Final Report

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Ethanol Fermentation of Mixed Sugar Streams Derived from Lignocellulosic Residues

Submitted to

Tennessee Valley Authority
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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)

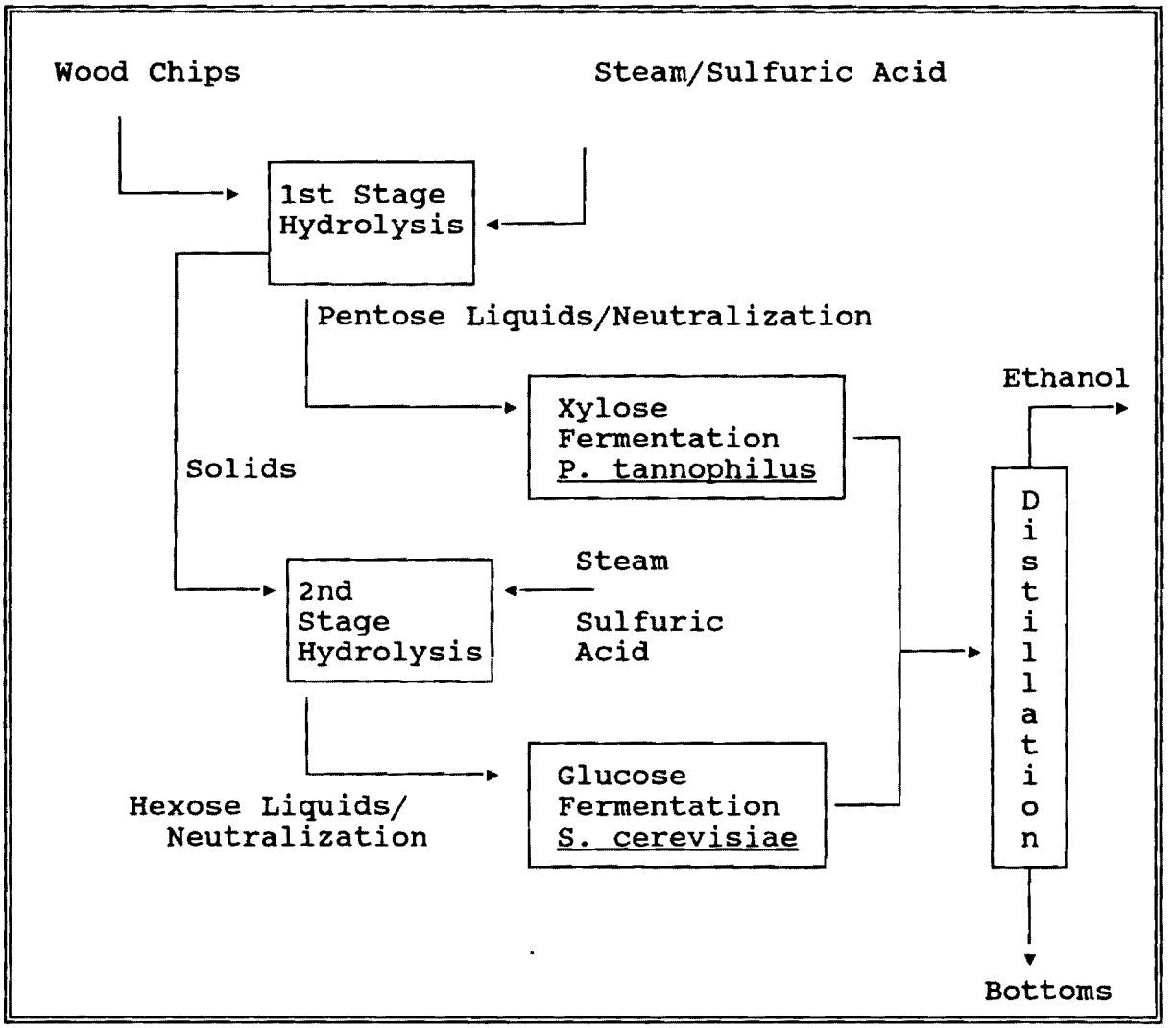
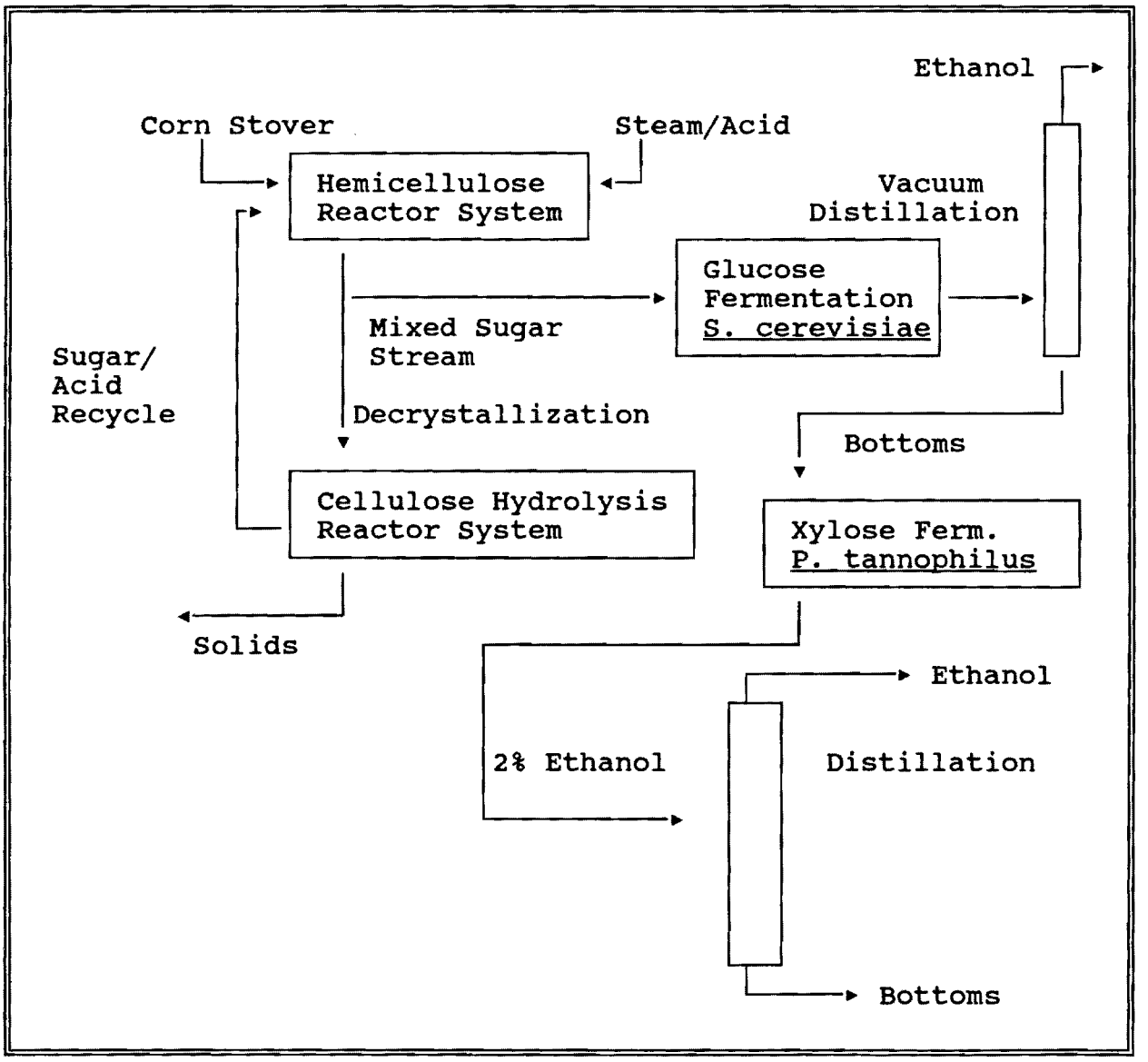


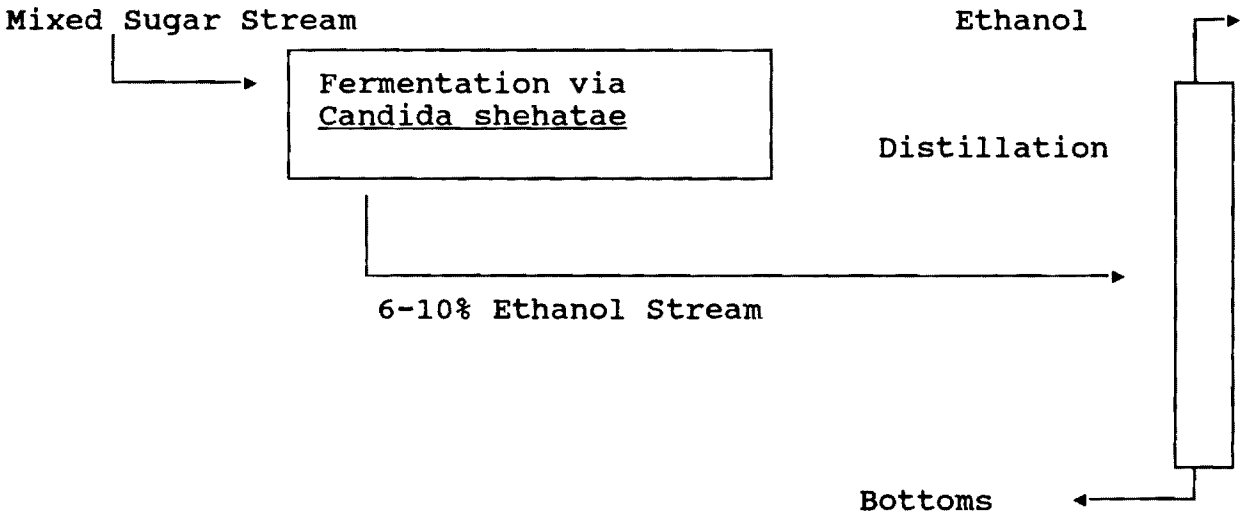
Figure 2. Flowsheet for Batch Fermentation of Concentrated Acid Hydrolyzate (Kastner, 1987; Barrier et al., 1985 and Mary Jim Beck, 1987)



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 x 10^{-12} compared to 9.4 x 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 x 10^{-12} compared to 12.12 x 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 X 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.

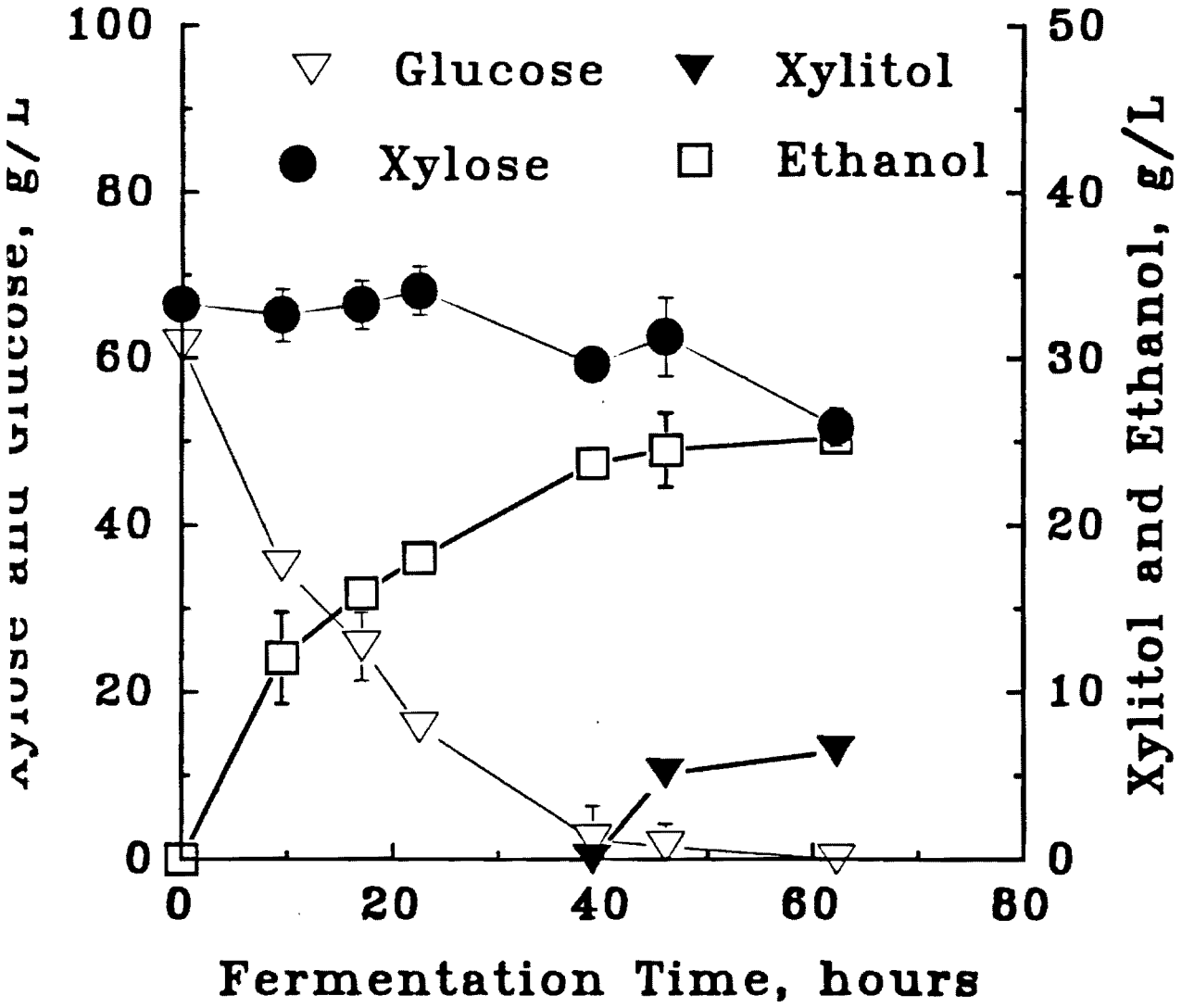


Figure 5 - Batch Fermentation of a mixture of 25% glucose and 75% D-xylose by *Candida shehatae* cells aerobically pre-grown on glucose.

