitrogen source.

D-xylose utilization and further ethanol production was inhibited by the high initial ethanol concentration. Only 15 g/L of xylose was consumed and 5 g/L of ethanol produced. In addition about 10 g/L of xylitol was formed.

Thus, it appears that ethanol concentrations of about 20 g/L may not be the upper limit in ethanol production. Parameters other than ethanol concentration may limit final ethanol levels.

Some initial work was also performed with mixed sugars (10/90, 20/80, 50/50 d-xylose to glucose). Each fermentation was performed with a total initial sugar concentration of about 100 g/L. These high cell density fermentations (inoculated from aerobic seed cultures grown in d-xylose) were performed in spinner flasks and were considered to be anaerobic. Figures 5, 6 and 7 show results for these mixed sugar fermentations.

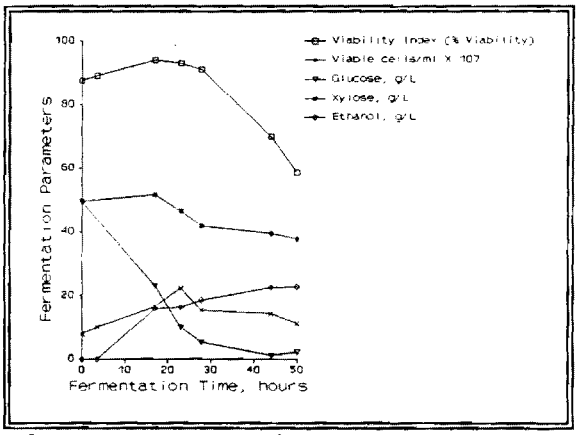

Figure 5 -- Mixed Sugar (50% Glucose and 50% d-Xylose) Fermentation in Spinner Flask.

The most notable results are those indicated in Figures 6 and 7. If the d-xylose to glucose ratio was 20/80 or lower, d-xylose was consumed first. After most of the d-xylose was consumed (a small residual amount was not utilized) glucose assimilation was initiated.

Regardless of the sugar ratio the final ethanol concentrations were about the same; 20 g/L. Also, the viability index began to decline 20 to 30 hours into the fermentation.

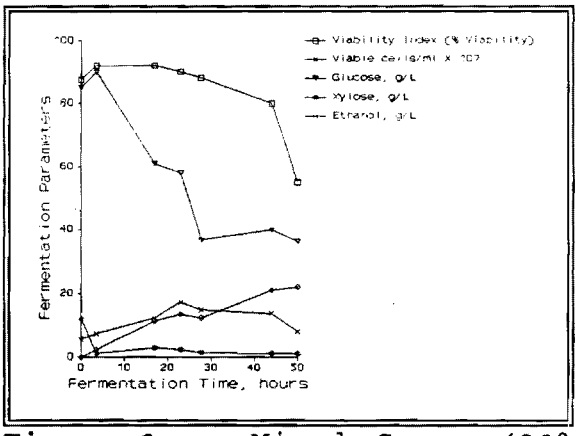

Figure 6 -- Mixed Sugar (90% Glucose and 10% d-Xylose) Fermentation in Spinner Flask.

Prolonged exposure to fermentative conditions has been qualitatively assessed to reduce cell viability (Alexander, et al., 1988). However, not until this work has the reduction in viability been quantified and correlated with fermentation parameters. Based on this data (and previous work) a hypothesis for the decline in cell viability was formulated.

It was hypothesized that *Candida shehatae* may not be able to synthesize essential amino acids under anaerobic conditions. Under anaerobic conditions precursors generated from the TCA cycle, are not available. These precursors are necessary for some amino acid synthesis. Thus, it was hypothesized that the addition of TCA cycle intermediates would enable the formation of these precursors under anaerobic conditions. In the next set of experiments this theory was applied to try and extend cell viability.

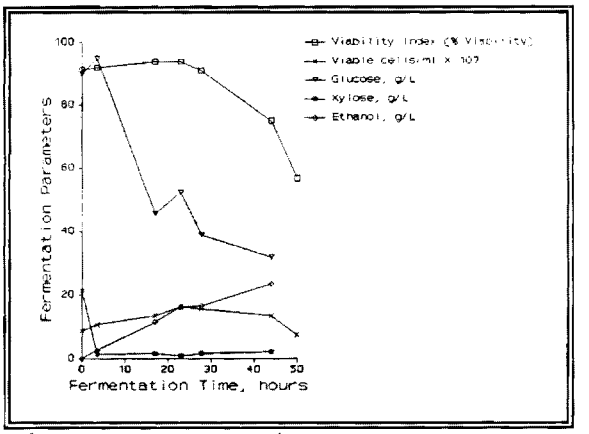

Figure 7 -- Mixed Sugar (80% Glucose and 20% d-Xylose) Fermentation in a Spinner Flask.

Experiments 7 - 12:

In this set of experiments, mixed sugar fermentations and pure d-xylose fermentations with and without TCA cycle intermediates were performed. All fermentations were performed in spinner flasks except for the TCA cycle fermentation which was performed in a benchtop fermentor. Each fermentation was inoculated with cells aerobically grown in the presence of d-xylose. Figures 8 through 13 show the results for these fermentations.

The most striking results can be seen in Figure 10, and in Figures 8 and 13. Figure 10 shows that in the fermentation with a 50/50 mixture of glucose and d-xylose, the two sugars were consumed simultaneously; no diauxy is apparent.

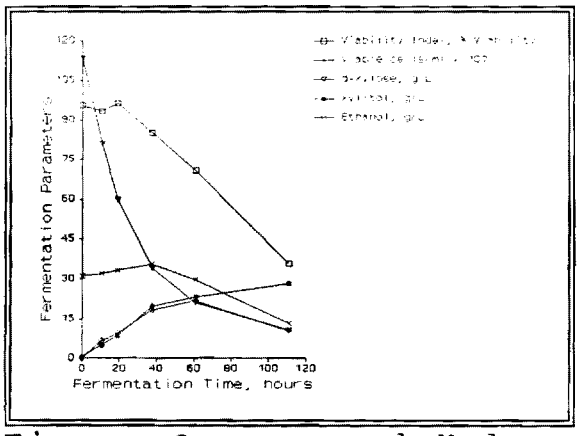

Figure 8 -- d-Xylose Fermentation in Spinner Flask

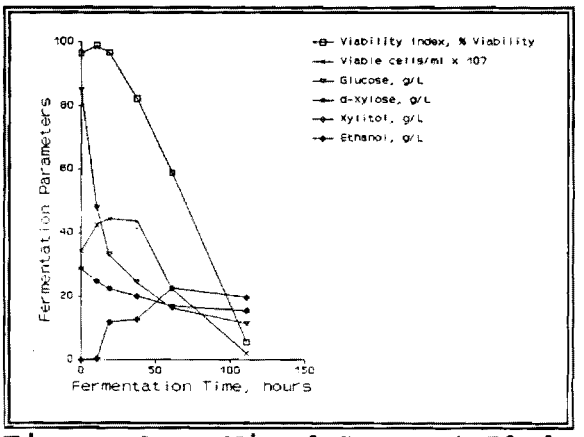

Figure 9 -- Mixed Sugar (25% d-Xylose and 75% Glucose) Fermentation in a Spinner Flask.

This behavior also occurred at other sugar ratios (see Figures 9 and 11).

Elimination of diauxy was a result of previously growing the cells in d-xylose under aerobic conditions. The aerobic conditions, combined with the presence of d-xylose, induced synthesis of enzymes required for d-xylose metabolism (and growth). This enabled simultaneous utilization of both d-xylose and glucose when the cells were

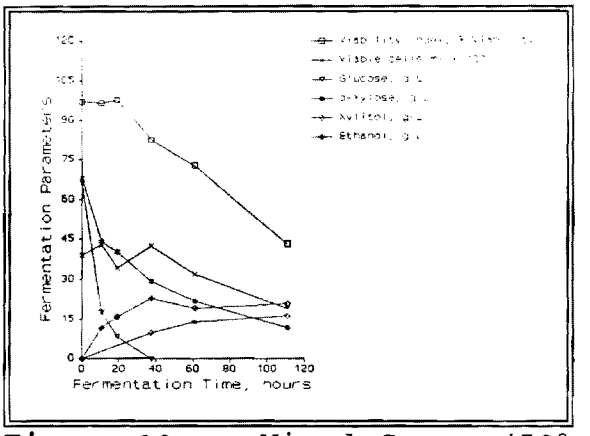

Figure 10 -- Mixed Sugar (50% d-xylose and 50% glucose) Fermentation in Spinner Flask.

transferred into a mixed sugar environment. Similar results have been obtained with the yeast, *Pachysolen tannophilus* (Slininger, P.J. and Bothast, 1987).

Figures 8 and 13 compare d-xylose fermentation with and without TCA cycle intermediates. Addition of the intermediates enhanced the fermentation in several ways.

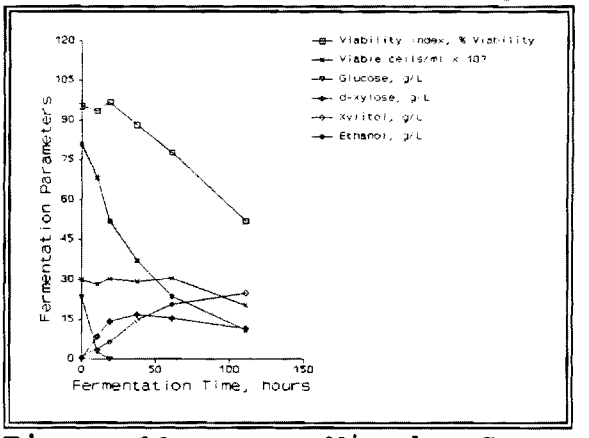

Figure 11 -- Mixed Sugar Fermentation (75% d-xylose and 25% glucose) in a Spinner Flask.

First, the final ethanol concentration was higher; 30 g/L compared to 23 g/L

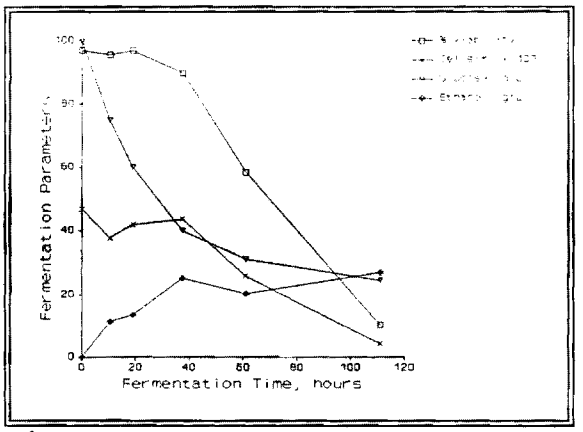

Figure 12 -- Pure Glucose Fermentation in Spinner Flask.

without the TCA cycle intermediates. Secondly, the xylitol level was lower (20 g/L) than in the fermentation without the TCA cycle intermediates (30 g/L). Thirdly, addition of the TCA cycle intermediates maintained a higher viability index for a longer time period. For

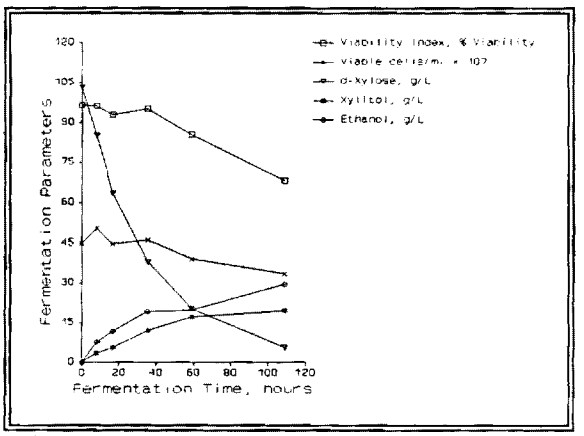

Figure 13 -- d-xylose Fermentation with Addition of TCA Cycle Intermediates 11 hours into the Fermentation; Fermentation was in a Benchtop Fermentor.

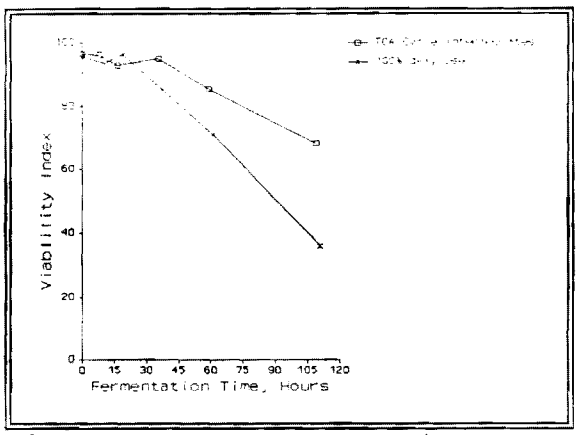

Figure 14 -- Comparison of Viability Index in d-Xylose Fermentation with and without TCA Cycle Intermediates.

example, 60 hours into the fermentation the viability index was about 90% (TCA) compared to 70% without TCA cycle intermediates (Figure 14).

These results suggest that addition of TCA cycle intermediates may be means to overcome the toxic effects of long-term exposure to anaerobic conditions. If the cell viability in mixed sugar fermentations can be extended this might enable higher final ethanol concentrations.

In addition, maintenance of cell viability under anaerobic conditions would enable fermentation at high cell densities (cell recycle or cell immobilization) without the continuous generation of viable cells (Alexander et al., 1988).

However, further experimental work is needed to verify that the addition of TCA cycle intermediates was independently responsible for maintenance of cell viability.

CONCLUSIONS and FUTURE PLANS

At this point in the research it would be premature to draw conclusions about the potential for industrial ethanol fermentation using *Candida shehatae*. Moreover, it would be premature to design a fermentation process based on data in the literature and in this work. More benchscale and pilot scale work is needed before that step is taken (Jefferies, 1985). However, conclusions can be drawn from this work which may point research in d-xylose and mixed sugar fermentations in the right direction.

Mixed sugar fermentations suffer from increased fermentations times due to diauxy. However, the data from this work show that diauxy can be eliminated by growing the cells in the presence of d-xylose under aerobic conditions. When transferred to a mixed sugar environment both d-xylose and glucose are consumed simultaneously.

It is apparent from this work that long term exposure to anaerobic conditions with a non-amino nitrogen source leads to a decline in cell viability. As a result the final ethanol concentration is limited to about 20 g/L. It is felt that the anaerobic conditions and a non-amino nitrogen source (such as $(NH_4)_2SO_4$ or Urea) inhibit the microorganisms ability to acquire or synthesize essential amino acids. This leads to the decline in cell viability, and consequently a limit on the final ethanol concentration.

It is thought that the decline in cell viability is in part due to the termination of the TCA cycle under anaerobic conditions. The addition of TCA cycle intermediates provides precursors for the synthesis of essential amino acids, which can then be used for protein synthesis. These preliminary data indicate that addition of TCA cycle intermediates helps to maintain the cell viability under anaerobic conditions. As a consequence higher final ethanol concentrations are achieved.

In mixed sugar fermentations, addition of TCA cycle intermediates combined with aerobically conditioning the cells in d-xylose may reduce fermentation times and increase final ethanol concentrations .

However, before these experiments are performed it must be verified that addition of TCA cycle intermediates was independently responsible for the increased maintenance of cell viability. Once this has been verified, the consumption pattern of the TCA cycle intermediates must be determined.

In addition to verifying the impact of the TCA cycle intermediates, the optimum conditions for aerobically growing the cells in d-xylose must be found. It is felt that the phase of growth is the most important variable in this step; early exponential phase being the best point to terminate the aerobic culture.

Variation in this step can lead to differences in the main fermentation (pure d-xylose and

mixed sugar fermentation), even if the main fermentation conditions are identical. This type of behavior can be seen by comparing Figures 5 and 10.

Figures 5 and 10 show data for two fermentations at 50% d-xylose and 50% glucose (high starting cell densities from aerobic cultures). As can be seen from the figures even though the main fermentation conditions were identical the results are different. The variation in results were attributed to differences in the aerobic culturing step. Based on this data it is apparent that the culture should be carefully monitored in terms of cell viability and growth phase. It is felt that the aerobic fermentation process should be terminated early in the exponential growth phase and at a high viability index (greater than 0.95). Therefore, future plans include the following: 1) find optimum conditions for aerobic conditioning of cells 2) use these conditions to complete the mixed sugar fermentation work and 3) determine TCA requirements for anaerobic mixed sugar fermentations.

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Wayman M. and S.T. Tsuyuki, Fermentation of Xylose to Ethanol by Candida shehatae, **Biotechnology and Bioengineering Symp.** No. 15, p.167, 1985.

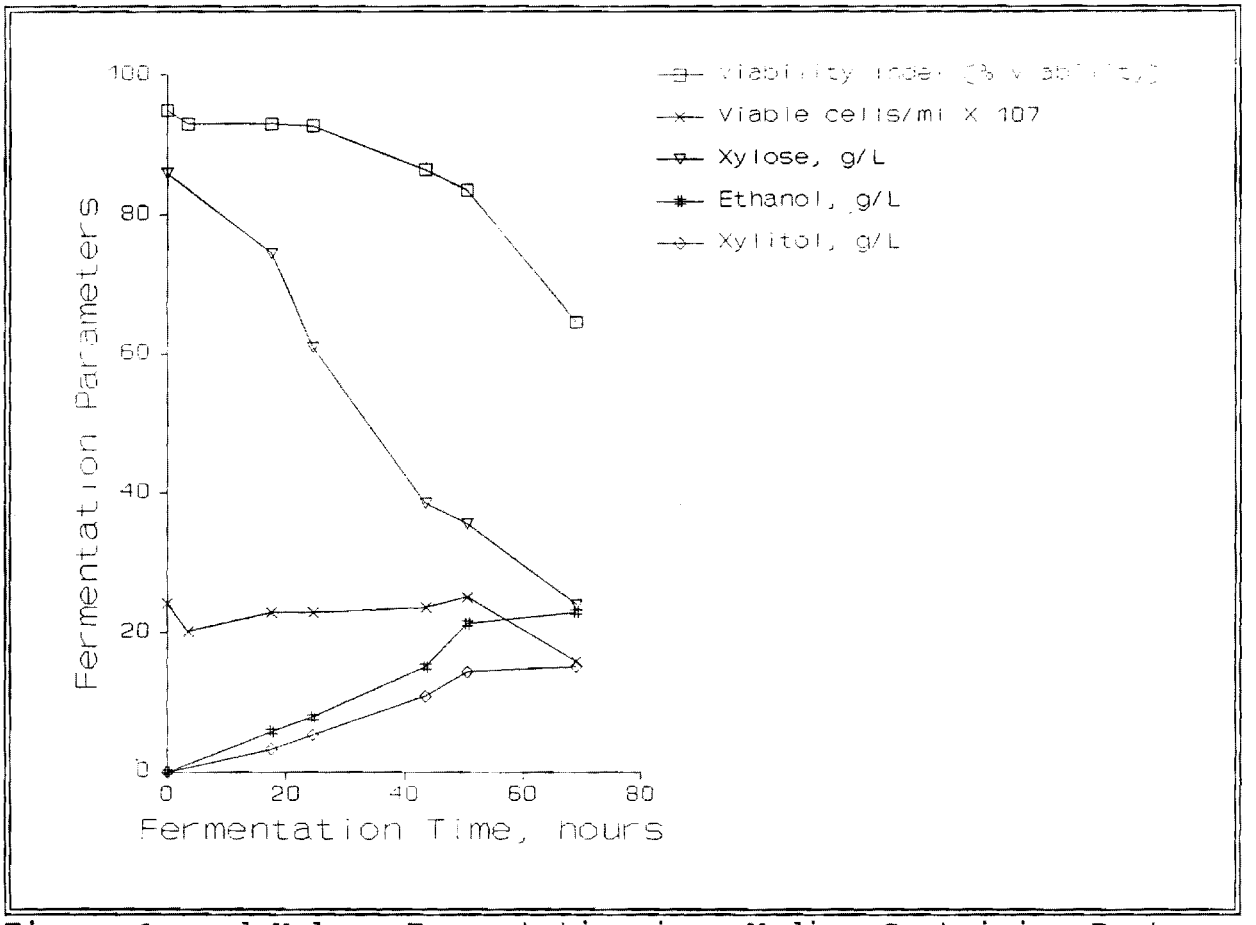


Figure 1 -- d-Xylose Fermentation in a Medium Containing Peptone; Fermentor was a Spinner Flask.

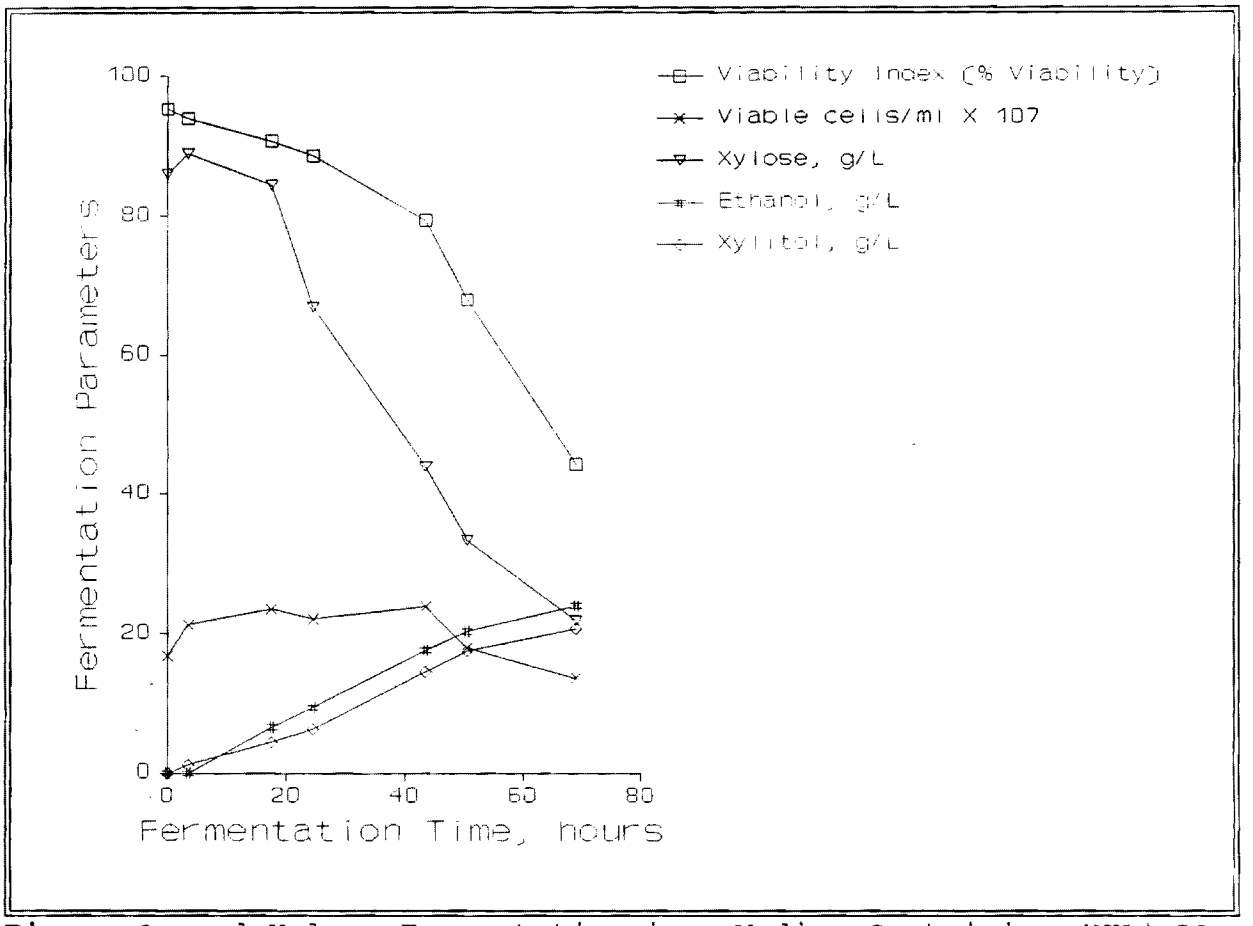


Figure 2 -- d-Xylose Fermentation in a Medium Containing $(NH_4)_2SO_4$; the Fermentor was a Spinner Flask.

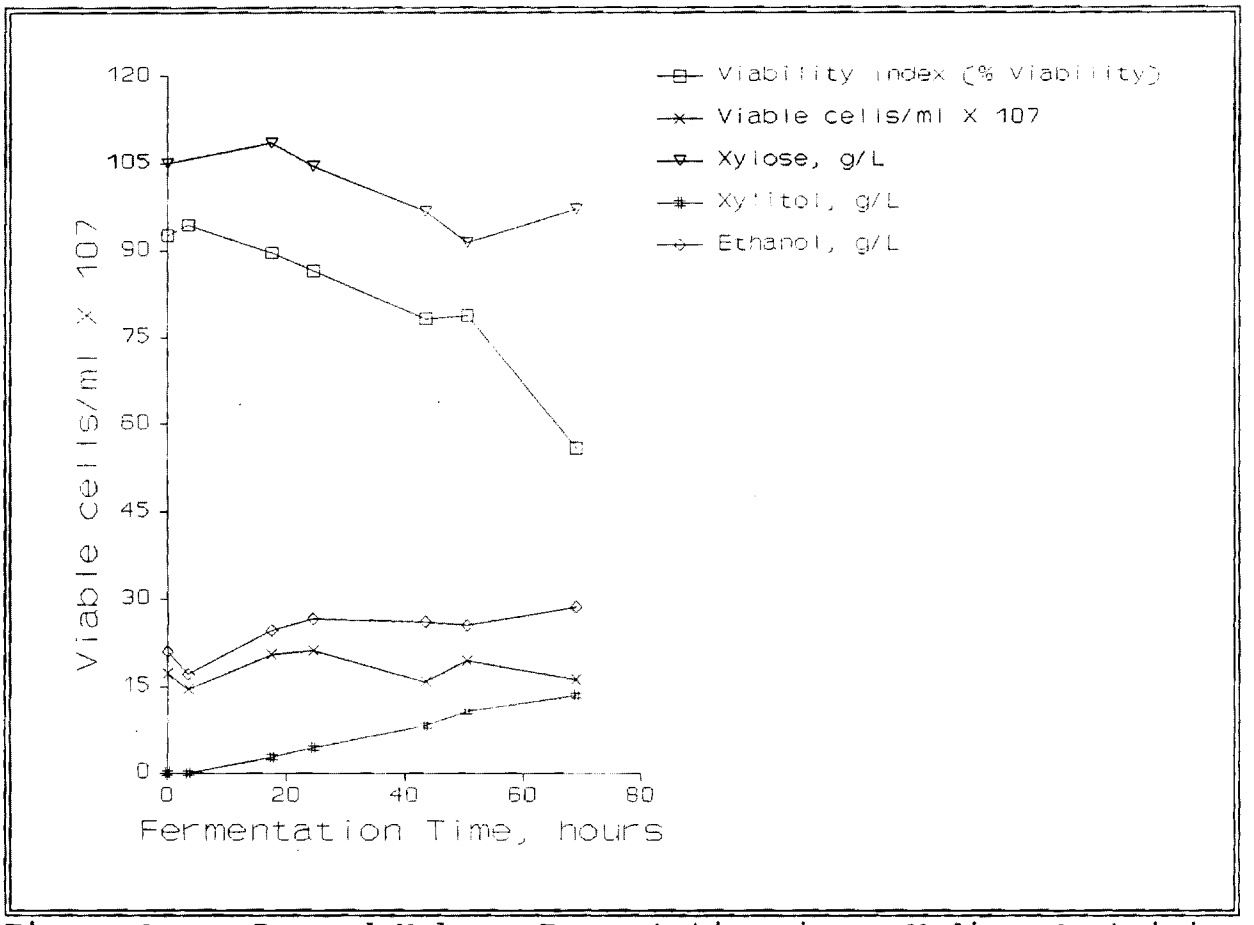


Figure 3 -- Pure d-Xylose Fermentation in a Medium Containing Peptone and an Ethanol Concentration of 20 g/L at Time Zero; Fermentor was a Spinner Flask.

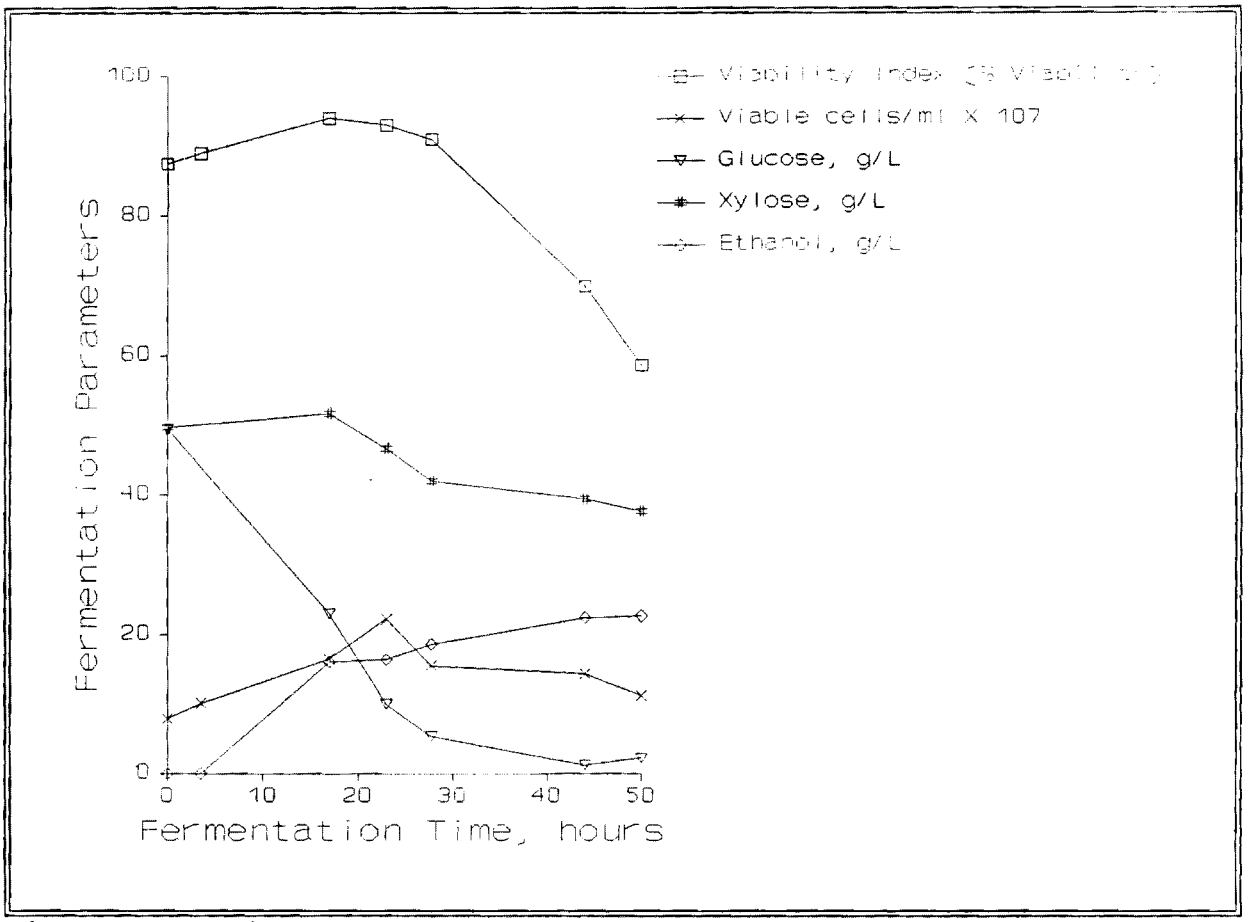


Figure 4 -- Mixed Sugar (50% Glucose and 50% d-Xylose) Fermentation in Spinner Flask.

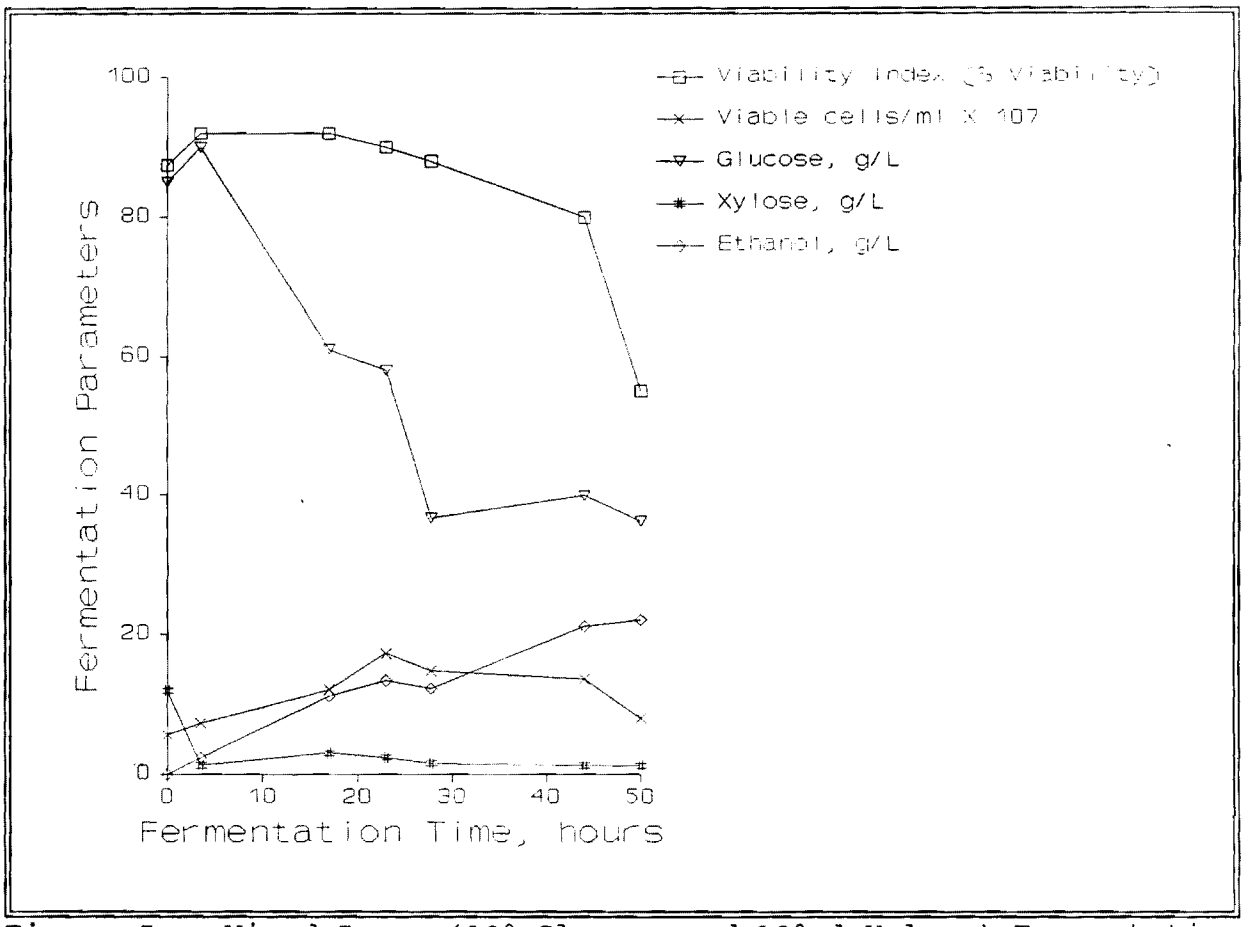


Figure 5 -- Mixed Sugar (90% Glucose and 10% d-Xylose) Fermentation in Spinner Flask.

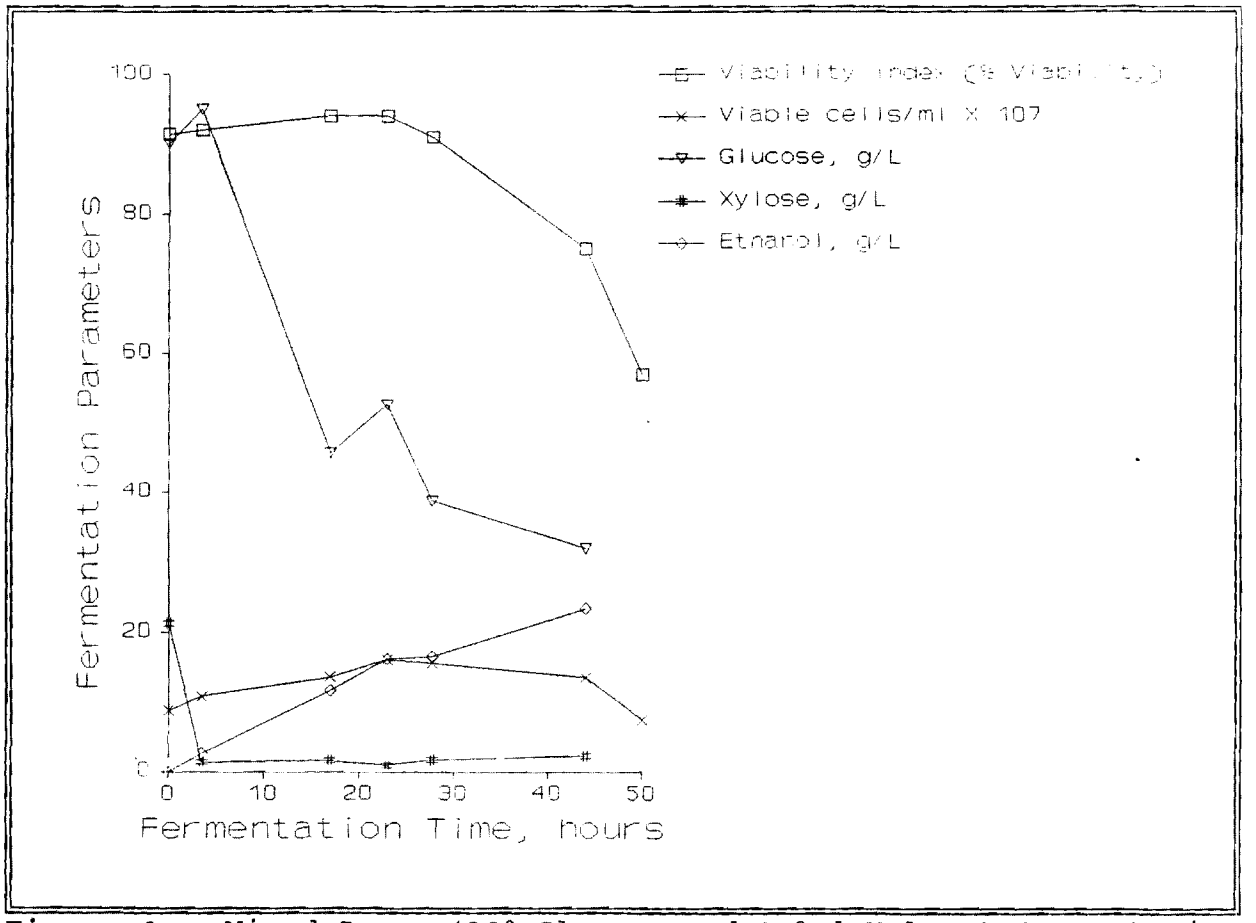


Figure 6 -- Mixed Sugar (80% Glucose and 20% d-Xylose) Fermentation in a Spinner Flask.

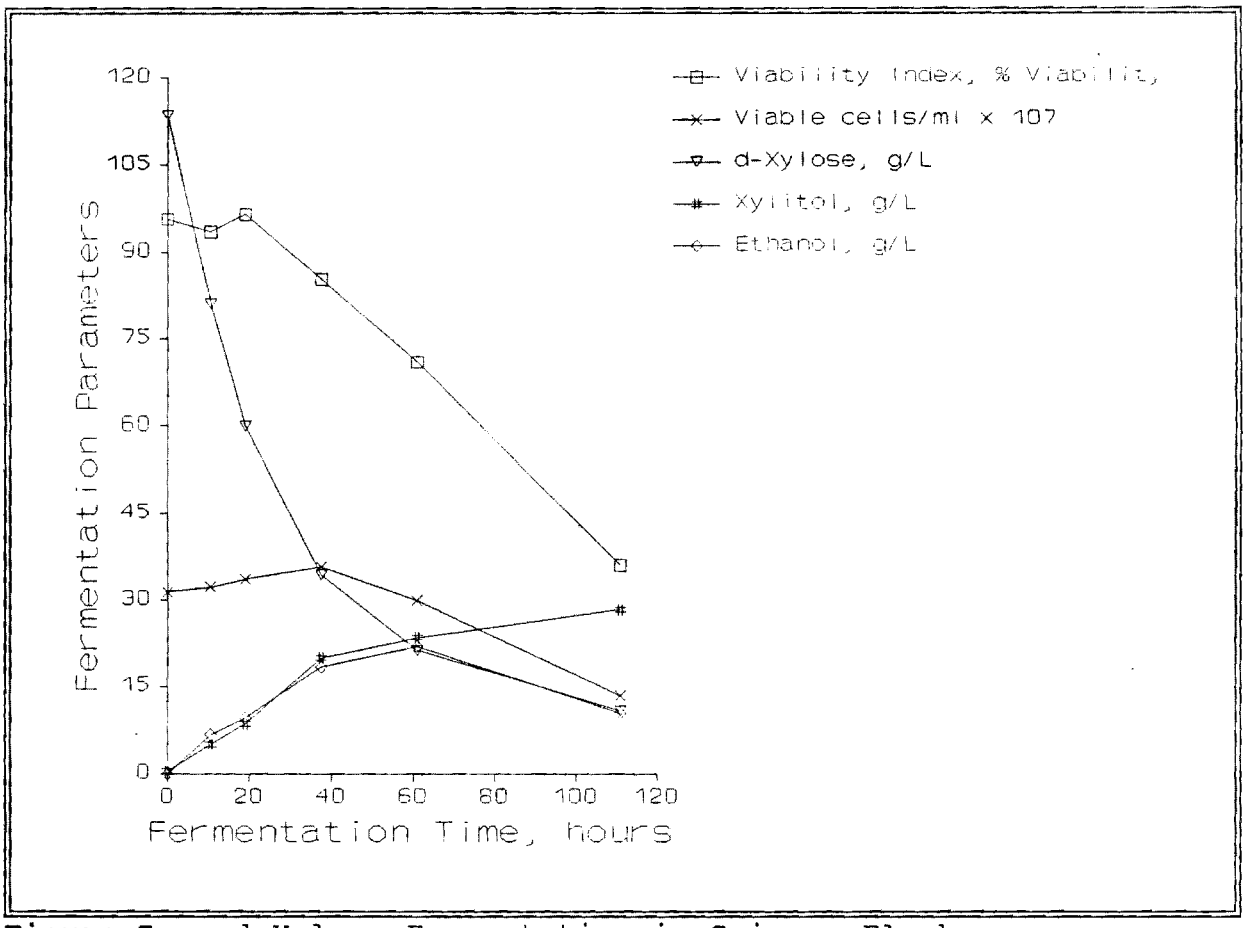


Figure 7 -- d-Xylose Fermentation in Spinner Flask

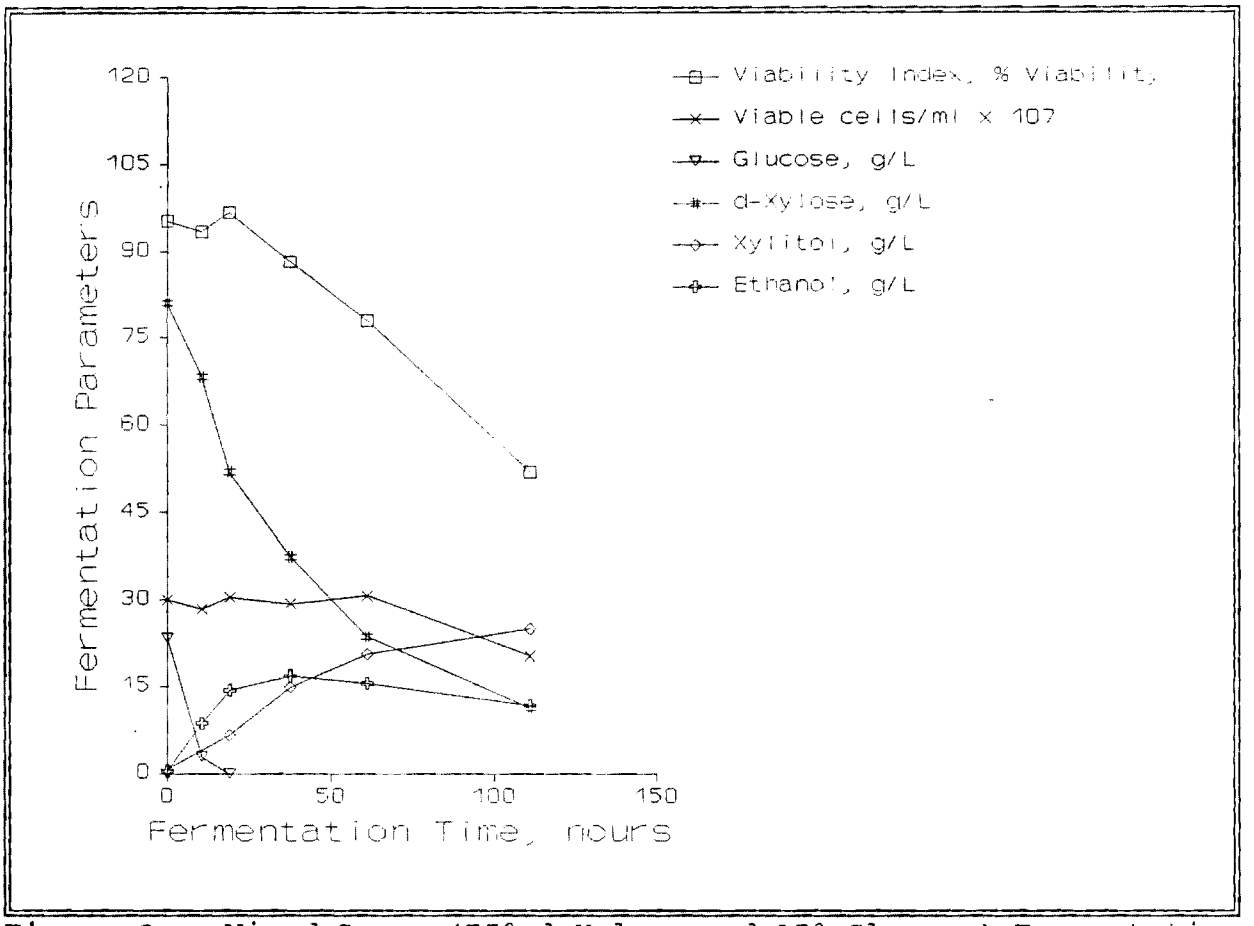


Figure 8 -- Mixed Sugar (75% d-Xylose and 25% Glucose) Fermentation in a Spinner Flask.

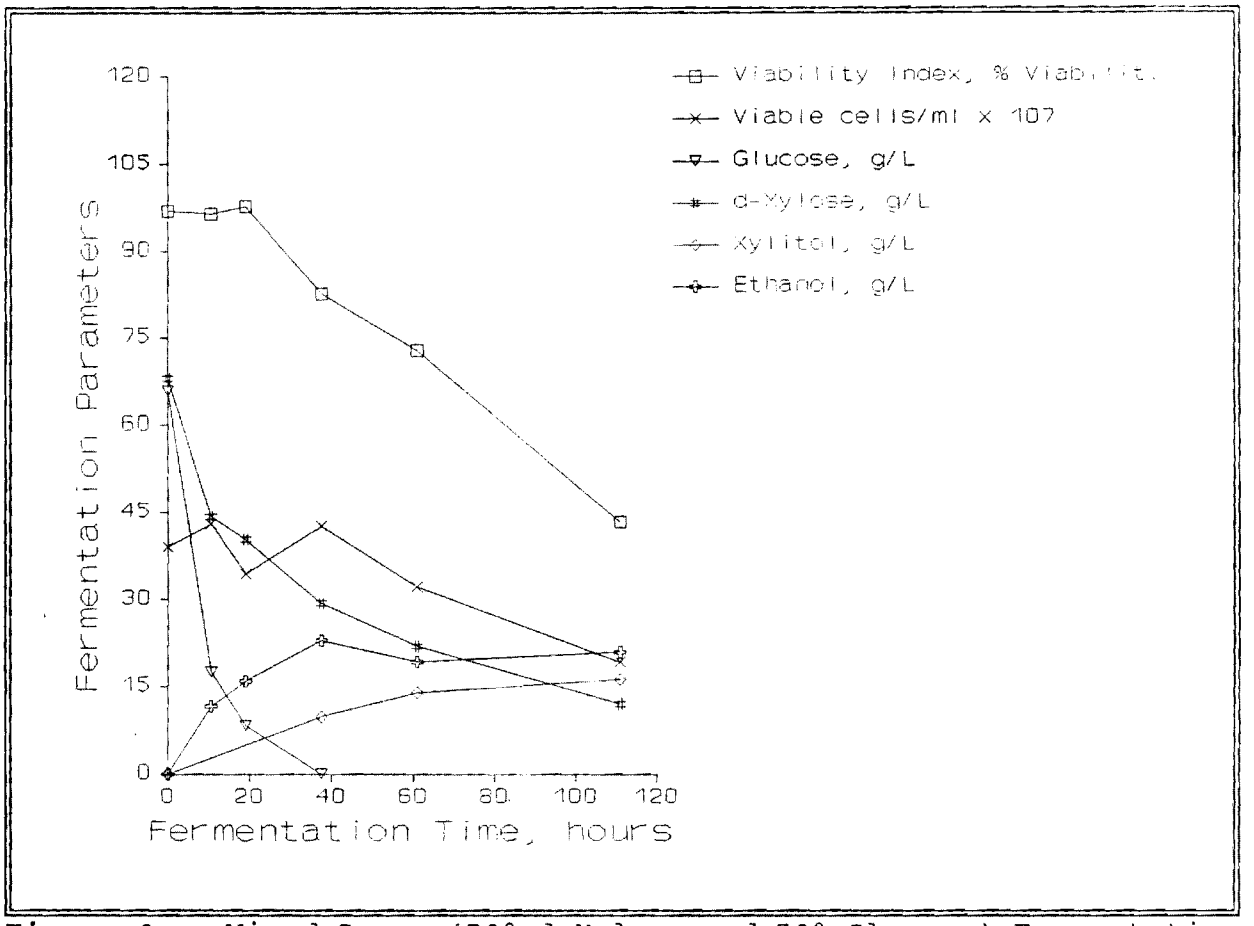


Figure 9 -- Mixed Sugar (50% d-Xylose and 50% Glucose) Fermentation in a Spinner Flask.

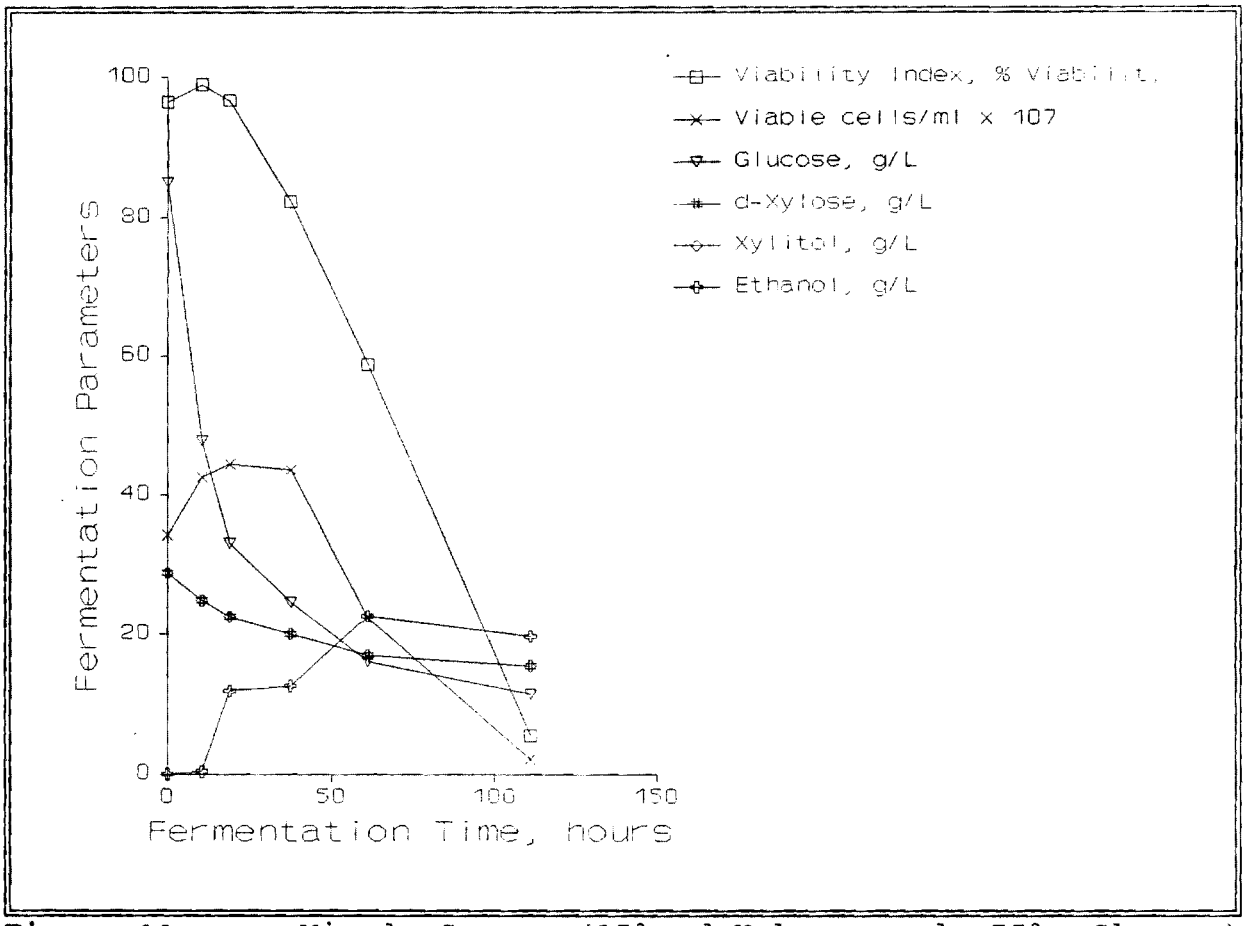


Figure 10 -- Mixed Sugar (25% d-Xylose and 75% Glucose) Fermentation in a Spinner Flask.

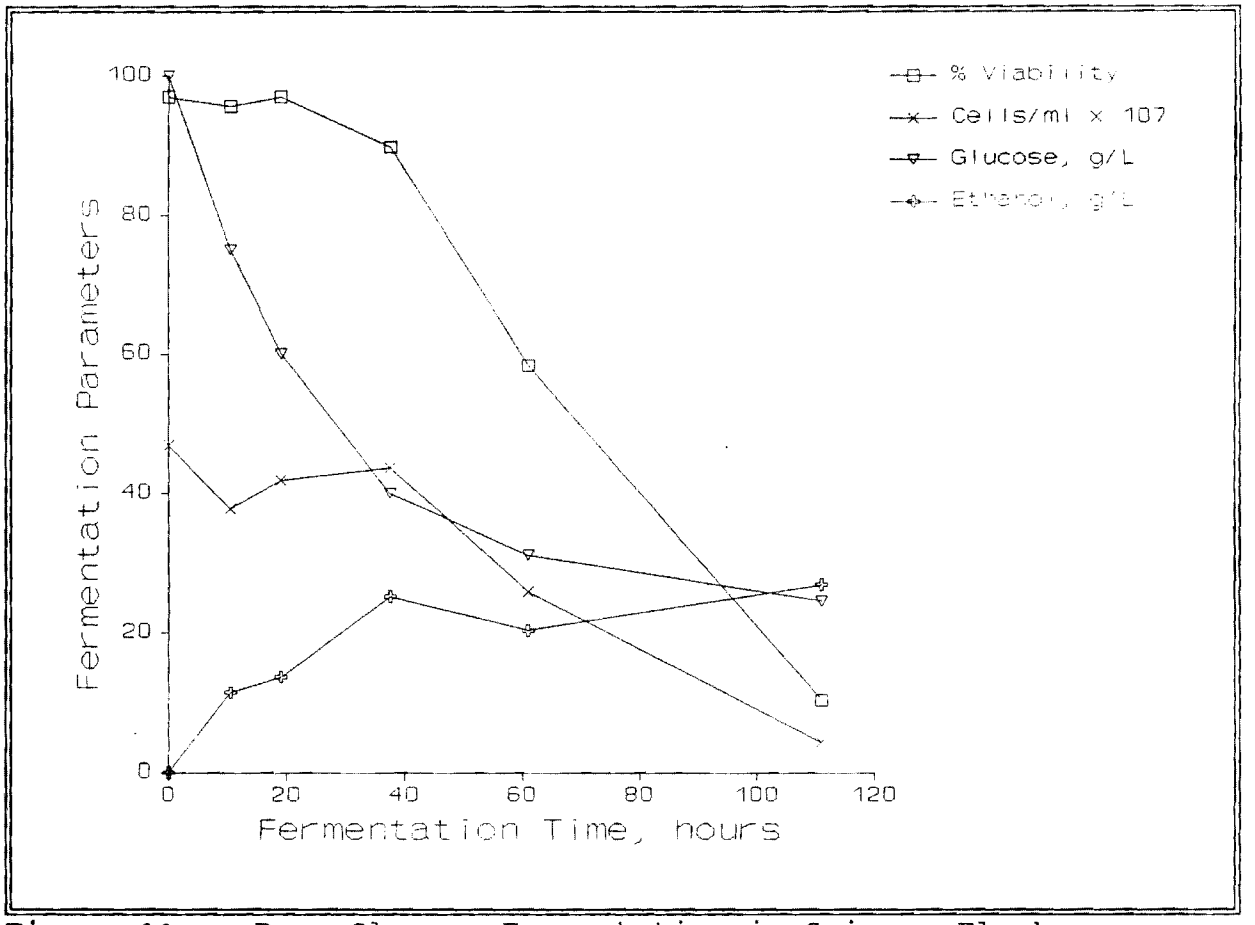


Figure 11 -- Pure Glucose Fermentation in Spinner Flask.

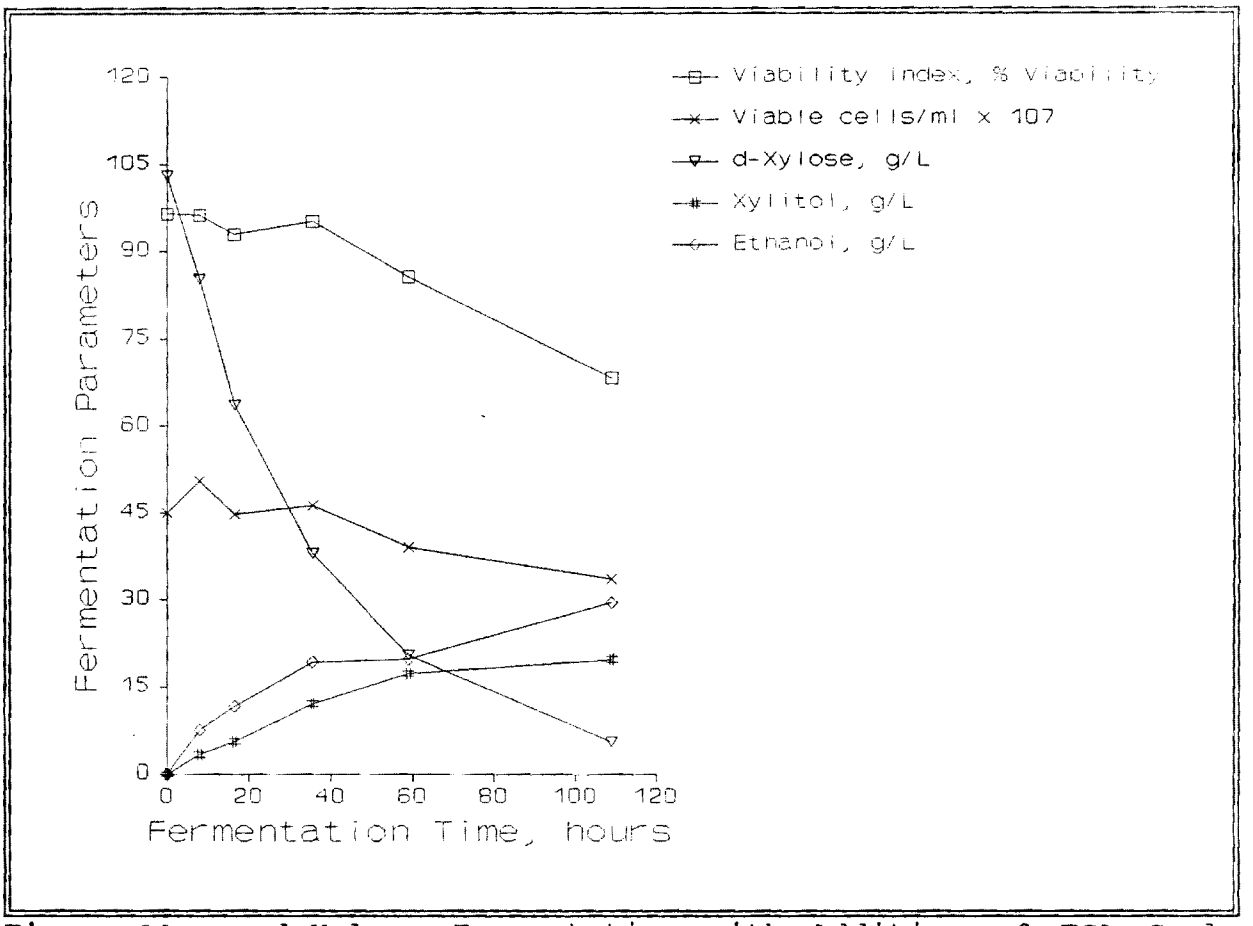


Figure 12 -- d-Xylose Fermentation with Addition of TCA Cycle Intermediates 11 Hours into the Fermentation; Fermentor was Benchtop.

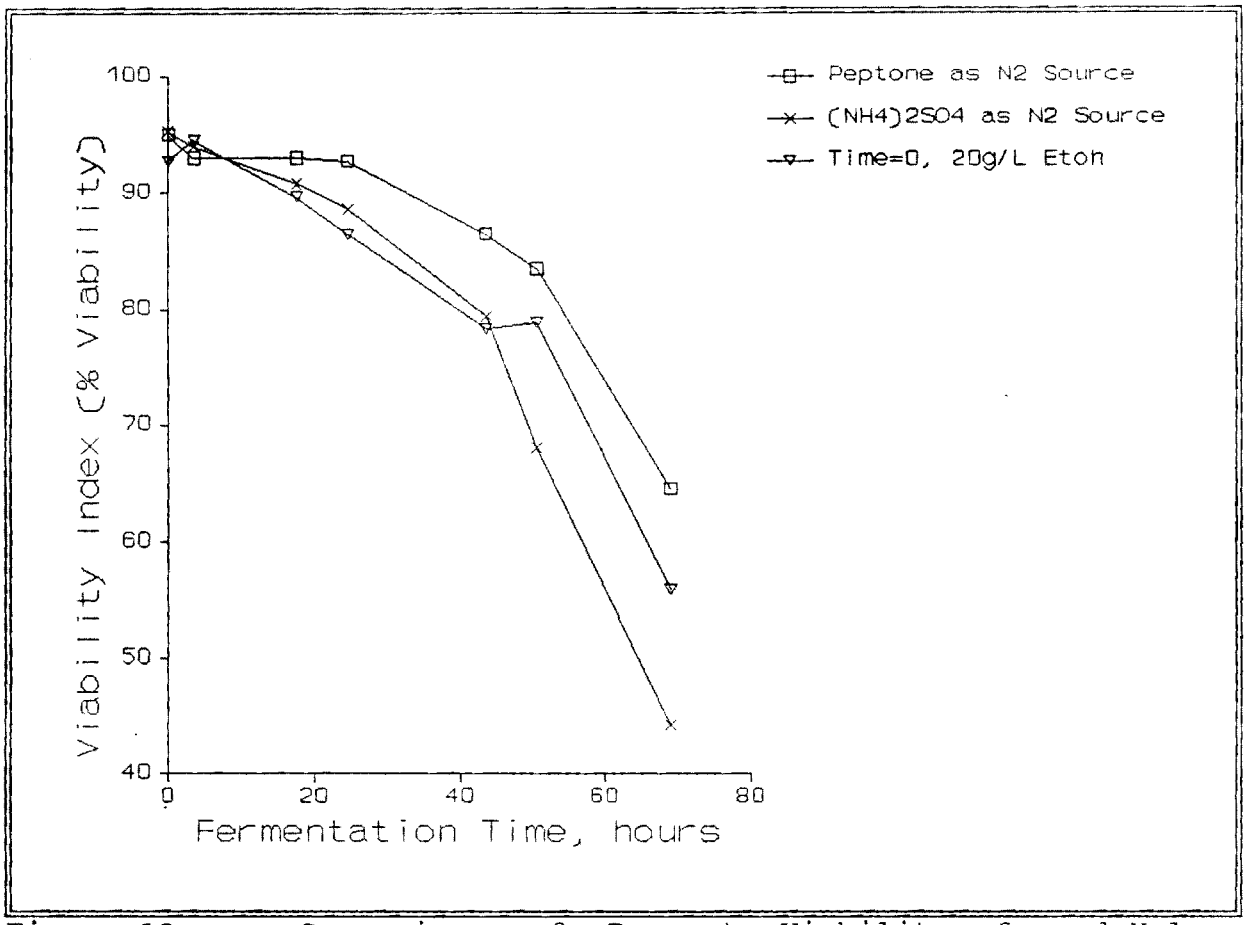


Figure 13 -- Comparison of Percent Viability for d-Xylose Fermentation in Two Different N_2 Sources and with an Initial Ethanol Concentration of 20 g/L; Fermentors were Spinner Flasks.

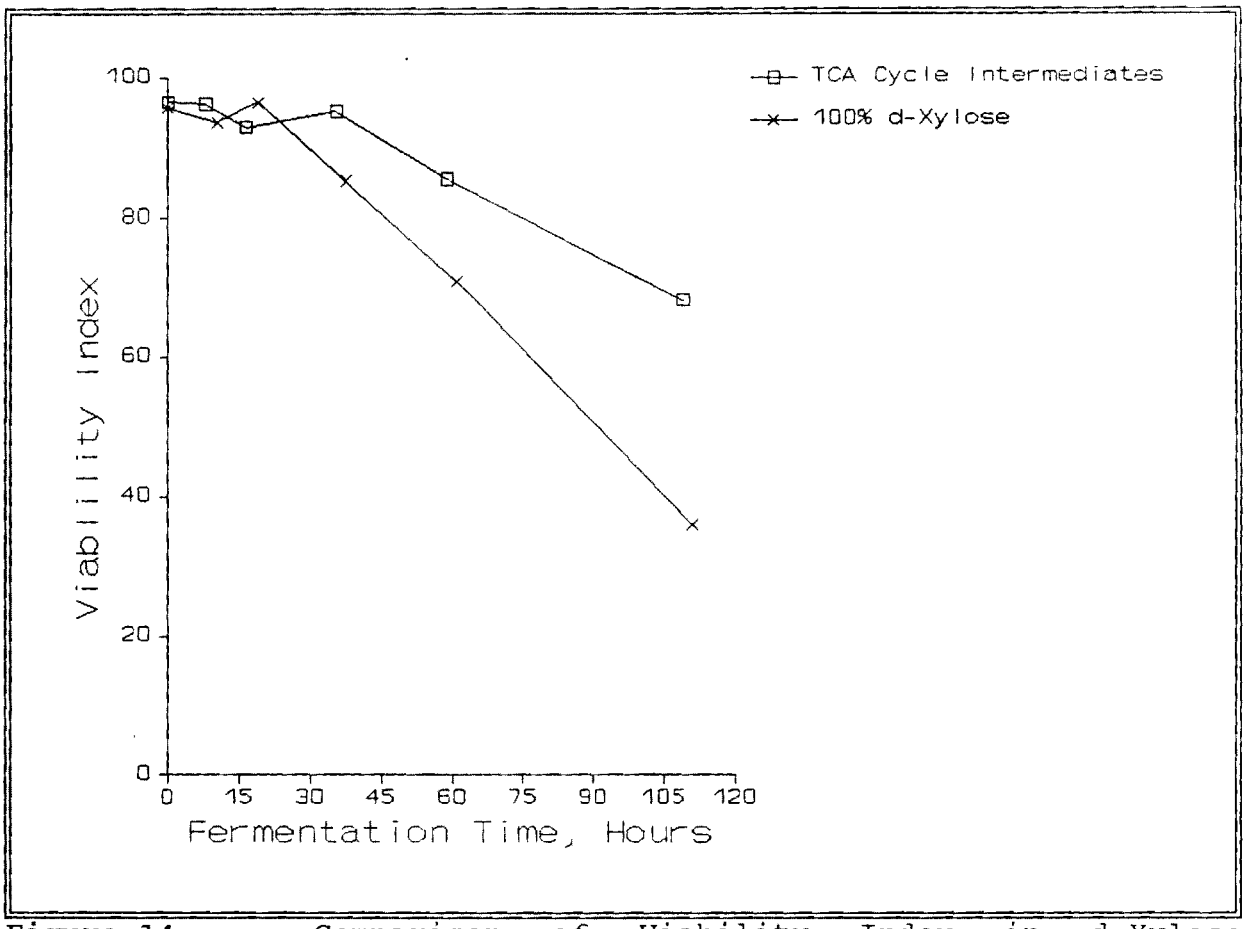


Figure 14 -- Comparison of Viability Index in d-Xylose Fermentation with and without TCA Cycle Intermediates.

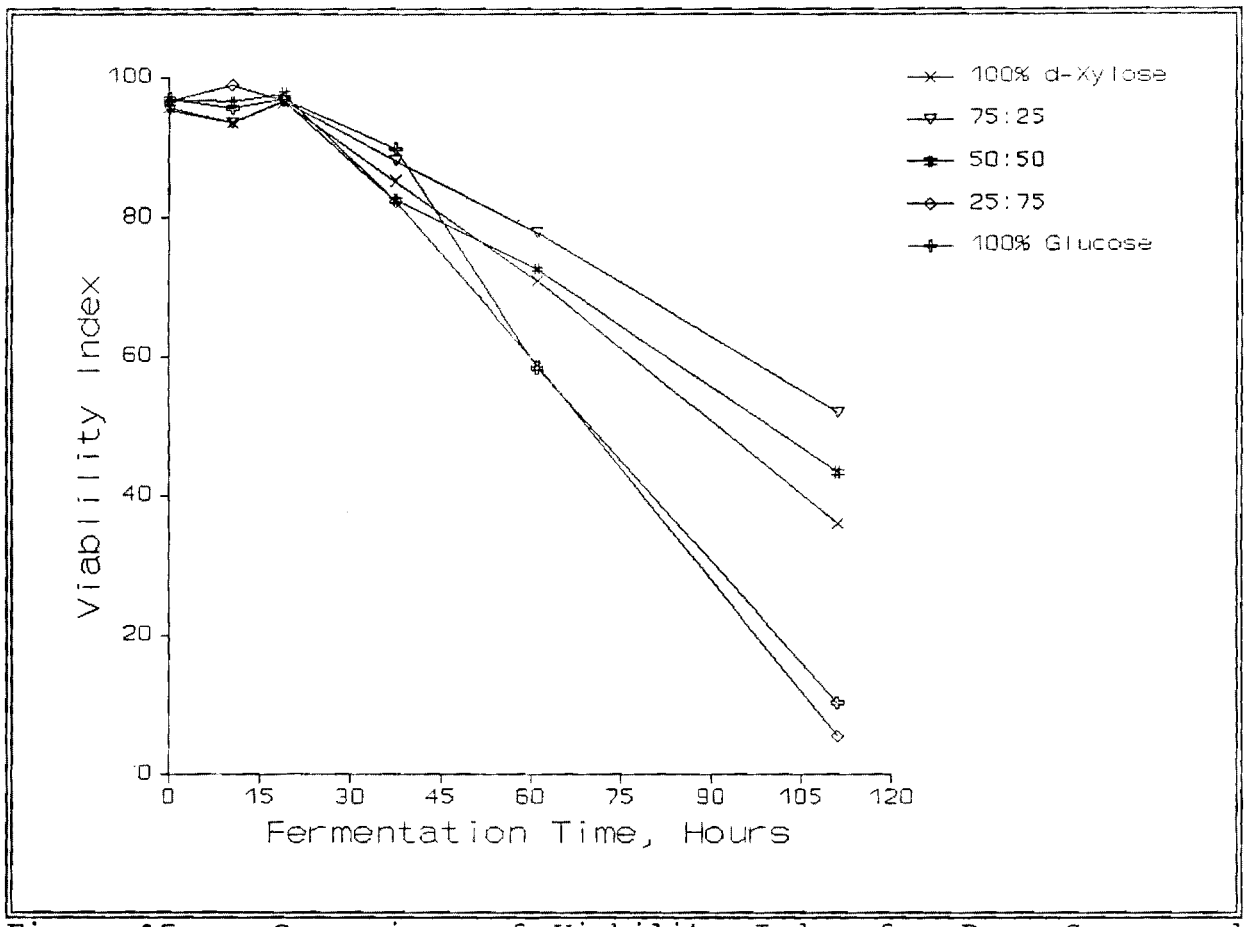


Figure 15 -- Comparison of Viability Index for Pure Sugar and Mixed Sugar Fermentations. Legend -- Top to Bottom is % d-Xylose : % Glucose

QUARTERLY PROGRESS REPORT

March 1, 1989 - July 10, 1989

ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC RESIDUES

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by
J.R. Kastner and Professor R.S. Roberts

School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

July 10, 1989

INTRODUCTION:

The data from the first phase of study indicate anaerobic conditions result in reduced cell viability and the production of xylitol (an unwanted by-product). This reduction in viability and by-product formation may have resulted from the lack of a key cofactor or growth factor in the medium. The lack of a cofactor or growth factor would limit the microorganisms ability to synthesize important proteins or utilize key enzymes. Use of a defined medium (which will contain cofactors and growth factors) may help maintain viability and enable the synthesis and use of key enzymes in the d-xylose pathway.

In addition to the possibility of maintaining cell viability, the use of a defined medium may enable the detection of a component or components responsible for long term cell viability. As opposed to an undefined medium (as used in the previous experiments) all components, and their concentrations, are specified in a chemically defined medium. Since all components are known, selective addition or subtraction of key components in the fermentation medium would provide a systematic method of identifying compounds required for long term cell viability (anaerobic conditions). Long term cell viability would result in higher ethanol yields and enable the use of continuous biological reactor designs; immobilized (packed bed) or cell recycle systems.

Experimental data from previous experiments also indicate that *Candida shehatae* produces large amounts of xylitol (by-product) under anaerobic

conditions. Data from the literature indicate two possible methods to circumvent by-product formation: 1) d-xylose fermentation via the yeast *Pichia stipitis*; several researchers have shown that this yeast produces little xylitol under anaerobic conditions (Prior et al., 1989) and, 2) addition of a hydrogen ion acceptor, such as acetone, to prevent formation of xylitol; Alexander, 1986 has demonstrated this principle with *Pachysolen tannophilus*.

MATERIALS AND METHODS:

Microorganism and growth conditions: Candida shehatae (ATCC 22984) was maintained on YM Broth Slants (2% Agar). A 24 hour slant was used to inoculate (rinse transfer) a 500 ml Erlenmeyer Flask (250 ml working volume). The seed culture medium consisted of d-xylose (20g/L), peptone (5g/L) and yeast extract (3g/L). The culture was shaken for 24 hours at 300 rpms. At the end of 24 hours, 100 ml was used to inoculate a New Brunswick Benchtop reactor; total working volume of 1000 ml. The medium used in this step was as reported in Kastner and Roberts; Phase I Report, March 31 1989; d-xylose concentration was 120 g/L and ammonium sulfate was 10 g/L.

Reactor Conditions: Aeration was maintained at 1.5 Liters/min. Agitation was varied in an attempt to maintain the percent dissolved oxygen level greater than 90% (500-900 rpms). Percent dissolved oxygen (D.O.) was measured via a New Brunswick Dissolved Oxygen Electrode (Galvanic) 900 Series Probe and Model- 40 Dissolved Oxygen Analyzer. The pH was monitored with an Ingold pH Electrode and periodically adjusted with 10N NaOH. The initial pH was 5.0 and the temperature was maintained at 30°C. Anaerobic conditions were imposed by reducing the agitation to 300 rpms and sparging dry nitrogen through the system until the %D.O. was zero.

RESULTS:

Defined Medium Fermentation:

Under aerobic conditions the pH dropped rapidly from 5.0 to 2.4 within the first 10 hours (pH was not automatically controlled); see Figure 1. As can be seen from Figures 1 and 2 under aerobic conditions (% D.O. > 50) large adjustments of pH were necessary. Figure 1 also shows that the viability index declined at low pH (under aerobic conditions) and after prolonged exposure to

anaerobic conditions; about 20 hours after nitrogen was sparged through the system the viability index declined.

After the pH was adjusted to 6.5 cell growth was rapid; viable cell density increased from 10 X 10^7 at 20 hours to about 50 x 10^7 cells/ml at 40 hours (Figure 2). Shortly after the % D.O. was set to zero cell growth leveled off (stationary phase) and by-products were formed (Figures 2 and 3). Once the stationary phase was reached xylitol and ethanol were formed at equal rates and in equal amounts (see Figure 3). The final concentration for both by-products was about 22 g/L.

Mixed Sugar Fermentation via Pichia stiptis:

A mixed sugar fermentation utilizing this yeast has been completed. HPLC analysis is currently being performed.

Mixed Sugar Fermentation via Candida shehatae:

More data on the ability to induce simultaneous sugar utilization is being collected. Two more additional mixed sugar fermentations are underway; one with cells conditioned on d-xylose and another with cells conditioned on glucose. Once HPLC analysis has been performed a paper will be written and submitted to **Biotechnology Letters**

Mixed Sugar Fermentation with Candida shehatae; Addition of a Hydrogen Ion Acceptor:

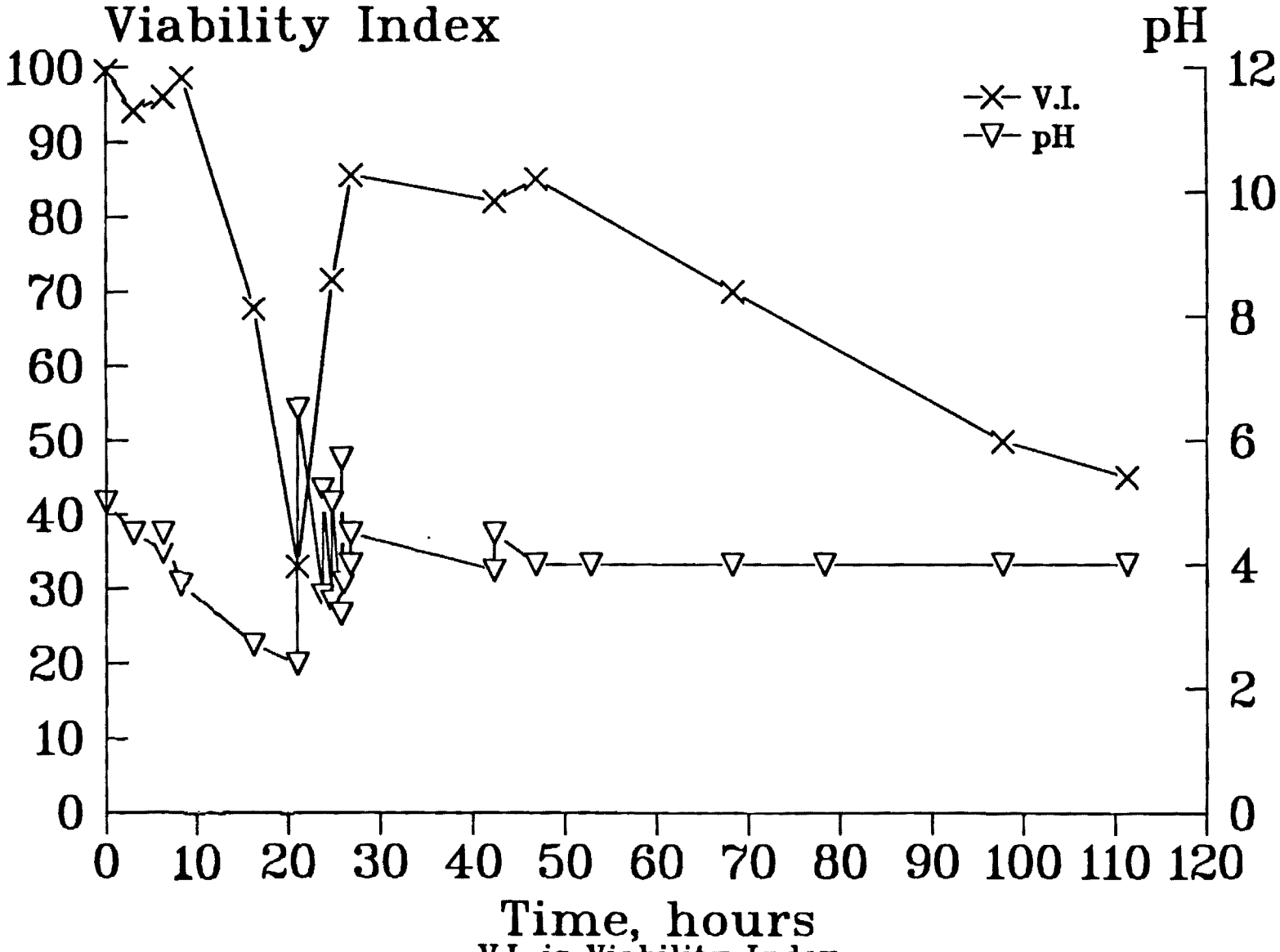
In order to increase ethanol levels and yields (prevent xylitol formation) in d-xylose fermentation, acetone will be added to the fermentation reactor. *Candida shehatae* will be aerobically conditioned on d-xylose and then inoculated into a mixed sugar environment with acetone. This small fermentation project is being performed by the Applied Biology Department (Georgia Tech). If ethanol yields and concentrations are increased further experimental work may be performed.

References:

Alexander N.J., Acetone stimulation of ethanol production from d-xylose by *Pachysolen tannophilus*, **Applied Microbiology and Biotechnology** (1986), 25:203-207.

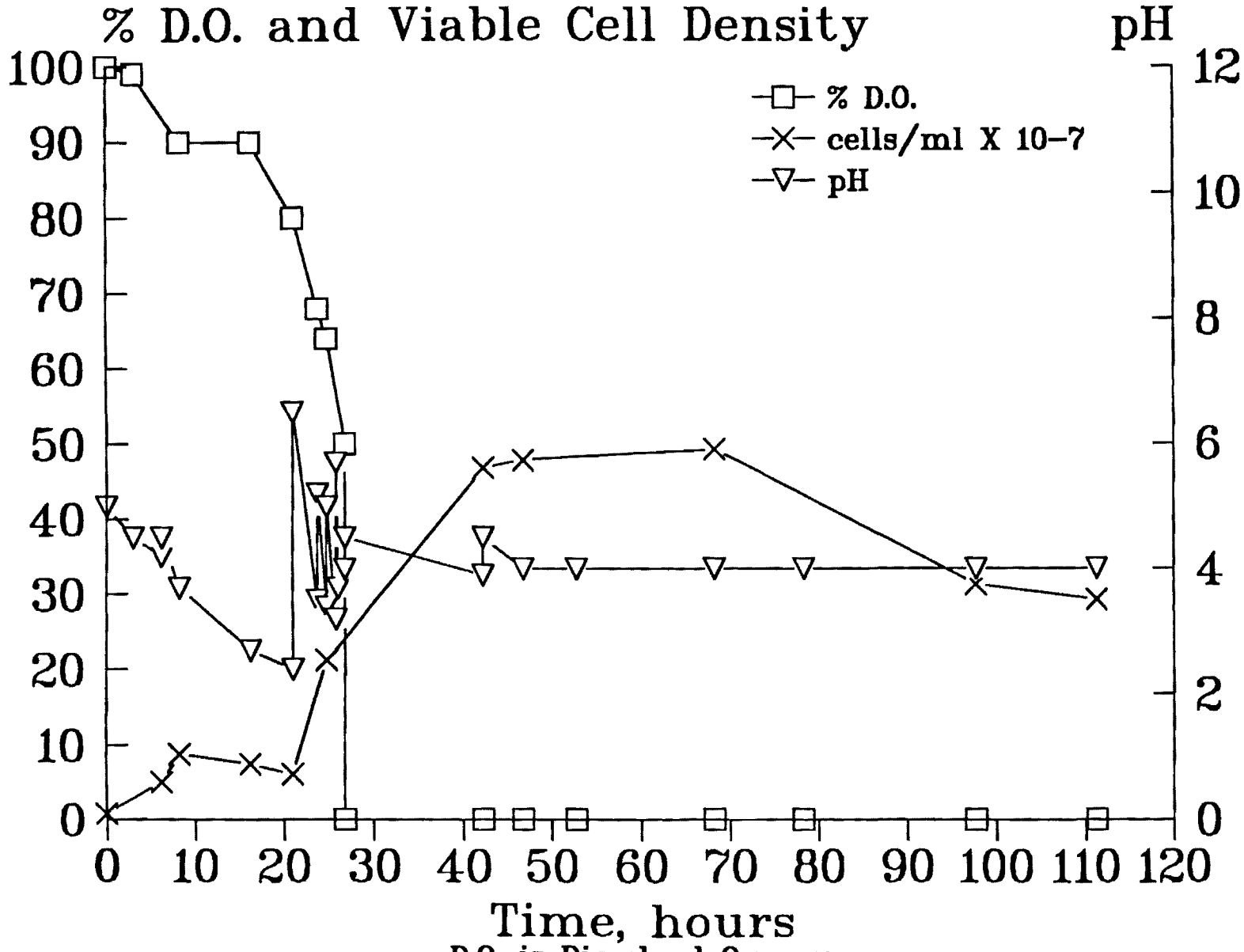
Prior B.A. et al, Fermentation of d-Xylose by the Yeasts *Candida shehatae* and *Pichia stiptis*: Prospects and Problems., **Process Biochemistry**, February 1989, p.21.

Figure 1 : d-Xylose Fermentation via Candida shehatae in a Defined Medium.



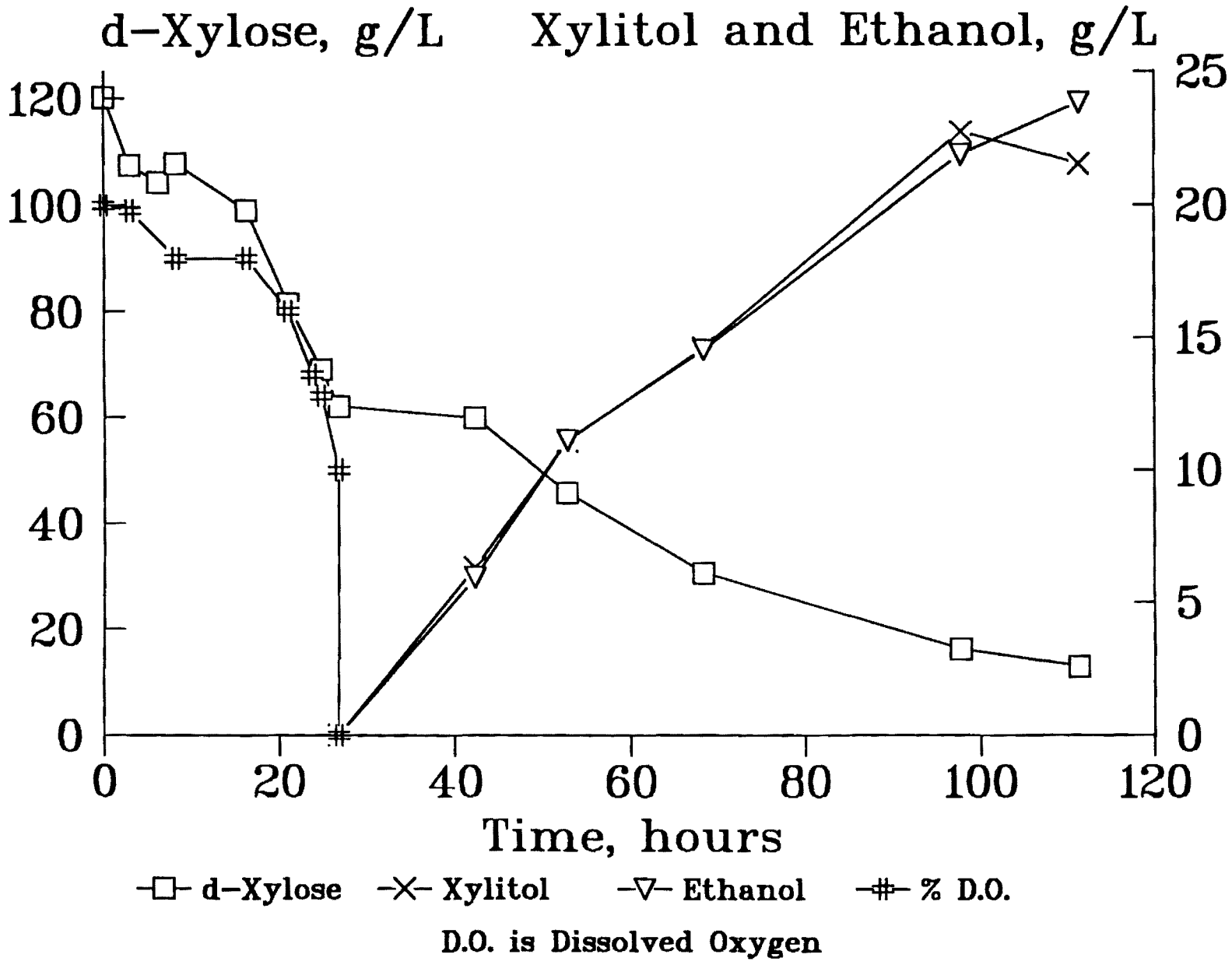
V.I. is Viability Index

Figure 2 : d-Xylose Fermentation via Candida shehatae in a Defined Medium.



D.O. is Dissolved Oxygen

Figure 3 : d-Xylose Fermentation via Candida shehatae in a Defined Medium.



QUARTERLY PROGRESS REPORT

June 30, 1989 - September 30, 1989
Contract # TV-74261A

ETHANOL FERMENTATION OF MIXED SUGAR STREAMS DERIVED FROM LIGNOCELLULOSIC RESIDUES

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by
J.R. Kastner and Professor R.S. Roberts

School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

October 10, 1989

INTRODUCTION

As previously reported, *Candida shehatae* cell viability declines and xylitol production begins under anaerobic conditions using the medium of Wayman and Tsuyuki (1985). Reduction in viability and xylitol formation may have been due to the lack of a key cofactor(s) or growth factor(s) in the medium. The lack of a cofactor or growth factor would limit the microorganisms ability to synthesize important proteins or utilize key enzymes. The medium of Wayman and Tsuyuki (1985) contained yeast extract and peptone both of which are chemically undefined. The amount and type of components are unknown in a chemically undefined medium. Therefore, it is not possible to identify the cofactor(s) required to maintain cell viability. To do this a chemically defined medium is required.

How can the defined medium be utilized? The composition of a chemically defined medium can be systematically varied and the effect of various medium compounds on cell viability can be determined (Kim and Roberts, 1987). Using these data a medium can then be formulated in which long term cell viability can be maintained. Using such a medium results in higher fermentation rates and yields. Continuous biological reactor systems such as immobilized cell reactors would require this type of medium for long term operation.

As previously reported, *Candida shehatae* produces large amounts of the unwanted by-product, xylitol, under anaerobic conditions. Xylitol formation can be possibly reduced by medium reformulation. Alexander (1986) found xylitol formation by *Pachysolen tannophilus* could be reduced by the addition of acetone. Xylitol formation could be reduced also by changing the fermentation microorganism. For example the yeast, *Pichia*

stipitis, produces little xylitol under anaerobic conditions (Prior et al., 1989). Both medium reformulation and change of microorganism are being investigated as a technique to reduce xylitol formation.

MATERIALS AND METHODS

Defined Medium:

The defined medium is based on a medium by du Preez (1983). The composition of the defined medium is shown in Table 1.

Table 1. Composition of the Defined Medium

Component	Concentration
Salts (g/L)	
$(NH_4)_2SO_4$	10
KH_2PO_4	10
$MgSO_4$	0.244
$CaCl_2*2H_2O$	0.05
Trace Elements (mg/L)	
$FeSO_4*7H_2O$	35
$MnSO_4*H_2O$	7
$ZnSO_4*7H_2O$	11
$CuSo_4*5H_2O$	1
$Na_2MoO_4*7H_2O$	1.3
$CoCl_2*6H_2O$	2.0
H_3BO_3	2
KI	0.35
$Al_2(SO_4)_3$	0.5
Vitamins (mg/L)	
Meso-Inositol	100
Ca-Pantothenate	20
Thiamine-HCl	5
Pyridoxine	5
Nicotinic Acid	5
p-Aminobenzoic	1
d-Biotin	0.1

Microorganism and growth conditions:

Candida shehatae and *Pichia stipitis* were maintained on YM Broth Slants (2% Agar). A 24 hour slant was used to inoculate (rinse transfer) a 500 ml Erlenmeyer Flask (250 ml working volume). The seed culture medium consisted of d-xylose (20g/L) in the defined medium. The culture was shaken for 24 hours at 300 rpm's. At the end of 24 hours, 100 ml was used to inoculate a New Brunswick Bioflow II reactor (total working volume of 1000 ml). The medium used in this step was the defined medium.