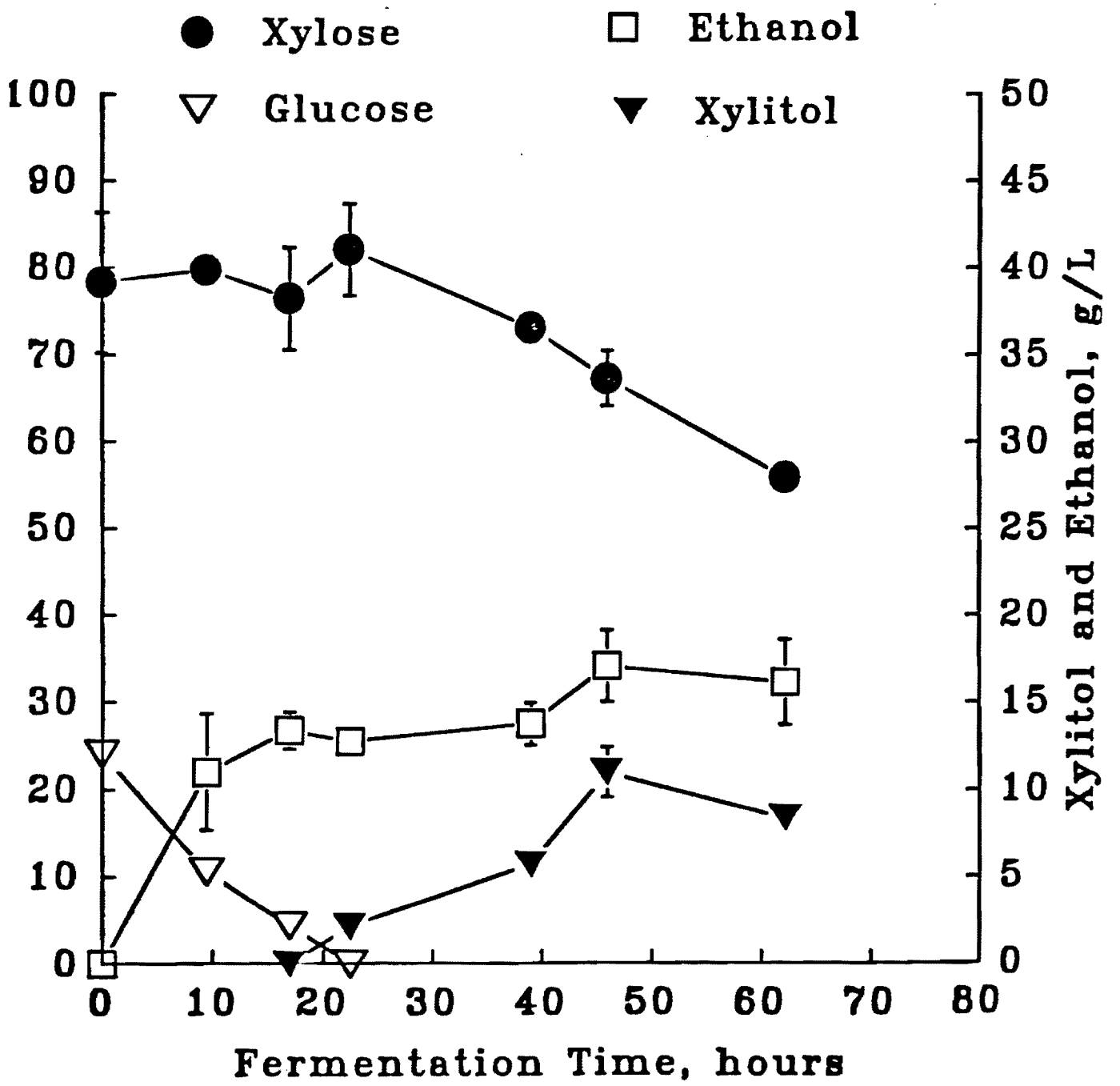


Figure 6 - Batch Fermentation of a mixture of 50% glucose and 50% D-xylose by *Candida shehatae* cells pre-grown aerobically on D-xylose.

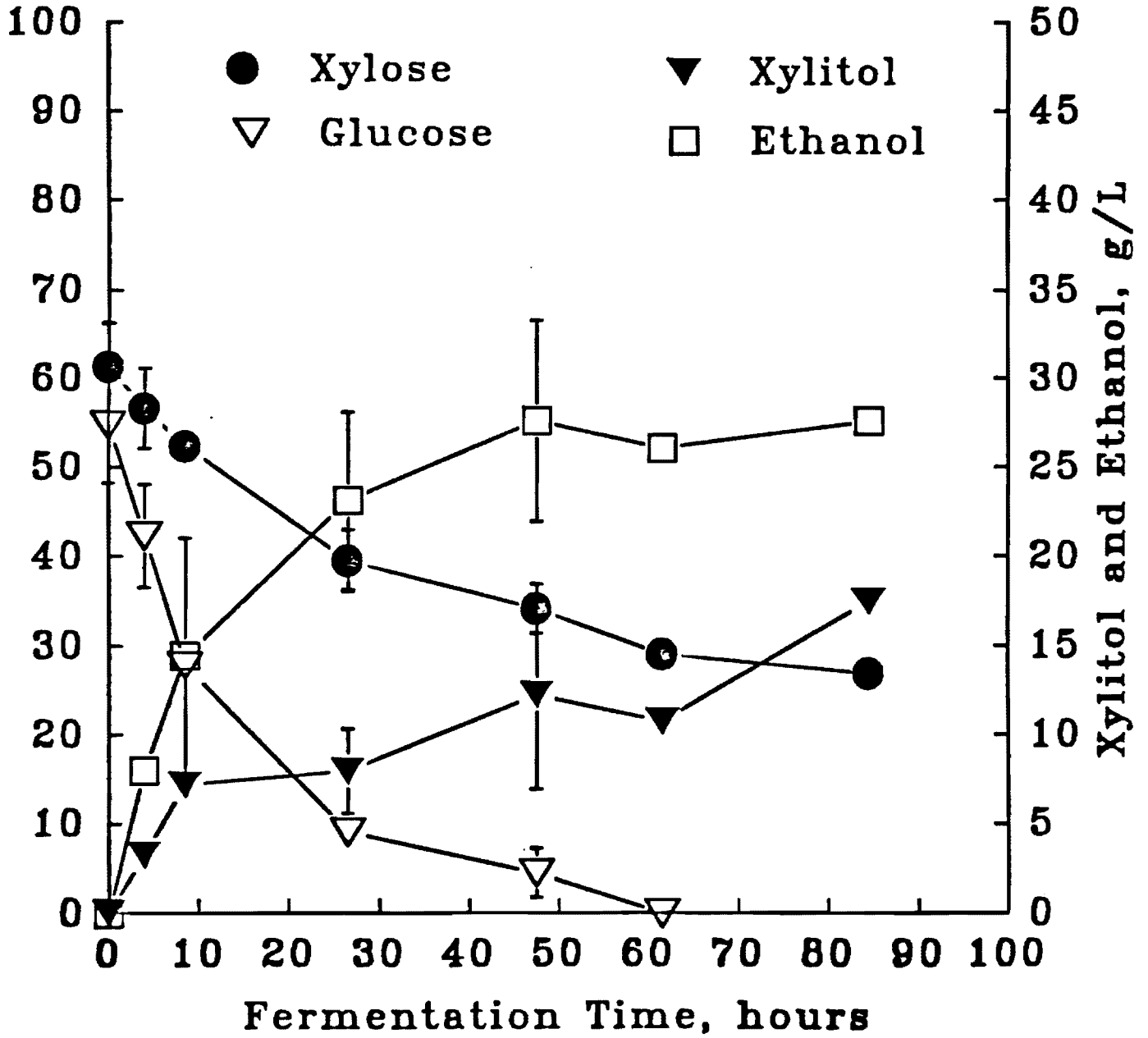


Figure 7 - Batch Fermentation of a mixture of 25% glucose and 75% D-xylose by *Candida shehatae* cells aerobically pre-grown on D-Xylose.

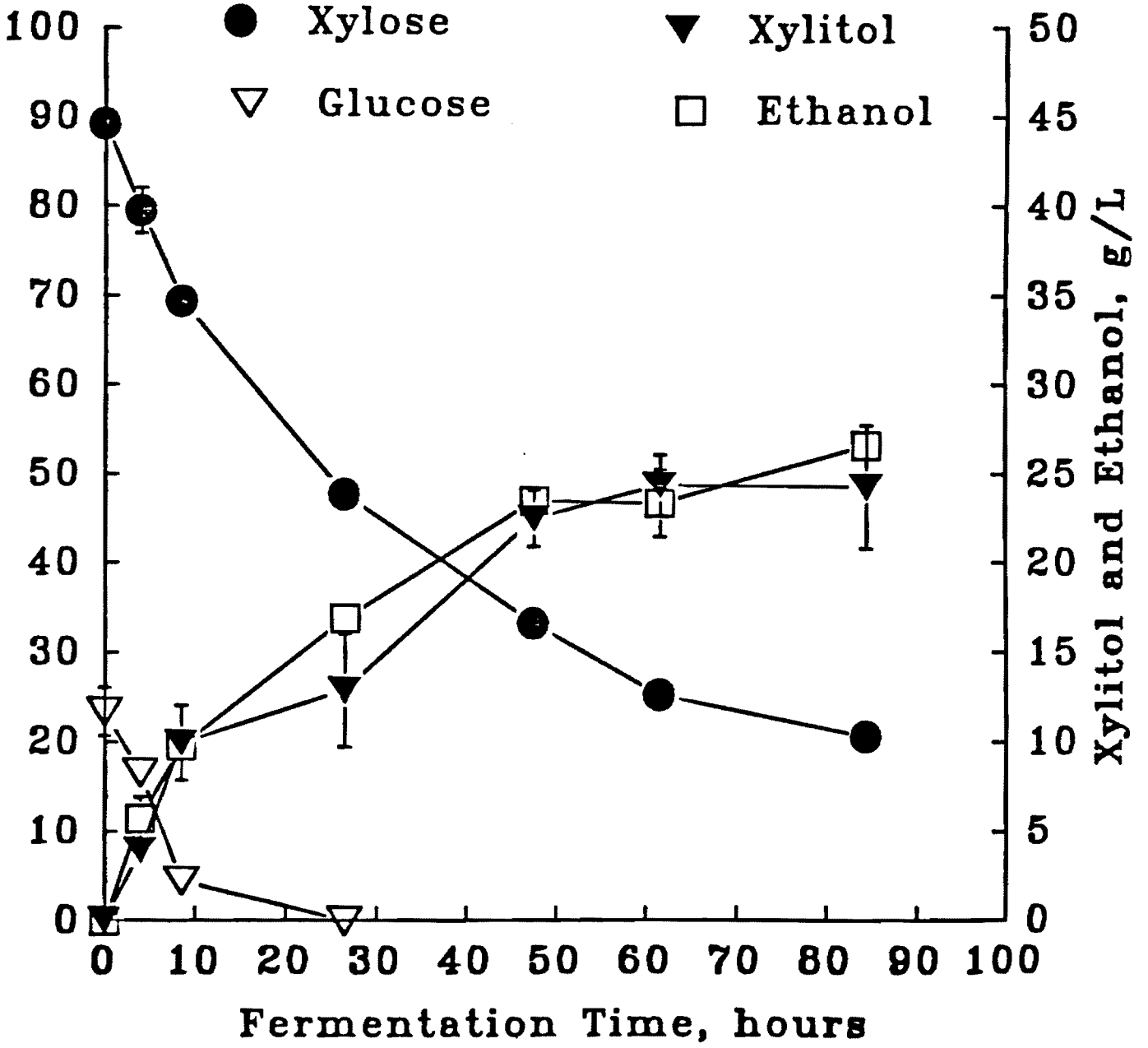


Figure 9 - Viability of *Candida shehatae* cells (as measured by methylene blue staining) in pure D-xylose and mixed sugar fermentations (cells were pre-grown on D-xylose).

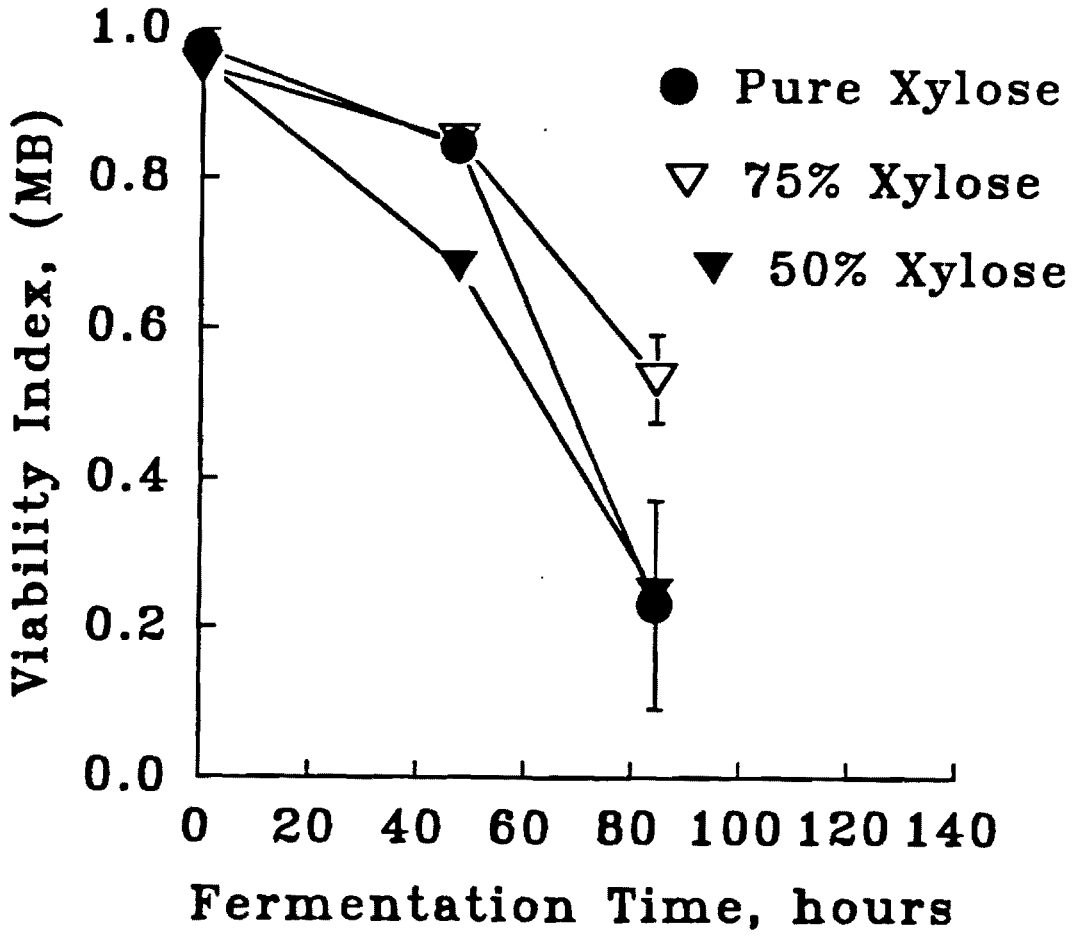
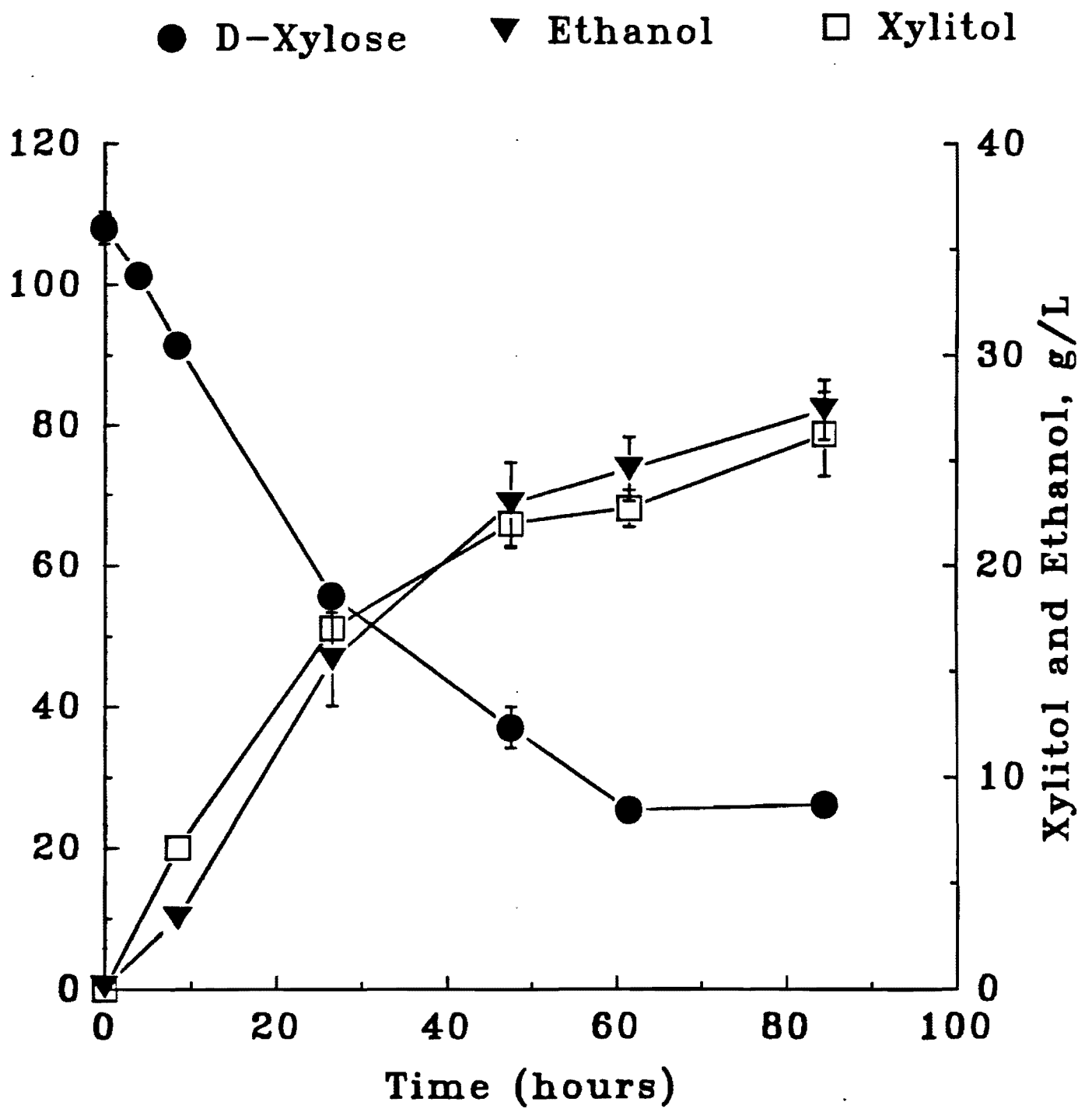