Reactor Conditions: Aeration was maintained at 1.4 Liters/min. Agitation was set at 550 rpm. The pH was monitored with an Ingold pH Electrode and controlled with a NewBrunswick Automatic pH Controller (Model pH-40). The initial pH was 4.5 and the temperature was maintained at 30°C. Anaerobic conditions were imposed by shutting off the air flow and reducing the agitation to 250 rpm.

RESULTS

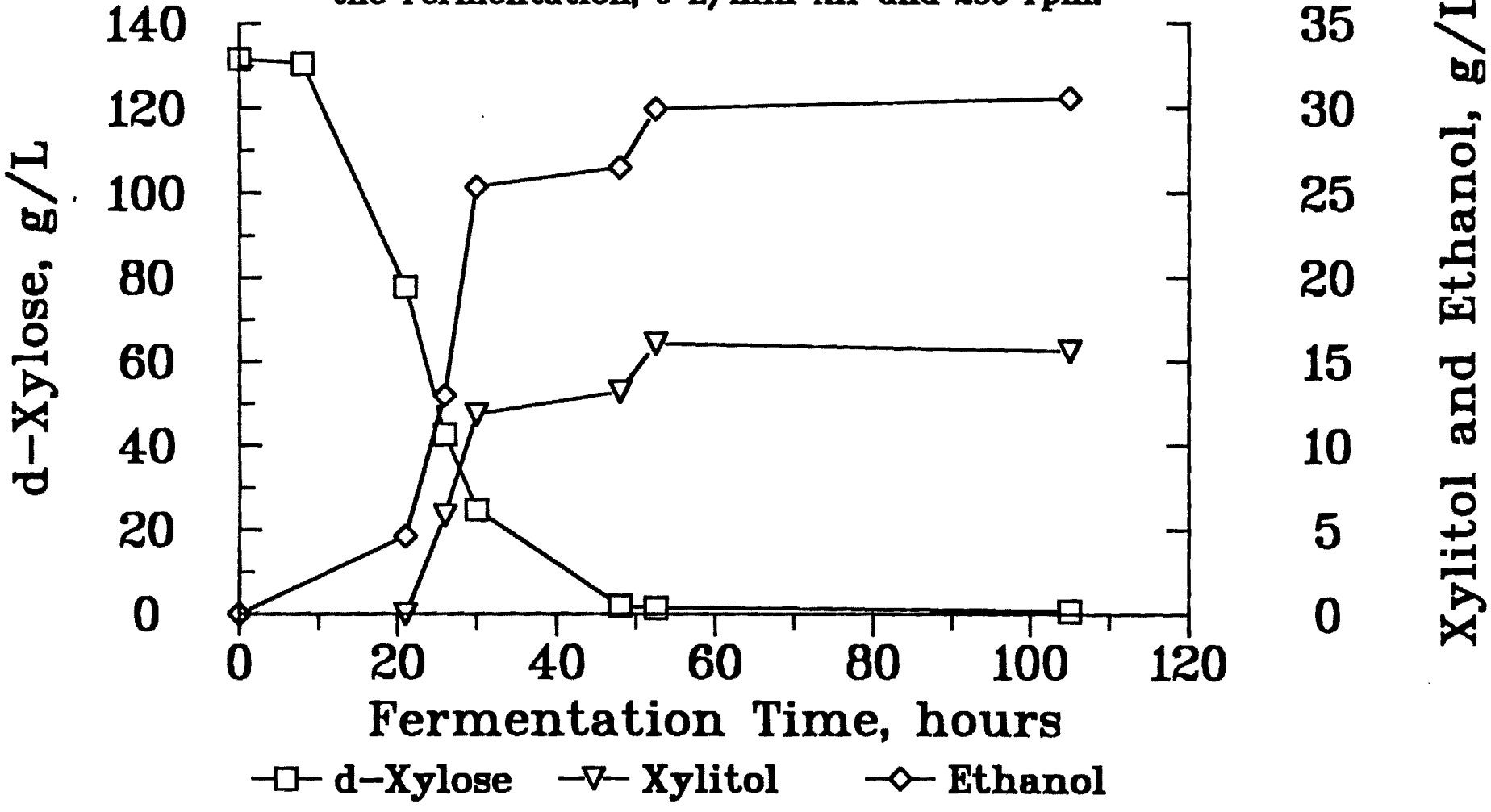
Defined Medium Fermentation:

In the initial experiments with the defined medium, the pH of the fermentation broth dropped rapidly (Quarterly Report: March 31, 1989 to June 30, 1989). This was due to a lack of pH control and a low concentration of buffer, KH_2PO_4. When the cells were switched from aerobic to anaerobic conditions the cell viability and d-xylose uptake rate declined rapidly. Large amounts of xylitol (equal to that of ethanol) were also formed. Since rapid changes in pH are known to effect negatively d-xylose fermentation, the defined medium experiments were repeated with pH controlled at 4.5.

If the pH was controlled (4.5) and a defined medium utilized, fermentation results were improved. When the cells were switched from aerobic to anaerobic conditions the d-xylose uptake rate was rapid (within 50 hours there was no residual d-xylose) and less xylitol was produced (15 g/L of xylitol and 30 g/L of ethanol); compare Figure 1 with Figure 2.

Figure 1 — d-Xylose Fermentation via Candida shehatae in a Defined Medium; Initial Condition was 1.4 L/min Air and 550 rpm.

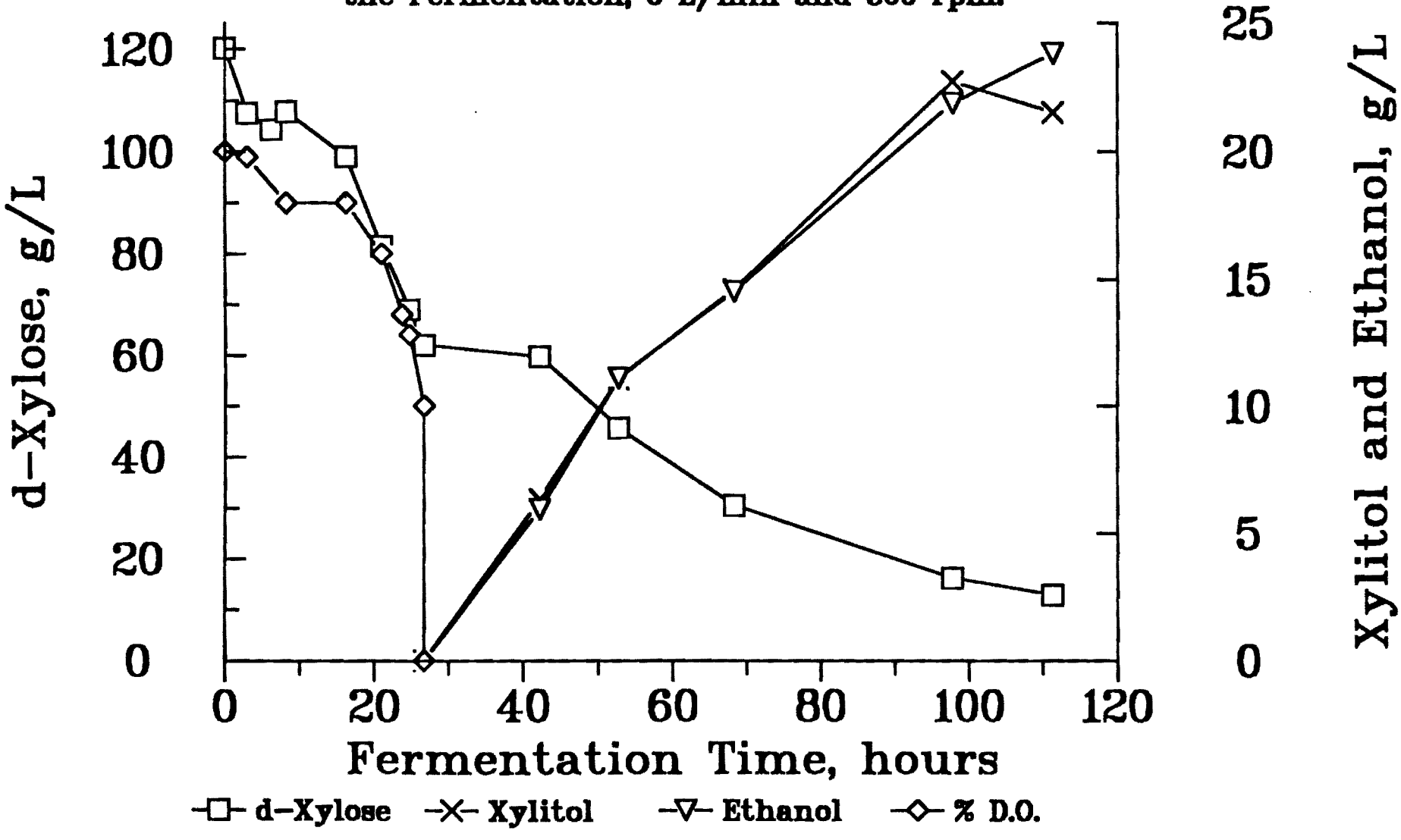
Anaerobic Conditions were imposed 21 hours into the Fermentation; 0 L/min Air and 250 rpm.



Experiment was not run in Duplicate.
The Maximum Viable Cell Density was 111 x 107 cells/ml.

Figure 2 : d-Xylose Fermentation via Candida shehatae in a Defined Medium; Initial Condition was 1.5 L/min and 500 rpm.

Anaerobic Conditions were imposed 24 hours into the Fermentation; 0 L/min and 300 rpm.



The pH was not Controlled in this Fermentation.

Also, the defined medium fermentation (constant pH) gave better results when compared to fermentations in an undefined medium (3g/L of Yeast Extract and 5g/L of $(NH_4)_2SO_4$). The results for this fermentation are shown in Figure 3. Here again it can be seen that large amounts of xylitol are produced (equivalent to that of ethanol) and d-xylose is not completely consumed.

The benefits of using a defined medium and controlling pH can also be seen if one compares viability indices. A comparision is shown in Table 2. The viability index (percentage of viable cells) remained the highest in the defined medium with pH control.

Table 2. -- A Comparision of Viability Indecies in Fermentations of Pure d-Xylose by *Candida shehatae.*

Fermentation Condition	Initial V.I.	Final V.I.
Defined Medium; pH Controlled at 4.5	1.0 (0 hrs)	0.83 (100 hrs)
Defined Medium; pH Not Controlled	1.0 (0 hrs)	0.6 (100 hrs)
Undefined Medium; pH Not Controlled	0.975(0 hrs)	0.23 (84.5 hrs)

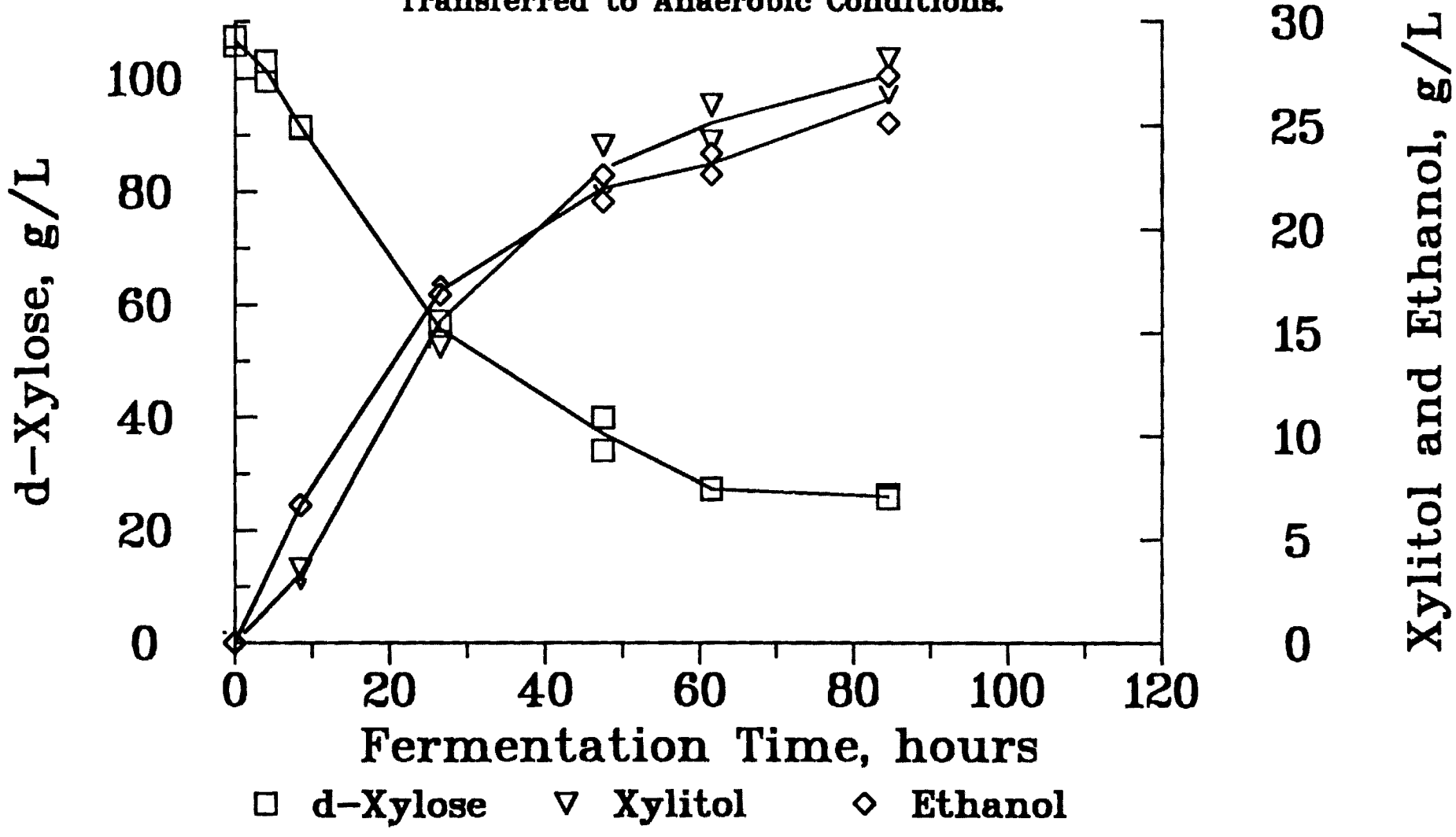
$$\text{Viability Index} = \frac{\#\ \text{viable cells (non-blue)}}{\#\ \text{viable cells (non-blue)} + \text{dead cells (blue)}}$$

Comparison of Candida shehatae and Pichia stipitis:

A defined medium fermentation with *Pichia stipitis* (pH controlled at 4.5) was also performed. Figure 4 shows these results. *Pichia stipitis* did not perform as well as *Candida shehatae*. The d-xylose uptake rate was much slower and a longer fermentation

Figure 3 — d-Xylose Fermentation via Candida shehatae in an Undefined Medium (Yeast Extract)

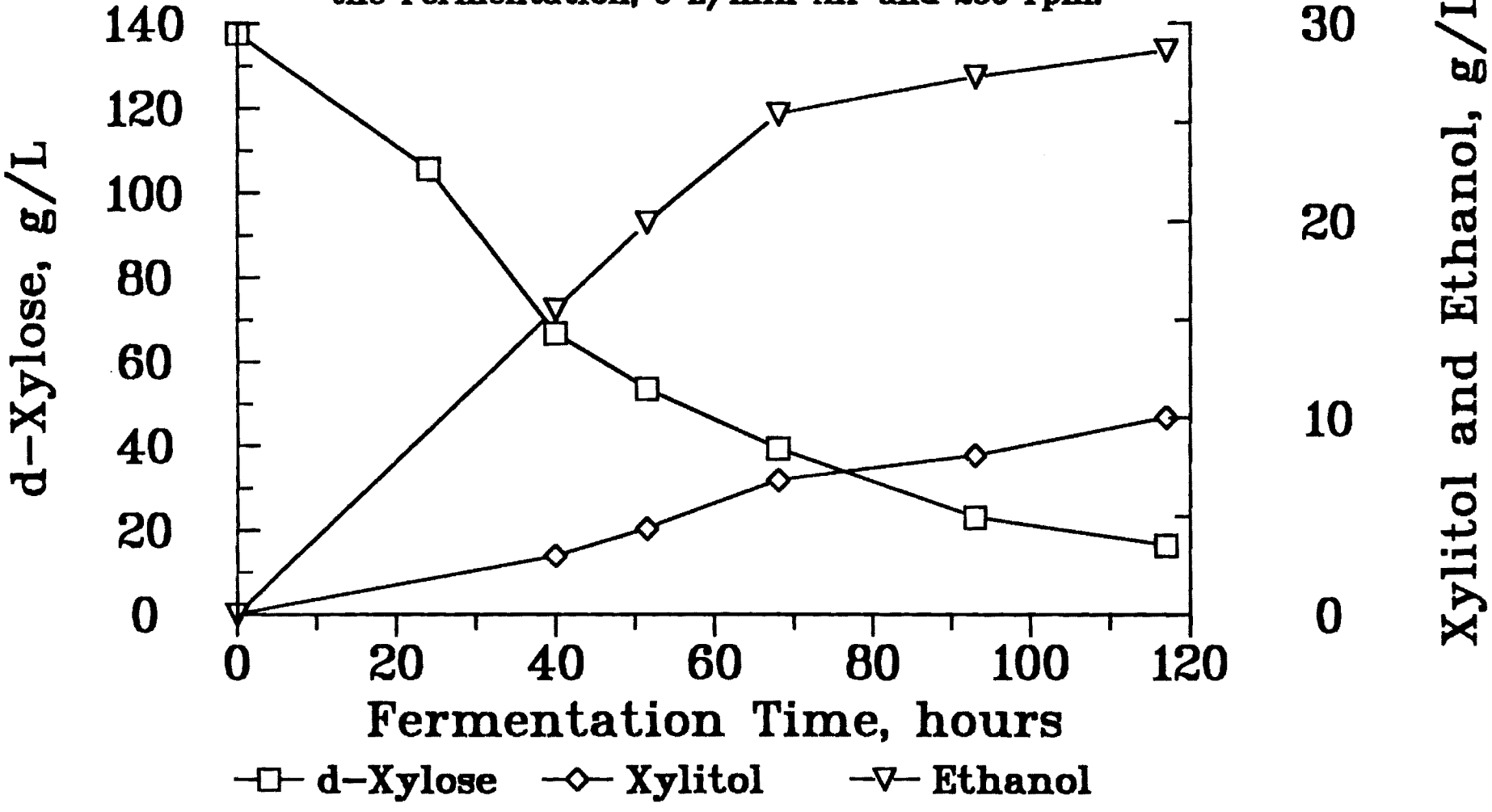
Cells were Aerobically Grown on d-Xylose and then Transferred to Anaerobic Conditions.



Experiment was run in Duplicate.
The Mean Starting Viable Cell Density was 27.5 x 107 cells/ml.

Figure 4 -- d-Xylose Fermentation via Pichia stipitis in a Defined Medium; Initial Condition was 1.4 L/min Air and 550 rpm.

Anaerobic Conditions were imposed 24 hours into the Fermentation; 0 L/min Air and 250 rpm.



Experiment was not run in Duplicate.
The Maximum Viable Cell Density was 140 x 107 cells/ml

time was required to reach the maximum ethanol concentration. When compared to *Candida shehatae*, the *Pichia stipitis* fermentation was incomplete; there was residual d-xylose at the end of the fermentation.

Mixed Sugar Fermentation via Pichia stipitis:

A mixed sugar fermentation utilizing this yeast has been completed. Analysis of the samples is being performed using HPLC.

Mixed Sugar Fermentation via Candida shehatae:

HPLC analysis has been completed. A paper has been submitted to **Biotechnology Letters**. A copy of the paper is provided in the Appendix. Figures in the paper are provided in full size.

Mixed Sugar Fermentation with Candida shehatae; Addition of a Hydrogen Ion Acceptor:

To increase ethanol levels and yields (prevent xylitol formation) in d-xylose fermentation, acetone was added to the fermentation reactor. *Candida shehatae* were aerobically conditioned on d-xylose and then inoculated into a mixed sugar environment with acetone. This small fermentation project has been completed. HPLC analysis is underway.

Future Work

Fed-Batch Fermentation:

The benefits of utilizing a defined medium will continue to be explored using fed-batch fermentation experiments. The experiments will be performed according to the following procedure. A 24 hour slant of *Candida shehatae* will be used to inoculate a seed culture (250 ml of defined medium). After 24 hours at 300 rpm's and 30°C, two separate 100 ml volumes will be used to inoculate two Multigen Batch Reactors (total volume of 500 ml). Each reactor will initially be operated under aerobic conditions (air sparging and agitation with pH control). When a certain cell density is reached anaerobic conditions will be imposed (nitrogen sparging). At this point and at 24 hour intervals medium will be added to each reactor. One reactor will receive d-xylose plus defined medium. The other will receive d-xylose, defined medium and TCA cycle intermediates.

Use of fed-batch fermentations will have several advantages over the previous experimental techniques. Under aerobic conditions the fed-batch experiments will achieve high cell densities. Anaerobic conditions can then be imposed without the tedious process of terminating the aerobic step, collecting the cells, spinning them down and then inoculating into shaker flasks or spinner flasks. Also, use of the Multigen Batch Reactors will allow pH control. Finally, under anaerobic conditions, the effect of supplementing the cells with different medium formulations can be monitored. The effect can be quantified by the viability index, ethanol levels and xylitol concentration.

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Appendix

SIMULTANEOUS FERMENTATION OF D-XYLOSE AND GLUCOSE BY *Candida shehatae*

Jim Kastner, School of Applied Biology
and R.S. Roberts*, School of Chemical Engineering

Georgia Institute of Technology
Atlanta, Georgia 30332-0100

SUMMARY

Mixtures of d-xylose and glucose were anaerobically fermented with the yeast *Candida shehatae*. Cells aerobically grown on glucose exhibited a sequential anaerobic utilization pattern; glucose first then d-xylose. Cells aerobically grown on d-xylose anaerobically consumed glucose and d-xylose simultaneously; no lag in d-xylose consumption was seen. The best results were obtained with cells aerobically grown on d-xylose and inoculated into a 75:25 mixture. 25 g/L of ethanol and 25 g/L of xylitol were obtained from 120 g/L of carbohydrates within 50 hours.

INTRODUCTION

Biomass, in the form of agricultural and wood residues, is composed of three biopolymers; hemicellulose, cellulose and lignin. The hemicellulose and cellulose biopolymers can be hydrolyzed using a mineral acid. The resulting hydrolyzate mainly consists of glucose and xylose. If the hydrolyzate is to be fermented, obviously a microorganism which can utilize both has the potential to give higher product yields.

In the case of ethanol fermentation, however, only some yeasts are capable of anaerobically fermenting d-xylose. At present *Candida shehatae* and *Pichia stipitis* are preferred to anaerobically ferment d-xylose (Prior, et al., 1989). These yeasts in the presence of d-xylose and glucose, will preferentially utilize glucose (du Preez, 1986). Once the glucose has been consumed enzymes for d-xylose catabolism must be synthesized which causes a lag period before d-xylose utilization begins. This increases fermentation times and lowers ethanol yields.

To circumvent this problem one might be able to induce the d-xylose enzymes before inoculation into a mixed sugar environment. If glucose does not severely repress d-xylose catabolism, this would allow simultaneous utilization of both sugars and alleviate the lag period. Slininger and Bothast (1987) utilized the yeast, *Pachysolen tannophilus*, to demonstrate this idea. Since *Candida shehatae* ferments d-xylose at a faster rate than

P. tannophilus, and gives higher ethanol yields, it would be of interest to test this principle using *C. shehatae*. This paper discusses the results for mixed sugar fermentations in which *C. shehatae* were aerobically grown on either d-xylose or glucose and used for anaerobic mixed sugar fermentation.

EXPERIMENTAL METHODS

Microorganism: A strain of *Candida shehatae* (ATCC 22984) was obtained from the American Type Culture Collection. It was maintained on YM agar slants (YM broth in 2% agar). Long-term stock cultures were maintained at 4°C, and subcultured at one to two month intervals.

Seed Cultures: Seed cultures were prepared by inoculating a YM Broth-2% Agar slant with *Candida shehatae* and incubating the slant at 30°C for 24 hours . A rinse transfer from the slant was then used to inoculate 250 ml of seed culture media in a 500 ml Erlenmeyer Flask. The media was similar to the media of Wayman and Tsuyuki (1985) and consisted of 3 g/l yeast extract, 5g/l peptone and 20 g/l of either d-xylose or glucose. The flask was placed in an incubator shaker (30°C, 300 rpms) for approximately 24 hours. The seed culture was then aseptically transferred to an aerobic batch fermentor.

An 8 liter New Brunswick batch fermentor (Model FS-607 Fermentation system with a Model F7-100 batch reactor) and a New Brunswick Multigen 2 Liter Benchtop Fermentor (F-2000) were used in the experiments. The Model FS-607 was operated under the following conditions; 1) three liters of media (2-3 ml of antifoam), 2) agitation at 500 - 600 rpms, 3) aeration of 2 liters/min and 4) a temperature of 30°C. Periodic samples were taken and microscopic observations performed to check for contamination. For the Mulitgen the following conditions were applied; 1) one liter of media, 2) agitation of 500-600 rpms, 3) an aeration rate of 1.3-1.6 liters/min, and 3) a temperature of 30°C. The media for both aerobic fermentors consisted of 6 g yeast extract/L, 10 g peptone/L and 50 g/L of either d-xylose or glucose.

Periodic samples were taken during the batch aerobic fermentations to determine the total and viable cell concentration and viability index. The aerobic batch fermentations were terminated when a cell concentration of 25-40 x 10^7 cells/ml was reached. At this point 200 - 250 ml aliquots were withdrawn and centrifuged at 3000 rpm for 15 - 20 minutes. The centrifuge bottles were then decanted and used to inoculate Bellco spinner flasks for the anaerobic fermentations.

Anaerobic Fermentations: The 500 ml Bellco spinner flasks contained two side ports (one plugged with cotton and the other with a rubber septum). Each reactor had a working volume of 250 ml. Agitation, approximately 250 revolutions per minute (rpm), was provided by a magnetic stirrer. The spinner flasks were housed in a Fischer Model 510 Semi-Automatic Isotemp CO_2 Incubator to maintain the temperature at 30°C.

The fermentations were performed with cells aerobically grown on either glucose or d-xylose. The anaerobic fermentation media consisted of 3g yeast extract/L, 5g ammonium sulfate/L and 120 g mixed sugars/L. The mixed sugars had nominal d-xylose to glucose ratios of 75:25 or 50:50 respectively. The initial viable cell concentration ranged between 26 and 35 x 10^7 cells/ml with a corresponding viability index between 0.95 and 0.98. Each fermentation was performed in duplicate and the experiments were replicated.

Analytical Methods: Periodically, two to five ml samples were taken and placed in ice. An aliquot of the sample was taken and viable cell counts determined by the method of Lee et al., (1981). The remaining sample was centrifuged at 5000 rpm for 15 minutes. The supernatant was decanted and stored in glass vials at 0°C. Xylose, glucose, xylitol and ethanol concentrations were determined by HPLC analysis using cellobiose (0.01 g/ml) as the internal standard. A Waters GPC-I, equipped with a Bio-Rad Aminex Ion Exclusion HPX-87H column and a differential refractometer, was used. The differential refractometer was set at 16x, and the mobile phase was 0.01N H_2SO_4 at a temperature of 65°C and a flowrate of 0.6 ml/min.

RESULTS AND DISCUSSION

For all of the mixed sugar anaerobic fermentations, the total cell concentration remained essentially constant at 25-36 X 10^7 cells/ml. However, viable cell concentration and viability index did decrease late in the fermentations (data not shown). The anaerobic conditions and the presence of ethanol effectively resulted in a stationary phase fermentation. Sreenath et al. (1986) and du Preez et al. (1984) reported similar results.

Cells aerobically grown on d-glucose and then used for an anaerobic mixed sugar fermentation showed a sequential utilization pattern as shown in Figures 1 A and B. At first glucose was exclusively consumed. Once glucose had been consumed d-xylose utilization began. However, the amount of d-xylose consumed was small and xylitol was produced. Similar carbohydrate consumption and by-product formation patterns were seen for the 75% d-xylose - 25% glucose and the 50% d-xylose - 50% glucose mixtures.

Cells aerobically grown on d-xylose and then used for an anaerobic mixed sugar fermentation had a simultaneous sugar utilization pattern as shown in Figures 2 A and B. For both the 75% d-xylose - 25% glucose and the 50% d-xylose - 50% glucose mixtures, the sugars were consumed simultaneously until the glucose was exhausted. The rate of glucose consumption (g/cell-hr) was approximately 1.6 times that of d-xylose consumption for the 75% d-xylose - 25% glucose mixture. In the 50% d-xylose - 50% glucose mixture the glucose consumption rate was 3.4 times that of d-xylose. During this time both ethanol and xylitol were formed. Once glucose had been exhausted d-xylose consumption continued and resulted in additional xylitol formation with little additional ethanol formation. The 50% d-xylose - 50% glucose ratio gave the highest ethanol production.

In *Pachysolen tannophilus*, enzymes for d-xylose utilization are induced by d-xylose but not glucose (Bolen et al., 1985). Also, glucose inhibits d-xylose catabolism by repressing the induction of d-xylose enzymes (Bicho et al., 1988). It appears that the

same mechanism occurs in *Candida shehatae* and can explain the experimental results.

Cells aerobically grown on glucose were not able to adequately utilize d-xylose. A severe lag in d-xylose catabolism was seen in these cells and when d-xylose utilization did begin it was incomplete. This is probably due to the initial lack of d-xylose catabolic enzymes and the difficulty of synthesizing these enzymes under anaerobic conditions.

If the cells were aerobically conditioned on d-xylose, there was no lag in d-xylose catabolism. Aerobically conditioning the cells on d-xylose induced the synthesis of the d-xylose catabolic enzymes. Since glucose catabolic enzymes are constitutive (always present) these cells were primed for utilization of both sugars. Although both sugars were concurrently consumed , the rate of d-xylose utilization did decrease with time and in both cases (75/25 and 50/50 d-xylose to glucose ratios) there was residual d-xylose. The change in the d-xylose utilization rate (and residual d-xylose) may have been due to glucose repression and/or anaerobic conditions in the fermentations; the two effects can not be separated by this work.

Acknowledgements

This research was funded by the Tennessee Valley Authority (TV-74261A) and the Georgia Tech Foundation.

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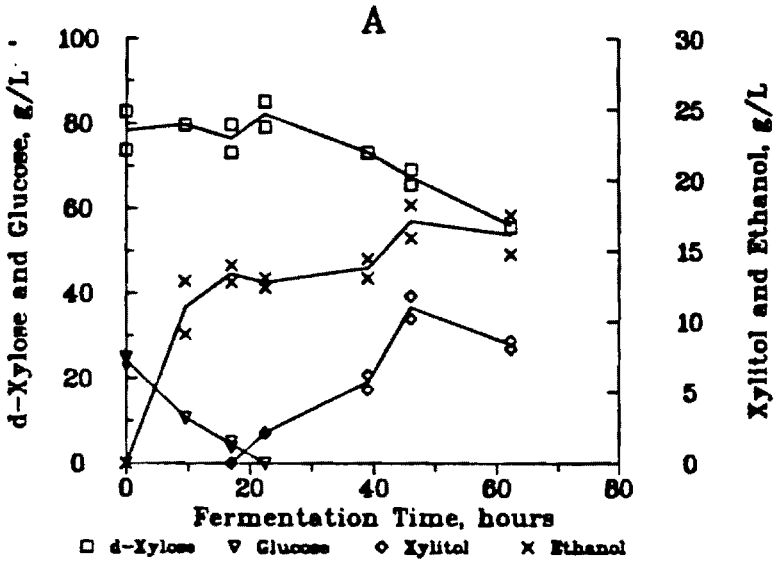


Figure 1A - Anaerobic Fermentation of a 75% d-xylose and 25% glucose mixture with *Candida shehatae* cells aerobically grown on glucose.
The graph was constructed by calculating the mean at each datum (duplicates) and connecting the points.

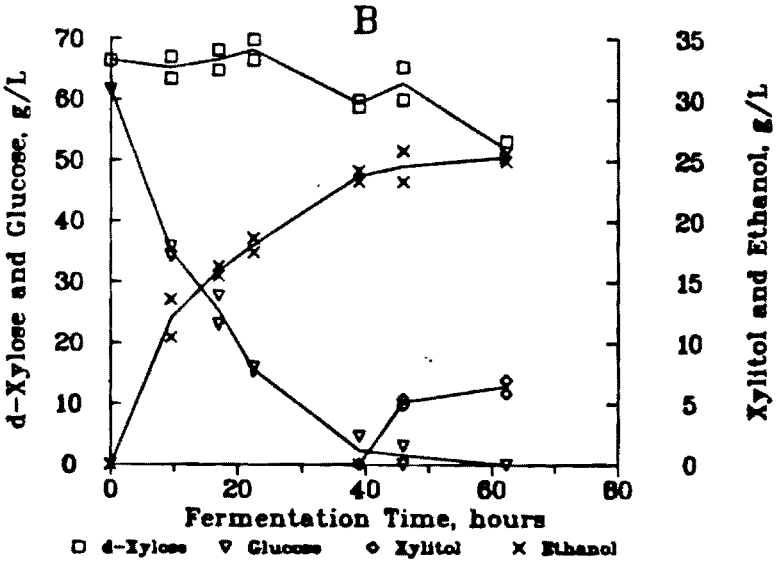


Figure 1B - Anaerobic Fermentation of a 50% d-xylose and 50% glucose mixture with *Candida shehatae* cells aerobically grown on glucose.
The graph was constructed by calculating the mean at each datum (duplicates) and connecting the points.

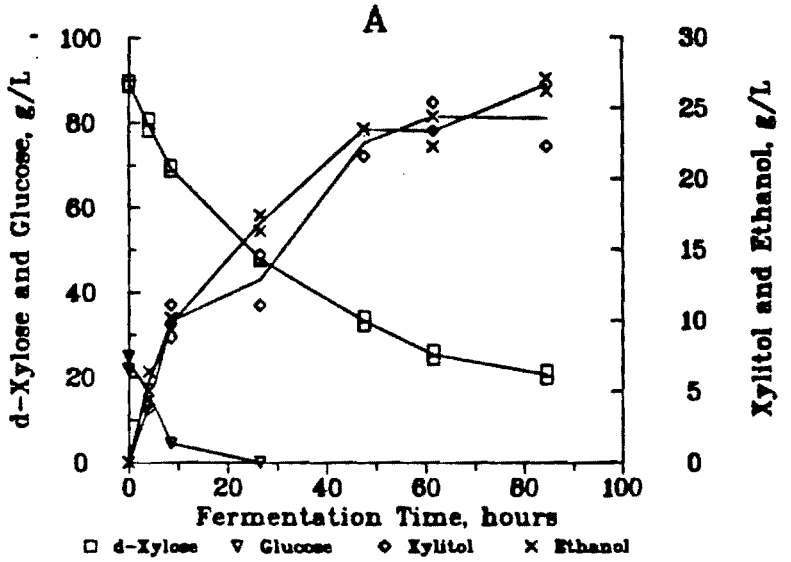


Figure 2A - Anaerobic Fermentation of a 75% d-xylose and 25% glucose mixture with *Candida shehatae* cells aerobically grown on d-xylose.
The graph was constructed by calculating the mean at each datum (duplicates) and connecting the points.

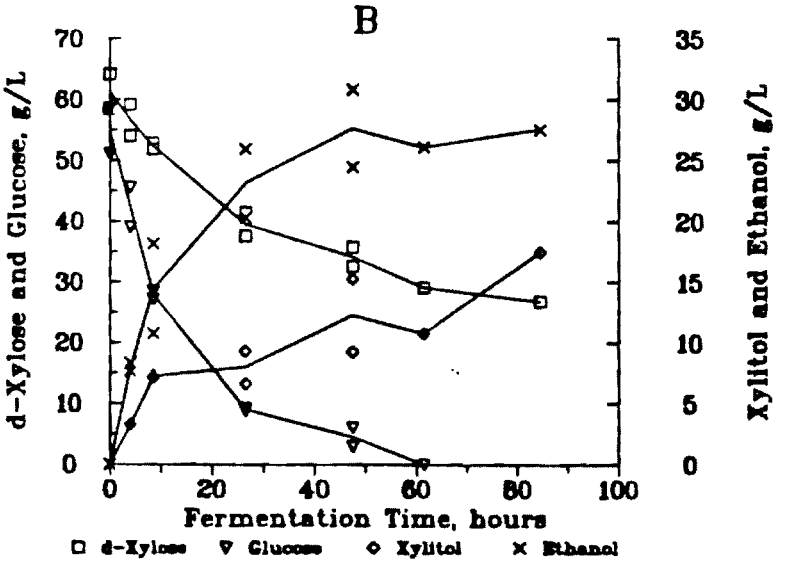
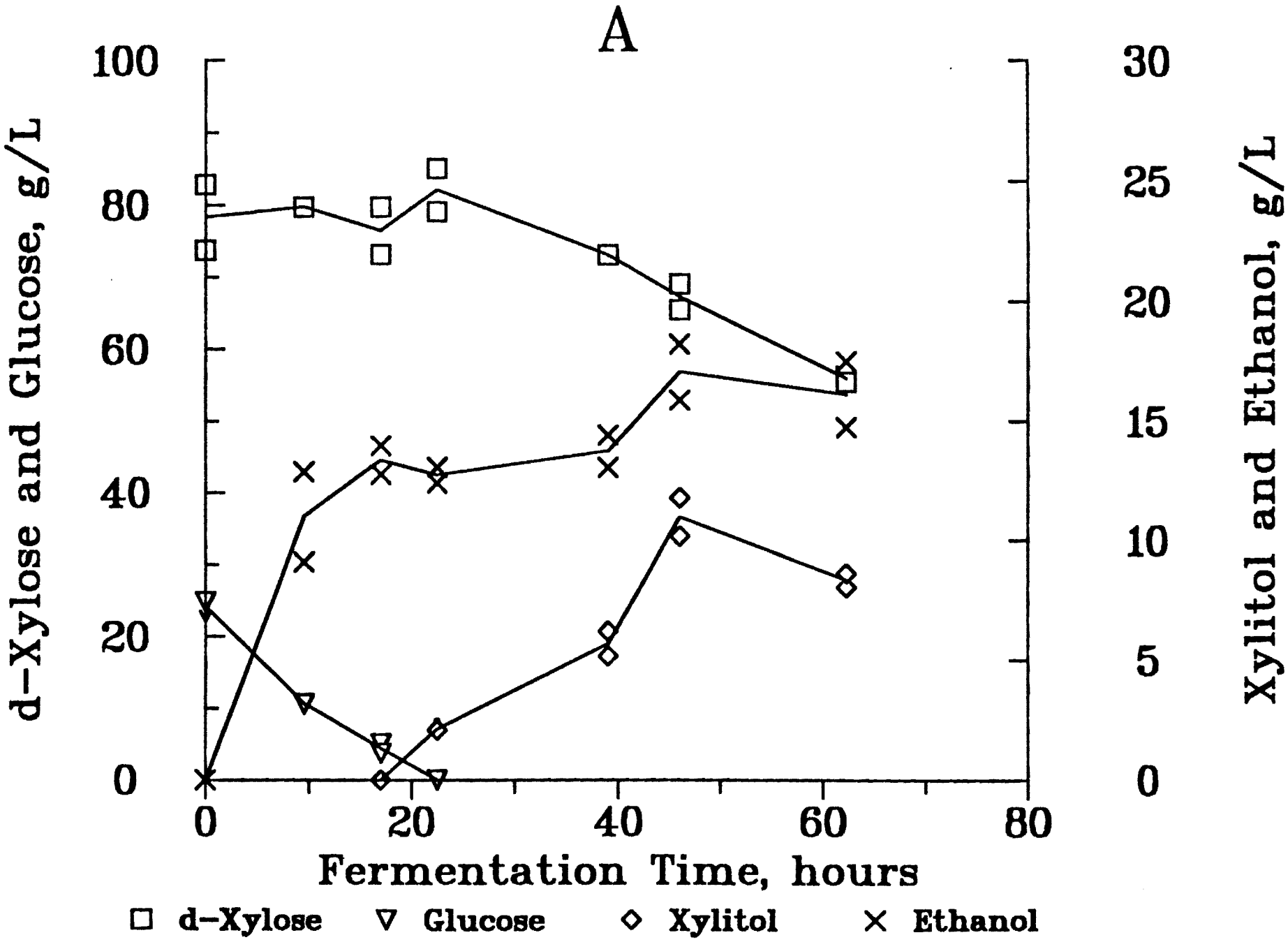
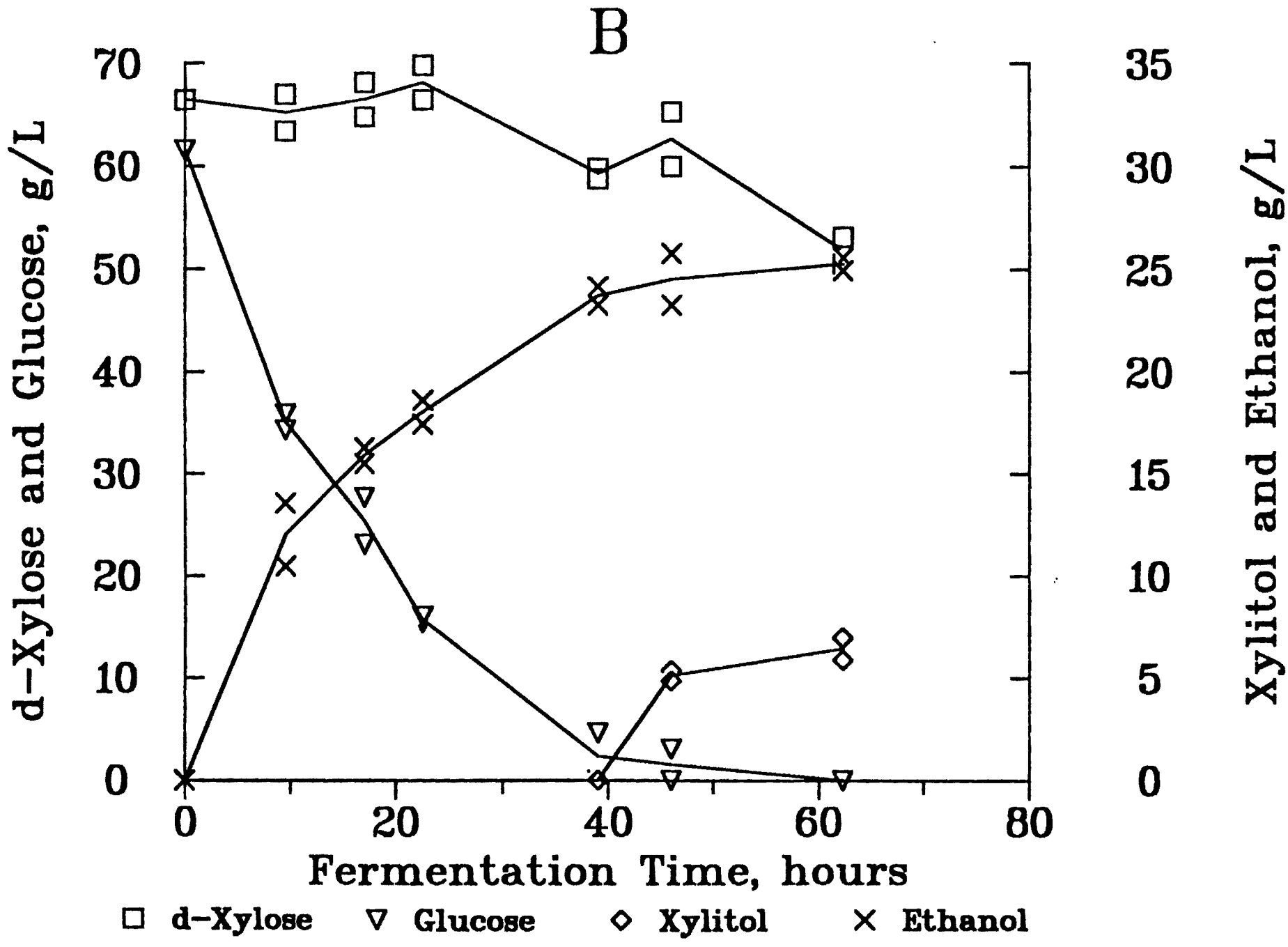
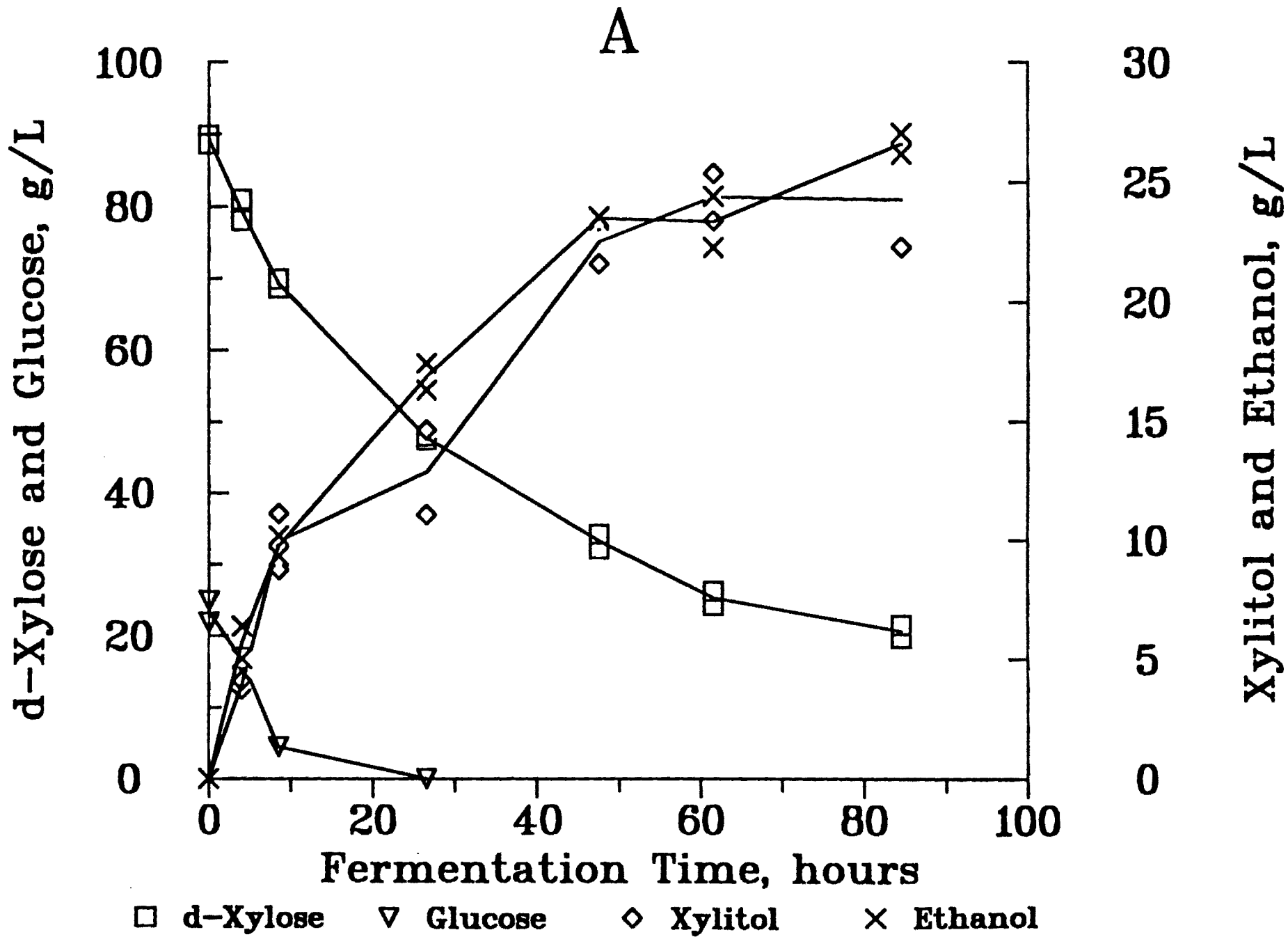


Figure 2B - Anaerobic Fermentation of a 50% d-xylose and 50% glucose mixture with *Candida shehatae* cells aerobically grown on d-xylose.
The graph was constructed by calculating the mean at each datum (duplicates) and connecting the points.


A
d-Xylose and Glucose, g/L
Xylitol and Ethanol, g/L
Fermentation Time, hours
0
20
40
60
80
100
0
5
10
15
20
25
30
d-Xylose
Glucose
Xylitol
Ethanol


B
d-Xylose and Glucose, g/L
Xylitol and Ethanol, g/L
Fermentation Time, hours
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30
35
0
20
40
60
80
d-Xylose
Glucose
Xylitol
Ethanol


A
d-Xylose and Glucose, g/L
Xylitol and Ethanol, g/L
Fermentation Time, hours
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5
10
15
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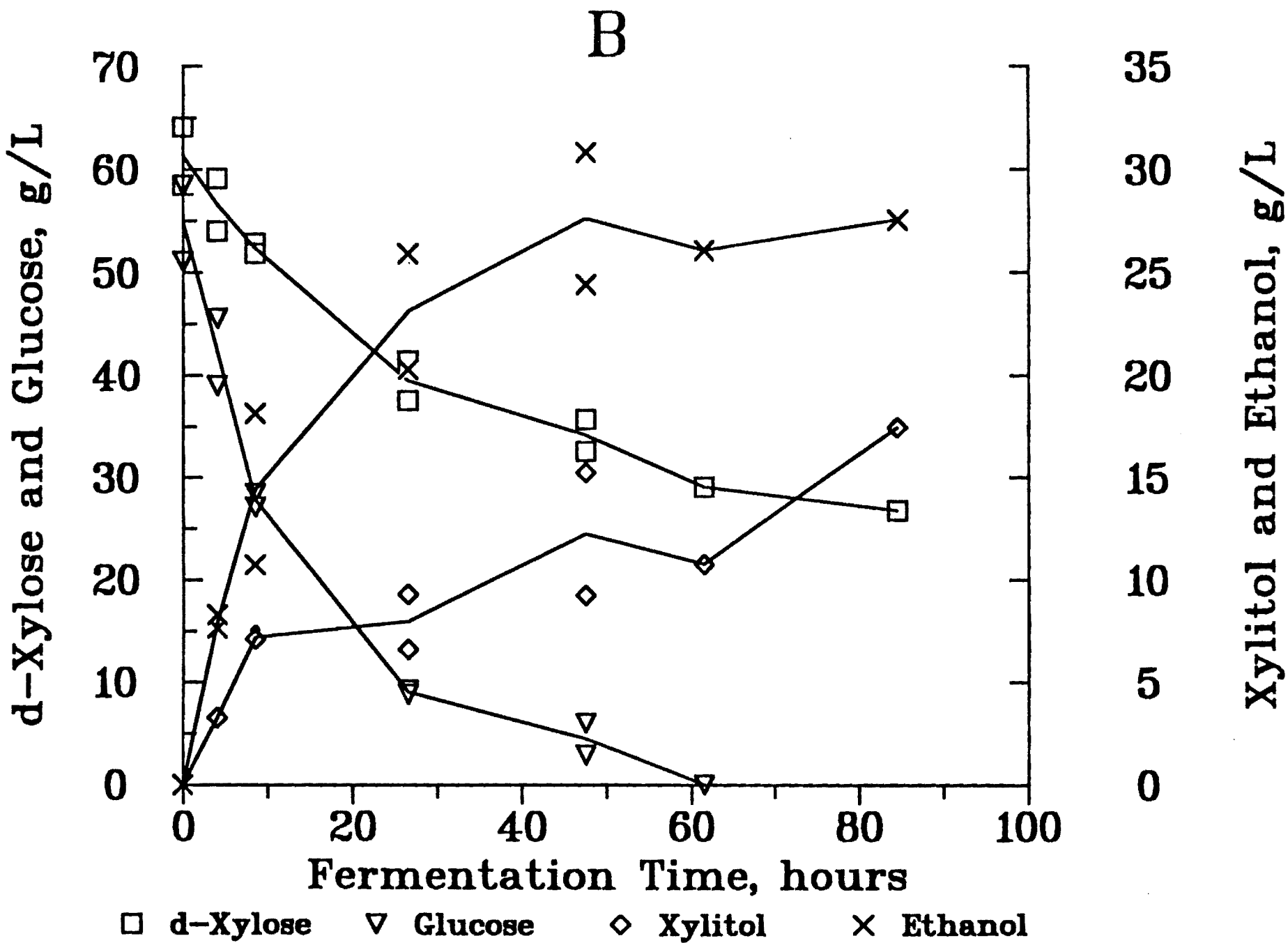
30
d-Xylose
Glucose
Xylitol
Ethanol


B
d-Xylose and Glucose, g/L
Xylitol and Ethanol, g/L
Fermentation Time, hours
0
10
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50
60
70
0
5
10
15
20
25
30
35
0
20
40
60
80
100
d-Xylose
Glucose
Xylitol
Ethanol

Quarterly Progress Report

October 1, 1989 - December 31, 1989
Contract # TV- 74261A

Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

Jim Kastner , School of Applied Biology
and Professor R.S. Roberts, School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

Summary

Fed batch fermentations were performed with the yeast *Candida shehatae*. The yeast cells were aerobically grown on D-xylose in a defined medium with no casamino acids. After 24 hours an anaerobic condition was imposed. At periodic intervals concentrated xylose solutions were added in a batch manner. It was found that cell stress caused an increase in xylitol formation. For example, 20g/L of ethanol and 30g/L of xylitol were produced from a fed batch fermentation in which the seed culture was stressed, compared to **40g/L of ethanol** and 15g/L of xylitol for one in which the seed culture was relatively not stressed. It was found that addition of the TCA cycle intermediates, *alpha*-ketoglutaric acid and oxaloacetic acid, under anaerobic conditions did not enhance cell viability nor increase ethanol levels. Restricted transport of these intermediates across the cell membrane may have been the reason for their lack of influence on cell viability.

Introduction

Dry cell weight (g biomass/L) has been used to determine the change in cell population in most ethanol fermentations using *Candida shehatae* This method measures total cell biomass, but gives no indication of cell viability. However, Kastner and Roberts (1989 A) have shown that cell viability is a very important parameter which must be monitored. In anaerobic fermentations with *Candida shehatae*, methylene blue staining was used to monitor cell viability (or the viability index as the percentage of viable cells). Under anaerobic conditions the cells did not grow (Figure 1) and most importantly, after a short exposure time to anaerobic conditions (20-40 hours) the viability index declined (Figure 1). This type of response to anaerobic conditions is quite different from the *Saccharomyces cerevisiae* traditionally used in ethanol fermentations.

Unlike the yeast *Saccharomyces cerevisiae*, *Candida shehatae* will not grow under anaerobic conditions on xylose or glucose (Neirinck, et al. 1984). However, *Saccharomyces cerevisiae* will grow on glucose under anaerobic conditions. Moreover, in xylose fermentations with *Candida shehatae*, the lack of oxygen causes a decline in cell viability. In *Saccharomyces cerevisiae* (it should be remembered that *Saccharomyces* can not ferment xylose) it takes a much longer time before a decline in cell viability is observed. These data (and data from the literature) suggest a fundamental difference between the two yeasts. It appears that *Saccharomyces cerevisiae* can synthesize components it needs for cell maintenance and growth without oxygen (at least for several generations); *Candida shehatae* can not!

To make sure that the lack of intermediates from oxygen metabolism were limiting *Candida shehatae*, use of a defined medium was proposed (Kastner and Roberts, 1989 B). This was done because in the previous fermentations an undefined medium (yeast extract and $(NH_4)_2SO_4$) was used. The undefined medium may not have contained growth factors (or autoclaving may have decomposed them) and trace elements required for growth. Use of the defined medium did improve batch xylose fermentation. The cell viability was higher and the ratio of ethanol to xylitol was increased. However, only about 30 g/L of ethanol was produced from 80g/L of xylose (Figures 1, 2 and 3). In this batch

fermentation xylose was depleted within 50 hours and cell viability was approximately 87 percent. This indicates that more ethanol could have been produced if additional xylose had been added in a fed batch manner.

A fed batch fermentation would also allow testing of long term exposure to anaerobic conditions. The composition of the medium could be varied and the effects of various medium compounds on cell viability and the ethanol to xylitol ratio could be determined. This report discusses the results for two types of fed batch, pure d-xylose fermentations.

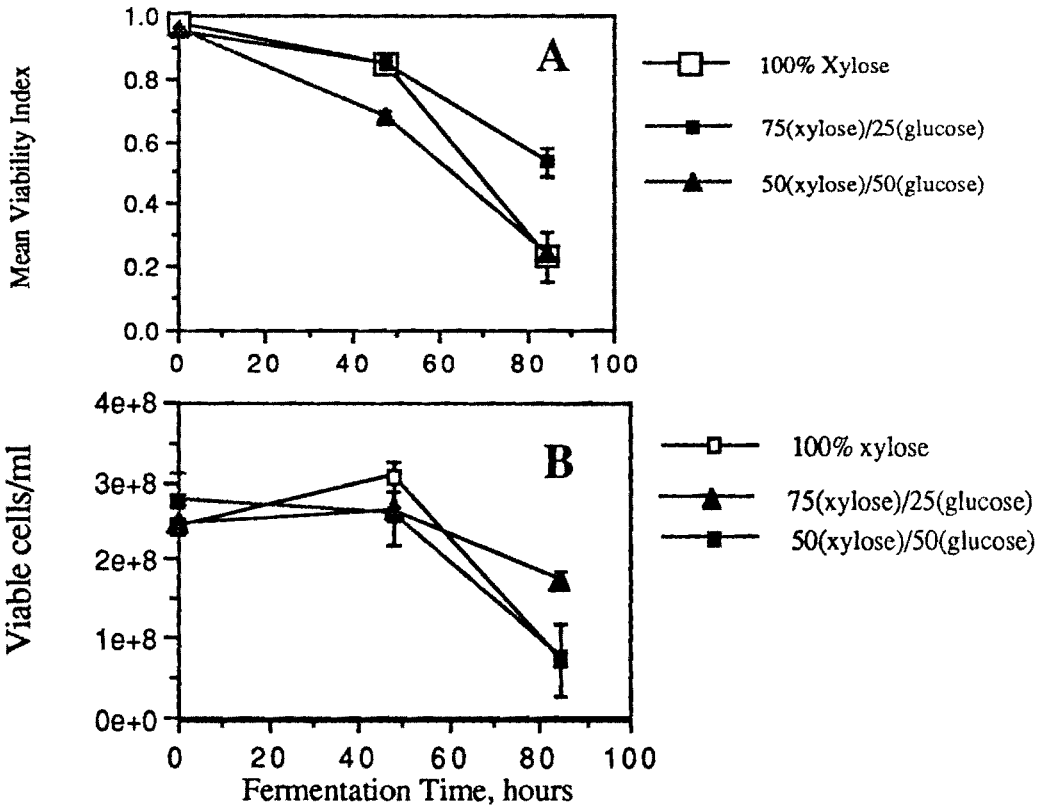

Figure 1. -- Pure D-xylose and mixed sugar fermentations under anaerobic conditions using the yeast *Candida shehatae* (Kastner and Roberts, 1990).

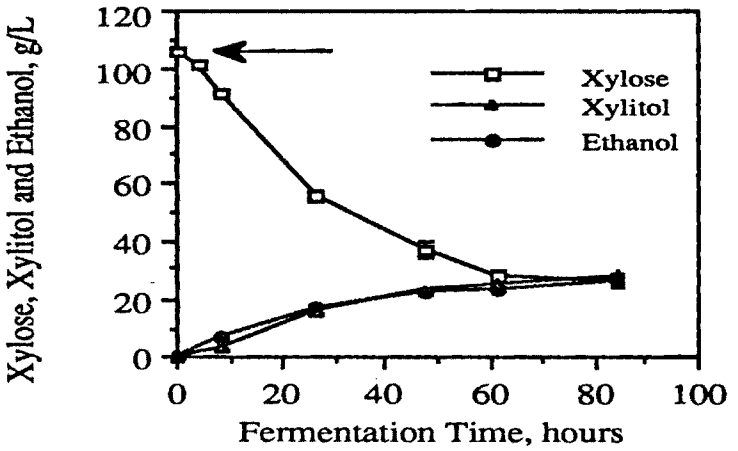

Figure 2. -- Pure D-xylose fermentation in an undefined medium using *Candida shehatae*. The conditions in this fermentation are the same as those described in Figure 1.

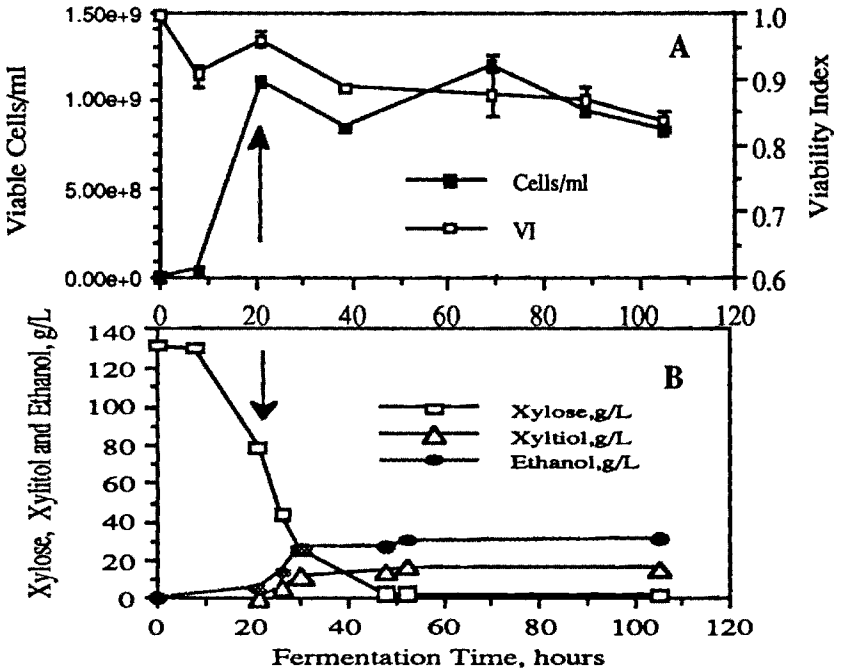

Figure 3. -- Batch xylose fermentation in a defined medium (no amino acids) with the pH controlled at 4.5. Figure A shows the viable cell population as determined by methylene blue staining. Figure B shows the xylitol and ethanol formation with time. Arrows indicate the point at which anaerobic conditions were imposed.

Materials and Methods

Defined Medium:

The defined medium was similar to that of du Preez (1983), except that citric acid and casamino acids were not added. Three stock solutions were prepared, a vitamin solution (1000X), salt solution (50X) and trace element solution (500X), (Table 3). The salt and trace element solutions were autoclaved, while the vitamin solution was cold filtered. One liter of medium contained 20ml of the salt solution, 2ml of the trace element solution and 1ml of the vitamin solution. The xylose concentration was 20g/L in the seed culture and 100g/L in the main fermentations. The nitrogen source solution contained $(NH_4)_2SO_4$ (10g/L), KH_2PO_4 (10g/L) and MgO_4*SO_4 (0.5g/L). This solution and the xylose solution were autoclaved separately and then aseptically combined.

Table 3 -- Preparation of the defined medium.

Solution	Composition	Conc.,g/L
Salt	$CaCl_2*2H_2O$	0.05
	$FeSO_4*7H_2O$	0.035
	$MnSO_4*H_2O$	0.007
	$ZnSO_4*7H_2O$	0.011
Trace Elements	$CuSO_4*5H_2O$	0.001
	$CoCl_2*6H_2O$	0.002
	$Na_2MoO_4*7H_2O$	0.0013
	H_3BO_3	0.002
	KI	0.00035
	$Al_2 (SO_4)_3$	0.0005
	meso-inositol	0.1
Vitamin	Ca-pantothenate	0.02
	Thiamine*HCl	0.005
	Pyridoxine*HCl	0.005
	Nicotinic Acid	0.005
	p-Aminobenzoic	0.001
	d-Biotin	0.0001

Microorganism and seed culture techniques:

A 24 hour slant was used to inoculate (rinse transfer) a 500 ml Erlenmeyer Flask (250 ml working volume). In experiments 1 and 2 (see Table 4) after 24 hours at 300rpms, 100ml of this culture was used to inoculate a New Brunswick Multigen Reactor. In experiments 3 and 4, 50 ml of this culture was used to inoculate two 500 ml Erlenmeyer flasks containing 67 g/L of xylose and 250 ml. These two seed cultures were incubated at 30^{0}C and 300rpms. After 24 hours all of the medium was used to inoculate the New Brunswick Multigen reactors.

In experiment #2, 100ml of a 30%(w/w) d-xylose solution was aseptically added at periodic intervals. In experiment #3 a 64%(w/w) d-xylose solution was used for this purpose (100 ml added at time). In experiment #4, 100 ml of a 64%(w/w) xylose solution, containing *alpha*-ketoglutaric acid and oxaloacetic acid, was added to the fermentor (5% w/w). All concentrated xylose solutions were cold filtered (0.2um filter). The pH was controlled at 4.5 (with 10N NaOH) and anaerobic conditions were imposed after approximately 24 hours by shutting off the air flow and reducing the agitation to 250 rpm.

Table 4 -- Experimental Conditions for D-Xylose Fermentations in Defined Media.

Experiment	S_o,g/L	Volume,ml	pH	T,^{0}C	Agitation	Aeration
1) Batch	50	1000	4.5	27	700	1.5 L/min
2) Fed Batch	100	500	4.5	28	750	1.5 L/min
3) Fed Batch	100	1000	4.5	30	700	1.5 L/min
4) Fed Batch (TCA)	100	1000	4.5	30	700	1.5 L/min
5) Batch, du Preez,1983	90	1500	4.5	30	400-1000	1.0 L/min

S_o indicates the initial xylose concentration

Analytical Methods:

Xylose, glucose and xylitol were analyzed by HPLC (Kastner and Roberts,1990). A Varian 3700 GC operated with helium as the mobile phase, a Poropak Q column (170°C) and a FID detector was used to quantify ethanol. Methanol was used as the internal standard.

Results and Discussion

Specific Growth Rate and Doubling Time:
(under aerobic conditions)

Industrial fermentations require high cell densities to achieve economical production rates. To achieve high cell densities the cells must be grown under aerobic conditions with an inexpensive nitrogen source. For this reason the defined medium contained $(NH_4)_2SO_4$ without expensive casamino acids. Cell densities have reached 1.0 X 10^9 cells/ml with use of this medium (Figure 3). Thus, in a qualitative sense, lack of amino acids in the medium did not reduce the maximum attainable cell population (under aerobic conditions).

To access the effect on growth (lack of amino acids) in a quantitative manner the specific growth rate was calculated from data in Figures 3 and 4. These values were then compared with data from fermentations performed in a medium with casamino acids - data from du Preez, 1983 (see Figure 3). These data are shown in Table 5.

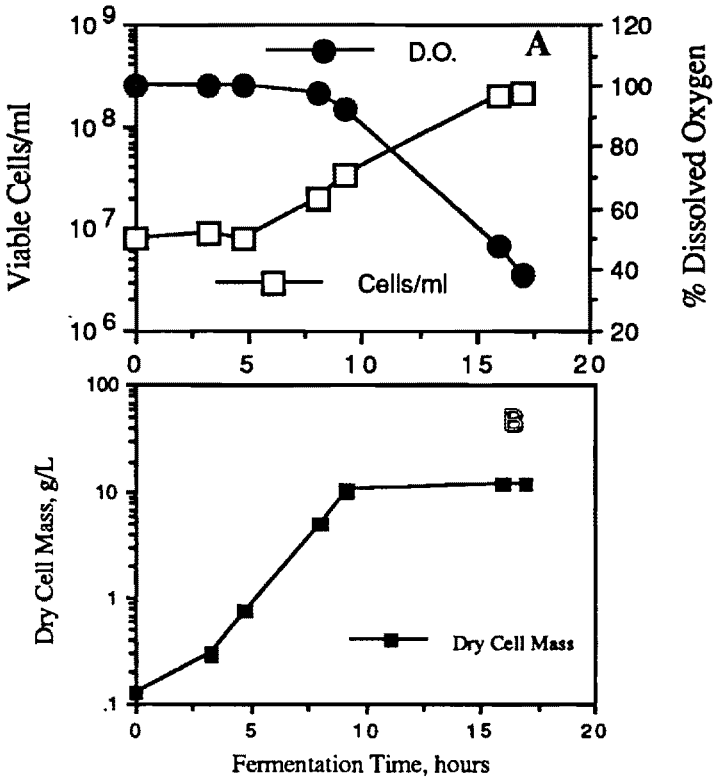

Figure 4. Growth of *Candida shehatae* under aerobic conditions in a defined medium. Figure A shows data for a defined medium with no casamino acids; Figure B was taken from du Preez, 1983 and portrays data for a medium with casamino acids. Reactor conditions were similar (see Table 4).

The specific growth rate can be calculated from a batch fermentation with certain assumptions. It is assumed the cells are in the exponential phase and the growth is balanced. If the growth is balanced a single parameter will describe the cell population - μ, the specific growth rate. The relationship between and the cell population is shown in Equation 1. In balanced growth μ is a constant, this allows Equation 1 to be solved (gives Equation 2). One can now plot ln X vs. time and the slope will give μ (or one can choose two points and directly solve the equation for μ). Also, one can solve Equation 2 for the characteristic doubling time of the yeast, t_d (see equation 3).

Equation 1) $$\frac{dX}{dt} = \mu X \rightarrow \int \frac{dX}{X} = \mu \int t$$

Equation 2) $$\ln X|_b^a = \mu \Delta t$$

Equation 3) $$t_d = \frac{\ln 2}{\mu}$$

where X = cells/ml or grams of cells/L
t = time, t_d = doubling time, hours

Table 5. - Comparison of specific growth rates and doubling times for batch xylose fermentations.

Conditions	Specific growth rate, hr^{-1}	Doubling Time, hr
Batch, defined medium with casamino acids (data from du Preez)	0.47	1.5
Batch, defined medium with no amino acids (data from Figure 2)	0.40	1.7
Batch, defined medium with no amino acids Data from Figure 3, two points in the exponential phase	0.45	1.5

As can be seen from Table 5, the specific growth rate (and doubling time) are similar for media with and without amino acids. These data indicate that the cells can synthesize all the amino acids required for growth under aerobic conditions.

Fed Batch Fermentations on D-xylose:

It is apparent from research in this lab (and the literature) that *Candida shehatae* need products of oxygen metabolism for growth and cell maintenance. More specifically, functional mitochondria are required. One function of the mitochondria is to produce precursors for biosynthetic pathways. Oxaloacetic acid and *alpha*-ketoglutaric acid are two such precursors which are produced in the mitochondria by the TCA cycle. It may be that under anaerobic conditions the precursors can't be provided as required. Transport of the TCA cycle intermediates across the inner mitochondrial membrane may also be a problem. These problems might be alleviated by adding the TCA cycle intermediates to medium.

In all the fed batch fermentations the cell viability began to decline when anaerobic conditions were imposed (Figures 5 and 6). Also, as can be seen in Figure 6 addition of oxaloacetic and *alpha*-ketoglutaric acid to the medium did not improve cell viabilities. It was thought that these two TCA cycle intermediates did not improve viability because they were not transported across the cell membrane. It may be that the cells need to be conditioned to take up the TCA cycle intermediates, or the form of carboxylic acids must be changed to promote their transport.

There was also a difference between the two control fermentations - xylose fermentations in a defined medium with no TCA cycle intermediates added. Each were run under similar conditions but the ethanol to xylitol ratios were different; 1.0 in first and 2.0 in the second (Figures 5 and 7). An attempt to explain the differences between the two fermentations will be made in the following paragraphs.

At time zero in the first fermentation (Figure 5) microscopic observation revealed elongated cells, however, at the same time period in the second fermentation (Figure 7) the cells were round and oval shaped. Elongated cells indicate a sign of stress and a potential change in metabolism, while round oval shaped cells signify healthy cells with a normal metabolism. This difference in cell morphology (shape) was attributed to a difference in cell culturing.

In the first fermentation a seed culture which contained 20 g/L of xylose in a defined medium was used (Materials and Methods). The low concentration of

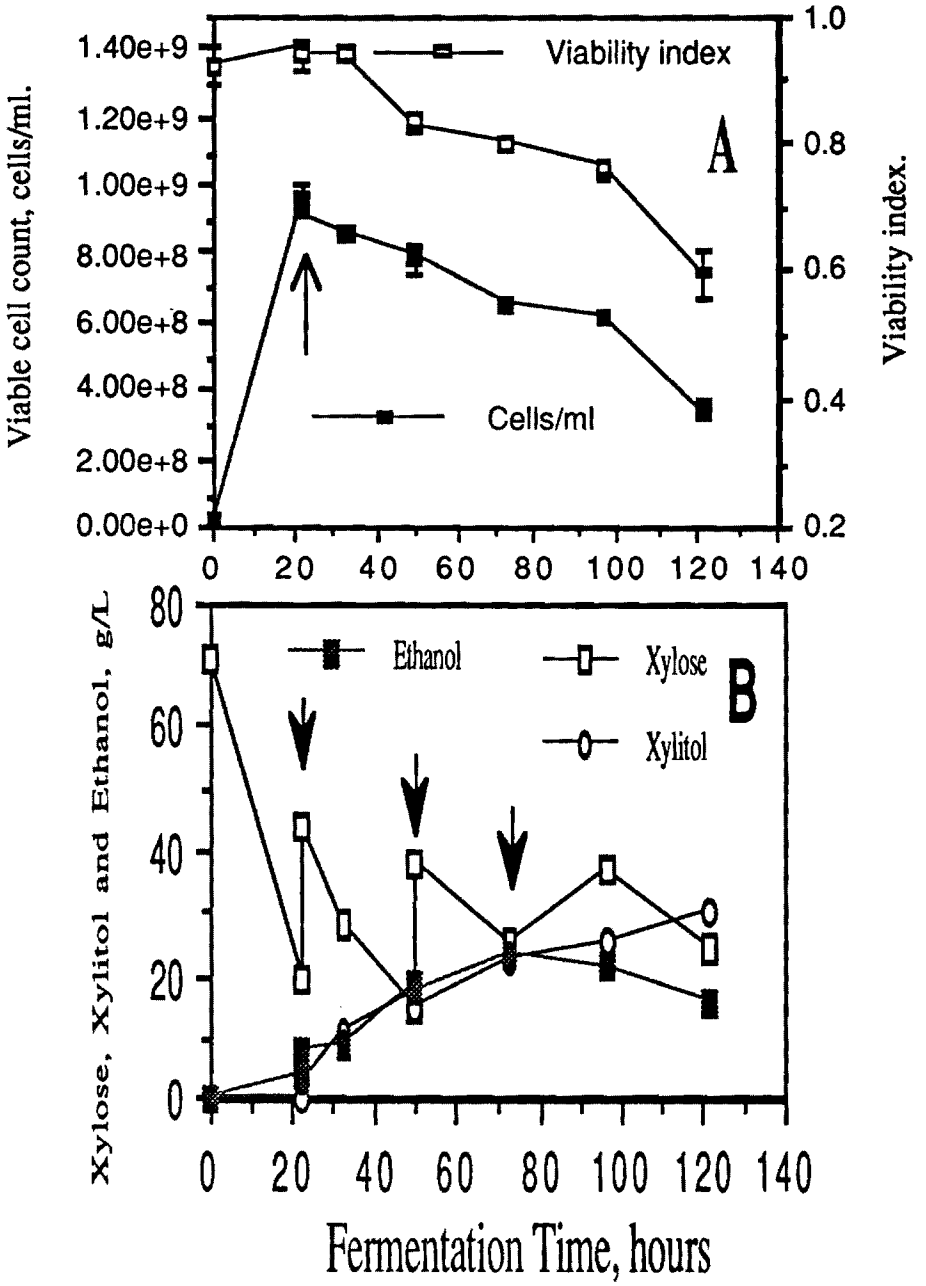

Figure 5. Fed batch xylose fermentation using *Candida shehatae*. Figure A shows the viable cell population determined by methylene blue staining. Figure B shows xylitol and ethanol formation as a function of time. The first arrow indicates when anaerobic conditions were imposed and medium was added. The other arrows indicate when medium was added in batch manner.

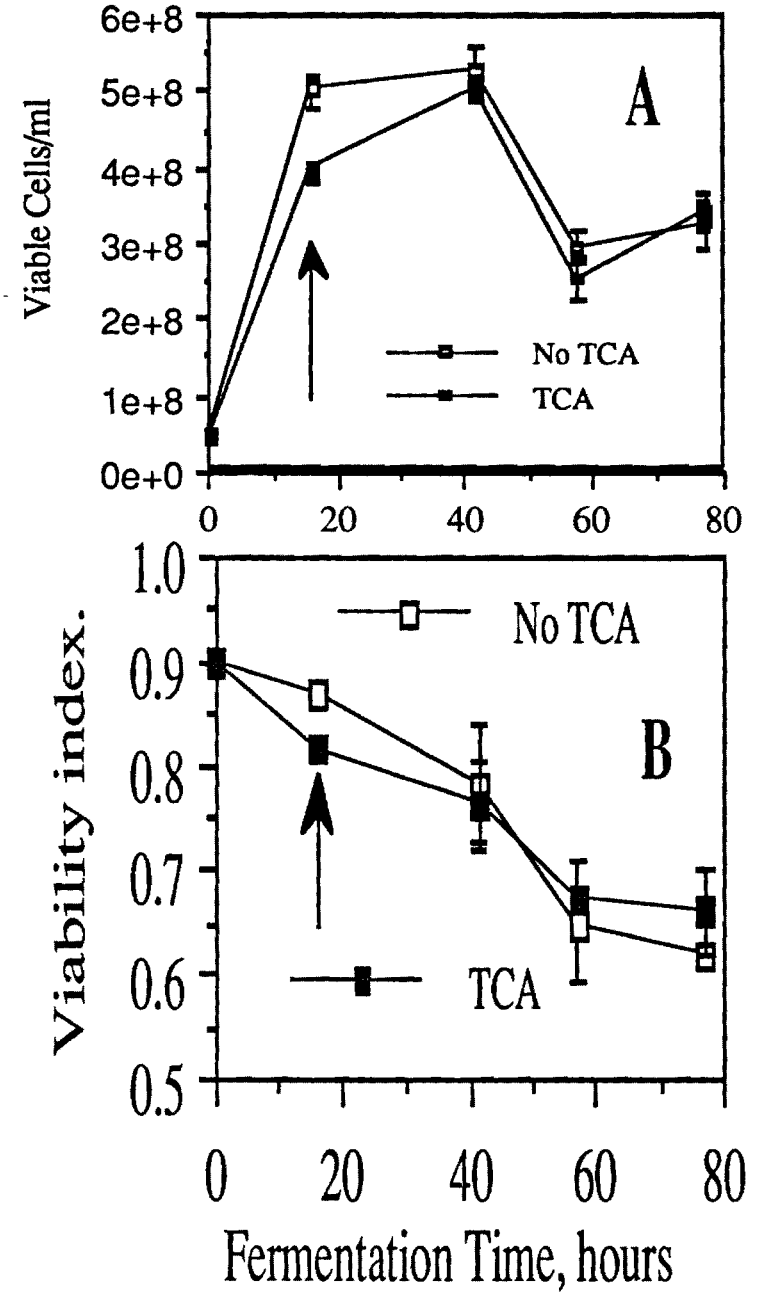

Figure 6. Fed batch fermentation in which TCA cycle intermediates were added to the reactor when anaerobic conditions were imposed (indicated by the arrow). A control fermentation with no TCA intermediates was also performed. Figure A shows the viable cell population and Figure B the change in the percentage of viable cells.

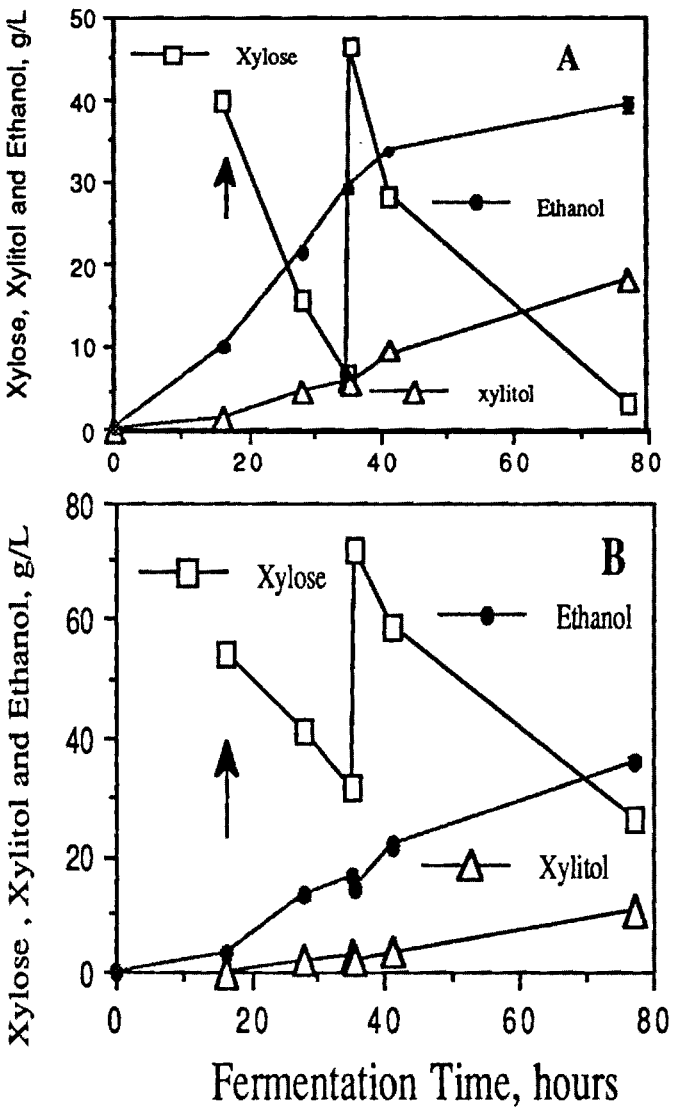

Figure 7. Fed batch fermentations in a defined medium using *Candida shehatae* . Figure A is the control reactor; no TCA cycle intermediates were added to the reactor. Figure B shows fermentation data for the reactor which received the TCA cycle intermediates. The arrows indicate when anaerobic conditions were imposed.

xylose and the small head space probably caused substrate and/or oxygen limitation. In the second fermentation an additional culturing step was used; this culture contained 67 g/L of xylose (Materials and Methods). This second step probably alleviated any stress incurred in the first seed culture. Thus, substrate and/or oxygen limitation apparently caused a change in cell metabolism (due to stress) which caused the higher levels of xylitol.

In the last two fed batch fermentations the final ethanol levels were about the same - 39 g/L in the control and 36 g/L in the reactor with the TCA cycle intermediates (Figure 7). The reactor with the TCA cycle intermediates did produce a lower amount of xylitol - 10.8 g/L compared to 18.1 g/L in the control. Here again this was probably due cell stress. In the control reactor the xylose level dropped to 5 g/L before more xylose was added (Figure 7). Such a low level of xylose could have induced cell stress and caused an increase in xylitol production.

Conclusions

If provided with oxygen, *Candida shehatae* can grow without amino acids in a defined medium. The medium must contain vitamins, salts, trace elements and a nitrogen source. The growth rate was slightly lower than in the medium with amino acids. But this is to be expected since the cells must use extra time, energy and carbon to synthesize the needed amino acids. Thus, simple nitrogen sources, such as inexpensive $(NH_4)_2SO_4$, can be used to grow these cells (under aerobic conditions).

Under anaerobic conditions, *Candida shehatae* did not grow. Moreover, cell viability declined in the defined medium (Figures 5 and 6). The drop in cell viability may have been due to ethanol and xylitol buildup or key components were still missing from the medium. To see if the medium was still the limiting factor, alpha-ketoglutarate and oxaloacetic acid were added to the medium.

Addition of TCA cycle intermediates did not improve cell viability nor increase ethanol concentrations. This may have been due to a lack of utilization because the intermediates could not be transported across the cell membrane. Each compound or class of compound has specific mechanisms to transport them across the cell membrane. These mechanisms usually require a membrane bound protein

and must be induced by the compound itself. It could be that the cells were not conditioned in the presence of oxaloacetic and *alpha*-ketoglutaric acid long enough to induce a transport protein. Further experiments will be performed to determine if transport is the problem.

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Quarterly Progress Report

December 31, 1989 - March 31,1990

Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

Jim Kastner, School of Applied Biology
and Professor R.S. Roberts, School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

Summary

Fed batch fermentations on glucose and *alpha*-ketoglutaric acid, and rapid batch fermentations on D-xylose were performed with the yeast *Candida shehatae*. Relative to the control ($(NH_4)_2SO_4$ and glucose), cells aerobically conditioned on *alpha*-ketoglutaric acid maintained cell viability for a longer time under anaerobic conditions. The cell viability was maintained between 0.8 and 0.98 for approximately 8 days of anaerobic conditions. However, the conditions of this fermentation apparently inhibited ethanol production (only 33 g/L). A fermentation (on glucose) was performed in which *alpha*-ketoglutaric and NH_4OH were added to the medium. This resulted in a final ethanol concentration of 46 g/L.

Fermentation times were significantly reduced by using a rapid batch method. A fermentation of D-xylose was accomplished in 7 hours. This fermentation produced 27 g/L of ethanol and 20 g/L of xylitol (initial cell density was 4×10^9 viable cells/ml). Another rapid batch fermentation was performed to test the effect of casamino acids. The fermentation which received the casamino acids produced more ethanol (42 g/L compared to 30 g/L). The fermentation times were longer (26 hours) because the starting cell densities were lower. However, xylitol levels were lower, approximately 10 g/L in both reactors.

Introduction

In previous experiments, fed batch fermentations on a defined medium without amino acids were performed with the yeast *Candida shehatae*. The fermentations were conducted in two phases. Initially, the cells were grown to a high concentration by sparging air through the reactor (aerobic). After a high cell density was reached the aeration was terminated and the agitation reduced (anaerobic). During the aerobic stage the cells consumed carbohydrate to produce more biomass. This lowered the amount of carbohydrate available to produce ethanol when anaerobic conditions were imposed. Thus, to increase the amount of carbohydrate available to produce ethanol, a concentrated xylose solution was added to the reactor. Compared to batch fermentations, ethanol levels were increased by this fed batch method (Table 1).

Table 1 -- Comparison of ethanol concentrations between batch and fed batch xylose fermentations (Kastner and Roberts, 1989 A).

Conditions	Fed Batch	Batch
Ethanol,g/L	40	30
Xylitol,g/L	15	15
Time,hours	80	50

Although ethanol levels have been improved, they are still not high enough for commercial production. Also, the fermentation times are too long (Table 1) and the cell viabilities continued to decline under anaerobic conditions. The decline in cell viability could have been due to inhibition by fermentation by-products (ethanol and xylitol) or due to the cells inability to synthesize biosynthetic precursors. It is also possible the two effects combined to inhibit the cells.

In yeast there exist a cell organelle called the mitochondria. Within the mitochondria are a group of enzymes which catalyze a set of reactions called the TCA cycle. One function of these enzymes is to produce biosynthetic precursors; two of which are *alpha*-ketoglutarate and oxaloacetic acid. It was theorized that the production of these precursors may have been reduced under anaerobic conditions. The reduction in precursors levels may have caused the decline in cell viability.

To test the latter theory, *alpha* -ketoglutarate and oxaloacetic acid were added to the medium. However, these TCA cycle intermediates had no effect on cell viability. It was theorized that these compounds were not utilized during the anaerobic fermentation due to restricted transport across the cell membrane. To see if transport was the problem, the fermentation experiments were redesigned.

A decision was made to add only *alpha*-ketoglutaric acid to the medium, and aerobically grow the cells in its presence. It was hoped that this would condition the cells to take up the intermediate when anaerobic conditions were imposed. In addition, the concentration of *alpha* -ketoglutaric acid was increased. This would make it easier to identify *alpha* -ketoglutaric acid by High Performance Liquid Chromatography (HPLC) analysis. The uptake of *alpha* -ketoglutaric acid could then be quantified from HPLC analysis.

Alpha -ketoglutaric acid is an intermediate in a cyclic pathway which is fed by products of glucose and xylose (carbohydrate) metabolism. Increased concentrations of *alpha* -ketoglutaric in the medium may change the carbohydrate metabolism of *Candida shehatae*. The change in metabolism can not be predicted since no experiments of this kind have ever been performed. For this reason it was decided to use glucose as the carbohydrate source. Unlike xylose, glucose is a carbohydrate source whose metabolic enzymes are constitutive; this means they are always present and do not have to be induced. Thus, if during the fermentation glucose utilization is inhibited, consumption can continue with no lag period if the inhibition is removed. If the glucose is completely consumed during the fermentation (and *alpha*-ketoglutaric acid remains), enzymes for the transport and catabolism of glucose will still be present. Thus, glucose can be utilized with no period of cellular adjustment after additional glucose is added to the medium.

As noted earlier, the fermentation times are too long for commercial ethanol production and the cell viabilities begin to decline after about 20-40 hours. Thus, it would be of interest to see if higher ethanol concentrations could be achieved within a 20-40 hour time frame. This type of fermentation could be called a rapid batch fermentation. A rapid batch fermentation would require very high cell populations in good shape (high percentage of viable cells) to complete the fermentation before cell viabilities began to drop.

This report discusses the results for a rapid batch fermentation on xylose and fed batch fermentations on *alpha*-ketoglutaric acid and glucose.

Materials and Methods

Defined Medium :

Four fed batch fermentations were performed. In the first experiment the defined medium was the same as previously reported (Kastner and Roberts, 1989 A). In the last three fermentations the medium preparation and composition was the same except citric acid was added (0.5 g/L). Two stock solutions were prepared; a salt and trace element solution, or mineral solution, at 20X and a vitamin solution at 250X. Both solutions were cold filtered. A liter of medium contained 50ml of the mineral solution and 4ml of the vitamin solution. The glucose concentration varied according to the seed culturing method and experimental design (Figures 1 through 4). The nitrogen source solution contained $(NH_4)_2SO_4$, 10g/L and KH_2PO_4, 10g/L. The glucose solution for the seed culture and main fermentations, and the nitrogen source solution were autoclaved. The concentrated glucose solutions used for batch feeding were cold filtered.

Seed Culture :

For a description of the seed culturing technique see Figures 1 through 4.

Main Fermentations :

The fed batch fermentations were performed in a New Brunswick Multigen Benchtop Fermentor (1.5 Liter working volume). The pH was controlled at 4.5 (Kastner and Roberts, 1989 B). See Figures 1 through 4 for the fermentation conditions.

Analytical :

alpha-Ketoglutaric acid was analyzed by HPLC. The methods were the same as previously reported (Kastner and Roberts, 1990). The HPLC column used could separate *alpha*-ketoglutaric acid from glucose and the by-products of the fermentation. A response factor (ratio of the *alpha*-ketoglutaric acid peak to that of the internal standard peak) was calculated for each sample taken. However, since a standard curve was not run, the uptake or depletion of *alpha*-ketoglutaric acid could be followed in a qualitative sense only. A standard curve is needed to calculate the concentration.

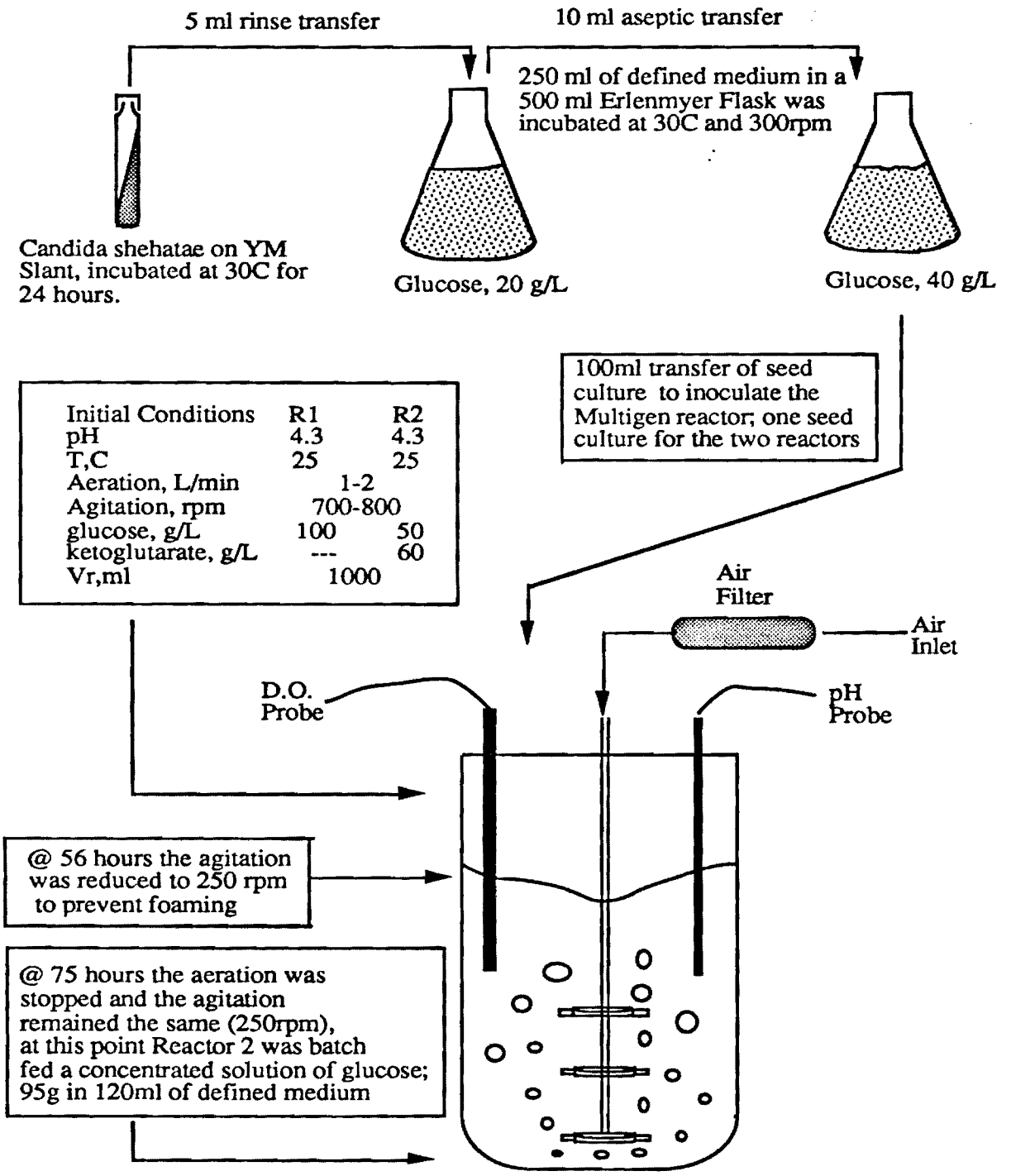


Figure 1 -- Seed culture method for fed batch fermentation of *alpha*-ketoglutaric acid and glucose.

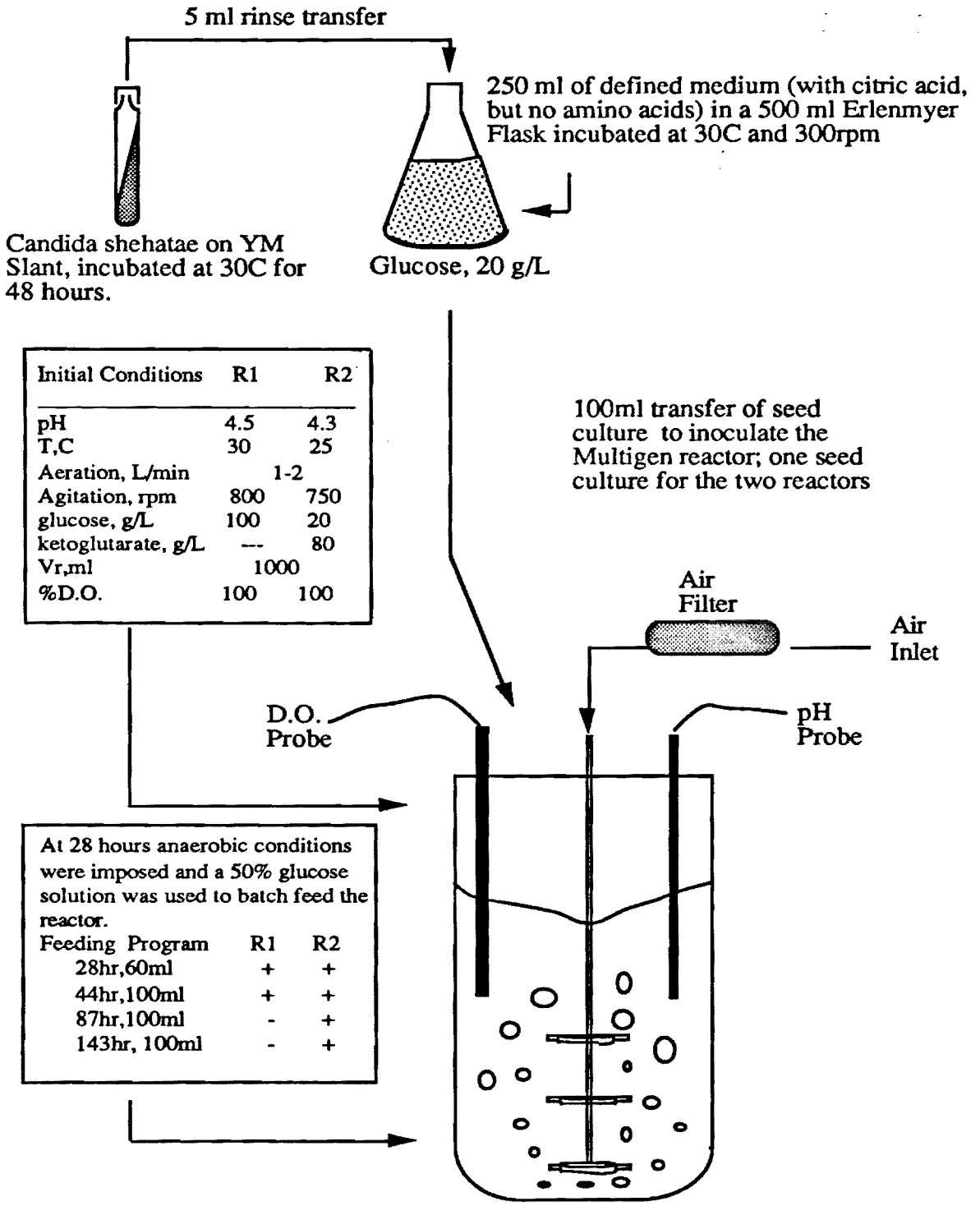
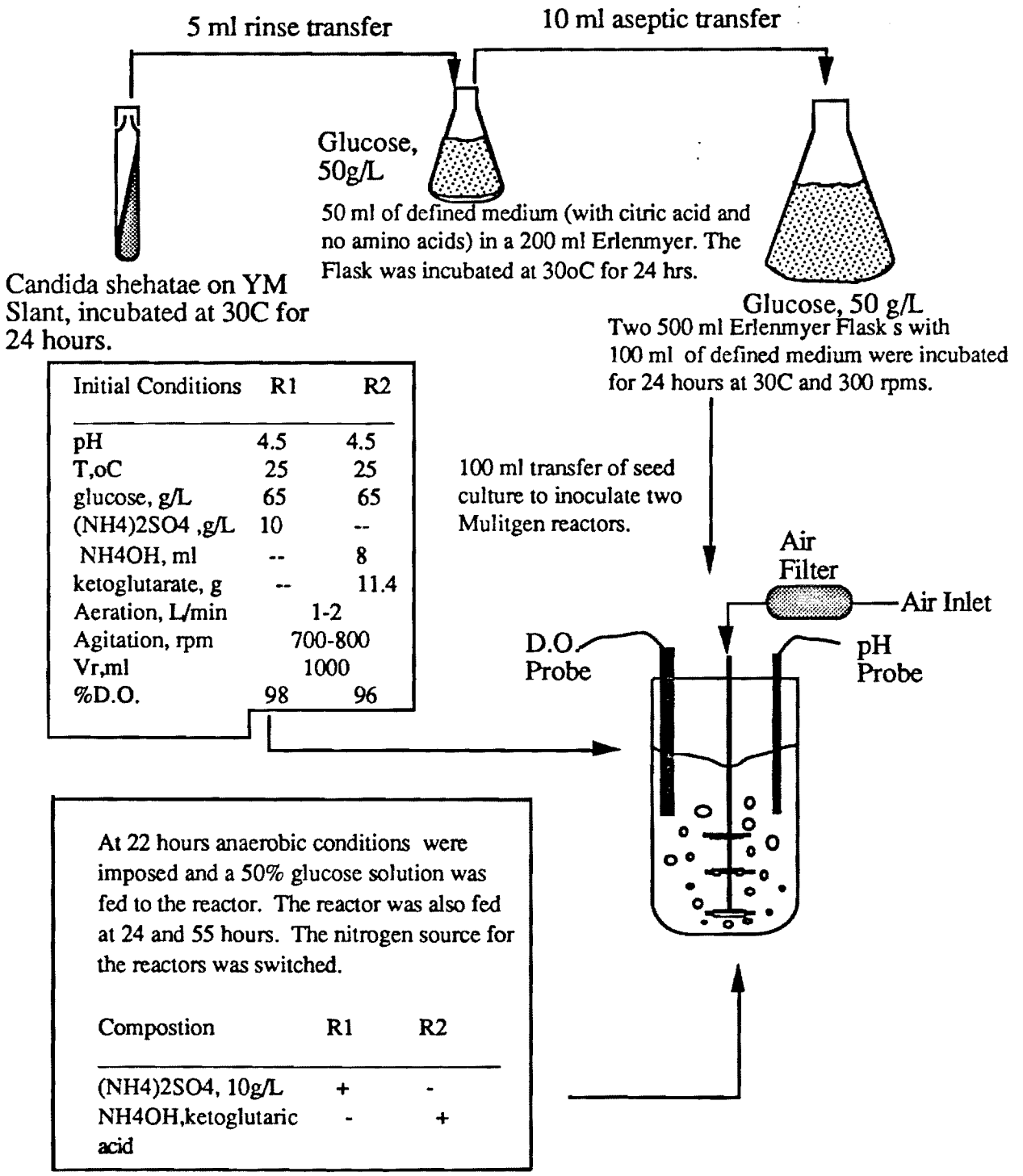


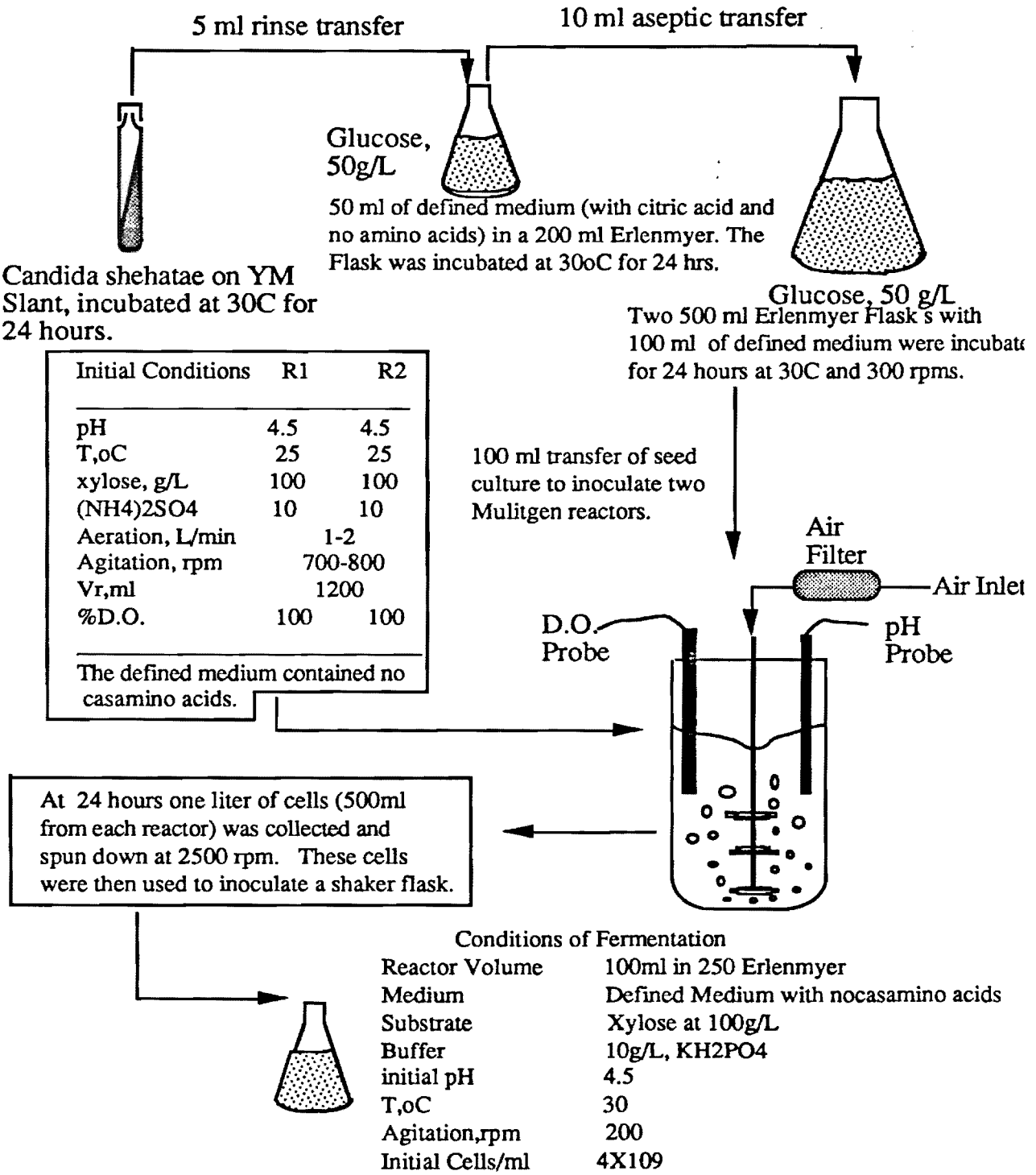
Figure 2 -- Seed culture method for fed batch fermentation of *alpha*-ketoglutaric acid and glucose.



Initial Conditions	R1	R2
pH	4.5	4.5
T,oC	25	25
glucose, g/L	65	65
(NH4)2SO4 ,g/L	10	--
NH4OH, ml	--	8
ketoglutarate, g	--	11.4
Aeration, L/min	1-2	
Agitation, rpm	700-800	
Vr,ml	1000	
%D.O.	98	96

Compostion	R1	R2
(NH4)2SO4, 10g/L	+	-
NH4OH,ketoglutaric acid	-	+

Figure 3 -- Seed culture method for fed batch fermentation of *alpha*-ketoglutaric acid and glucose.



Initial Conditions	R1	R2
pH	4.5	4.5
T,oC	25	25
xylose, g/L	100	100
(NH4)2SO4	10	10
Aeration, L/min	1-2	
Agitation, rpm	700-800	
Vr,ml	1200	
%D.O.	100	100

The defined medium contained no casamino acids.

Figure 4 -- Seed culture method for rapid batch fermentation of D-xylose.

Results and Discussion

Fed Batch Fermentations of alpha-Ketoglutaric acid and Glucose :

Fed batch fermentations were performed on *alpha*-ketoglutaric acid and glucose. In previous fermentations on D-xylose under anaerobic conditions, the cell viability declined shortly after anaerobic conditions were imposed. *Alpha*-ketoglutaric acid, a dicarboxylic acid generated in the TCA cycle, was added to see if cell viability could be maintained under anaerobic conditions. However, to stimulate cell viability the dicarboxylic acid must be utilized by the cell. This requires transport across the cell membrane. To promote transport through the cell membrane, the cells were aerobically grown on *alpha*-ketoglutaric acid and glucose. To verify that the dicarboxylic acid was transported across the cell membrane, High Performance Liquid Chromatography (HPLC) analysis was performed on the fermentation samples (Materials and Methods). A response factor, the ratio of the *alpha*-ketoglutaric acid peak to that of the internal standard peak, was calculated for each sample taken. A decrease in the response factor represents a decline in the concentration of *alpha*-ketoglutaric acid (Materials and Methods).

In any fermentation in which the effect of different variables are tested, a control must be performed. To test the effect of *alpha* -ketoglutaric acid (experimental reactor) a control fermentation was run along with an experimental fermentation. The control reactor contained a medium identical to that in the experimental reactor, except *alpha* -ketoglutaric acid was not present (glucose only). Thus, the only difference between the two reactors should be that one contained alpha-ketoglutaric acid and the other did not. Therefore, all other operating conditions should be the same. For instance, the temperature, pH, reactor volume, agitation, aeration rates, and the glucose concentration should be similar in both reactors.

Unfortunately, in the first experiment (Figure 1), the control reactor became substrate limited before additional glucose was added to the reactor. Direct comparisons could not be made between the control (no *alpha* -ketoglutaric acid) and the experimental reactor (contained *alpha* -ketoglutaric acid). The drop in viability relative to the experimental reactor was probably due to substrate limitation (Figures 5 and 6).

In the experimental reactor (*alpha*-ketoglutaric acid), the glucose concentration also dropped to zero before additional glucose was added (Figure 6, 75 hour mark). However, the viability did not decline rapidly (Figure 6). In fact, from 50 hours to 75 hours the viability was approximately 0.9. These data suggest that *alpha*-ketoglutaric acid helped maintain cell viability. To maintain

viability *alpha*-ketoglutaric acid must have been consumed. However, the response factor for *alpha*-ketoglutaric acid was constant during the first 75 hours (Figure 7). Thus, a very small amount of *alpha*-ketoglutaric acid must have been consumed. So small that the decline in *alpha*-ketoglutaric acid could not be detected.

After a batch of concentrated glucose was added to the experimental reactor at the 75 hour point, and anaerobic conditions were imposed, the response factor dropped. This was due to a dilution effect. However, from the 75 hour point to the 125 hour point, the response factor decreased (Figure 7). This indicates *alpha*-ketoglutaric was consumed under anaerobic conditions. Over the same period the viability index was maintained at 0.9. The viability index declined from 0.9 to 0.85 over the next 25 hours. During this period the response factor increased. These data suggest that if *alpha*-ketoglutaric acid is consumed it can help maintain cell viability. These data also indicate that an increase in the *alpha*-ketoglutaric acid concentration is a sign of decreased cellular activity.

In the second experiment (Figure 2) neither fermentation (control or experimental reactor) was substrate limited before additional glucose was added to the reactors (Figures 8 and 9). Thus any differences in viability, viable cells/ml and ethanol levels could be attributed to the presence of *alpha*-ketoglutaric acid.

After 48 hours (28 hours of which was aerobic) the viable cell density in each reactor was about the same, 2×10^8 cells/ml. Beyond this point no cell growth occurred in Reactor 1, but growth did occur in Reactor 2 (Figure 8 and 9). These data suggest that *alpha*-ketoglutaric acid stimulated growth under anaerobic conditions.

Relative to the control, the presence of *alpha*-ketoglutaric acid (experimental reactor) appeared to maintain the cell viability at a higher level for a longer time. As shown in Figure 10, after four days the viability index was 0.85 in the experimental reactor compared to 0.6 in the control reactor. The difference between the two reactors increased as the fermentation progressed. After eight days the viability index was 0.8 in the experimental reactor compared to 0.3 in the control reactor (Figure 10).

The increased cell viability in the experimental reactor was apparently due to utilization of *alpha*-ketoglutaric acid under both aerobic and anaerobic conditions. During the first 28 hours (aerobic phase), the response factor for *alpha*-ketoglutaric acid declined from 1.4 to 1.2 and the glucose concentration dropped from 15 to 5 g/L. The data also indicate that both *alpha*-ketoglutarate and glucose were consumed under anaerobic conditions (Figure 11).

In addition to the effect of *alpha*-ketoglutaric acid, on cell viability it was important to note the effect on ethanol production. Both the control and experimental reactors produced the same level of ethanol (33 g/L). However, the time needed to reach this concentration was shorter in the control reactor. To reach 33 g/L of ethanol 8 days were required for the control compared to 16 days for the experimental reactor (Figures 8 and 9). These data suggest the high concentration of *alpha*-ketoglutaric acid (Materials and Method; Figure 2) inhibited ethanol production.

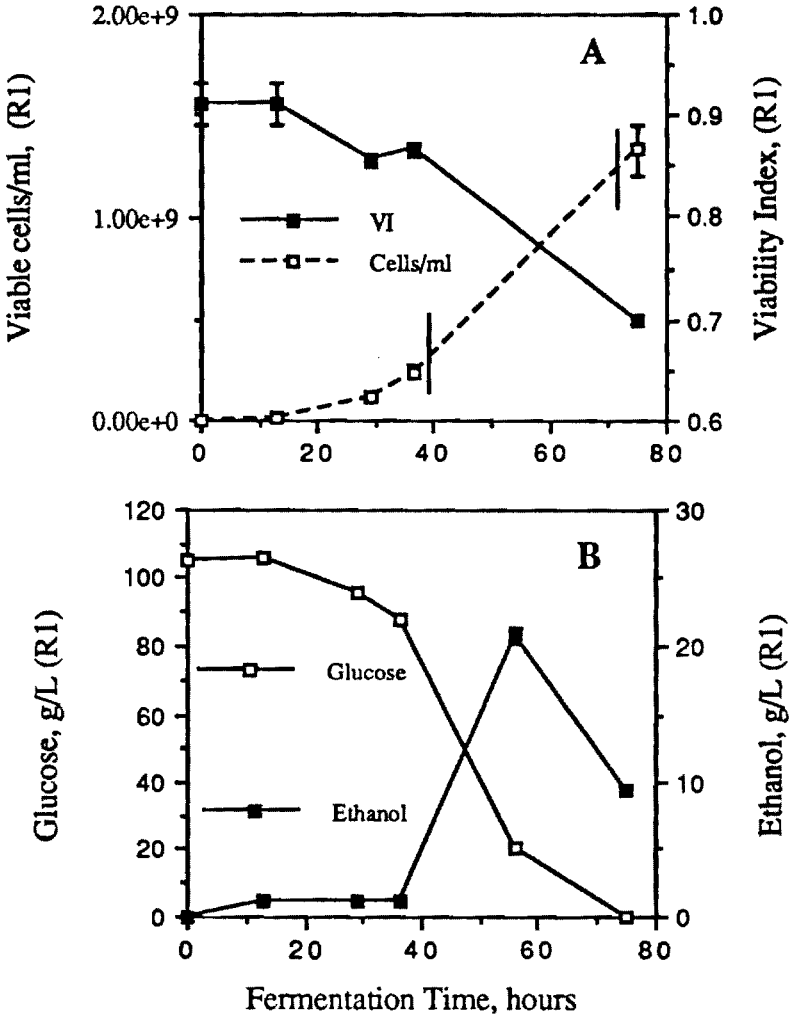


Figure 5 -- Batch fermentation of glucose (control reactor) by *Candida shehatae* . Figure A shows the change in the viable cell population and viability index. Figure B shows the pattern of glucose consumption and ethanol production. The bars in Figure A indicate that cell counts were not taken during this period; thus the maximum cell density obtained was not calculated. Figure B also indicates that ethanol was consumed; this was possible because anaerobic conditions had not been imposed until 75 hours into the fermentation. The fermentation was terminated at this point because the cell viability had dropped to 0.70.

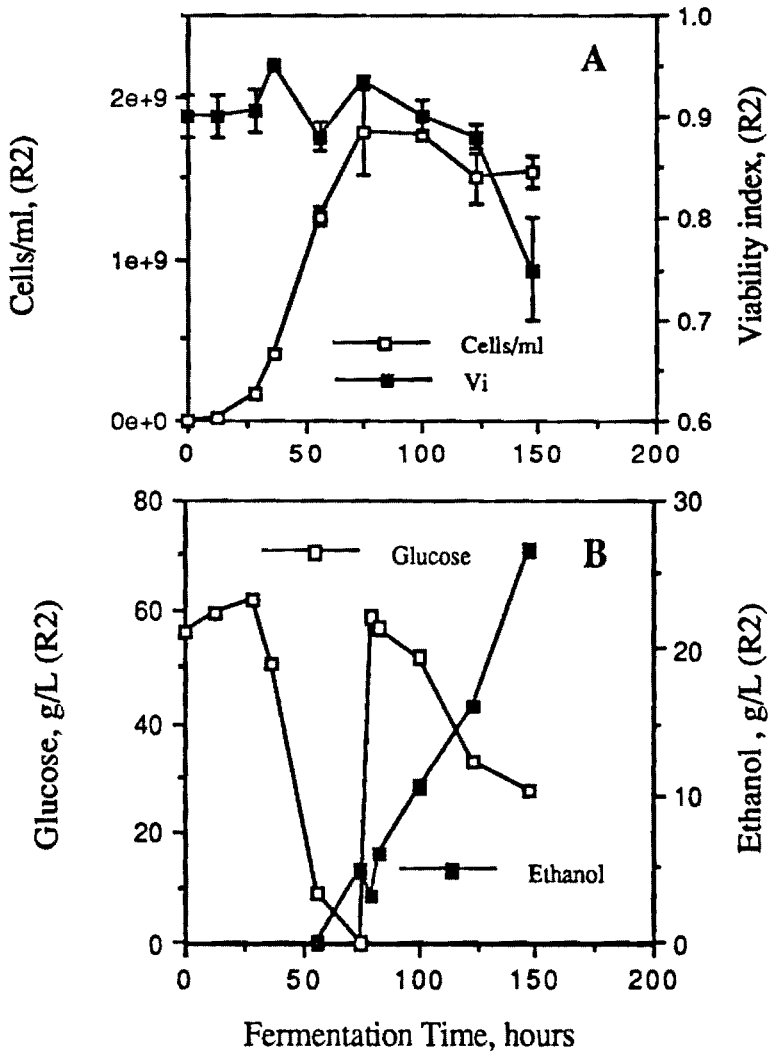


Figure 6 -- Fed batch fermentation of *alpha*-ketoglutaric acid (60 g/L) and glucose by *Candida shehatae* (experimental reactor). Figure A shows the change in the viable cell population and viability index. Figure B shows the pattern of glucose consumption and ethanol production. After 75 hours the air sparging was stopped and the agitation reduced. At this point a concentrated glucose solution was added to the reactor.

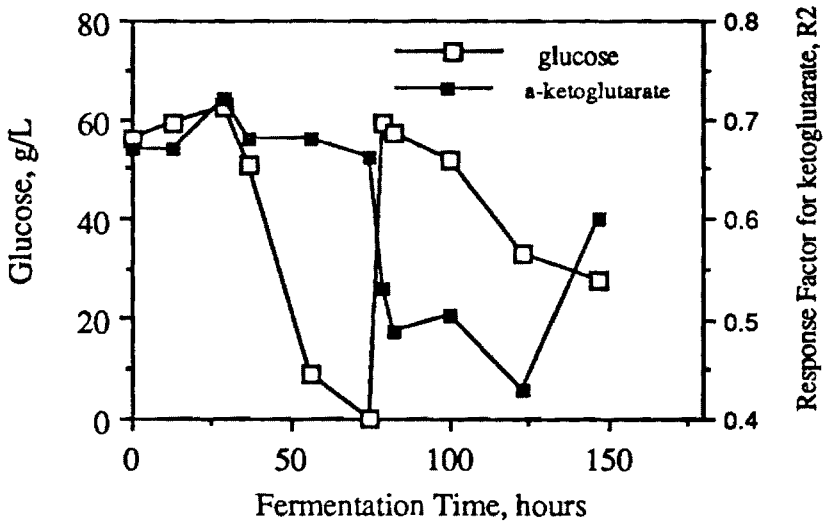


Figure 7 -- Fed batch fermentation of *alpha* -ketoglutaric acid (60 g/L) and glucose by *Candida shehatae* (experimental reactor). After 75 hours the air sparging was stopped, the agitation reduced. At this point concentrated glucose solution glucose was added to the reactor. The figure shows the change in glucose concentration and the response factor for *alpha* -ketoglutaric acid. The response factor is the ratio of the *alpha* -ketoglutaric acid peak to the internal standard peak (Materials and Methods).

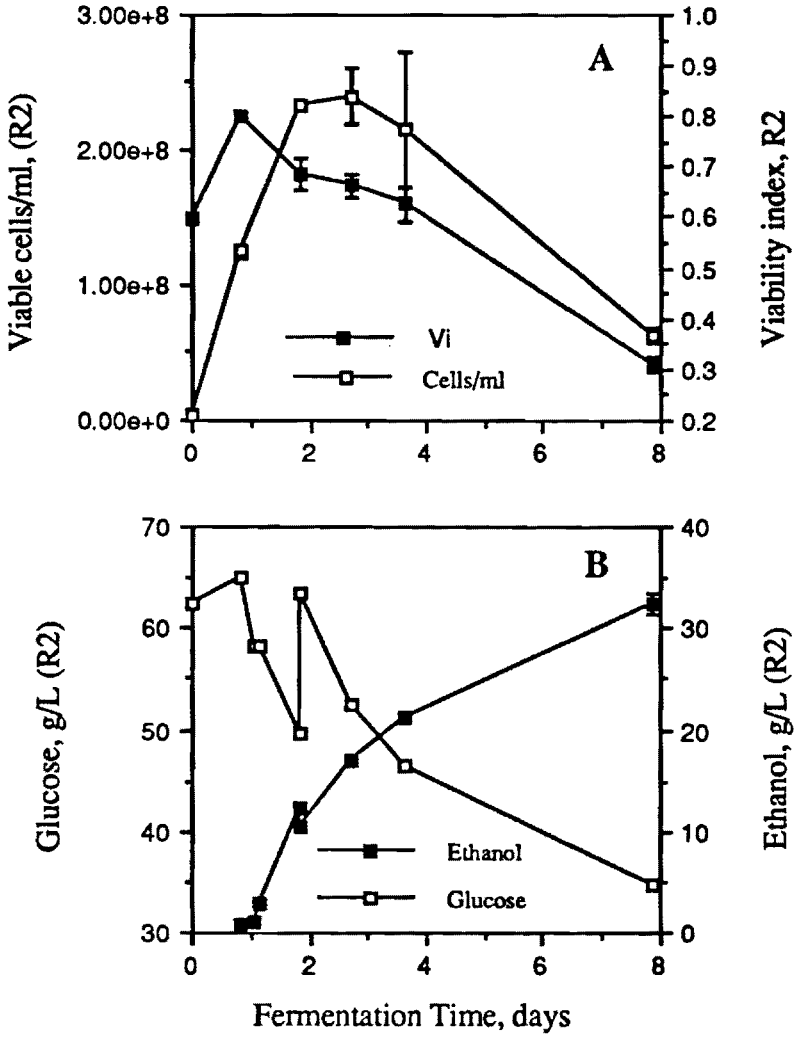


Figure 8 -- Batch fermentation of glucose (control reactor) by *Candida shehatae*. Figure A shows the change in the viable cell population and viability index. Figure B shows the pattern of glucose consumption and ethanol production. Anaerobic conditions were imposed 28 hours into the fermentation and a concentrated solution of glucose added to the reactor (indicated by arrows).

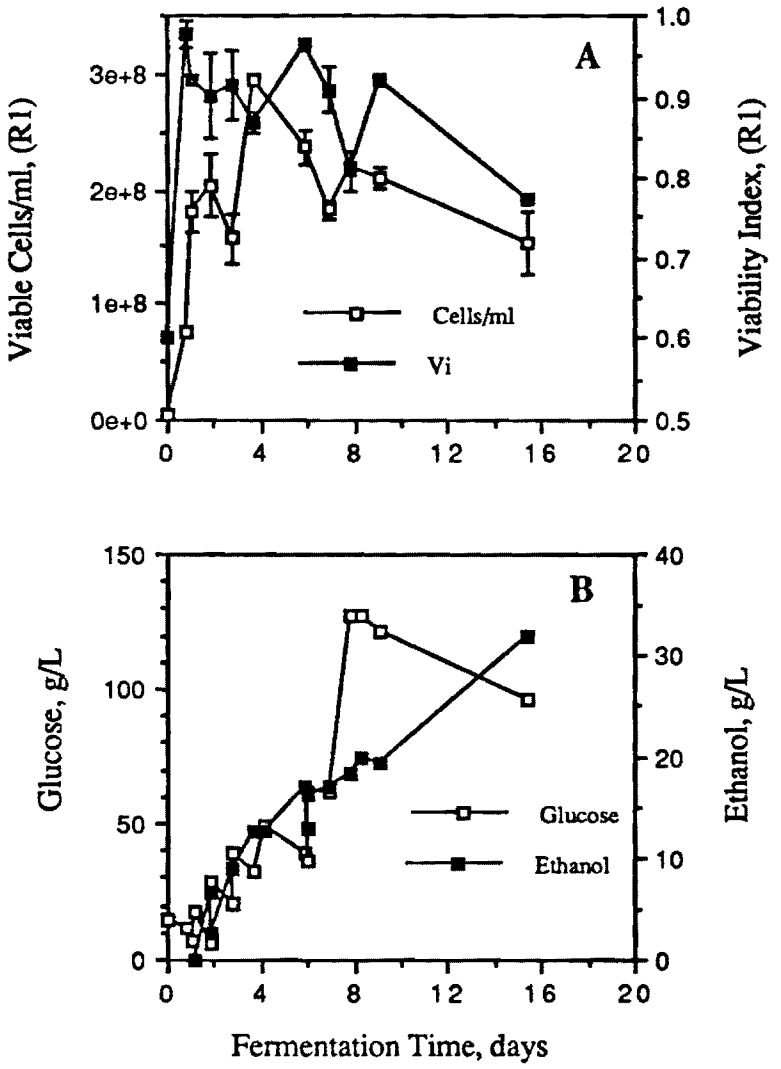


Figure 9 -- Fed batch fermentation of *alpha*-ketoglutaric acid (80 g/L) and glucose by *Candida shehatae*(experimental reactor). Figure A shows the change in the viable cell population and viability index. Figure B shows the pattern of glucose consumption and ethanol production. After 75 hours the air sparging was stopped and the agitation reduced. At this point a concentrated glucose solution was added to the reactor.

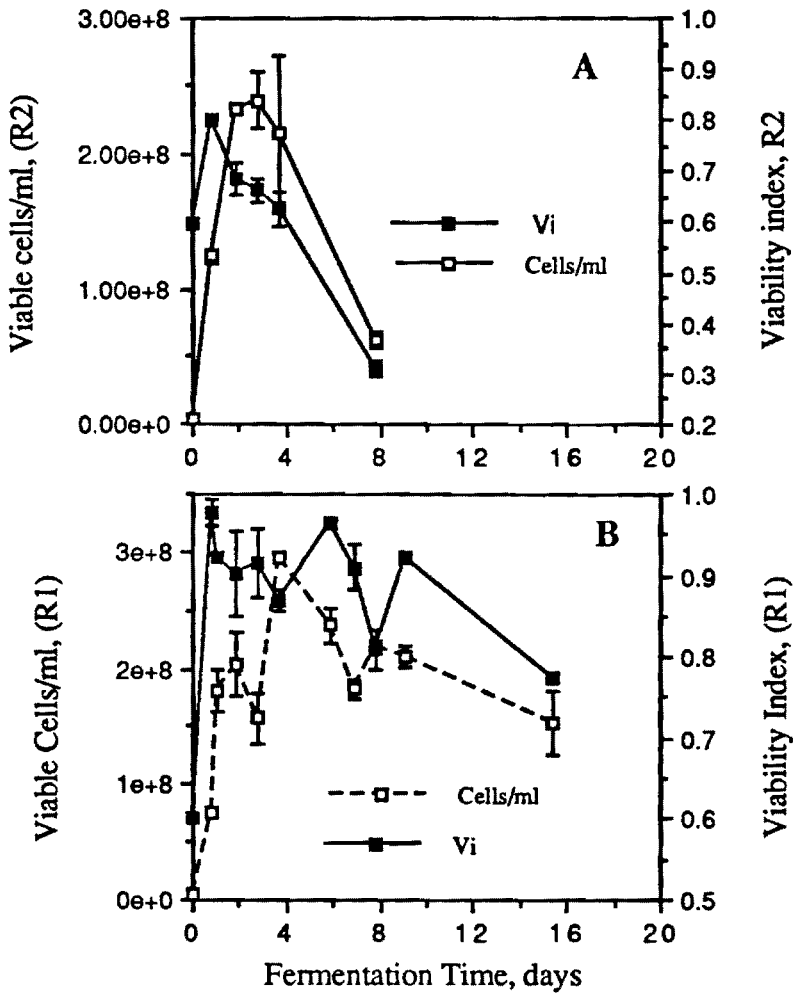


Figure 10 -- Fed batch fermentations of glucose, 65 g/L (control, Figure A) and glucose, 20 g/L and *alpha*-ketoglutaric acid, 80 g/L (experimental, Figure B). The figure compares the difference in the viability indices and viable cell densities between the two reactors. Figure A shows data for the control and Figure B data for the experimental reactor.

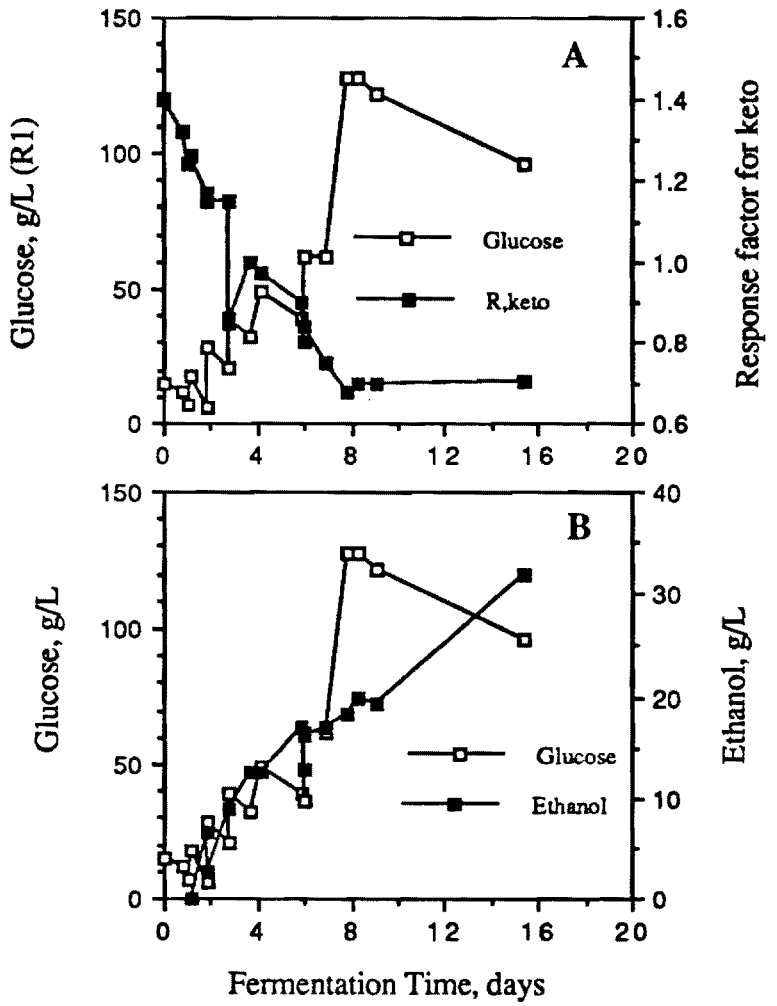


Figure 11 -- Fed batch fermentation of glucose, 20 g/L and *alpha* -ketoglutaric acid, 80 g/L (experimental). Figure A shows the change in the response factor for *alpha* -ketoglutaric acid and Figure B the formation of ethanol compared with the glucose concentration.

Fed Batch Fermentations on Alpha-ketoglutaric acid and Glucose With Different Nitrogen Sources :

In this experiment the concentration of *alpha* -ketoglutaric acid was lowered. In addition a different nitrogen source was used. Initially reactor one (R1) contained $(NH_4)_2SO_4$ and no *alpha*-ketoglutaric acid. Reactor two (R2) contained NH_4OH and *alpha*-ketoglutaric acid (Materials and Methods, Figure 3). This was done for several reasons.

Previous work showed that high concentrations of *alpha*-ketoglutaric combined with a conditioning step could positively effect cell viability (under anaerobic conditions). However, the high concentration of *alpha* -ketoglutaric acid inhibited ethanol production. Also, a commercial fermentation would not be feasible due to the high concentration of *alpha* -ketoglutaric acid and the required conditioning step. Thus, the fermentation must be designed to produce high amounts of ethanol with prolonged cell viability. However, the fermentation must be performed with low concentrations of *alpha* -ketoglutaric acid and no cell conditioning step.

To prolong cell viability, *alpha* -ketoglutaric acid must be utilized. This would require transport across the cell membrane (by a specific protein carrier) without conditioning the cells. To do this *alpha* -ketoglutaric must be recognized by a constitutive carrier protein. A constitutive carrier protein is always synthesized and does not have to be induced. To transport *alpha* -ketoglutaric acid without cell conditioning (inducement of carrier) and at low concentrations a carrier protein must recognize the dicarboxylic acid. An attempt was made to change the form of alpha-ketoglutaric acid such that it would recognized by a carrier protein. For this reason NH_4OH was used as the nitrogen source. It was hoped that the ammonium ion would complex with the dicarboxylic acid (*alpha* -ketoglutaric acid) and be transported as a nitrogen source.

There was little difference between reactors 1 and 2 during the aerobic phase (first 24 hours). The cell densities reached the same level in each reactor; 2.5×10^8 cell/ml and the viability indices were also similar, about 0.9 (Figure 12). However, when the reactors became anaerobic the similarities ceased.

At this point (24 hours) a concentrated solution of glucose was added to both reactors (100 ml). Reactor 1 received a solution containing *alpha*-ketoglutaric acid (11 g/ 8ml of NH_4OH) and NH_4OH (8ml/1000ml) and Reactor 2 a solution containing $(NH_4)_2SO_4$, 10g/L. Growth continued in both reactors, but for a longer time in Reactor 2. After 50 hours (25 hours under anaerobic conditions) the cell concentration reached 5.0×10^8 cells/ml in Reactor 1 (bottom of error bar, Figure 12). After 50 hours the cell density reached 4.0×10^8 cells/ml in Reactor 2. At this point

cell growth ceased in Reactor 1, but continued in Reactor 2. Another sample was taken from Reactor 2 after 75 hours. The cell density had reached 5 x 10^8 cells/ml. Thus, as in the previous fermentation, *alpha*-ketoglutaric acid apparently stimulated cell growth under anaerobic conditions.

For the first 75 hours of the fermentation the cell viabilities in both reactors ranged between 0.95 and 0.8 (50 hours under anaerobic conditions). After this point the cell viability in Reactor 2 dropped rapidly compared to Reactor 1 (Figure 12). The rapid drop in cell viability effected the glucose uptake and ethanol production.

Ethanol levels were the same, about 29 g/L, after 75 hours, and the viability index was approximately 0.9 (both reactors). However, in Reactor 2, after 100 hours the glucose uptake stopped (40 g/L of residual glucose). Also, the ethanol production leveled off (35 g/L) and the viability decreased to 0.6 (Figure 12 and 13). In Reactor 1 metabolic activity continued for approximately 150 hours beyond this point. After 250 hours, 46 g/L of ethanol had been produced and 20 g/L of glucose had not been consumed. The final viability index was 0.7 (Figure 12 and 13). The addition of *alpha*-ketoglutaric (with NH_4OH) to Reactor 1 prolonged cell viability under anaerobic conditions. This enabled the cells to produce more ethanol and increase the final ethanol concentration (relative to Reactor 2).

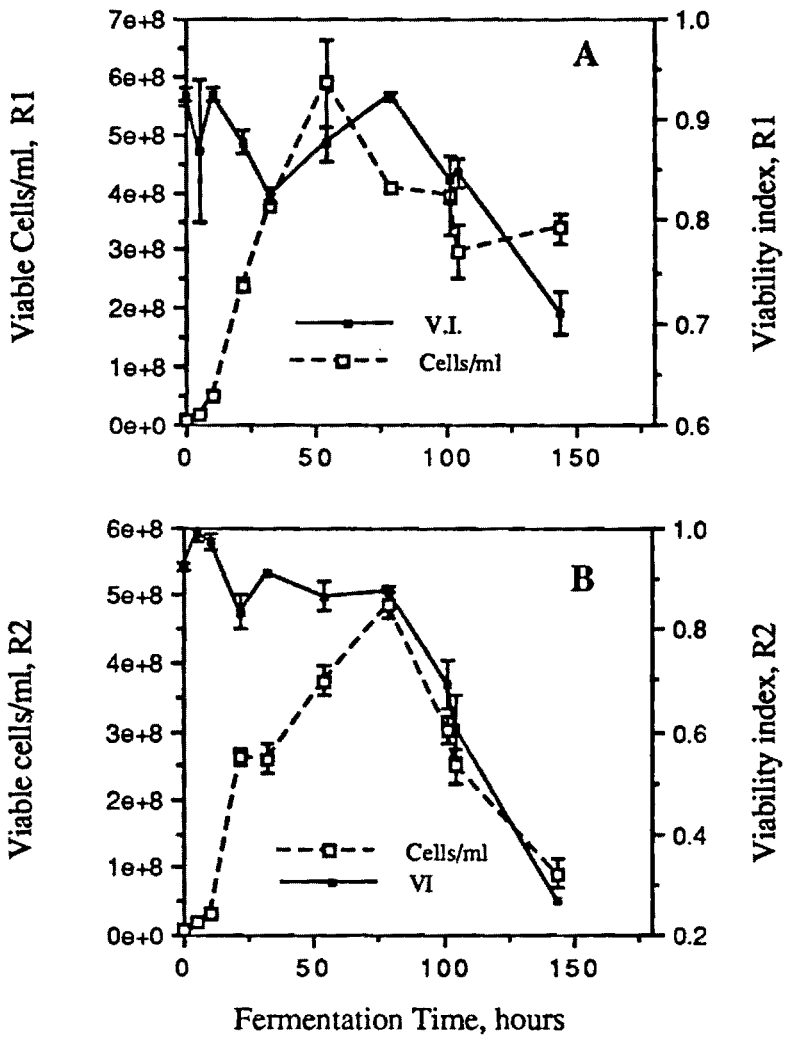


Figure 12 -- Comparison of viability index and viable cell density for fed batch fermentations of glucose and *alpha*-ketoglutaric acid. Figure A shows the results for Reactor 1 and Figure B shows the result for Reactor 2 (see Materials and Methods for condition of each reactor).

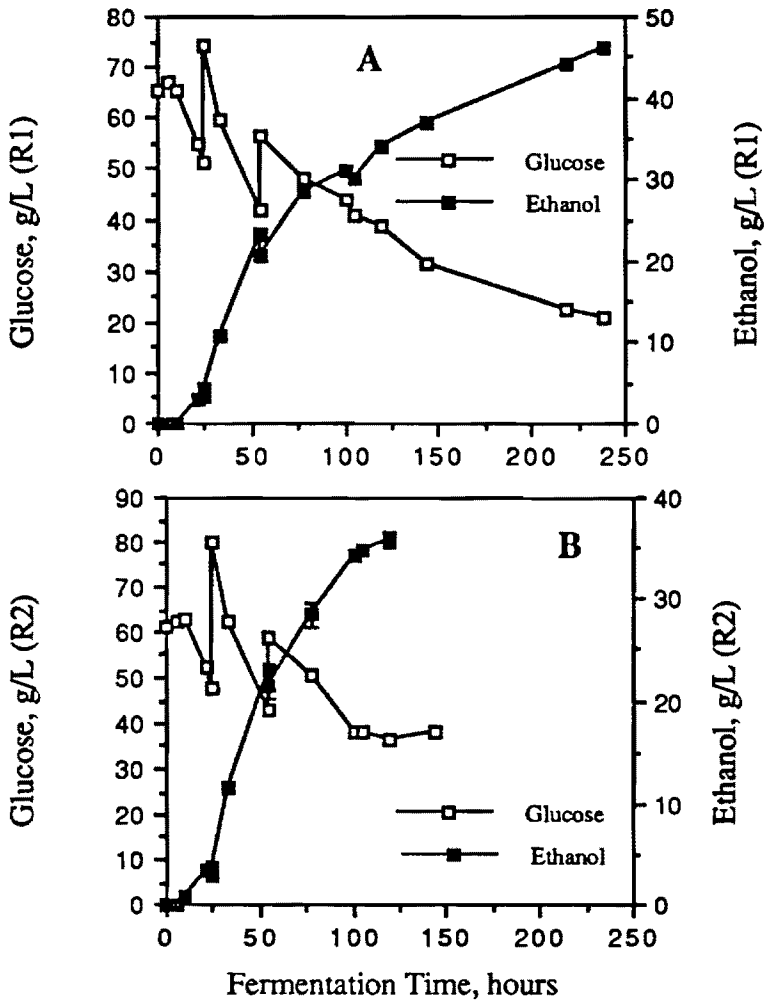


Figure 13 -- Profile of glucose and ethanol levels for fed batch fermentations of glucose and *alpha*-ketoglutaric acid. Figure A shows the results for Reactor 1 and Figure B shows the result for Reactor 2 (see Materials and Methods for condition of each reactor).

If *alpha*-ketoglutaric did stimulate cell viability in Reactor 1, *alpha* -ketoglutaric must have been utilized by the cell. The fermentation samples were analyzed by HPLC analysis to see if utilization could be detected. The peak associated with *alpha* -ketoglutaric acid was separated and identified on a HPLC chromatogram. The chromatogram is a strip chart showing the individual peaks of the components in the sample (Figure 14 and 15). Once the peak was identified the ratio of its area to that of an internal standard peak area was calculated. This ratio is called the response factor for *alpha* -ketoglutaric acid (Materials and Methods). A decline in the response represents a decline in *alpha* -ketoglutaric acid. HPLC analysis indicated that the *alpha*-ketoglutaric acid peak disappeared during the fermentation in Reactor 1. At time zero in Reactor 1, *alpha*-ketoglutaric was not present. When the first batch feeding was performed the *alpha*-ketoglutaric acid peak appeared at the 22 hour mark (Figure 14). Two and one half hours later another batch was added to the reactor. HPLC analysis of a sample at this point indicated the *alpha*-ketoglutaric acid peak had disappeared (Figure 14). This could have been due to a dilution effect and not actual consumption. Thus, the uptake of *alpha*-ketoglutaric acid could not be verified. In Reactor 2 *alpha*-ketoglutaric was not consumed (Figure 15). At time zero in this fermentation *alpha*-ketoglutaric acid was present (Figure 15). When the reactor was batch fed at the 22 hour mark the *alpha*-ketoglutaric acid peak disappeared (Figure 15). However, the peak reappeared in later samples (Figure 15)!

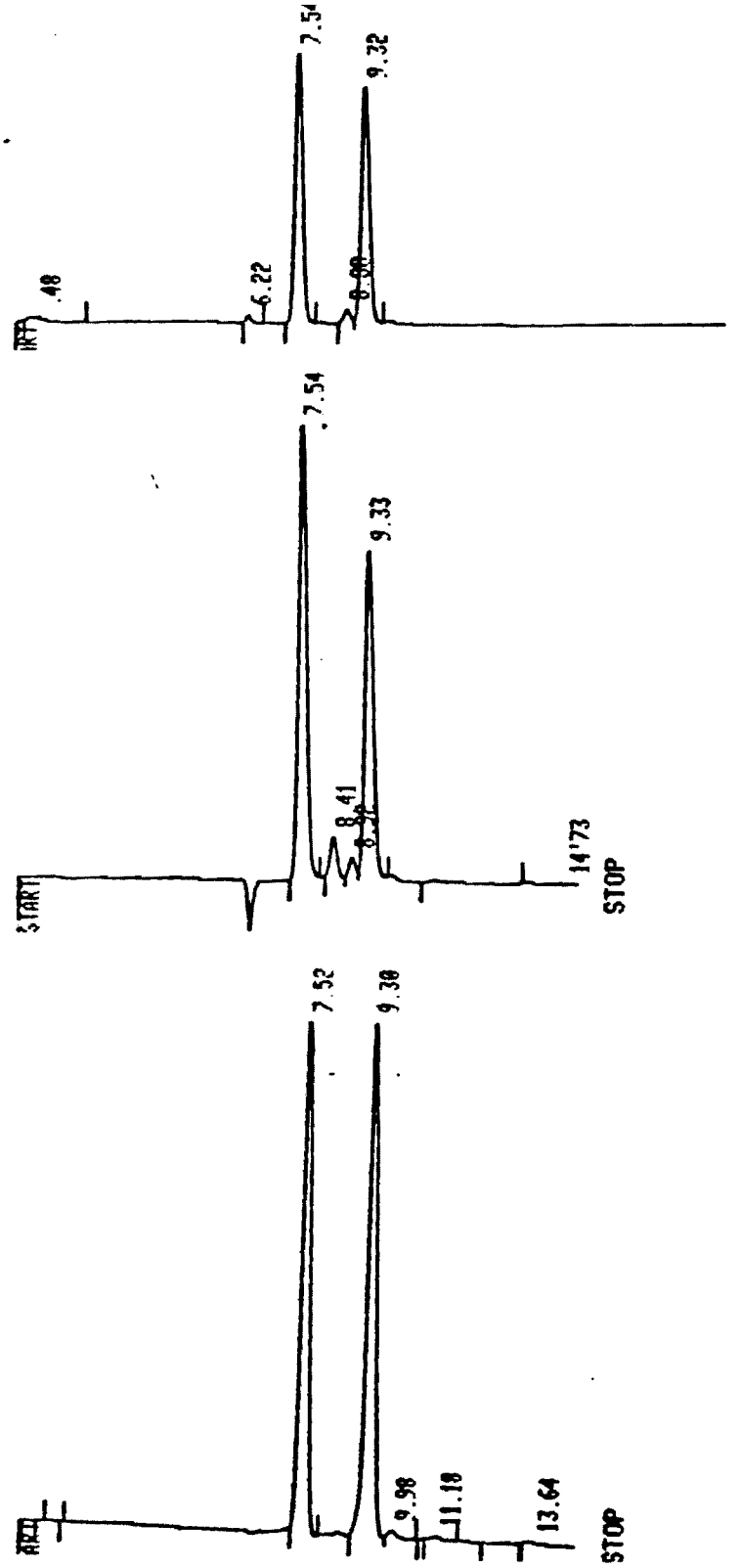


Figure 14 -- HPLC chromatogram of samples taken from Reactor 1 (Material and Methods). The top chromatogram is for a sample taken at 5.25 hours, the middle for a sample taken at 22 hours and the bottom for a sample taken at 24.5 hours. The peak at 7.54-7.58 minutes is cellobiose (internal standard), 8.41 is *alpha*-ketoglutaric acid, 8.88-8.92 is an unknown, and 9.32-9.38 is glucose. It should be noted that each sample was diluted by a factor of 10 before analysis.

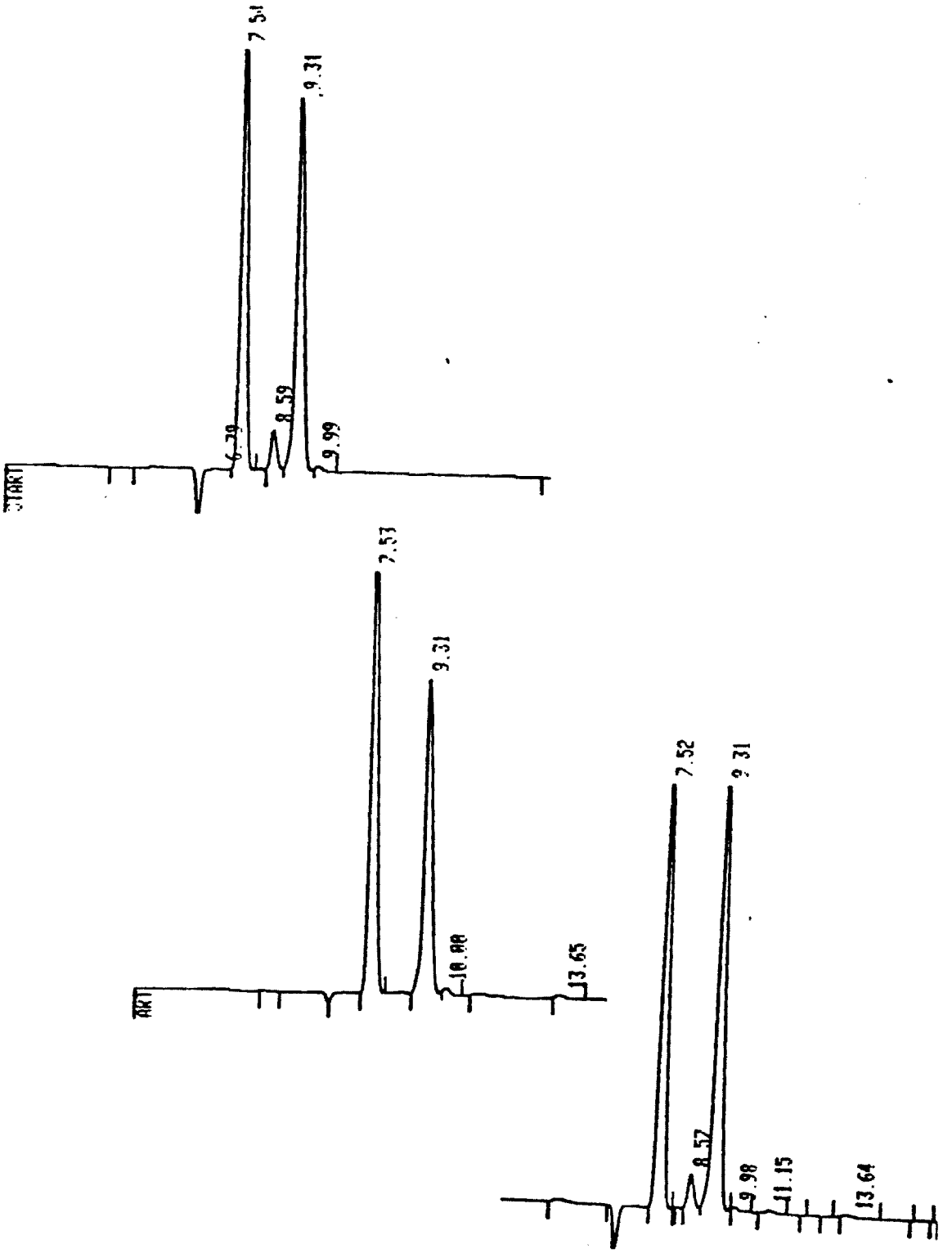


Figure 15 -- HPLC chromatogram of sample taken from Reactor 2 (Material and Methods). The top chromatogram is for a sample taken at 5.25 hours, the middle for a sample taken at 22 hours and the bottom for a sample taken at 24.5 hours. The peak at 7.54-7.58 minutes is cellobiose (internal standard), 8.41 is *alpha*-ketoglutaric acid, 8.88-8.92 is an unknown, and 9.32-9.38 is glucose. It should be noted that each sample was diluted by a factor of 10 before analysis.

Rapid Batch Fermentation of D-Xylose

The time required to obtain maximum ethanol levels is a major drawback with the previous fermentations. Thus, a rapid batch fermentation was designed to ferment xylose within 24 hours (Materials and Methods, Figure 4). The fermentation was started with 4 x 10^9 viable cells/ml (viability index was 0.9) and a xylose concentration of approximately 50 g/L. Within 5 - 7 hours all the xylose had been consumed and 20 g/L of xylitol and 27 g/L of ethanol were produced. Also, the viability index was still 0.9 (Figure 16). At this point the reactor was batch fed with a concentrated xylose solution and the xylose concentration reached 80 g/L (Figure 16).

From the 7 hour mark to the end of the fermentation (24 hours), 40 g/L of xylose was consumed. However, very little ethanol was produced beyond this point. In fact at the end of the fermentation the xylitol concentration (30 g/L) was higher than the ethanol concentration (27 g/L). It is also important to note that the viability index declined from 0.9 to 0.35 (Figure 16). Substrate limitation apparently caused stress which induced the drop in viability and increased xylitol formation.

Another rapid batch fermentation was performed to see if higher ethanol levels could be achieved. In this fermentation a defined medium was used, but unlike the previous fermentation, casamino acids were added to the medium (a control without casamino acids was also run). The initial cell densities were not as high as in the previous experiment (Table 2). Therefore, the time required to reach the maximum ethanol level was longer (Figures 16 and 17).

Table 2 -- Rapid D-xylose fermentation by *Candida shehatae.*

Defined Medium	Time, hours	Viable cells/ml	Viability Index
No Casamino	0	0.91 x 10^9	0.98
Casamino	0	6.6 x 10^9	0.98
No Casamino	26		0.36
Casamino	26		0.33

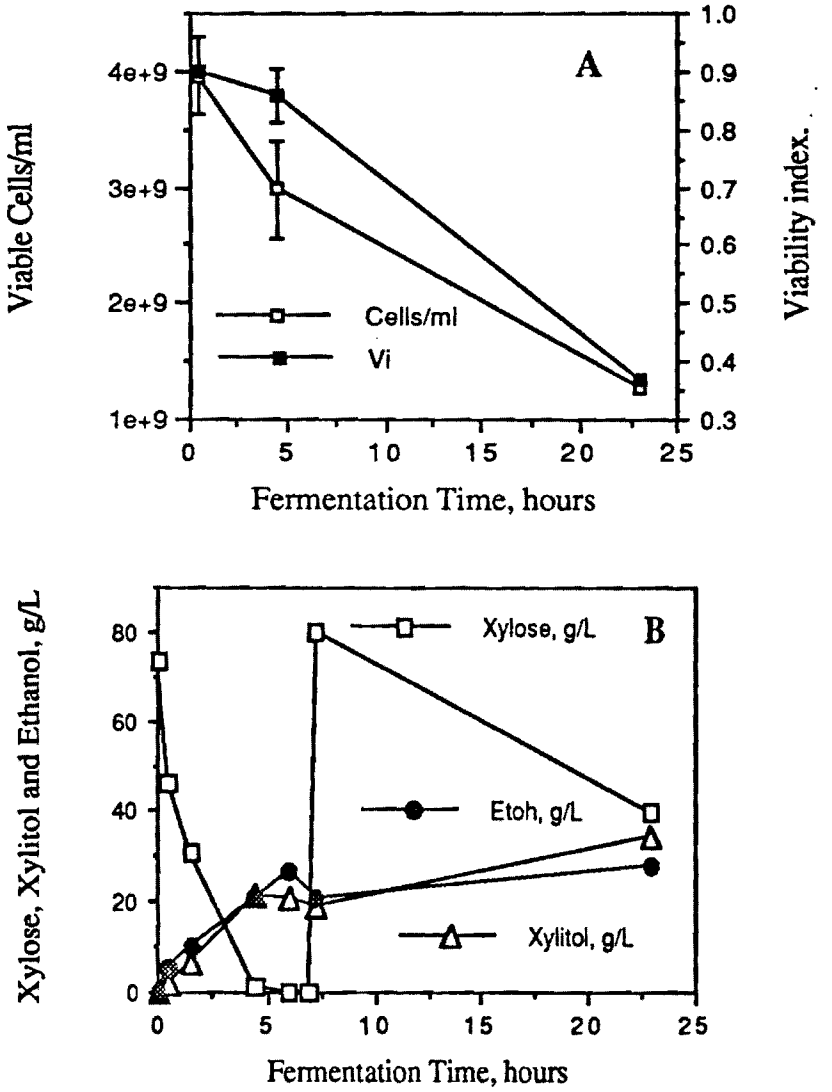


Figure 16 - Rapid batch fermentation of D-xylose using *Candida shehatae* on a defined medium. Figure A shows the viable cell density and viability index for the fermentation. Figure B shows the xylose consumption and ethanol production patterns for the fermentation.

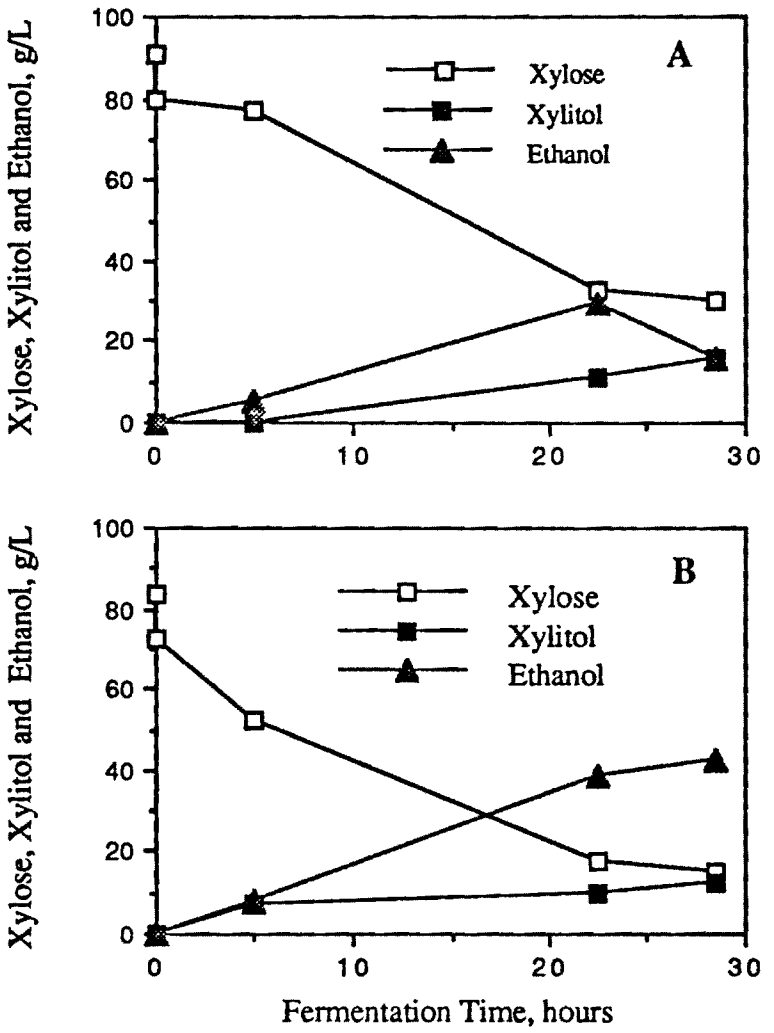


Figure 17 -- Rapid batch fermentation of D-xylose using *Candida shehatae* . Figure A shows the results for fermentation on a medium without casamino acids (control reactor) and Figure B with casamino acids (experimental reactor).

Differences Between Fermentations With and Without Casamino acids in the Medium:

In the control reactor the maximum ethanol level was reached in 23 hours (30 g/L). In the reactor with casamino acids, 42 g/L of ethanol was produced after 26 hours (Figure 17). The final concentration of xylitol in the reactor with casamino acid was slightly lower than in the control (Figure 17, 12.6 g/L compared to 15.8 g/L). Apparently the addition of casamino acids, increased the amount of ethanol produced from xylose. Casamino acids also decreased the amount of xylitol produced from xylose (under anaerobic conditions). Unfortunately, viability indices were calculated only at the beginning and at the end of the fermentation. Thus, the effect of the casamino acids on cell viability could not be ascertained.

Conclusions

The objective of this research was to develope a fermentation method to maintain cell viabilities under anaerobic conditions. This was accomplished by aerobically conditioning the cells on a high concentration of *alpha*-ketoglutaric acid. This step improved cell viabilities under anaerobic conditions. The conditioning step enabled *alpha* -ketoglutaric acid to be transported across the cell membrane. HPLC analysis confirmed that the dicarboxylic acid was utilized by the yeast under both aerobic and anaerobic conditions. However, the high concentration of *alpha* -ketoglutaric acid inhibited ethanol production.

The inhibition of ethanol production and the cell conditioning step would restrict a commercial fermentation based on the use of *alpha* -ketoglutaric acid. Thus a fermentation method must be found which does not require a conditioning step and inhibit ethanol production. A set of experiments was performed to see if these problems could be surmounted.

The concentration of *alpha* -ketoglutaric was lowered to prevent ethanol inhibition. Also, an attempt was made to change the form of *alpha*-ketoglutaric acid. For this reason NH_4OH was added to *alpha*-ketoglutaric acid. It was hoped that the NH_4OH would complex with *alpha*-ketoglutaric acid and be recognized by a transport protein located in the cell membrane. Thus, the cells would not have to be conditioned for transport to occur. Cells were aerobically grown on a defined medium with $(NH_4)_2SO_4$ and glucose and then batch fed NH_4OH with *alpha*-ketoglutaric acid. These cells performed better than cells conditioned on *alpha*-ketoglutaric acid. HPLC analysis indicated that the cells conditioned on *alpha* -ketoglutaric acid (in the presence of $(NH_4)_2SO_4$) did not utilize *alpha*-ketoglutaric acid. HPLC analysis could not confirm if *alpha*-

ketoglutaric acid was consumed by the cells with NH_4OH as the nitrogen source. Better results (higher ethanol levels and prolonged cell viability) were obtained with *alpha*-ketoglutaric acid and NH_4OH. However, the results can not be attributed to *alpha*-ketoglutaric acid. A control fermentation (NH_4OH only) must be performed.

Another objective of this research was to decrease the fermentation times. This was accomplished by using a rapid fermentation method (batch). Yeast cells were aerobically grown to a high cell density. These cells were then used to ferment D-xylose within 24 hours (under anaerobic conditions). Ethanol levels of 42 g/L were achieved. A drawback to these fermentations was a decline in cell viability; the cells can not be reused. Further research needs to be done to design a rapid fermentation in which cell viabilities remain high.

References

1. Kastner, J.K. and Roberts, R.S. (1989 A), Quarterly Progress Report October 1, 1989 - December 31, 1989. Contract #TV-74261A.

2. Kastner, J.K. and Roberts, R.S. (1989 B), Quarterly Progress Report June 30, 1989 - September 30, 1989. Contract #TV-74261A.

3. Kastner, J.K. and Roberts, R.S. (1990), Simultaneous Fermentation of D-Xylose and Glucose by *Candida shehatae*. Biotechnology Letters, 12, 1, 57-60.

Quarterly Progress Report

April 1, 1991 -- May 1, 1991

Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

Jim Kastner and Professor Jack Jones, School of Applied Biology
and Professor R.S. Roberts, School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

Experiments Performed

Microorganism: *Candida shehatae* was used in all fermentation experiments. All experiments were run in duplicate (except were noted).

Effect of Xylose Concentration on Xylose Fermentation:

One drawback to xylose fermentors has been the low levels of ethanol produced. One possible way to increase ethanol levels is to increase the initial substrate concentration. However, little research has been performed to test the effect of increasing the initial sugar concentration. Three different initial xylose concentrations were tested. *Candida shehatae* was used to batch ferment xylose at 75-100 g/L, 125-150 g/L and 175-200 g/L. The medium used in these fermentations was chemically defined and contained no amino acids. These experiments were performed in erlenmeyer flasks.

Mixed Sugar Fermentations on Defined Medium:

Previous research has shown that *Candida shehatae* will ferment xylose and glucose simultaneously. The fermentations were performed under anaerobic conditions and with cells pre-grown on xylose. Growth did not occur under these conditions (or growth was extremely limited). These experiments were performed to see if growth would result in the repression of xylose utilization in a mixed sugar fermentation. Also, cell viability data was collected and compared to a control fermentation (pure xylose). This was done to see if a mixed sugar fermentation had an advantage over a pure xylose fermentation. These experiments were performed in erlenmeyer flasks on a defined medium without amino acids.

Comparison between Xylose and Glucose Fermentations:

Cell viability in the mixed sugar fermentations declined at a slower rate than in the pure xylose fermentations (see Figure 4A). As will be discussed later, viability did not decline in the mixed sugar fermentation until glucose had been depleted. Therefore it was decided to compare xylose and glucose fermentations. A defined medium without casamino acids was used. Experiments were conducted in both shaker flasks (no pH control) and New Brunswick benchtop fermentors with pH control (at 4.5). The sugar uptake, growth rate, ethanol produced and cell viability were measured. This was done to see if there were any distinct differences between glucose and xylose fermentations.

Effect of Casamino Acids on Xylose Fermentations:

Amino acids are required to maintain cell viability. Cell viability will decline if cells can not synthesize amino acids. Under oxygen limited conditions amino acid synthesis may not occur or may be severely limited. Amino acids were added to the defined medium to test their effect on cell viability (and other parameters). In preliminary experiments, asparagine and glutamine were added. A higher percentage of viable cells resulted when the two amino acids were added. The final cell density was also higher. However, there was a distinct difference in the final pH. In the reactor with the amino acids the pH started at 6.5 and ended at 6.4. The reactor without the amino acids started at 6.5 and ended at 3.9. The lower pH could have caused the decline in the viability. Thus the positive effect of the amino acids could have been due to their buffering capacity and not their uptake and utilization. To separate the effects a fermentation without amino acids, but with increased buffering capacity, was performed.

Summary of Results

Effect of Xylose Concentration on *Candida shehatae* (Defined Medium):

The higher xylose concentration, 175-200 g/L, reduced the growth rate of *Candida shehatae* (see Figure 1A). The uptake of xylose was also slower at the higher initial xylose concentration (Figure 1). Cell viability declined in all three cases (measured by methylene blue stain, see Figure 1B). However, the decline was more rapid for the lower xylose concentrations (see Figure 1B). It was theorized that the decline in viability was due to depletion of oxygen from the medium. The growth rate of cells at 175-200 g/L was lower than the other concentrations. Thus the rate of oxygen depletion was lower. This resulted in the slower rate of decline in cell viability.

Other parameters may have influenced cell viability. Substrate limitation could have caused the more rapid decline in cell viability at the lower initial xylose level, 75 g/L (see Figure 1). This was not the case in the other fermentations. There was a minimum of 25 g/L of residual xylose at the end of the fermentations.

Ethanol may have also had an effect on cell viability. The average ethanol level was 25 g/L in each reactor after about 100 hours (see Figure 2). However, the rate of ethanol formation was slower at the higher initial xylose level. This may have caused or contributed to the slower decline in ceil viability for this fermentation.

Mixed Sugar Fermentations on Defined Medium:

The growth rates were similar for both the mixed sugars and pure xylose fermentations (see Figure 4B). The reactors with higher initial sugar concentrations did reach higher cell concentrations. Cell viability declined with time in all reactors (see Figure 4A). However, the decline in cell viability was not as rapid for the mixed sugar fermentations. Xylose and glucose were consumed simultaneously in the mixed sugar fermentations (Figures 4 and 4C). However, glucose was consumed at a faster rate. After about 50 hours glucose was depleted (in both reactors). Cell viability declined after glucose had been consumed (this was noted by Mary Jim in her last letter). This has prompted us to compare xylose and glucose fermentations. Ethanol levels were highest in the mixed sugars with an initial total carbohydrate level of about 75 g/L (see Figure 6).

Glucose and Xylose Fermentations:

Shaker Flasks:

Glucose and xylose fermentations were performed in shaker flasks in a defined medium (without amino acids). There was little difference between the fermentations. The rate of cells produced and the viability index profile were similar (see Figures 7 and 9). Ethanol, sugar and by-product analysis has not been completed.

Multigen Fermentors (not run in duplicate):

Glucose and xylose fermentations were performed in New Brunswick Multigen Fermentors. The **pH was controlled at 4.5** using concentrated NaOH and the dissolved oxygen was monitored. The reactors were not aerated. Since the reactors were not aerated, the percent dissolved oxygen dropped rapidly (see Figure 10). For this reason the fermentations were considered to be anaerobic. After the first 24 hours growth was minimal (see Figure 11). In that same period the viability dropped from 0.98 to 0.92 on glucose and to 0.85 on xylose. However, as shown in Figure 12, the viability slowly increased. In the glucose fermentation, after 250 hours the viability was about 0.95. HPLC and GC analysis has not been completed. Contamination was observed late in the xylose fermentation.

Effect of Casamino Acids on Xylose Fermentation:

These fermentations were performed in shaker flasks. Vitamin free casamino acids, a mixture of amino acids, were used. This was done for two reasons. First, casamino acids have been used in the defined mediums outlined in the literature. Secondly, it is not known which, if any, amino acids are required by *Candida shehatae*. Specific amino acids will be tested, if casamino acids stimulate ethanol fermentation.

The concentrations of KH_2PO_4 and K_2HPO_4 were increased from 5 g/L to 10 g/L each. This was done to increase the buffering capacity of the reactors without casamino acids. Two sets of experiments were performed. The first experiment had two reactors (in duplicate) one with casamino acids and the other with the increased buffer concentration. In the second experiment an additional reactor was run. This reactor was a control and had a lower buffer capacity. It contained 5 g/L of KH_2PO_4 and K_2HPO_4 and no casamino acids.

In the first experiment all measured parameters were similar for each fermentation. The growth rate was about the same (see Figure 13). The cell viability was about 0.85 at 100 hours for both reactors (see Figure 14). The pH started at 6.5 and remained above 5 after 100 hours for both reactors (see Figure 15). Xylose uptake and xylitol production were similar for both reactors (Figures 16 and 17). In the second experiment all measured parameters between the casamino acid and higher buffer capacity reactor were similar. There were differences between the control (lower buffer capacity) and the other two reactors. The viability index declined faster in the reactor with the lower buffer capacity (Figure 19). Also, the final pH was lower than in the other two reactors (3.0 compared to 4.5, see Figure 20). Carbohydrate, ethanol and xylitol analysis has not been completed.

Future Work

Cell viability was reported to decline in previous fermentations. The decline in cell viability was attributed to anaerobic conditions (lack of oxygen). Anaerobic conditions cause a change in yeast metabolism. Ethanol and CO_2 are produced when oxygen becomes rate limiting. CO_2 dissolves in H_2O and produces H_2CO_3. H_2CO_3 can dissociate to form HCO_3^- and H^+. This can lower the pH.

Shaker flasks have been the reactor of choice for most fermentations performed in the literature (and in our lab). In shaker flask experiments pH can not be held constant. In an attempt to control pH, buffers are added to the system. However, even the additional buffers can not maintain a constant pH (this is quite evident from the data presented in this report and the March, 1991 Progress Report).

The data indicate that pH or a change in pH effects cell viability. Factors beside pH may also contribute to the decline in cell viability. The effect of pH on viability must be separated from other variables. To do this pH must be controlled. Therefore, most experiments will now be performed with pH control. The following set of experiments are going to be performed within the next two months.

1) Comparison of xylose and glucose fermentations under anaerobic conditions and a pH of 4.5.

2) Xylose, glucose and mixed sugar fermentations at different pH's. Three different pH's will be tested, 6.0, 4.5 and 3.5. These fermentations will be performed in a NewBrunswick benchtop fermentor under anaerobic conditions.

4) Effect of casamino acids on xylose, glucose and mixed sugar fermentations under anaerobic conditions. The pH will be controlled at 4.5.

Figures for Effect of Xylose Concentration on Xylose Fermentation

Figure 1A -- Total Cells/ml Produced in Pure Xylose Fermentations by Candida shehatae (Performed in Shaker Flasks).

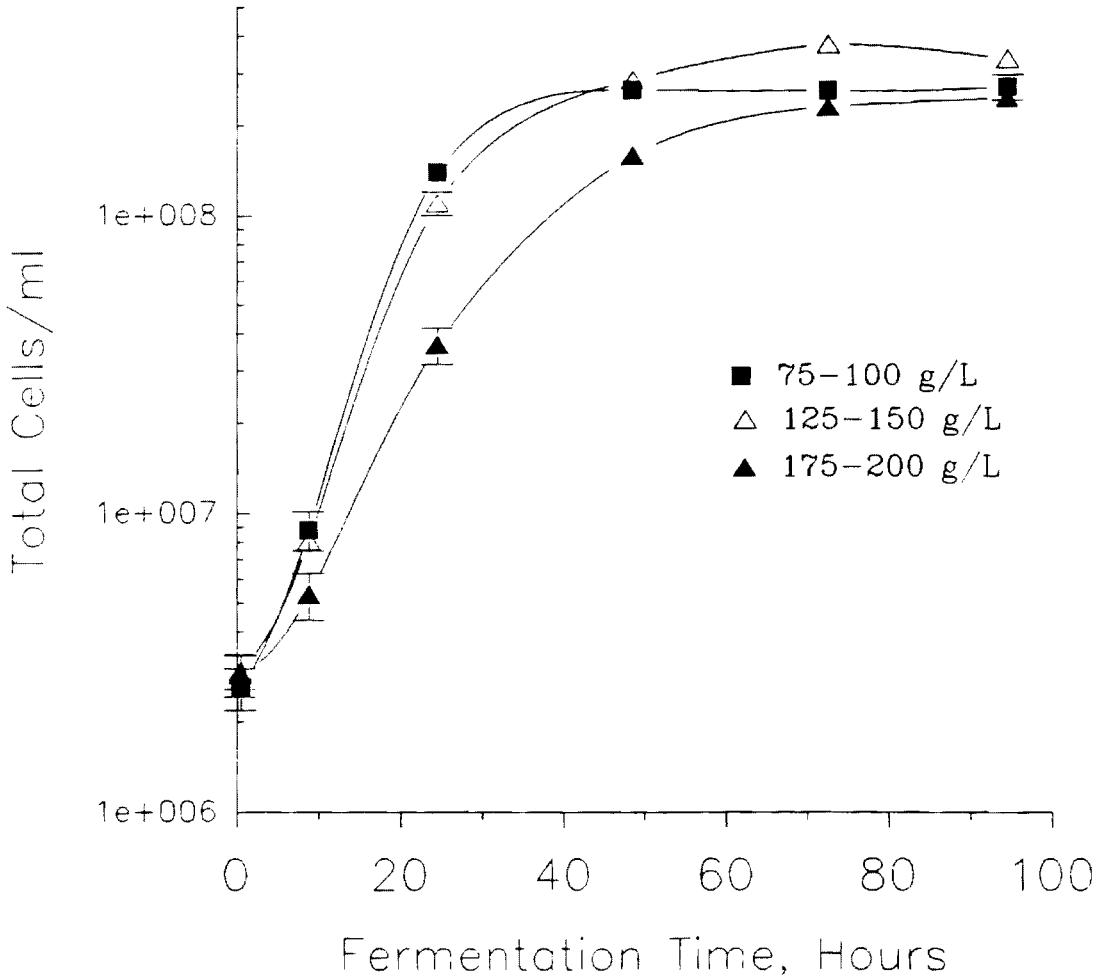


Figure 1 -- Xylose Uptake in Pure Xylose Fermentations by Candida shehatae (Performed in Shaker Flasks).

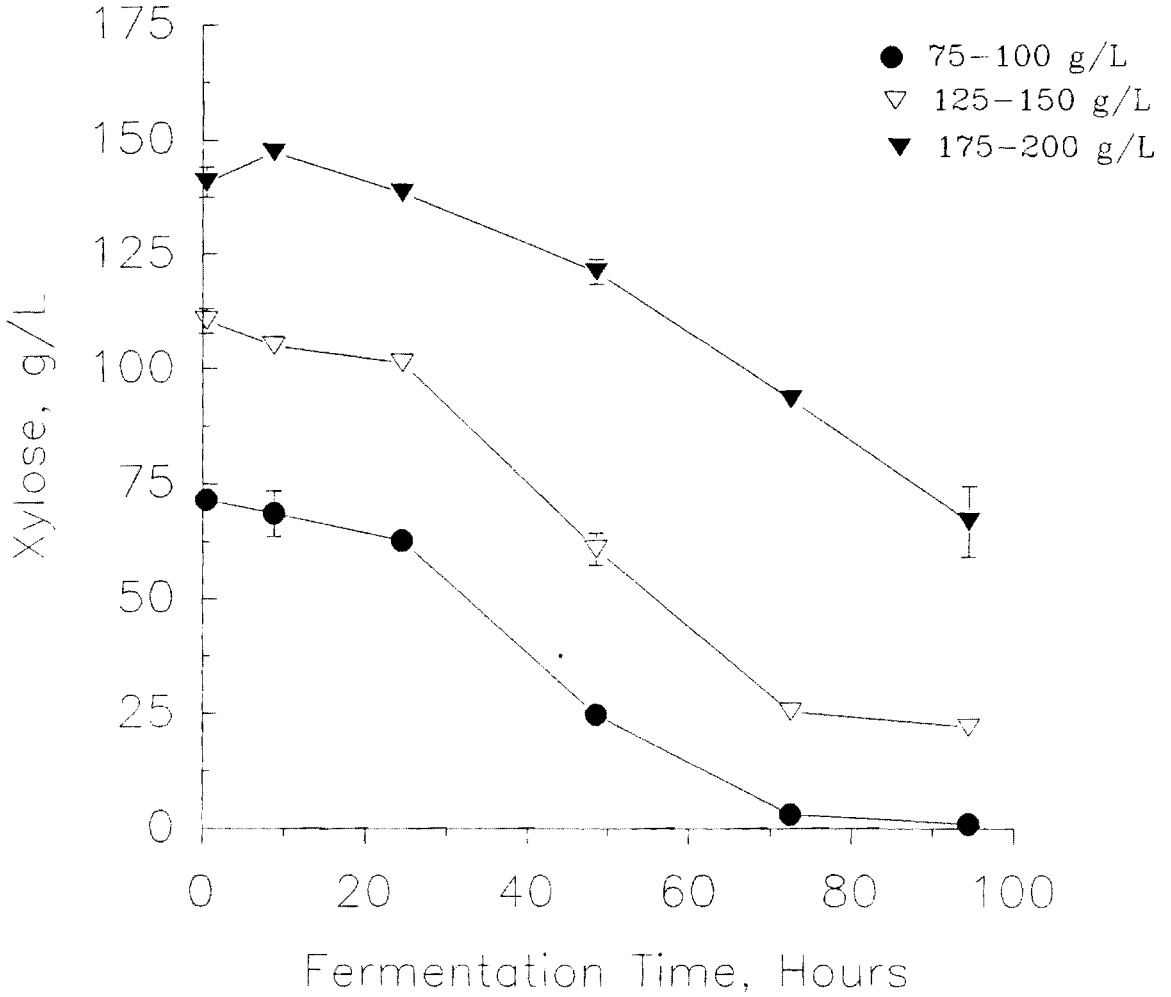


Figure 2 -- Ethanol Production in Pure Xylose Fermentations by Candida shehatae (Performed in Shaker Flasks).

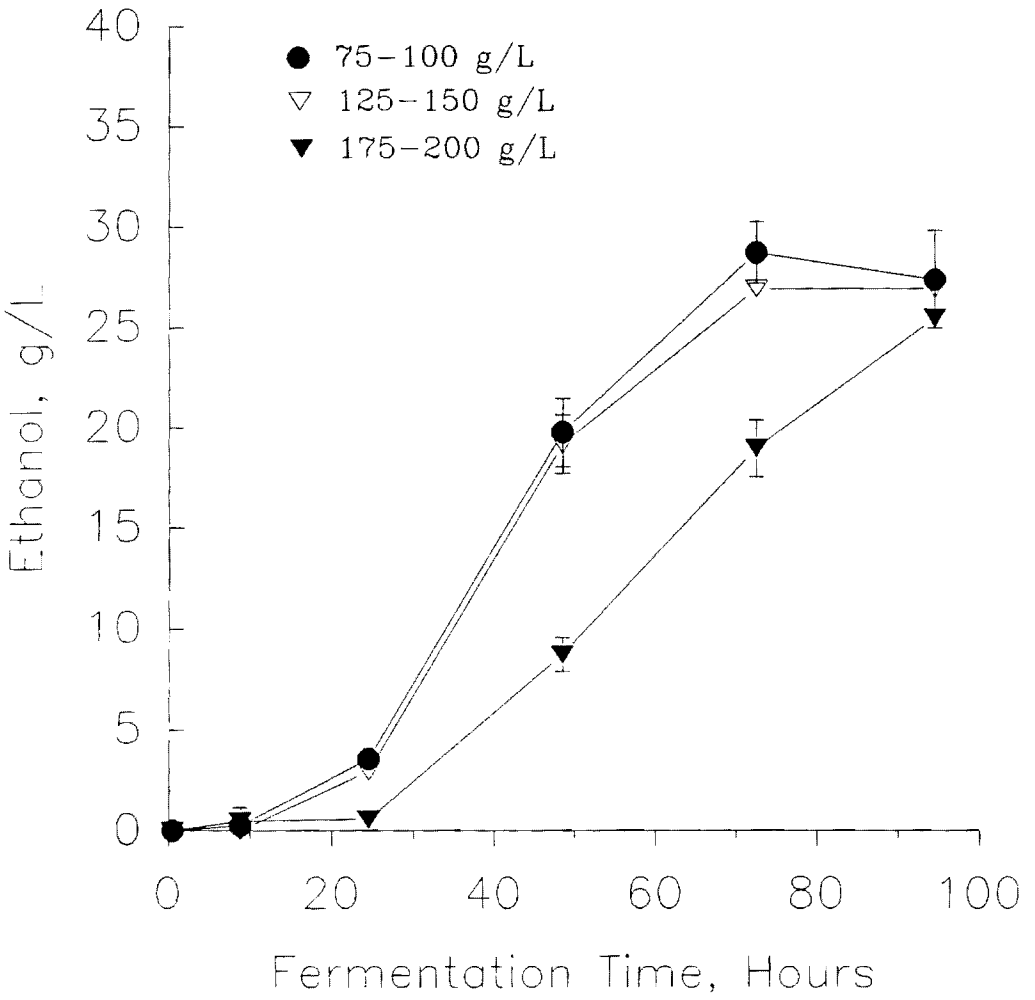


Figure 3 -- Xylitol Production in Pure Xylose Fermentations by Candida shehatae (Performed in Shaker Flasks).