Figure 8 - Batch Fermentation of pure D-xylose by *Candida shehatae* cells aerobically pre-grown on D-Xylose.



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.

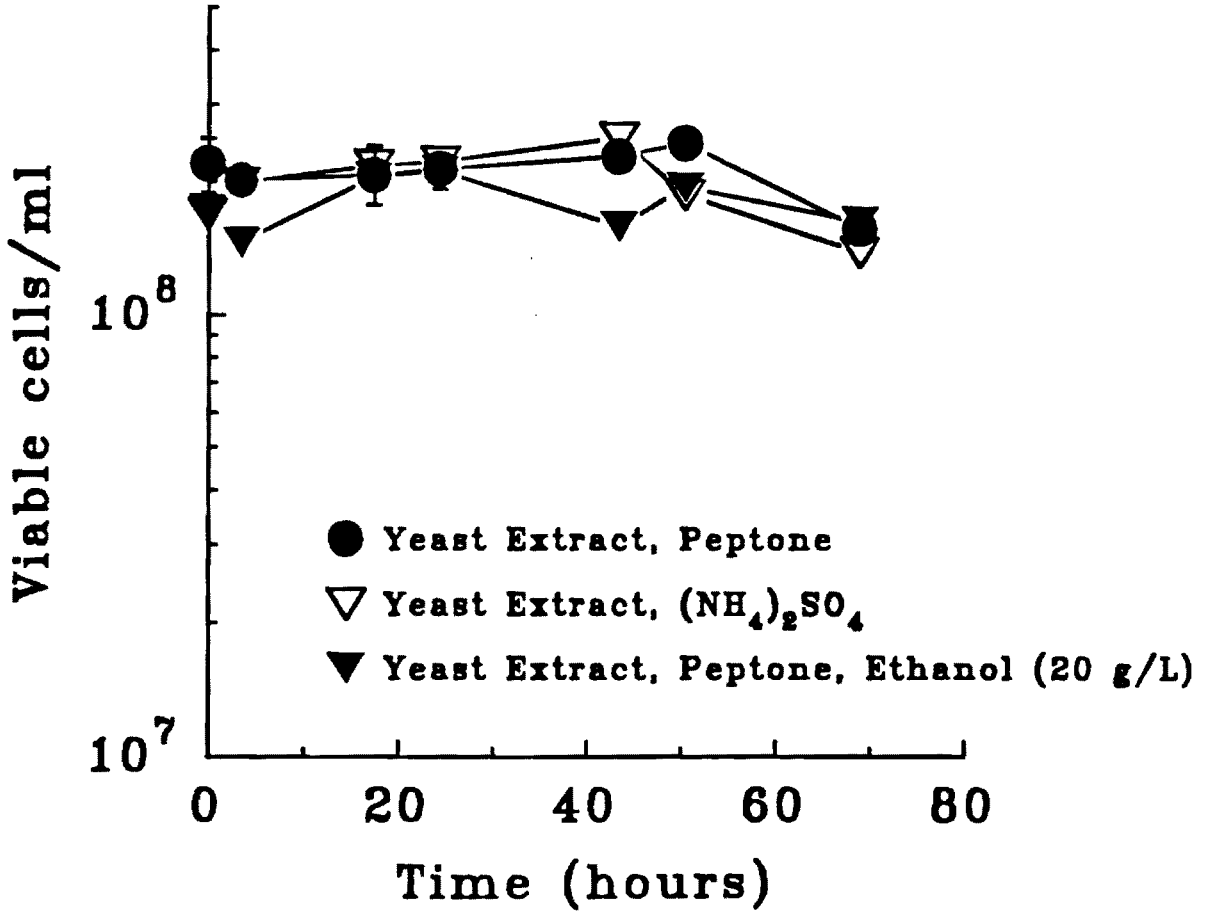


Figure 11 - D-Xylose Fermentation on Yeast Extract and Peptone as the Nitrogen Source.

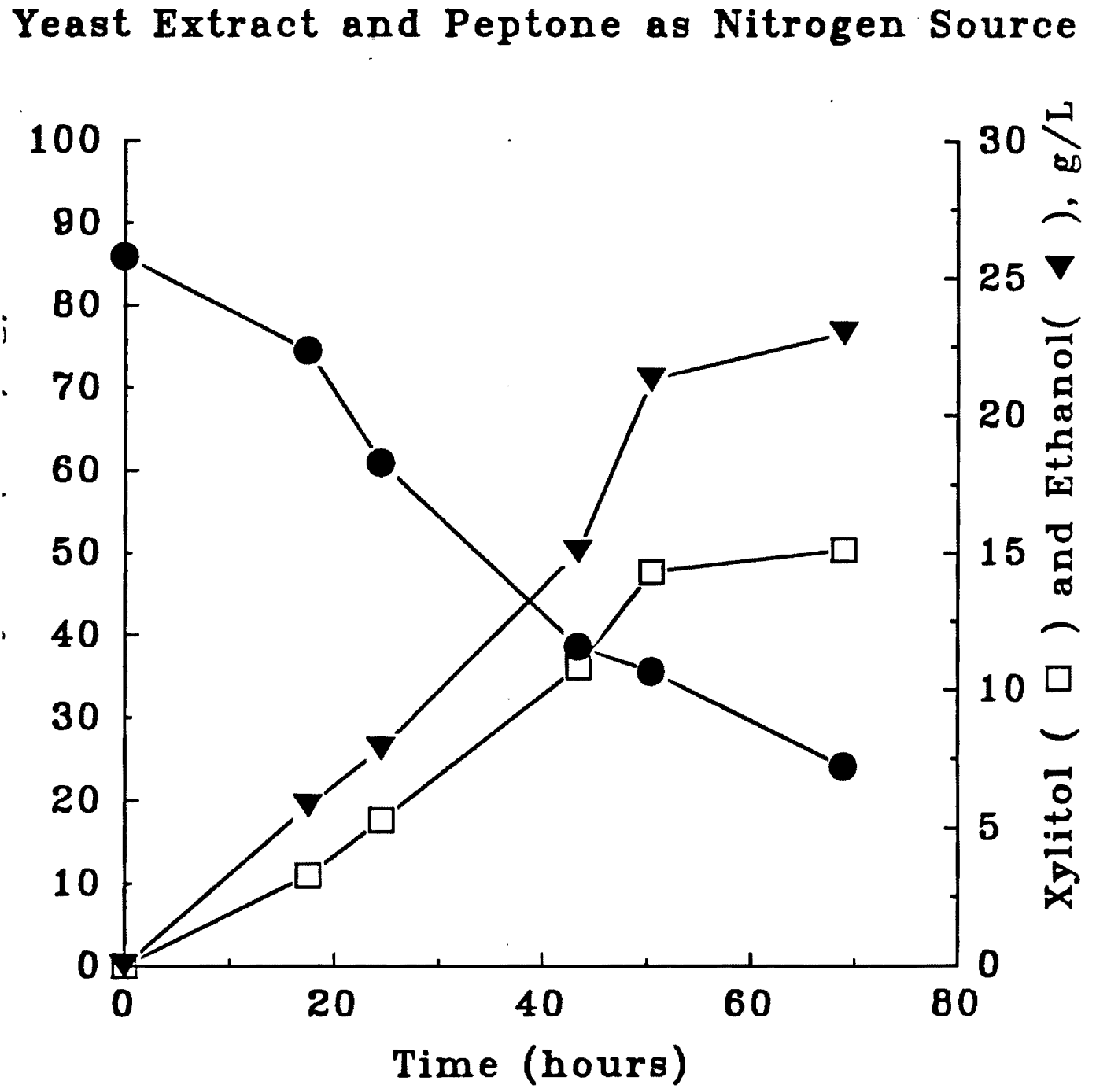


Figure 12 - D-Xylose Fermentation on Yeast Extract and $(NH_4)_2SO_4$ as the Nitrogen Source.

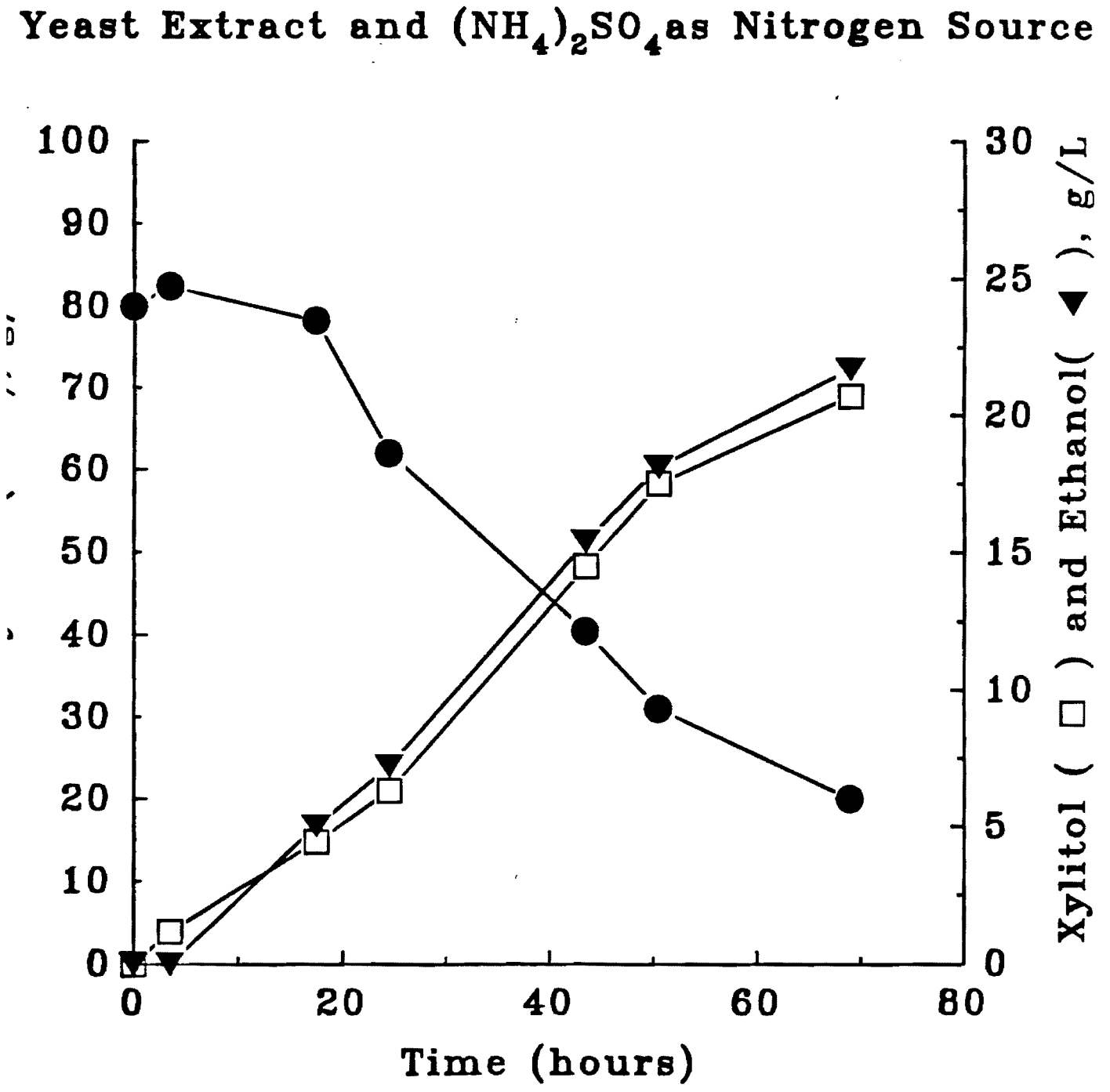


Figure 13 - D-Xylose Fermentation on Yeast Extract and Peptone as the Nitrogen Source with 20 g/L of Added Ethanol at Time Zero.

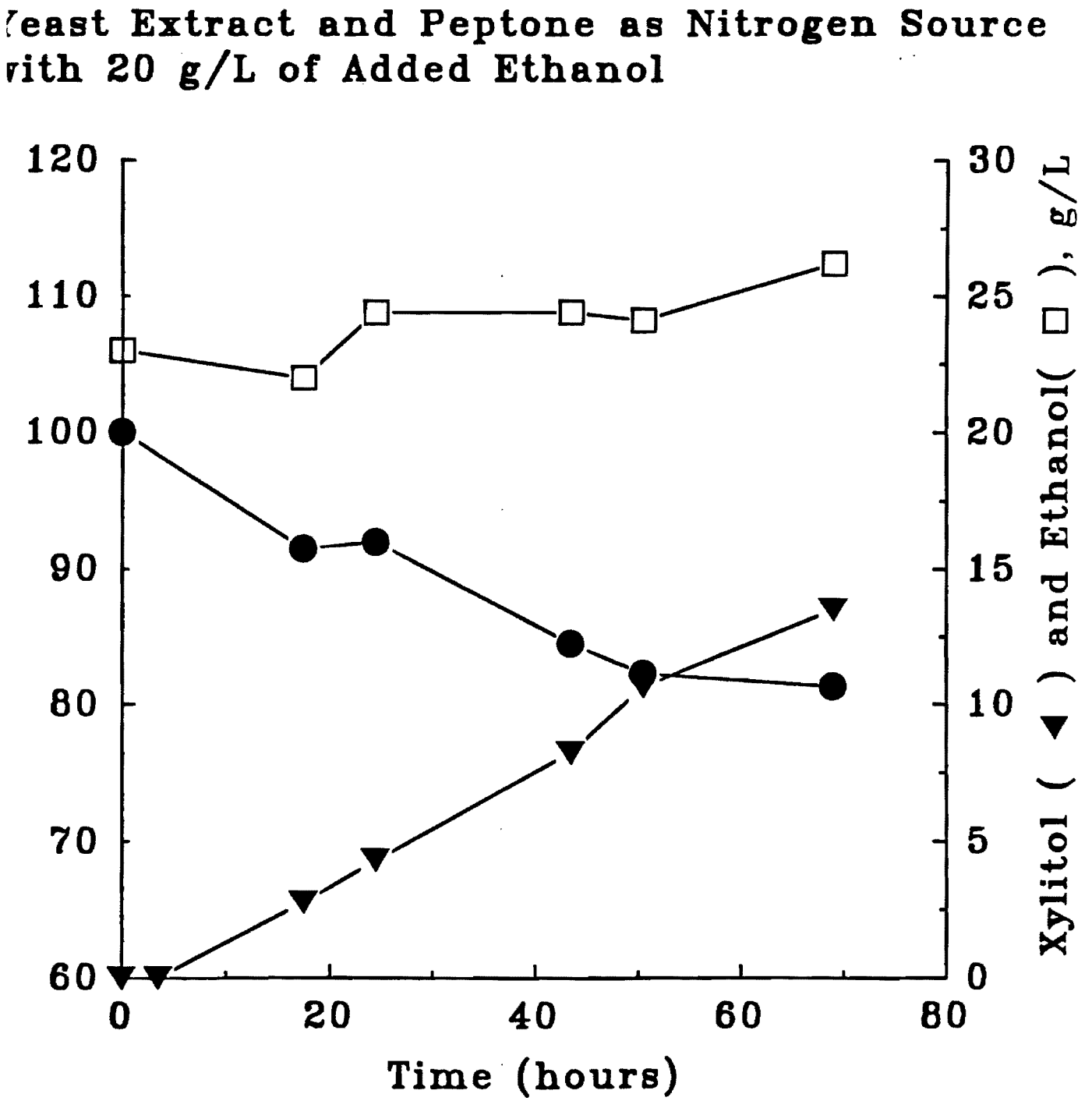
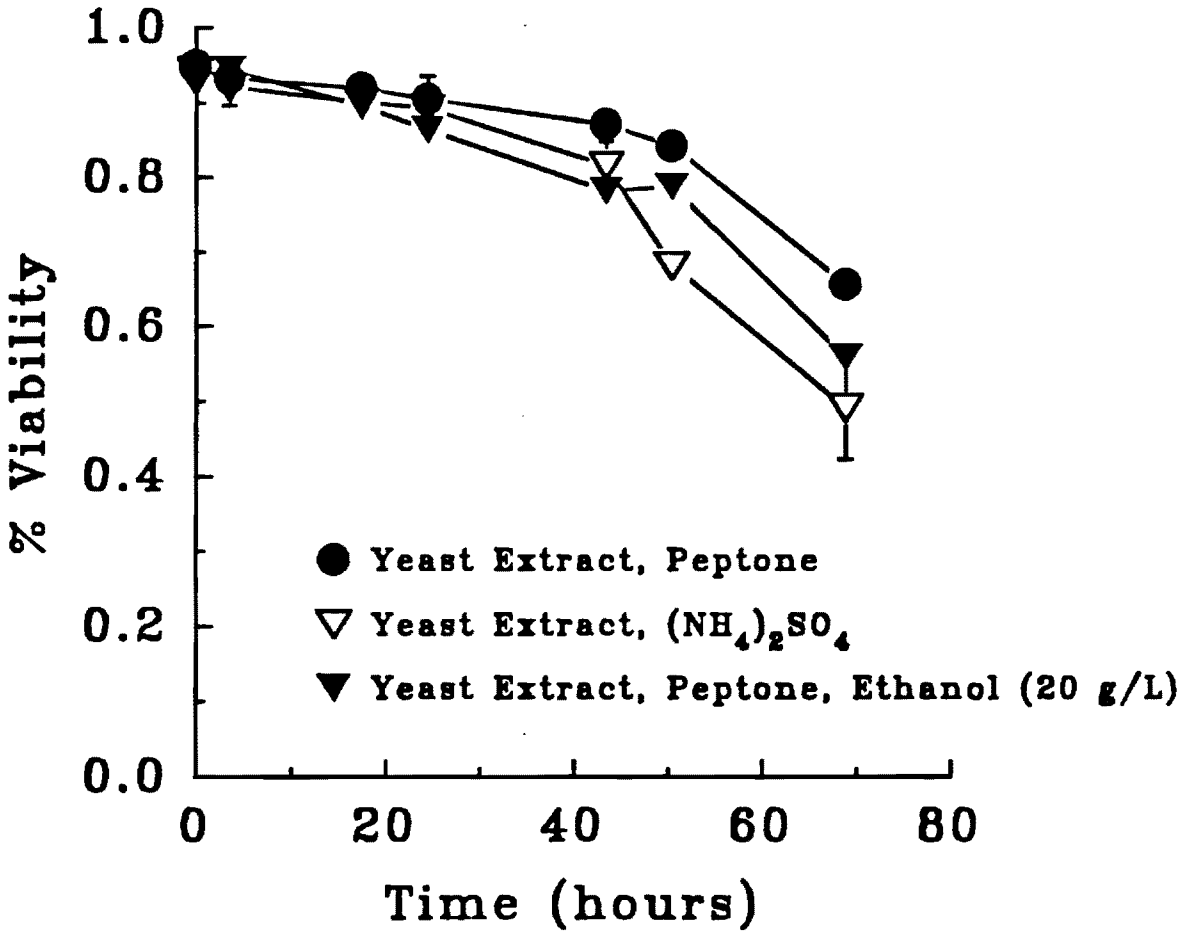


Figure 14 - Effect of Different Nitrogen Sources and Added Ethanol on Cell Viability During D-Xylose Fermentations.



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.

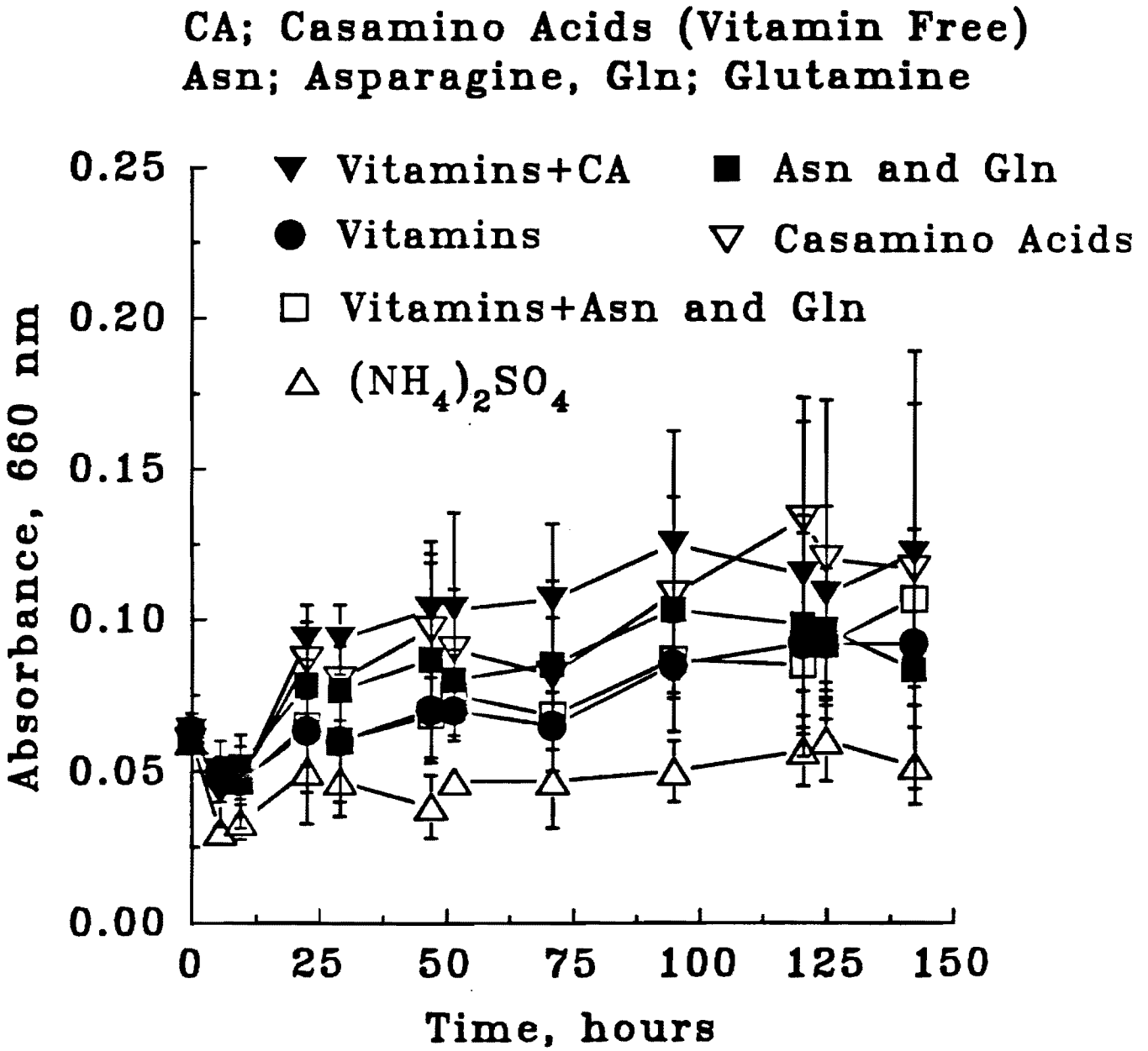


Figure 16 - Anaerobic Growth of Saccharomyces cerevisiae on Glucose.

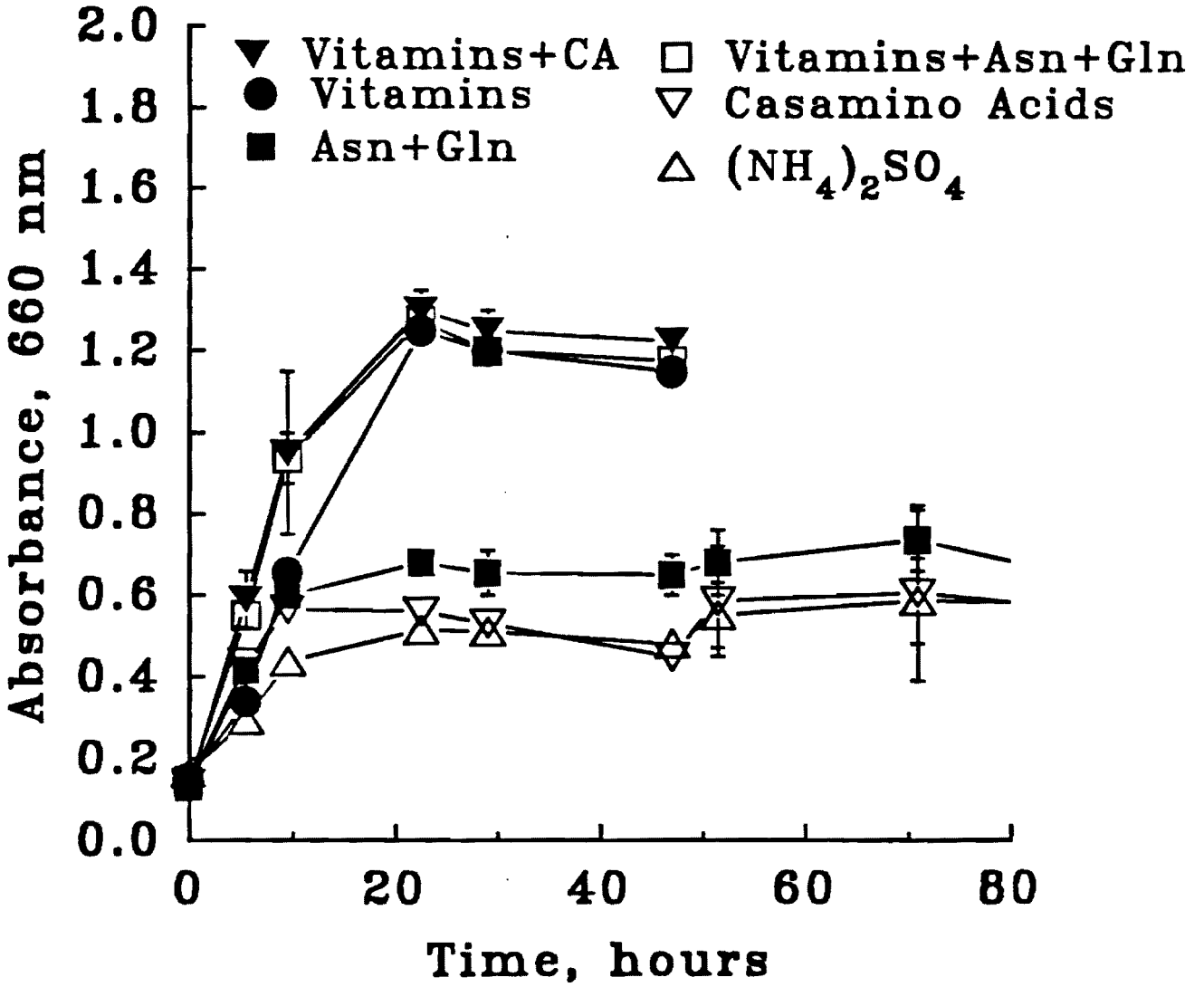
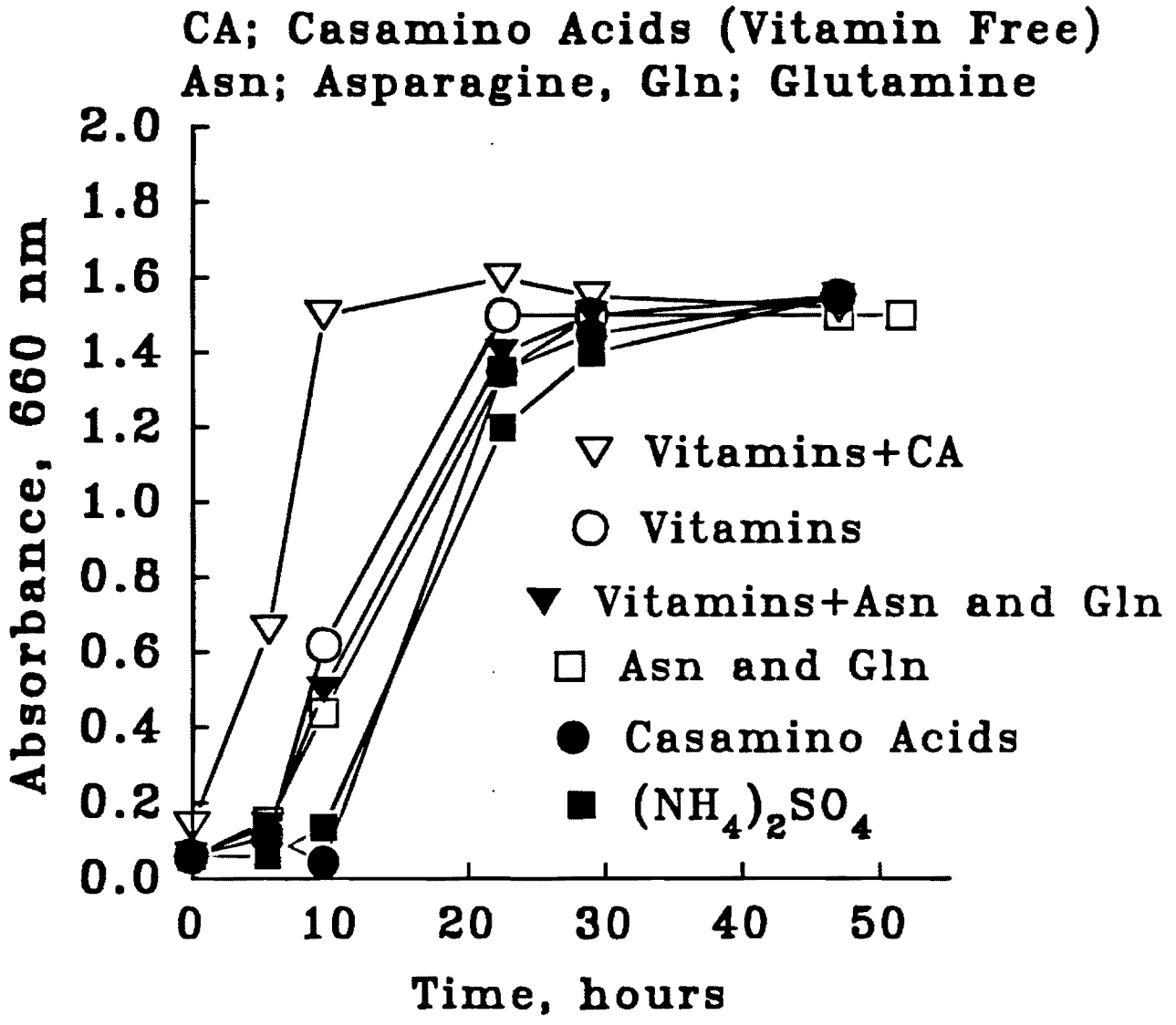