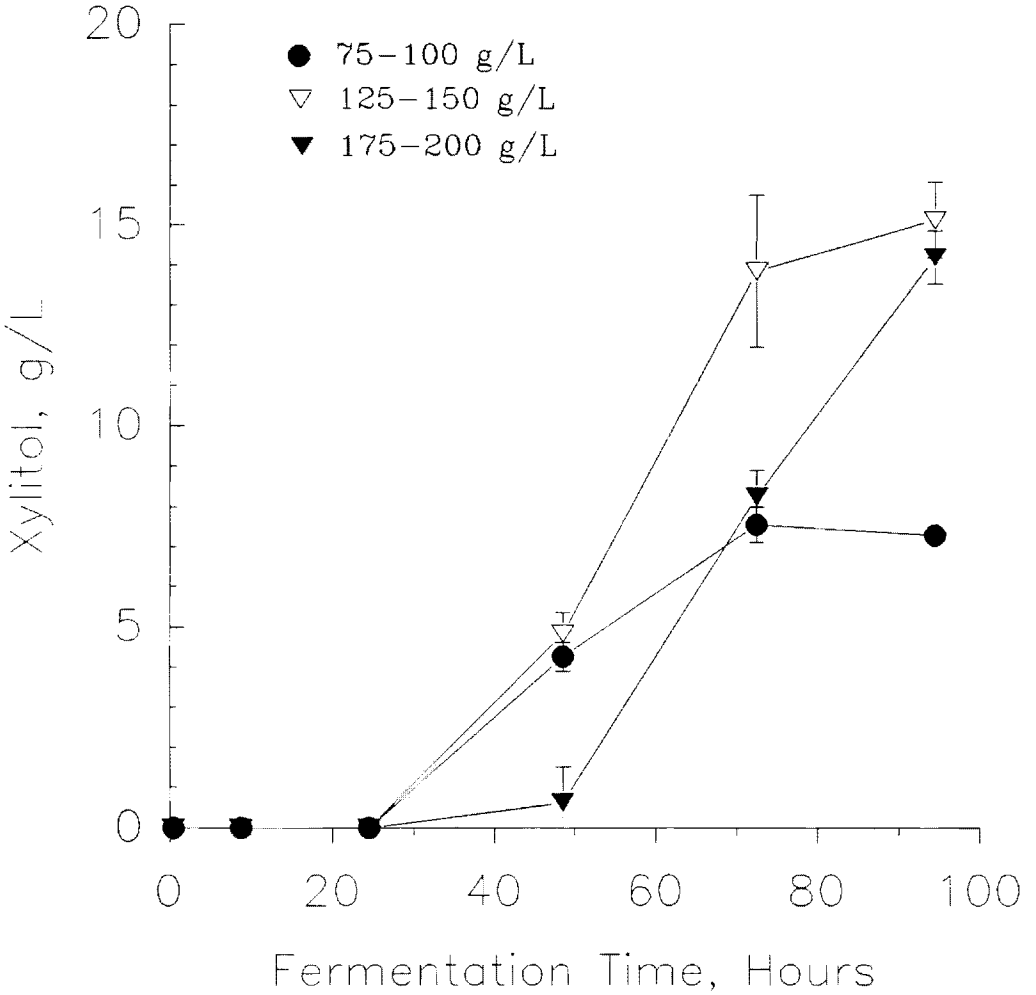


Figure 12 -- The Change in pH as a function of Xylose Concentration in an Ethanol Fermentation by Candida shehatae

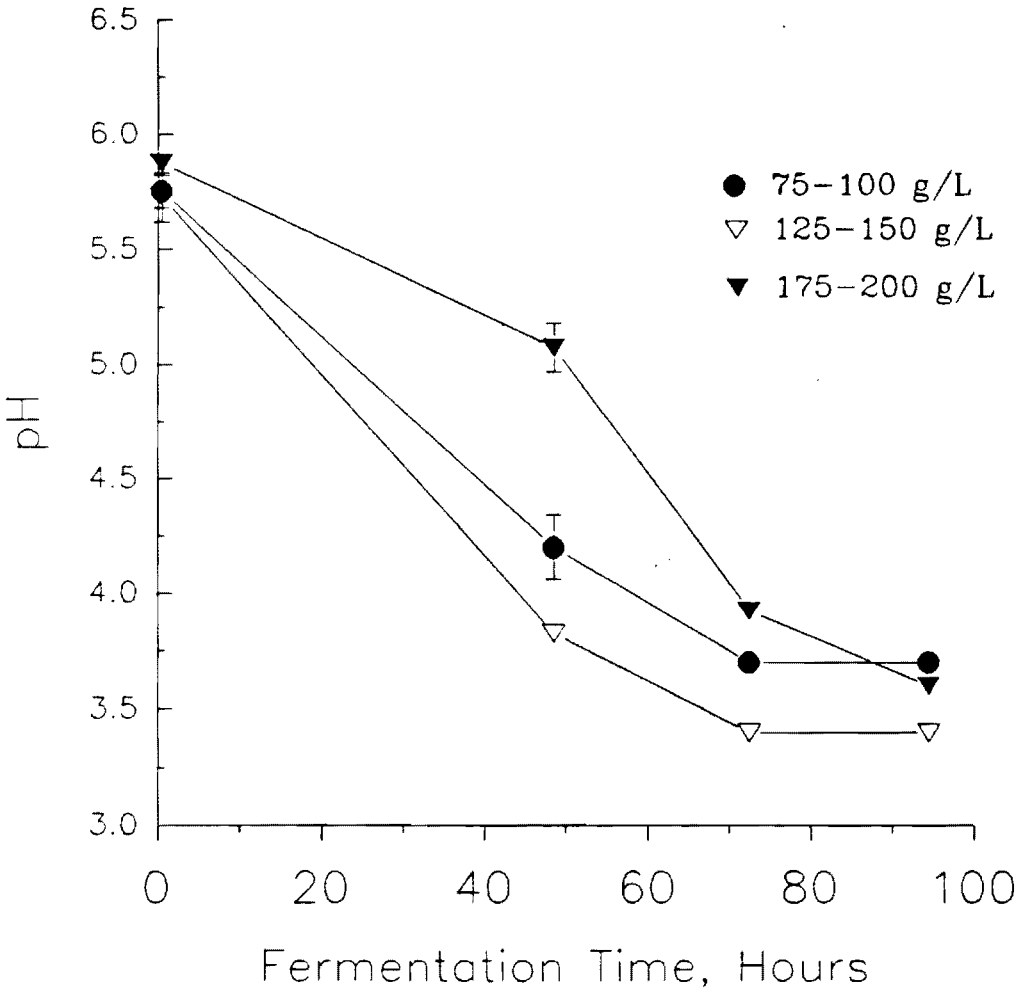
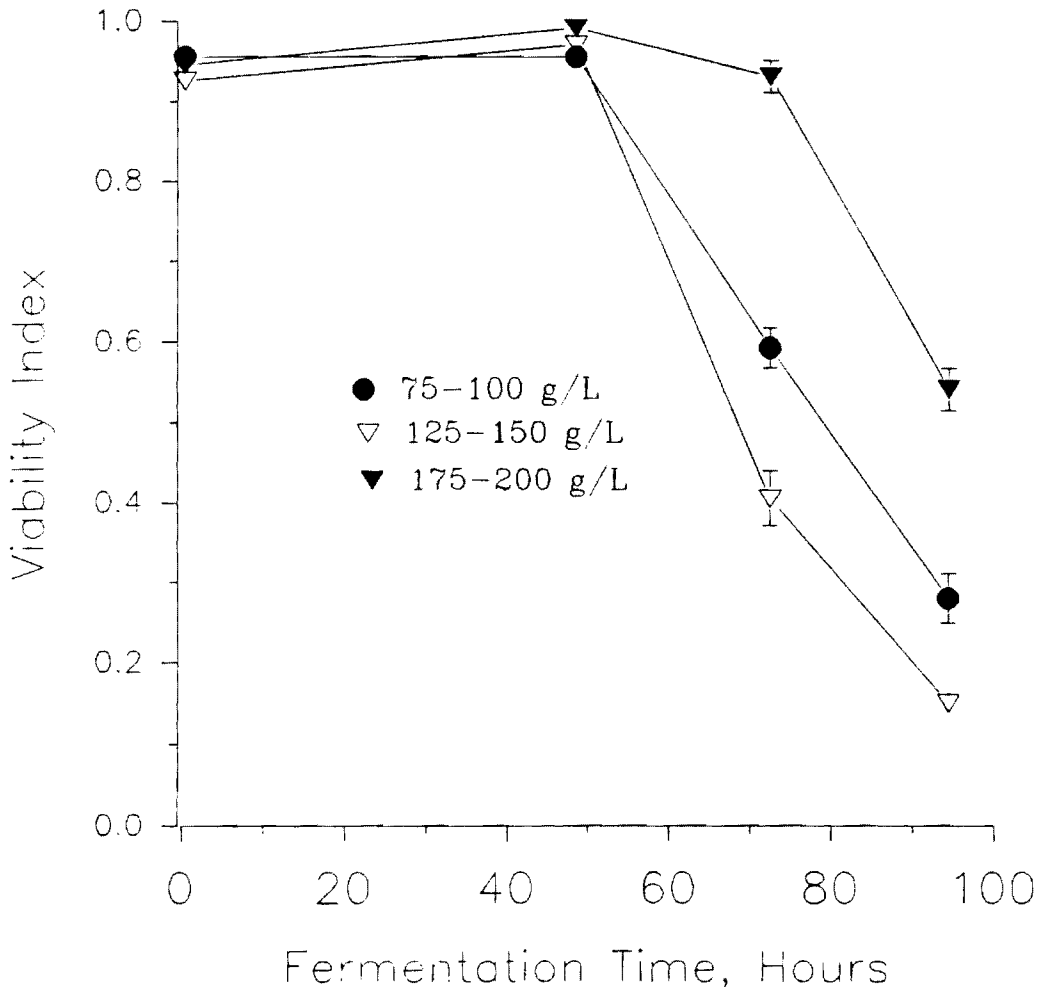


Figure 1B -- Change in Viability Index for Pure Xylose Fermentations by Candida shehatae (Performed in Shaker Flasks).



Figures for Mixed Sugar Fermentations on Defined Medium

Figure 4B -- Total Cells/ml Produced in Xylose and Mixed Sugar Fermentations by Candida shehatae (Performed in Shaker Flasks).

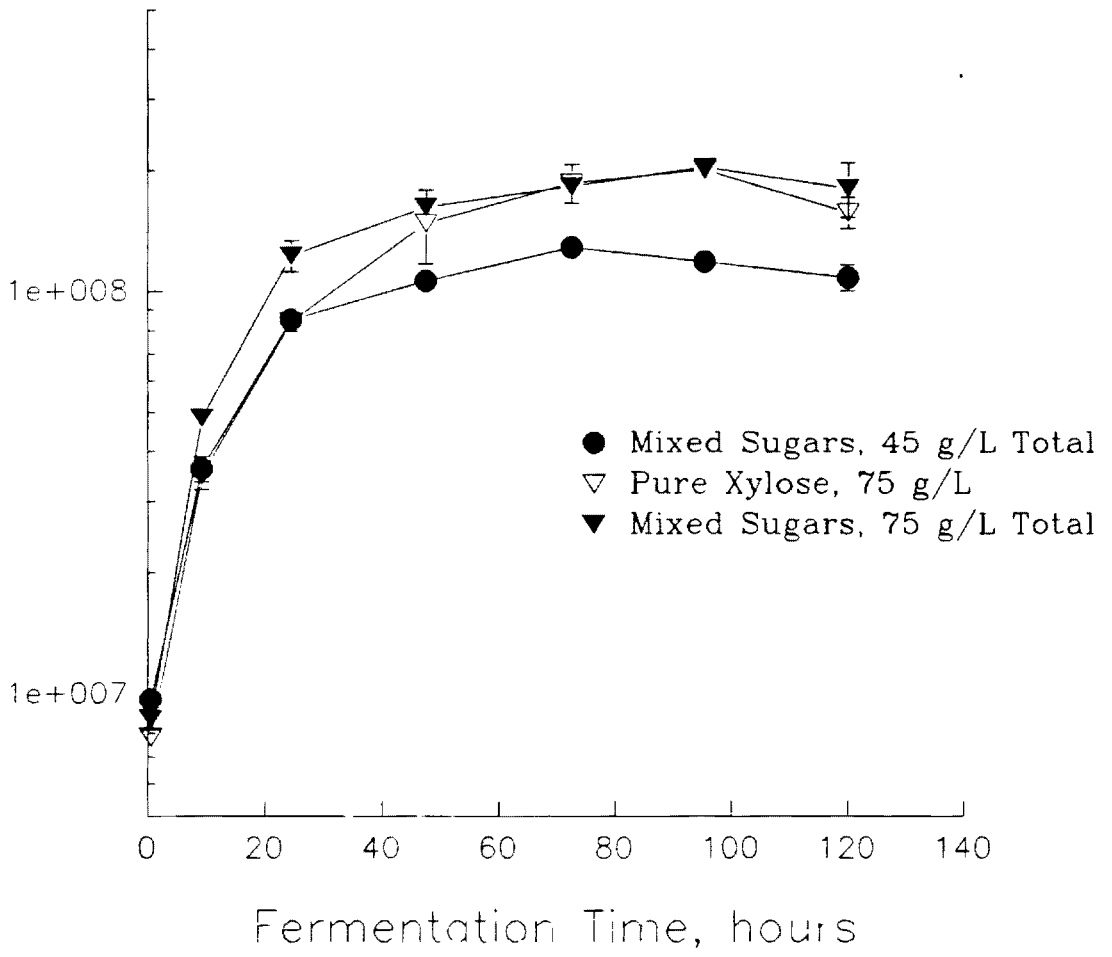


Figure 5 -- Uptake of Xylose in a Fermentation by Candida shehatae (Performed in Shaker Flasks).

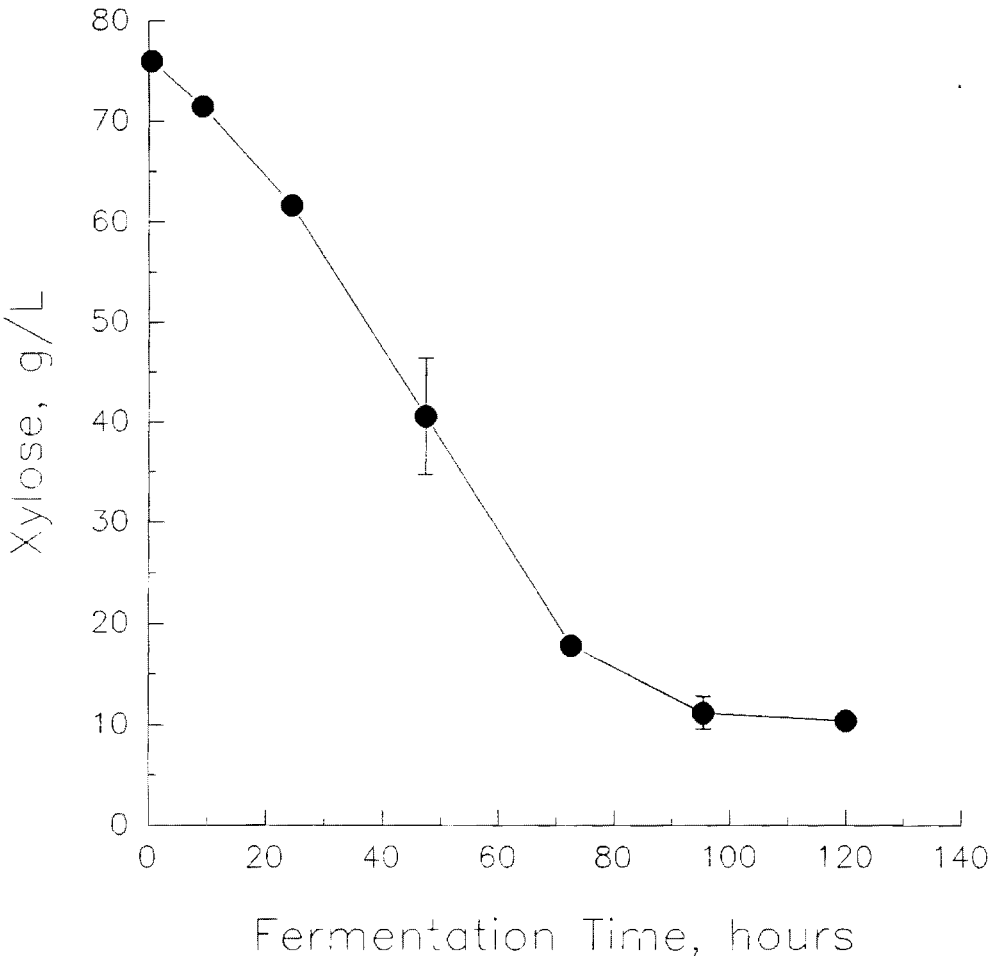
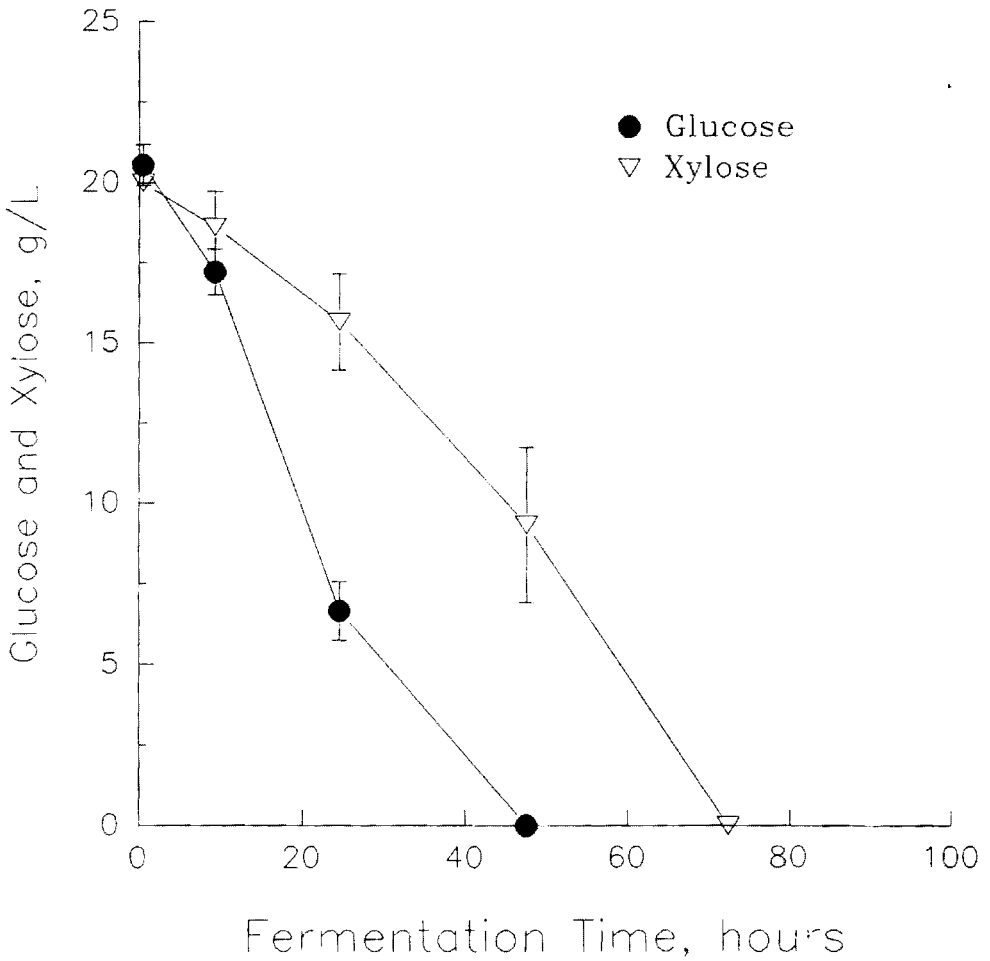


Figure 4 -- Uptake of Xylose and Glucose in a Fermentation by Candida shehatae (Performed in Shaker Flasks).



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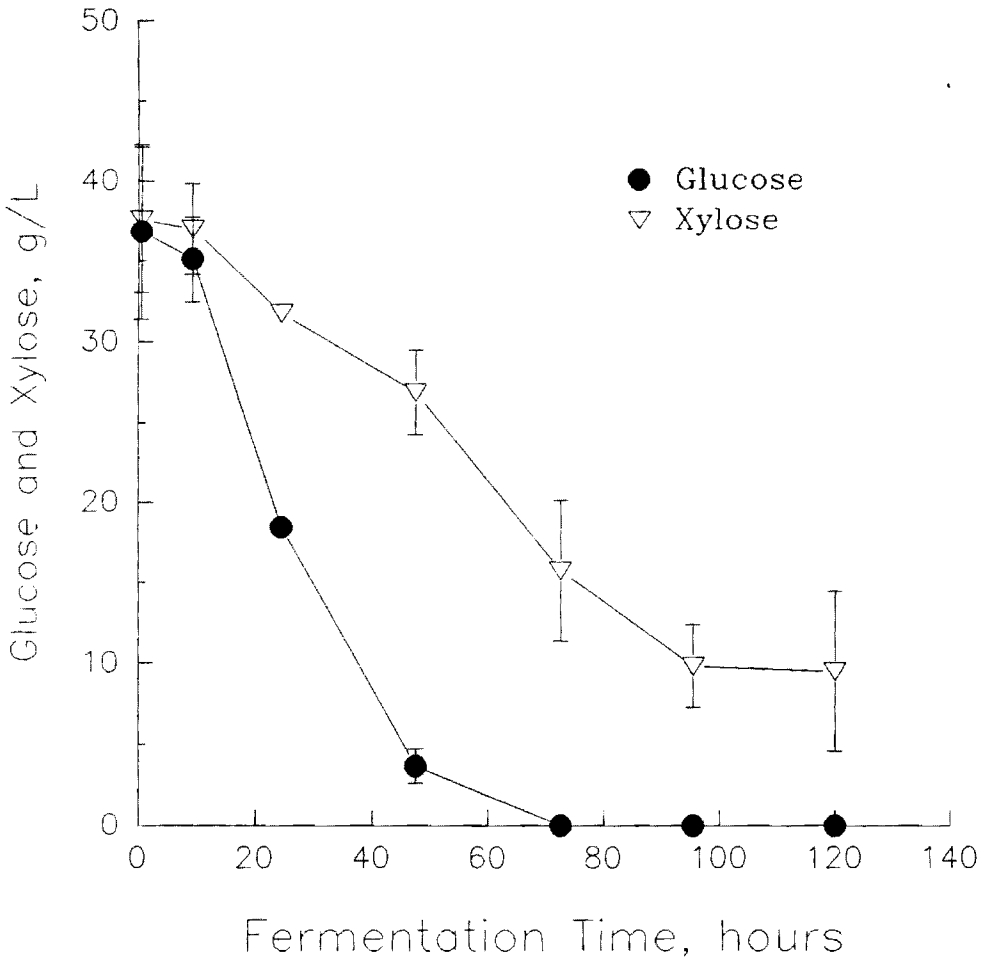

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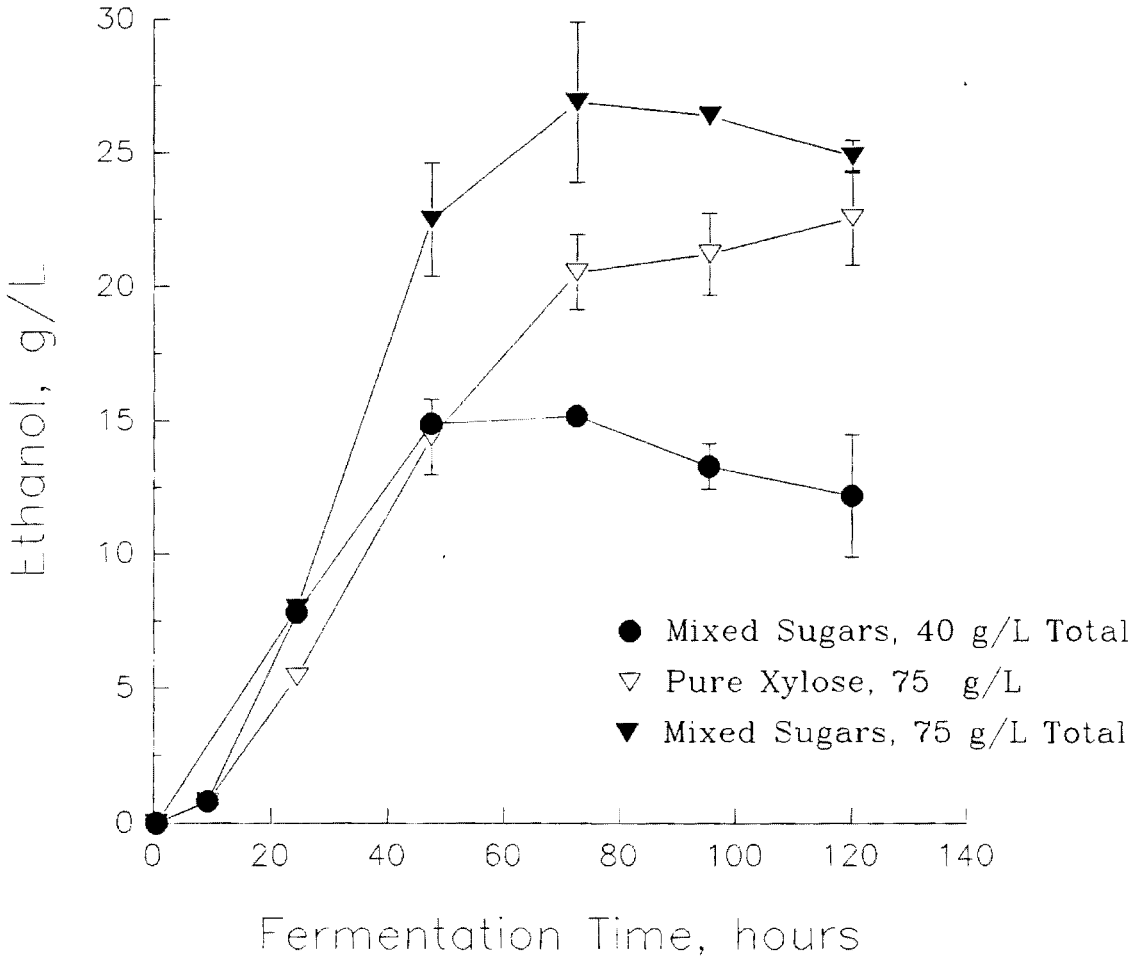
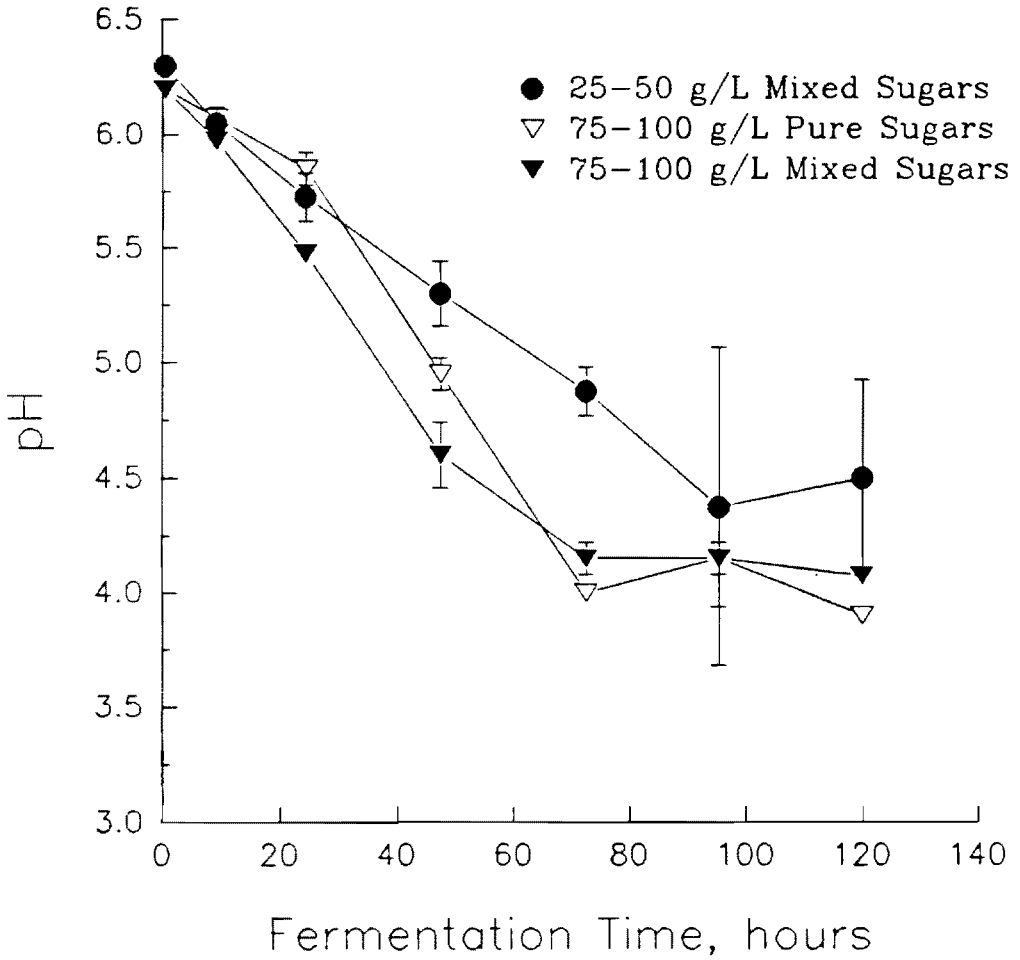


Figure 14 -- pH Change in Mixed Sugar (50% Glucose and 50% Xylose) and Pure Xylose Fermentation by Candida shehatae



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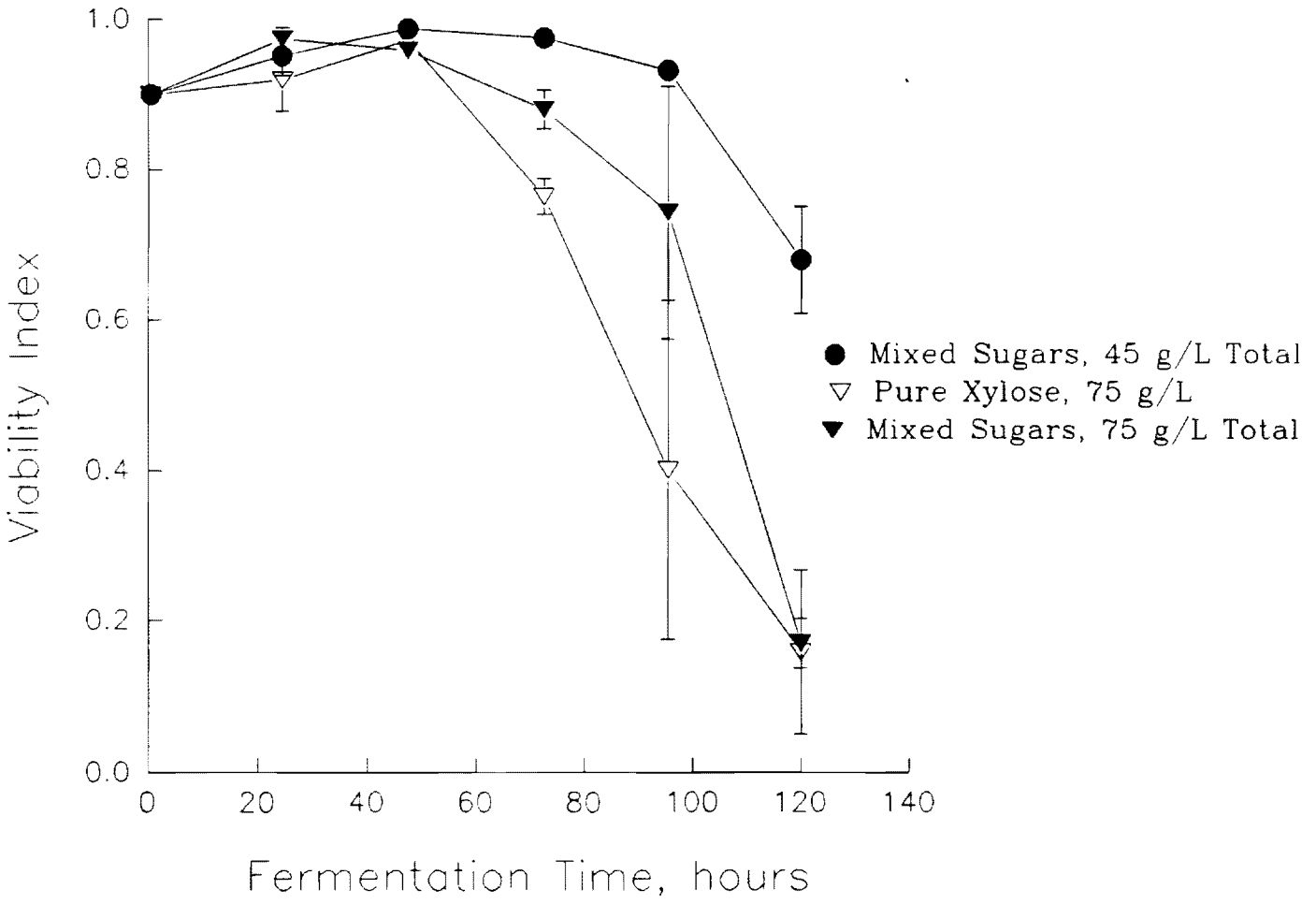

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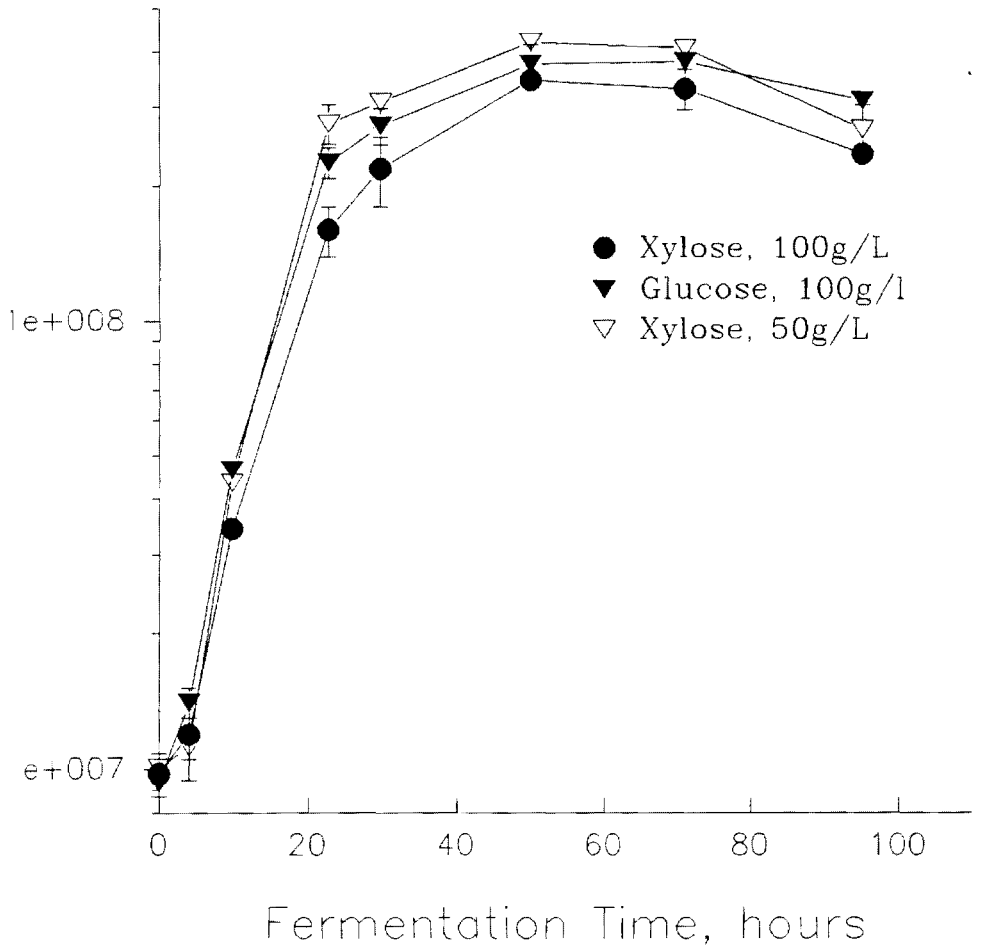

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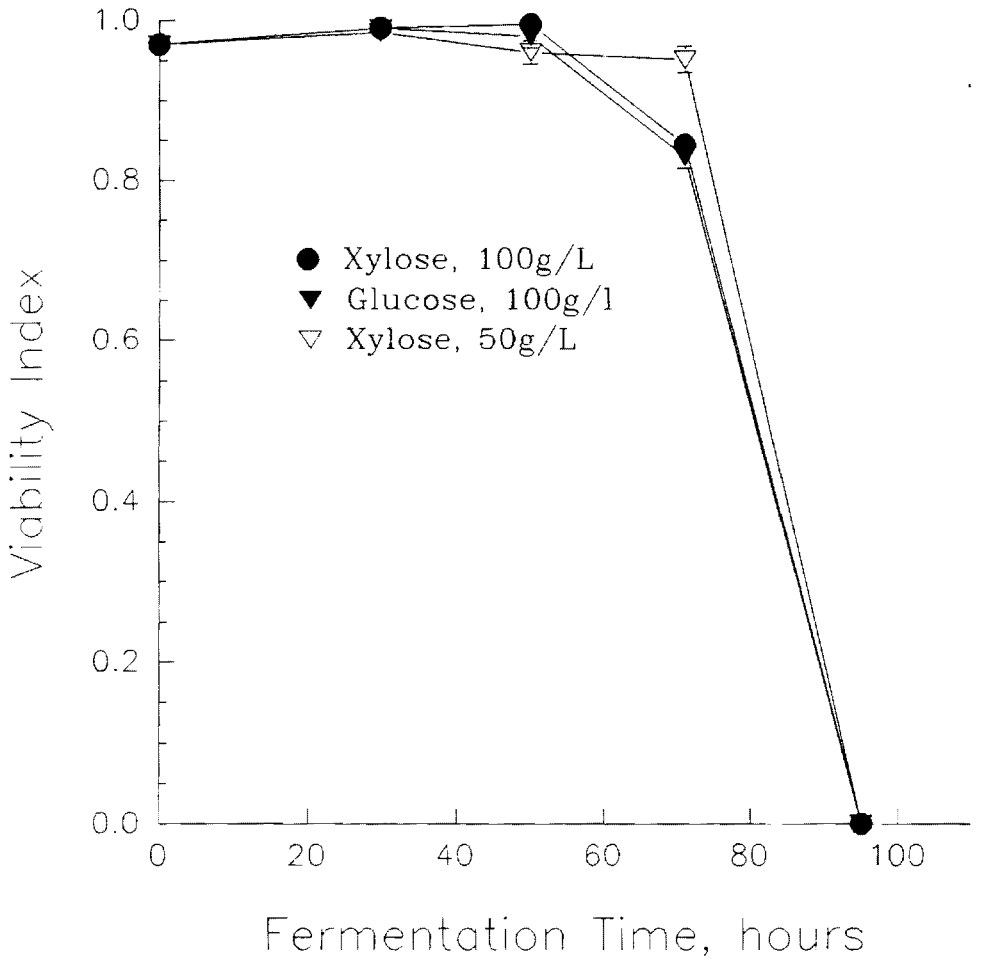

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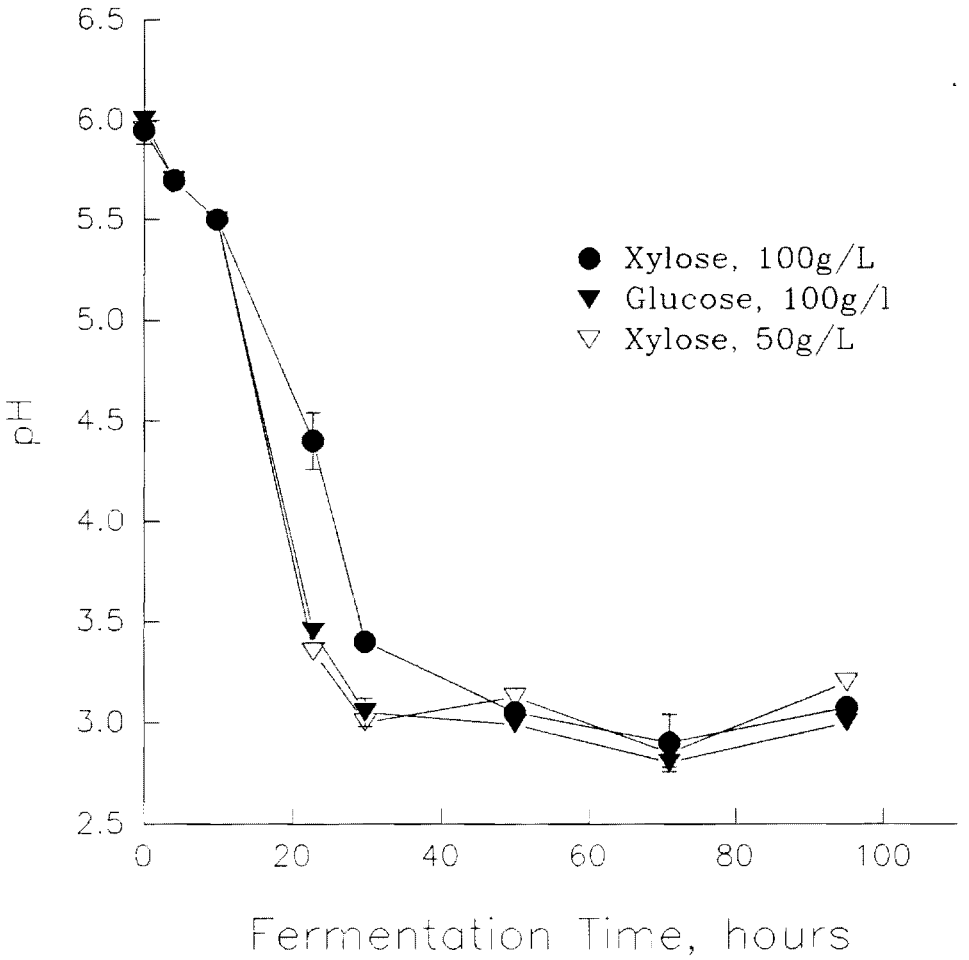

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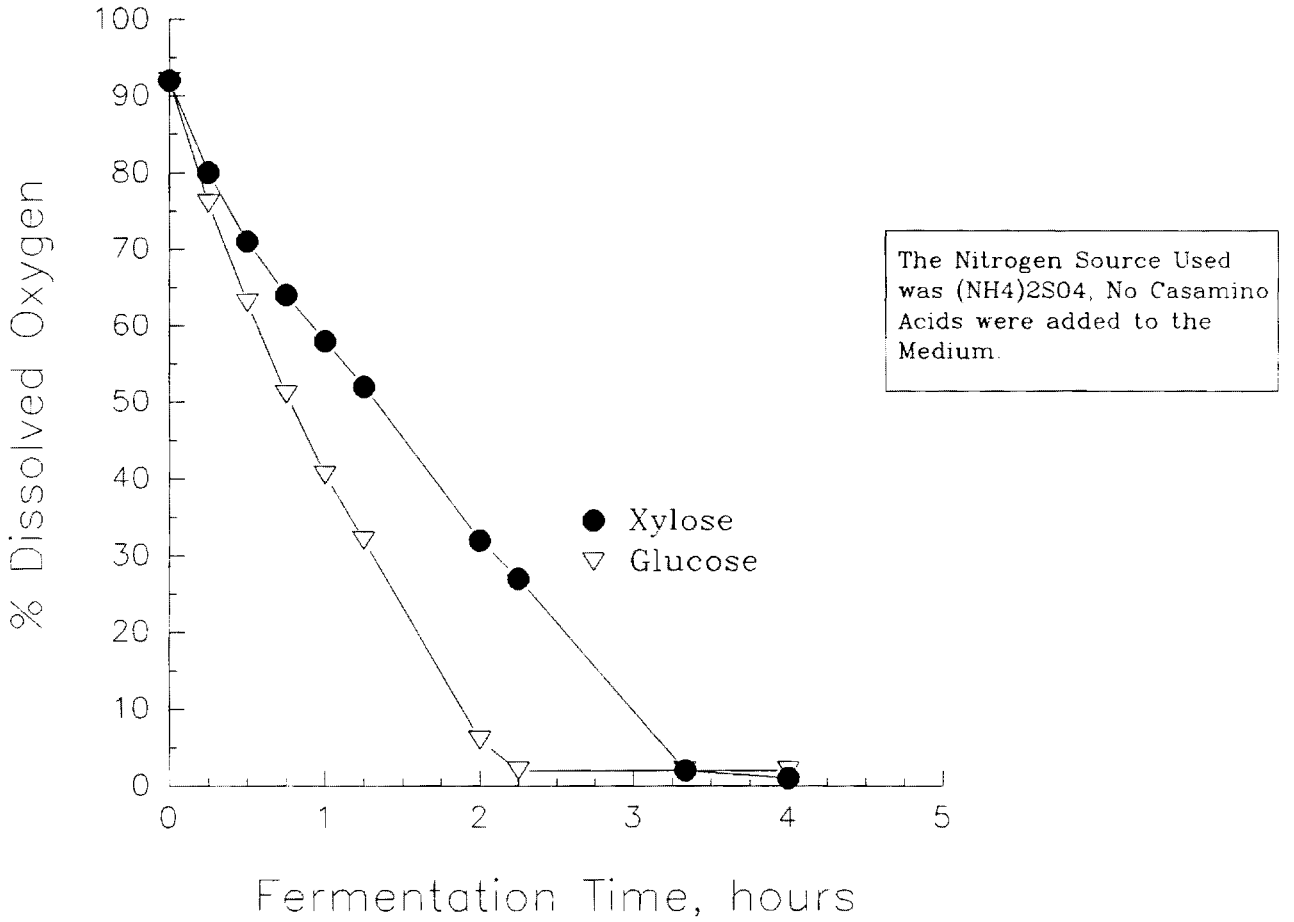
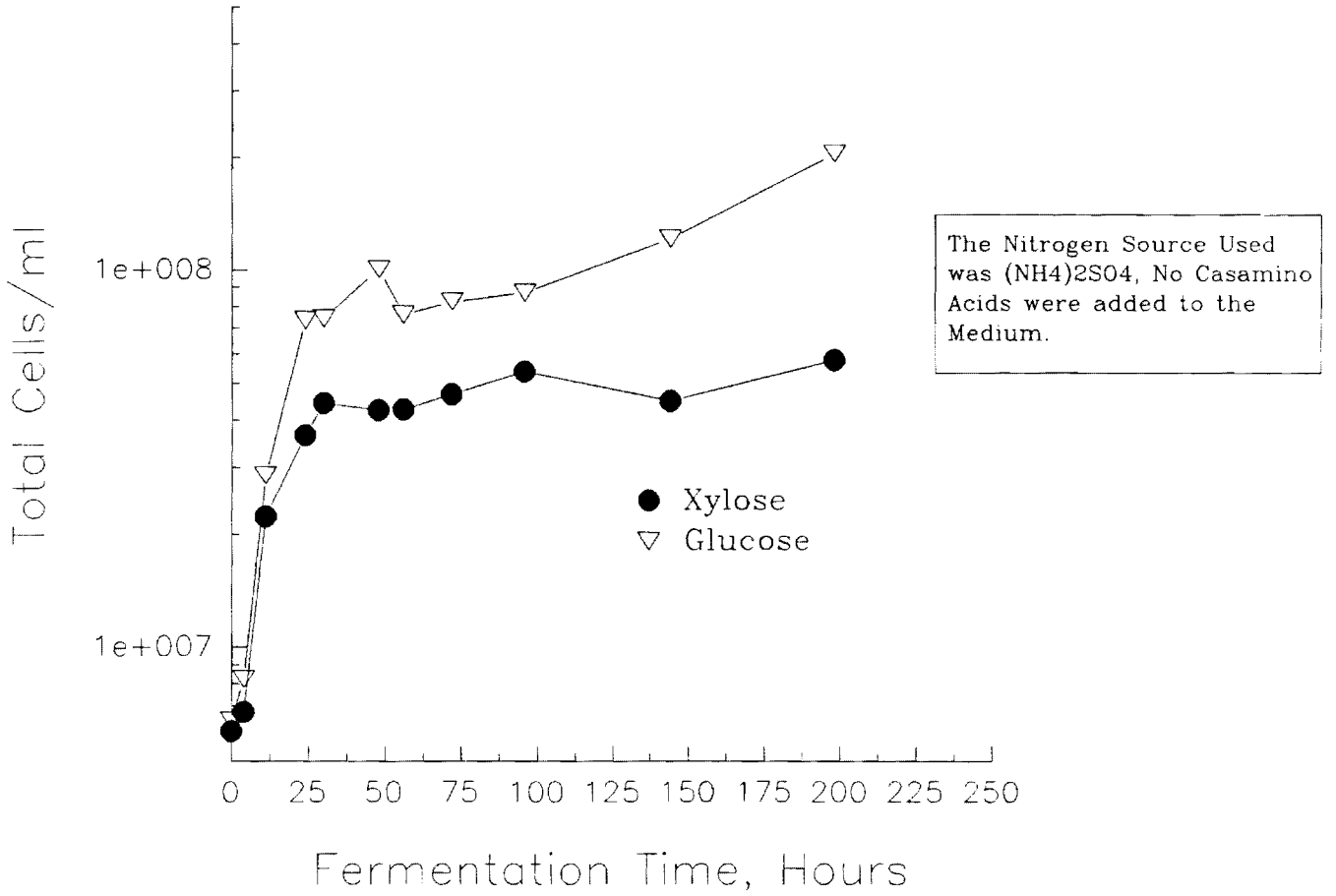


ıre 11 -- Total cells/ml Produced for Xylose and Glucose Fermentations by Candida shehatae. The pH was controlled at 4.5 and Temperature maintained at 30 C.



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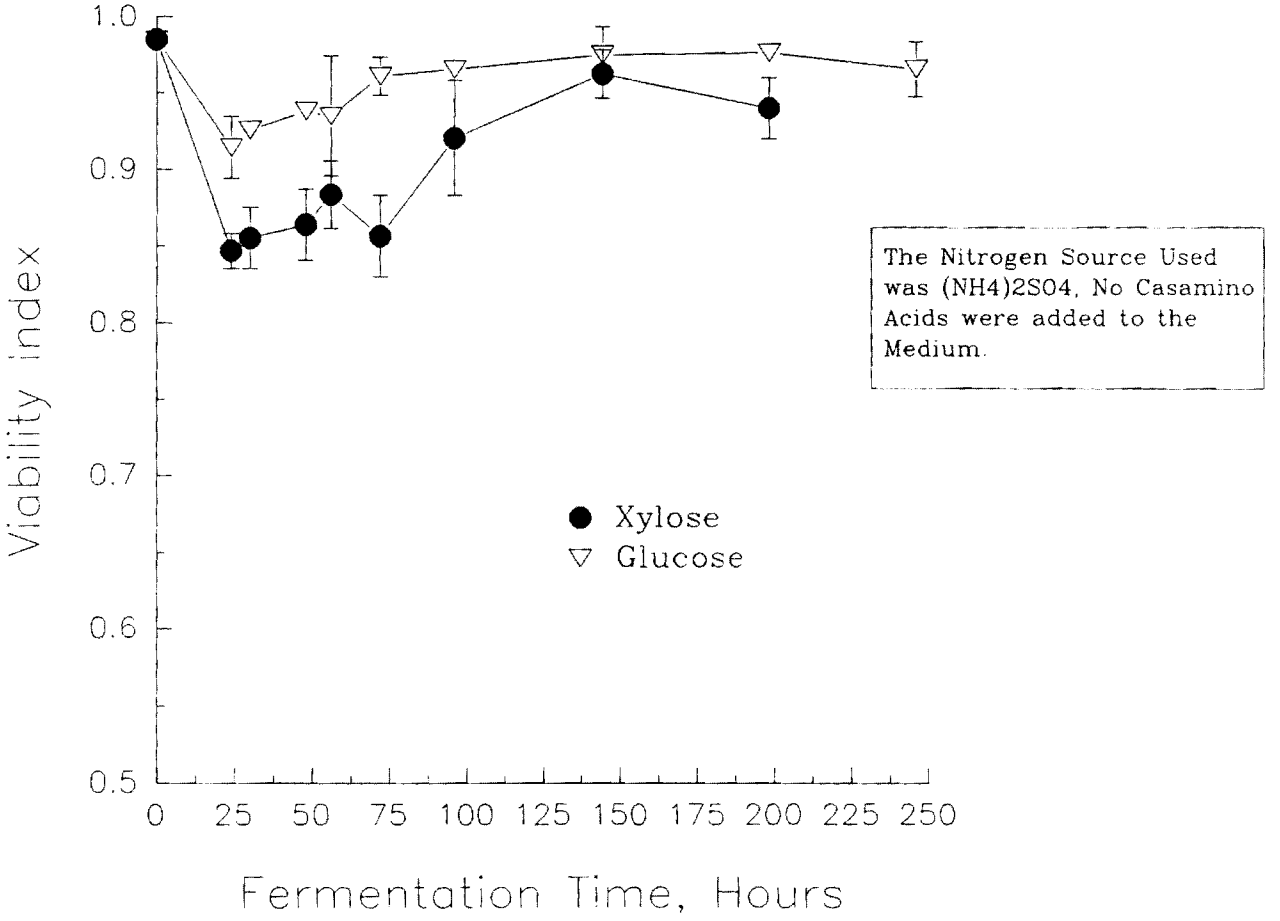

Effect of Casamino Acids on Xylose Fermentations in Shaker Flasks

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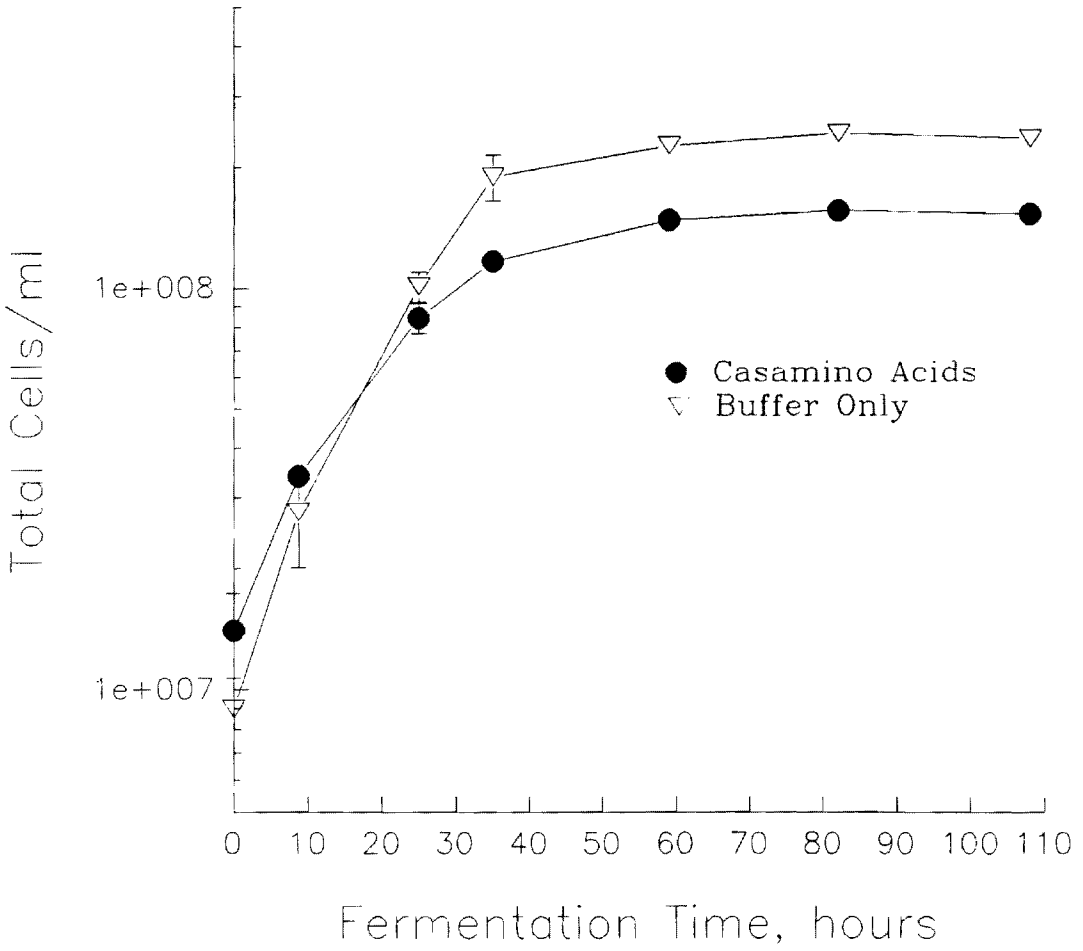

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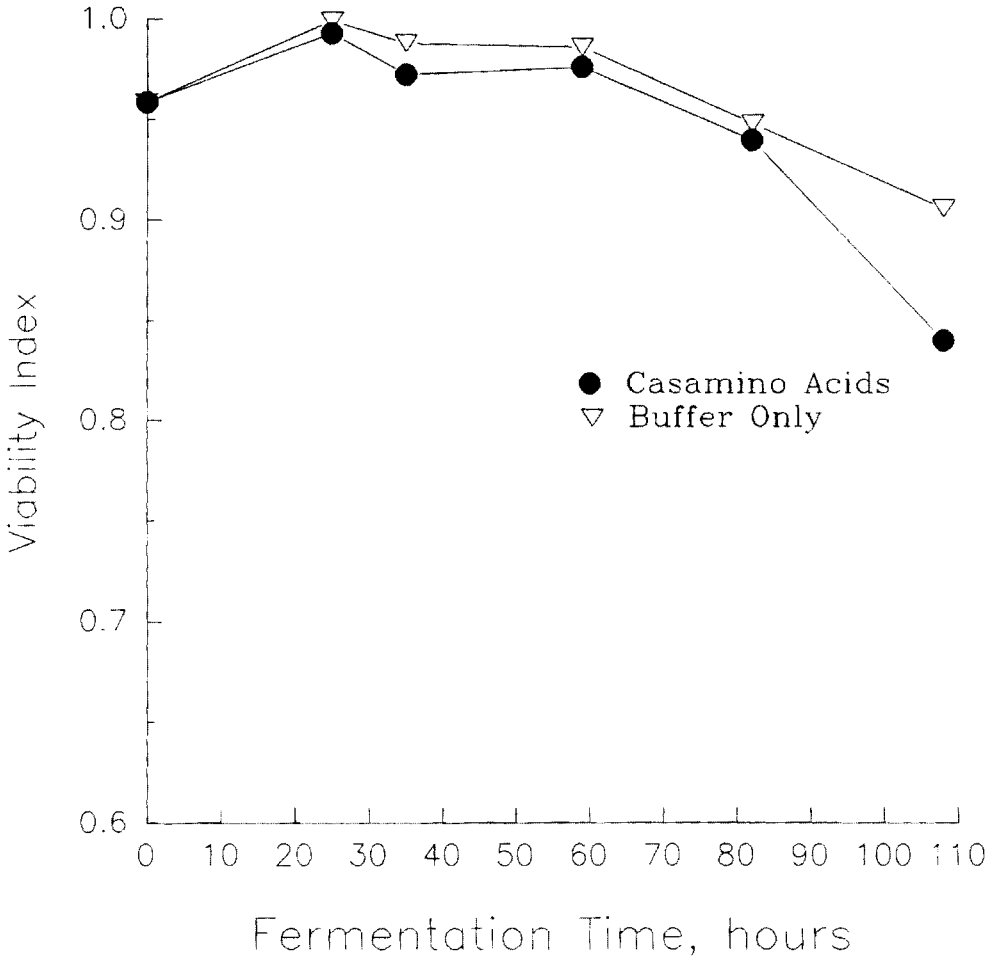

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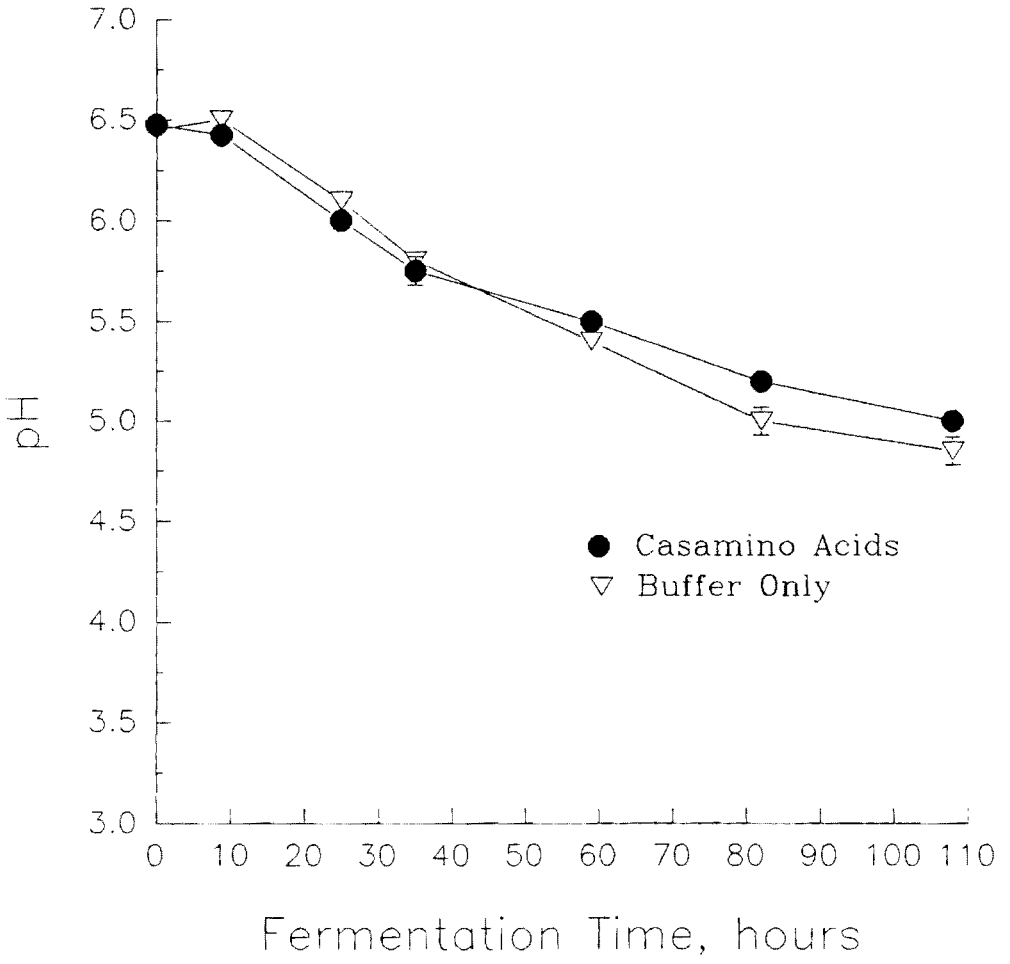

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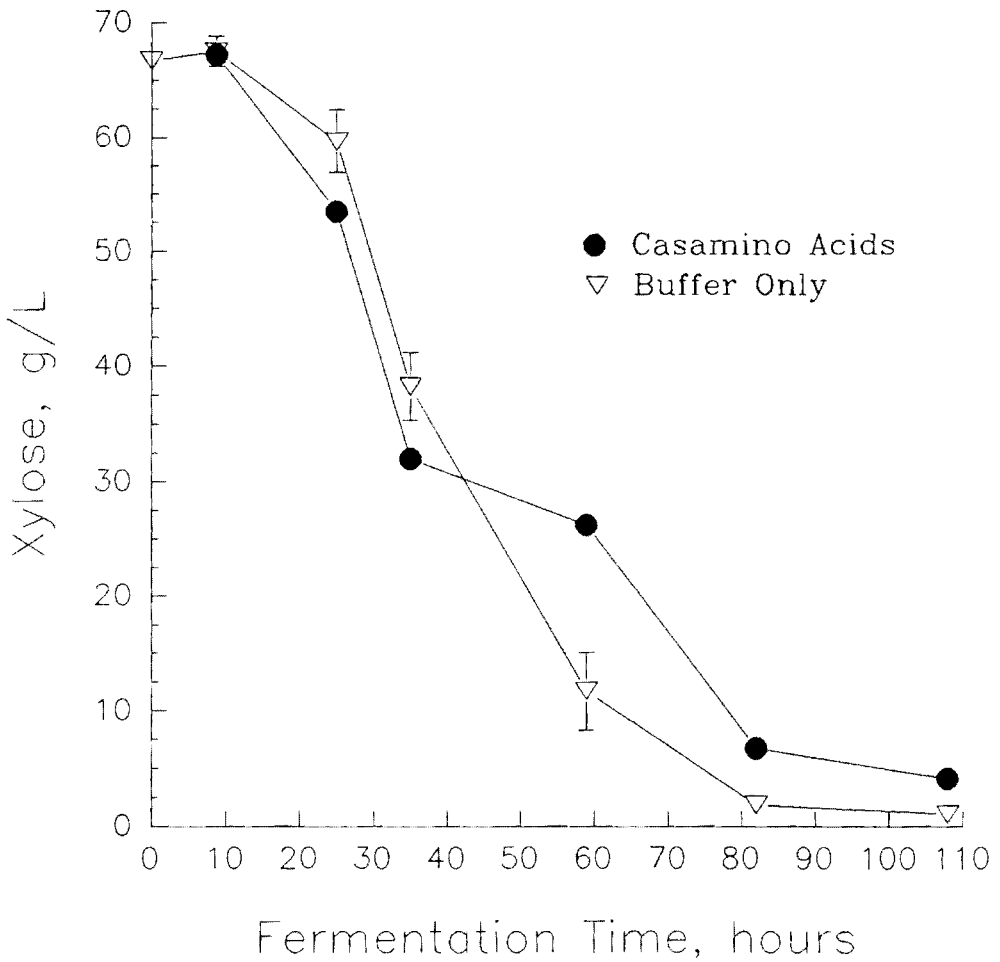

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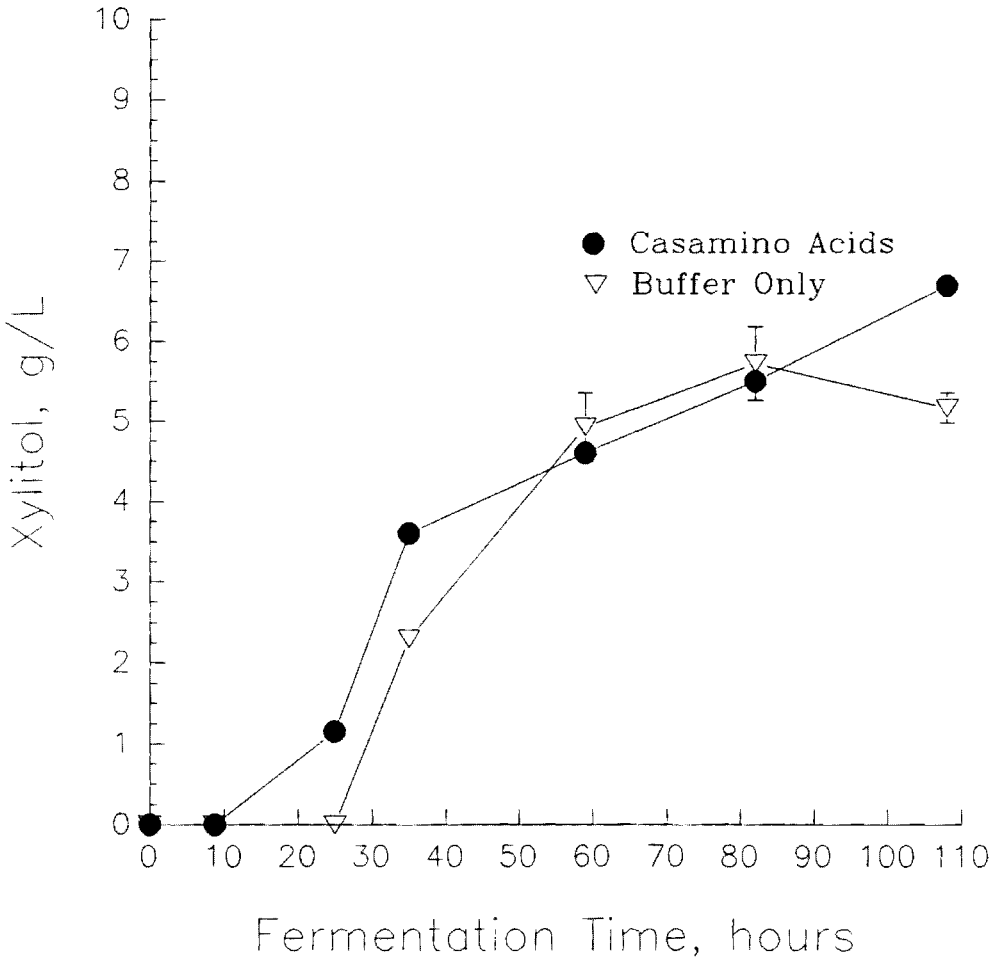

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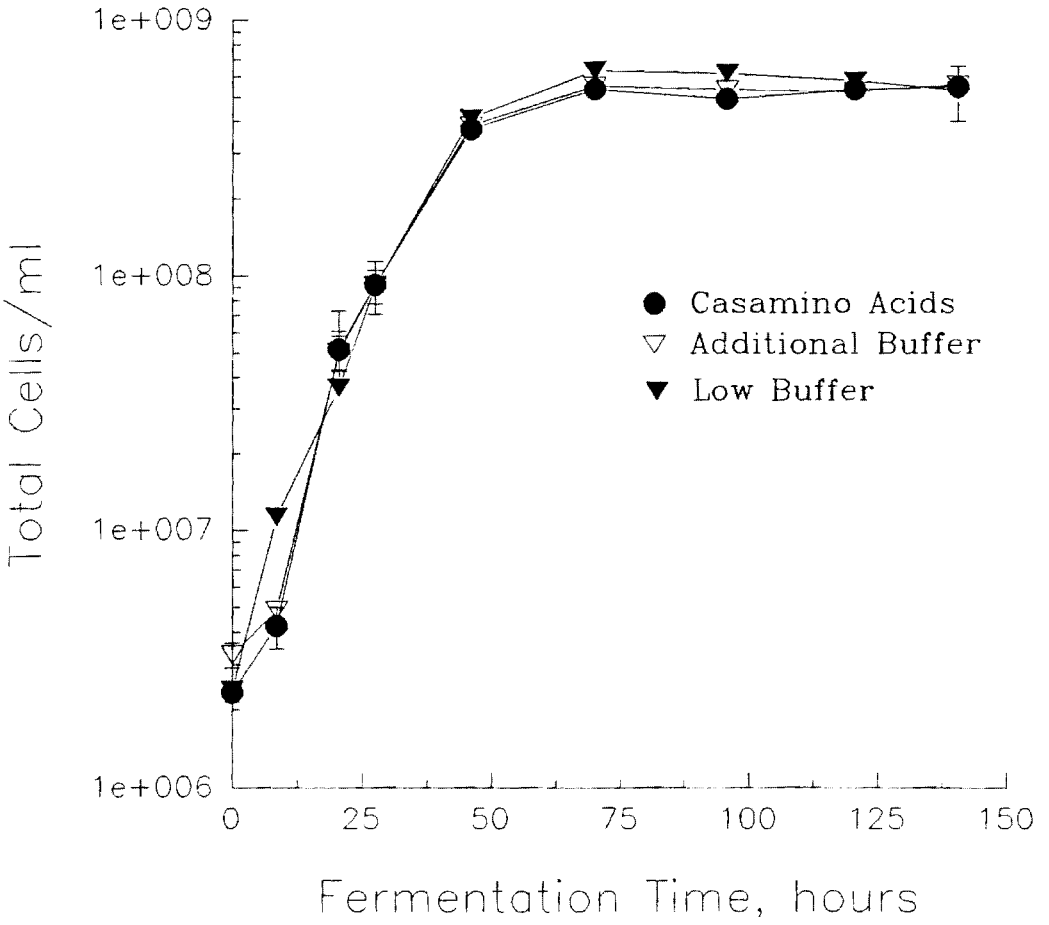

Figure 19-- Change in Viability Index for Xylose Fermentations by Candida shehatae with and without Casamino Acids in a Defined Medium.

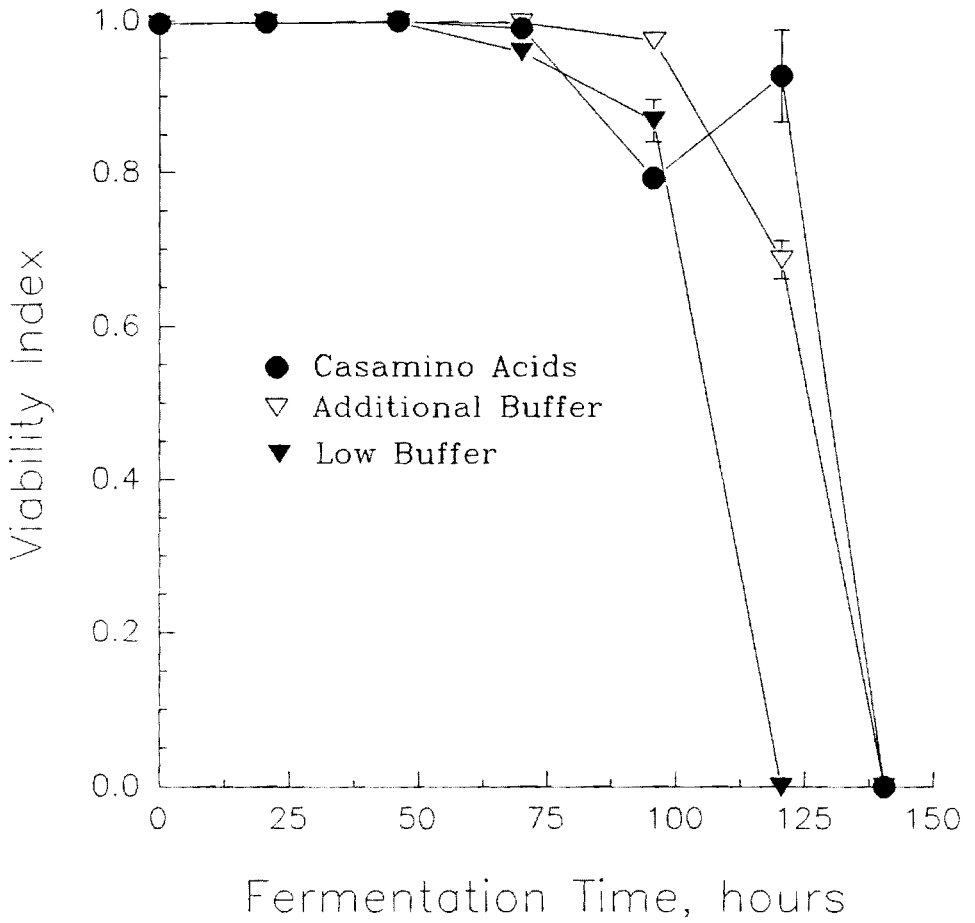

Figure 20 -- Change in pH for Xylose Fermentations
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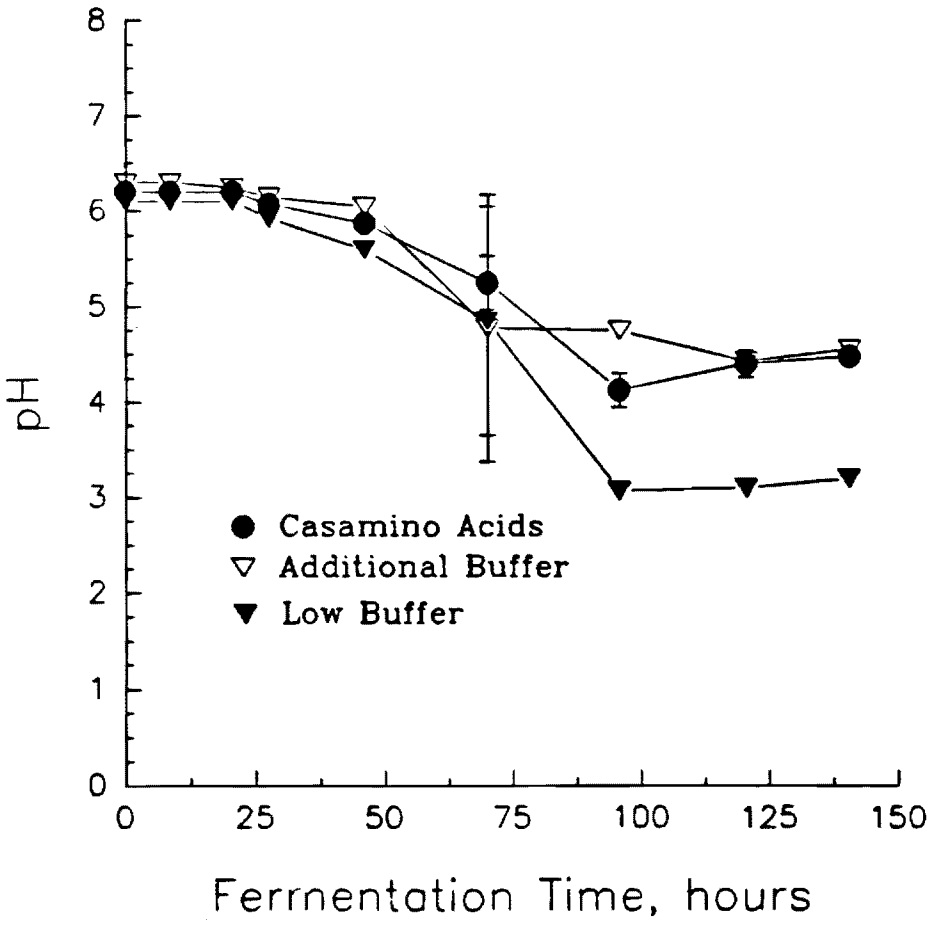

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Final Report

April 1, 1990 - June 30, 1991

Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
Biomass Branch
Muscle Shoals, Alabama 35600

Submitted by

Jim Kastner and Professor Jack Jones, School of Applied Biology
and Professor R.S. Roberts, School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

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1. SIGNIFICANCE AND APPLICATION

a. Introduction

Lignocellulosics from agricultural residues, woody residues and municipal solid wastes are a potential source of liquid fuels (ethanol) and are produced in large quantities in the United States. For example, in 1975, 14.0 million tons of Municipal Solid Wastes, 30.0 million tons of Agricultural Residues, and 16.8 million tons of Forestry Residues were generated. (Cowling, 1975). Lignocellulosics are composed of lignin, cellulose and hemicellulose.

Cellulose is a polymer of glucose and hemicellulose is a polymer which can contain a significant amount of D-xylose. The cellulose and hemicellulose fractions can be converted into their monomers by acid or enzymatic hydrolysis, resulting in a feedstock composed mostly of glucose and D-xylose. Since hemicellulose constitutes a significant fraction of the waste residues (Tables 1 and 2), the percentage of D-xylose in this mixture can range from 20-50%, depending on the lignocellulosic source and the process used to generate the sugars. To economically produce ethanol from waste residues the xylose fraction must be fermented.

Table 1 - Composition of Different Lignocellulosic Sources (Bierman, 1983)

Source	Cellulose wt%	Hemicellulose wt%	Lignin wt%
Municipal Solid Wastes	61	22	9
Softwood	45-50	25-40	25-35
Hardwood	40-55	24-40	18-25

Table 2 - Composition of Agricultural Residues (Magee and Kosaric, 1985)

Waste/By-Product	Cellulose wt%	Hemicellulose wt%	Lignin wt%
Bagasse	45-55	25-27	19-21
Rice Straw	32-53	21-24	12-25
Wheat Straw	31-51	23-38	18-21
Corn Stover	35-40	30-35	14-15
Barley Straw	35-40	17-20	13-14

Several processes have been described in the literature to ferment the pentose sugars:

1) Conversion of xylose to xylulose by xylose isomerase and consequent fermentation by *Saccharomyces cerevisiae*. (Gong et al., 1981)

2) Utilization of a thermophilic bacterium such as *Thermoanaerobacter ethanolicus* capable of fermenting xylose. (Paul, 1981; Wiegel and Puls, 1985)

3) Ethanol production by the xylose fermenting yeast, *Pachysolen tannophilus*. (Schneider, et al., 1983)

Each of the above fermentation processes suffers from disadvantages which presently limits their use. For example, the conversion of xylose to xylulose by an isomerase and consequent fermentation by the yeast *Saccharomyces cerevisiae* is a two step process. The two step process is required because the isomerase is of bacterial origin and requires different optimum operating conditions (pH and temperature) than the yeast. Thermophilic bacteria fermentation is impeded by feedback inhibition and mixed solvent production which limits final ethanol concentration. Finally, *Pachysolen tannophilus* fermentation of xylose results in low ethanol concentrations due to feedback inhibition at much lower concentrations then in *Saccharomyces cerevisiae* fermentation. The low concentration of ethanol combined with relatively low yields make the recovery of ethanol from this fermentation not economically feasible.

b. Current Fermentation Design

Of the three processes, direct xylose fermentation by a yeast shows the greatest promise for short term incorporation into a large scale process (Jefferies, 1983). A two-stage, batch fermentation process is currently proposed to produce ethanol from the mixture of hexose and pentose. Since most organisms ferment hexose preferentially, *Saccharomyces cerevisiae* will be employed to ferment any glucose present initially, while *Pachysolen tannophilus* will be used to ferment residual xylose. Figures 1 and 2 depict current batch fermentation designs for sugar streams produced by the dilute and concentrated acid hydrolysis of lignocellulosic residues. For each of these designs, the concentration of ethanol obtained from the fermentation of the xylose is very low. The

recovery of this low concentration ethanol is very costly and significantly increases the manufacturing cost of producing ethanol from lignocellulosic materials.

Figure 1. Flowsheet for Batch Fermentation Process of Dilute Acid Hydrolyzate (TVA Biomass Program Brochure, Muscle Shoals, Alabama and Teledyne Wah Chang Albany Brochure, 1986)

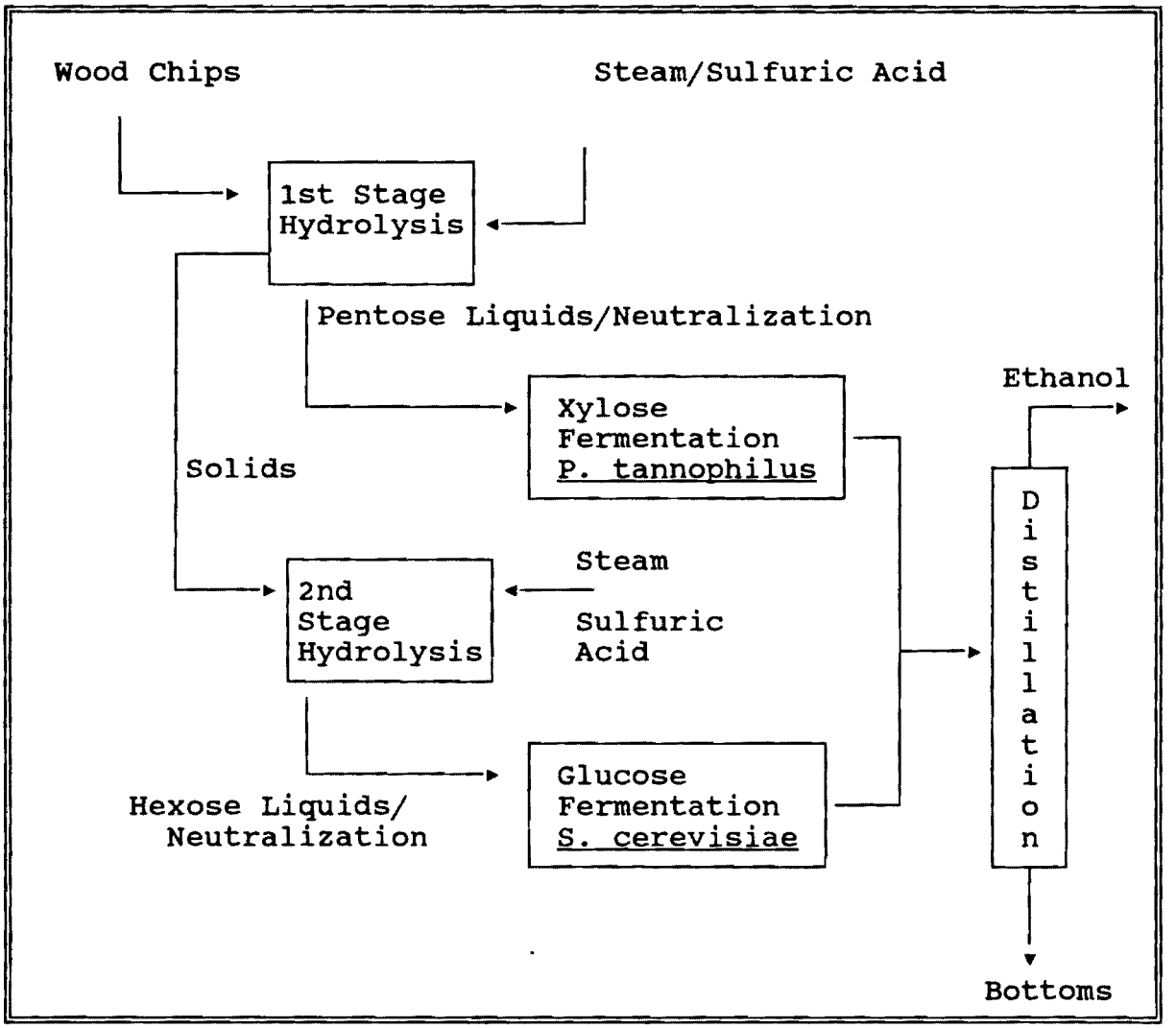
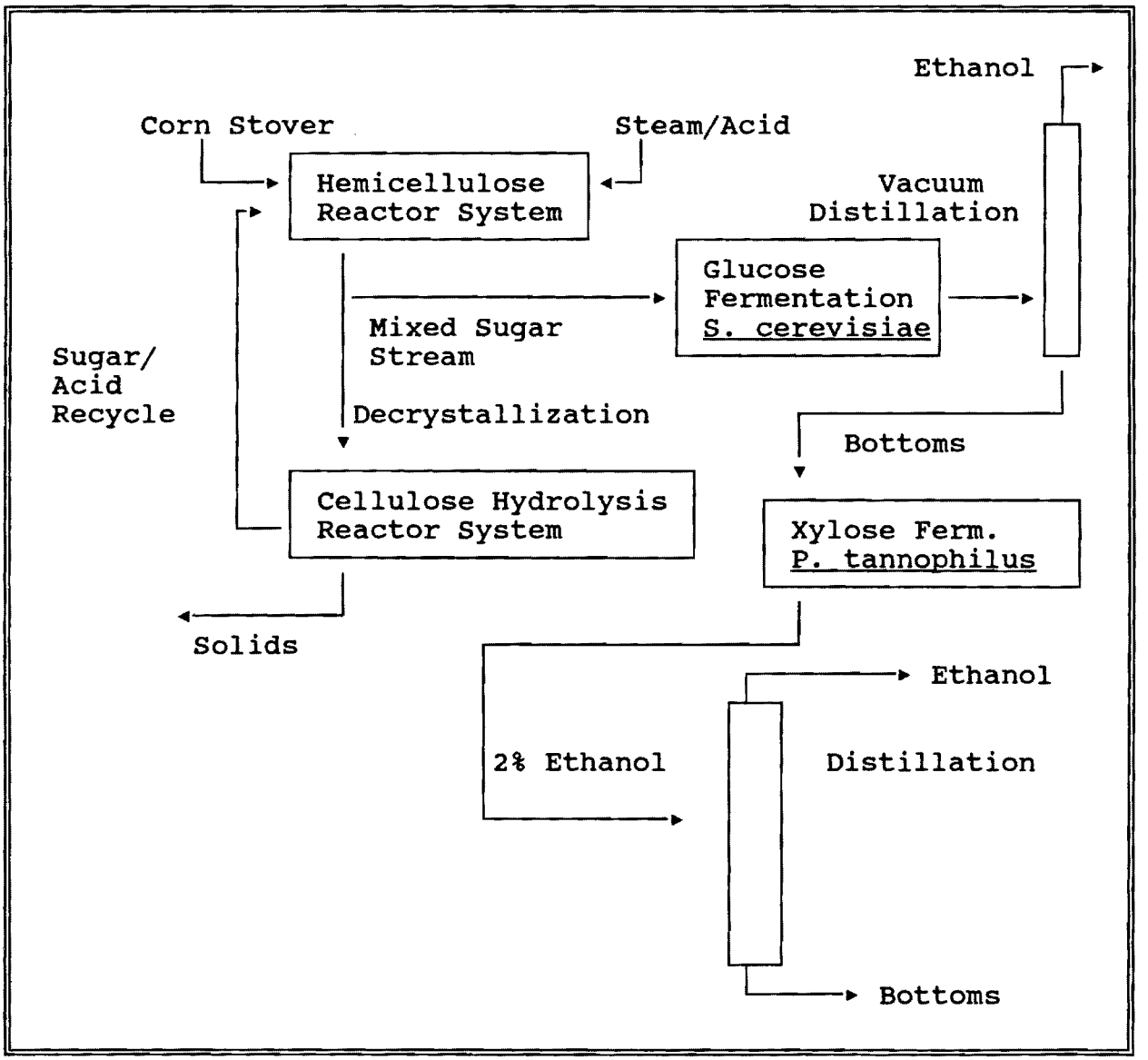


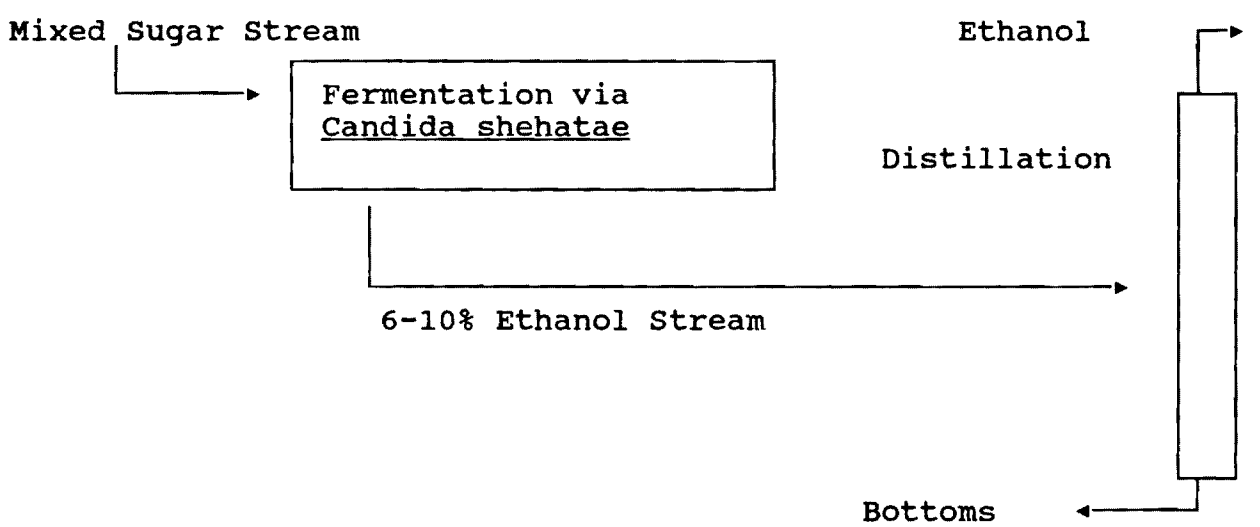
Figure 2. Flowsheet for Batch Fermentation of Concentrated Acid Hydrolyzate (Kastner, 1987; Barrier et al., 1985 and Mary Jim Beck, 1987)



c. Research Relevancy: Mixed Sugar Fermentation

The recovery of the low concentration ethanol significantly increases manufacturing costs in the current fermentation design. If the concentration of the ethanol from the xylose fermentation can be increased, the overall economics of the process will be improved. Advantages would also be gained if mixed sugars could be fermented in a single step. A one stage fermentation coupled with high ethanol concentrations would reduce the equipment cost in the fermentation area (reduce the number of batch fermentors required) and decrease the cost of ethanol distillation (compare Figures 1, 2, and 3).

Figure 3. Flowsheet for Mixed Sugar Fermentation



Recently the yeast, *Candida shehatae*, was shown to be a better D-xylose fermentor than *Pachysolen tannophilus*. *C. shehatae* fermented D-xylose to higher a ethanol level and a higher ethanol yield, in a shorter time than *P. tannophilus*. However, little information is available on *Candida shehatae's* ability to ferment mixed sugars. Many microorganisms ferment mixed sugars in a sequential pattern; glucose is consumed first followed by other carbohydrates present. Usually there is a lag after glucose depletion before utilization of the other carbohydrates begin and sometimes the utilization is incomplete. This behavior, sometimes called a diauxic lag, can increase fermentations times, reduce yields and make the fermentation uneconomical.

A fermentation method must be developed to overcome the diauxic lag. If a single stage mixed sugar fermentation is to be designed, additional data are needed for the fermentation of xylose and glucose mixtures by *C. shehatae*. Data on such process variables as pH, effect of ethanol, oxygen levels, medium composition and carbohydrate consumption rates are required. The effect of these process variables on *C. shehatae* will indicate if the yeast can be used in a large-scale fermentation of D-xylose and mixed sugars.

2. PRELIMINARY EXPERIMENTS - SUMMARY OF RESULTS

a. Mixed Sugar Fermentations Under Anaerobic Conditions on an Undefined Medium:

Mixtures of D-xylose and glucose were anaerobically fermented with the yeast *Candida shehatae*. Cells aerobically grown on glucose exhibited a sequential anaerobic utilization pattern; glucose was consumed first then D-xylose (Figures 4 and 5). Cells aerobically grown on D-xylose anaerobically consumed glucose and D-xylose simultaneously; no lag in D-xylose consumption was seen (Figures 6 and 7). Although no lag in D-xylose utilization occurred, the D-xylose consumption rate was dependent on the glucose concentration. The initial rate of D-xylose consumption (g/cell-hr) was 9.85×10^{-12} compared to 9.4×10^{-12} for glucose in the 75% D-xylose - 25% glucose mixture (90g/L D-xylose and 25 g/L glucose). In the 50% D-xylose -50% glucose mixture (60 g/L of D-xylose and 55 g/L of glucose) the initial D-xylose consumption rate was 3.96×10^{-12} compared to 12.12×10^{-12} for glucose.

A pure D-xylose fermentation was also performed to compare data with the mixed sugar fermentations (Figure 8). These cells utilized D-xylose at a rate of 7×10^{-12} g/cell-hr and produced 27 g/L of ethanol and 28 g/L of xylitol within 85 hours from 107 g/L of D-xylose. As in the other fermentations D-xylose utilization was incomplete and cell viability declined late in the fermentation. staining) declined late in the fermentations (Figure 8 and 9).

The best results for mixed sugar fermentations were obtained with cells aerobically grown on D-xylose and inoculated into a 75% D-xylose:25 % glucose mixture. All of the glucose was utilized and 78% of the D-xylose was utilized. 25 g/L of ethanol and 25 g/L of xylitol were obtained from 95 g/L of carbohydrates within 85 hours.

Figure 4 - Batch Fermentation of a mixture of 50% glucose and 50% D-xylose by *Candida shehatae* cells pre-grown aerobically on glucose.

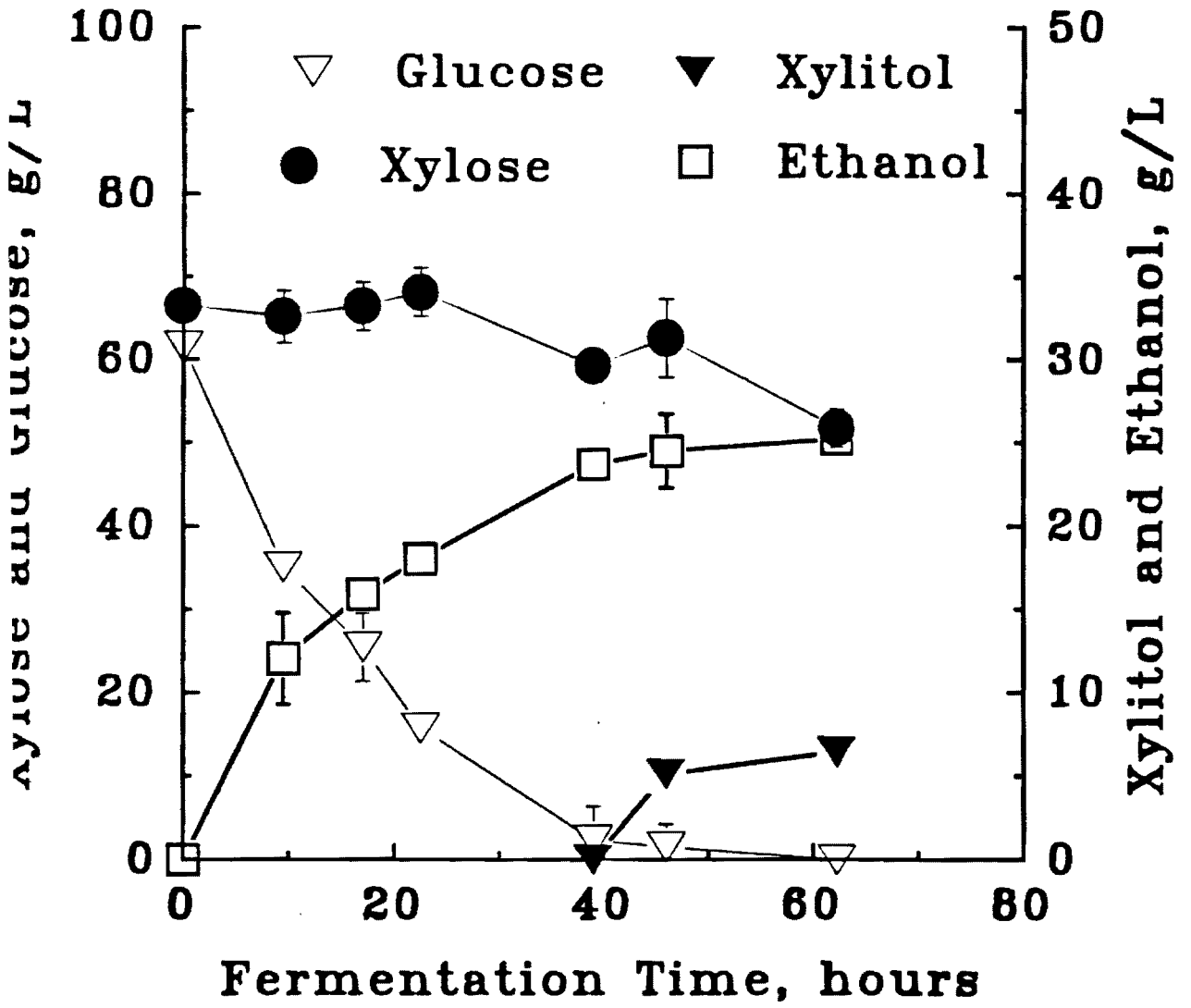


Figure 5 - Batch Fermentation of a mixture of 25% glucose and 75% D-xylose by *Candida shehatae* cells aerobically pre-grown on glucose.