Figure 17 - Aerobic Growth of *Candida shehatae* on Glucose.



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.

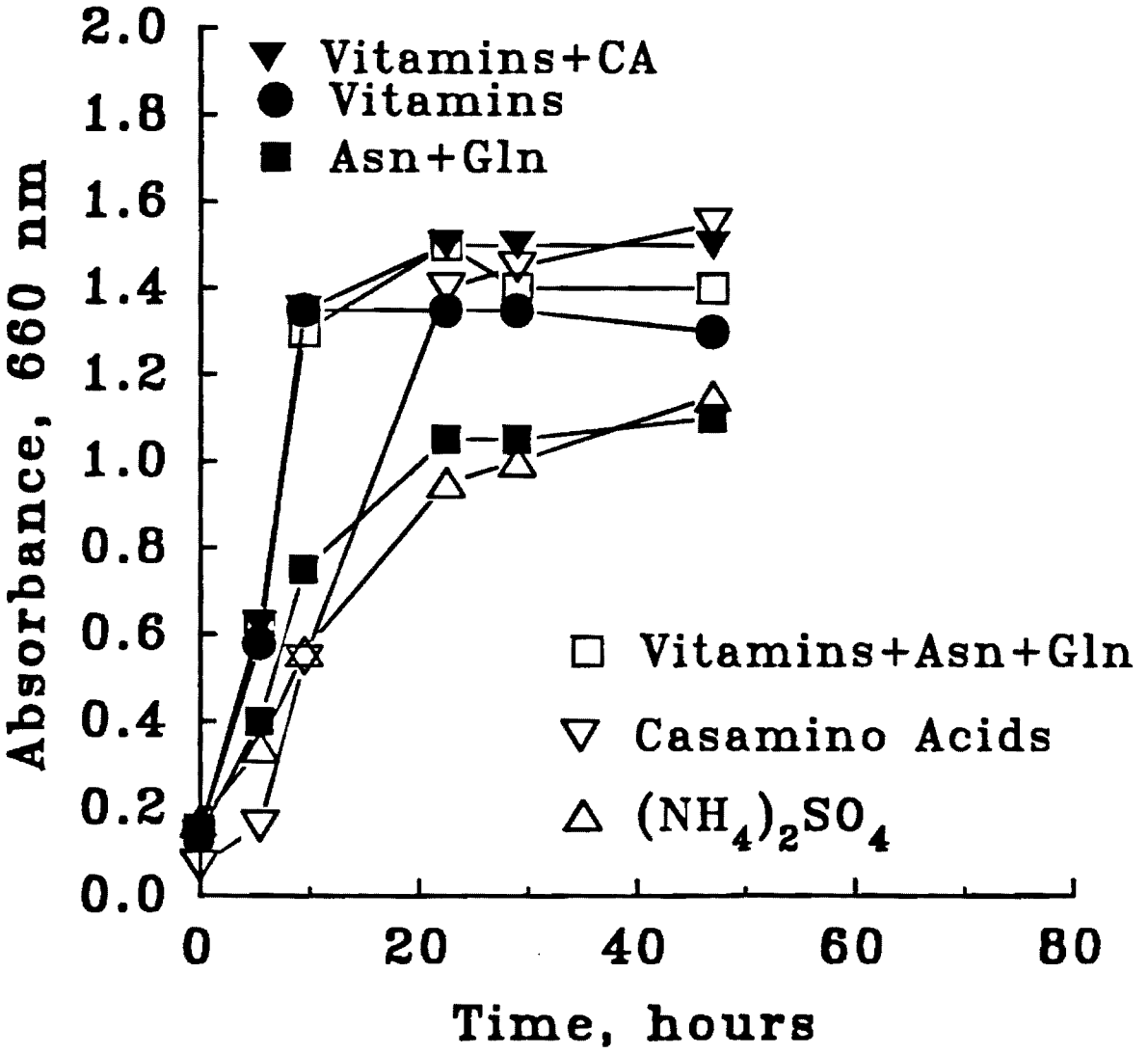


Figure 19 - Comparison of Anaerobic and Aerobic Growth on Vitamins and Casamino Acids for *Candida shehatae*.

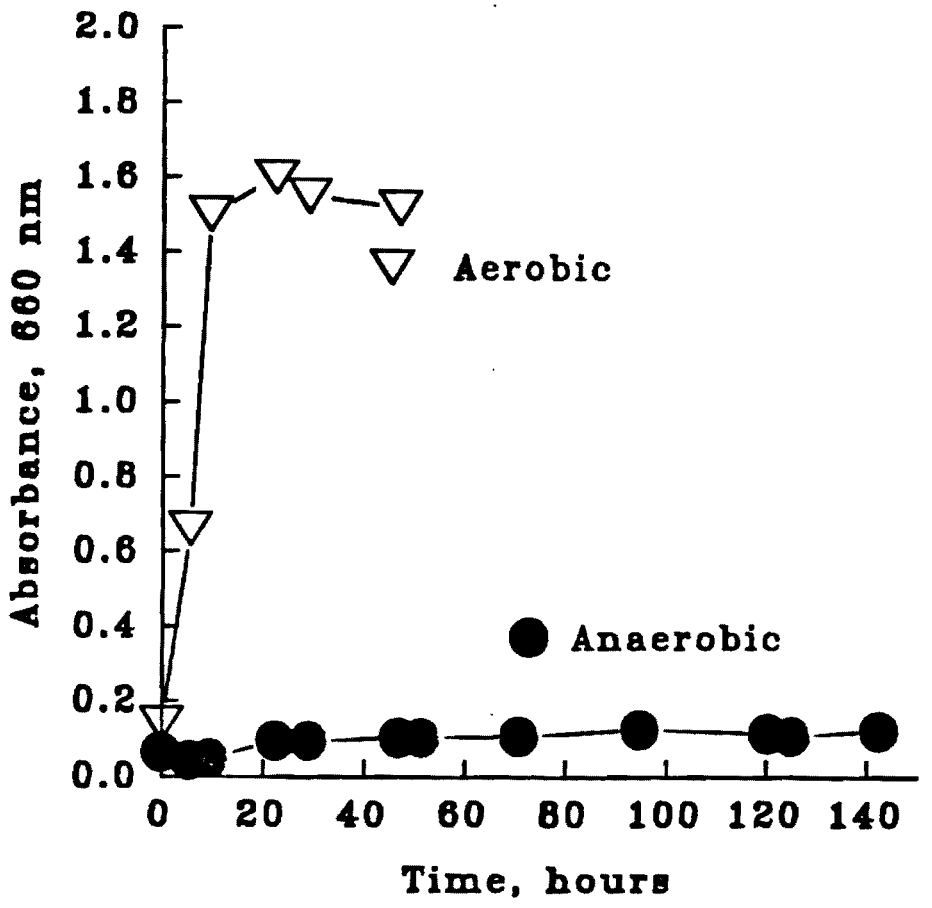


Figure 20 - Comparison of Anaerobic and Aerobic Growth on Vitamins and Casamino Acids for *Saccharomyces cerevisiae*.

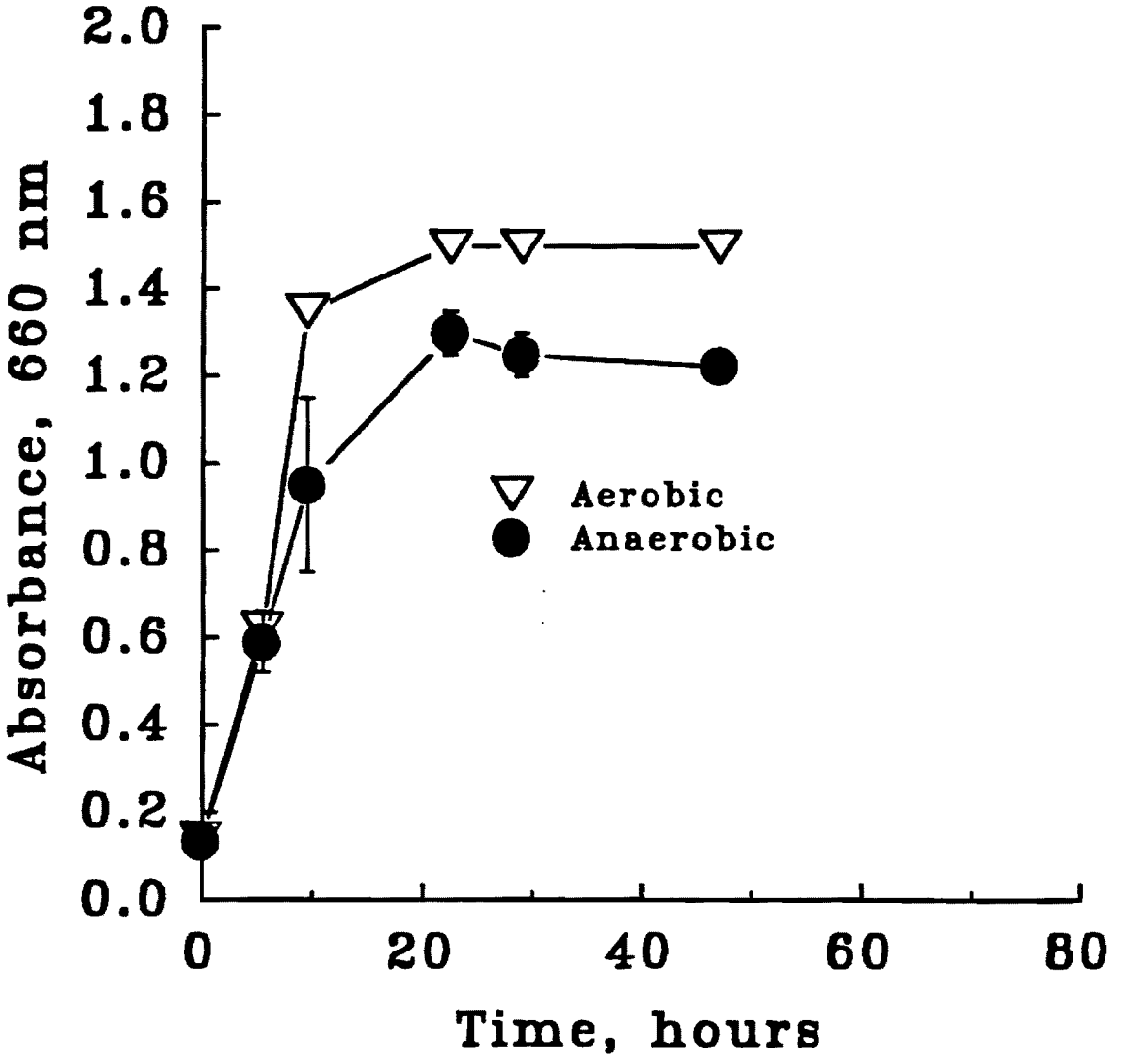
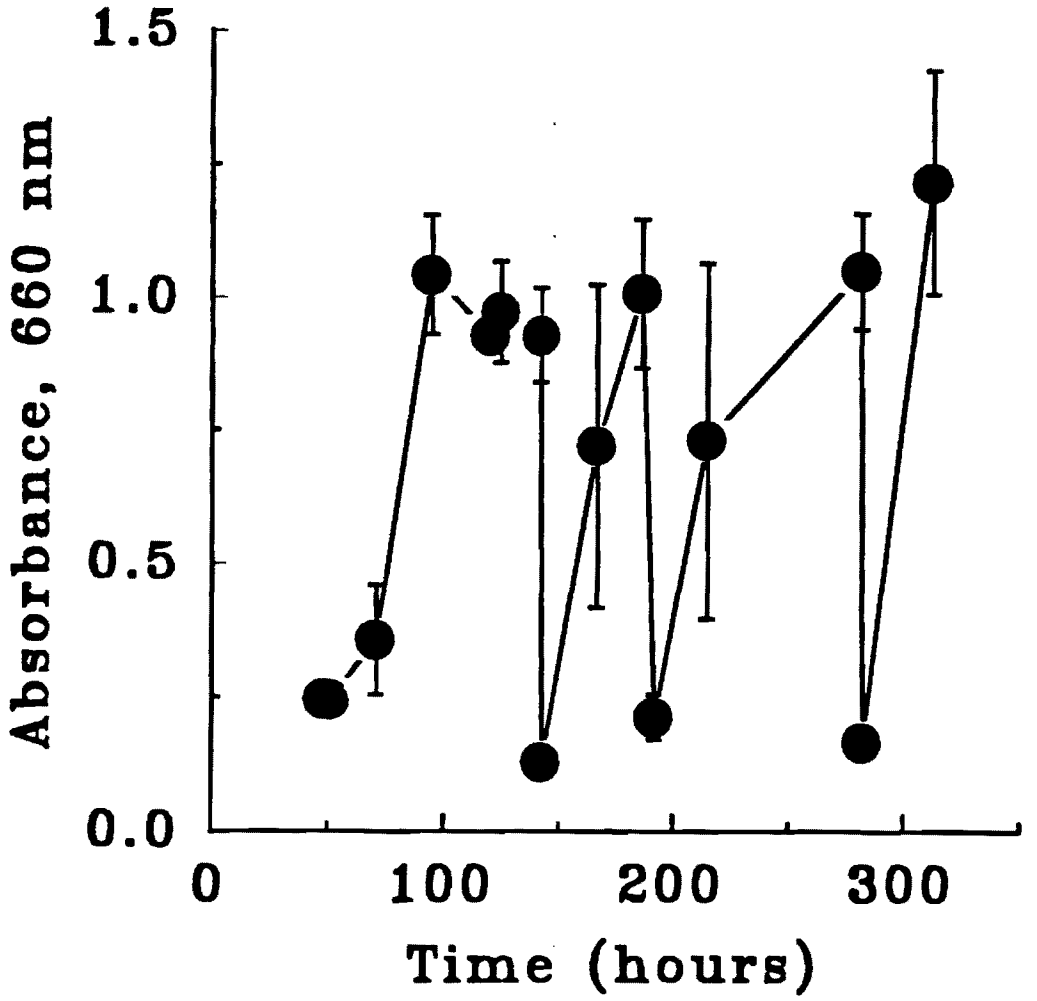


Figure 21 - Anaerobic Growth of *Saccharomycescerevisiae* on a Defined Medium with Vitamins and Casamino Acids and Glucose as the Substrate (the cultures were periodically transferred to new tubes to prevent substrate limitation).



(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.

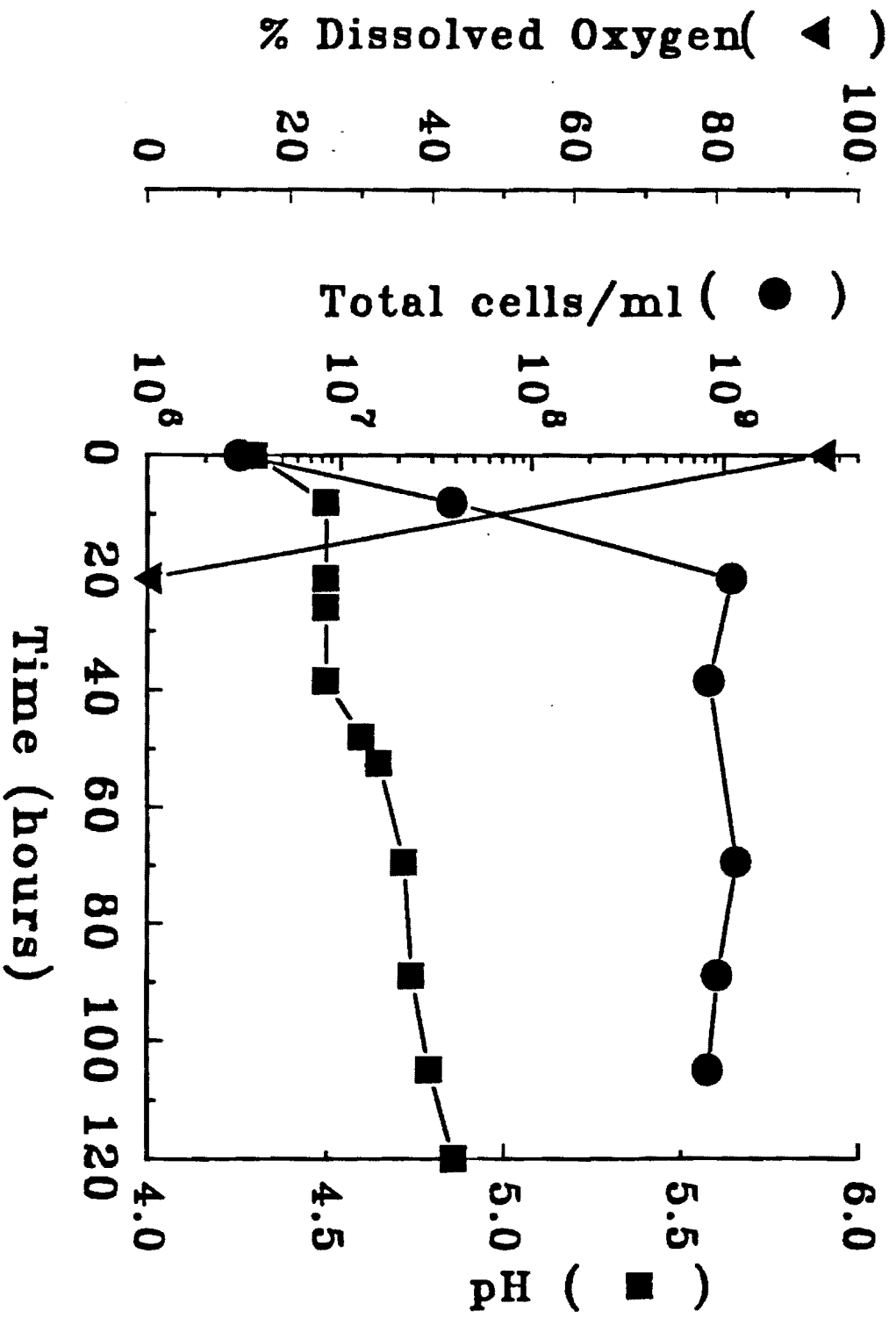


Figure 23 - Batch D-Xylose Fermentation on a Defined Medium by *Candida shehatae*.

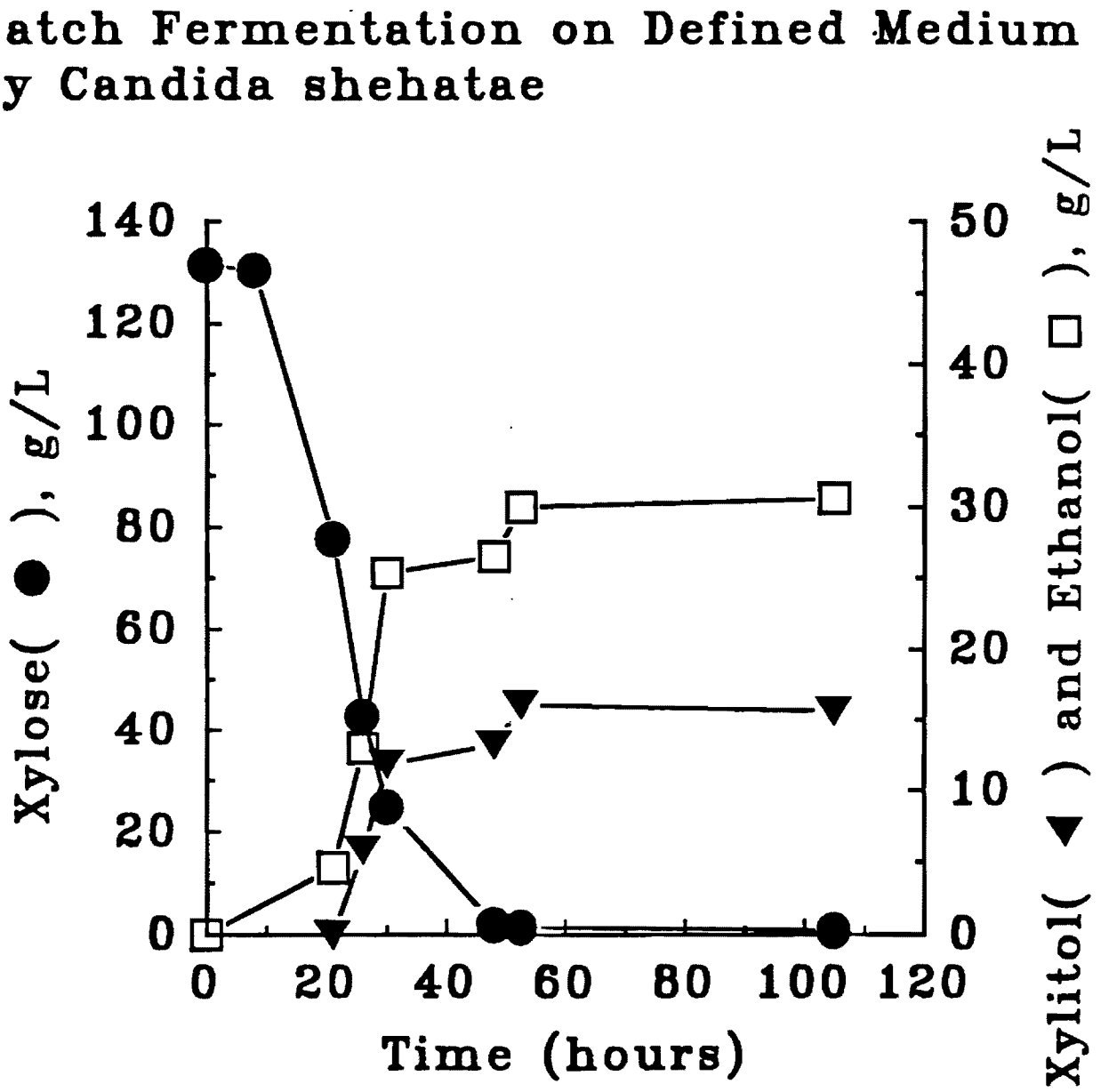


Figure 24 - Cell Viability of *Candida shehatae* During a Batch D-Xylose Fermentation on a Defined Medium.

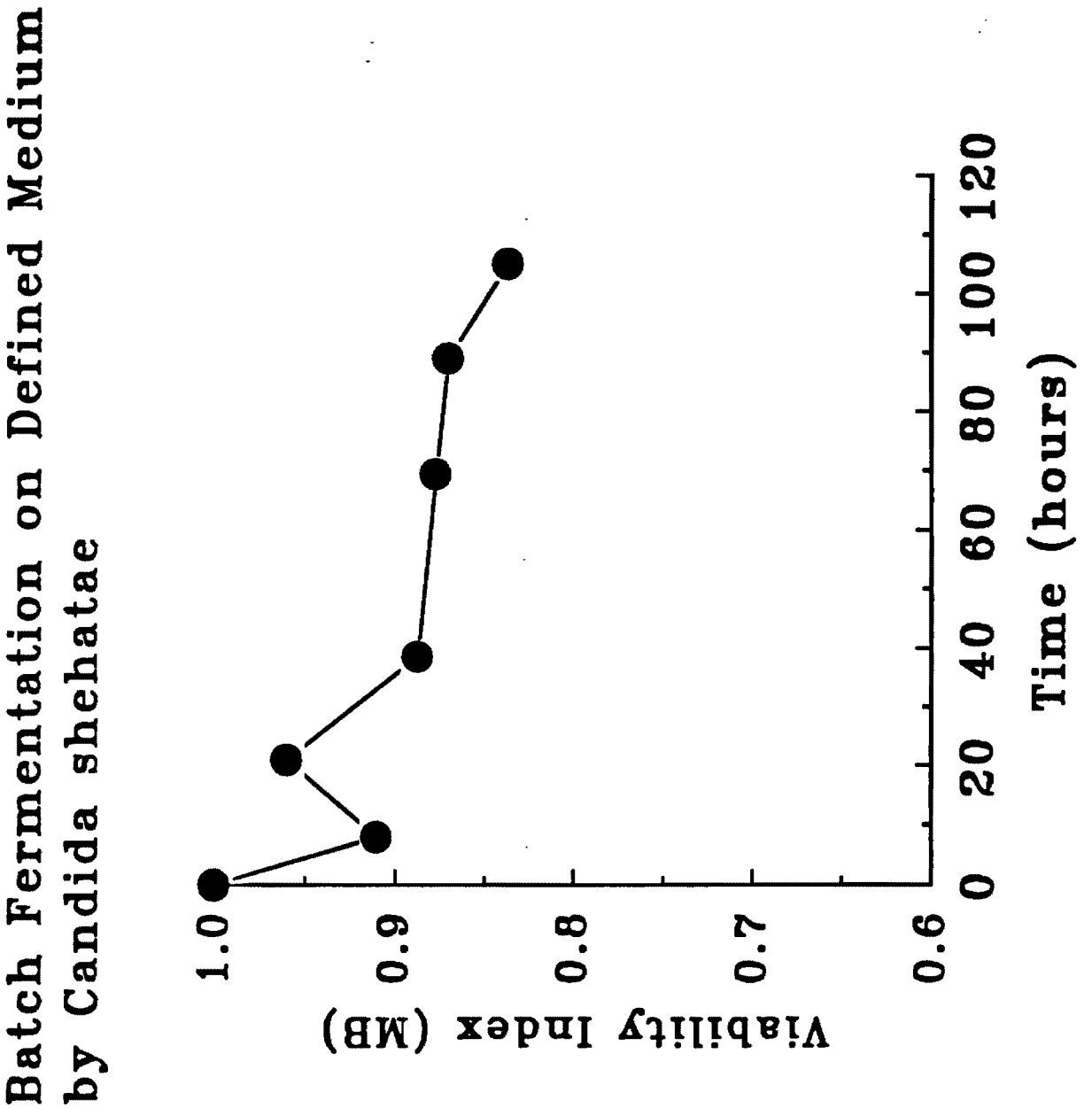


Figure 25 - Batch D-Xylose Fermentation on a Defined Medium by *Pichia stipitis*.