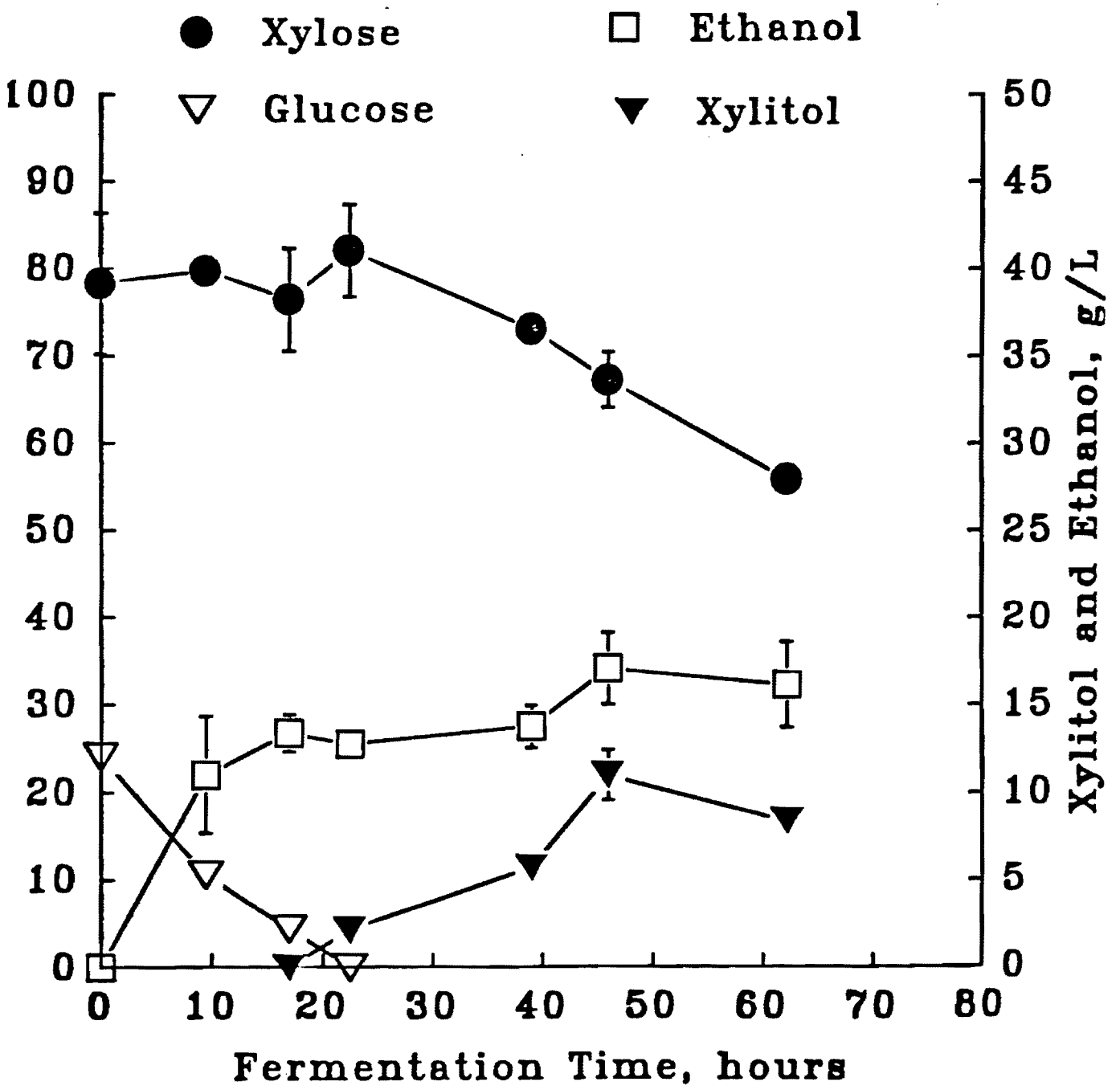


Figure 6 - Batch Fermentation of a mixture of 50% glucose and 50% D-xylose by *Candida shehatae* cells pre-grown aerobically on D-xylose.

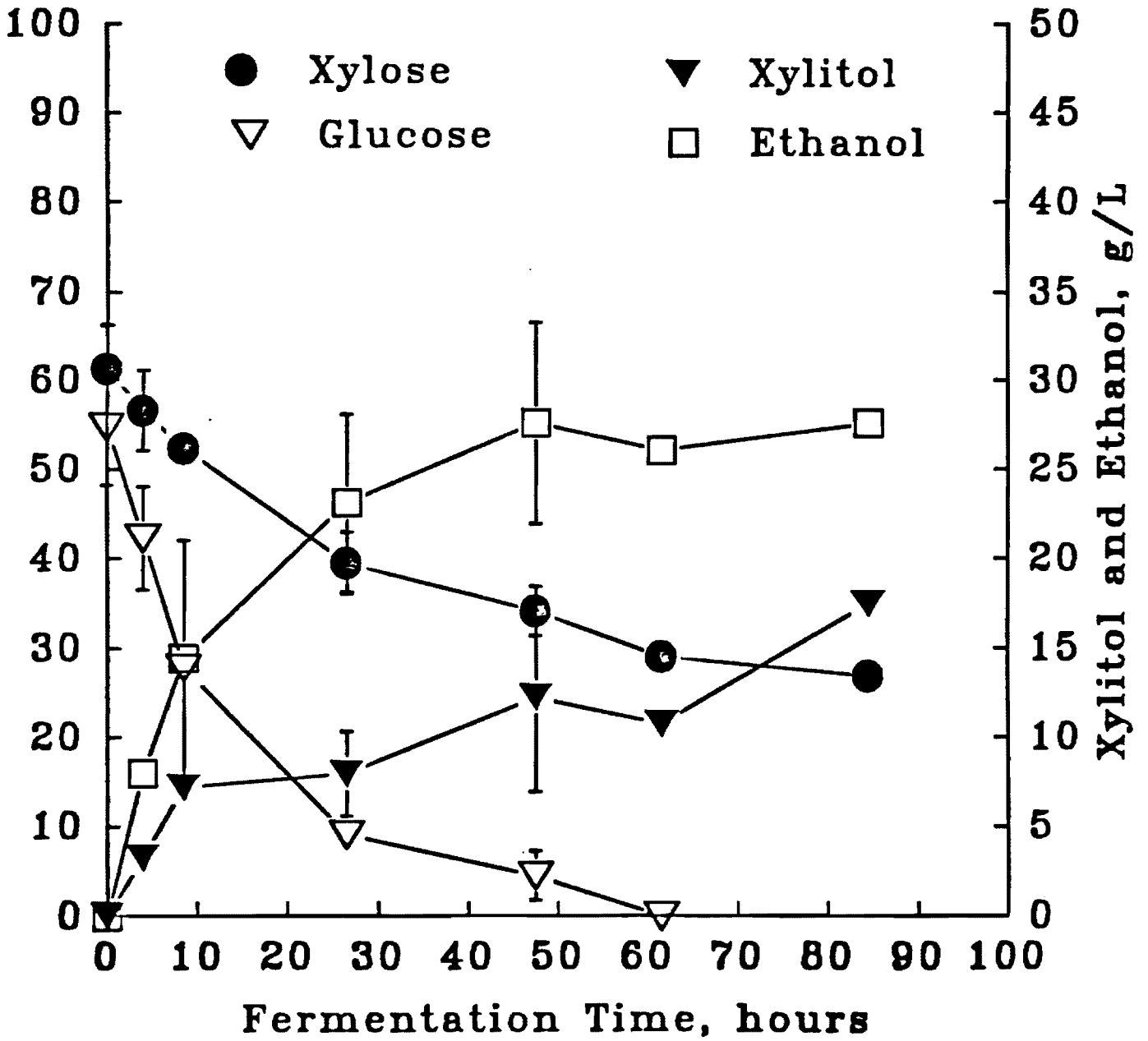


Figure 7 - Batch Fermentation of a mixture of 25% glucose and 75% D-xylose by *Candida shehatae* cells aerobically pre-grown on D-Xylose.

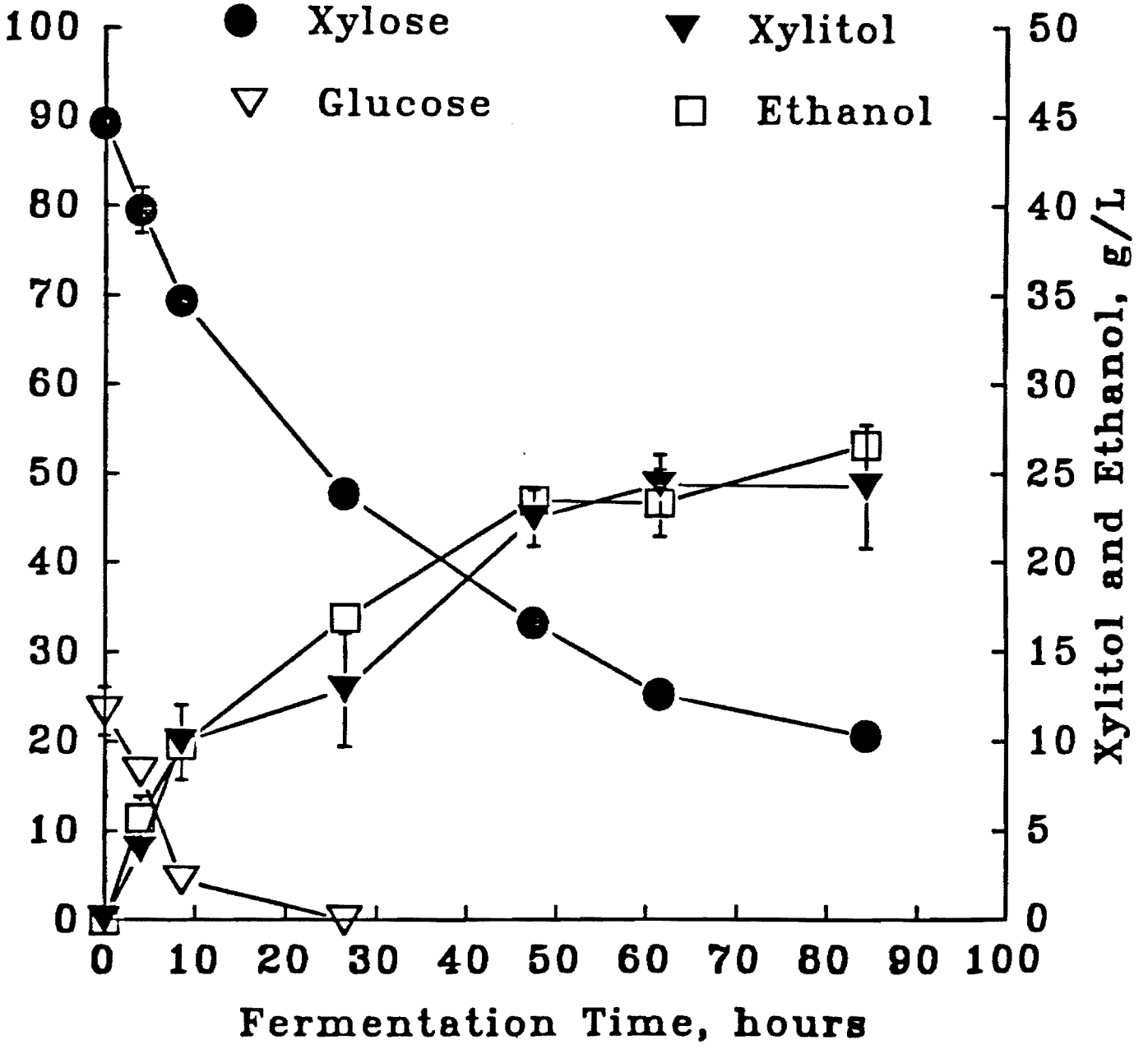


Figure 9 - Viability of *Candida shehatae* cells (as measured by methylene blue staining) in pure D-xylose and mixed sugar fermentations (cells were pre-grown on D-xylose).

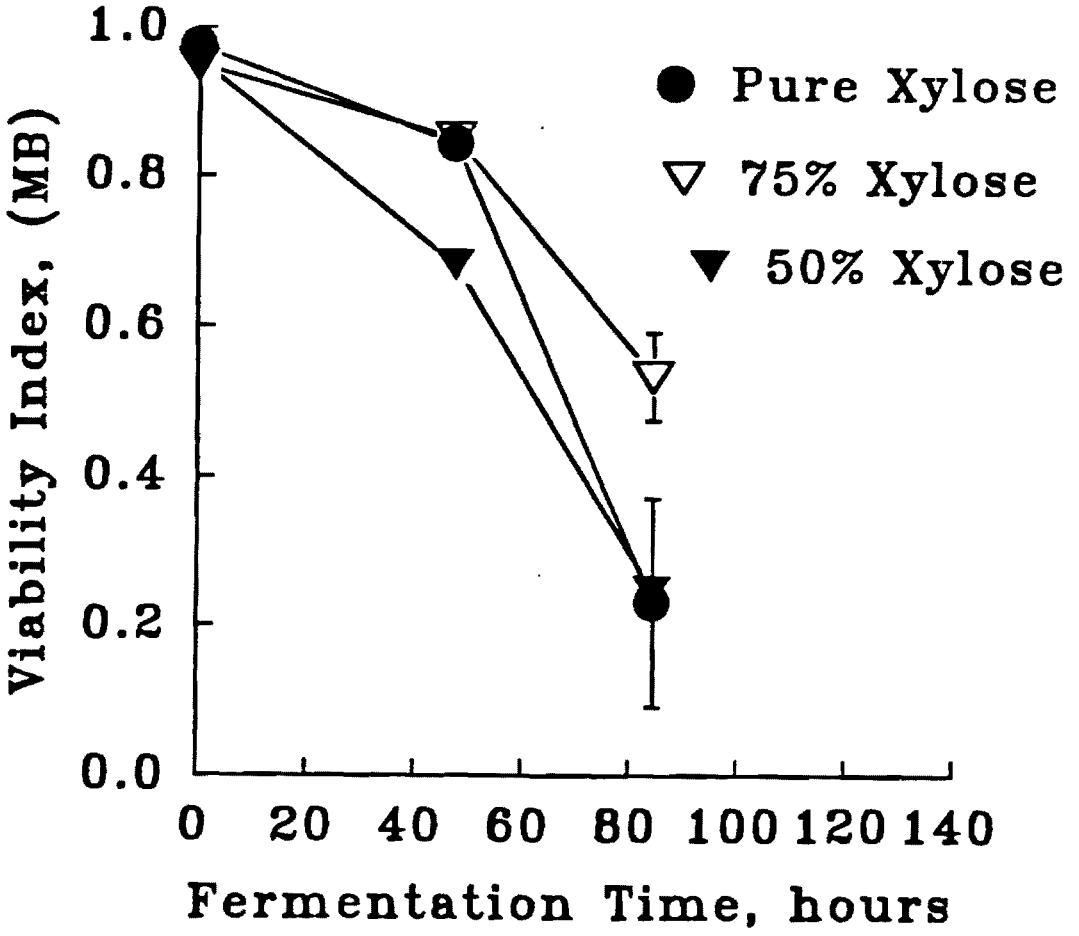
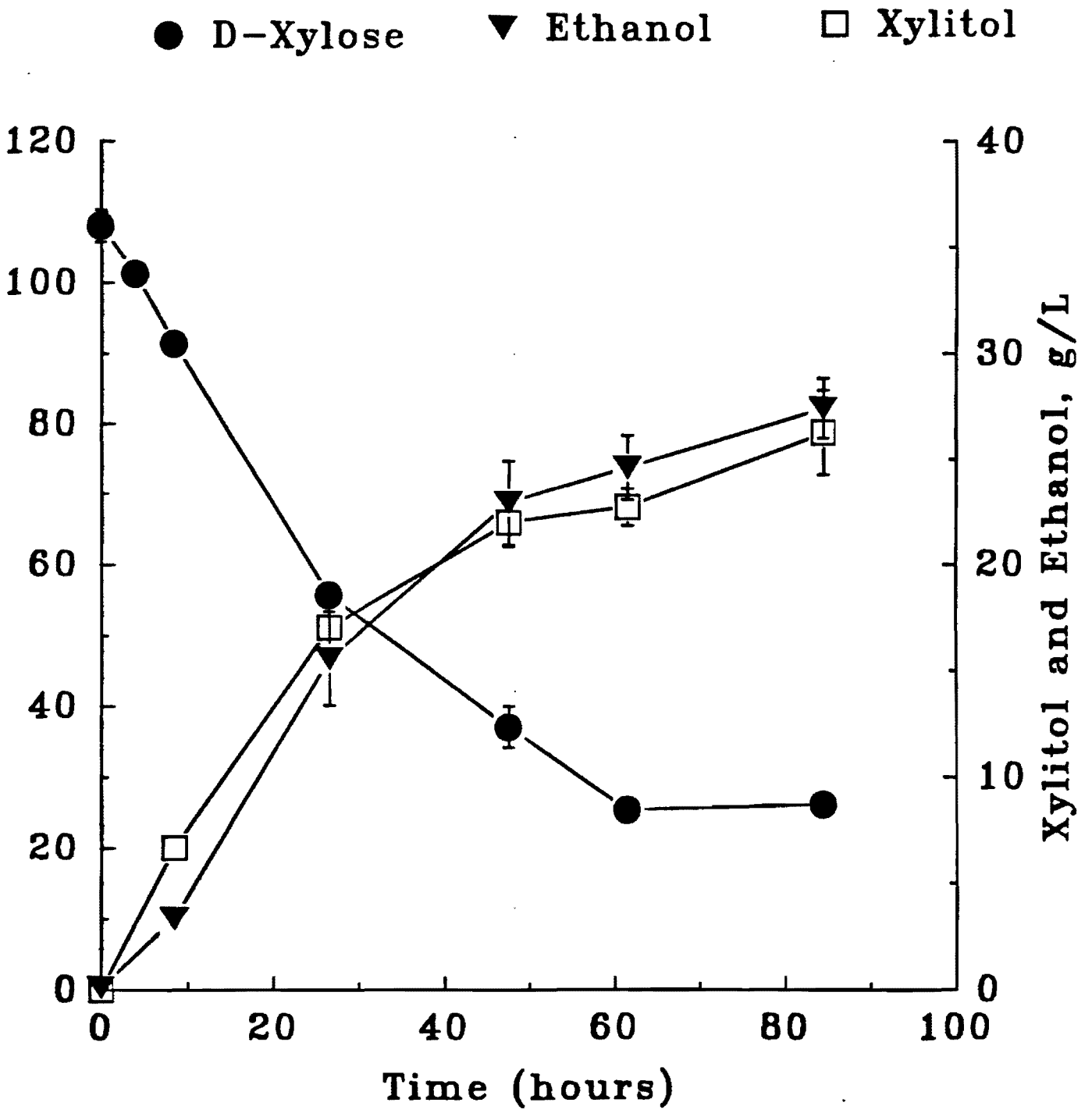


Figure 8 - Batch Fermentation of pure D-xylose by *Candida shehatae* cells aerobically pre-grown on D-Xylose.



b. Variation of Nitrogen Source in Anaerobic D-Xylose Fermentations in an Undefined Medium:

The effect of two different nitrogen sources and the effect of added ethanol were tested on D-xylose fermentations by *Candida shehatae* (See Table 3). As can be seen from Figure 10, growth (cell replication) did not occur regardless of the nitrogen source. When compared to peptone, ammonium sulfate resulted in more xylitol production (see Figures 11 and 12). The addition of ethanol (20 g/L) inhibited D-xylose utilization and further ethanol production. Only 20 g/L of D-xylose was consumed (from 100 g/L D-xylose) and 5 g/L of ethanol and 12.5 g/L of xylitol were produced (Figure 13). Cell viability declined in the fermentation with added ethanol but the rate of decline was no faster than in the fermentation with ammonium sulphate but no added ethanol (Figure 14). When compared to peptone, ammonium sulfate resulted in a faster decline in cell viability (Figure 14).

Table 3 - Medium Composition Used in D-Xylose Fermentations.

	Components Added, g/L					
Reactor	Yeast Extract	Peptone	$(NH_4)_2SO_4$	Ethanol	$Y_{E/S}$	$Y_{X/S}$
1	3	--	5	--	0.286	0.26
2	3	5	--	--	0.32	0.24
3	3	5	--	18	0.25	0.625

$Y_{E/S}$ = Grams of ethanol produced per gram of xylose consumed.
$Y_{X/S}$ = Gram of xylitol produced per gram of xylose consumed.

Figure 10 - Effect of different nitrogen sources and added ethanol on cell population levels during D-xylose fermentations.

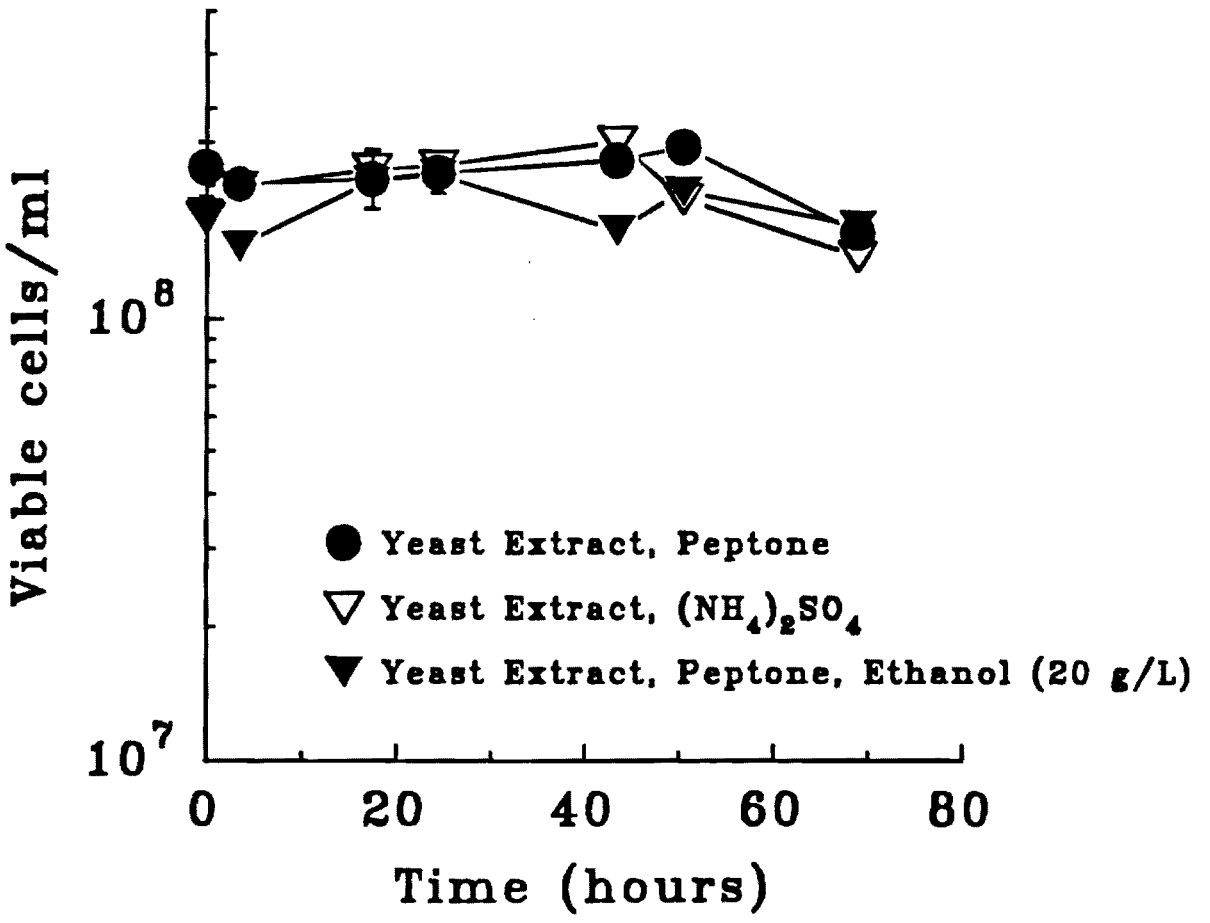


Figure 11 - D-Xylose Fermentation on Yeast Extract and Peptone as the Nitrogen Source.

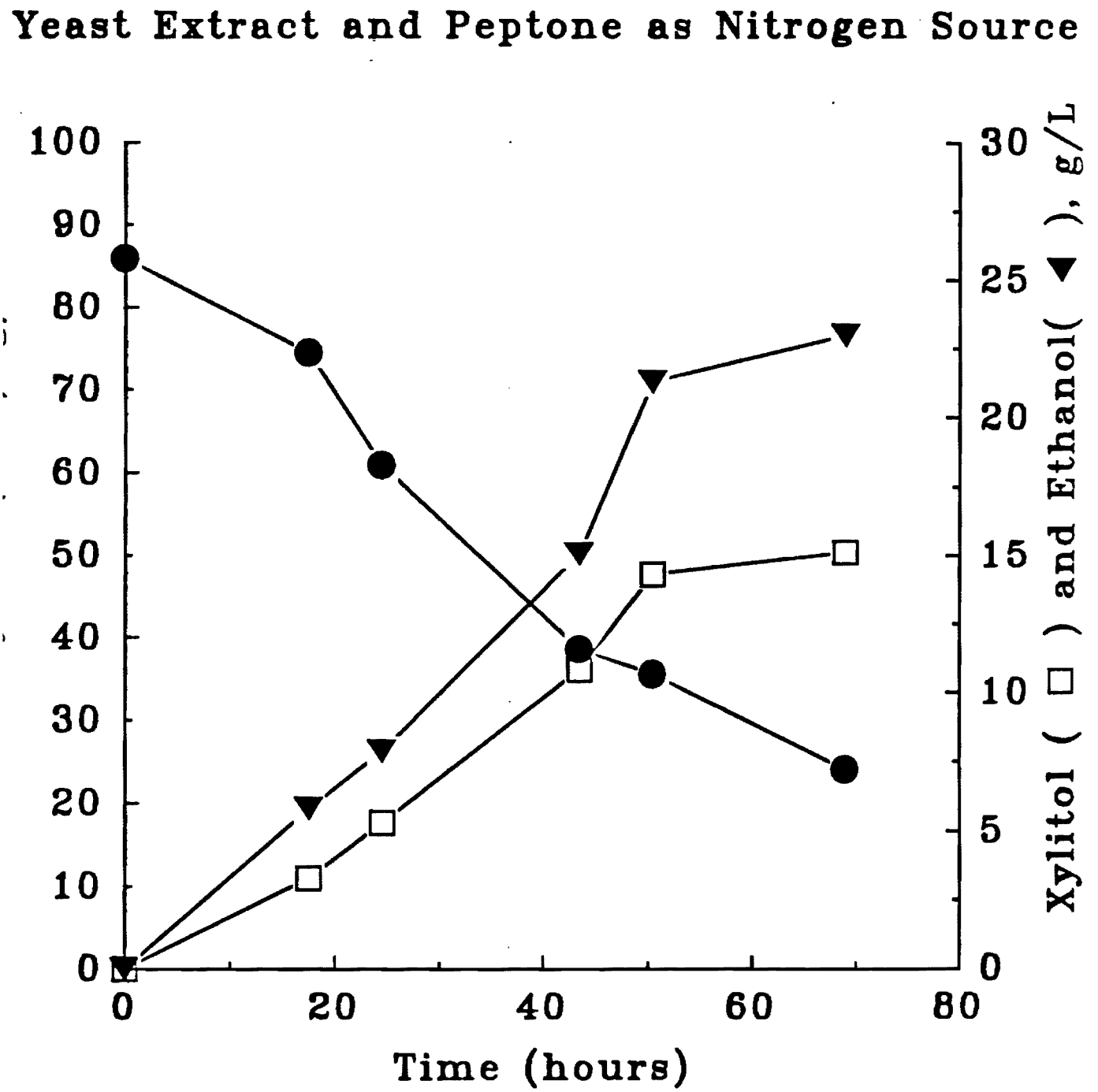


Figure 12 - D-Xylose Fermentation on Yeast Extract and $(NH_4)_2SO_4$ as the Nitrogen Source.

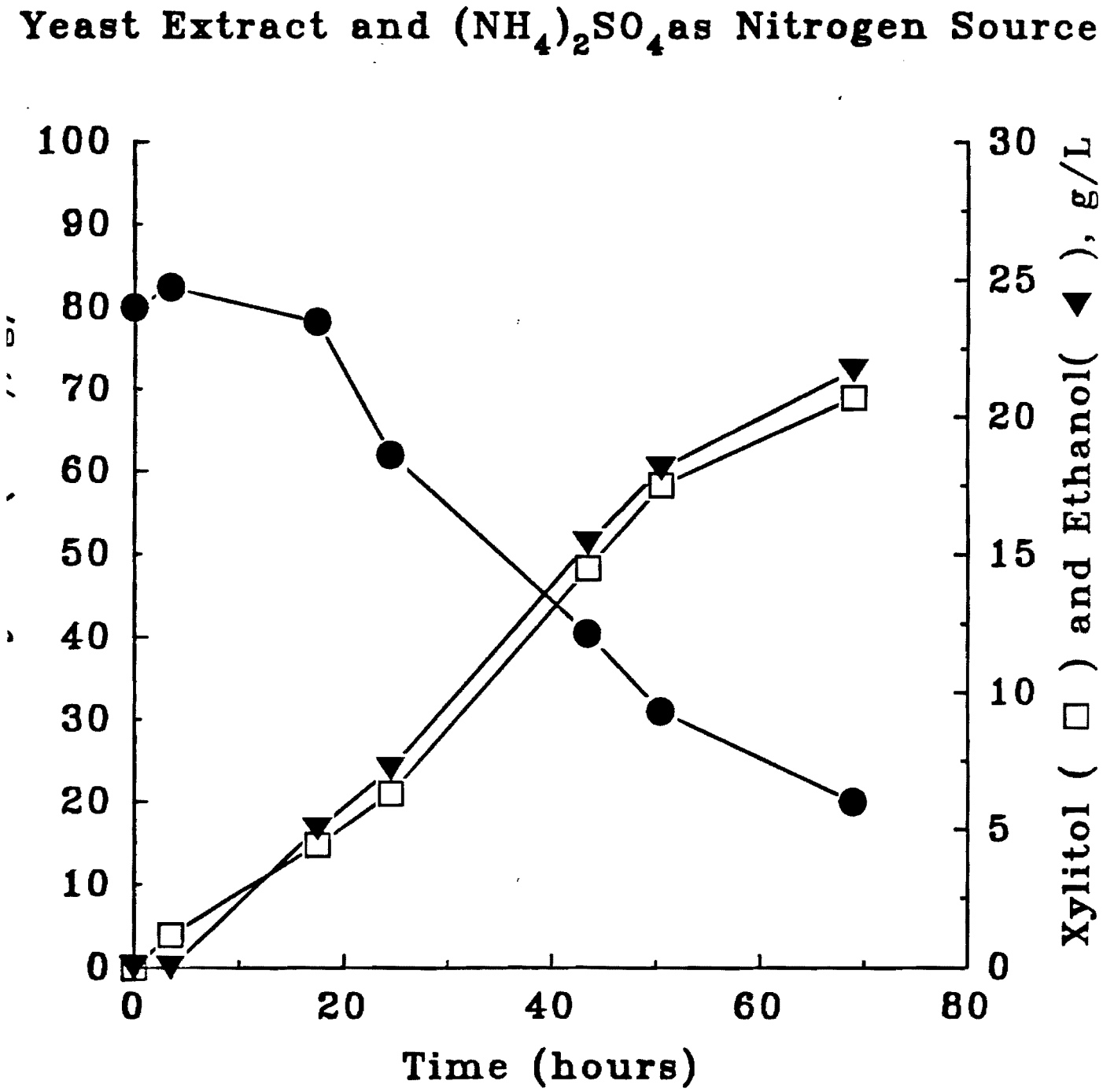


Figure 13 - D-Xylose Fermentation on Yeast Extract and Peptone as the Nitrogen Source with 20 g/L of Added Ethanol at Time Zero.

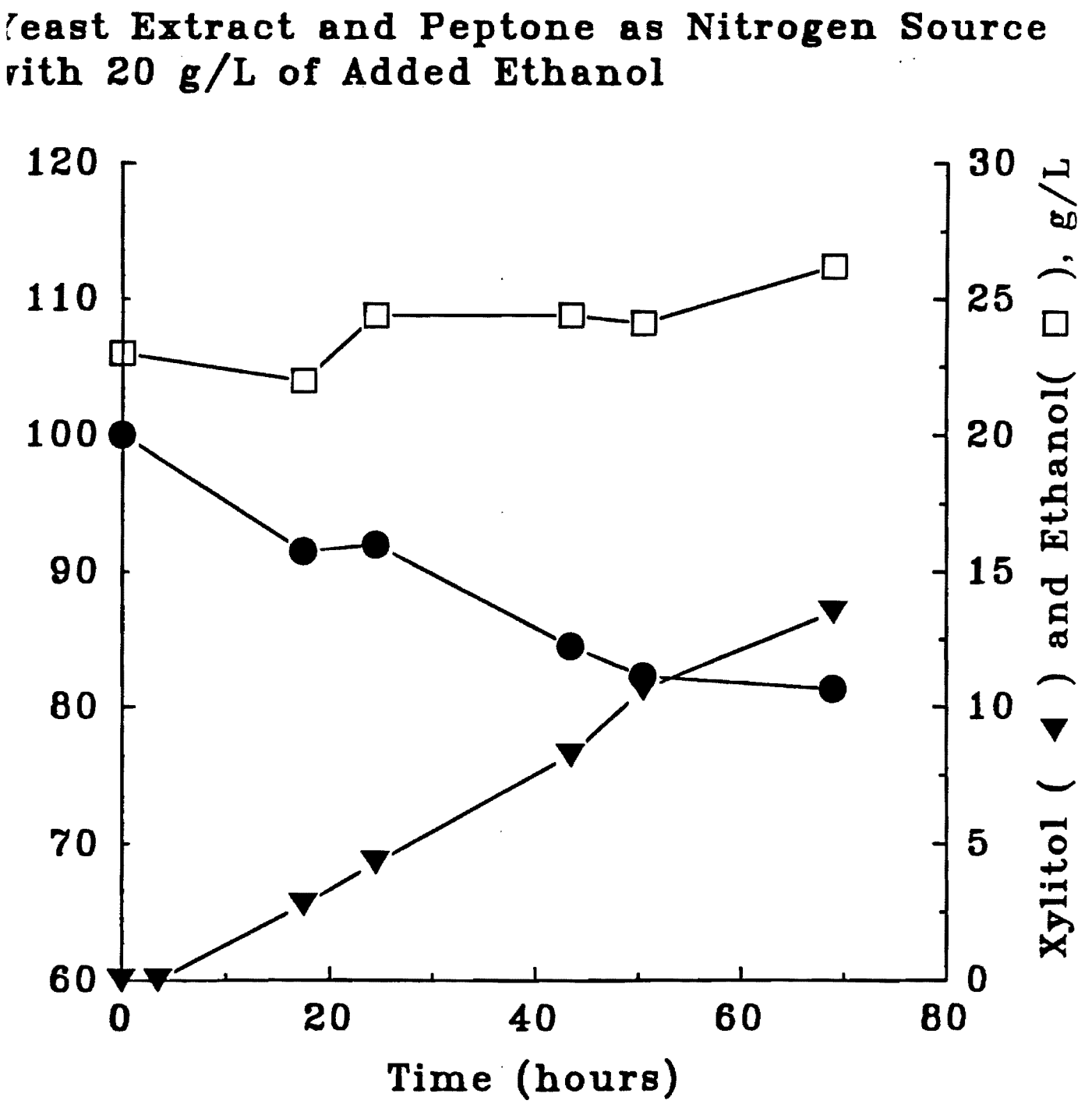
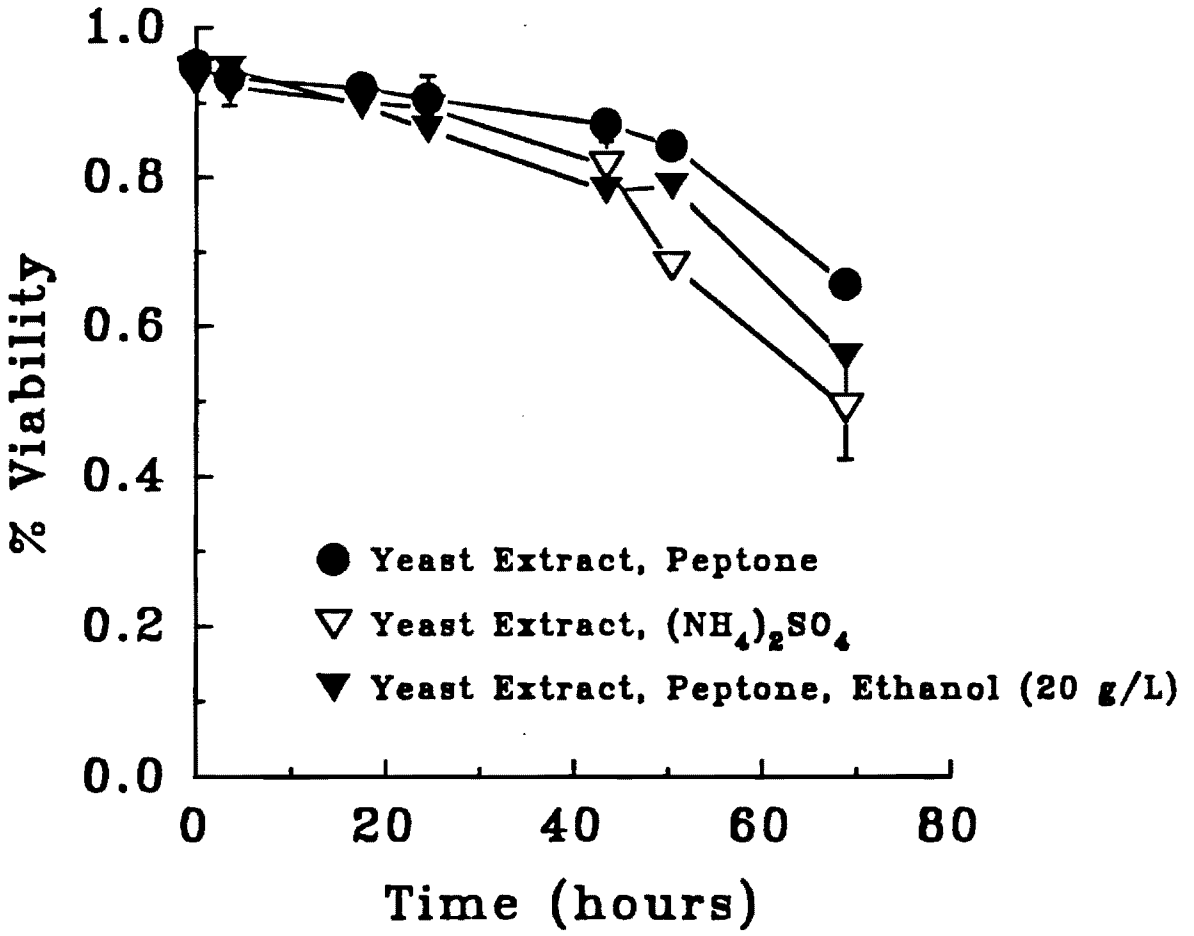


Figure 14 - Effect of Different Nitrogen Sources and Added Ethanol on Cell Viability During D-Xylose Fermentations.



c. Conclusions and Rational for the Next Research Step

If preconditioned on D-xylose, *Candida shehatae* can simultaneously ferment D-xylose and glucose to ethanol. The consumption rate of D-xylose was dependent on the glucose concentration; the rate of D-xylose consumption decreased when the initial glucose concentration was increased. Also, D-xylose utilization was incomplete (whether the cells were preconditioned on D-xylose or glucose) and correlated with the decline in cell viability. *Candida shehatae* did not grow (in terms of cell replication) with yeast extract, peptone or ammonium sulphate as the nitrogen source. The reason for the lack of growth and for the decline in cell viability was unclear. The lack of growth may have been due to a oxygen limitation (anaerobic conditions) or possibly due to a nutritional limitation. A nutritional limitation could have also caused the decline in cell viability and incomplete D-xylose utilization.

A defined medium was formulated (Table 4), to see if the fermentations were nutrient limited and if D-xylose metabolism could be altered to produce less xylitol. The type of nitrogen source did seem to affect the amount of xylitol formation. Less xylitol, a by-product of anaerobic D-xylose fermentation, was formed with peptone as the nitrogen source compared to $(NH_4)_2SO_4$. A defined medium will help identify the component(s) in peptone responsible for this change in metabolism. Also, if the previous medium was nutrient limited, a defined medium may increase ethanol levels (greater than 25 g/L) and increase the rate of ethanol production.

Table 4. Composition of the Defined Medium Used in D-Xylose Fermentations

Component	Concentration, per liter
D-xylose	50 g (seed culture)
	100 g (fermentations)
$(NH_4)_2SO_4$	10 g
citric acid	0.5 g
mineral salts and trace elements	
KH_2PO_4	2.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.05 g
$FeSO_4 \cdot 7H_2O$	35 mg
$MnSO_4 \cdot H_2O$	7 mg
$ZnSO_4 \cdot 7H_2O$	11 mg
$CuSO_4 \cdot 5H_2O$	1 mg
$CoCl_2 \cdot 6H_2O$	2 mg
$Na_2MoO_4 \cdot 2H_2O$	1.3 mg
H_3BO_3	2 mg
KI	0.35 mg
$Al_2(SO_4)_3$	0.5 mg
vitamins	
thiamine•HCl	5 mg
pyridoxine•HCl	5 mg
nicotinic acid	5 mg
p-aminobenzoic acid	1 mg
meso-inositol	100 mg
Ca-pantothenate	20 mg
d-biotin	0.1 mg

d. Test of Medium Supplements for Stimulation of Anaerobic Growth Controlled Batch D-Xylose

In this set of experiments different compounds were added to the defined medium (Table 4) and tested to see if they would stimulate anaerobic growth of *Candida shehatae* on glucose. If a compound had been found which stimulated growth its effect on D-xylose metabolism would have been tested. The results for anaerobic conditions were compared to data from aerobic conditions for *C. shehatae* and with results for *Saccharomyces cerevisiae* (Brazilian Usina alcohol strain).

The inoculum for these experiments was prepared on the defined medium (glucose). Anaerobic tubes were prepared by sparging with N_2 and inoculations were performed inside an anaerobic chamber. The fermentations were performed on 20 g/L of glucose. Each component tested was added to a base medium (Table 4) which contained biotin, pantothenate, inositol, nicotinic acid, p-aminobenzoic acid, pyridoxine, and thiamine. A novel mixture of vitamins was tested and consisted of lipoic acid, riboflavin, folic acid and vitamin B12.

These vitamins did not stimulate anaerobic growth in *Candida shehatae* (as measured by light scattering or absorbance at 660 nm, Figure 15). Casamino acids and the individual amino acids, asparagine and glutamine, did not stimulate anaerobic growth in *C. shehatae* (Figure 15). It was found that *S. cerevisiae* grew well under anaerobic conditions if given vitamins (Figure 16). Casamino acids or asparagine plus glutamine were found to stimulate anaerobic growth in *S. cerevisiae* (Figure 16). Under aerobic conditions, *Candida shehatae* could grow to a high cell density (as measured by absorbance at 660 nm) without vitamins and amino acids (Figure 17). However, vitamins did stimulate growth and

Figure 15 - Anaerobic Growth of *Candida shehatae* on Glucose.

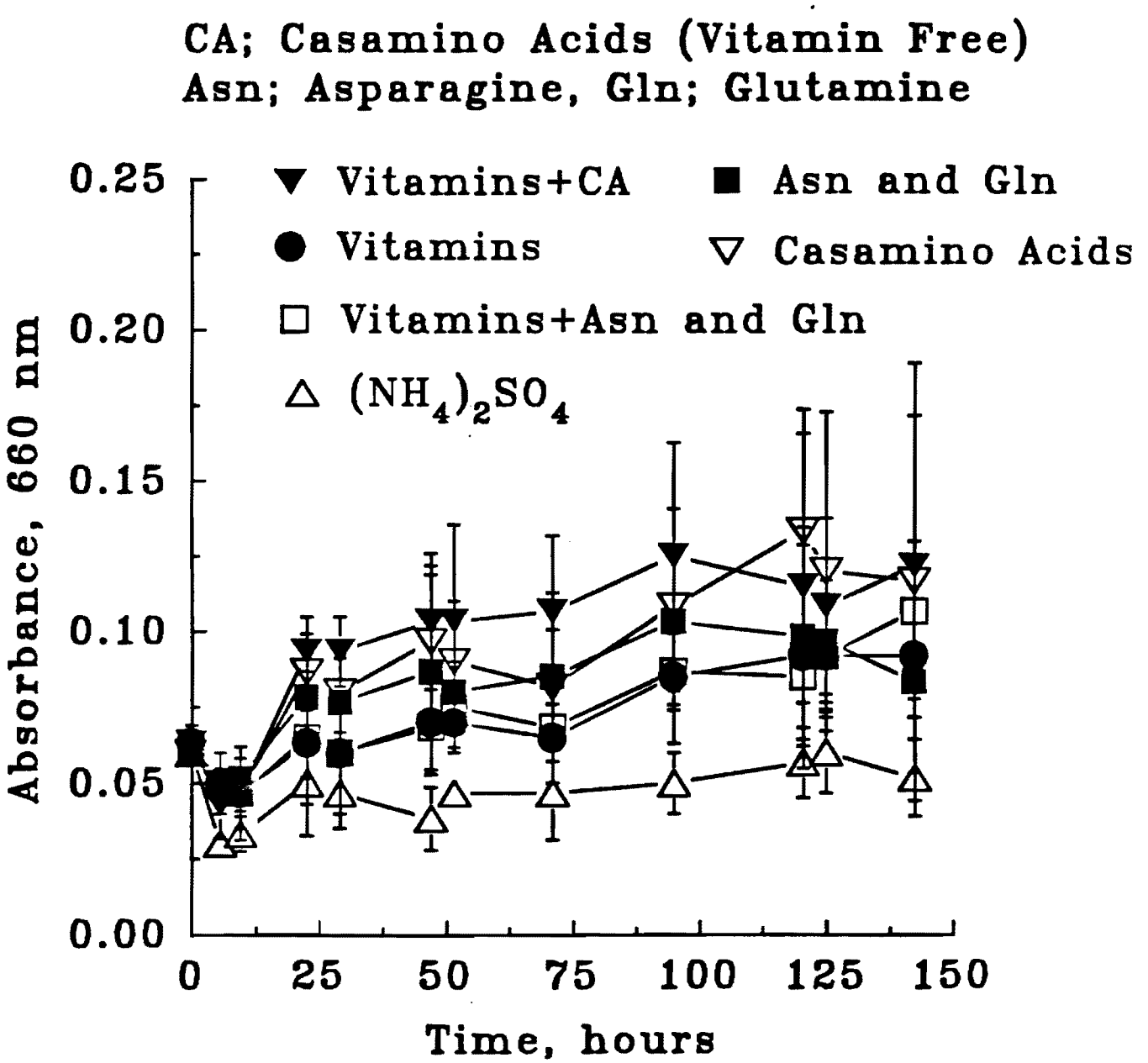


Figure 16 - Anaerobic Growth of Saccharomyces cerevisiae on Glucose.

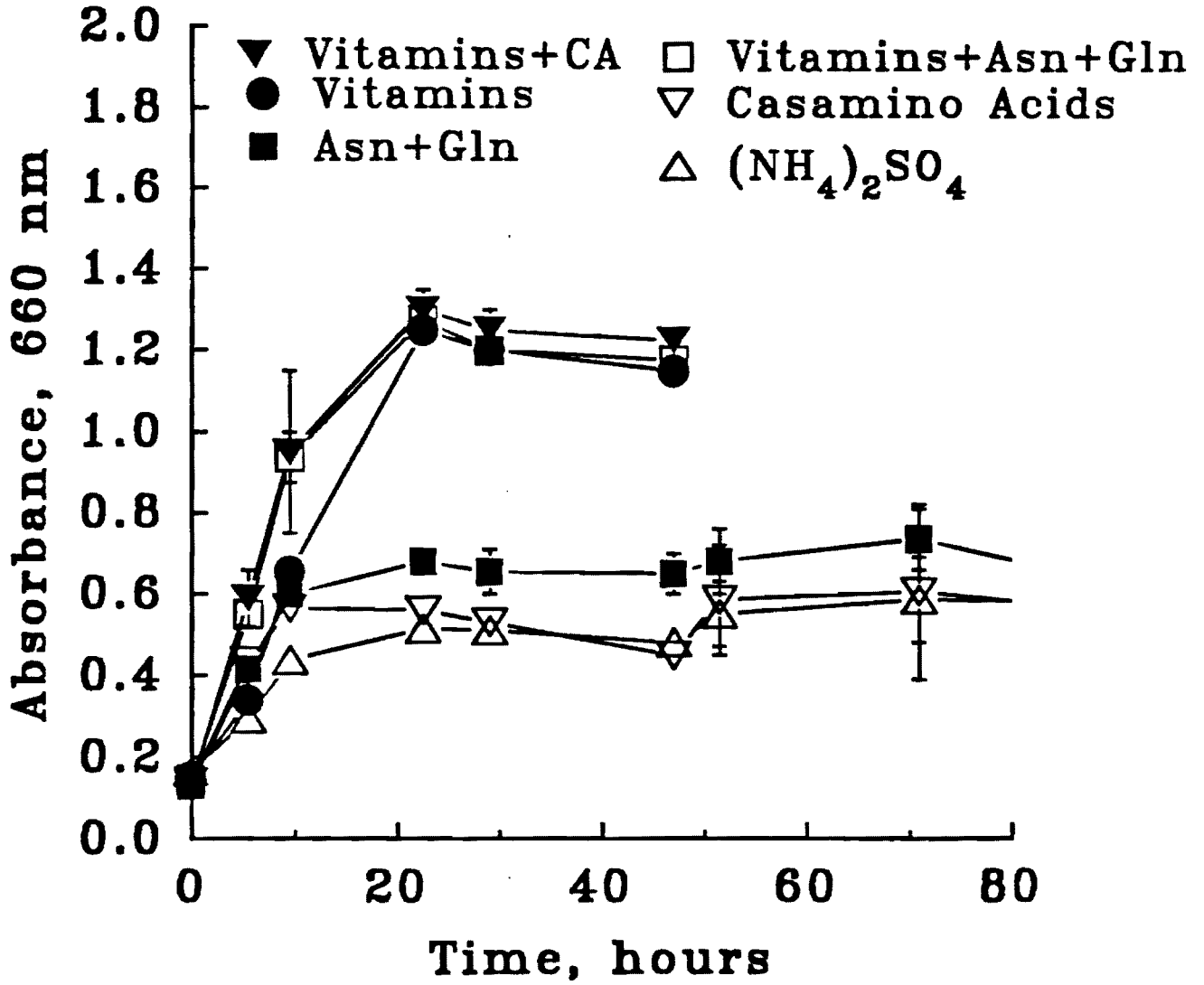
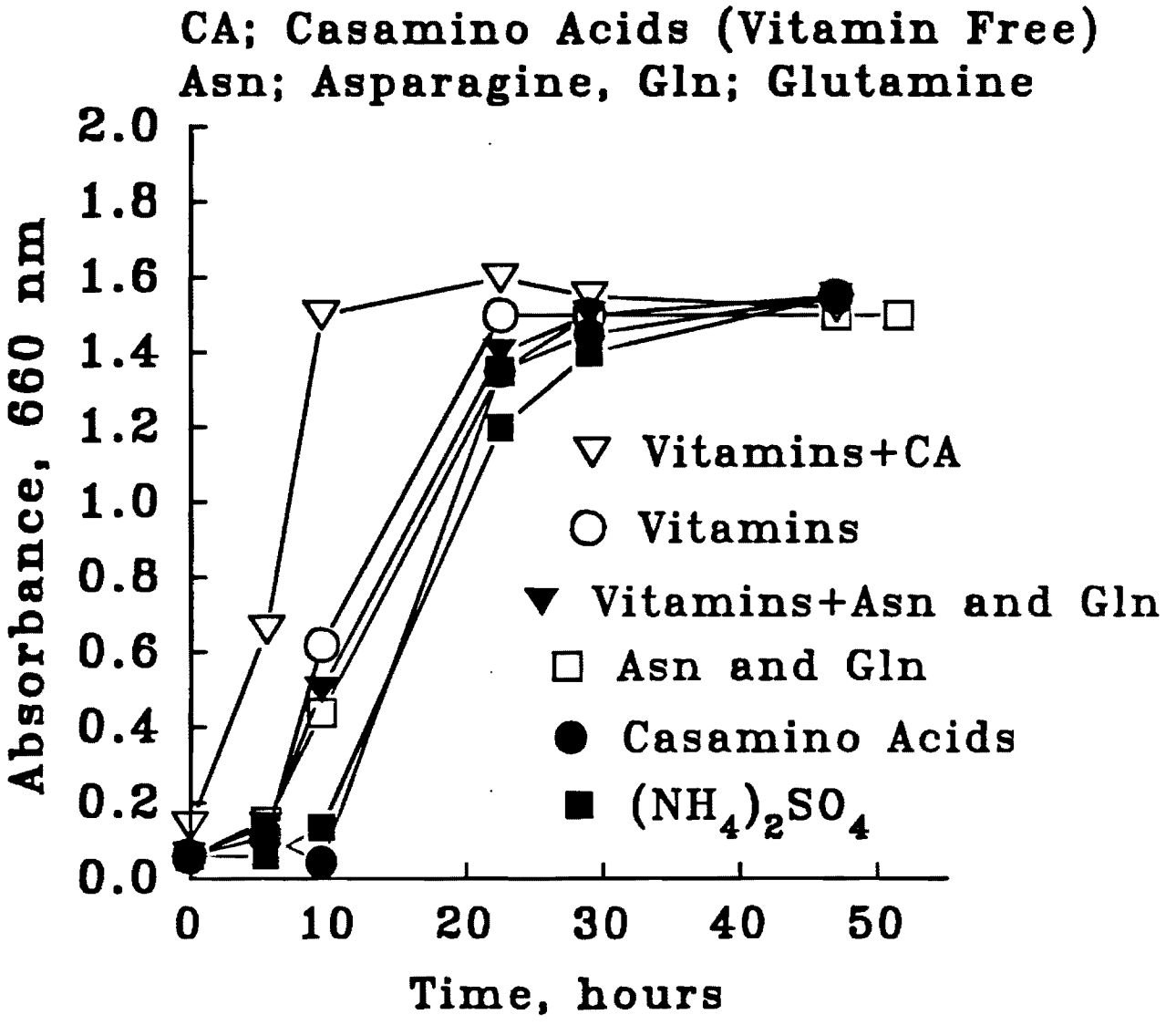


Figure 17 - Aerobic Growth of *Candida shehatae* on Glucose.



reduced the lag time before the onset of growth. *Saccharomyces cerevisiae* grew well with vitamins and casamino acids but grew poorly on glutamine and asparagine and $(NH_4)_2SO_4$ (Figure 18). Compared to *Candida shehatae*, under aerobic conditions, *Saccharomyes cerevisiae* did not grow as well without vitamins and a source of amino acids.

The data show that there is a fundamental metabolic difference between *C. shehatae* and *Saccharomyces cerevisiae* under anaerobic conditions. *Saccharomyces cerevisiae* can grow under anaerobic conditions while the growth of *Candida shehatae* under anaerobic conditions is severely limited or completely inhibited (compare Figures 19 and 20). In addition, it was found that *Saccharomyces cerevisiae* could grow extended periods of time without oxygen (up to 300 hours in the presence of low ethanol concentrations, Figure 21).

e. Batch Fermentations with a Defined Medium: pH and temperature controlled D-xylose fermentations by *C. shehatae* and *P. stipitis*

Batch fermentations were performed on a defined medium (Table 4) with *Candida shehatae* and *Pichia stipitis*. *Pichia stipitis* is another yeast which can ferment D-xylose. *Pichia stipitis* is reported to produce lower levels of xylitol than *Candida shehatae*. *Candida shehatae* or *Pichia stipitis* cells were inoculated into a NewBrunswick batch reactor at a relatively low cell density. The reactor was aerated at 1.4 liters/min and agitated at 550 rpm. The reactor had a working volume of 1 liter. The temperature was controlled at 30°C and the pH was controlled at 4.5. As the cell density increased the percent dissolved oxygen (%D.O.) decreased and after approximately 20-24 hours the %D.O. reached zero

Figure 18 - Aerobic Growth of *Saccharomyces cerevisiae* on Glucose.

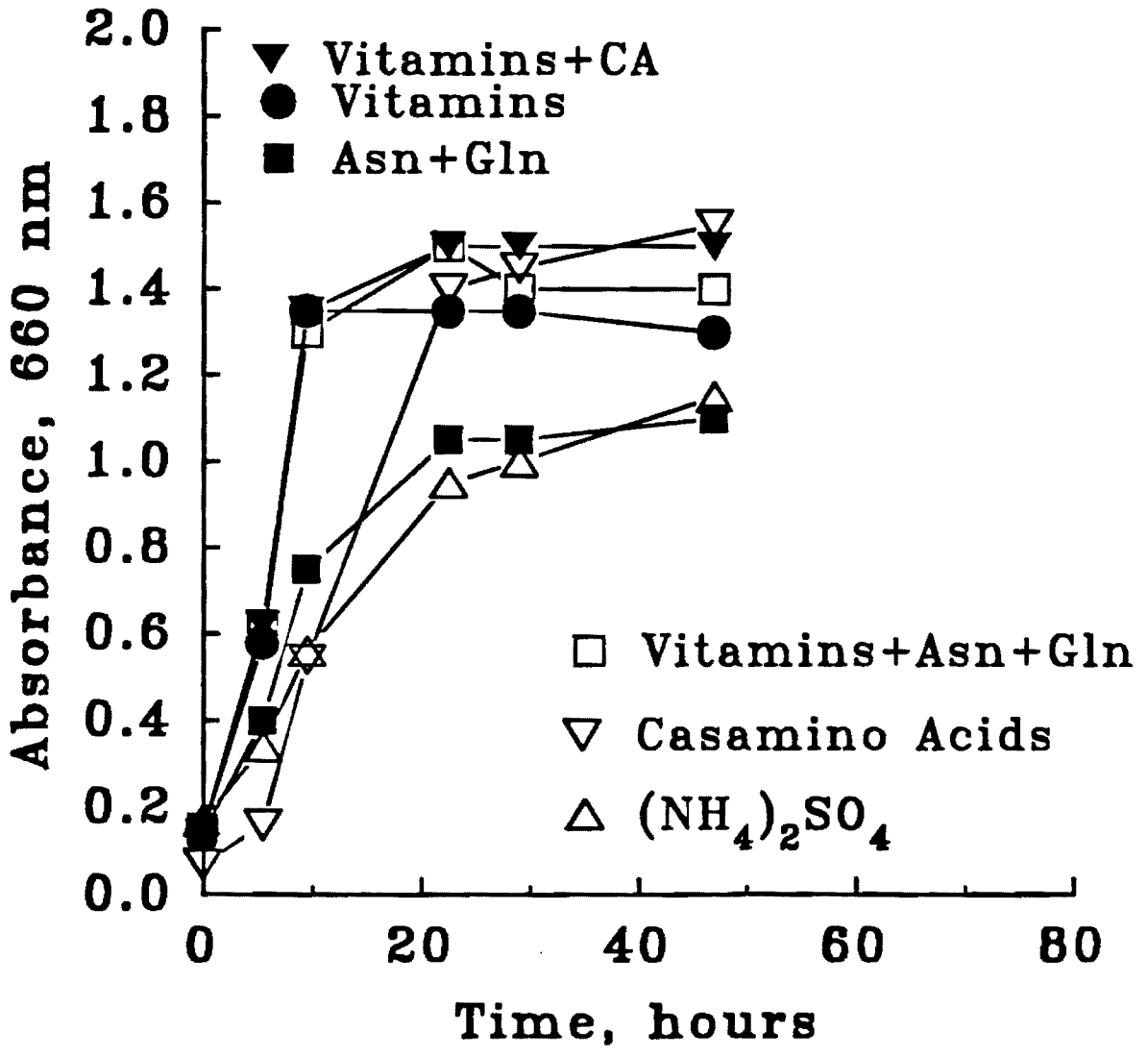


Figure 19 - Comparison of Anaerobic and Aerobic Growth on Vitamins and Casamino Acids for *Candida shehatae*.

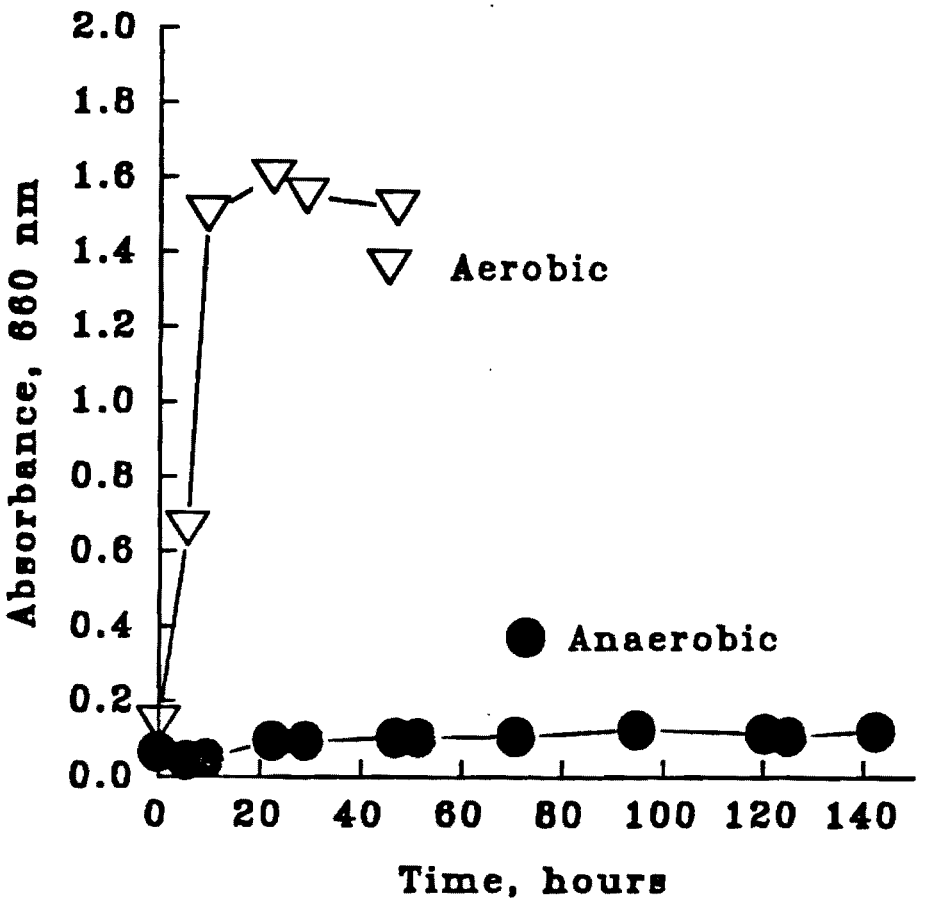


Figure 20 - Comparison of Anaerobic and Aerobic Growth on Vitamins and Casamino Acids for *Saccharomyces cerevisiae*.

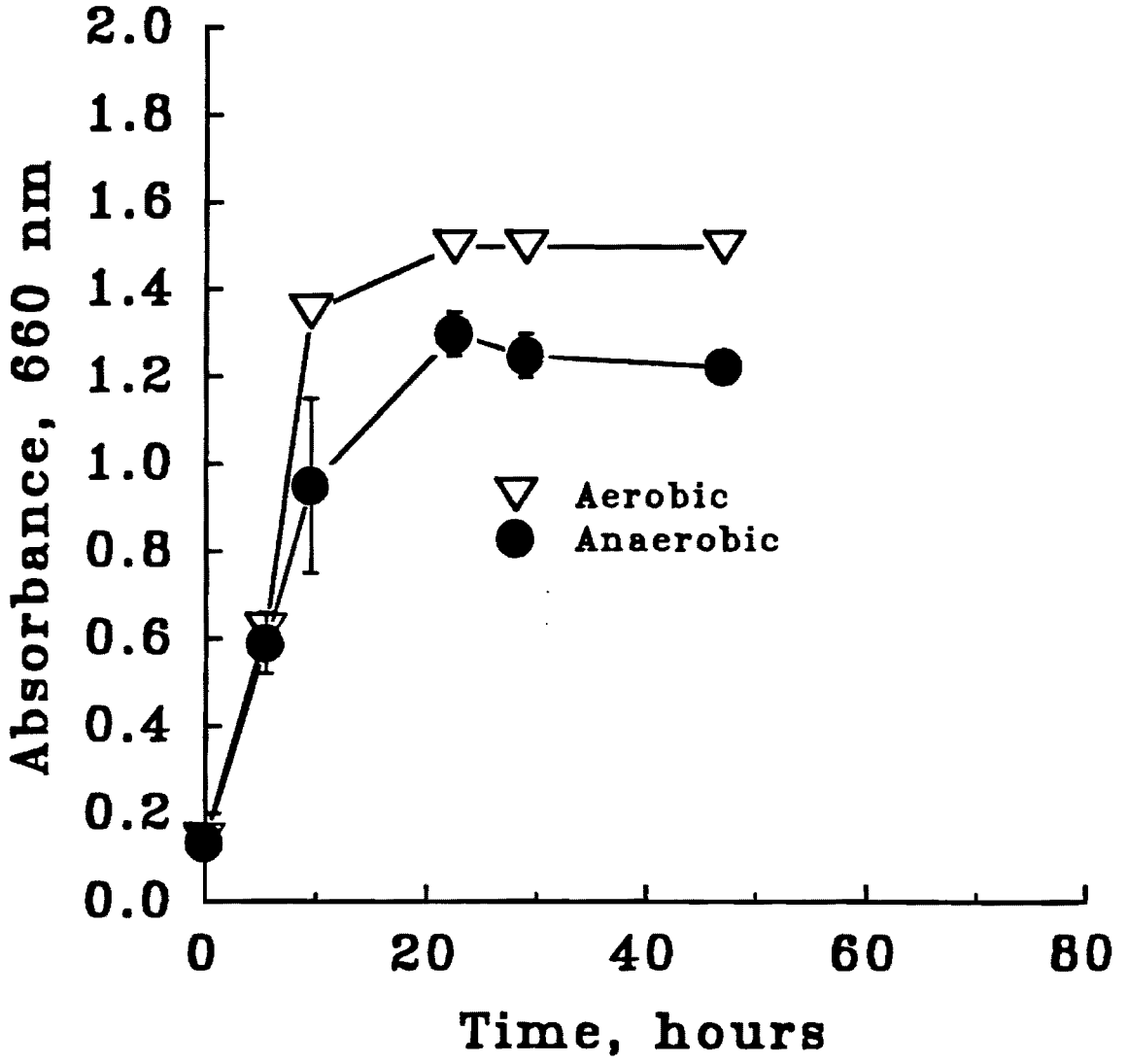
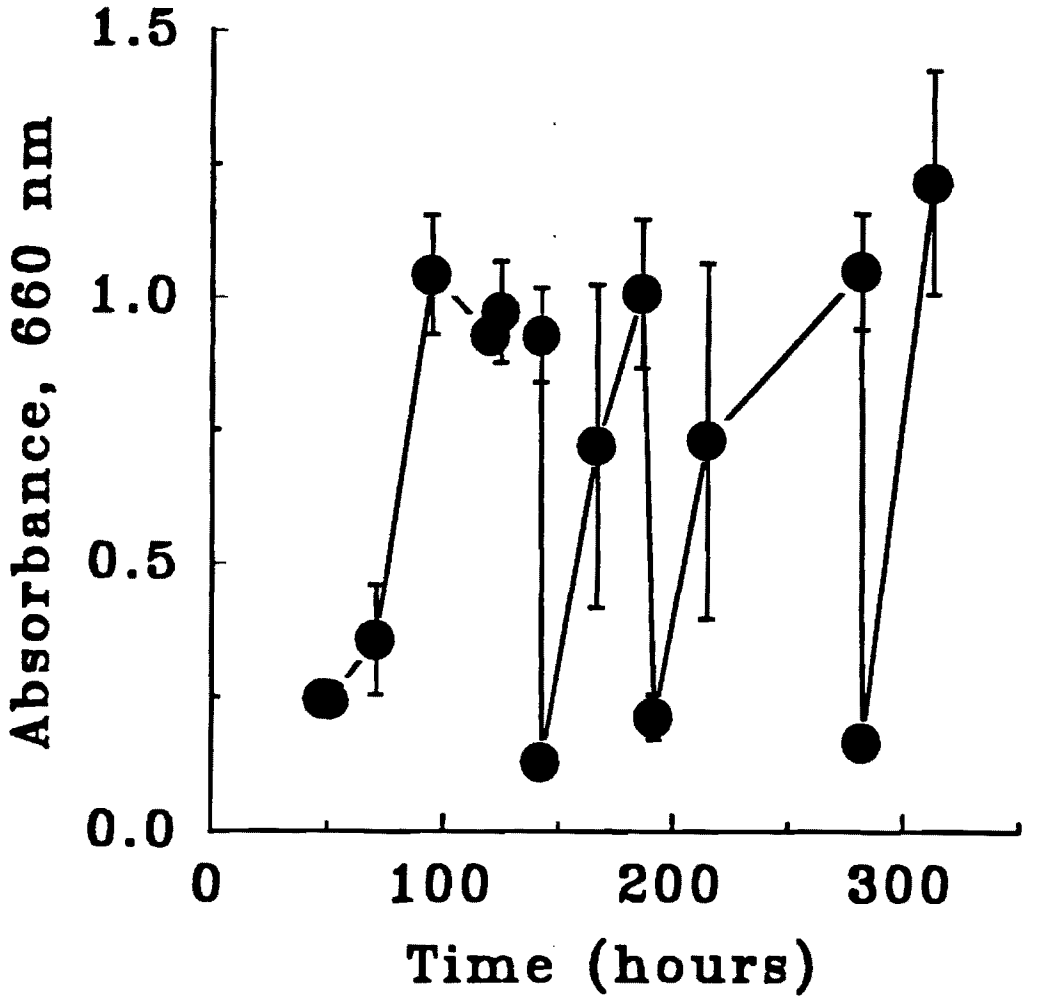


Figure 21 - Anaerobic Growth of *Saccharomycescerevisiae* on a Defined Medium with Vitamins and Casamino Acids and Glucose as the Substrate (the cultures were periodically transferred to new tubes to prevent substrate limitation).



(Figure 22). At this point aeration was terminated and the agitation reduced to 250 rpm. Cell viability was monitored by methylene blue staining.

Candida shehatae completely utilized the D-xylose within 50 hours to produce 27 g/L of ethanol and 13 g/L of xylitol (Figure 23). The yield of ethanol and xylitol, calculated from 21 hours to 50 hours was 0.29 g ethanol/ g xylose consumed ($Y_{E/S}$) and 0.17 g xylitol/g xylose consumed ($Y_{X/S}$) respectively. The rate of xylose utilization, calculated from 21 hours to 30 hours was 6 X 10^{-12} g/cell-hr for *Candida shehatae*. Cell viability declined late in the fermentation as measured by methylene blue staining (Figure 24; Lee et. al., 1981).

Pichia stipitis achieved approximately the same cell density as *Candida shehatae* (Figure 25). *Pichia stipitis* did not completely utilize the D-xylose and its D-xylose utilization rate was slower than that of *C. shehatae* under anaerobic conditions; *P. stipitis* consumed D-xylose at a rate of 0.78 X 10^{-12} g/cell-hr (Figure 26). The lower D-xylose utilization rate of *P. stipitis* resulted in a longer fermentation time (120 hours) and incomplete D-xylose utilization. *Pichia stipitis* did produce a lower amount of xylitol (10 g/L and $Y_{X/S}$=0.112 g xylitol/g xylose consumed) and 28 g/L of ethanol. The cell viability of *P. stipitis* at the end of fermentation (120 hours) was 0.70 which was much lower than the value of 0.85 for *C. shehatae* (compare Figures 24 and 25).

Figure 22 - Batch D-Xylose Fermentation by *Candida shehatae* on a Defined Medium.

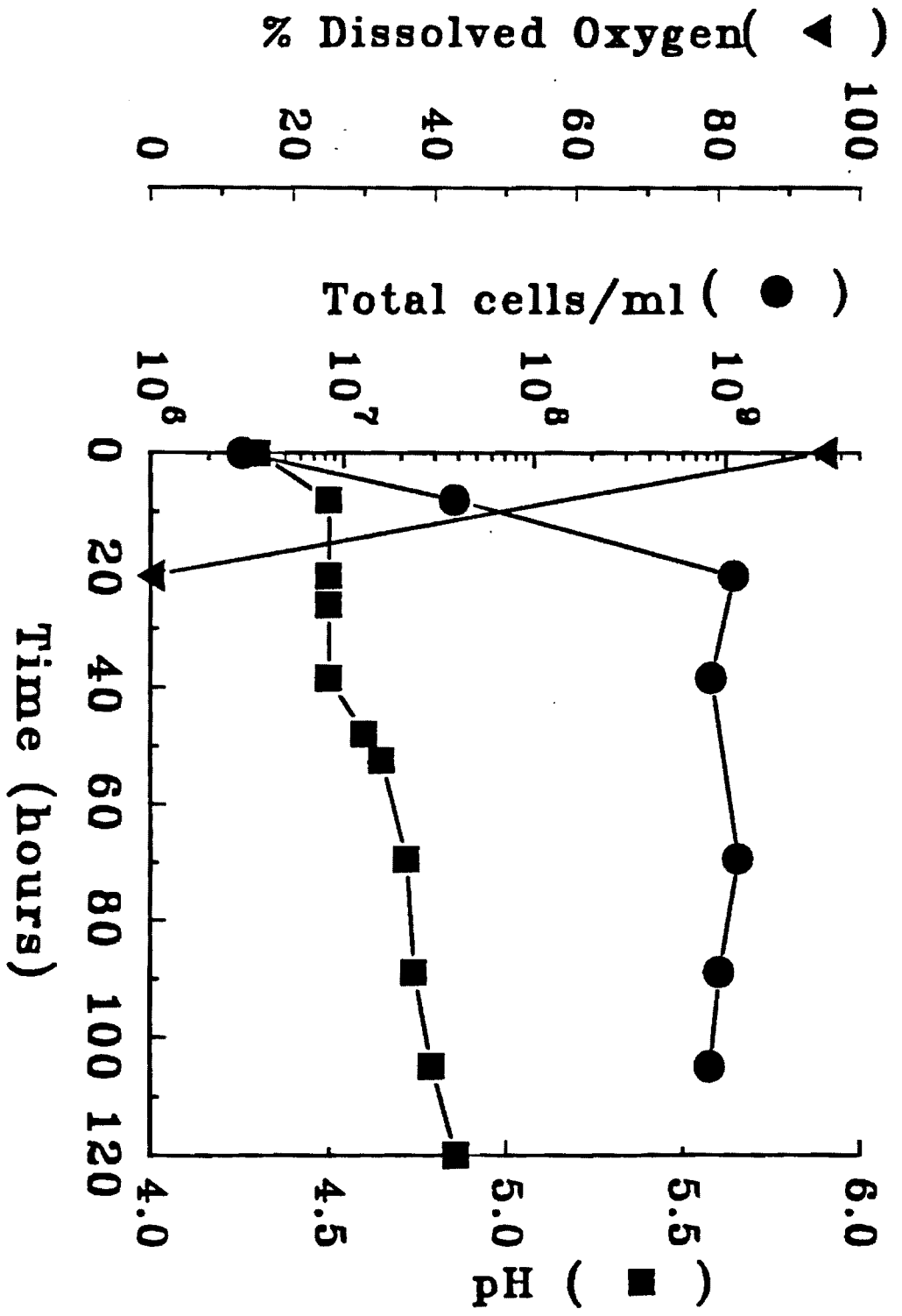
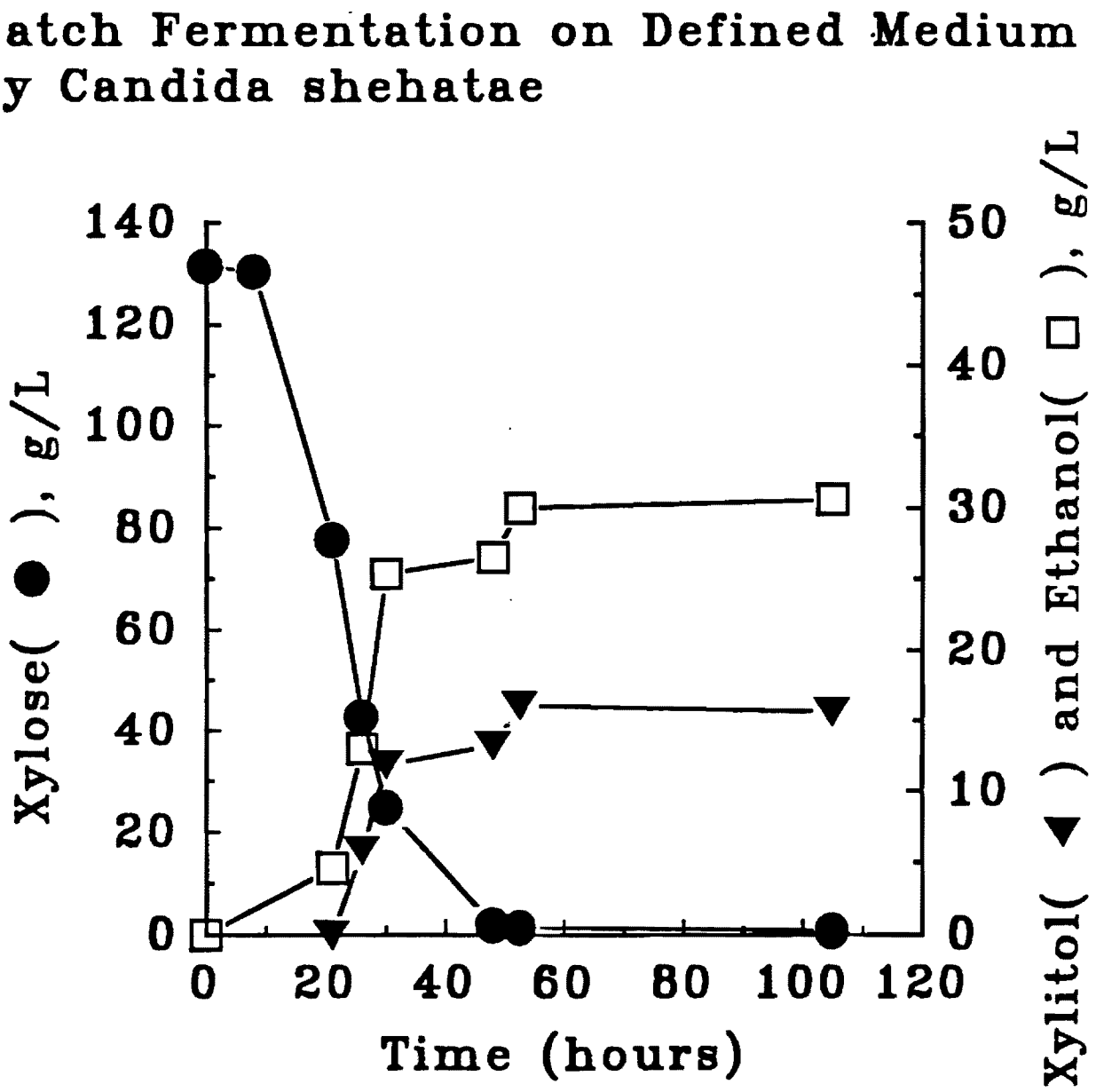


Figure 23 - Batch D-Xylose Fermentation on a Defined Medium by *Candida shehatae*.



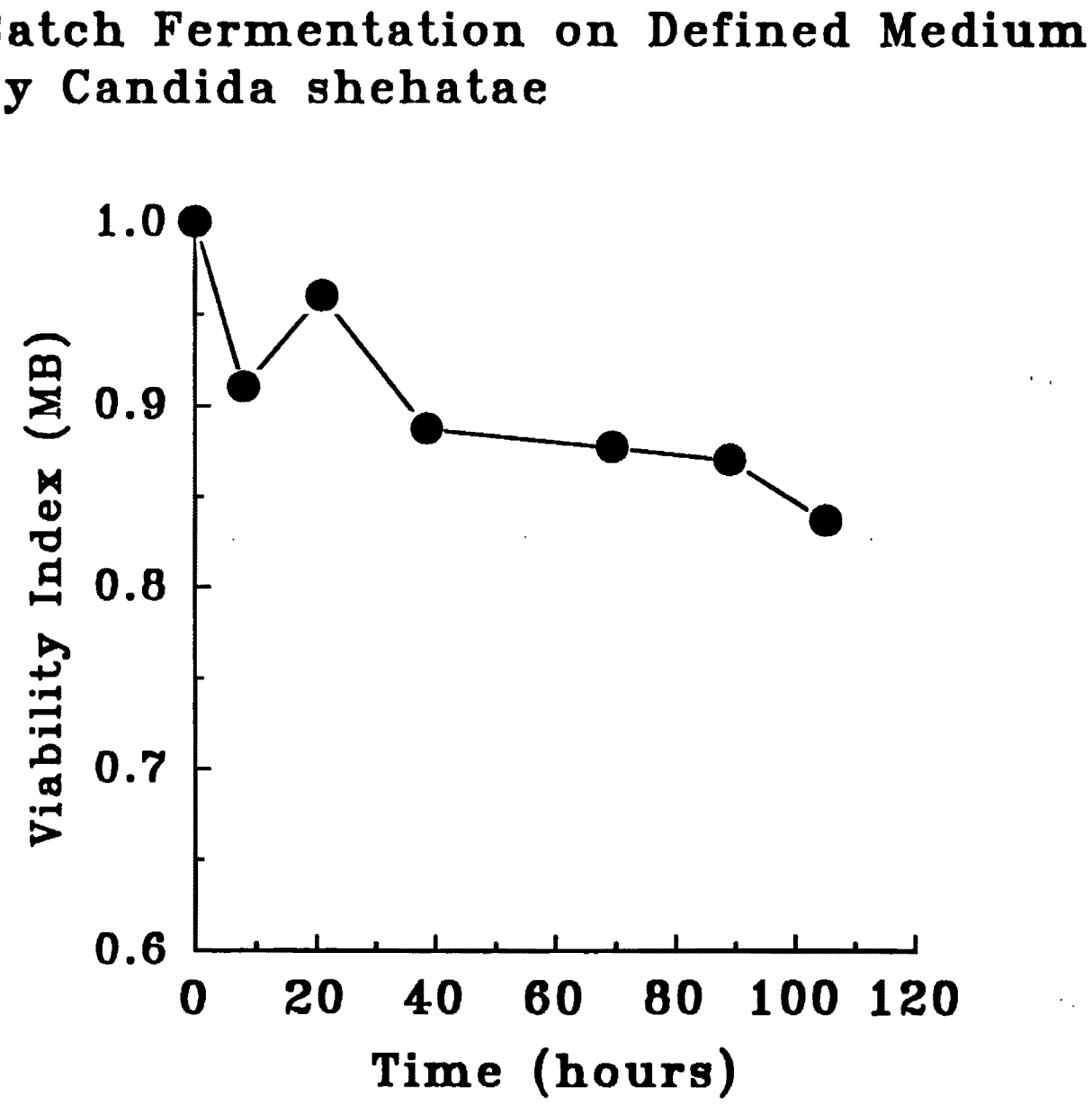


Figure 24 - Cell Viability of *Candida shehatae* During a Batch D-Xylose Fermentation on a Defined Medium.

Figure 25 - Batch D-Xylose Fermentation on a Defined Medium by *Pichia stipitis*.

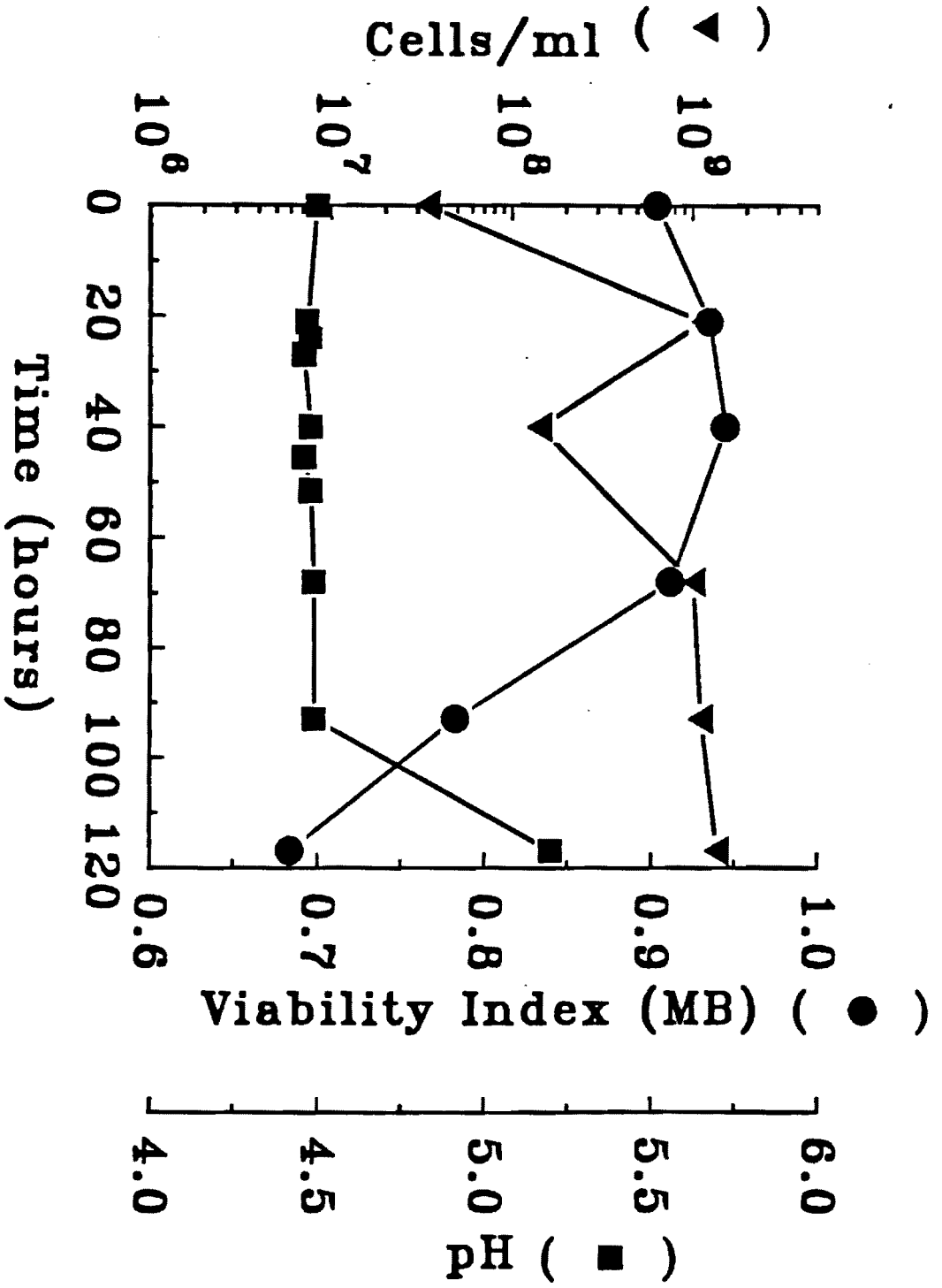
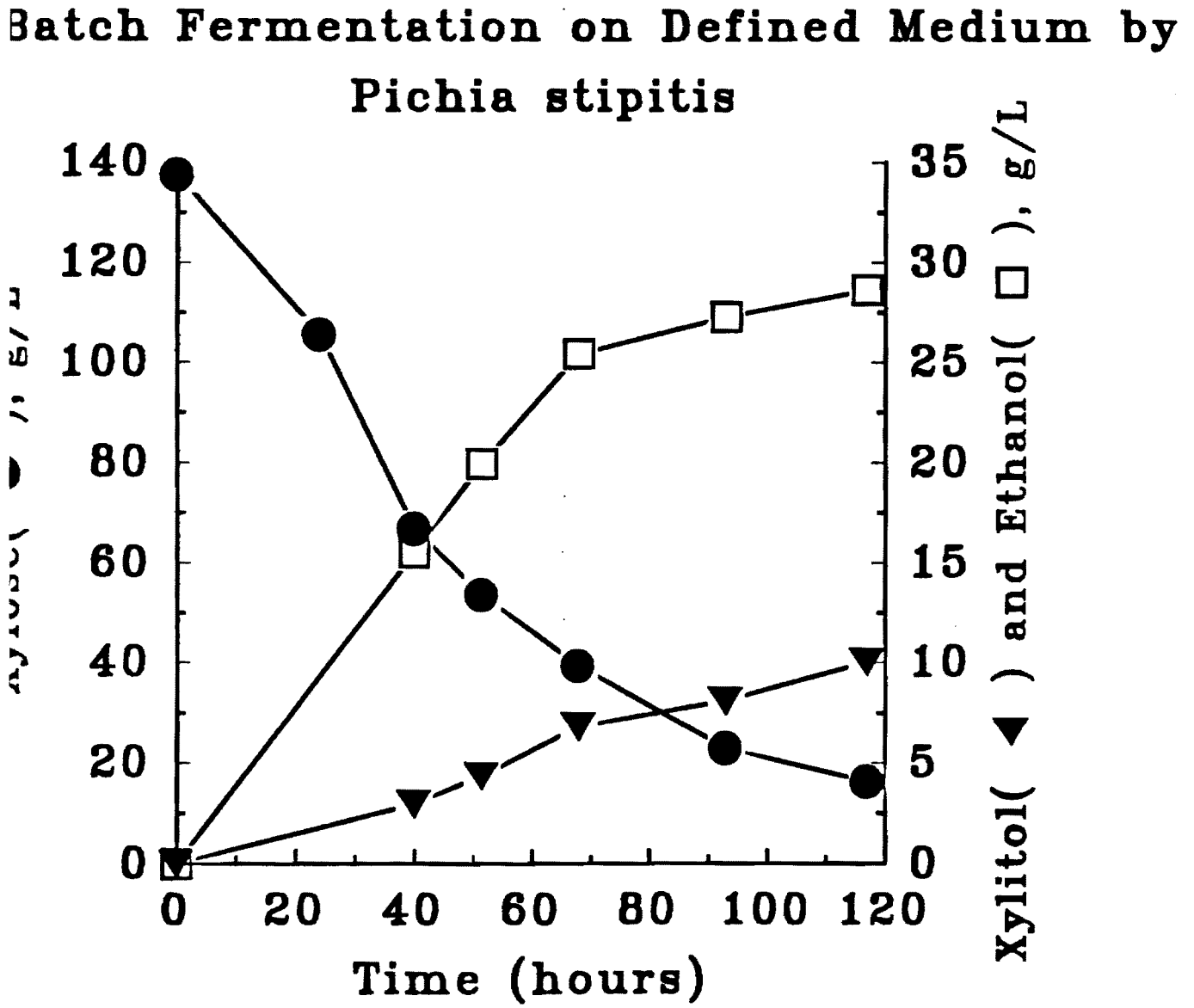


Figure 26 - Batch D-Xylose Fermentation on a Defined Medium by *Pichia stipitis*.



f. Effect of D-Xylose Concentration on Fermentations

Xylose fermentations using various microorganisms have produced low ethanol concentrations. One possible way to increase ethanol concentrations is to increase the initial substrate concentration. However, little research has been performed to test the effect of increasing the initial sugar concentration. In a series of experiments, three different initial xylose concentrations were tested. *Candida shehatae* was used to batch ferment xylose at 75 g/L, 112 g/L and 140 g/L. The medium used in these fermentations was chemically defined and contained no amino acids. These experiments were performed in erlenmeyer flasks with a cotton plug on top.

The higher xylose concentration, 140 g/L, reduced the intial growth rate of *Candida shehatae* (Figure 27). The uptake of xylose was also slower at the higher initial xylose concentration (Figure 29). The higher D-xylose concentration also reduced the initial rate of ethanol and xylitol production early in the fermentation (Figure 30 and 31). The initial rate of decline in pH was slower at the higher initial D-xylose concentration, another indication that this high level of D-xylose inhibited *C. shehatae* (Figure 32).

Figure 27 - Effect of D-Xylose Concentration on growth of *Candida shehatae*.

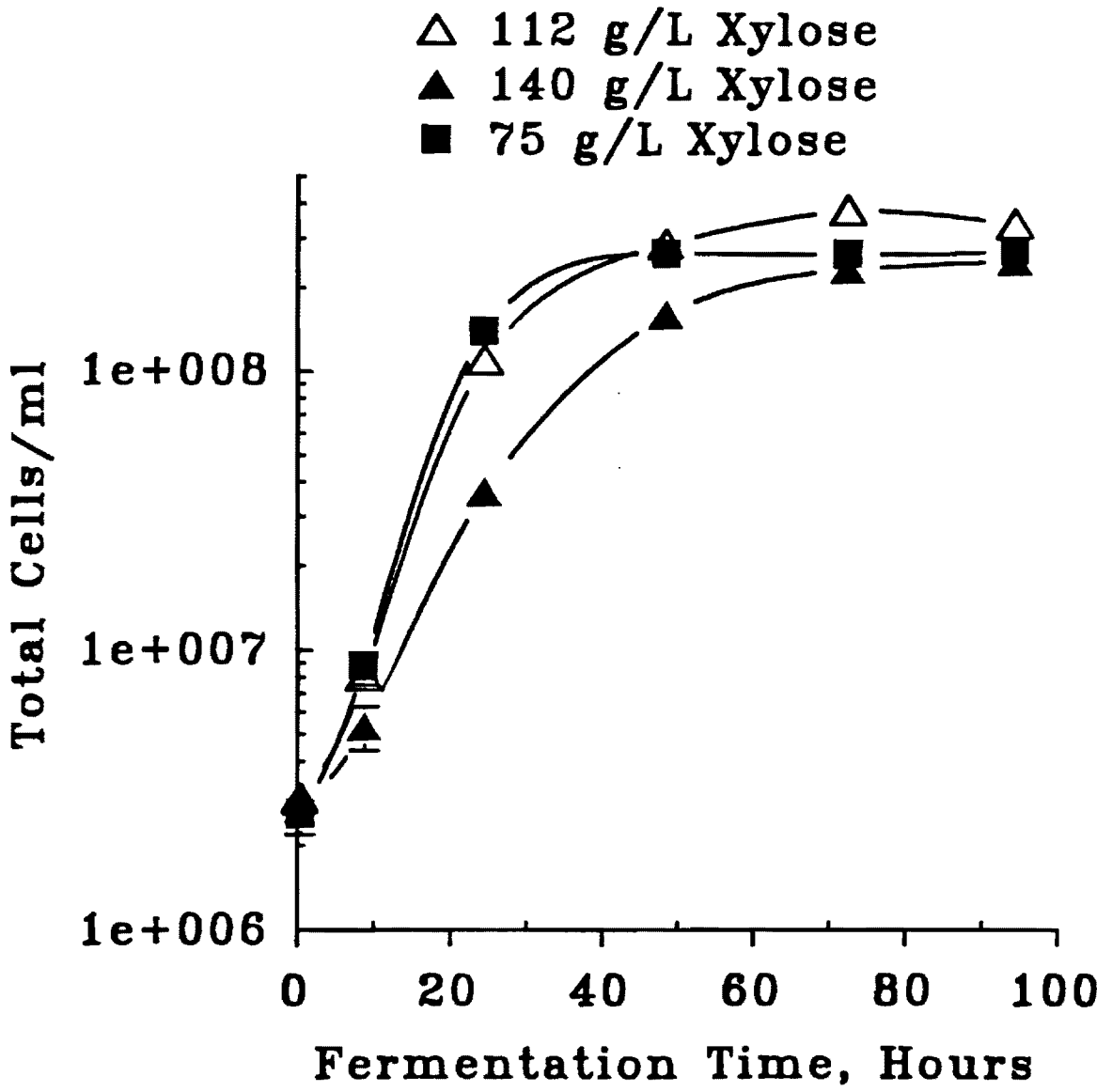


Figure 29 - Effect of D-Xylose Concentration on the Utilization Rate of D-Xylose.

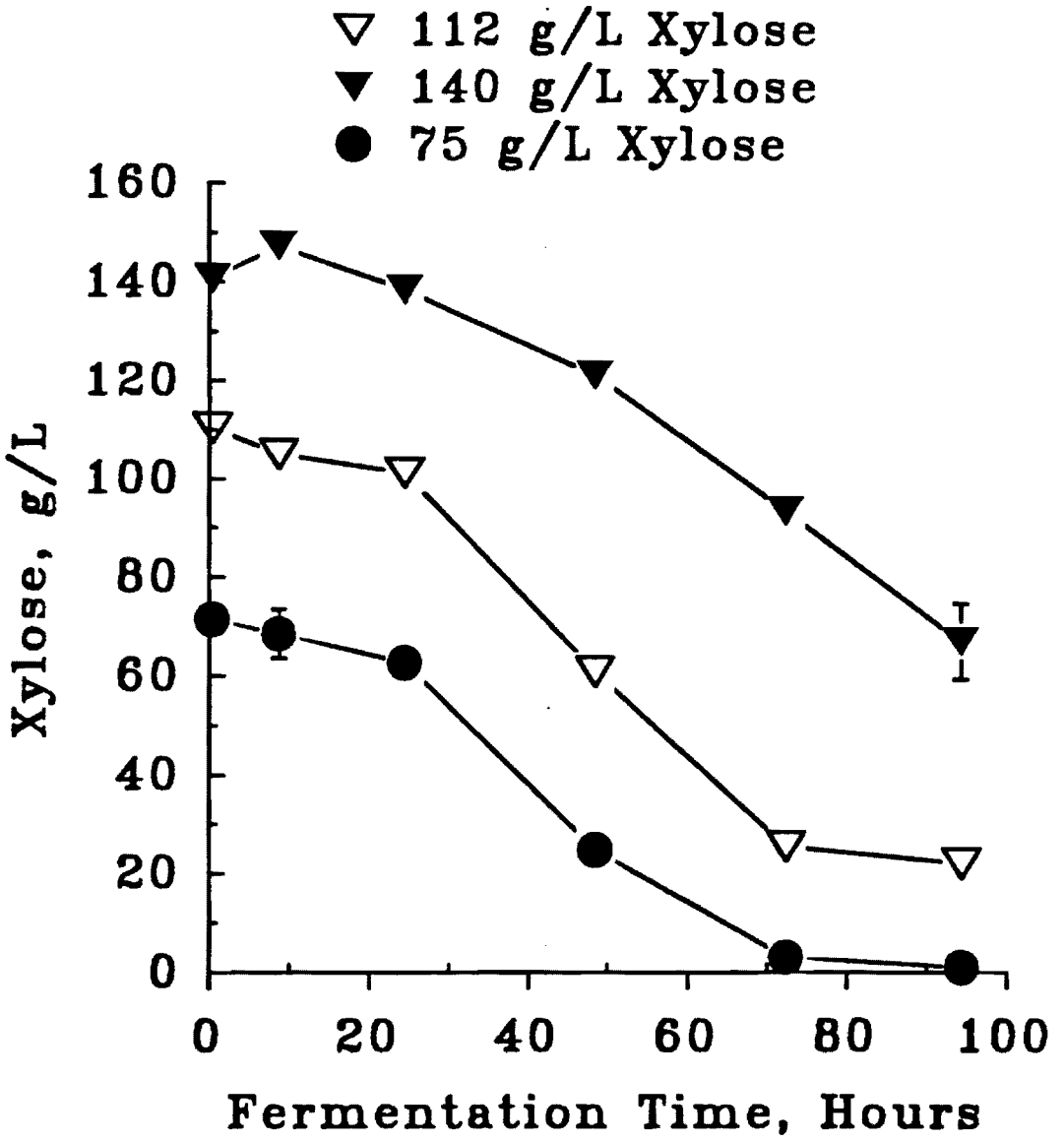


Figure 30 - Effect of D-Xylose Concentration on Ethanol Production by *Candida. shehatae*.

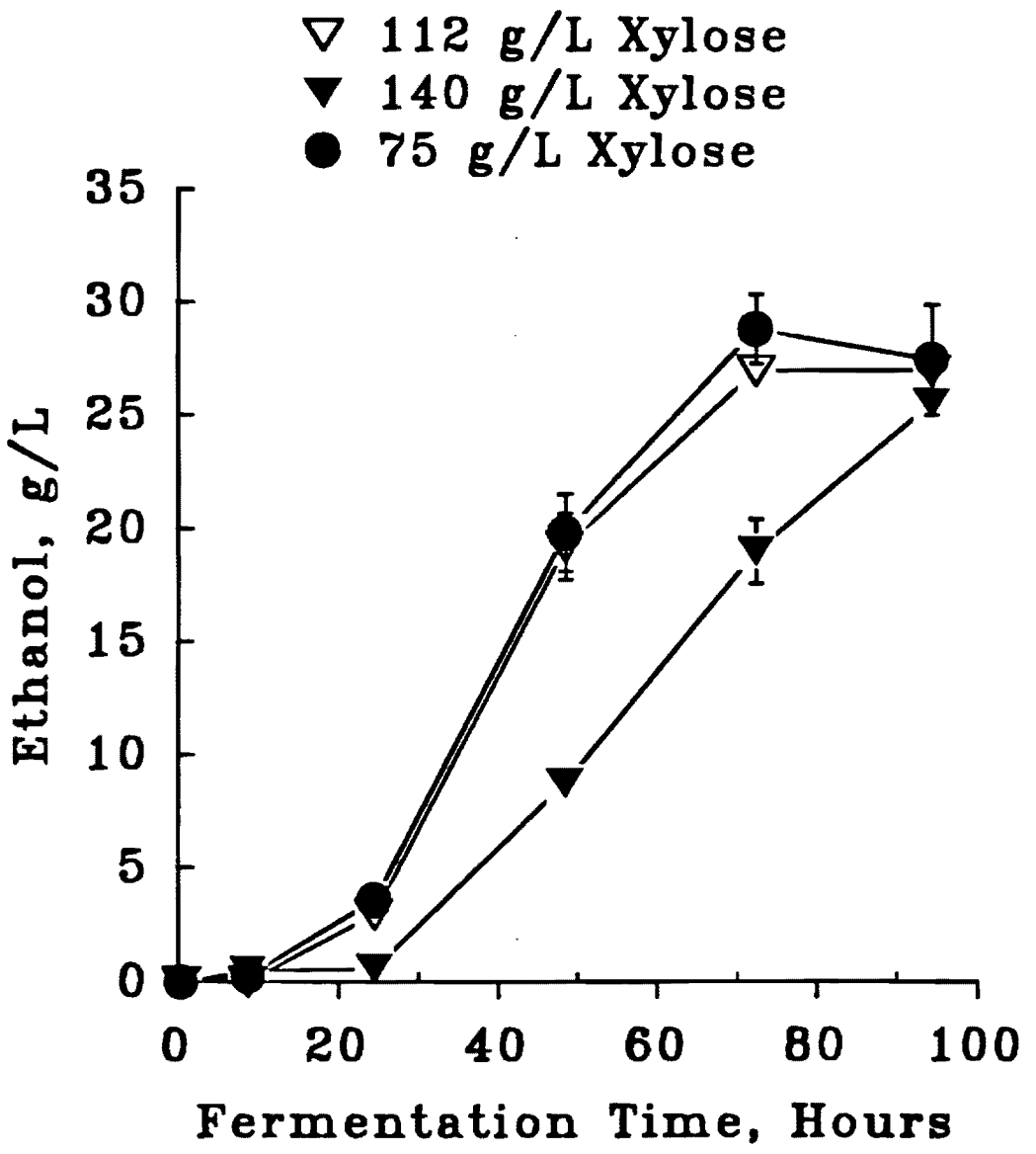


Figure 31 - Effect of D-Xylose Concentration on Xylitol Production by *C. shehatae*.

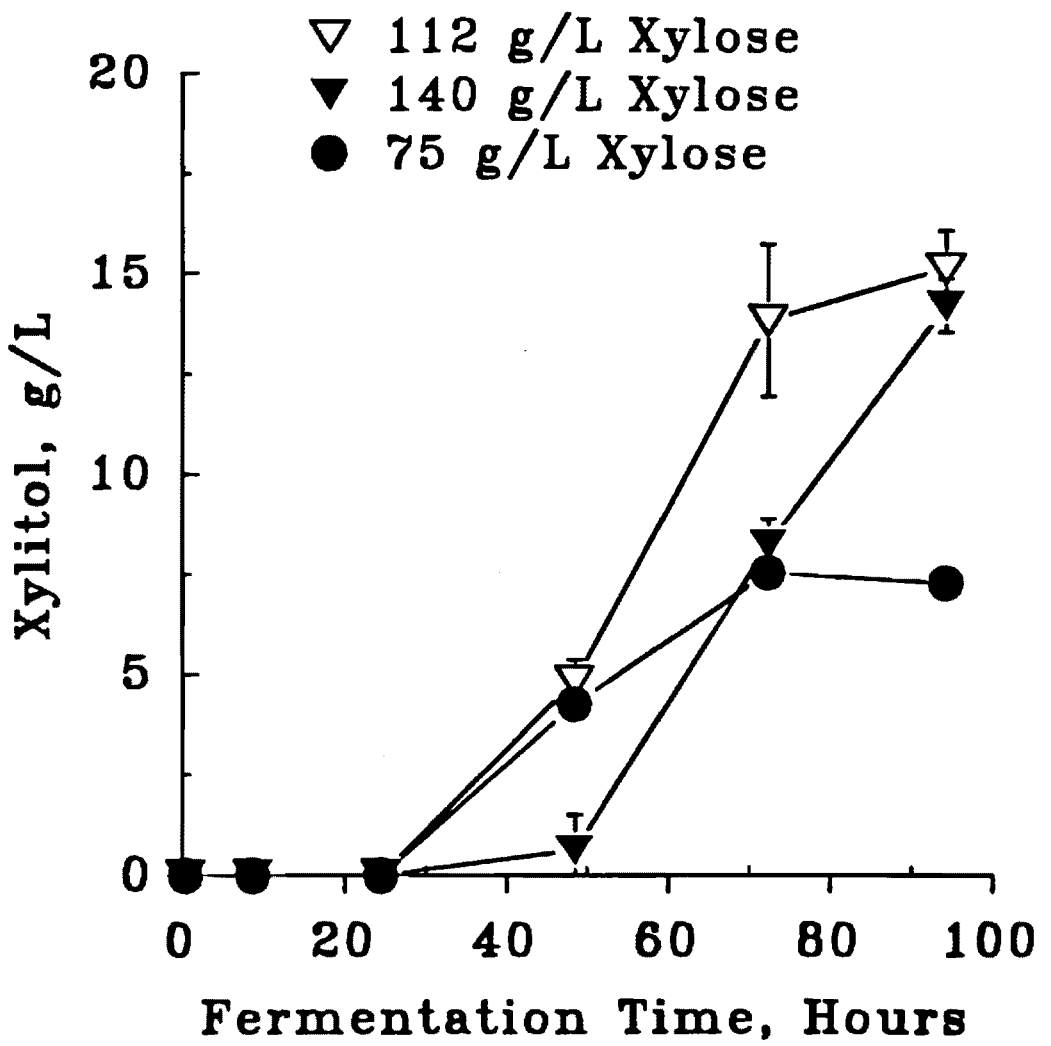
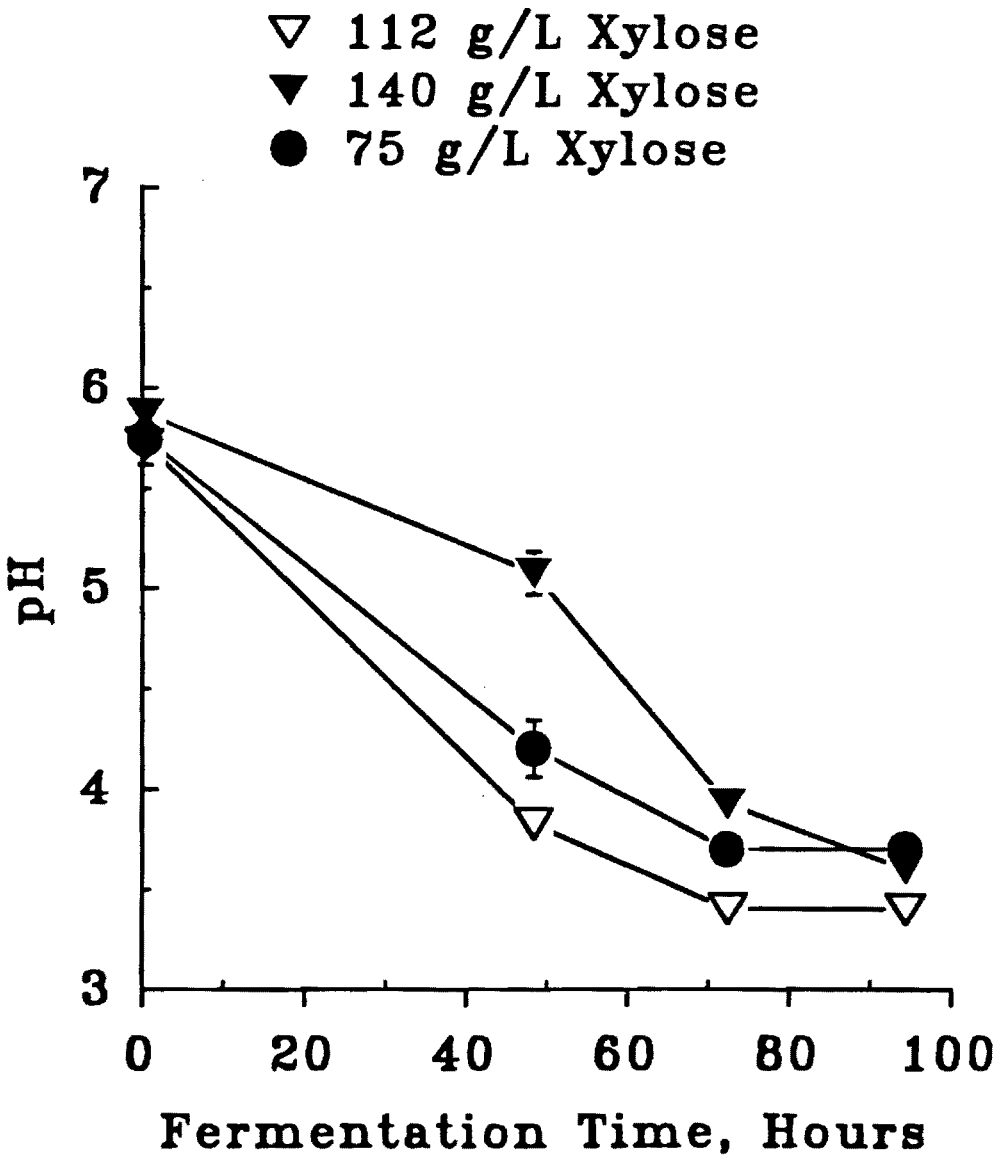


Figure 32 - Change in pH During Fermentations at Different Initial D-Xylose Concentrations.

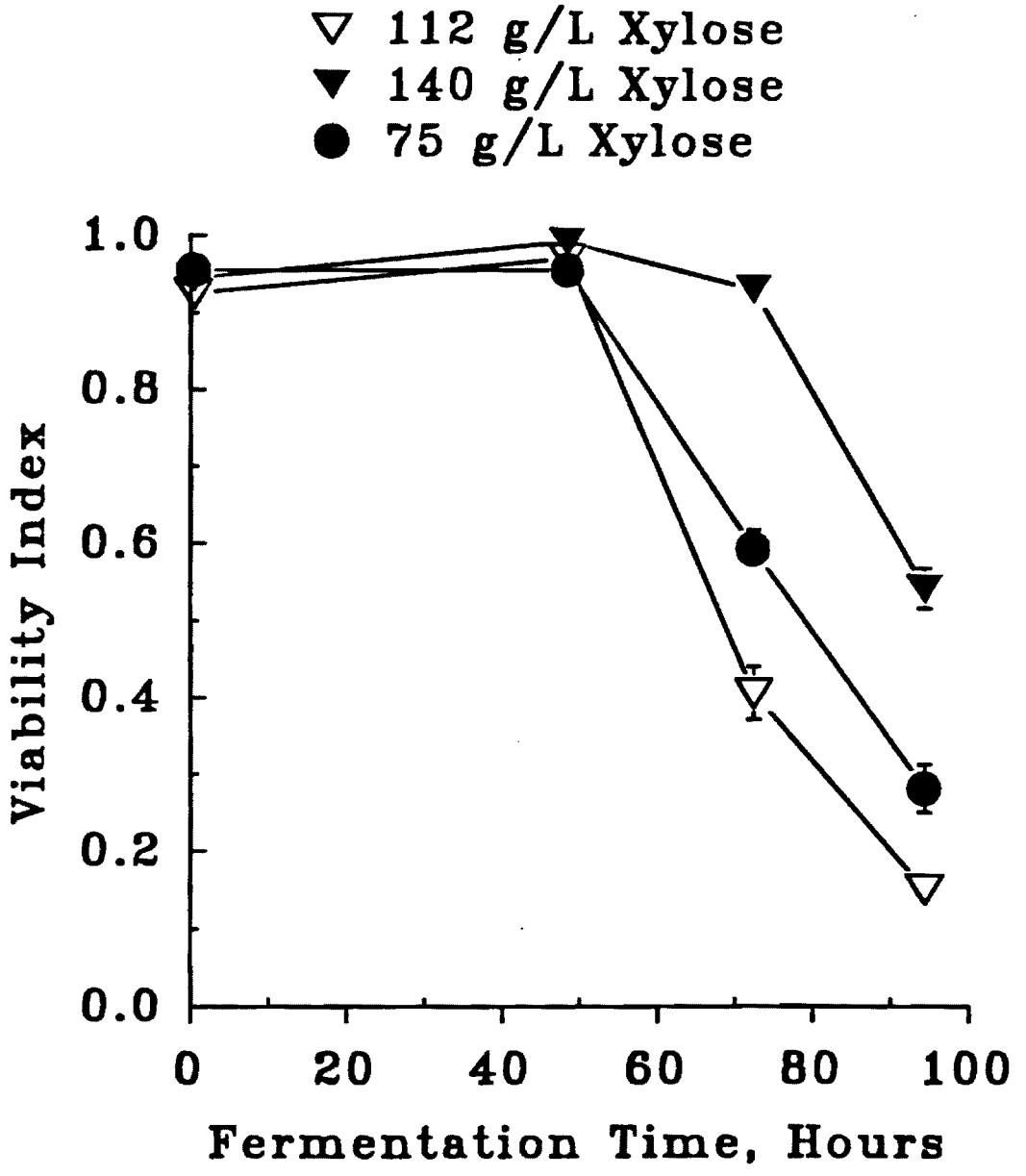


f. Effect of D-Xylose Concentration on Fermentations:

After an initial period, cell viability declined for the three initial concentrations of D-xylose (measured by methylene blue stain; Figure 33). However, the decline was more rapid after 50 hours for the lower xylose concentrations of 75 g/L and 112 g/L. The decline in viability may have been due to depletion of oxygen from the medium. The growth rate of cells at 140 g/L was lower than the other concentrations and thus the rate of oxygen depletion was lower. The slower rate of decline in oxygen may have resulted in the slower rate of decline in cell viability.

Other factors may have influenced cell viability. Substrate limitation may have caused the more rapid decline in cell viability at the lower initial xylose level, 75 g/L (see Figure 29). This was not the case with the initial concentrations of 112 and 140 g/L of D-xylose. There was greater than 25 g/L of residual xylose at the end of these fermentations. Ethanol may have also had an effect on cell viability. The average ethanol level was 25 g/L in each reactor after about 100 hours (see Figure 30). However, the rate of ethanol formation was slower at the higher initial xylose level. This may have caused or contributed to the slower decline in cell viability for this fermentation.

Figure 33 - Effect of D-Xylose Concentration on Cell Viability.



g. Mixed Sugar Fermentations on Defined Medium Under Growth Conditions

Previous research has shown that *Candida shehatae* will ferment xylose and glucose simultaneously (Kastner and Roberts, 1989). These fermentations were performed under anaerobic conditions and with cells pre-grown on xylose. Growth did not occur under these conditions (or growth was extremely limited). It was the objective of these experiments to see if growth would result in the repression of xylose utilization in a mixed sugar fermentation. Also, cell viability data was collected and compared to a control fermentation (pure xylose). This was done to see if a mixed sugar fermentation had any advantage over a pure xylose fermentation in terms of long term cell viability. These experiments were performed in erlenmeyer flasks on a defined medium without amino acids.

The growth rates were similar for both the mixed sugars and pure xylose fermentations (Figure 34). The reactors with higher initial sugar concentrations did reach higher cell concentrations. Cell viability declined with time in all reactors (Figure 35). However, the decline in cell viability was not as rapid for the mixed sugar fermentations. Xylose and glucose were consumed simultaneously in the mixed sugar fermentations (Figures 36 and 37). However, glucose was consumed at a faster rate and the rate of D-xylose utilization was reduced by the presence of glucose (Figure 38). Ethanol levels were highest in the mixed sugars with an initial total carbohydrate level of about 75 g/L (Figure 39). Initially the pH levels in all reactors were 6.25 and declined to a level between 4.0 and 4.5 (Figure 40) .

Figure 34 - Growth of *Candida shehatae* During D-Xylose and Mixed Sugar Fermentations.

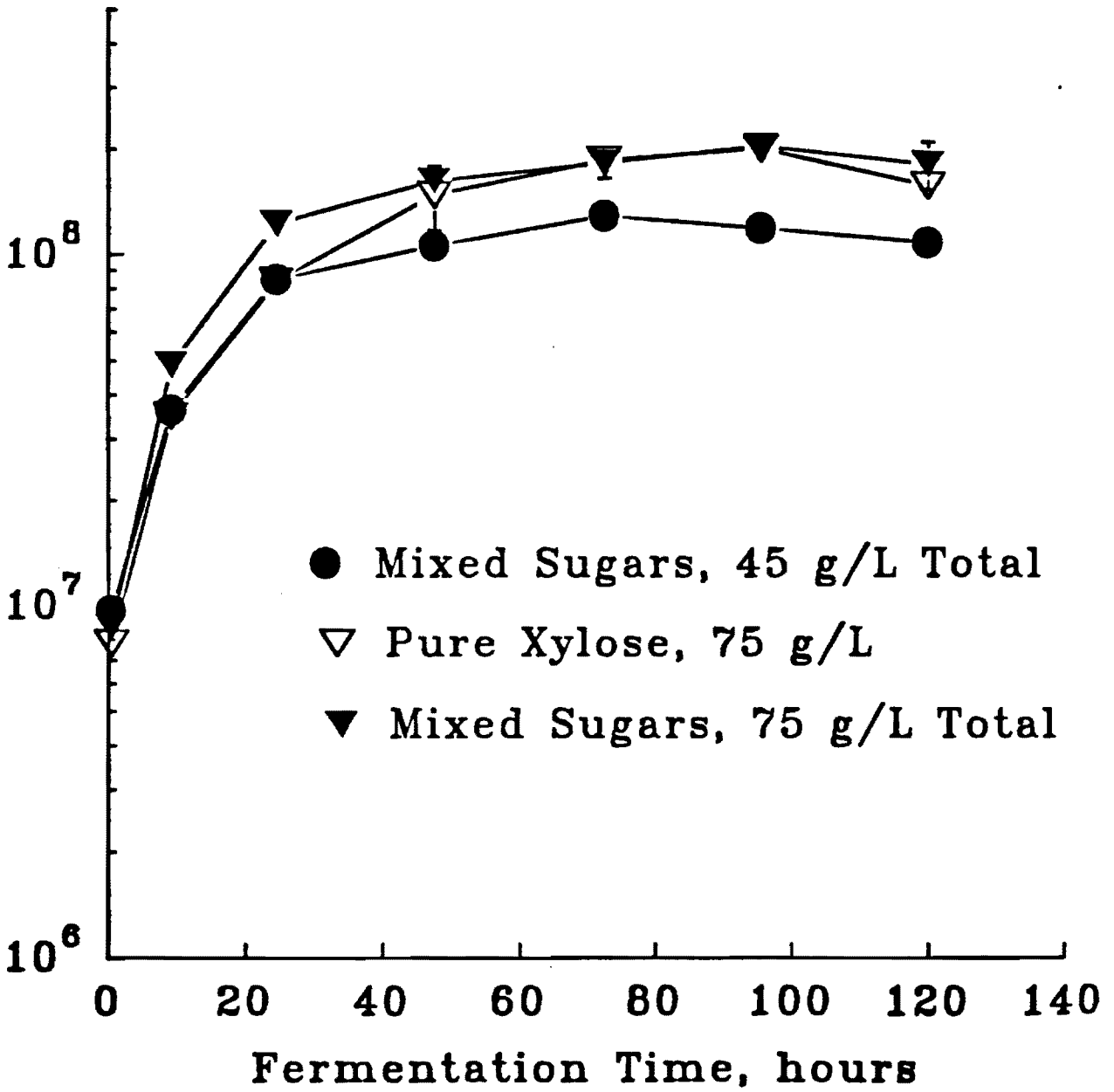


Figure 35 - Change in Cell Viability During D-Xylose and Mixed Sugar Fermentations.

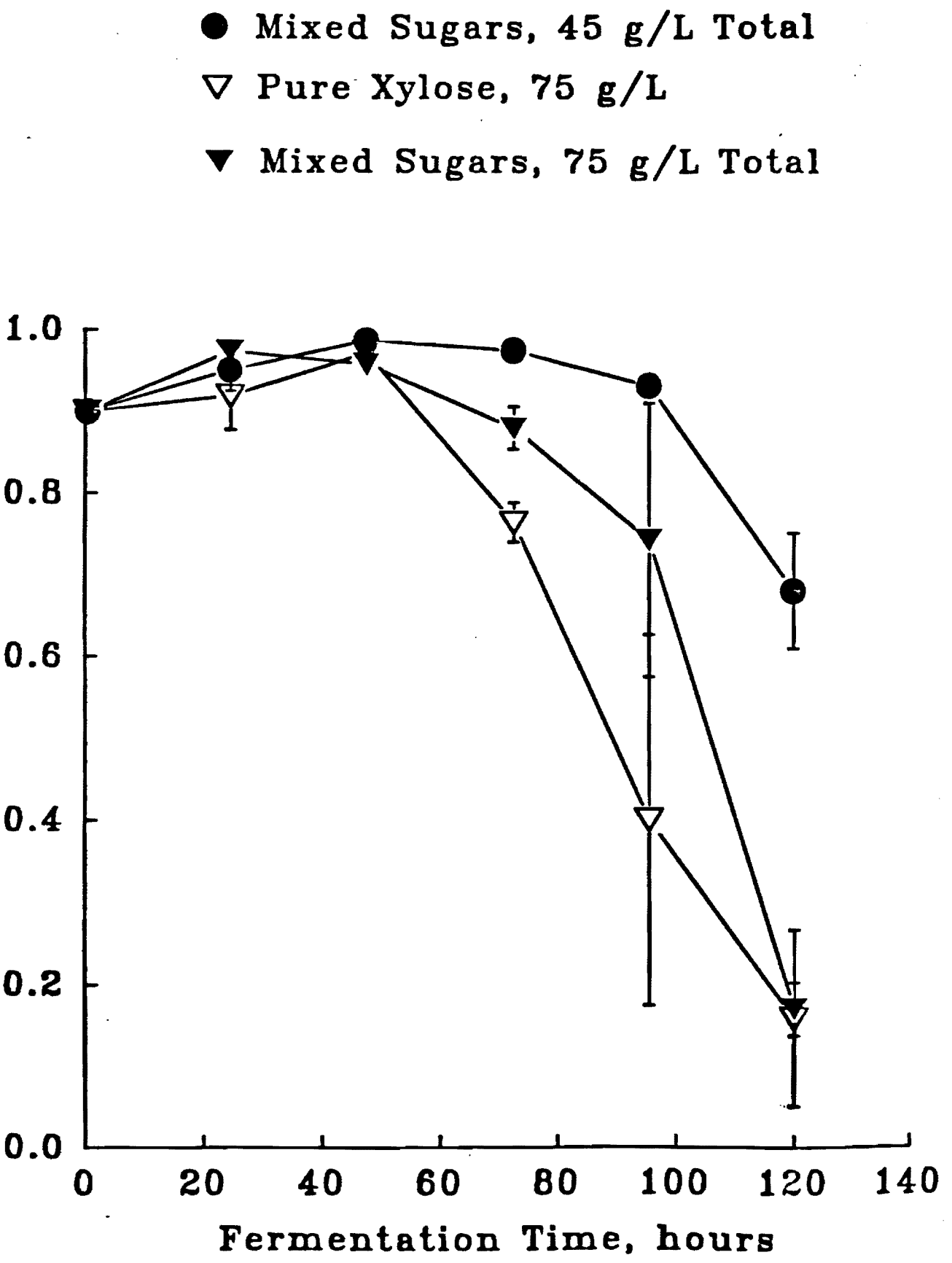


Figure 36 - Simultaneous Utilization of Glucose and Xylose in a Batch Reactor with 50% Glucose and D-Xylose (40 g/L Total) at Time Zero.

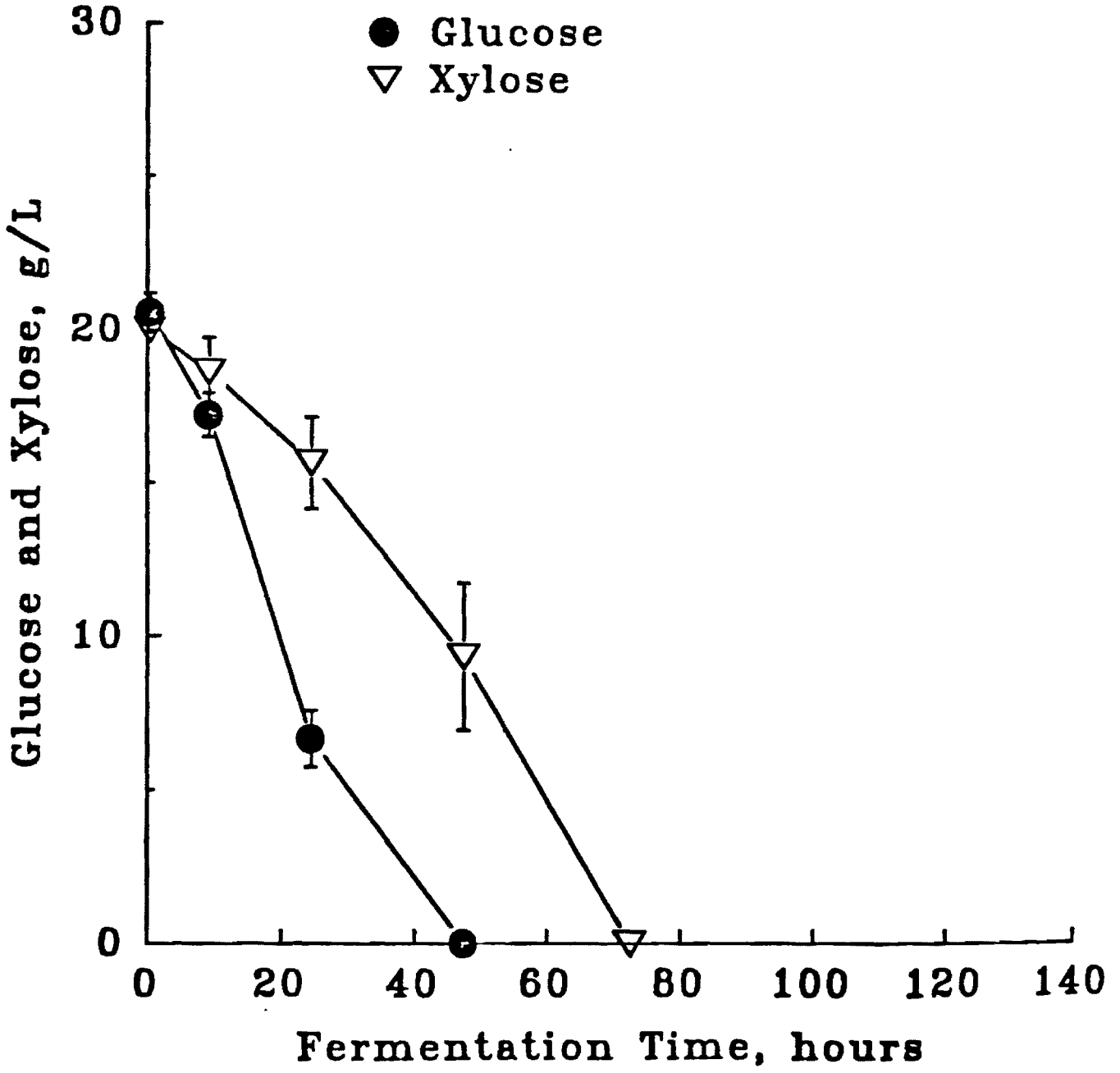


Figure 37 - Simultaneous Utilization of Glucose and Xylose in a Batch Reactor with 50% Glucose and D-Xylose (76 g/L Total) at Time Zero.

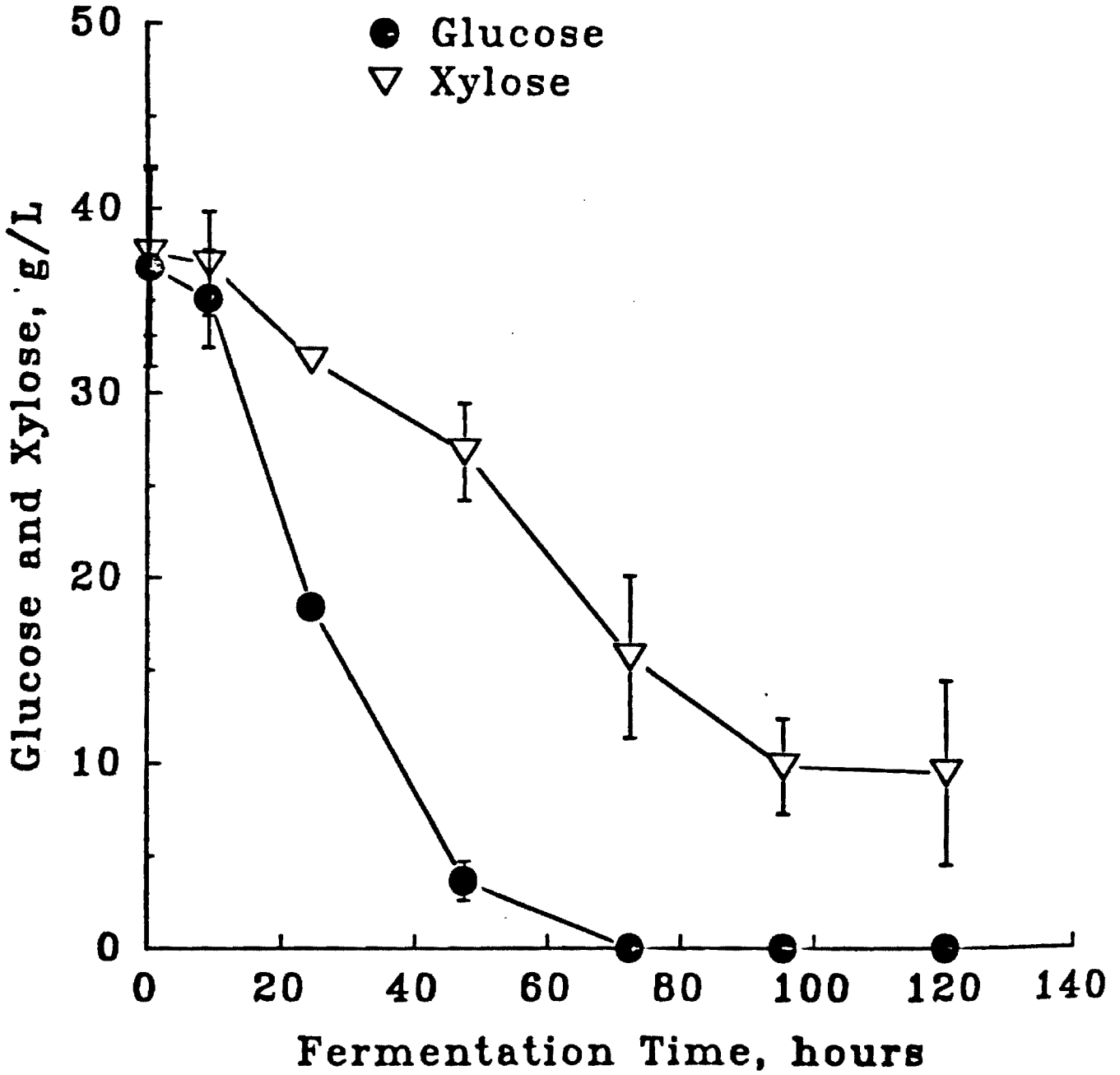


Figure 38 - Effect of Glucose Level on D-Xylose Uptake in a Mixed Sugar Fermentation.

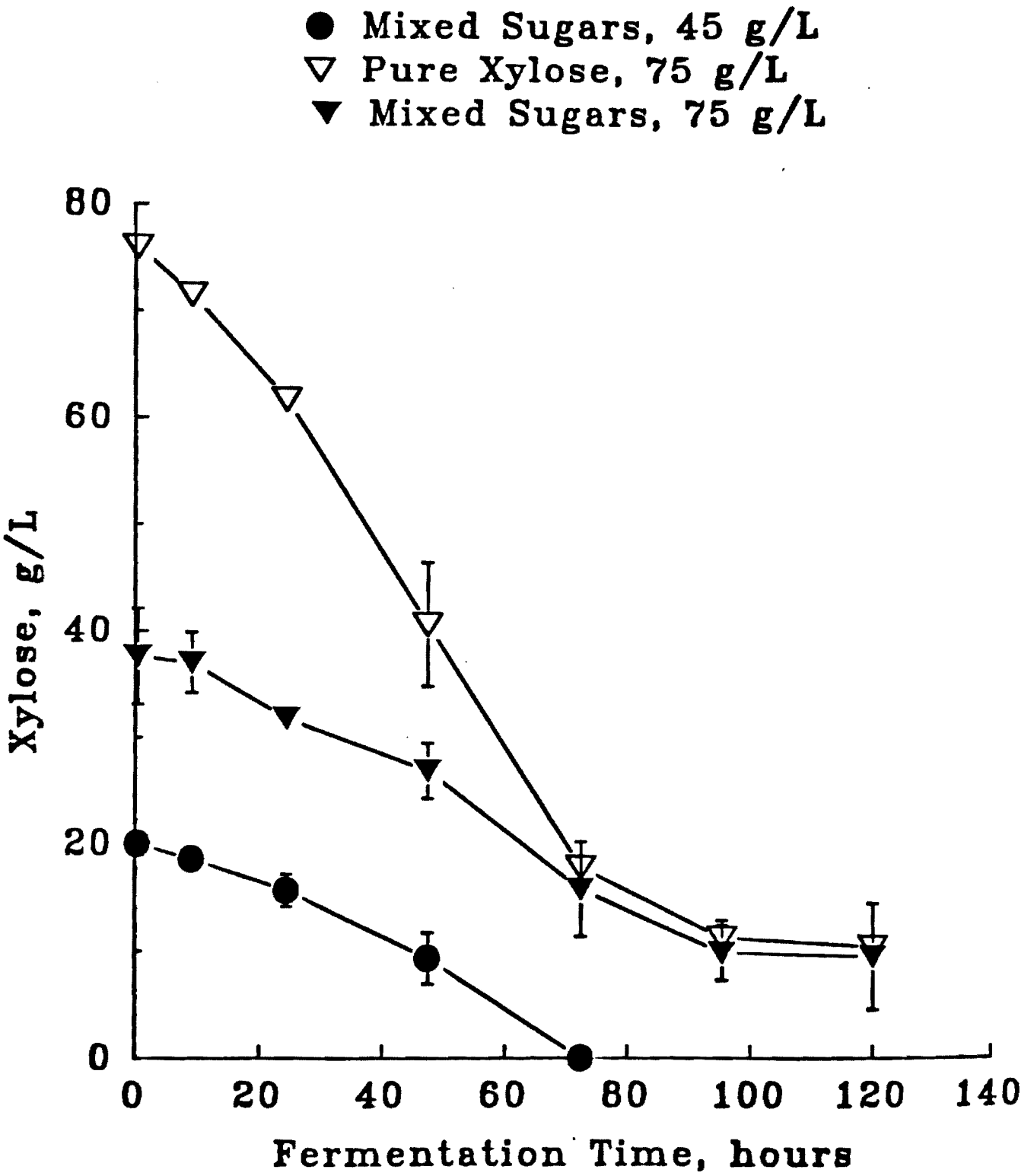


Figure 39 - Ethanol Production in Mixed Sugar and Pure D-Xylose Fermentations on a Defined Medium.

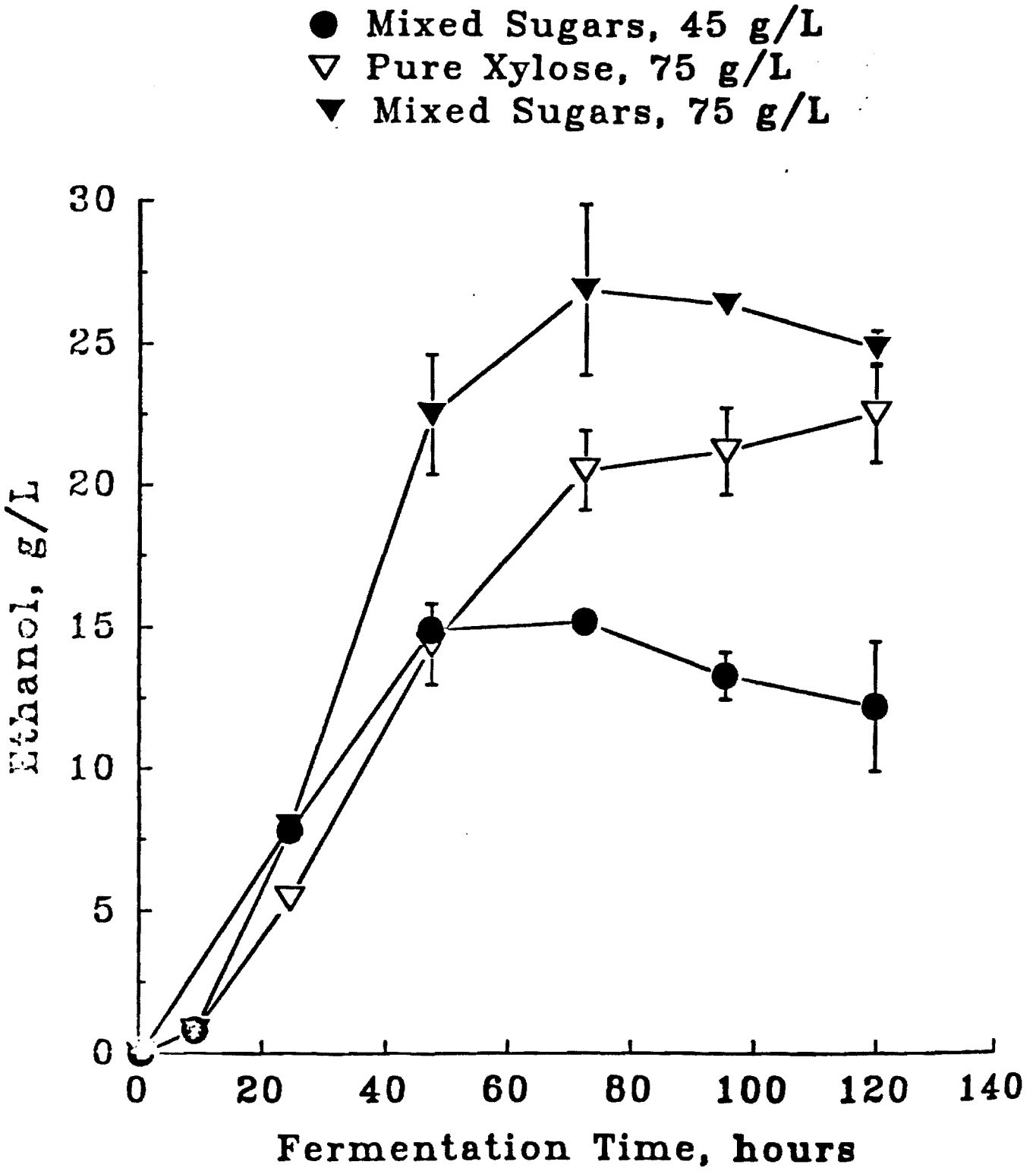
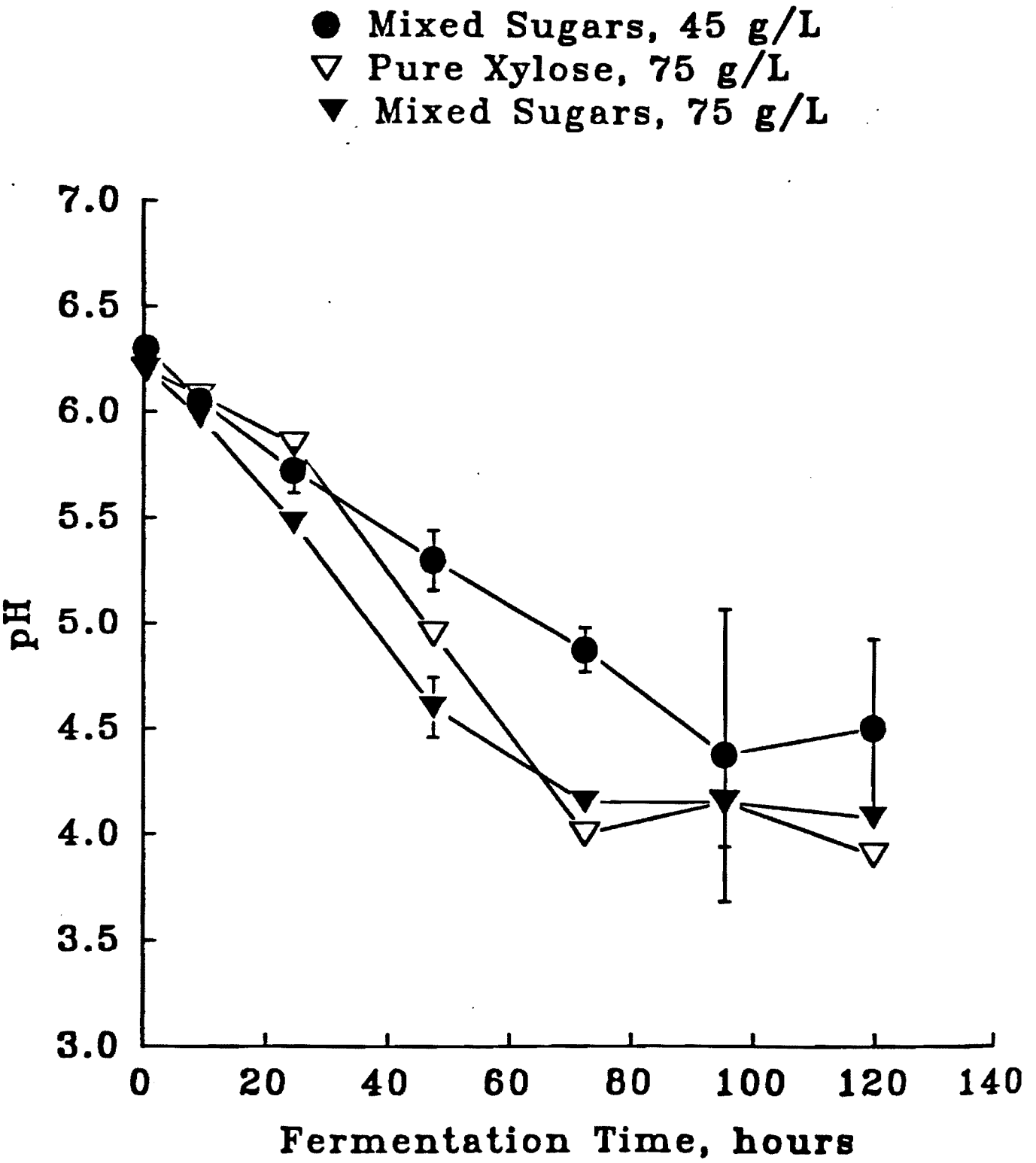


Figure 40 - Change in pH During Mixed Sugar and Pure D-Xylose Fermentations on a Defined Medium.



As described earlier the cell viability declined with time in all reactors. The data indicated that viability was correlated possibly with an exhaustion of glucose in the medium. After about 50 hours glucose was depleted (in both reactors with mixed sugars) and after glucose had been consumed cell viability began to decline. This prompted us to compare anaerobic glucose and D-xylose fermentations in terms of viability. It should be noted that the decline in cell viability also correlated with an increase in ethanol and xylitol and a decrease in pH. These parameters could have also affected cell viability.

h. Comparison of Glucose and D-Xylose Fermentations on a Defined Medium

In previous experiments cell viability began to decline after glucose had been consumed. This prompted us to compare xylose and glucose fermentations. Fermentations were performed on D-xylose and glucose in a defined medium (without casamino acids). In one set of fermentations *Candida shehatae* cells were inoculated into erlenmeyer flasks with cotton plugs. In the other fermentation *C. shehatae* cells were inoculated into a NewBrunswick benchtop fermentor in which the pH was controlled at 4.5.

Shaker Flasks:

Candida shehatae cells were inoculated into shaker flasks with 100 g/L of D-xylose, 100 g/L of glucose and 50 g/L of D-xylose. The growth rate and final cell densities were the same for all three conditions (Figure 41). The change in cell viability (as measured by methylene blue staining) and pH was the same for all three conditions (Figure 42 and 43).

Figure 41 - Pure Glucose and D-Xylose Fermentations in Shaker Flasks.

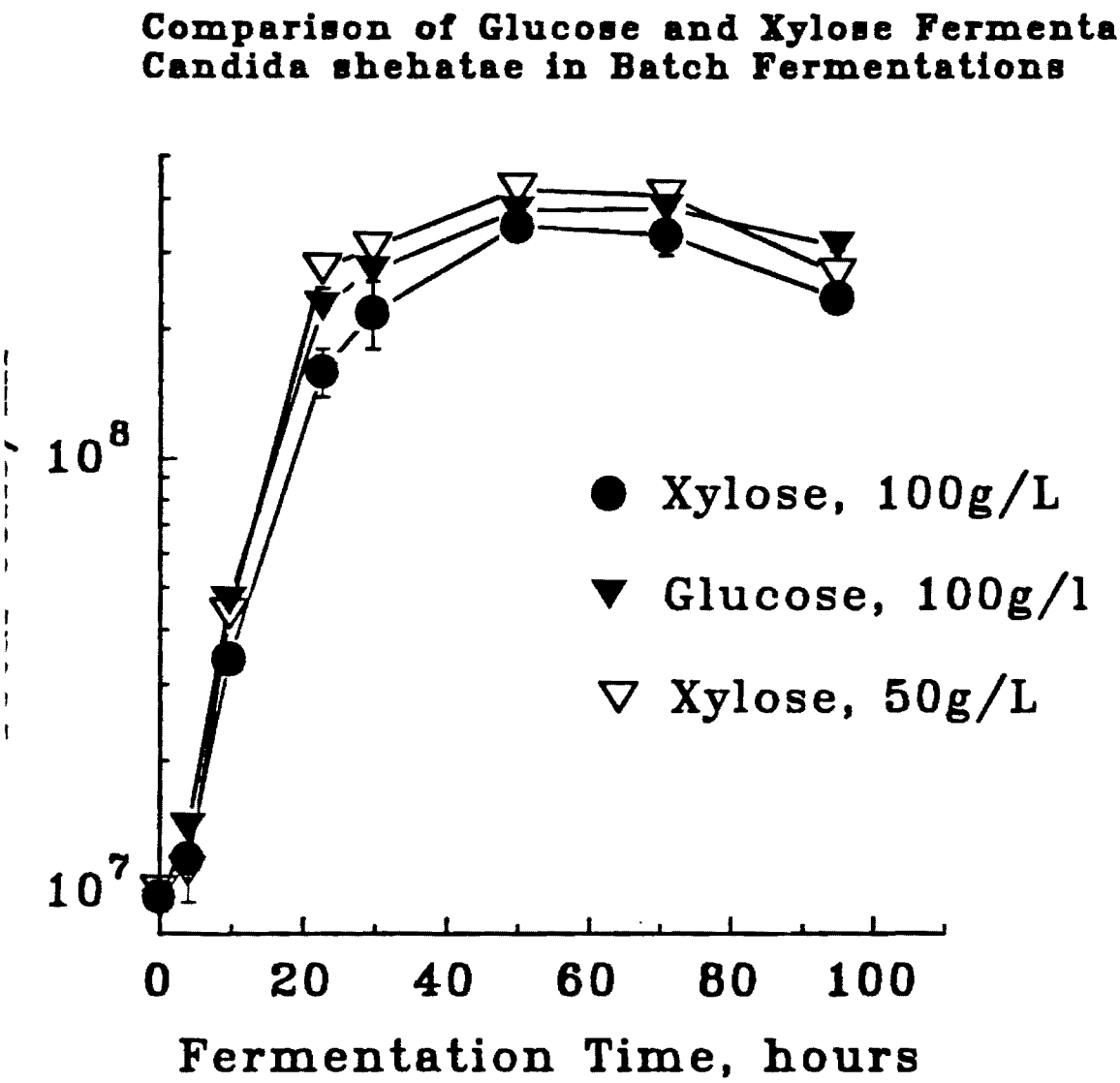


Figure 42 - Change Cell Viability as Measured by Methylene Blue Staining During Glucose and D-Xylose Fermentations by *Candida shehatae*.

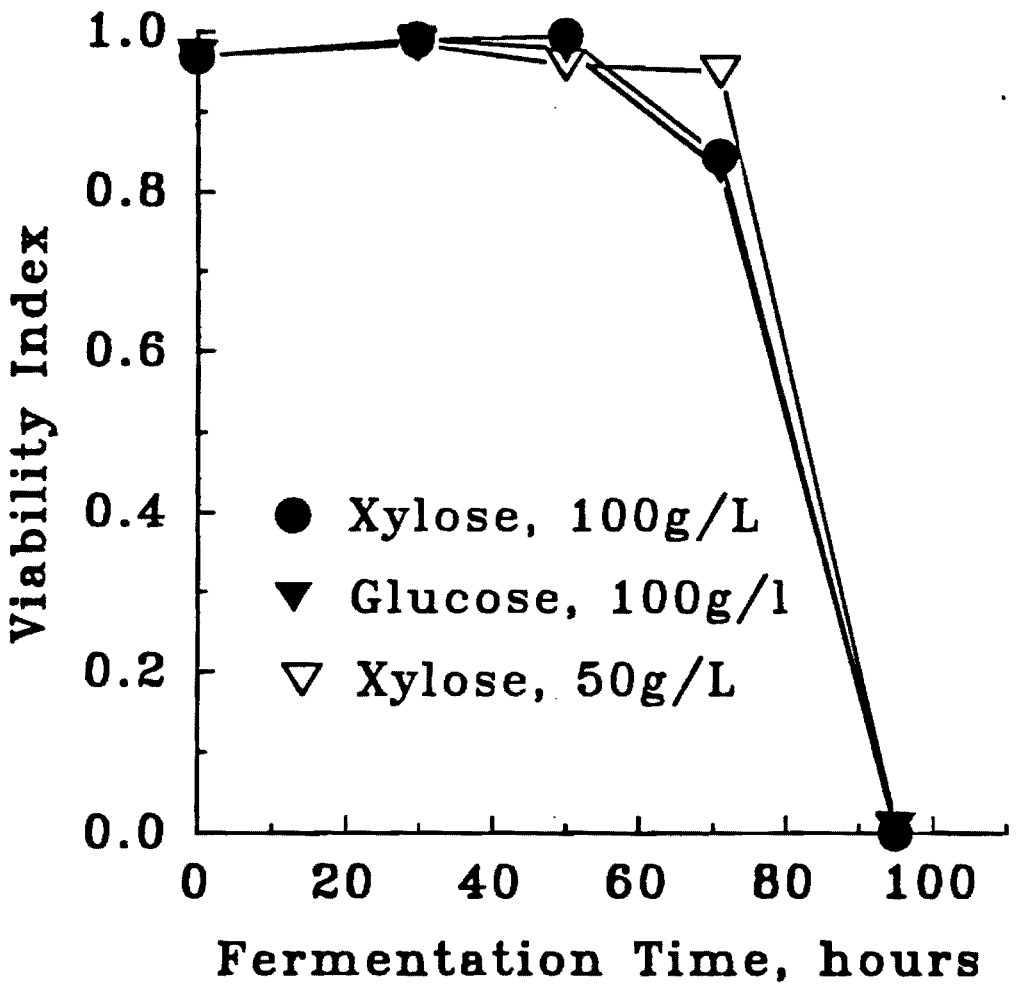
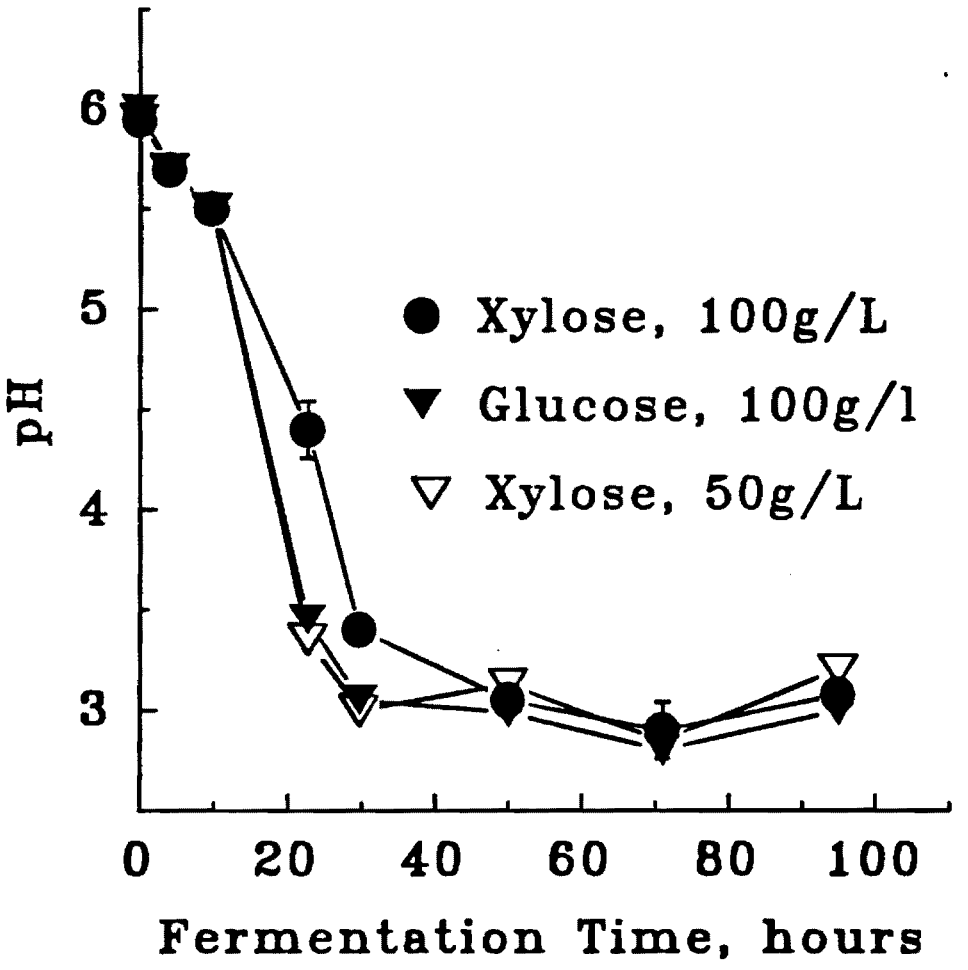


Figure 43 - Change in pH During Glucose and D-Xylose Fermentations by *Candida shehatae* in Shaker Flasks.



Benchtop Fermentor:

Cells were inoculated into a New Brunswick Multigen fermentors in which pH was controlled at 4.5. The reactors were not aerated and dissolved oxygen (D.O.) was monitored. Since the reactors were not aerated, the % D.O. dropped to zero within the first 3 hours (Figure 44). However, after the % D.O. dropped the cells growing on D-xylose doubled 2.54 times and the cells growing on glucose doubled 3.7 times (see Figure 45). This data is inconsistant with previous work which showed that growth did not occur or was severely limited by lack of oxygen.

After the first 24 hours growth was minimal and in that same period the viability (as measured by methylene blue staining) dropped from 0.98 to 0.92 on glucose and to 0.85 on D-xylose (Figure 46). However, the viability slowly increased; in the glucose fermentation after 250 hours the viability was 0.95 and at 200 hours into the D-xylose fermentation it was 0.95.

i. Effect of Casamino Acids on Batch D-Xylose Fermentations by *C. shehatae*

Previously, anaerobic fermentations were performed in a defined medium with a source of amino acids (casamino acids) to see if this would stimulate anaerobic growth of *Candida shehatae*. The presence of amino acids did not stimulate growth as measured by absorbance at 660 nm. However, the effect of casamino acids on cell viability was not determined. In this experiment casamino acids were added to a defined medium to test

Figure 44 - Change in Dissolved Oxygen During D-Xylose and Glucose Batch Fermentations in a Benchtop Fermentor without Aeration.

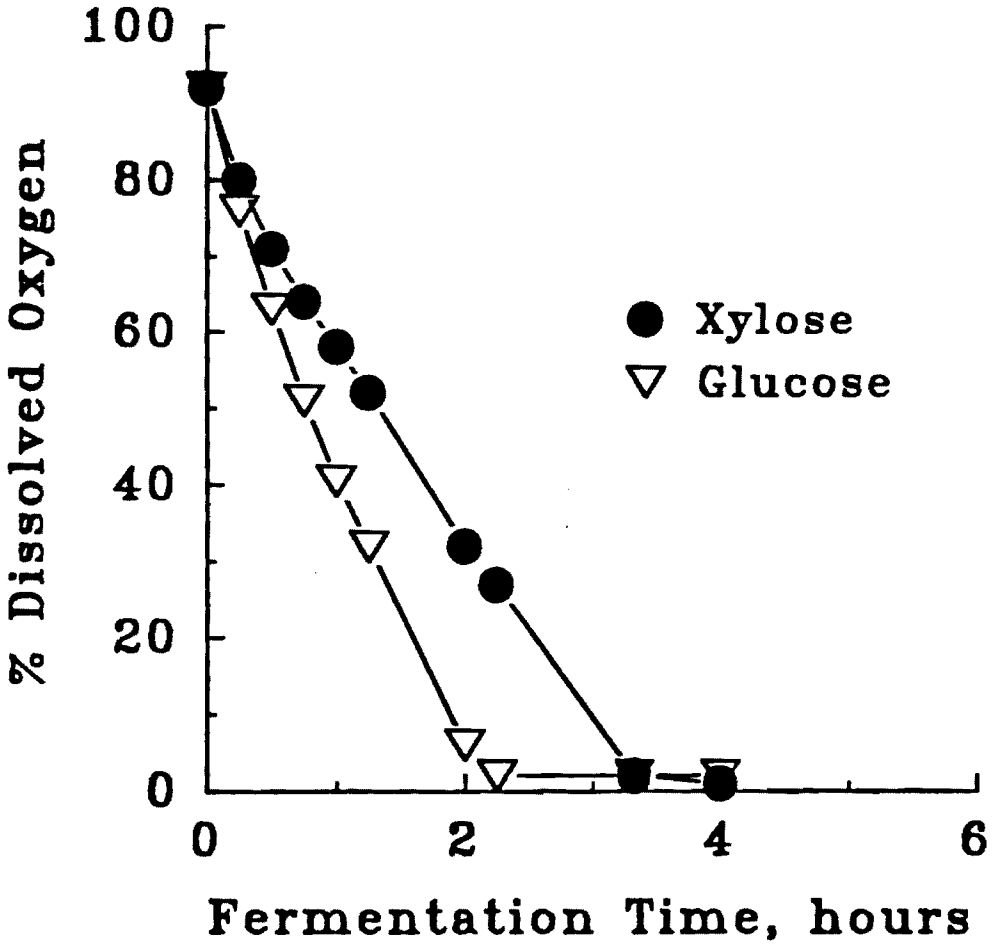


Figure 45 - Effect of Oxygen Limitation on the Growth of *Candida shehatae* on Glucose and D-Xylose.

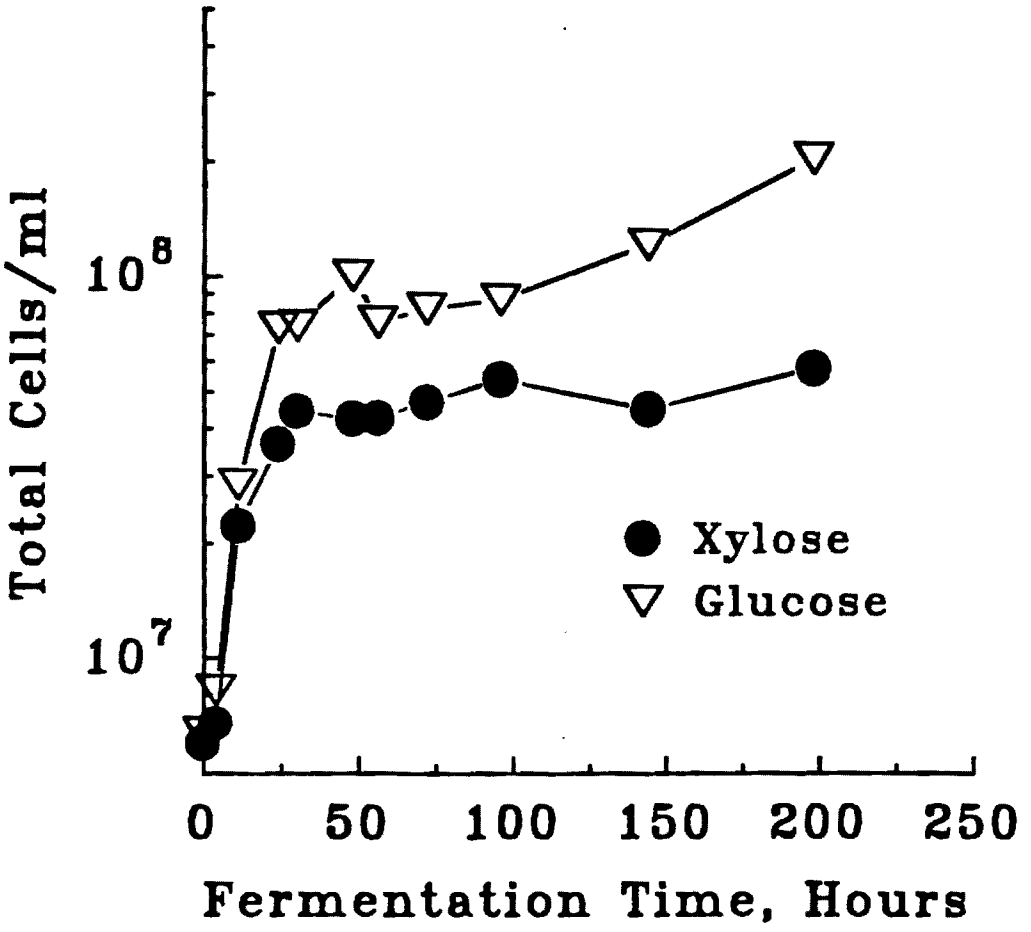
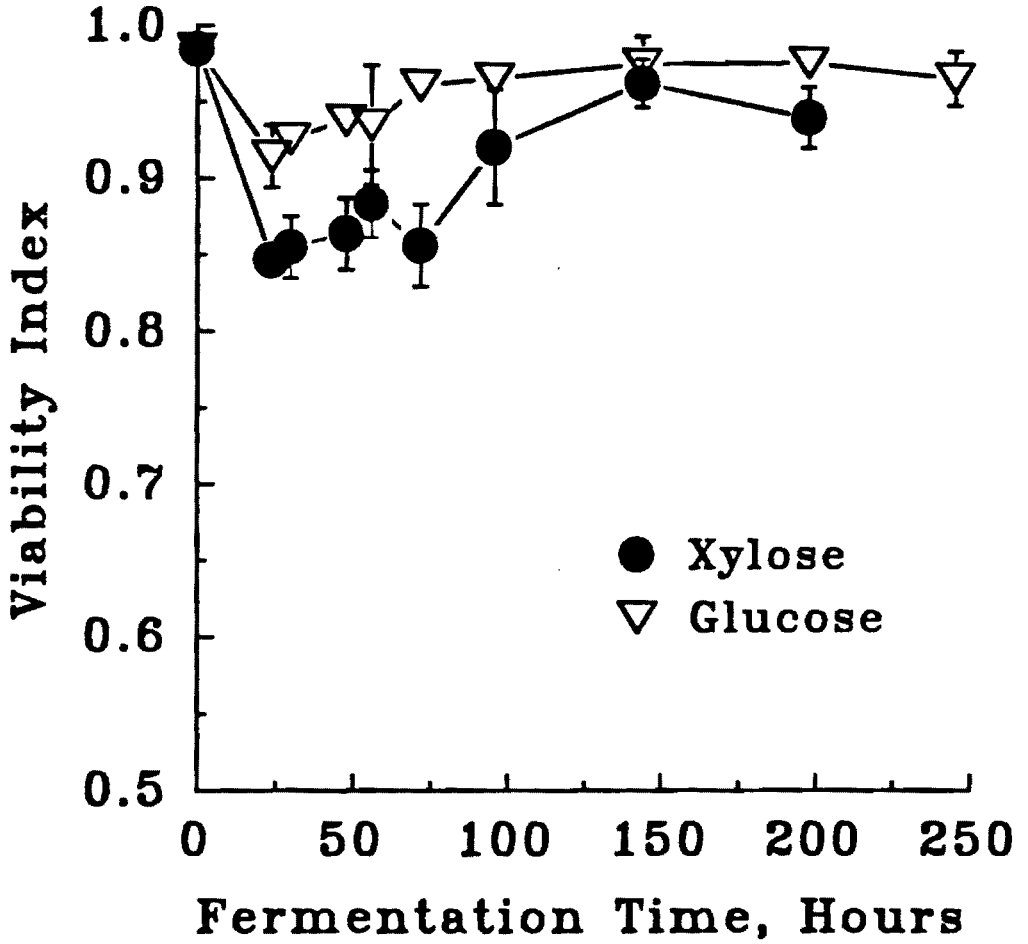


Figure 46 - Effect of Oxygen Limitation on the Viability of *Candida shehatae* on Glucose and D-Xylose.



their effect on cell viability. Additional experiments were performed, in which the medium had a low and high buffering capacity, but did not contain casamino acids in the medium (Table 5).

Table 5 - Concentration of Casamino Acids and Phosphate Buffer in Defined Medium

	Casamino Acids, g/L	KH_2PO_4, g/L	K_2HPO_4, g/L
Experimental	5	-	-
High Buffer	-	10	10
Low Buffer	-	5	5

As in previous experiments the presence of casamino acids did not stimulate growth. The final cell densities were about the same for the high buffer capacity and casamino acids (Figure 47). Also, the data showed that there was no difference in results between the fermentations on casamino acids and the reactor with additional buffering capacity. The rate of xylose utilization, xylitol formation, decline in viability and decline in pH was the same (Figures 48-51).

In the second experiment, a comparison of casamino acids, low buffer concentration and high buffer concentration, the presence of casamino acids did not stimulate growth compared to the other conditions (Figure 52). However, the decline in cell viability and pH was more rapid in the reactor with a lower buffering capacity (Figure 53 and 54). These data indicate that pH may have an impact on cell viability and that the positive effect casamino acids have on cell viability could be due to its buffering capacity.

Figure 47 - Effect of Casamino Acids on the Growth of *Candida shehatae*.

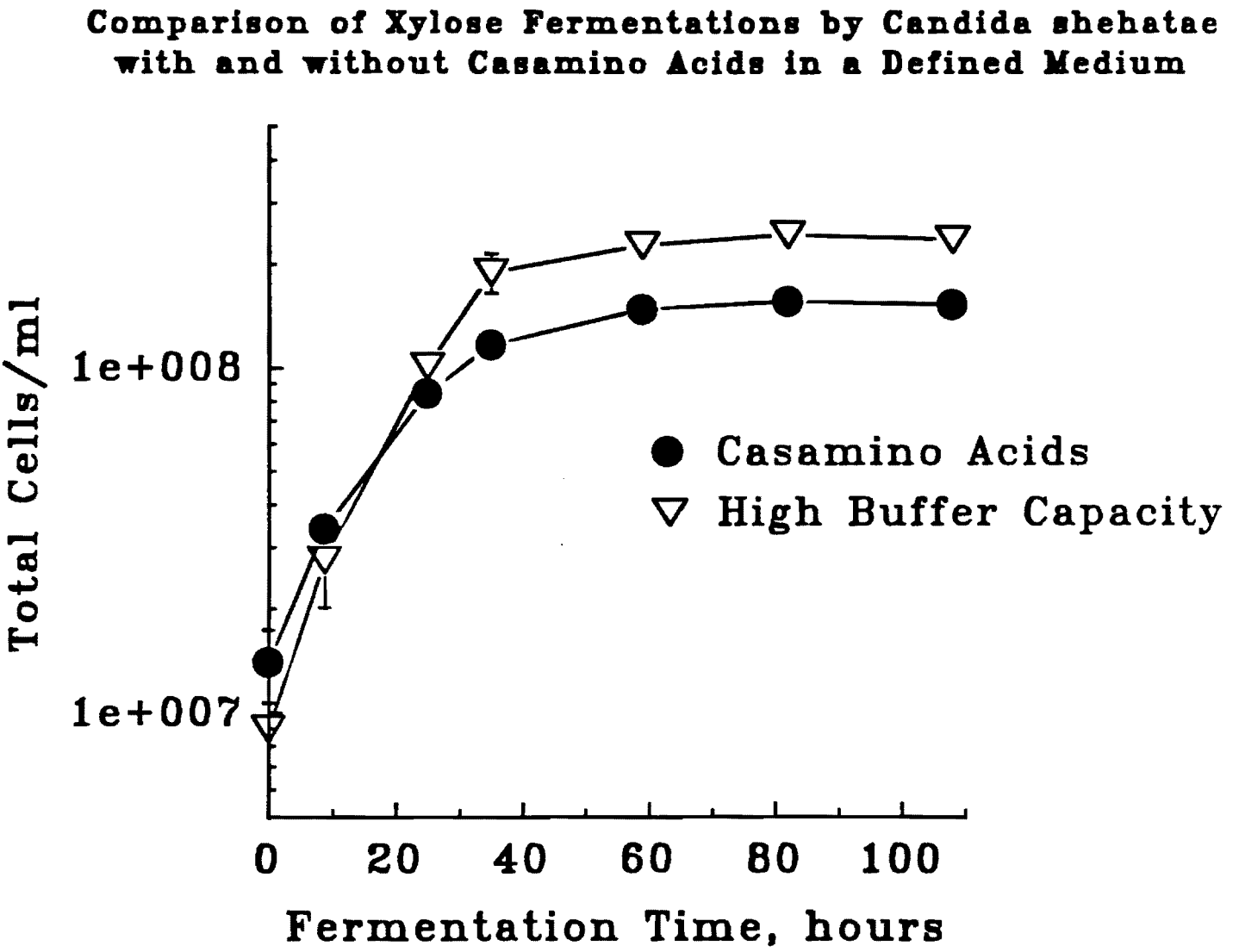


Figure 48 - Effect of Casamino Acids on D-Xylose Uptake.

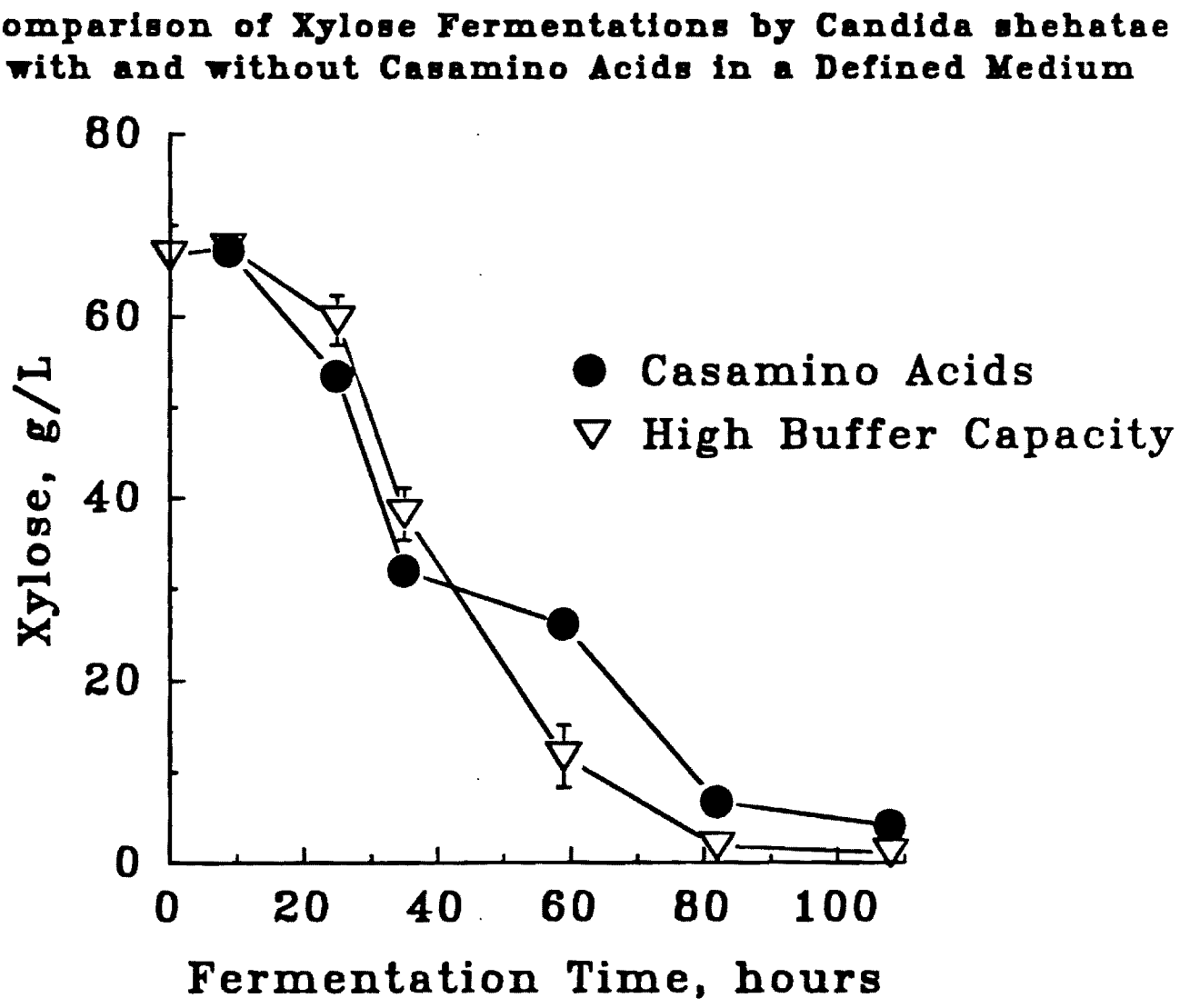


Figure 49 - Effect of Casamino Acids on Xylitol Formation.

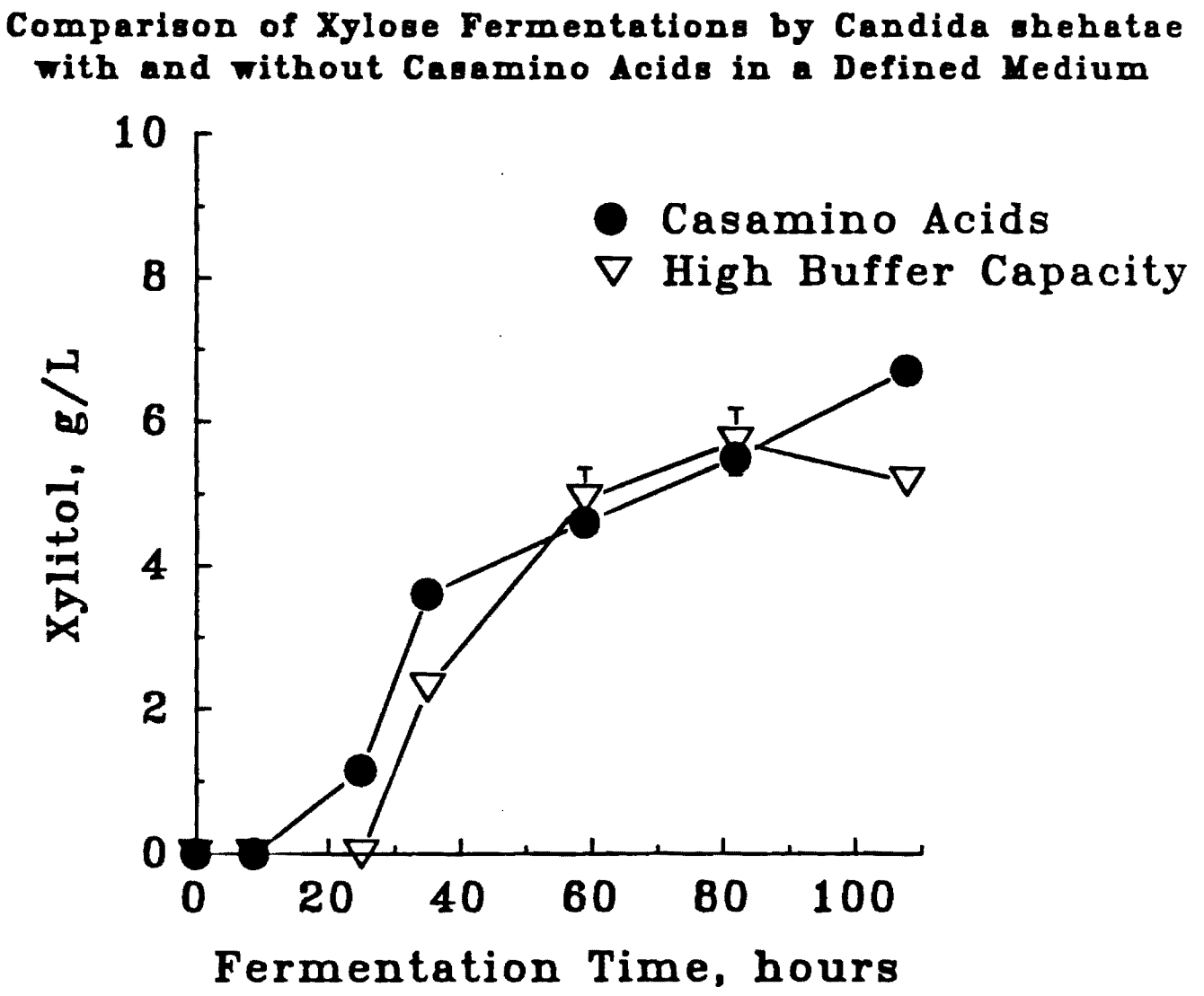


Figure 50 - Effect of Casamino Acids on Cell Viability.

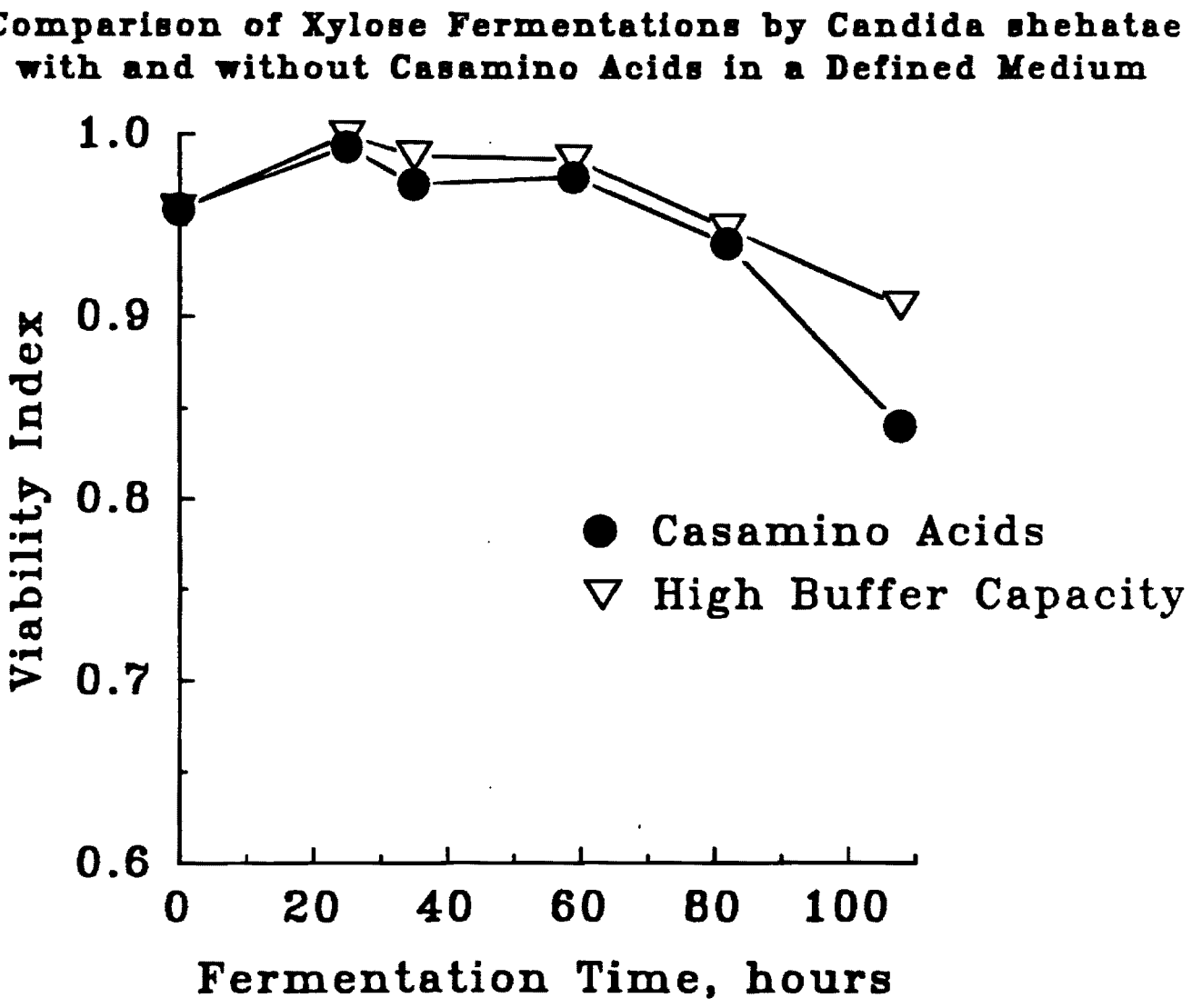


Figure 51 - pH Change In Defined Medium with Casamino Acids and a High Buffering Capacity (10 g/L K_2HPO_4 and 10 g/L of KH_2PO_4).

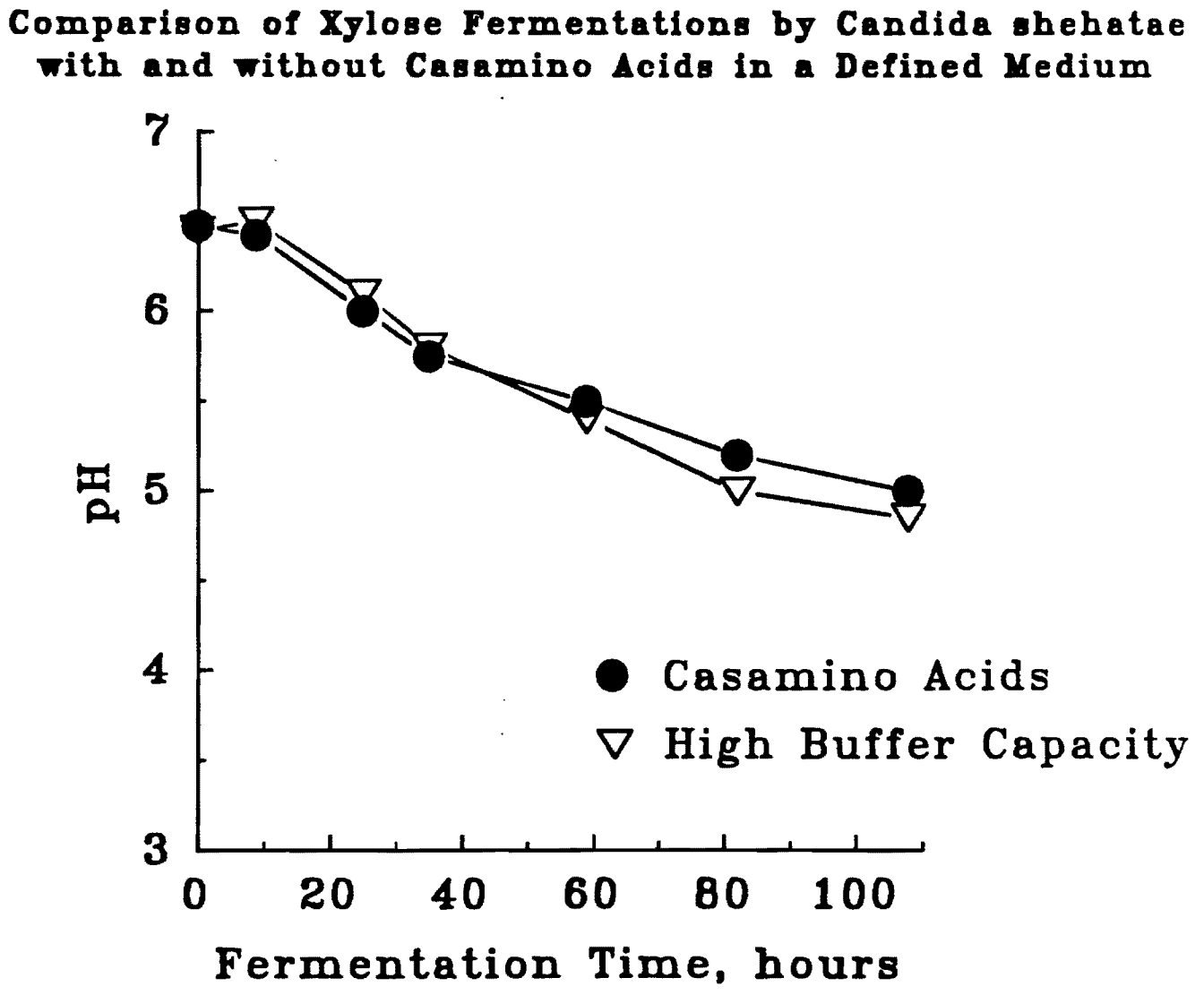


Figure 52 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and 5 g/L KH_2PO_4) on Cell Growth.