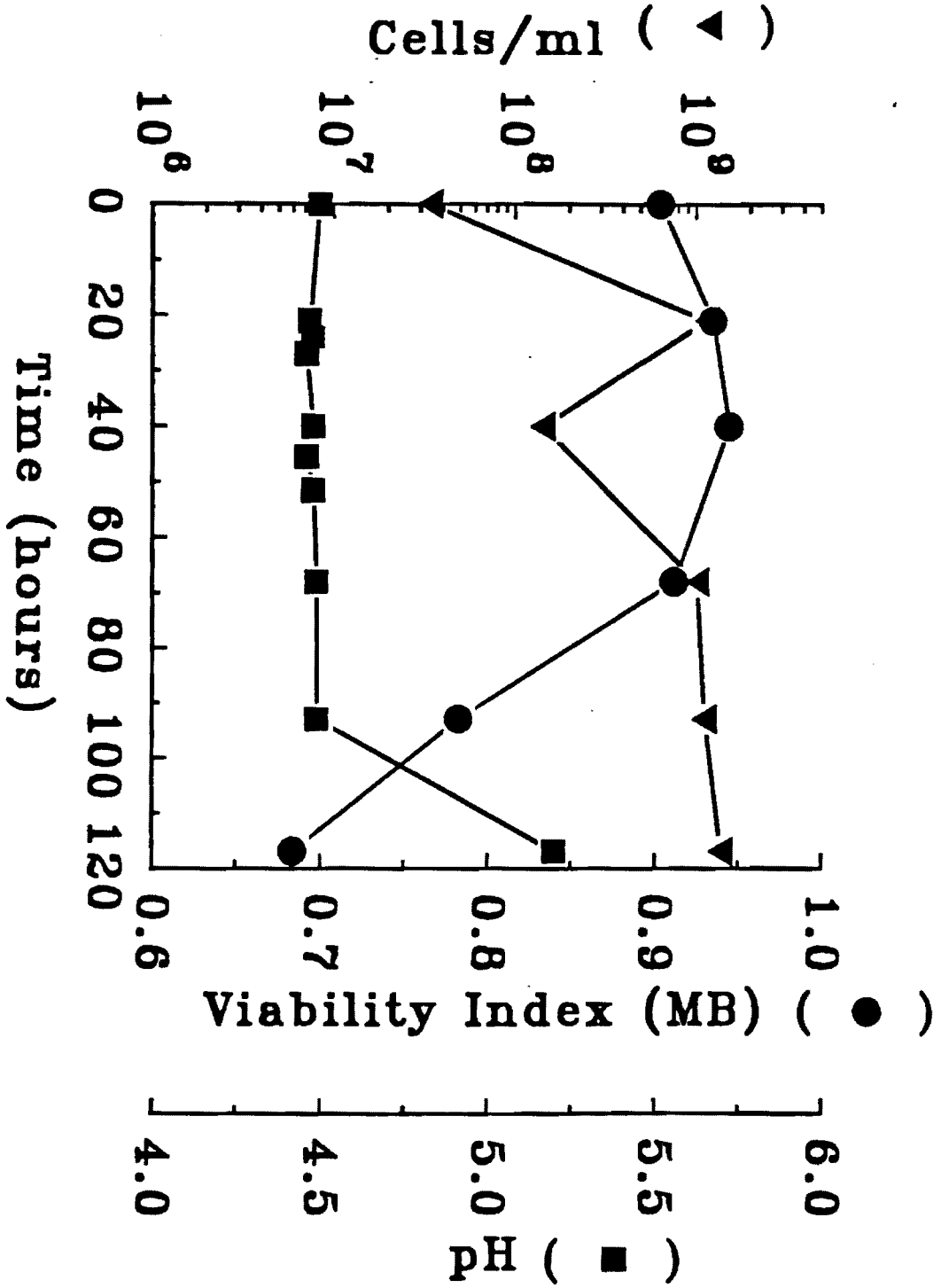
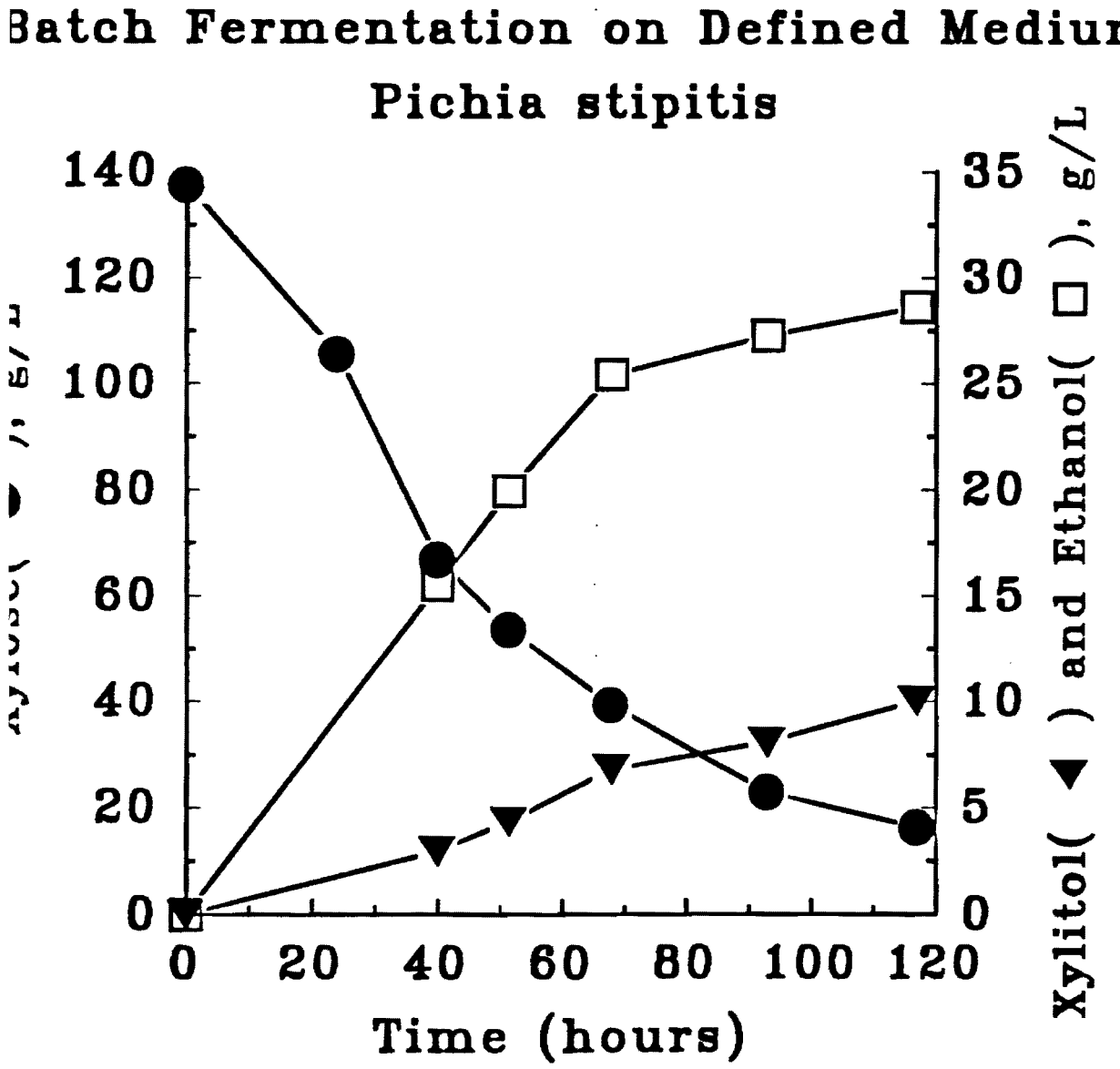


Figure 26 - Batch D-Xylose Fermentation on a Defined Medium by *Pichia stipitis*.



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*.

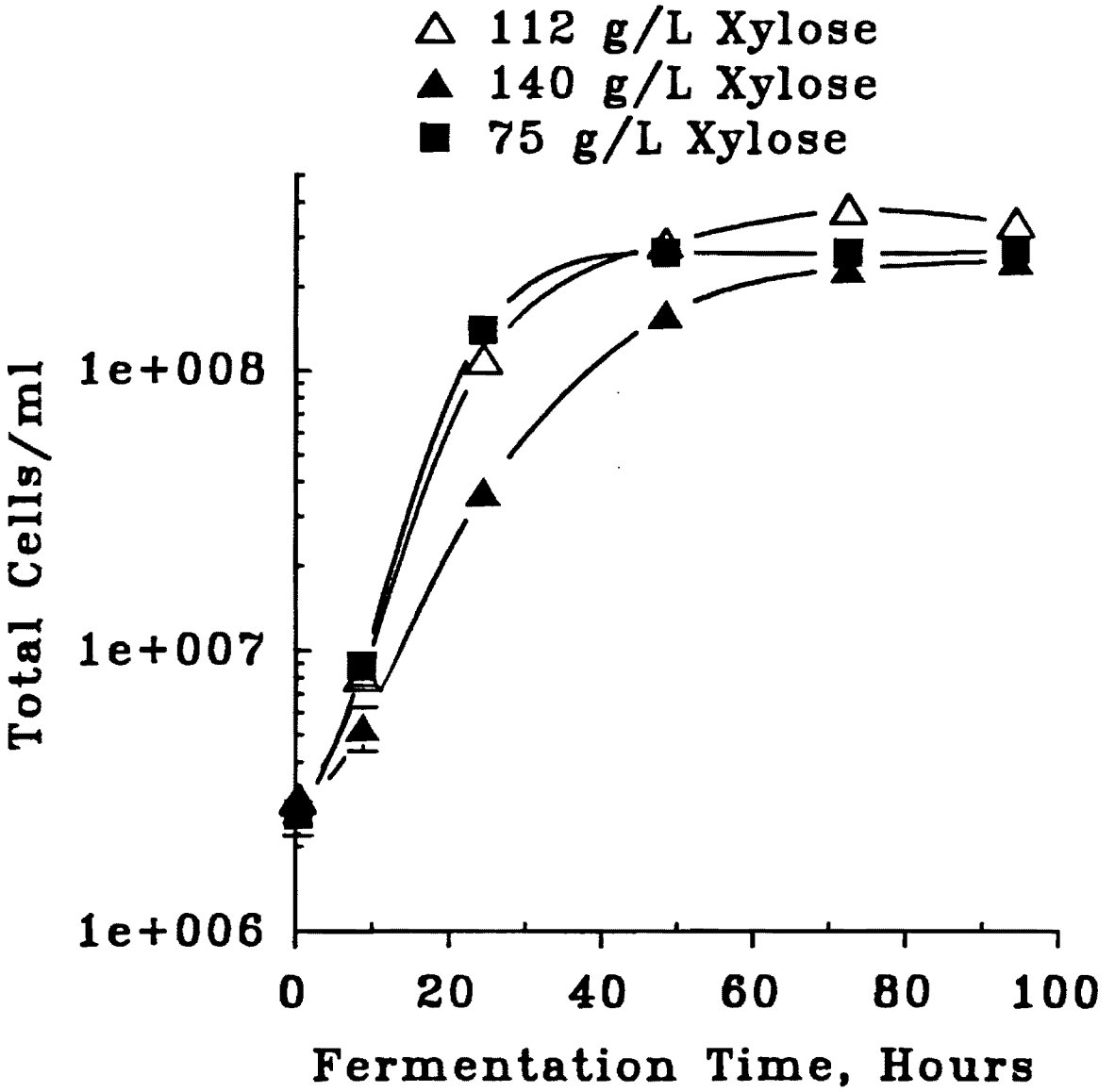


Figure 29 - Effect of D-Xylose Concentration on the Utilization Rate of D-Xylose.

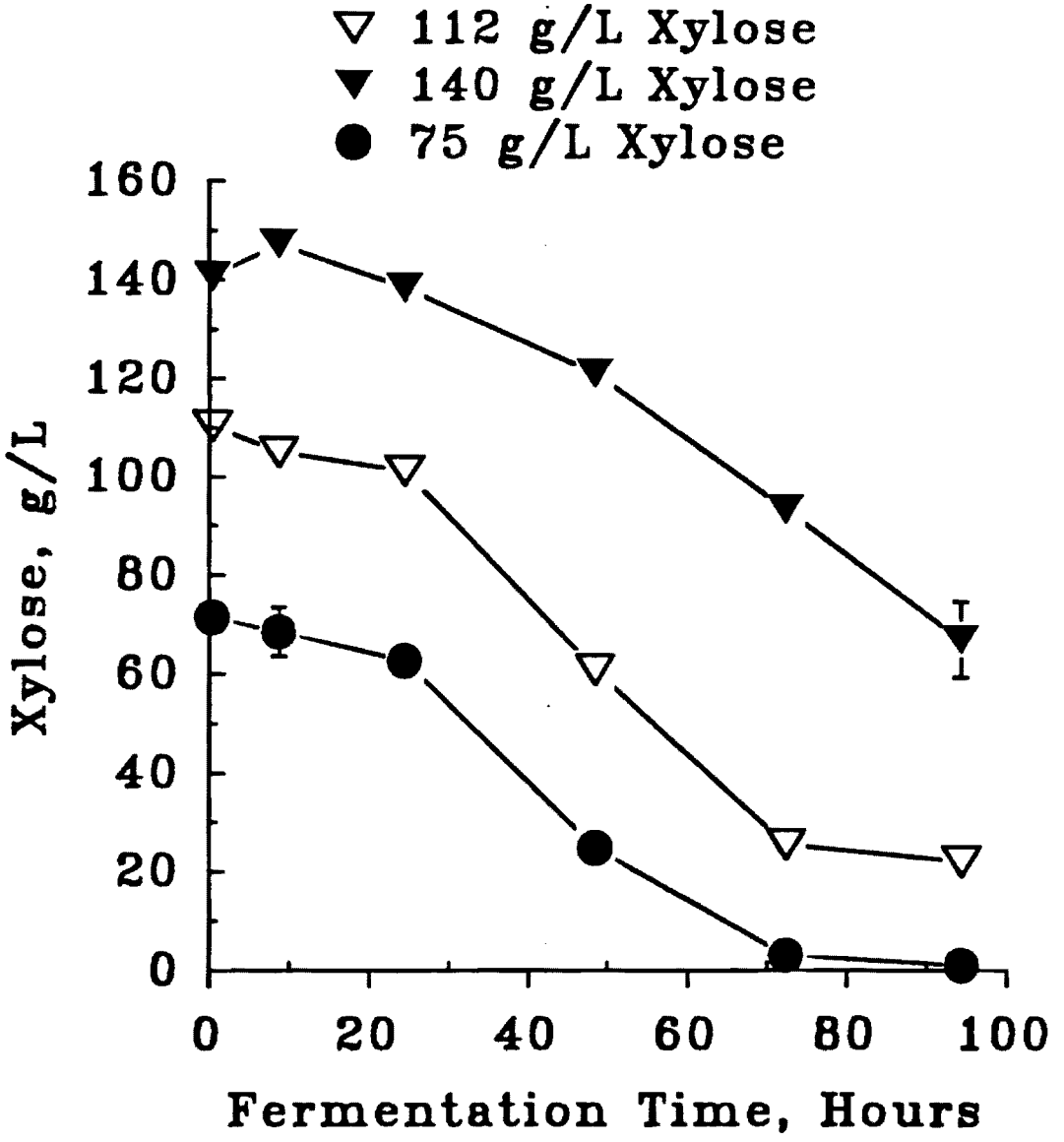


Figure 30 - Effect of D-Xylose Concentration on Ethanol Production by *Candida. shehatae.*