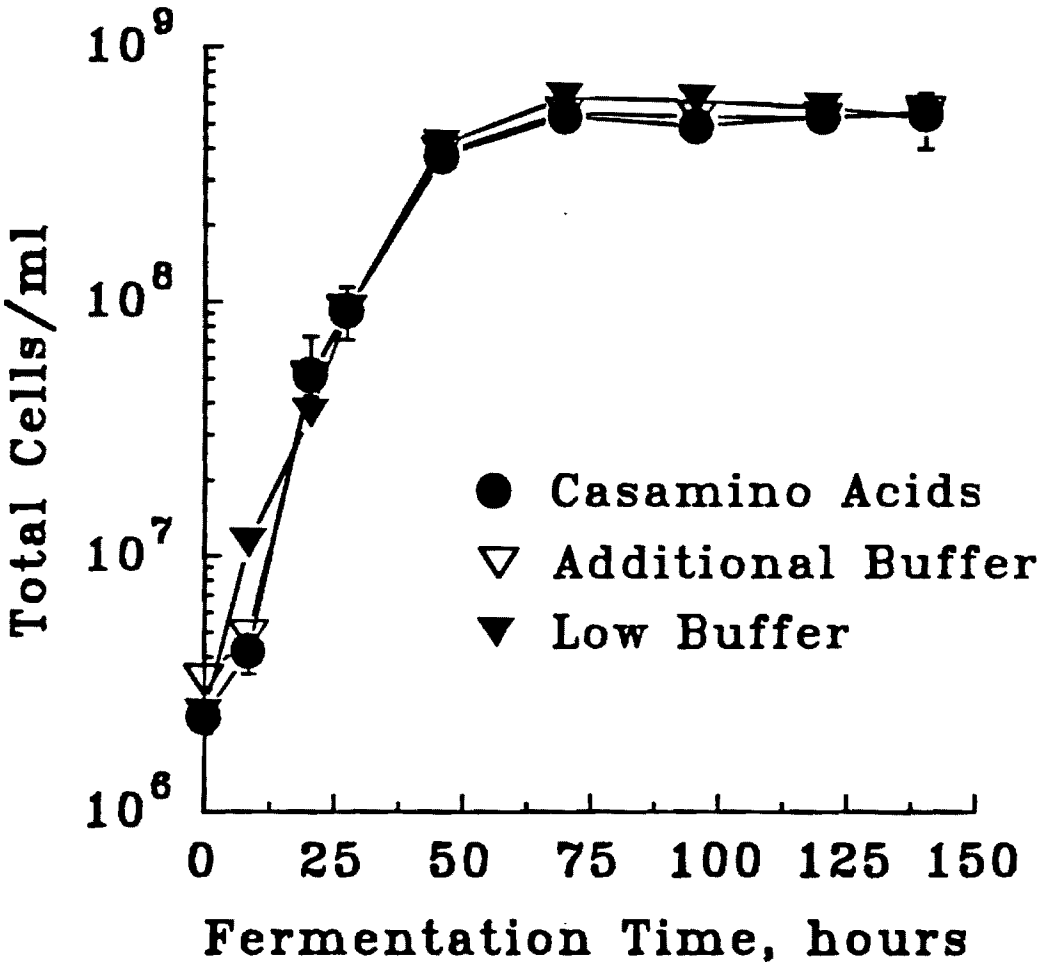


Figure 53 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and KH_2PO_4) on Cell Viability.

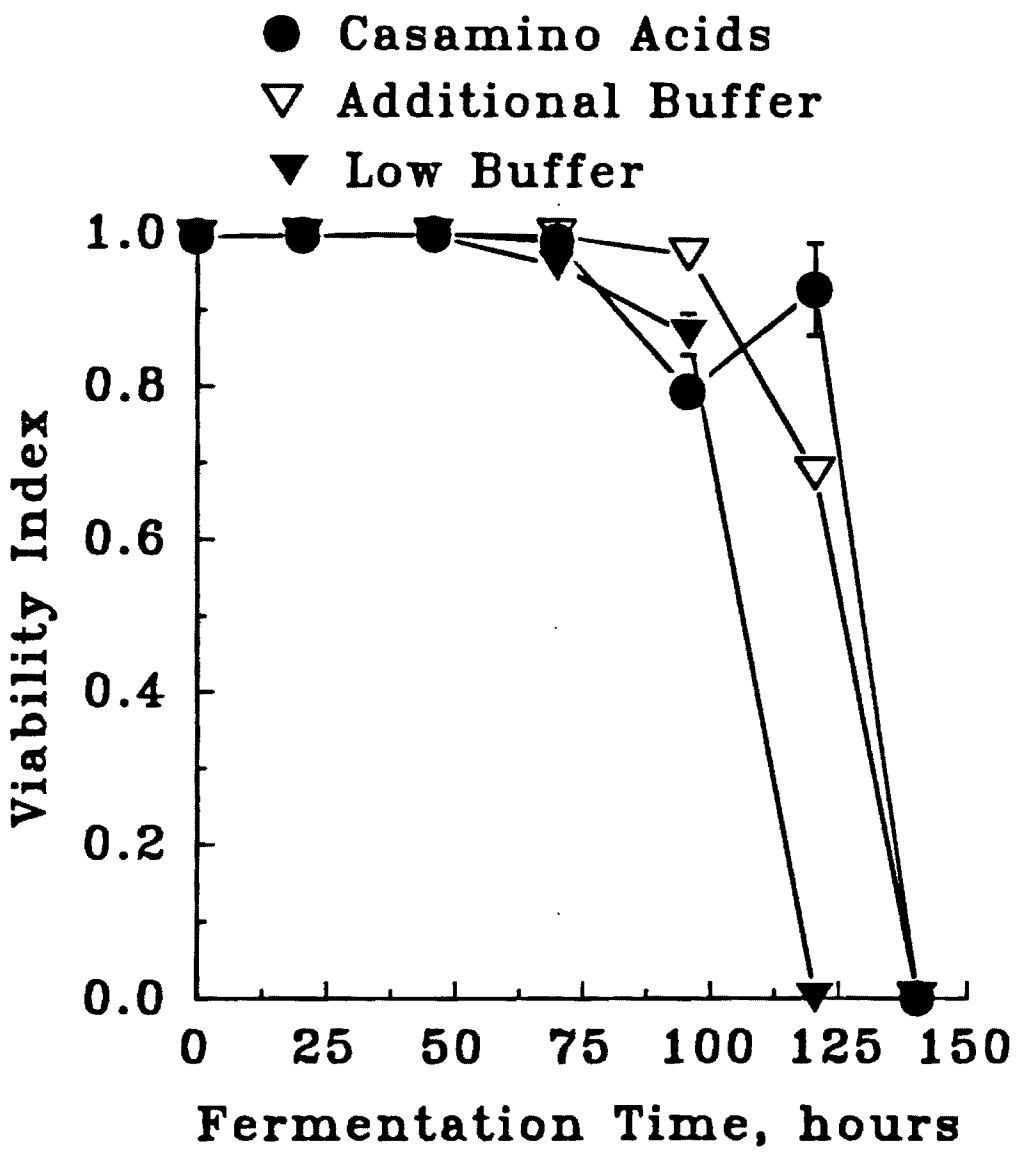
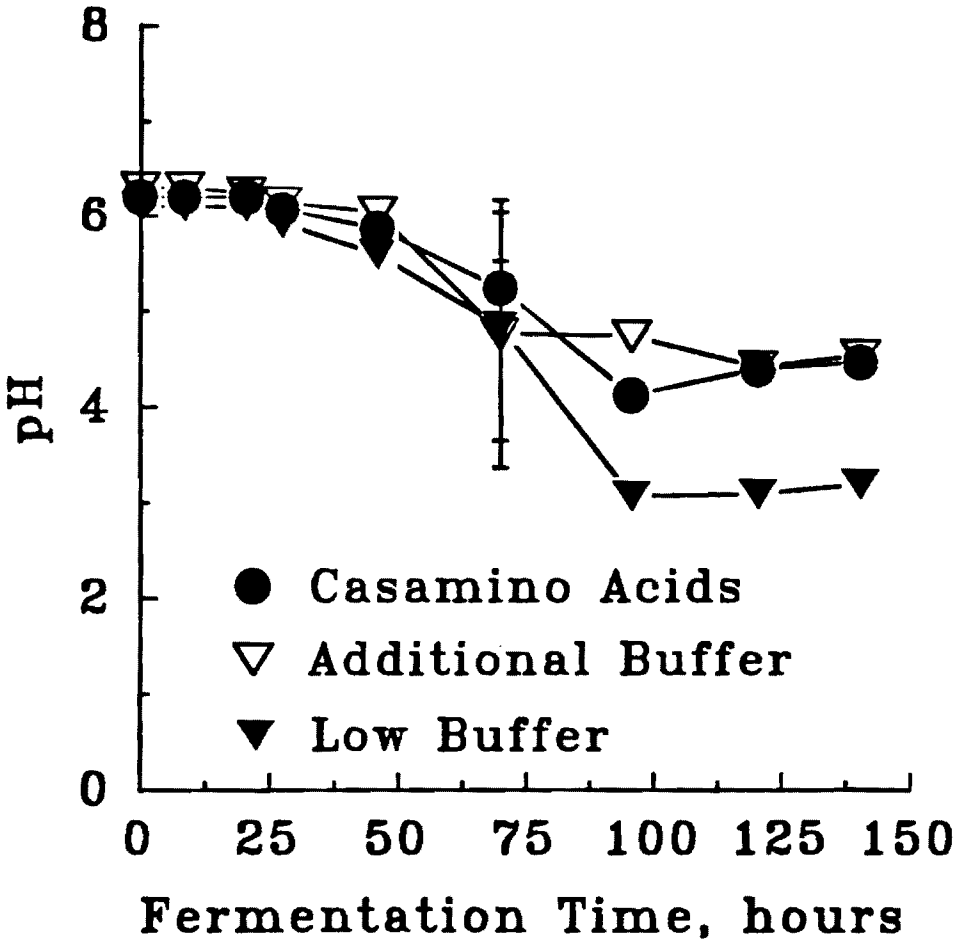


Figure 54 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and KH_2PO_4) on pH During D-Xylose Fermentations.



j. Continuous Fed-Batch D-Xylose Fermentation by *Candida shehatae*: Effect of Casamino Acids

Fed batch fermentations were performed on a defined medium (Table 4) with and without casamino acids. *Candida shehatae* cells were inoculated into a New Brunswick batch reactor at a relatively low cell density. The reactor was aerated at 2 liters/min and agitated at 800 rpm. The reactor had a working volume of 1 liter. The temperature was controlled at 30°C and the pH was controlled at 4.5. As the cell density increased the percent dissolved oxygen (%D.O.) decreased and after approximately 20-24 hours the %D.O. reached zero (Figure 55). At this point aeration was terminated and the agitation reduced to 400 rpm. A concentrated solution (60%) of D-xylose was pumped into the reactor after anaerobic conditions were imposed. Cell viability was monitored by methylene blue staining and plate counts.

Both reactors reached the same cell density; the presence of casamino acids did not stimulate aerobic nor anaerobic growth of *Candida shehatae* (Figure 55). The presence of casamino acids did seem to initially inhibit or reduce the D-xylose utilization rate. This resulted in the higher D-xylose level in the reactor with casamino acids (Figure 56). After 60 hours the D-xylose utilization decreased (as seen by the increase in the xylose levels in both reactors; Figure 56), indicating that the cells were inhibited. The inhibition could have been caused by ethanol or the anaerobic conditions.

With $(NH_4)_2SO_4$ as the nitrogen source approximately 42 g/L ethanol and 25 g/L of xylitol were produced (Figure 57). In the presence of casamino acids 35 g/L of ethanol and 25 g/L of xylitol were produced (Figure 58). Cell viability began to decline in both reactors late in the fermentations after about 100 hours (Figures 59 and 60).

Figure 55 - Viable Cell Concentration and Dissolved Oxygen Level During a Fed-Batch D-Xylose Fermentation by *Candida shehatae*.

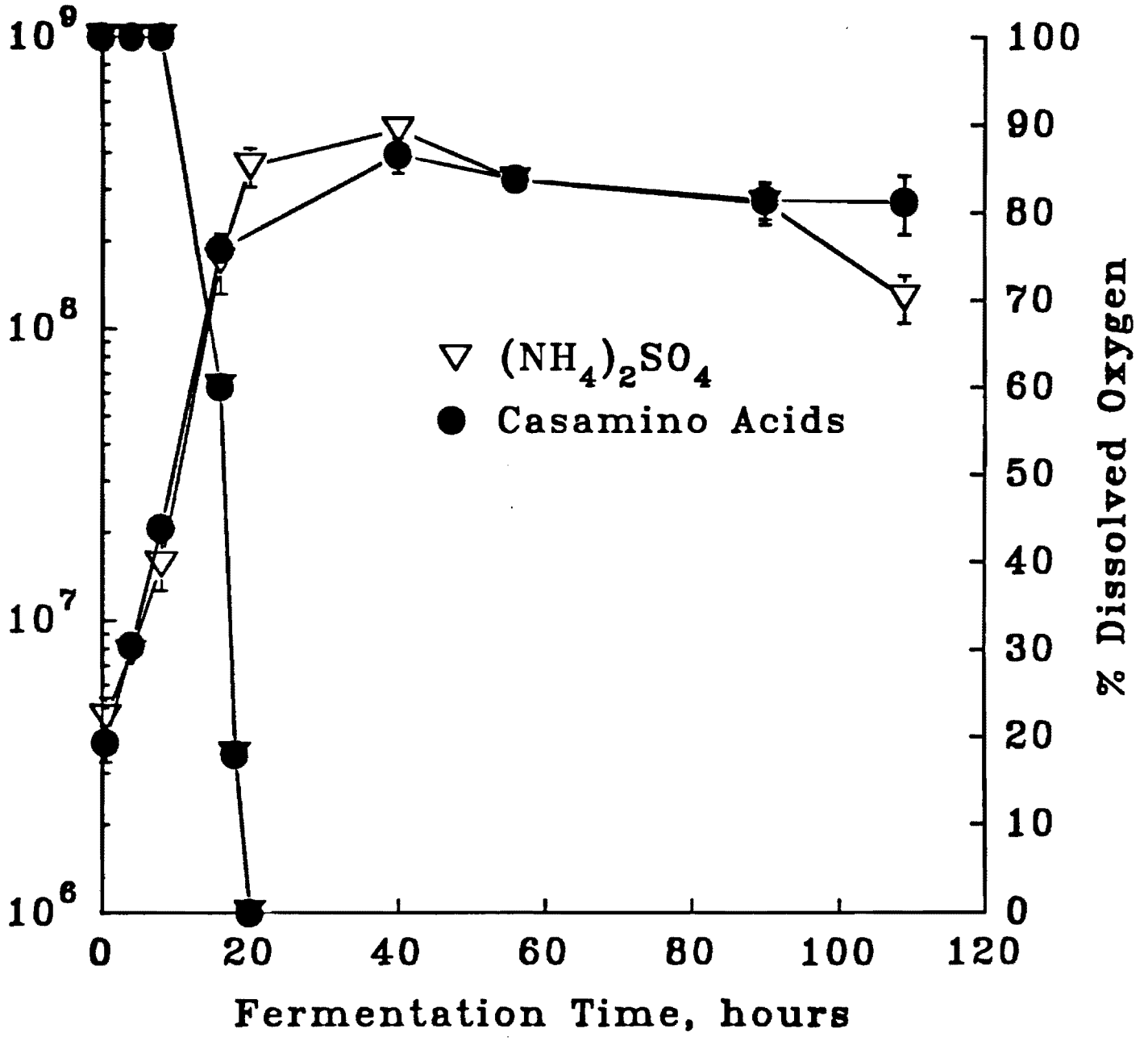


Figure 56 - D-Xylose Levels During a Fed-Batch Fermentation.

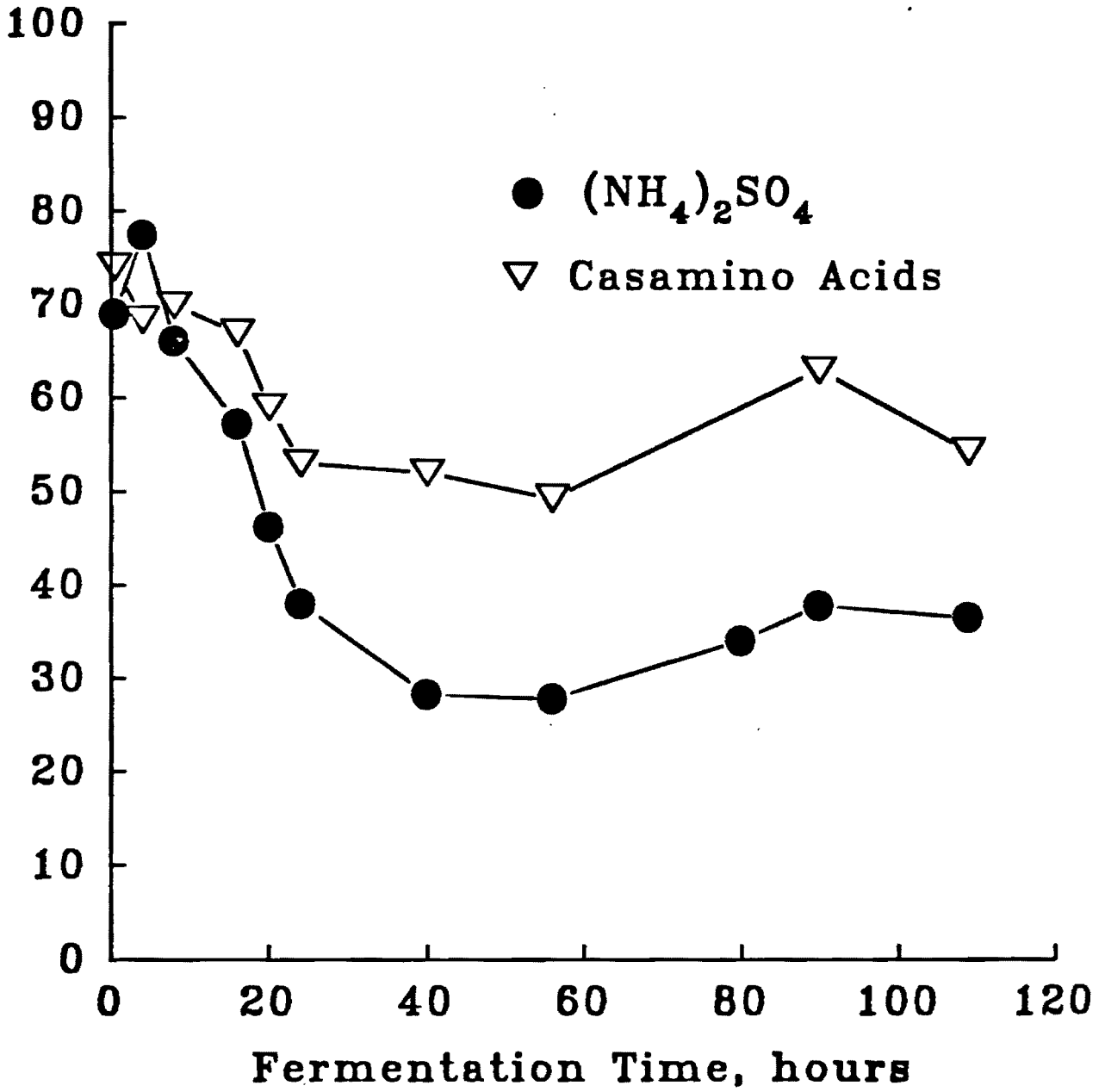


Figure 57 - Ethanol and Xylitol Production in Fed-Batch Fermentation with $(NH_4)_2SO_4$ as the Nitrogen Source.

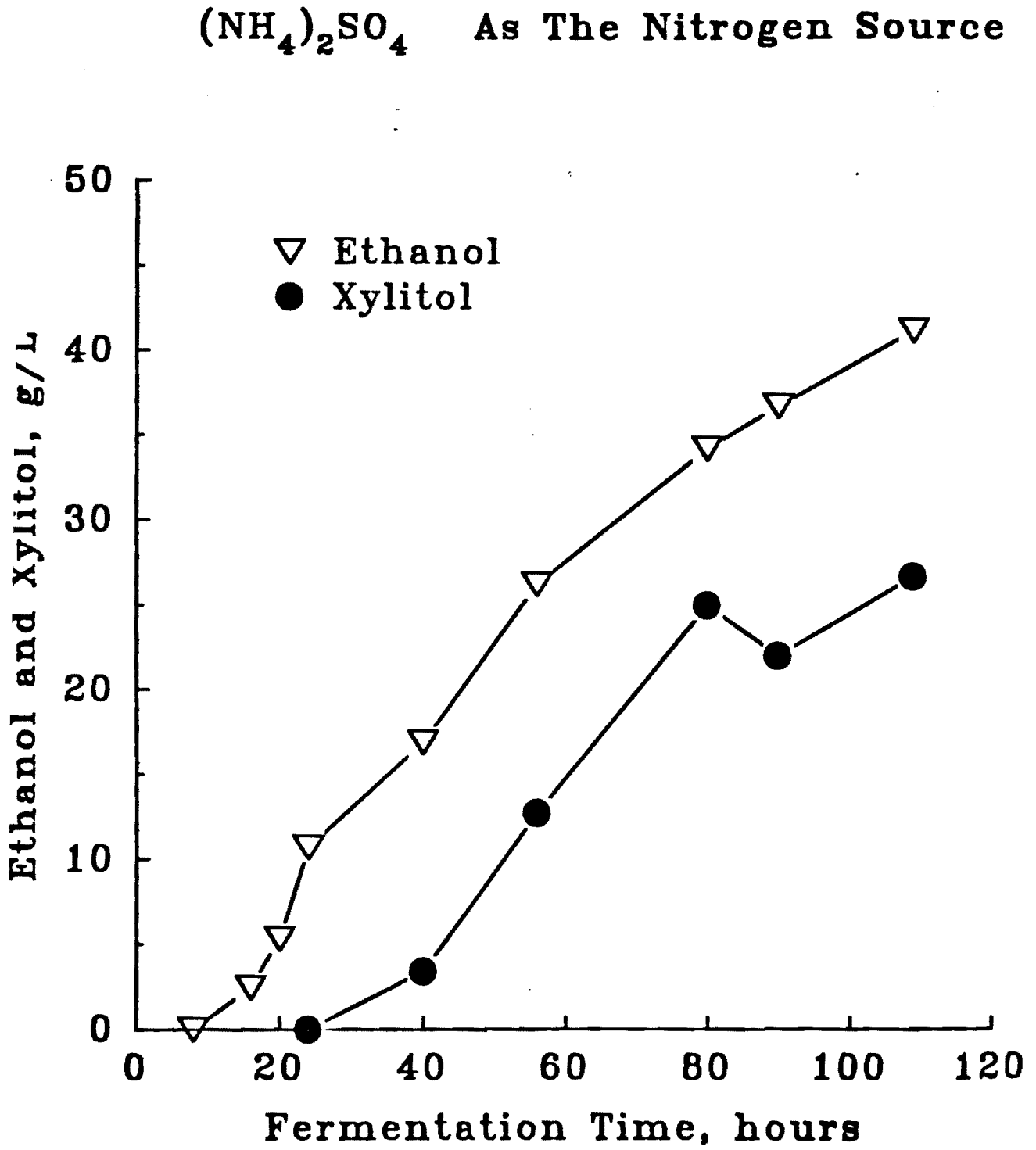


Figure 58 - Ethanol and Xylitol Production in Fed-Batch Fermentation with Casamino Acids as the Nitrogen Source.

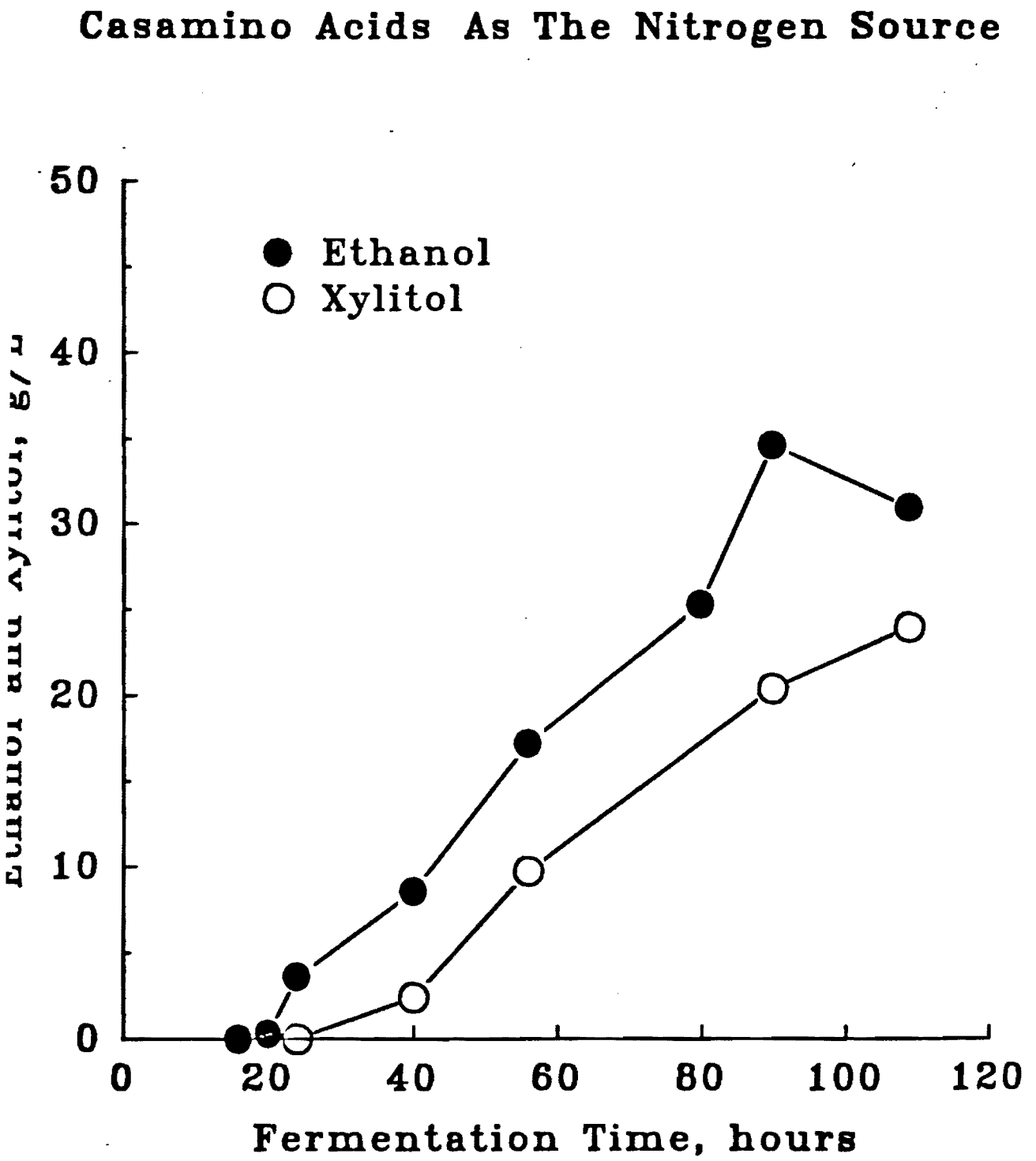


Figure 59 - Effect of $(NH_4)_2SO_4$ as the Nitrogen Source on Cell Viability.

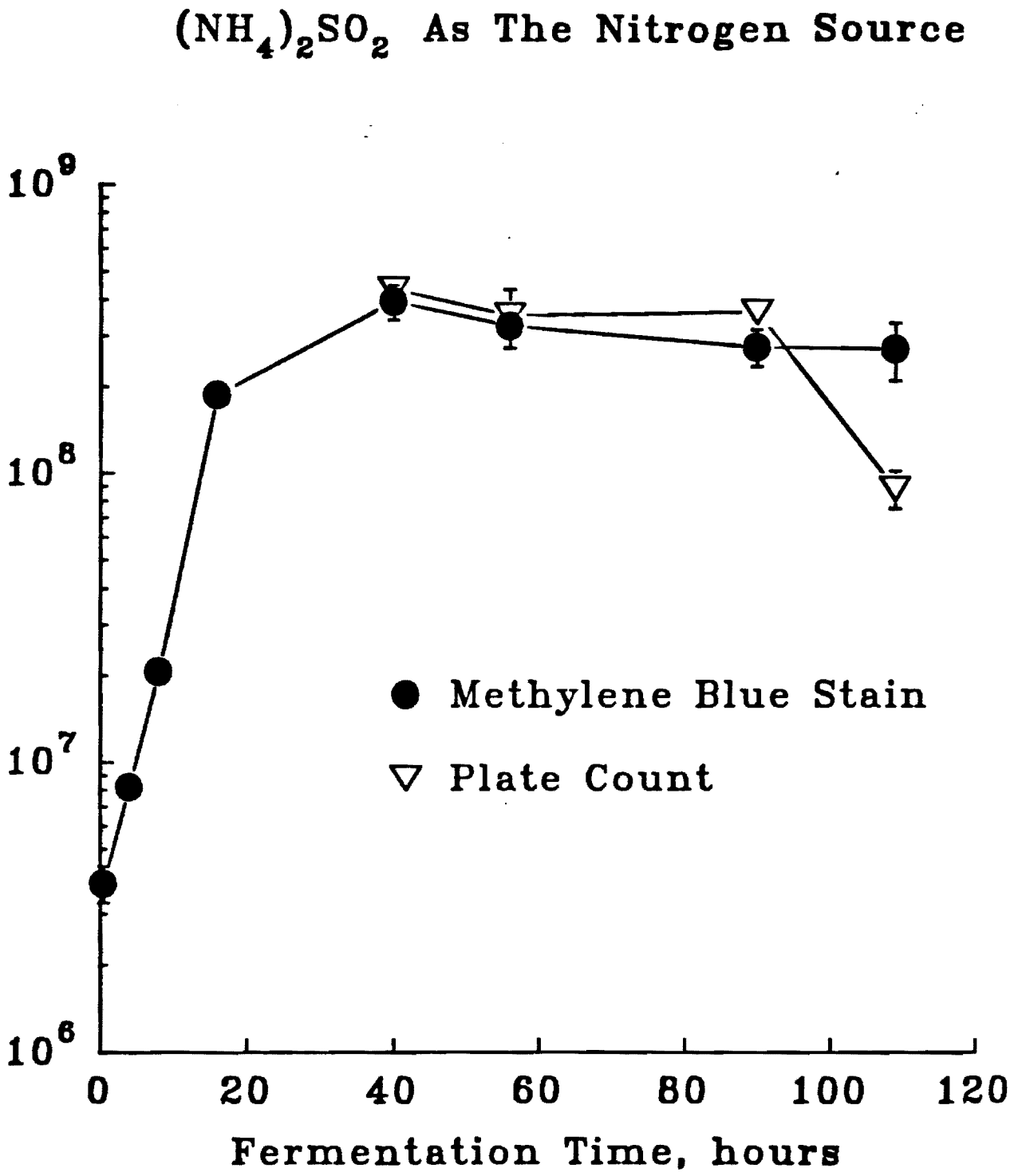
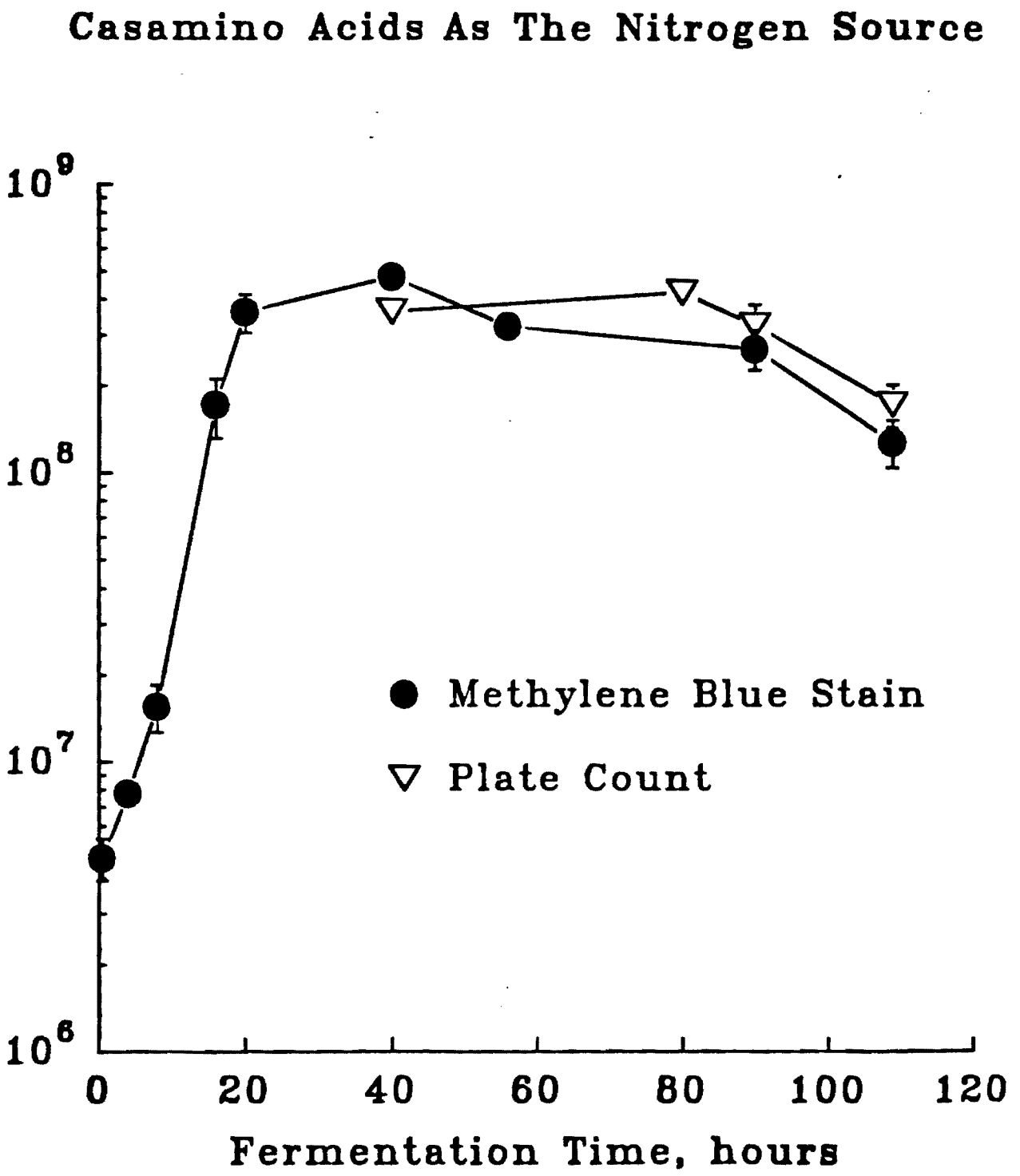


Figure 60 - Effect of Casamino Acids as the Nitrogen Source on Cell Viability.



k. Effect of pH on Fed-Batch D-Xylose Fermentation:

Fed batch fermentations on a defined medium (Table 4) were performed at two different pHs under anaerobic conditions. *Candida shehatae* cells were inoculated into a New Brunswick batch reactor at a relatively low cell density (Figure 61). The reactor was aerated at 2 liters/min and agitated at 600 rpm. The reactor had a working volume of 1 liter and the temperature was controlled at 30°C. Initially the pH was 5.4 in both reactors, but was allowed to drop to 4.5 in one reactor and to 2.5 in the other and controlled at these levels (Figure 62). Dissolved oxygen (% D.O.) was not monitored during the fermentation. Once the stationary phase was reached air sparging was ceased, the agitation was set to 400 rpm, and N_2 was sparged through the reactors. Periodically, a concentrated solution (60%) of D-xylose was pumped into the reactor to maintain the substrate level. Cell viability was monitored by methylene blue staining and plate counts.

The final cell densities achieved in the reactors were dependent on the pH. A pH of 2.5 resulted in 7×10^8 total cells/ml compared to 1.5×10^9 total cells/ml for a pH of 4.5 (Figure 61). The lower pH apparently inhibited the growth of *Candida shehatae*. The lower pH also decreased the D-xylose utilization rate toward the end of the fermentation. However, D-xylose utilization was incomplete at both pHs (Figure 63). The slowdown in D-xylose utilization correlated with a decline in cell viability. Cell viability declined in both fermentations, but was faster and greater for the fermentation performed at a pH of 2.5 (Figure 64).

More ethanol was produced at a pH of 4.5; the fermentation with a pH of 2.5 produced 30 g/L compared to 45 g/L for a pH of 4.5 (Figure 65). The ethanol yield

Figure 61 - Effect of pH on Cell Growth During a Fed-Batch D-Xylose Fermentation.

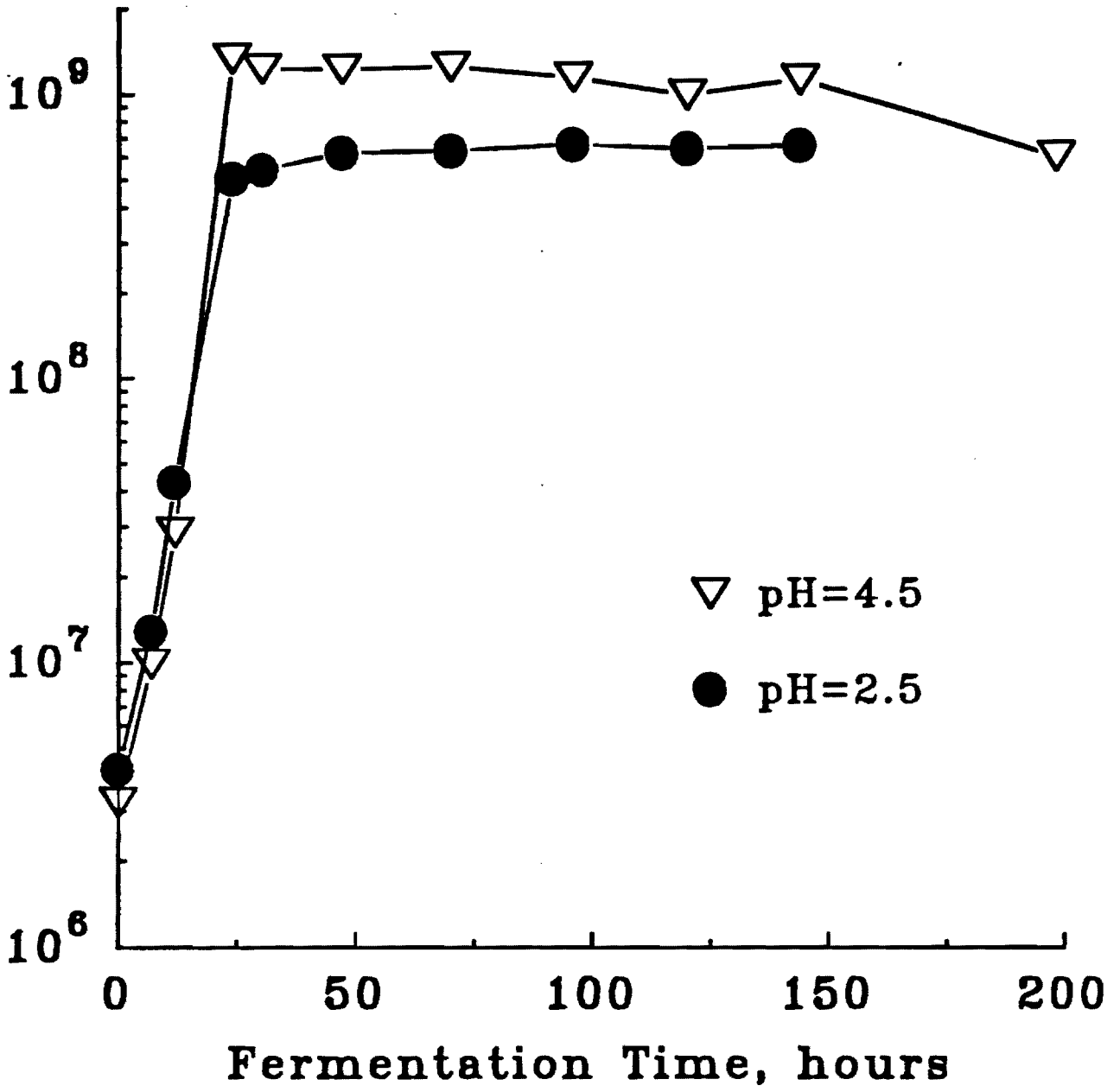


Figure 62 - pH Levels in Fed-Batch D-Xylose Fermentation by *Candida shehatae*.

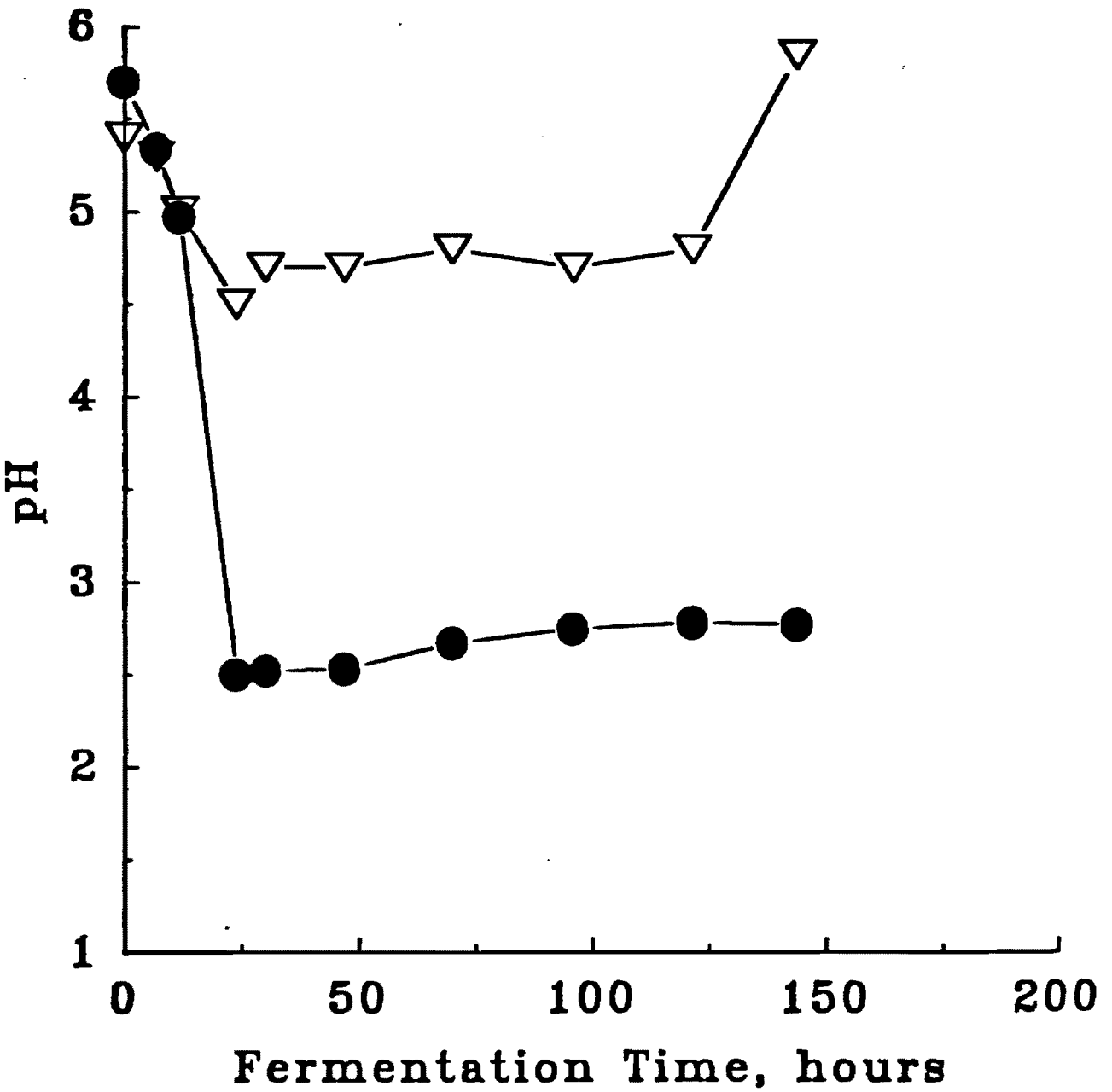


Figure 63 - Effect of pH on D-Xylose Utilization During a Fed-Batch Fermentation.

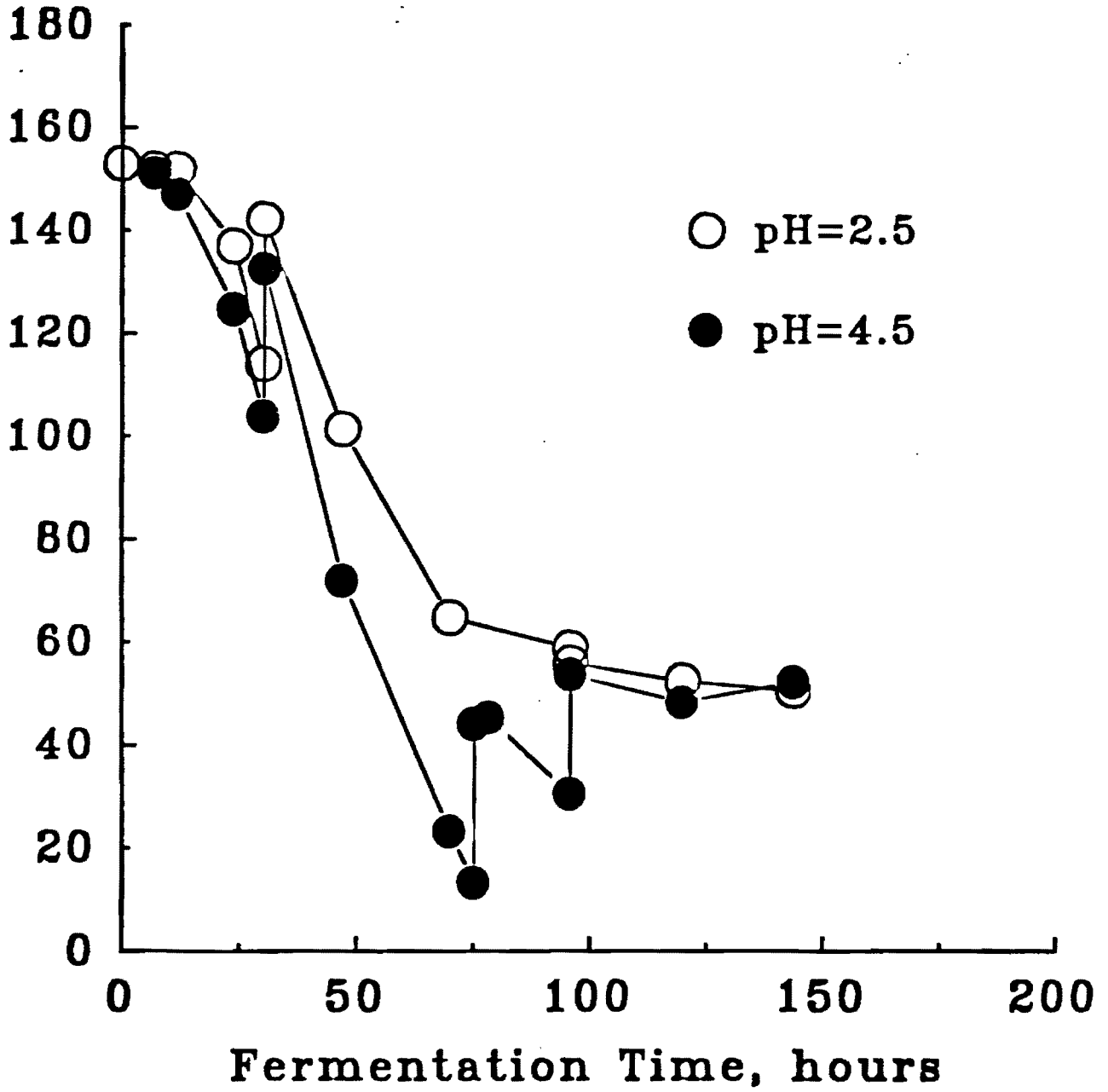


Figure 64 - Effect of pH on Cell Viability During a Fed-Batch Fermentation.

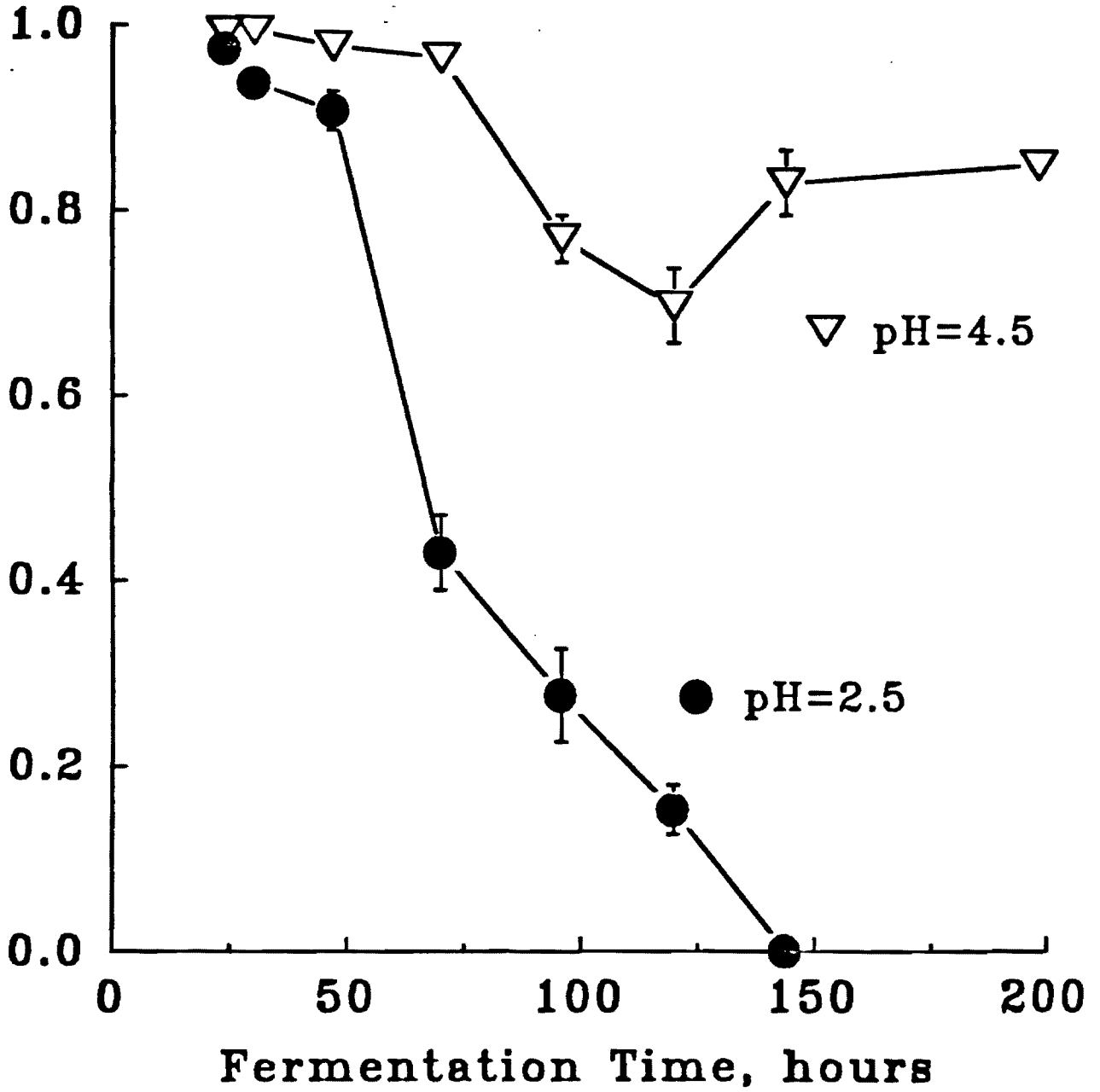
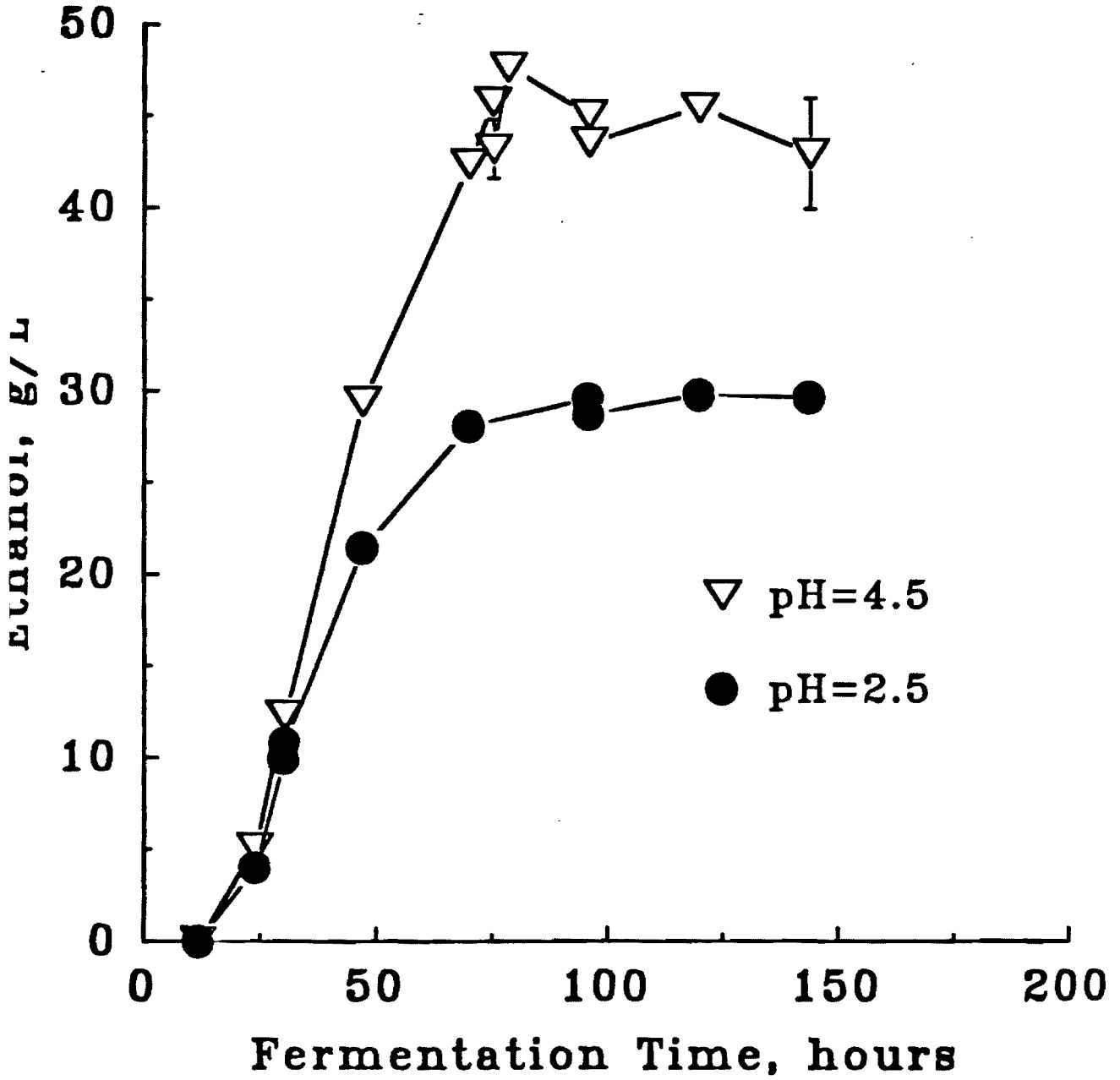


Figure 65 - Effect of pH on Ethanol Production in a Fed-Batch Fermentation.



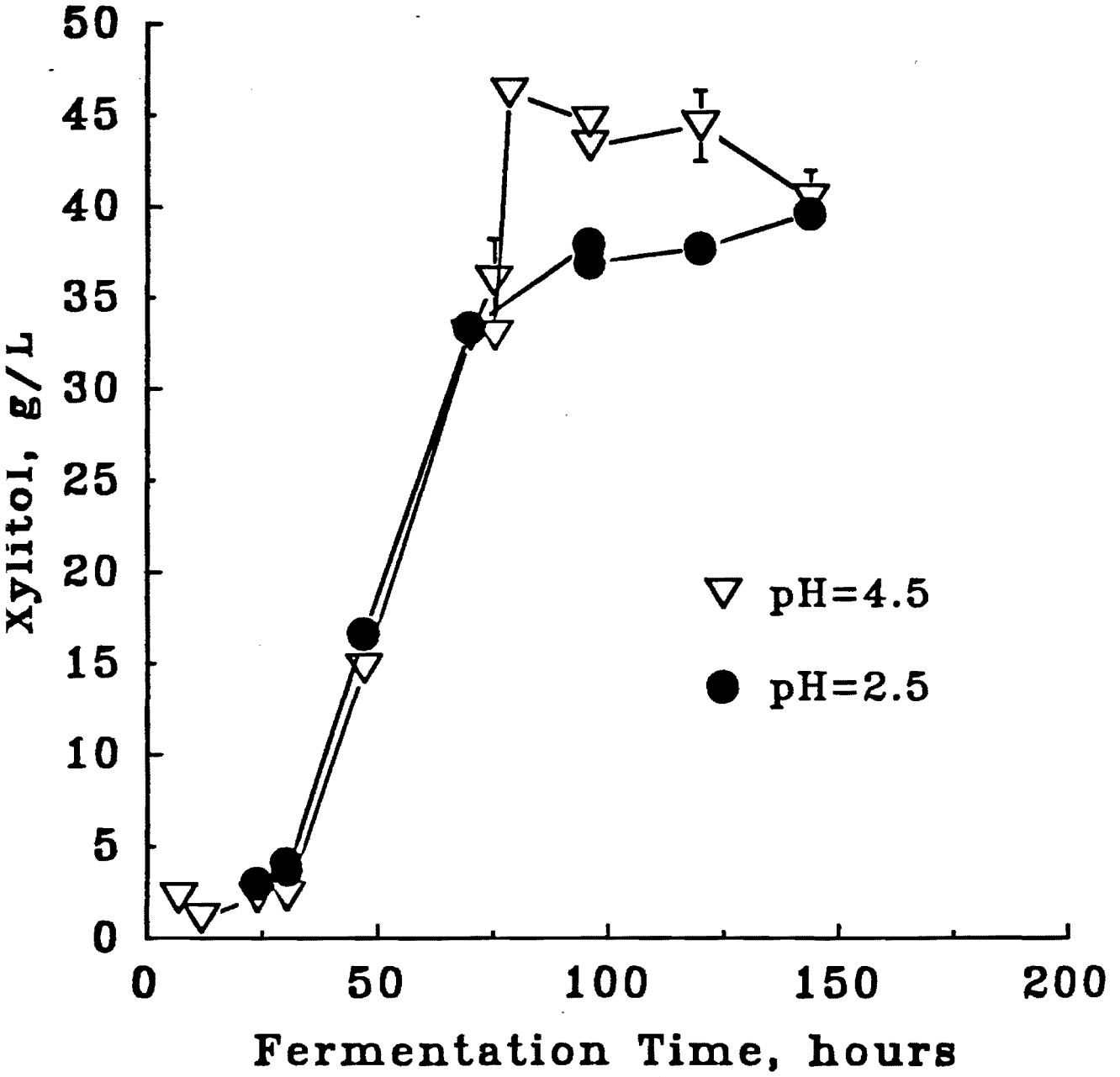
(grams of ethanol produce per gram of xylose consumed under anaerobic conditions) was 0.28 for a pH of 4.5 compared to 0.21 for a pH of 2.5. Also, more xylitol was produced at a pH of 4.5 compared to a pH of 2.5 (Figure 66).

l. Effect of Added Ethanol on D-Xylose Fermentations by *Candida shehatae*:

The data clearly show that *Candida shehatae* cells decline in viability under anaerobic conditions (as measured by methylene blue staining and plate counts). There are many metabolic changes which occur under anaerobic conditions which may contribute to the decline in cell viability. Under anaerobic conditions, *Candida shehatae* produce ethanol and xylitol, both of which may affect viability. Also, in batch fermentations without control instrumentation pH will decline due to CO_2 production and pH can also affect cell viability. Nutrient limitation(s) can also cause a decline in viability. Metabolic changes, such as a reduction of ATP production or lack of key intermediate synthesis can contribute to a decline cell viability.

The data up to this point indicate that the defined medium used without casamino acids was not nutrient limiting and therefore probably did not contribute to the decline in viability of *Candida shehatae*. The data does indicate that fermentation by-products, low pH levels and anaerobic conditions may cause or contribute to the decline in viability. In all cases viability declined as ethanol and xylitol levels increased, as the pH declined and after longterm exposure to anaerobic conditions. Experiments were designed to try to separate and identify the factor(s) responsible for the decline in cell viability of *Candida shehatae*.

Figure 66 - Effect of pH on Xylitol Production in a Fed-Batch Fermentation.



In these experiments the effect of added ethanol on cell viability was tested. The D-xylose fermentations were performed in 500 ml Erlenmeyer flasks in an incubator shaker (Eviron-Shaker, LabLine) at 30°C and 250 rpm. The working volume was 250 ml (total volume). Initial ethanol concentrations were 0, 25 and 50 g/L. These concentrations were obtained by adding the appropriate amount of cold-filtered 95% ethanol to each reactor. Distilled water was added to the control reactors instead of ethanol. All reactors were initially inoculated with $3.05 \pm 0.533 \times 10^6$ total cells/ml. Each flask was equipped with a rubber stopper and septum; this was done to achieve anaerobic conditions. A sterile 5 ml syringe filled with cotton was inserted into each septum to allow CO_2 evolution and prevent pressure build up. Liquid samples were withdrawn from the experimental flasks through the septum using a sterile 5 ml syringe. The initial pH in each reactor was 6.1 ± 0.03. Previous data showed that cell viability declined at much faster rate for a pH of 2.5 compared to 4.5. A pH of 6.0 at time zero would prevent the pH from declining below 4.5. Each fermentation was performed in duplicate and the experiments replicated.

Periodically, five ml samples were aseptically collected from the experimental flasks and immediately placed on ice. An aliquot of each sample was serially diluted in Ringer's solution. These samples were then spread plated (in triplicate) on to Difco (Detroit, Michigan) YM Broth in 2% (w/v) agar. The plates were incubated at 30°C and counted at 48, 72 and 96 hours. The number of colonies per plate ranged between 20 and 400. Viable cell counts were also determined by methylene blue staining using the method of Lee et al., 1981

For each fermentation sample, between 200-1000 cells were counted using a hemacytometer (Bright-Line, American Optical, Buffalo, New York) for each sample. Cells that were stained blue or light blue were scored as non-viable and cells that were clear (unstained) were scored as viable. Buds or daughter cells less than the size of the mother cell were not counted, while cells that remained attached and of approximate equal size were counted. Flocculation and aggregation of cells was not observed in our experiments.

Two different viability indices were calculated, one based on methylene blue and the other based on the plate counts. A methylene blue viability index was calculated as the number of unstained cells divided by the number of unstained plus stained cells. A viability index based on plate counts was calculated as the number of colony forming units per ml divided by the total cells/ml. Total cell counts were performed using an Elzone Model 80XY Particle Counter (Particle Data, Inc., Elmhurst, IL.). A saline solution (8.5 g/L NaCl and 0.2 g/L EDTA) was used to dilute the samples. The saline solution was continuously filtered through two 0.2 μm filters in series. The appropriate dilutions of samples were made to reduce coincidental passage and ranged from 1/51 to 1/201. Coincidental passage was maintained between 0.2 and 1.8 percent of the count. Cell counts were performed with a 30 μm orifice and 100 μl volumetric section. Each sample was counted a minimum of three times and results averaged for calculation of the total cells/ml.

The particle counter was also used to obtain cell volume distributions for each sample. The cell volume distribution was calibrated with 2.02 μm and 5.1 μm latex beads

at a log scale of 12, a current of 6 and a gain of 1. All counts and size distributions were performed with these settings. Cell volume distribution data were acquired on 128 channels using an IBM PC XT. The pH of an aliquot of each sample was taken with a Fisher Accumet Model 140A. The pH meter was periodically calibrated using buffers of pH 4 and 7. The remaining sample was centrifuged at 4053 * G (5000 rpm) for 15 minutes and/or passed through a 0.45 μm filter. The supernatant was decanted and stored in glass vials at 0°C. Xylose, glucose, xylitol and ethanol concentrations were determined by HPLC as previously reported (Kastner and Roberts, 1989).

Initial ethanol levels of 25 and 50 g/L completely inhibited *C. shehatae* fermentation of D-xylose under oxygen-limited conditions. As shown in Figure 67, there was no growth of *C. shehatae* in the reactors supplemented with ethanol. In the control experiments (no added ethanol), a ten-fold increase in cell number was observed during the initial stages of the fermentation, but continued cell growth was limited by oxygen availability. In cultures with added ethanol, D-xylose was not consumed. Also, no ethanol and xylitol, predominant products of anaerobic D-xylose fermentation, were produced (Table 6). The pH remained constant in these reactors (6.1 ± 0.03), also indicating that there was no fermentative activity in the reactors with added ethanol. In the control experiments D-xylose was consumed (41.4 ± 3.3 g/L), but its utilization was incomplete, and both ethanol

Figure 67 - Effect of Added Ethanol on Cell Growth.

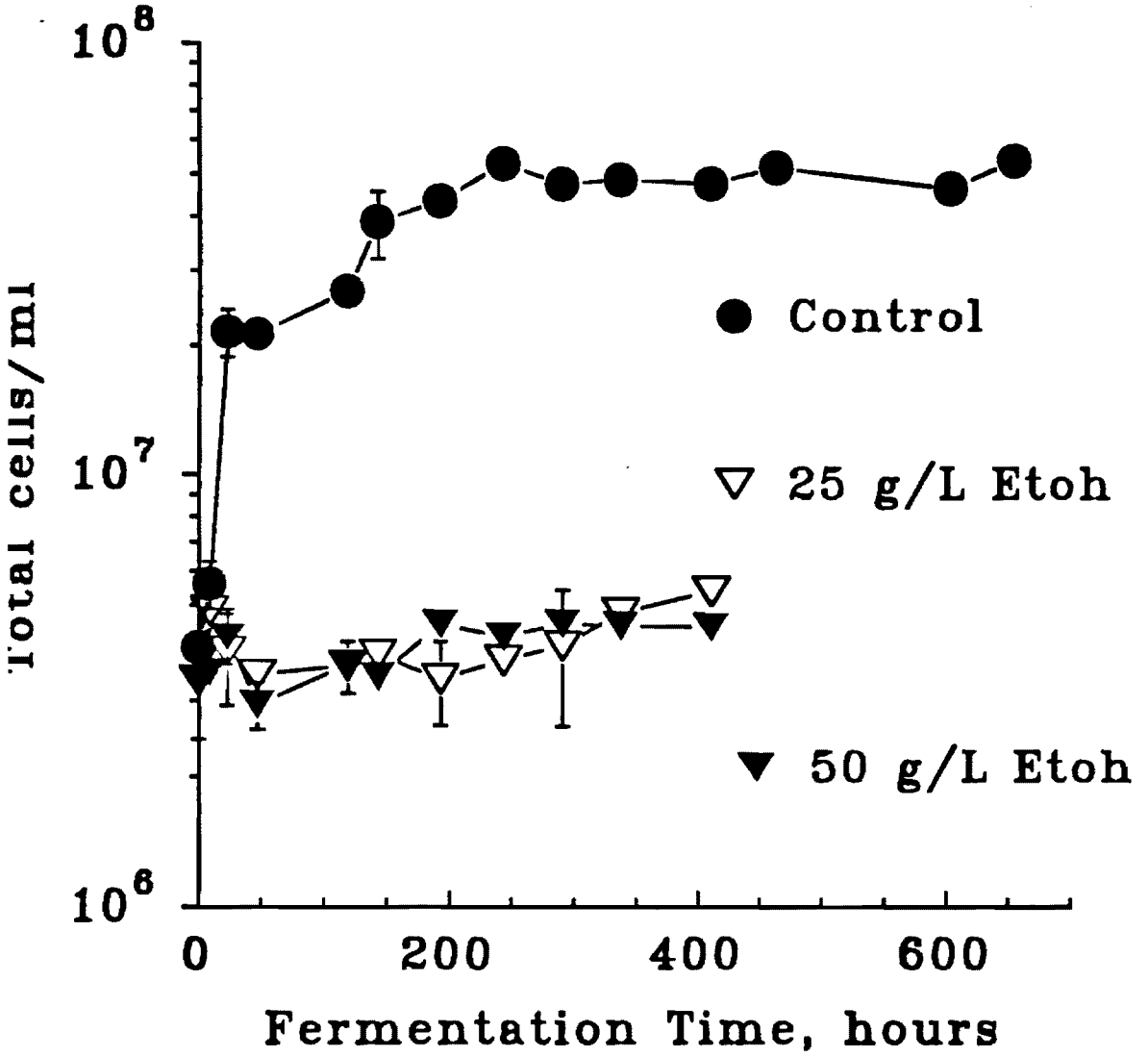


Table 6. Effect of added ethanol on oxygen-limited D-xylose fermentations by *Candida shehatae*.

Ethanol (g/L)	t_f (hours)	Used Sugars (%)	Ethanol Produced (g/L)	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)
0	655	37.5	13.0 ± 0.63	0.3 ± 0.002	0.22 ± 0.02
25	400	0.0	0.0	0.0	0.0
50	400	0.0	0.0	0.0	0.0

t_f : Time over which ethanol and xylitol yields were calculated.

$Y_{p/s}$: Ethanol yield from D-xylose; grams of ethanol produced per gram of consumed D-xylose.

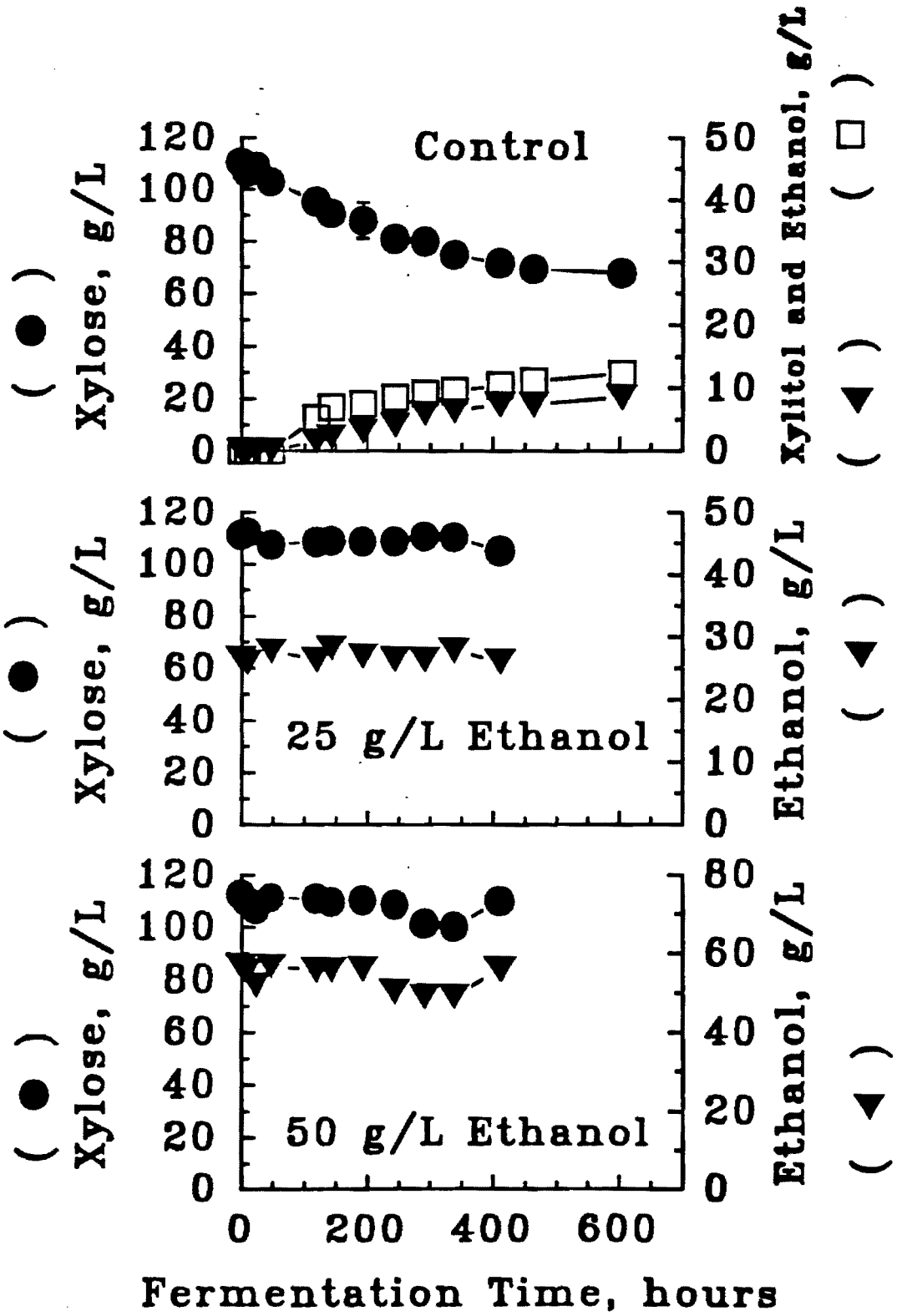
$Y_{x/s}$: Xylitol yield from D-xylose; grams of xylitol produced per gram of consumed D-xylose.

(13 ± 1.1 g/L) and xylitol (9.25 ± 0.1 g/L) were produced (Figure 68 and Table 6). In the control experiment the pH declined from 6.1 (0 hours) to 5.75.

Added ethanol caused a rapid decline in cell viability as measured by methylene blue staining and plate counts. The loss of cell viability was most rapid for experiments with 50 g/L of ethanol, followed by 25 g/L of ethanol and the control (Figure 69). In each experiment, the percent viability measured by plate counts declined at a faster rate than the percent viability measured by methylene blue staining (Figure 69). Thus, viability measurements using the plate count method were more sensitive to the effect(s) of ethanol. These data are similar to results reported for *Saccharomyces cerevisiae* and may be explained in the following manner (Dasari et al, 1990).

Plate counts and methylene blue staining are two different measures of cell viability (Jones, 1987). In the plate count method, only viable cells replicate and grow to form a colony on a suitable medium after dilution. Thus, lack of growth and colony formation may be due either to cell death or inhibition of essential growth processes, such as, cell division, protein synthesis, nutrient transport, energy generation, etc. The basis of methylene blue staining method is that viable cells either exclude the dye (and thus avoid staining) or reduce that dye which does enter the cell to its colorless form. A cell which stains blue (non viable cell) is considered to have a membrane which is permeable to the dye and is unable to maintain the dye in its reduced state. Therefore, non-viable cells stain as a result of irreversible cell damage and/or cell death. The data suggest that the added ethanol first caused a rapid termination in cellular replication processes, as indicated by the plate count measurements. Following inhibition of cellular replication, cell damage

Figure 68 - Effect of Added Ethanol on D-Xylose Utilization and Ethanol and Xylitol Production.



and/or cell death resulted, as demonstrated by the methylene blue staining method (Figure 69).

In addition to the changes in cell viability, the total and mean cell of volume of *C. shehatae* decreased in the reactors with added ethanol. The cell volume distributions for *C. shehatae* cells exposed to an initial ethanol concentration of 50 g/L is presented in Figure 70. Initially, the cell volume distribution curve was broad, indicating that the inoculum was derived from a growing cell population. The cell distributions narrowed and shifted downward towards smaller cell volumes as the experiment progressed (Figure 70). This effect is also evident in Figure 71, which shows the rapid decline in mean cell volume for experiments with added ethanol (25 and 50 g/L). The mean cell volume declined to a greater extent and at a faster rate when the cells were exposed to higher ethanol concentrations.

One possible explanation for the decline in mean cell volume and total cell volume is that the added ethanol caused leakage of metabolites and/or inorganic compounds across the cell membrane. Ethanol concentrations greater than 90 g/L have been shown to increase membrane permeability to metabolites in *Saccharomyces cerevisiae* (Salgueiro et al, 1988). This effect may develop in *Candida shehatae* at lower ethanol concentrations, if there are significant differences in membrane structure/composition between *C. shehatae* and *S. cerevisiae*. If this is the case, nutrient supplements could be added to the fermentation medium to replenish those lost by diffusion. Increased concentrations of vitamins, amino acids and magnesium ions have been shown to have a

Figure 69 - Effect of Added Ethanol on Cell Viability as Measured by Plate Counts and Methylene Blue Staining.

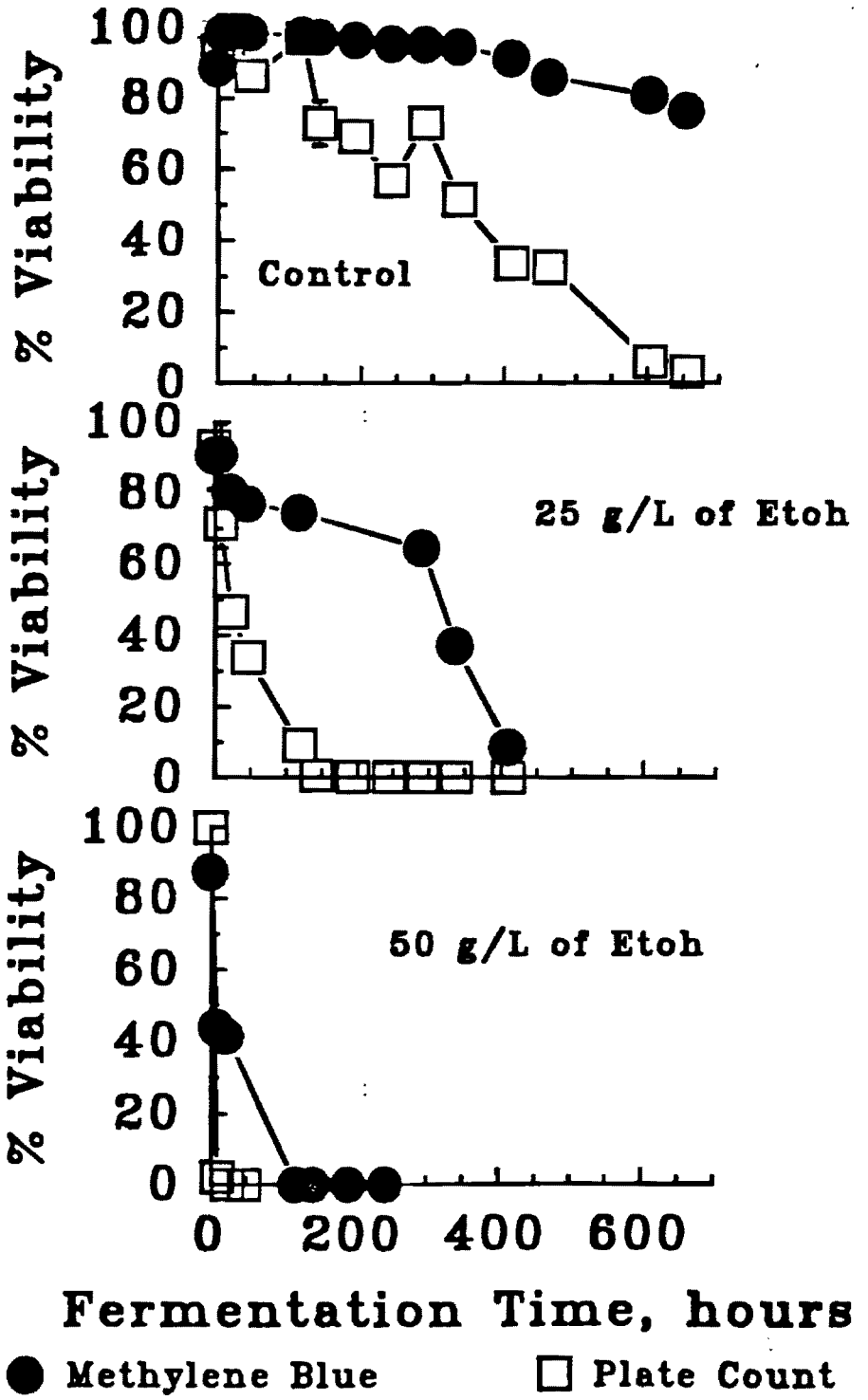


Figure 70 - Effect of Added Ethanol on Cell Volume Distribution for Cells Exposed to 50 g/L of Ethanol at Time Zero.

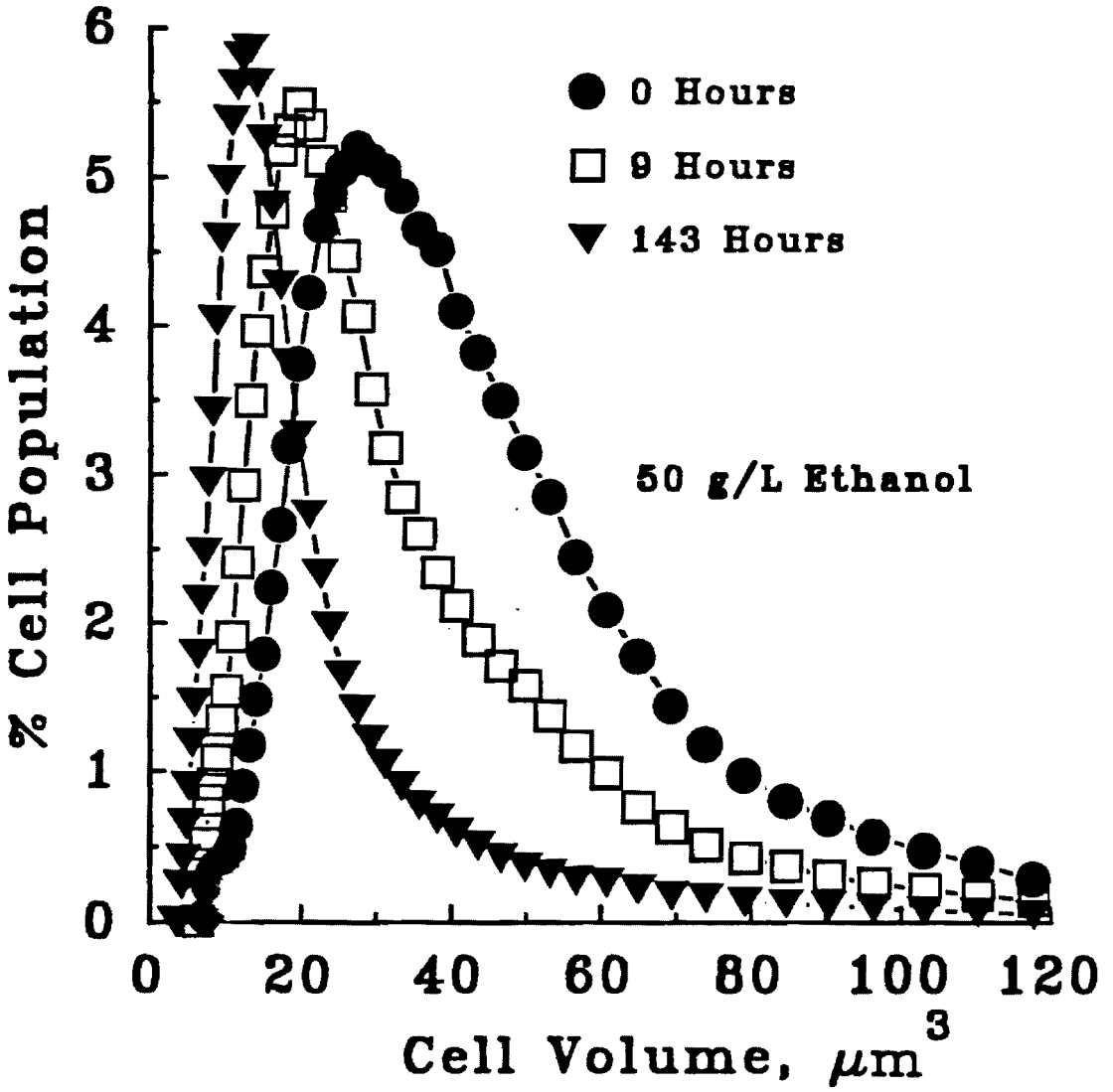
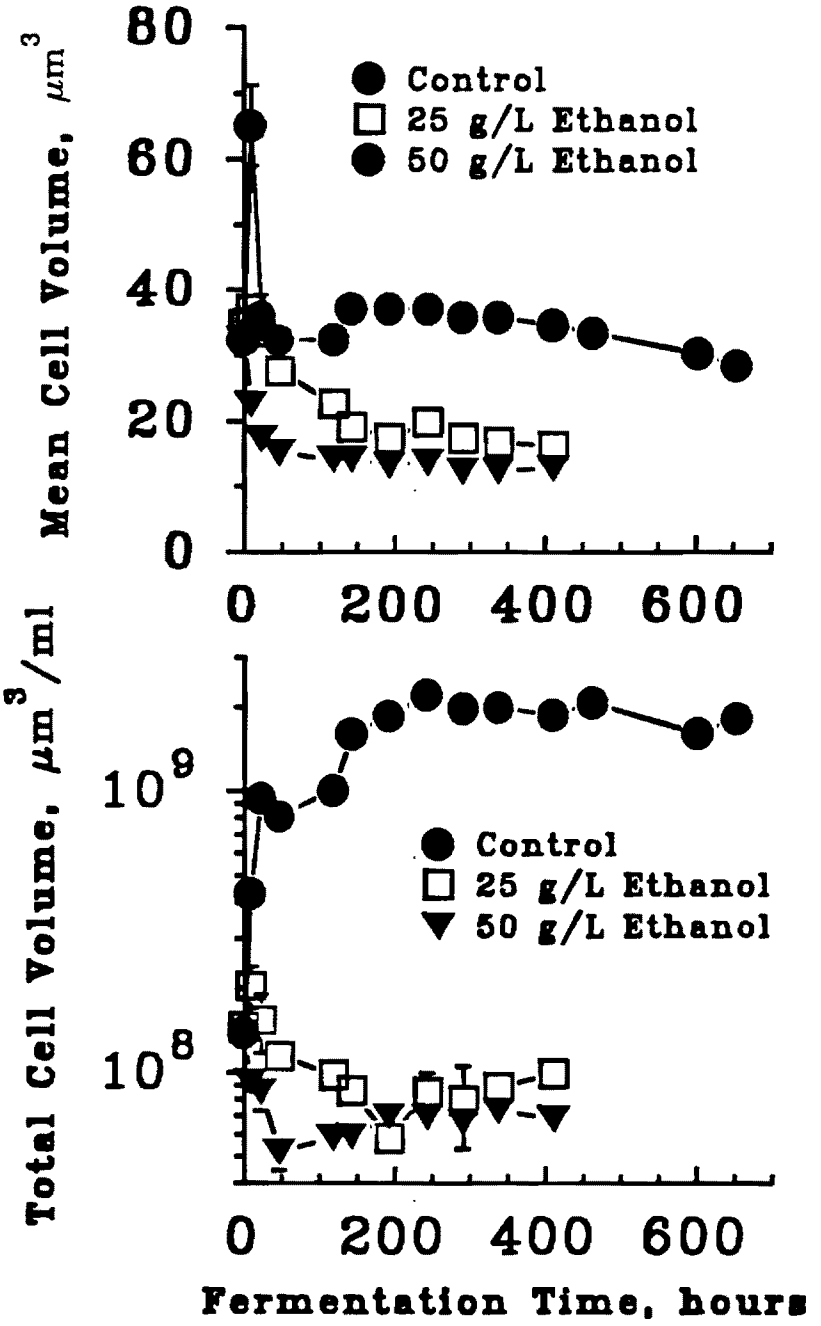


Figure 71 - Effect of Added Ethanol on Average Cell Volume and Total Cell Volume.



positive effect on ethanol fermentations by *Saccharomyces cerevisiae* (Dombek et al, 1986 and Salgueiro et al, 1988).

The added ethanol could also have disrupted membrane bound proteins required for nutrient transport. D-Xylose was not consumed in the experiments with added ethanol (25 and 50 g/L of ethanol). Ethanol has been shown to inhibit glucose and ammonium transport in *Saccharomyces cerevisiae* (Leao and van Uden, 1983 and Lee et al, 1981).

Although not as drastic, the percent viability (measured by plate counts) also declined in the control experiment (no added ethanol, Figure 69). The reason for this decline appears to involve a different mechanism than in the experiments with added ethanol. The cell volume distribution remained broad throughout the course of the control fermentation (data not shown). In the control fermentation there was an initial increase in mean cell volume followed by a decrease (Figure 71), after which the mean cell volume remained relatively constant. The total cell volume increased exponentially early in the fermentation and stabilized for the remainder of the experiment (Figure 71). These data suggest that there was insignificant cytoplasmic leakage during the course of the control fermentation. The final ethanol levels in the control experiments were lower than in experiments with added ethanol (Table 6). Also, the change in pH was small; it declined from 6.1 to 5.75 over 655 hours. The cell viability measured by methylene blue staining remained relatively high throughout the fermentation (Figure 69). These data show that the accumulated ethanol and/or the slight pH change caused minimal damage to *C. shehatae* in the control experiments and did not contribute to the decline in cell viability as measured by the plate counts. Apparently, another factor contributed to the decline in

cell viability.

The onset of the decline in cell viability (as measured **by the plate counts**) correlated with the time of growth termination (compare Figures 67 and 69). *Candida shehatae* requires oxygen for growth (Ligthelm et al, 1988) and it is likely that cessation of growth was due to the anoxic conditions which prevailed throughout the rest of the fermentation. The prolonged exposure of the cells to anaerobic conditions probably resulted in, or contributed to the decline in cell viability. The observation that *Candida shehatae* cannot replicate in the absence of oxygen suggests that functional mitochondria are required for cell growth and the maintenance of cell viability.

In contrast to *C. shehatae*, strains of *Saccharomyces* can grow under oxygen-limited and/or anaerobic conditions (Ingram and Buttke, 1984 and data from this research). The ability to grow under anaerobic conditions may be a requisite for high ethanol tolerance. The fact that *C. shehatae* can not grow under anaerobic conditions may have a direct relationship with its ethanol tolerance. The fermentation and cell viability data show that *C. shehatae* has a lower tolerance to externally added ethanol compared to *S. cerevisiae*. The viability of *S. cerevisiae* cells exposed to 50 g/L of ethanol declined 2.8 times slower than *C. shehatae* cells (measured by methylene blue staining under similar conditions (Lee et al, 1981). The lower ethanol tolerance of *C. shehatae* could be due to a difference in cell membrane structure/compositionand/or because *C. shehatae* can not grow under anaerobic conditions.

m. Effect of Added Xylitol:

These fermentations were identical to the fermentations in which ethanol was added except xylitol was added at concentrations of 25 and 50 g/L. Addition of xylitol had no apparent effect on the metabolism and cell viability of *Candida shehatae*; there was apparent affect on cell growth (Figure 72). As shown in Table 7 the ethanol and xylitol yields were approximately the same for each fermentation condition (except for the xylitol yield at 50 g/L). The cell viability declined in all reactors at the same rate (Figures 73 and 74). As in the previous experiments (effect of added ethanol), cell viability declined at a faster rate when measured by plate counts (compared to methylene blue staining; compare Figures 73 and 74).

Table 7 - Effect of Added Xylitol on D-Xylose Fermentation

Experiment	Time (hr)	%Substrate Utilized	Ethanol Produced	$Y^1_{E/S}$	$Y^2_{X/S}$
Control	507	35.5	9.8	0.25	0.21
25 g/L	507	37.5	10.4	0.27	0.21
50 g/L	507	37.5	10.7	0.25	0.14

[1] $Y_{E/S}$; grams of ethanol produced per gram of xylose consumed
[2] $Y_{X/S}$; grams of xylitol produced per gram of xylose consumed

n. Fed-Batch Mixed Sugar Fermentations; Effect of Casamino Acids:

Fed batch mixed sugar fermentations were conducted on a defined medium (Table 4) with and without casamino acids. *Candida shehatae* cells were inoculated into a New Brunswick batch reactor at a relatively low cell density (1 x 10^7 cells/ml). The reactor

Figure 72 - Effect of Added Xylitol on the Cell Growth of *Candida shehatae* under Anaerobic Conditions.

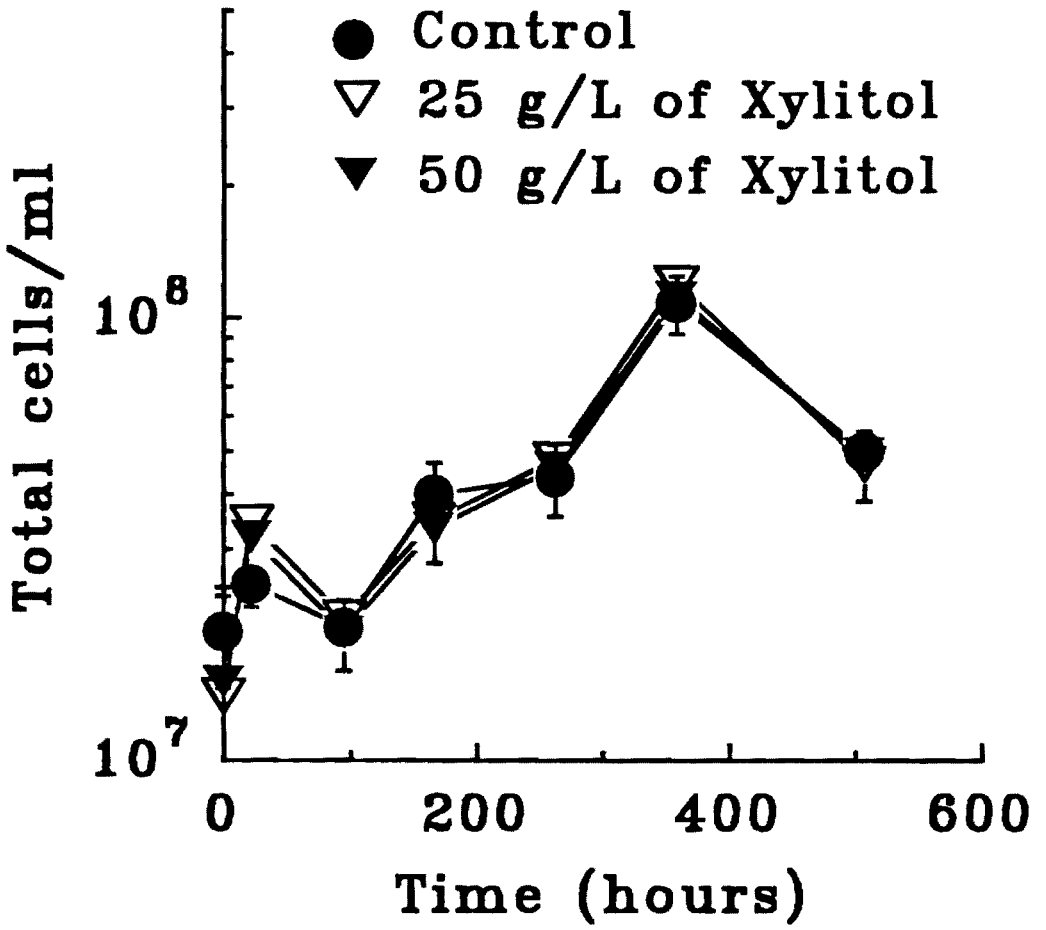


Figure 73 - Effect of Added Xylitol on the Cell Viability (as Measured by Methylene Blue Staining) of *Candida shehatae* under Anaerobic Conditions.

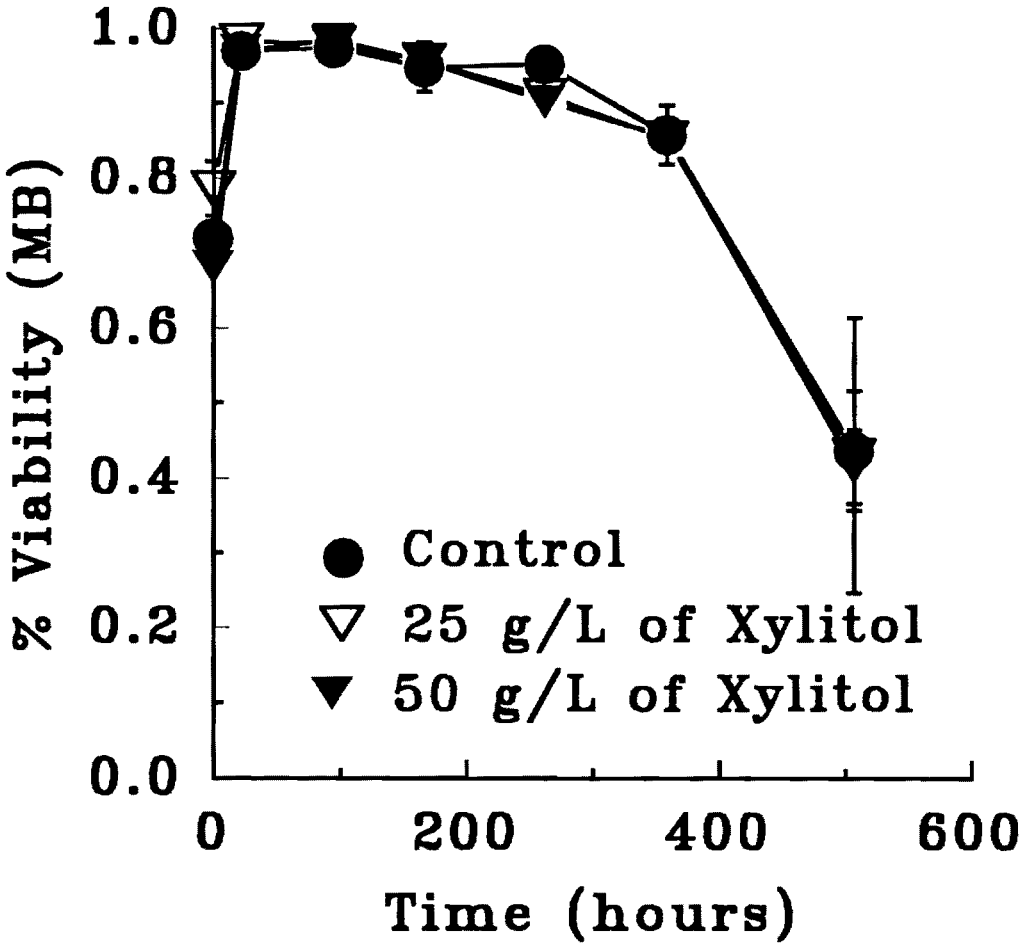
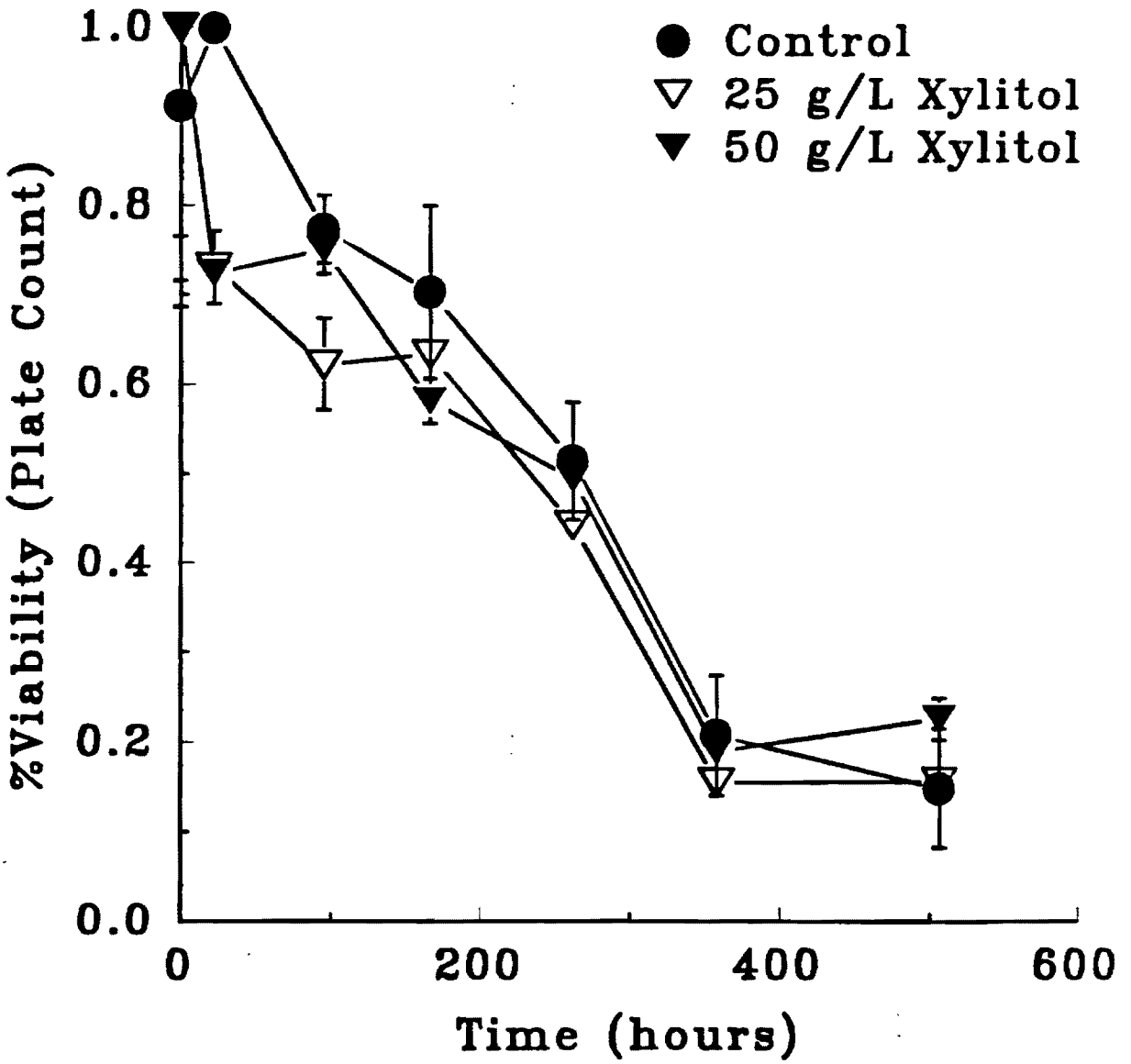


Figure 74 - Effect of Added Xylitol on Cell Viability as Measured by Plate Counts.



was aerated at 1.5 liters/min and agitated at 700 rpm. The reactor had a working volume of 1 liter. The temperature was controlled at 30°C and a pH of 4.5. Dissolved oxygen (% D.O.) was not monitored, but once the stationary phase was reached air sparging was ceased, the agitation was set to 400 rpm, and N_2 was sparged through the reactors. At this point a concentrated solution (60%) of glucose was pumped into the reactor to achieve a mixture of D-xylose and glucose. Also at this point one reactor received a concentrated solution of casamino acids to bring the casamino acid level to 2.5 g/L. Cell viability was monitored by methylene blue staining only.

Both reactors reached the same cell density (1.6 X 10^9 cells/ml); the presence of casamino acids did not stimulate aerobic nor anaerobic growth of *Candida shehatae* (Figure 75). In both reactors D-xylose and glucose were utilized to a low level and there was very little difference in final ethanol and xylitol levels (Figures 76 and 77 and Table 8). Cell viability, as measured by methylene blue staining, declined to about 70% in both fermentations (Figure 78).

Table 8 - Fed Batch Mixed Sugar Fermentations by *Candida shehatae*

Reactor	% Utilization		Ethanol	$Y^1_{E/S}$
	Xylose	Glucose	Produced, g/L	
+ CA[2]	95.4	100	45	0.37
- CA[2]	91.5	100	48	0.38

[1]$Y_{E/S}$; grams of ethanol produced per gram of xylose and glucose consumed

[2]CA; Casamino Acids (Vitamin Free), -; without and +; with

Figure 75 - Effect of Casamino Acids on a Fed-Batch Mixed Sugar Fermentation by *Candida shehatae*.

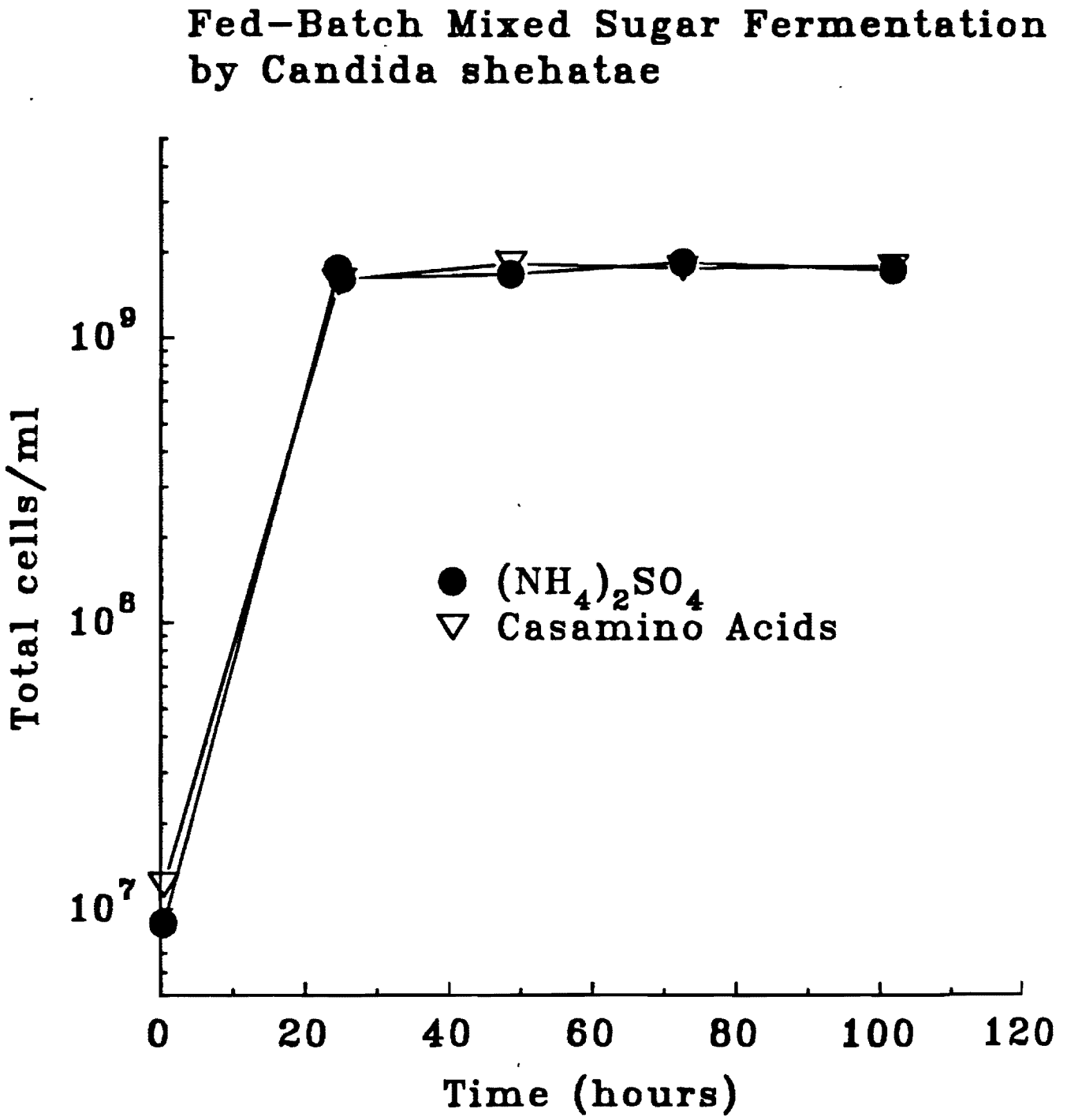


Figure 76 - Xylose Utilization and Ethanol and Xylitol Production in a Mixed Sugar Fermentation with $(NH_4)_2SO_4$ as the Nitrogen Source.

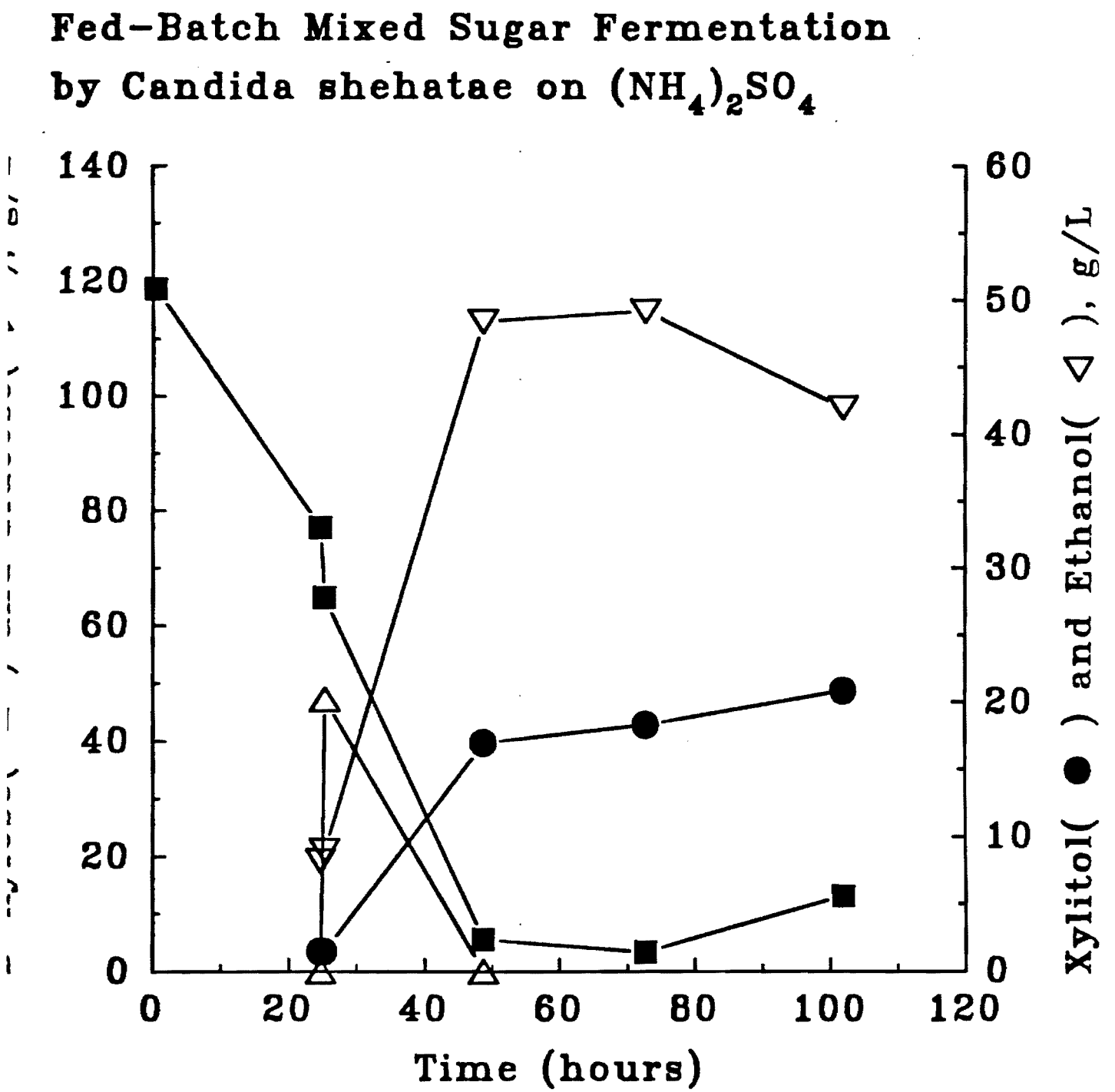


Figure 77 - Xylose Utilization and Ethanol and Xylitol Production in a Mixed Sugar Fermentation with $(NH_4)_2SO_4$ and Casamino Acids as the Nitrogen Source.

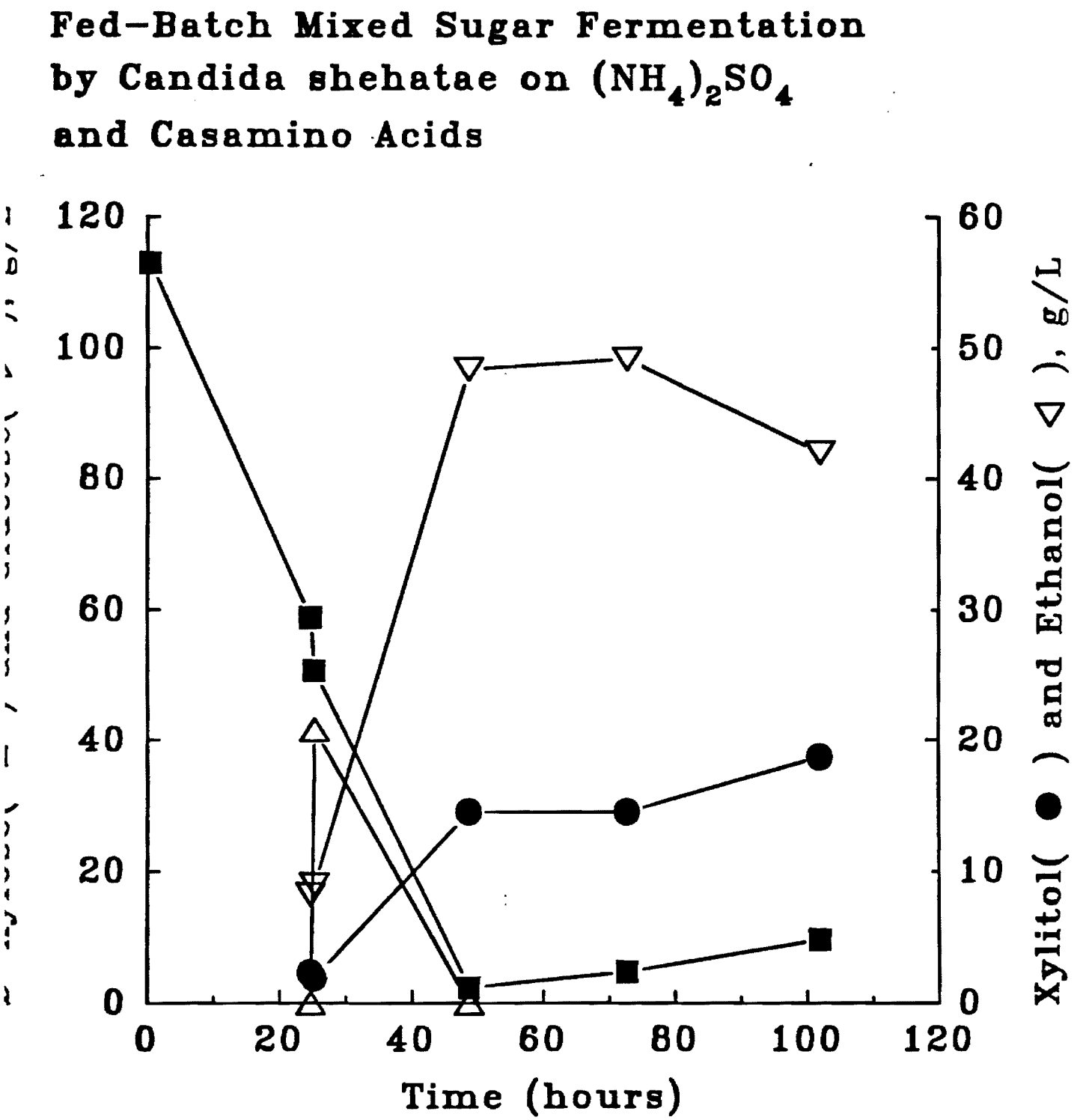
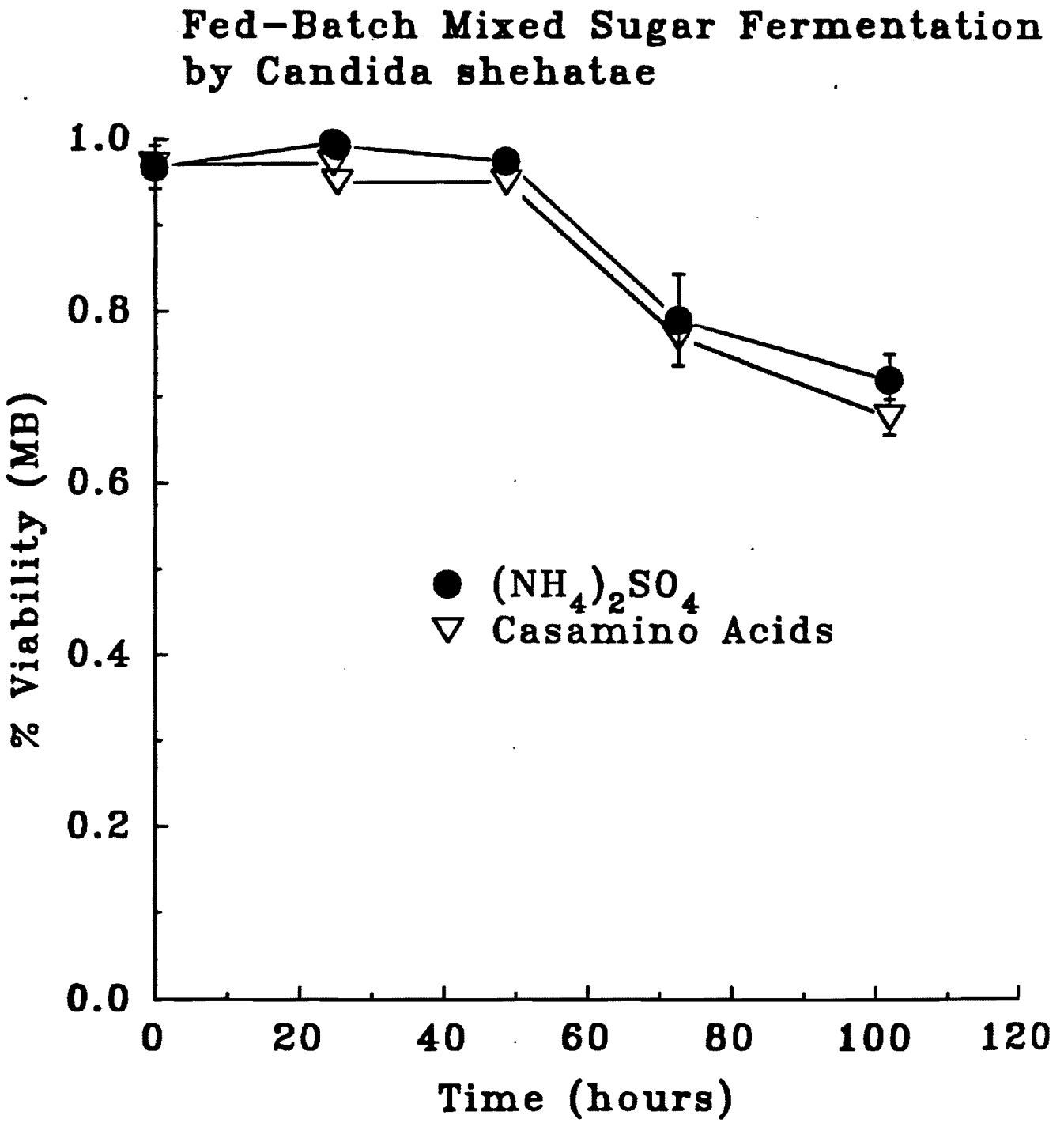


Figure 78 - Effect of Casamino Acids on Cell Viability in a Mixed Sugar Fed-Batch Fermentation.



o. Continuous Mixed Sugar Fermentations:

Previous data have shown that *Candida shehatae* can simultaneously utilize D-xylose and glucose under both non-growth and growth conditions. These experiments were performed in batch reactors and suggest that *Candida shehatae* can utilize mixed sugar substrates on a continuous basis in a CSTR (continuous stirred tank reactor). Thus, *Candida shehatae* could be used to produce ethanol and/or single cell protein in a chemostat or CSTR from waste mixed sugar streams. Aerobic fermentations of a mixed sugar stream were performed in a chemostat to study the kinetics of xylose and glucose utilization, and biomass formation.

Aerobic fermentations were performed in a New Brunswick Bioflow IIc Continuous Stirred Tank Reactor. The inlet feed consisted of a defined medium (Table 4), 0.2% antifoam, 2 g/L of D-xylose and 1.8 g/L of glucose. The inlet pH was 6.0 and the outlet pH was measured to be 3.5. The temperature was controlled at 30°C and the dissolved oxygen was maintained above 50% saturation.

Xylose was utilized in the presence of glucose under all conditions (from a retention time of 8.33 to 3.03 hours). As the retention time was decreased xylose began to appear in the outlet stream (Figure 79). This was due to the fact that the rate of D-xylose utilization did not increase as the retention time was decreased (Figure 80). The optimum productivity of biomass (cells/ml-hr) occurred at a retention time of 4.2 hours.

Figure 79 - Continuous Aerobic Fermentation of a Mixed Sugar Stream by *Candida shehatae* on a Defined Medium with $(NH_4)_2SO_4$ as the Nitrogen Source (pH=3.5, T=30°C).

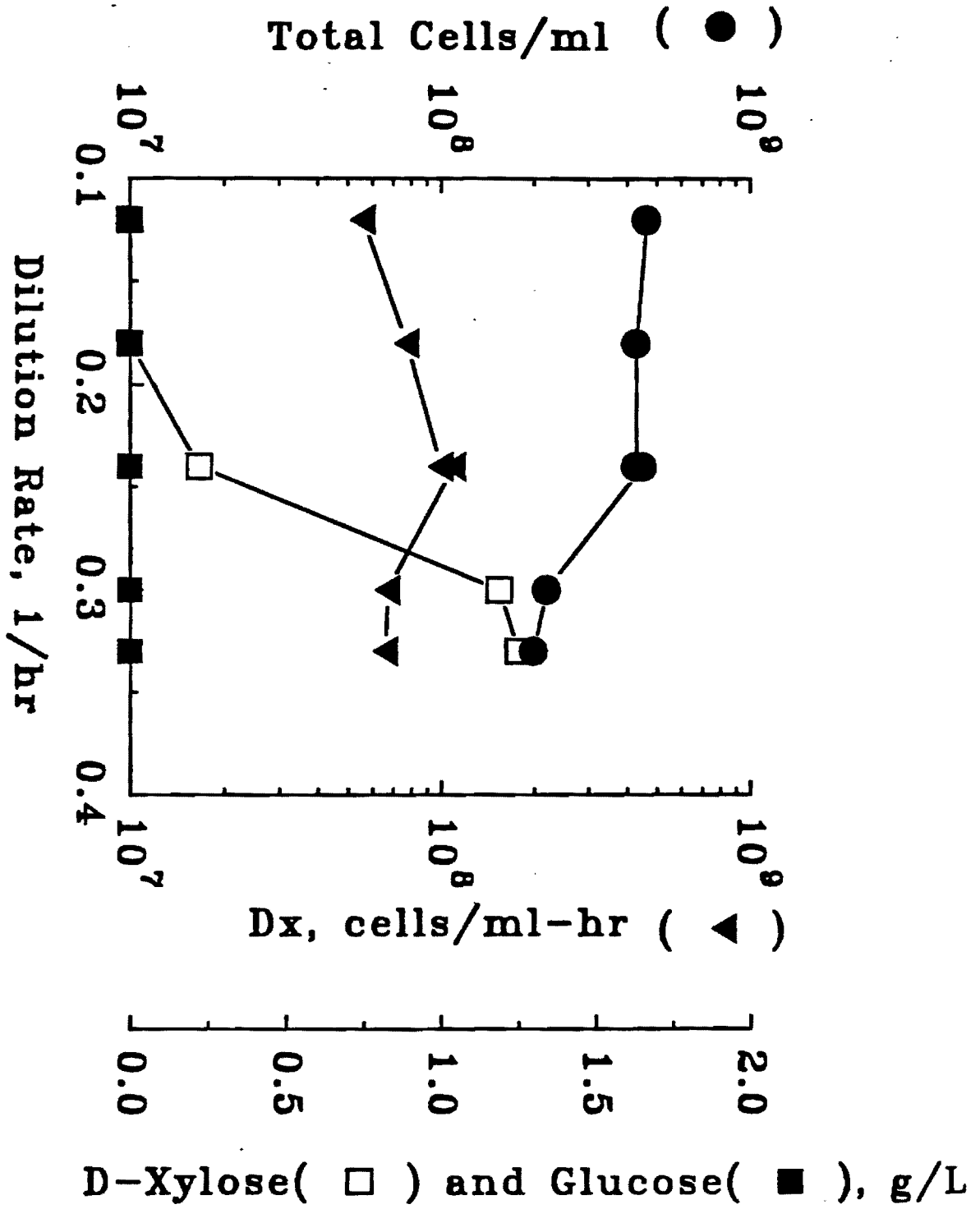
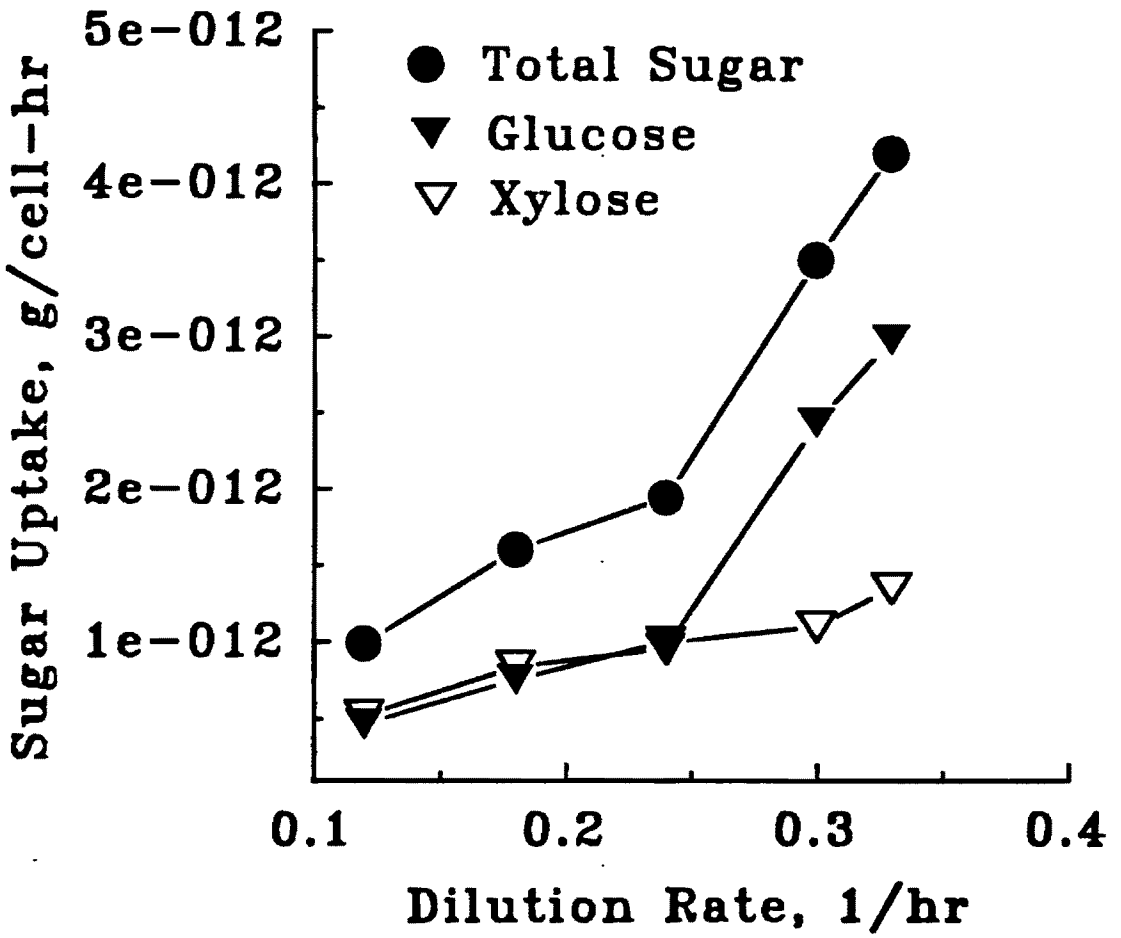


Figure 80 - Approximate Rate (g consumed per cell per hour) of Xylose, Glucose and Total Sugar Utilization in a Continuous Aerobic Fermentation of Mixed Sugar Stream.



CONCLUSIONS

If grown aerobically on D-xylose, *Candida shehatae* can simultaneously utilize D-xylose and glucose under anaerobic conditions (non-growth) to produce ethanol. This preconditioning step decreases the fermentation time and increases the amount of D-xylose consumed from a mixed sugar substrate (glucose and D-xylose). It was also found that *Candida shehatae* could simultaneously utilize xylose and glucose under semi-aerobic conditions (growth conditions) to produce ethanol. Recently our lab has shown that *Candida shehatae* will continuously utilize a mixed sugar stream, consisting of glucose and xylose, in a continuous stirred tank reactor (chemostat) under aerobic conditions. These data indicate that glucose does not repress xylose metabolism (or that repression is minimal) in *Candida shehatae*.

It should be noted that the chemostat experiments on a mixed sugar stream were not performed with funds from the TVA grant. However, the experiments and resultant data are in the interest of TVA's research goals. The data indicate that *Candida shehatae* may have an advantage over yeasts currently used to produce single cell protein (or other products) from mixed sugar streams which contain D-xylose. In the CSTR experiments *C. shehatae* utilized 100% of the glucose and 87.5% of the D-xylose at a dilution rate of 0.24 hr^{-1} (retention time of 4.2 hours) and at a dilution rate of 0.18 hr^{-1} utilized all of the sugars. However, *Candida utilis* is reported to utilize only 93-95% of the reducing sugars (it is assumed that the residual sugar is D-xylose) at a dilution rate of 0.10 hr^{-1} (see University of Mississippi Waste Utilization Report in Experimental Facility Testing Report by TVA in February 1986).

For ethanol fermentations by *Candida shehatae*, it is clear from the data that fed-batch fermentations are superior to batch fermentations. In fed-batch fermentations cells are grown aerobically to high cell densities and then allowed to go anaerobic. High cell densities increase ethanol productivity and reduce fermentation times. Substrate is periodically added to the reactor to prevent substrate limitation and increase the amount of ethanol produced. This method produced ethanol levels up to 50 g/L within 60 hours from a mixed sugar substrate. This represents a significant improvement over the 25 g/L of ethanol produced within 50 hours by *C. shehatae* in a batch reactor from a mixed sugar substrate. It is also clear from the fed-batch fermentation data that a pH level of 2.5 inhibits anaerobic D-xylose fermentation. More research is needed to understand the effect of pH on *C. shehatae* under anaerobic conditions in the presence of ethanol.

The 50 g/L of ethanol produced in the fed-batch reactors is the maximum concentration we have been able to obtain with *C. shehatae*. The data from the effect of added ethanol may explain why *C. shehatae* can not produce higher levels of ethanol. Exposure of *Candida shehatae* cells to added ethanol concentrations of 25 g/L or higher leads to inhibition of cellular growth/division and eventual cell death. The rapid decline in cell volume and total cell volume, in the presence of ethanol concentrations of 25 g/L or higher, indicates that *C. shehatae* cell membrane permeability is affected by ethanol. The lack of D-xylose consumption also demonstrates that ethanol has an inhibitory effect on *C. shehatae* metabolism. Mechanisms have been discussed as to how ethanol effects *C. shehatae*, but more data are needed to draw concrete conclusions.

Further experiments are needed to better understand the effect of ethanol on *C. shehatae* metabolism. Experiments could be performed to determine the lipid membrane composition of *C. shehatae* during aerobic, anoxic and prolonged anoxic fermentations. Once determined the lipid composition could be compared to an ethanol tolerant strain of *S. cerevisiae*. This would help formulate a mechanism for the effect of ethanol on *C. shehatae*. If there is a distinct difference in membrane composition between *C. shehatae* and *S. cerevisiae*, the medium could be supplemented with the key component(s) and its effect tested. Also, fermentation conditions could be modified to stimulate the production of the essential membrane component(s).

Most of the data indicate that growth of *C. shehatae* is either completely or severely inhibited by the lack of oxygen. However, one set of data indicate that several doubling periods occur under anaerobic conditions before a stationary phase is reached (see section 2h). Further experiments are needed to clarify the difference in the data and to better understand the short term effect of the lack of oxygen availability on *C. shehatae* metabolism.

Exposure of *Candida shehatae* cells to long-term anaerobic conditions led to inhibition of cellular growth/division and eventual cell death, as measured by plate counts. Plate counts were a more sensitive indicator of the inhibitory effects of anaerobic conditions and should be used to correlate the effect of other physiological parameters in future D-xylose fermentations. The inability of *C. shehatae* to grow in the absence of oxygen (or the severe inhibition of growth without oxygen) may contribute to its low ethanol tolerance. Research is needed to understand the role of oxygen in cellular growth

and viability in *C. shehatae*. Also, the function of the mitochondria and a better understanding of metabolic pathways and their regulation are needed.

RECOMMENDATIONS

Genetic Engineering:

Genetic engineering may be required to achieve rapid improvements in ethanol fermentations from D-Xylose. To achieve economical ethanol fermentations, final ethanol levels must be increased (> 50 g/L) and productivity increased (g of ethanol produced per liter of reactor per hour). Recently several attempts have been made to genetically engineer microorganisms to produce ethanol from D-xylose. The xylose isomerase gene (bacterial origin) was transferred into *Saccharomyces cerevisiae* and *Schizsaccharomyces pombe* (Sarthy A.V., et al, 1987; Amore R., et al., 1989; Chan et al., 1989) . It was hoped that this gene would be expressed (xylose isomerase enzyme) and would enable the two yeasts to convert xylose to xylulose. These two yeasts can convert xylulose to ethanol and give high ethanol yields and productivity (compared to D-xylose fermentors such as *Candida shehatae*). However, the xylose isomerase enzyme was not active within these yeast and this genetic engineering attempt appears to have been unsuccessful. Genes from *Zymomonas mobilis* (pyruvate decarboxylase and alcohol dehydrogenase II) have been transferred into *E. coli* (Lawford and Rousseau, 1991). This has improved ethanol fermentation from D-xylose by *E. coli*. However, there are drawbacks to ethanol fermentation using this genetically engineered microorganism. An extremely rich medium is required (Luria Broth) and as glucose and D-xylose concentrations increase ethanol

conversion efficiency decreases. One genetic engineering approach which has not been attempted and which may overcome the problems outlined above is protoplast fusion. A proposal will be sent to TVA describing the use of protoplast fusion to enhance the fermentative ability of *Candida shehatae*.

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Final Report Phase II and Phase III Proposal

April 30, 1990

Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
Biotechnology Research Center
Muscle Shoals, Alabama 35600

Submitted by

Jim Kastner, School of Applied Biology
and Professor R.S. Roberts, School of Chemical Engineering
Georgia Institute of Technology
Atlanta, Georgia 30332-0100

Introduction

Biomass as Energy :

Biomass is a renewable energy source which is currently underutilized. Large quantities of biomass are available in the form of Municipal Solid Wastes, Agricultural Residues and Forestry Residues. For example, in 1975, 14.0 million tons of Municipal Solid Wastes, 30.0 million tons of Agricultural Residues, and 16.8 million tons of Forestry Residues were generated (Cowling, 1975).

Biomass is composed of three biopolymers, hemicellulose, cellulose and lignin. To obtain energy from biomass it must be broken down into fermentable sugars. The hemicellulose and cellulose biopolymers can be hydrolyzed using a mineral acid. The result is a solution which mainly consists of glucose and xylose. These sugars can then be fermented to ethanol and then used as a liquid fuel or fuel supplement.

Reactor Design, Continuous vs. Batch:

To produce ethanol on a large scale a bioreactor reactor must be designed with a low operating cost and low capital cost. Low operating costs necessitate a simple, continuous process that completely utilizes the sugar. The process should also require a small amount of energy input. Low capital costs require high productivity and a bioreactor which is mechanically simple to design and operate. Based on these criteria, a continuous stirred tank reactor (CSTR) has many advantages over a batch reactor.

In a CSTR a sterilized sugar stream is processed in a well mixed reactor. A constant reactor volume is maintained by setting the flow rate of the exit stream equal to that of the feed stream. Yeast cells in the reactor consume the sugar to produce ethanol and cells. Cells must be produced to replace those cells lost in the exit stream (a steady-state is achieved when the growth and washout rate are equal).

A batch fermentation process is discontinuous. The reactor is filled to a set volume and then inoculated with a yeast seed culture. The yeast cells consume the sugar to produce ethanol and cells. At the end of the fermentation the contents are emptied and the cycle started again.

Potentially, operating costs can be lower in a CSTR process because there are less steps involved. Capital cost is lower in a CSTR because the required reactor volume is 1/3 that of a batch reactor (Maiorella, et al., 1981). In addition ethanol productivity is higher in a CSTR process. Although the CSTR has advantages over a batch reactor, improvements can be made on the CSTR process.

A CSTR fermentation process has several limitations. Cell densities (g active biomass/L) are only about 10-12 g/L (Maiorell et al., 1981). Such low cell densities limit ethanol production. Ethanol also limits the productivity because of its toxic effect on the cells. Reactor designs which increase the cell density or alleviate ethanol toxicity (or both) can improve upon the CSTR fermentor.

A Continuous Cell Recycle reactor can increase cell density. In this reactor the cells are separated from the exit stream and recycled into the reactor. Immobilized cell reactors obtain high cell densities by anchoring the cells on a surface or immobilizing the cells inside a polymer bead. In the vacuum and extractive fermentors the ethanol is rapidly removed to reduce its toxic effect on the cells (this also allows high cell densities). To produce ethanol in an economical manner, the cells in these reactors must remain viable for long periods. Unless the cells remain viable their catalytic properties are lost.

Anaerobic Effect :

Long term stability (viability) is a very important feature for commercial ethanol production. For ethanol production to be commercially feasible the catalytic properties of the cells must be continuously reused. For example, in a fed-batch fermentation the cells are aerobically grown to a high cell density. Anaerobic conditions (no oxygen) are then

imposed and additional sugar is added to the reactor for ethanol production. However, at the end of the batch cycle a substantial number of cells are dead. Cell death is due do to a combination of effects. The toxic effect of ethanol, and a depletion of nutrients and sugar can lead to cell death. Also, anaerobic conditions (lack of oxygen) can cause cell death. Thus, the cells can not be reused, and more substrate, time and money are required to achieve another fermentation.

In continuous fermentation processes substrate limitation does not occur. However, ethanol can inhibit cell growth and lead to cell death. Also, long term exposure to anaerobic condition can lead to cell death. A loss in cell viability would lead to a decrease in ethanol productivity and necessitate a shutdown of the reactor.

Previous research has shown that oxygen is required for long term operation. In the development of the Vacuum Fermentation Process, Cysewski and Wilke (1977), had to sparge pure oxygen through the reactor to maintain a long term ethanol fermentation. The fermentation was conducted under a vacuum (51 mm Hg) to boil away the ethanol. The ethanol concentration in the fermentation broth was maintained at 35 g/L and the overhead vapor contained 200 g/L of ethanol. An ethanol productivity of 80 g/L/hr was achieved with *Saccharomyces cerevisiae*. The researchers did not ascertain why oxygen was needed for long term operation of the reactor.

If the cells can not survive without oxygen, what are the products of oxygen metabolism which are required to maintain cell viability? Research has established why oxygen is needed for anaerobic ethanol fermentations, at least in the yeast *Saccharomyces cerevisiae*. Under anaerobic conditions yeast can not synthesize ergosterol and unsaturated fatty acids. However, these compounds are required for growth and cell maintenance (Andreasen and Stier, 1953 and 1954). They also help to reduce the toxic effect of ethanol. Thus, it is very important to study the effect of anaerobic conditions (lack of oxygen) on any ethanol fermentation.

Xylose Fermentation, Results from Phase I:

Anaerobic Fermentation of D-Xylose and Mixed Sugars :

As previously described, biomass can be broken down into D-glucose and D-xylose. For economical production of ethanol both sugars must be utilized. However, only some yeasts are capable fermenting D-xylose. At present *Candida shehatae* and *Pichia stipitis* are preferred to ferment D-xylose (Prior, et al., 1989). Research by Kastner and Roberts (1989 A) centered on D-xylose and mixed sugar fermentations by *Candida shehatae*. The fermentations were performed under anaerobic conditions using an **undefined medium**. This was done to test the effect of a lack of oxygen on cell viability. The effect on other variables, such as ethanol and other by-product levels, sugar uptake and cell growth, could also be tested.

Dry cell weight (g biomass/L) has been used to determine the change in cell population in most ethanol fermentations using *Candida shehatae*. This method measures total cell biomass, but gives no indication of cell viability. Kastner and Roberts (1989 A) showed that cell viability is a very important parameter which must be monitored. Methylene blue staining was used to monitor cell viability (or viability index, percentage of viable cells). Under anaerobic conditions the cells did not grow on D-xylose. Also, after a short exposure time to anaerobic conditions (20-40 hours) the viability index declined. The viability index is the number of viable cells divided by the total number of cells. A decline in the viability index indicates that cellular activity has declined. Thus, a decrease in cellular activity resulted in incomplete utilization of D-xylose.

Mixtures of D-xylose and D-glucose were fermented with the yeast *Candida shehatae* under anaerobic conditions. Cells aerobically grown on glucose exhibited a sequential utilization pattern. These cells consumed glucose first then D-xylose. Cells aerobically grown on D-xylose consumed glucose and D-xylose simultaneously; no lag in D-xylose consumption was seen (Kastner and Roberts, 1990). However, as in the D-xylose fermentations, cell viabilities declined and D-xylose consumption was incomplete.

Thus, anaerobic conditions impose stress on *Candida shehatae* cells. This stress manifests itself in the form of **xylitol** as a fermentation by-product (equal to the amount of ethanol) and a decline in cell viability.

Effect of Ethanol:

Candida shehatae do not grow on D-xylose or a mixture of D-glucose and D-xylose under anaerobic conditions (Kastner and Roberts, 1989 A). In addition, *Candida shehatae* do not grow on pure D-glucose under anaerobic conditions (Ligthelm et al., 1988). Ethanol is a product of anaerobic conditions and could inhibit cell growth. However, at time zero in these fermentations ethanol was not present. Thus the inhibition of growth, at least in the early stages of the fermentation, was not due to ethanol. *Candida shehatae* grow on D-xylose and D-glucose, if the medium is aerated. Under these conditions, ethanol does not inhibit growth until a concentration of 28 g/L is reached (at 32^0C, du Preez et al., 1987). These data show that the lack of oxygen inhibits cell growth before ethanol does.

Research by Kastner and Roberts (1988) showed that *Candida shehatae* cell viability declines under anaerobic conditions. The drop in viability occurred 20 - 40 hours into the fermentation. In these fermentations as the ethanol concentration increased the cell viability decreased. The maximum ethanol concentration ranged between 20 and 25 g/L. These data suggest that ethanol in addition to a lack of oxygen, may have contributed to the decline in cell viability. Thus, an attempt was made to see if the two effects could be separated.

A D-xylose fermentation was performed in which ethanol was added at time zero (18 g/L). The decline in cell viability was no more rapid than in fermentations with no ethanol at time zero. These data suggested the decline in cell viability was not due to ethanol (at ethanol concentrations of about 20 g/L), but due to anaerobic conditions.

Apparently, *Candida shehatae* can not synthesize a key intermediate (or intermediates) required for cell growth and maintenance without oxygen.

All the previous fermentations were performed with an undefined medium (yeast extract and $(NH_4)_2SO_4$). This medium may not have contained growth factors (or autoclaving may have decomposed them) and trace elements required for growth and cell maintenance. To make sure that the lack of intermediates from oxygen metabolism were limiting *Candida shehatae*, use of a defined medium was proposed (Kastner and Roberts, 1989 A).

Summary of Results for Phase II of Research

Use of A Defined Medium :

In a defined medium the concentration of each component added is known. Thus, a component's effect on the fermentation can be tested. This can be done by adding or subtracting a component from the medium. The changes in cell viability and ethanol levels, can then be compared to a control fermentation. A defined medium can enable the detection of a component or components needed for long term cell viability under anaerobic conditions (stability of bioreactor).

The defined medium was formulated after du Preez (1983). It contained salts, trace elements and vitamins. The nitrogen source used was $(NH_4)_2SO_4$ and **casamino acids were not added**. The vitamin solution was cold filtered to prevent degradation of the vitamins. Use of this medium improved D-xylose fermentations.

In previous fermentations with an undefined medium, the cells were grown to a high cell density with oxygen. They were then transferred to spinner flasks or shaker flasks for fermentation without oxygen. The fermentations with the defined medium were carried out in one reactor. The cells were grown to a high cell density by sparging oxygen through the reactor. After about 24 hours the oxygen addition was terminated and fermentation began.

Use of the defined medium (**without amino acids**) did not limit growth under aerobic conditions. A very high cell density was achieved by adding oxygen (1×10^9 cells/ml). However, as in the previous fermentations, when oxygen was not added cell division ceased. Although cell division stopped, D-xylose continued to be utilized until it was depleted.

Thus, unlike previous D-xylose fermentations, the substrate (D-xylose) was completely utilized. Cell densities in the previous fermentations ranged between 2.0 - 4.0 $\times 10^8$ cells/ml. This is much lower than the 1×10^9 cells/ml achieved in the fermentation with the defined medium. The volumetric D-xylose consumption rate, g D-xylose consumed/L-hr, was more rapid in the fermentor with the defined medium (5.93 compared to 1.96). These data suggest complete D-xylose utilization was due to higher cell densities and not to the defined medium. However, if the D-xylose consumption rate was calculated on a per cell basis, g D-xylose consumed/cell-hr, the uptake rates were similar. The D-xylose uptake rate was 7×10^{-12} for the undefined medium and 6×10^{-12} for the undefined medium. Therefore, the defined medium provided conditions which allowed complete D-xylose utilization.

The cell viability remained higher for a longer time than in previous fermentations. This allowed the cells to remain active and completely consume the D-xylose. Also, the ratio of ethanol to xylitol was increased (Kastner and Roberts, 1989 B). However, only about 30 g/L of ethanol was produced from 80g/L of D-xylose. In this batch fermentation, D-xylose was depleted within 50 hours and cell viability was approximately 87 percent. This indicates that more ethanol could have been produced if additional xylose had been added in a fed-batch manner. A fed-batch fermentation would also allow testing of long term exposure to anaerobic conditions.

Anaerobic Effect with Defined Medium, Fed- Batch Fermentations:

The fed-batch fermentation did increase the amount of ethanol produced from D-xylose. An ethanol concentration of 40 g/L was attained in 80 hours. However, as in the previous fermentations, cell division ceased and cell viability declined without oxygen (Kastner and Roberts, 1989 B). These data indicated a key intermediate of oxygen metabolism (or pathway required to produce an intermediate) was limiting *Candida shehatae*.

Saccharomyces cerevisiae can grow (divide) on D-glucose without oxygen for several generations. However, after this point, *Saccharomyces cerevisiae* can not synthesize sterols and unsaturated fatty acids without oxygen (Andreasen and Stier, 1953 and 1954). Sterols and unsaturated fatty acids must be added to stimulate growth (Macy and Miller, 1983). Growth occurs for several generations without oxygen. This is because turnover (degradation) of the sterols and unsaturated fatty acids in the membrane is slow. However, as the cell population increases under anaerobic conditions the concentration of sterols and unsaturated fatty acids in the cell membrane decrease. Thus, at some point a critical concentration is reached and growth (division) becomes inhibited.

Unlike *Saccharomyces cerevisiae* , *Candida shehatae* (and other pentose fermenting yeast, such as *Pachysolen tannophilus* and *Pichia stipitis*) can not grow on D-xylose or D-glucose without oxygen (Ligthelm et al., 1988). The maximum specific rate (g dry biomass/per hour - g dry biomass) was 0.30 hr^{-1} for *Saccharomyces cerevisiae* under anoxic conditions (on glucose with no oxygen). The maximum specific growth rate for *Pachysolen tannophilus*, *Pichia stipitis* and *Candida shehatae* was 0.03 hr^{-1}, 0.07 hr^{-1} and 0.005 hr^{-1} respectively (under anoxic conditions on D-glucose). The growth rates on D-xylose were 0.008 hr^{-1}, 0.003 hr^{-1} and 0.003hr^{-1} for *Pachysolen tannophilus* , *Pichia stipitis* and *Candida shehatae* under anoxic conditions.

The cells in the previous fermentations were semi-aerobically grown on a defined medium (vitamins and casamino acids). This should have allowed synthesis of sterols and

unsaturated fatty acids. The cells were then used to inoculate flasks under oxygen-limited or anoxic conditions. Therefore, growth was not inhibited because of a lack of sterols and unsaturated fatty acids. These data indicate that *Candida shehatae* (and probably the other pentose fermenting yeast) are inhibited without oxygen for another reason.

Data from research on other pentose fermenting yeast may give insight to the problem. *Pachysolen tannophilus* is another yeast which can ferment D-xylose. *Pachysolen tannophilus* uses a pathway similar to *Candida shehatae* to produce ethanol from D-xylose. The response of this yeast to anaerobic conditions is the same as *Candida shehatae*. It does not grow on D-xylose and produces xylitol under anaerobic conditions (no research has studied the effect of anaerobic conditions on cell viability in *Pachysolen tannophilus*). In *Pachysolen tannophilus* oxygen is required for growth. Under anaerobic conditions *Pachysolen tannophilus* can not grow on either D-glucose or D-xylose. Without oxygen, these carbohydrates can not be incorporated into biosynthetic precursors (Neirinck et al., 1984). In addition petite mutants of *Pachysolen tannophilus* could not be isolated.

Petite mutants are yeast which have a deletion in their mitochondrial genes. The mitochondria is a cell organelle which functions to produce biosynthetic precursors and ATP. Located in the mitochondria are the enzymes of the TCA cycle, the electron transport system and ATP synthesis. A deletion in the mitochondrial gene renders the mitochondria non-functional. These mutants can not synthesize the mitochondrial proteins or enzymes required for respiration. However, the yeast can grow without oxygen via fermentation. *Saccharomyces cerevisiae* is an example of a petite positive yeast. *Saccharomyces cerevisiae* uses acetaldehyde as an electron acceptor to produce ethanol and produces ATP by substrate level phosphorylation (under anaerobic conditions). Stable petite mutants of *Candida shehatae* can not be isolated (Jeffries, 1984). This indicates that *Candida shehatae* (and *Pachysolen tannophlius*) require functional mitochondria for growth and cell maintenance.

Under aerobic conditions the mitochondria function to generate ATP and produce biosynthetic precursors. ATP is formed from the electron transport system and oxidative phosphorylation. The biosynthetic precursors are formed in the TCA cycle and utilized when needed (Figure 1).

In the TCA cycle, several biosynthetic precursors are synthesized. Two of which are oxaloacetic acid and *alpha*-ketoglutaric acid. These two dicarboxylic acids can then be converted to amino acids, aspartic and glutamic acid. Amino acids are the building blocks of protein and are required for cellular function.

Without oxygen the enzymes of the electron transport system (ETS) are not present. Thus, the electrons generated in the TCA cycle can not be transported down the ETS and accepted by oxygen. This shuts the TCA cycle down. Also, ATP can not be generated in the mitochondria because a proton gradient is not generated. Instead, ATP is produced by substrate level phosphorylation. However, a method must be available which furnishes biosynthetic precursors.

In facultative organisms such as *E. coli*, without oxygen the TCA cycle does not operate as a cycle, but as a branched biosynthetic pathway (Figure 2). Under anaerobic conditions, the TCA cycle is branched because *alpha*-ketoglutarate dehydrogenase is absent. In addition succinate dehydrogenase is not present. For this reason two enzymes must be available under anaerobic conditions, fumarate reductase and pyruvate carboxylase. Pyruvate carboxylase fixes CO_2 to pyruvate to form oxaloacetate. Oxaloacetate can then serve as a branch point for the TCA pathway or a precursor to the amino acid, aspartic acid.

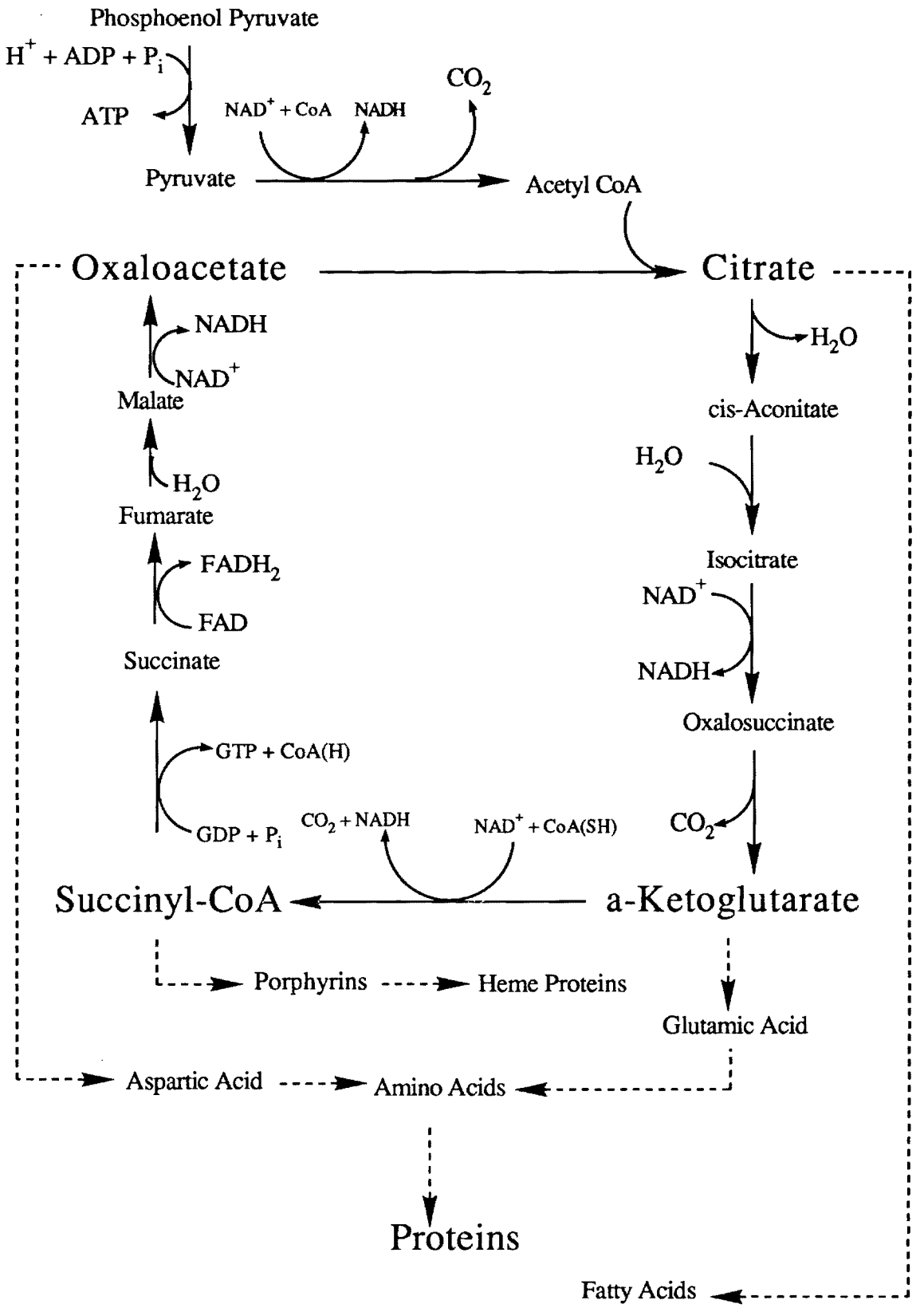

Figure 1 -- Pathway showing the TCA (Tri-Carboxylic Acid) cycle under aerobic conditions.

Oxaloacetate can be converted to succinate down a reductive pathway or converted to *alpha*-ketoglutaric acid down an oxidative pathway. However, the two pathways do not operate independently but are tightly coupled. Completion of the two pathways depends on the reduction of fumarate to succinate by fumarate reductase. Without this step NAD^+ can not be regenerated and pyruvate can not be oxidized to *alpha*-ketoglutaric acid. A NAD^+/NADH imbalance would shut down the two pathways and prevent synthesis of biosynthetic precursors.

Candida shehatae may not be able to synthesize an enzyme or enzyme system to reduce fumarate under anaerobic conditions. This would effectively shut down TCA pathway and prevent the formation of biosynthetic precursors. Biosynthetic precursors, such as *alpha*- ketoglutarate, oxaloacetate, citrate (acetyl-CoA) and succinate (succinyl-CoA), serve as intermediates in the formation of amino acids, fatty acids and porphyrins (Figure 1). Without these intermediates the macromolecules required for cell maintenance and growth can't be synthesized. This could be the reason *Candida shehatae* do not grow on D-xylose or D-glucose. A lack of biosynthetic precursors would also cause a decline in cell viability.

The most logical solution to this problem is to add oxygen to the medium. However, there are several problems to this approach. Oxygen is a key regulatory factor in *Candida shehatae* metabolism. Too much oxygen causes cells to funnel carbohydrates (D-xylose and D-glucose) into more biomass (more cells) and produce very little ethanol. Too little oxygen causes cell death.

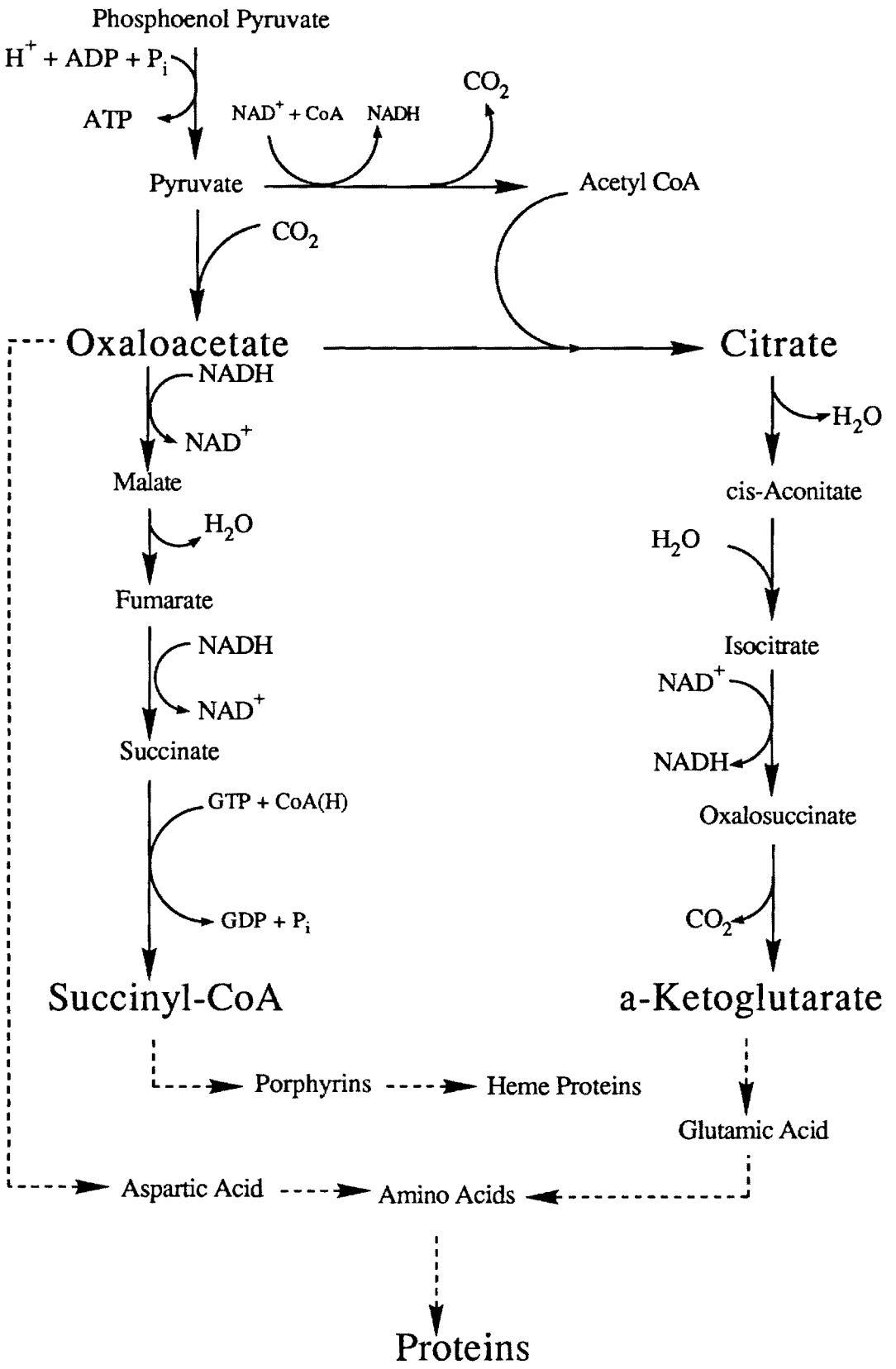

Figure 2 -- Generic TCA pathway for facultative microbes under anaerobic conditions.

Thus, the critical amount of oxygen required for cell maintenance must be quantified. In most fermentations the critical oxygen level is very small. In fact, the critical dissolved oxygen level in *Candida shehatae* is close to the detection limit of currently available dissolved oxygen probes (du Preez et al., 1988). This makes control of a fermentation in which oxygen is added very difficult.

D-Glucose and Alpha-ketoglutaric acid Fermentations:

A second approach to maintain cell viability is to add the components required for cell maintenance to the medium. For reasons previously discussed *alpha*-ketoglutaric was added to the medium. The effect of this biosynthetic precursor was then monitored. For each fermentation, cell viability and xylitol and ethanol levels were quantified.

In the first set of fermentations *alpha*-ketoglutaric acid was not added until anaerobic conditions had been imposed. In these fed-batch fermentations on D-xylose *alpha*-ketoglutaric had no impact. The change in cell viability was the same for both the control (no *alpha*-ketoglutarate) and the experimental reactor (Kastner and Roberts, 1989 B). In both fermentations the cell viability declined from 0.9 to 0.65 in 60 hours. It was theorized that *alpha*-ketoglutarate did not effect cell viability because it was not utilized. The fermentation conditions were redesigned to promote the uptake of alpha-ketoglutarate by the cells (Kastner and Roberts, 1989 C).

For reasons discussed in Kastner and Roberts (1989 C) glucose fermentations were performed with *Candida shehatae*. The cells were aerobically grown on a mixture of D-glucose and *alpha*-ketoglutaric acid (experimental reactor, the control had no *alpha*-ketoglutarate). After about 24 hours anaerobic conditions were imposed and D-glucose was added in fed-batch manner. Relative to the control reactor, the presence of *alpha*-ketoglutarate maintained the cell viability at a higher level for a longer time. After four days, the viability index in the control reactor was 0.6. In the experimental reactor (with *alpha*-ketoglutarate) the viability index was 0.8 after eight days.

Rapid Batch Fermentations on D-Xylose:

The time required to obtain maximum ethanol levels is a major drawback with the previous fermentations. To improve fermentation times, cell were first grown to a very high cell density. The high cell densities were achieved by sparging oxygen through the reactor. After about 24 hours the cells were spun down and inoculated into shaker flasks. The results of these fermentations were encouraging.

The first D-xylose fermentation was performed without casamino acids in the defined medium. The starting cell density was 4 x 10^9 cells/ml with a viability index of 0.90. As a result, D-xylose was completely consumed within 5-7 hours. 27g/L of ethanol and 20 g/L of xylitol were produced (Kastner and Roberts, 1989 C). A second fermentation was performed to compare the effect of casamino acids on the fermentation.

In these fermentations casamino acids apparently stimulated ethanol production. The control reactor (no casamino acids) produced 30 g/L of ethanol and 15.8 g/L of xylitol within 23 hours. The experimental reactor (casamino acids) produced 40 g/L of ethanol and 13 g/L of xylitol in 26 hours. Although casamino acids did stimulate ethanol production, the final cell viabilities were the same in both reactors. The viability index was 0.33 with casamino acids and 0.36 without casamino acids (the initial viability index was 0.98 in both reactors). Because viability indices were calculated only at the beginning and end, the effect of casamino acids on cell viability could not be ascertained. Further research is required on the effect of amino acids on *Candida shehatae* fermentations.

Research Plan for Phase III

Industrial ethanol fermentations require, long term yeast viability, rapid fermentation rates and high ethanol concentrations. Phase III research will study ways to obtain these requirements for *Candida shehatae* fermentations. The effect of four amino acids (valine, asparagine, glutamine, leucine) on cell viability will be tested. Further

research on the effect of TCA cycle intermediates on cell viability will also be tested. Rapid fed-batch fermentations will be performed. These fed-batch fermentations will be conducted to see if higher ethanol levels can be achieved within short time frames (60 g/L of ethanol in 24 hours). If a fermentation method is found which satisfies the above requirements, an Extractive D-xylose fermentation will be performed (provided time and research money is available). The details of the amino acid and rapid fed-batch fermentations will follow. An explanation of the Extractive Fermentor also follows.

Effect of Specific Amino Acids :

Unfortunately, the viability of *Candida shehatae* cells (and probably other pentose fermenting yeast) declines under anaerobic conditions (after 20-40 hours). All evidence points to the yeasts inability to produce biosynthetic precursors without oxygen. Two possible solutions are to add oxygen to the medium or add the biosynthetic precursors to the medium. For reasons previously discussed addition of oxygen does not seem to be a feasible alternative (especially for large scale fermentations).

Kastner and Roberts (1989 C), as well as other researchers (Jeffries, 1985) have shown that organic nitrogen sources stimulate ethanol production. Casamino acids, peptone and yeast extract are examples of organic nitrogen sources. Early work by Kastner and Roberts (1988) indicated that peptone improved cell viabilities.

Yeast extract, peptone and casamino acids are undefined nitrogen sources. This means the exact amount and type of amino acids in these nitrogen sources are not known. If only a few amino acids are required, they may not be in the right concentration or ratio. Also, there may be other components in the nitrogen source besides amino acids (salts, vitamins, trace elements etc.). Thus, a positive effect on cell viability and ethanol levels under anaerobic conditions could not be solely attributed to the amino acids. Therefore, we propose to study the effect of specific amino acids on anaerobic fermentations by *Candida shehatae*.

Previous research indicates that *Candida shehatae* can synthesize all the amino acids required for growth under aerobic conditions (Kastner and Roberts, 1989 B). However, under anaerobic conditions, growth stops and cell viability declines. These data imply that biosynthetic precursors from the TCA pathway are not synthesized. These biosynthetic precursors are oxaloacetate, *alpha*-ketoglutarate, acetyl-CoA (derived from citric acid) and succinyl-CoA (derived from succinate).

What amino acids should be tested? Four amino acids that feed into the TCA cycle will be tested (for an initial study). Two amino acids, generated from oxaloacetate and *alpha*-ketoglutarate, will be tested. Also, two amino acids, which can be converted to acetyl-CoA and succinyl-CoA , will be studied.

Oxaloacetate is converted to aspartic acid and *alpha*-ketoglutarate is converted to glutamic acid. Both are amino acids required for protein synthesis. We propose to add two amino acids, asparagine and glutamine, to the medium which can be easily converted to aspartic and glutamic acid.

Acetyl-CoA is required for fatty acid, phospholipid and sterol synthesis. Fatty acids, phospholipids and sterols are important constituents of the cell membrane. We propose to add an amino acid which can be converted to acetyl-CoA, leucine .

Succinyl-CoA is required for porphyrin synthesis. Porphyrins are essential components of heme proteins. These proteins function to transfer electrons in oxidation /reduction reactions. Valine can be converted to succinyl-CoA and will also be added to the medium.

TCA Cycle Intermediates:

If *Candida shehatae* cells are conditioned on *alpha*-ketoglutaric acid, cell viability remains high for long periods under anaerobic conditions. There were several drawbacks to this fermentation. The fermentations were conducted on D-Glucose and not D-xylose.

Further research is needed to see if the same results can be obtained on D-xylose fermentations. If so, two other problems must be addressed.

The conditioning step required time and a high concentration of *alpha*-ketoglutaric. Furthermore ethanol production was inhibited. To economically ferment D-xylose, the conditioning step must be eliminated, the level of *alpha*-ketoglutaric optimized and ethanol inhibition alleviated. Research on these problems will continue.

Candida shehatae may require other TCA cycle intermediates beside *alpha*-ketoglutaric acid. Information as to which intermediates are needed will be derived from D-xylose fermentations on specific amino acids. An estimate of the required intermediate concentration can also be obtained.

Rapid Fed-Batch Fermentations:

According to Jeffries (1983), fermentations of D-xylose must reach 6%(w/v) ethanol within 36 hours to be economical. Kastner and Roberts (1989 C) achieved a batch D-xylose fermentation within 5-7 hours (Kastner and Roberts, 1989 C). However, the ethanol concentration only reached 2.7%(w/v) because the D-xylose was completely consumed within the 5-7 hour period. We propose to feed D-xylose at periodic intervals during the fermentation. Periodic feeding will prevent substrate exhaustion and increase ethanol concentrations.

Extractive Fermentor:

Ethanol is toxic to yeast. Thus, when a certain ethanol level is reached in a typical bioreactor the cells are inhibited and begin to die. In an Extractive Fermentor, ethanol is removed as it is produced by contact with a liquid (called the extractant). This can lower the ethanol concentration in the bioreactor to the point where the cells are not inhibited. High reactions rates (complete utilization of concentrated sugar streams) and rapid ethanol

production rates can be achieved. Also, if the liquid extractant is much less volatile than ethanol the two can be easily separated.

The success of the Extractive Fermentor depends upon several process variables. The selection of the liquid extractant is very important. Since the extractant comes in contact with the yeast, it must be non-toxic to the yeast. The extractant must be selective for ethanol and have a high distribution coefficient for ethanol. Also, the extractant can not form an emulsion with the fermentation broth. This would prevent transfer of nutrients and sugars to the cells.

The Extractive Fermentor can be designed to immobilize the cells in the reactor. The extractant and fermentation broth (liquid containing yeast cells) are brought into contact. The fluids are mixed and then separated into two phases (two different zones of liquid). The top (organic) phase contains the extractant and ethanol and the bottom (aqueous) phase contains the yeast cells (Figure 3). Extractant and ethanol can be continuously removed from the top phase and sent to a distillation unit. There the extractant and ethanol are separated and the extractant reutilized. The yeast cells remain in the bottom phase and never leave the reactor (unless designed to do so). Thus, the cells are immobilized within the reactor.

This method of immobilizing the cells is entirely different from other techniques. In other techniques the cells are grown on a surface or attached by chemical means to a surface (matrix). In some cases cells are immobilized within a polymer bead (Na-alginate is an example). These techniques reduce the cell surface area available for sugar and ethanol transport. They also introduce large mass transfer resistances, especially to gas transfer. In these immobilized systems the cells on the surface are viable. These cells are viable because nutrients and sugar are readily available. However, cells beneath the surface are stressed due to nutrient and sugar limitations (these limitations are due to large mass transfer resistances).

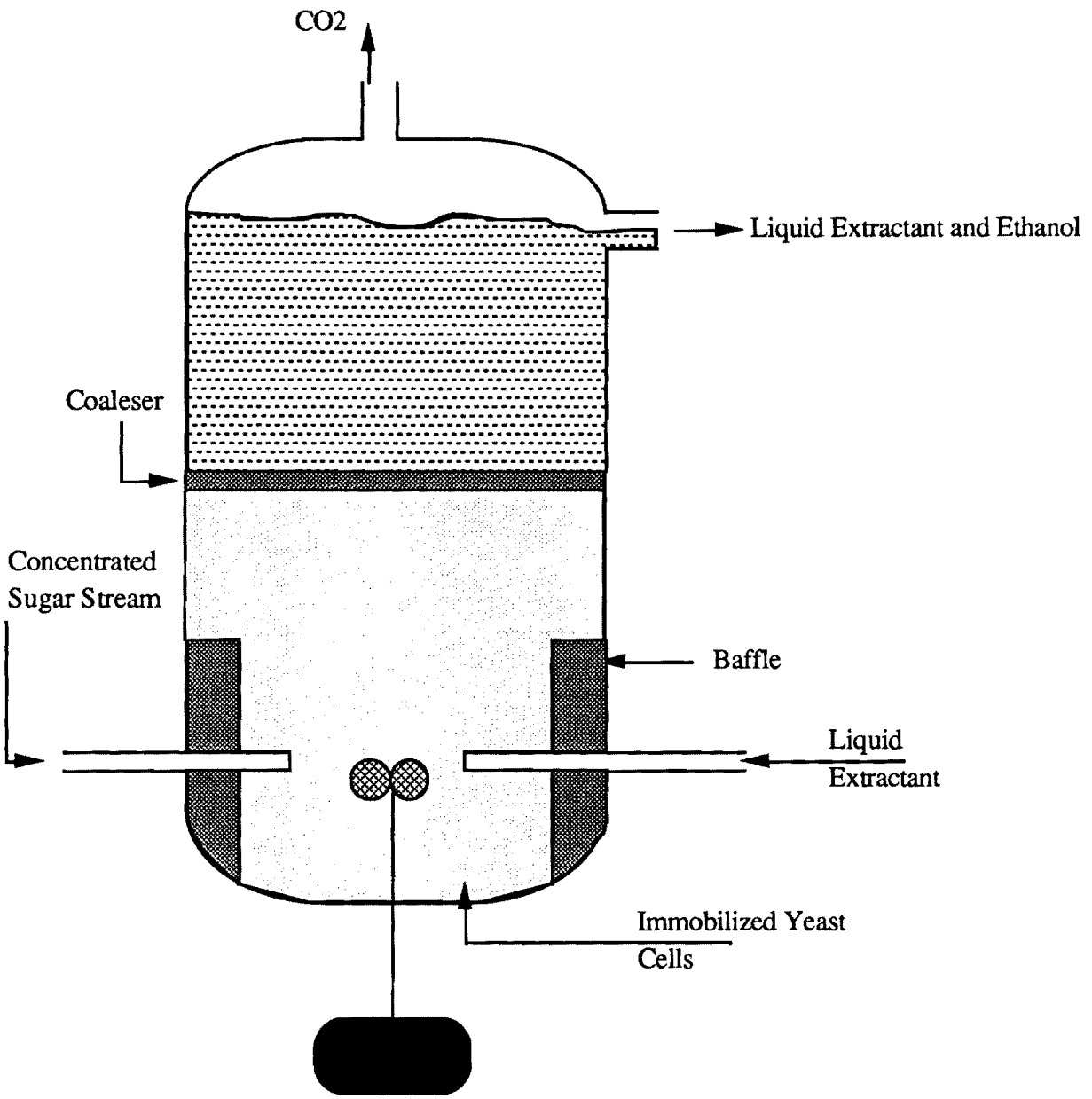

Figure 3 -- Drawing of Extractive Fermentor. This drawing was designed only to portray the characteristics of an Extractive Fermentor. The actual design of an industrial Extractive Fermentor could be quite different.

In the Extractive Fermentor the cells are not attached to a matrix but are suspended in an aqueous environment. Thus, the entire surface area of the cell is exposed to the medium. Since the cells do not grow as a film or inside a matrix, substrate and nutrients can be easily transferred to the cells. Agitation of the medium can provide a well mixed reactor in which mass transfer resistances can be eliminated (if an emulsion is not formed).

Extractants with the previously described characteristics have been found. In fact, a Continuous Extractive Fermentor has been tested by Kollerup and Daugulis (1986). These researchers achieved an ethanol productivity of 115 g/L/hr from 159 g/L of glucose (*Saccharomyces cerevisiae*). To this date no one has attempted to use an Extractive Fermentor to produce ethanol **from xylose (or mixed sugars)**. In addition the long term stability (cell viability and effect of extractant on yeast cells) of the Extractive Fermentor has not been ascertained.

Experimental Methods and Procedures

Microorganism : *Candida shehatae* (ATCC 22984) will be maintained at $4^{O}C$ on YM agar slants. Glucose will be replaced by D-xylose in the YM broth. The cultures will be subcultured at one to two month intervals.

Defined Medium : The medium will be formulated after du Preez (1983). Stock solutions of minerals, vitamins and amino acids will be cold filtered. Ammonium sulfate will be used as the inorganic nitrogen source ($(NH_4)_2SO_4$).

Seed Cultures : See Figures 4 and 5 for seed culturing method.

Analytical Methods : Cell viability will be determined as in Kastner and Roberts (1990). Carbohydrates and xylitol will be quantified by HPLC analysis (Kastner and Roberts, 1990). Ethanol concentration will be quantified by GC analysis (Kastner and Roberts, 1989 B).

Experimental Plan :

Amino Acids :

The effect of amino acids on *Candida shehatae* fermentations will be compared to control fermentations. Control fermentations will be run under identical conditions but will not have amino acids in the medium. Fermentations will be performed on D-glucose and D-xylose. Initial carbohydrate concentrations will be 100 g/L. Duplicates will be run when possible. A flowsheet for this experiment is shown in Figure 4 .

Rapid Fed-Batch Fermentations :

Initially, rapid fed-batch fermentations will be performed without amino acids in the defined medium. If amino acids are found to maintain cell viability under anaerobic conditions, they will be included in the defined medium. Duplicates will be run when possible. Fermentations will be performed on D-xylose, D-glucose and mixed sugars. Initial carbohydrate concentrations will be 100 g/L. The mixed sugar ratio will simulate a municipal solid waste stream (75% D-Glucose:25% D-Xylose). A flowsheet for this experiment is shown in Figure 5.

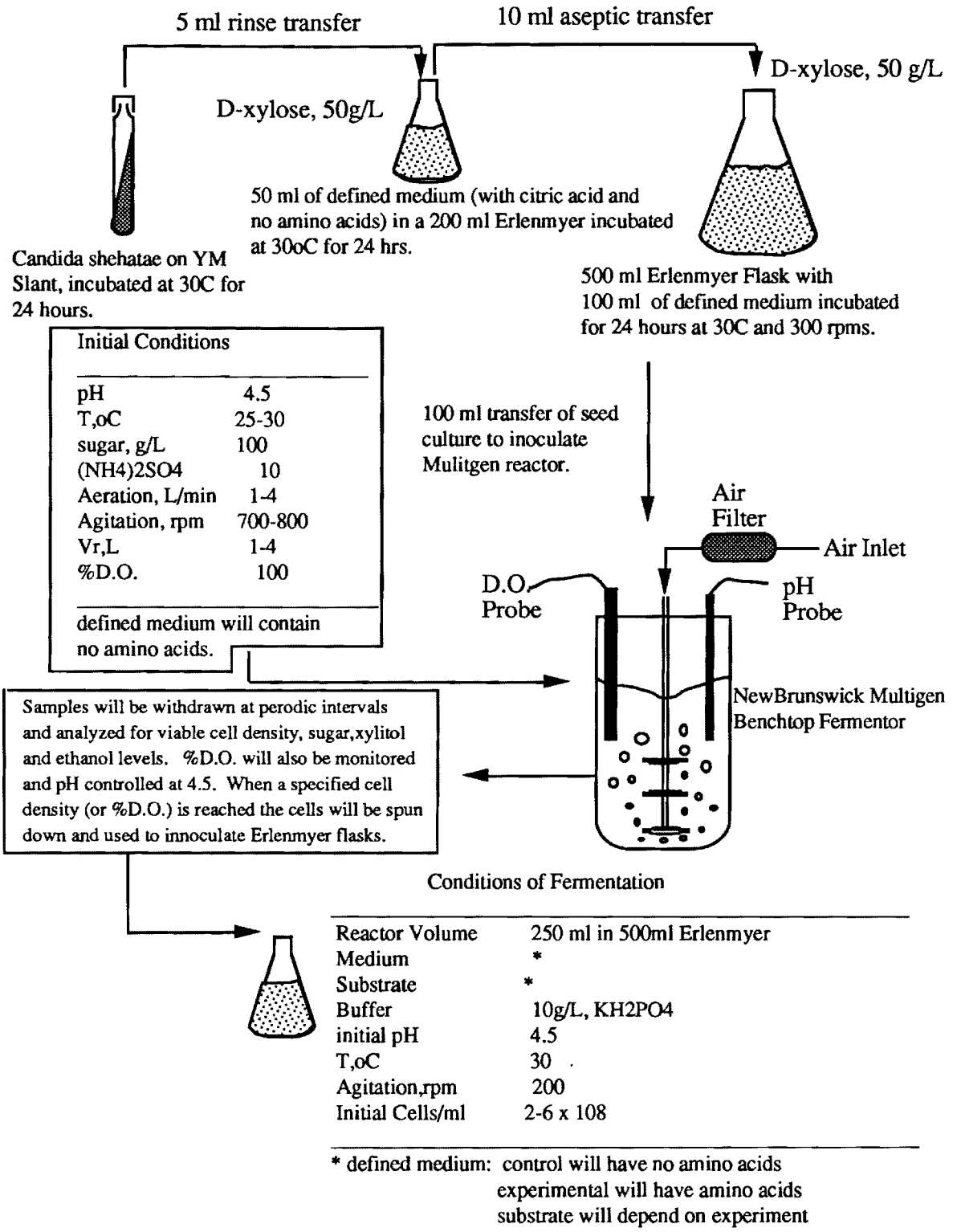

Figure 4 -- Flowsheet for *Candida shehatae* fementations with amino acids in the defined medium.

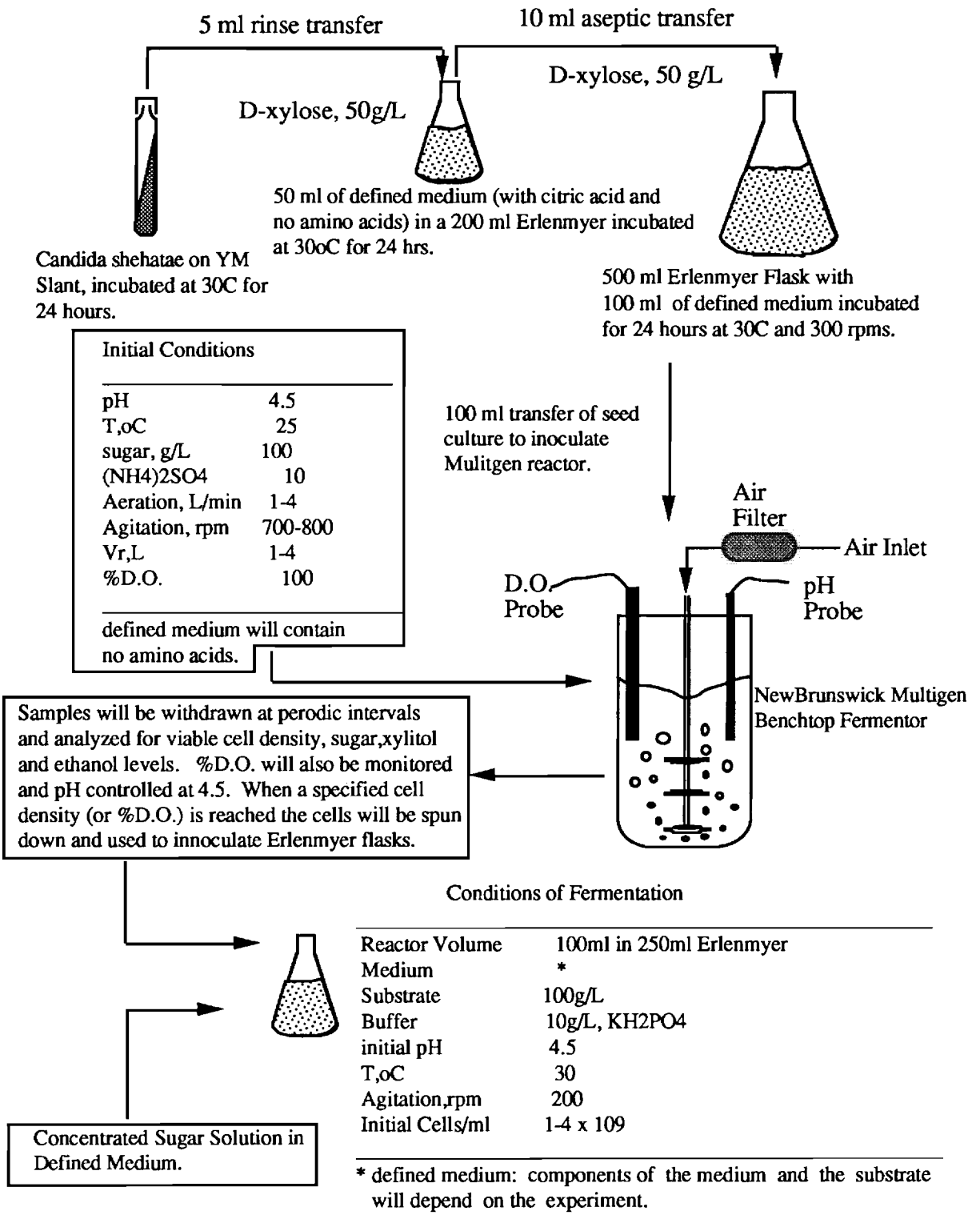

Figure 5 -- Flowsheet for rapid fed-batch fermentations in a defined medium.