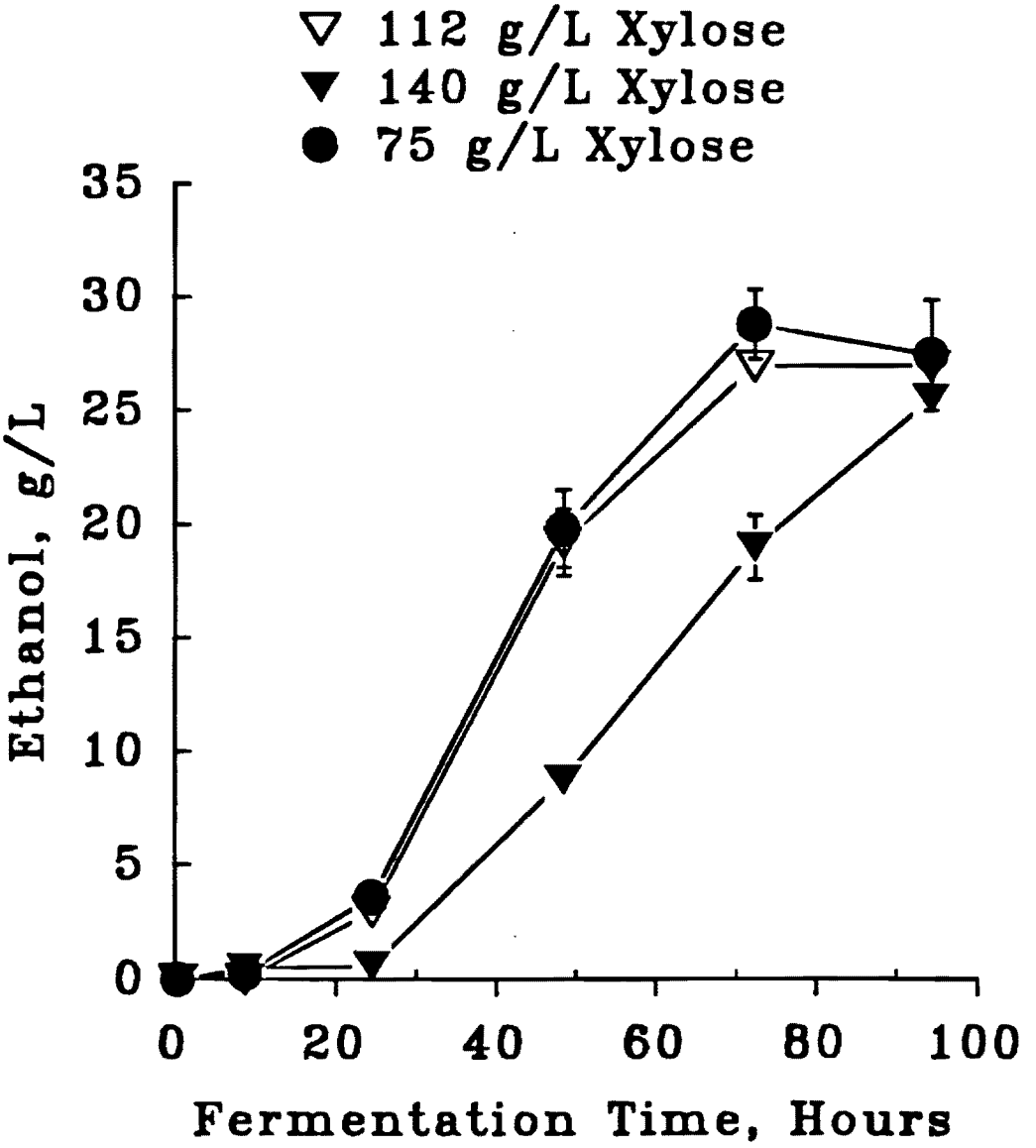


Figure 31 - Effect of D-Xylose Concentration on Xylitol Production by *C. shehatae*.

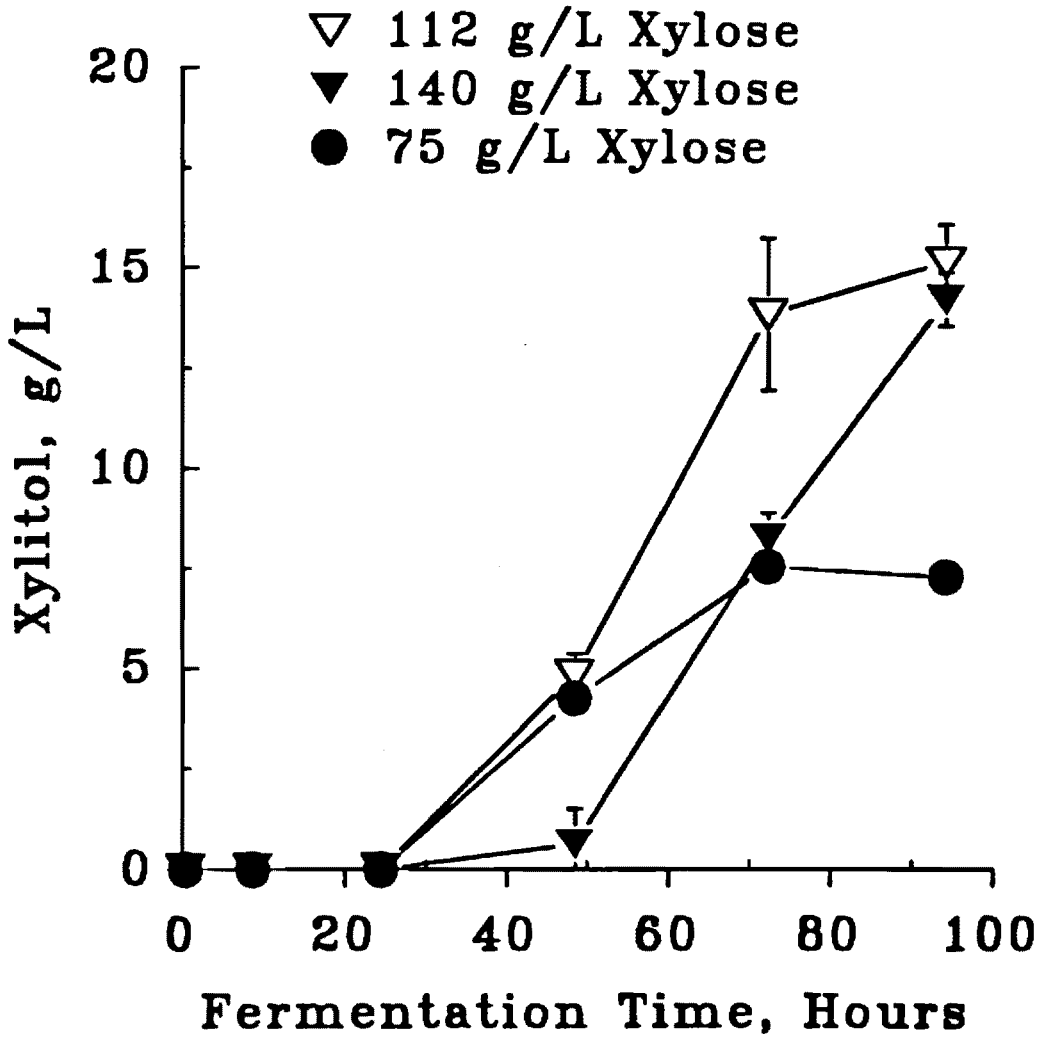
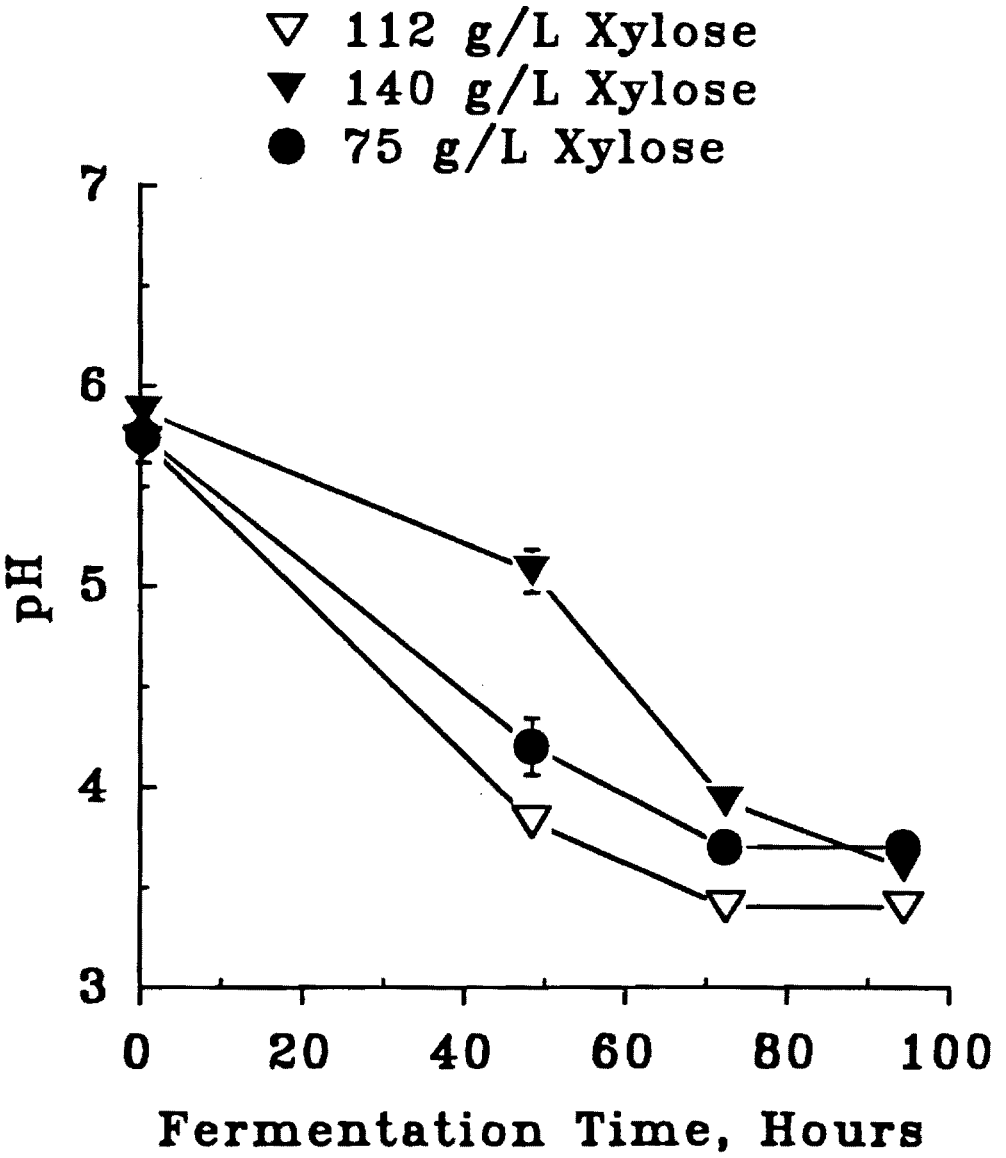


Figure 32 - Change in pH During Fermentations at Different Initial D-Xylose Concentrations.

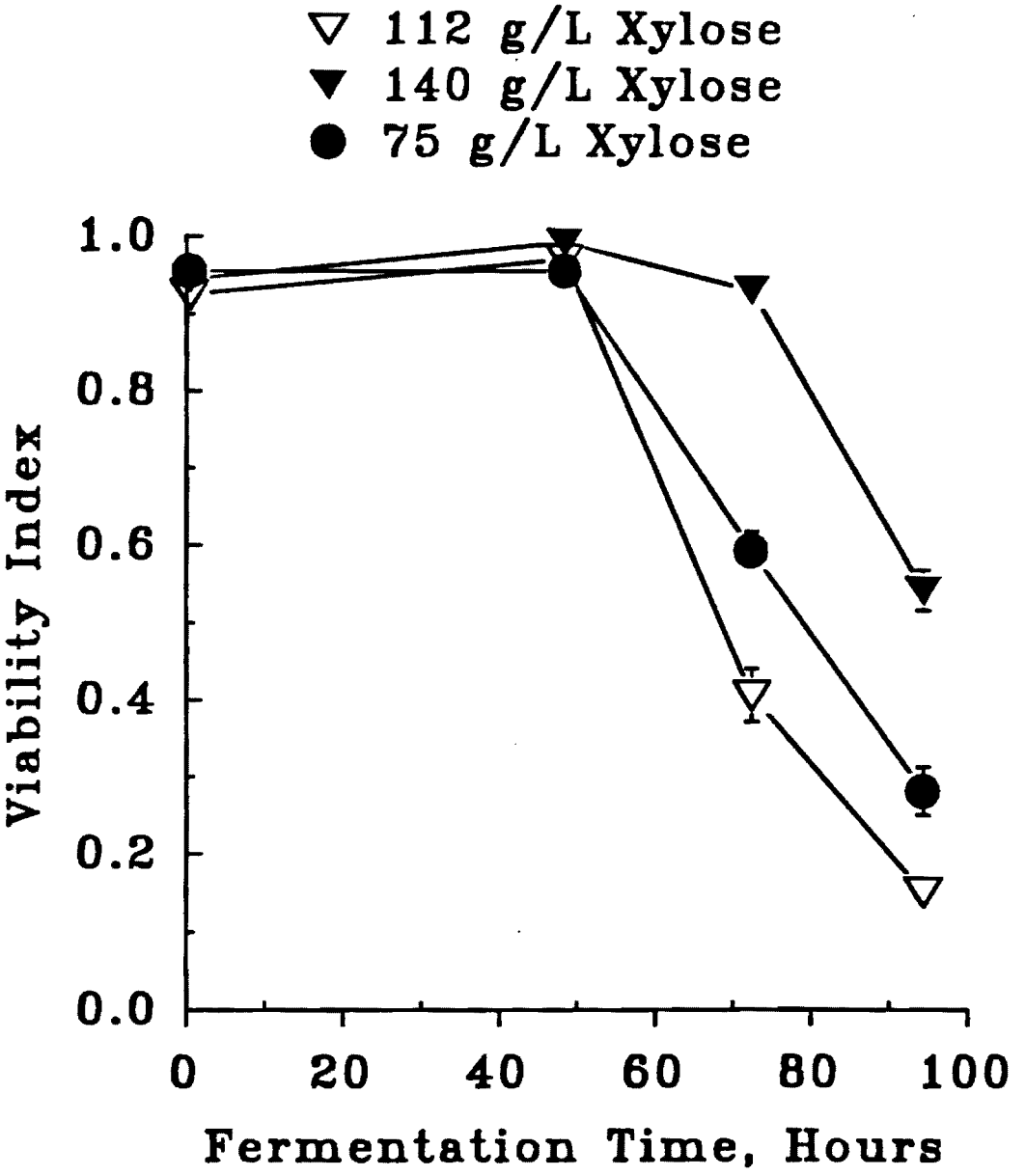


f. Effect of D-Xylose Concentration on Fermentations:

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.

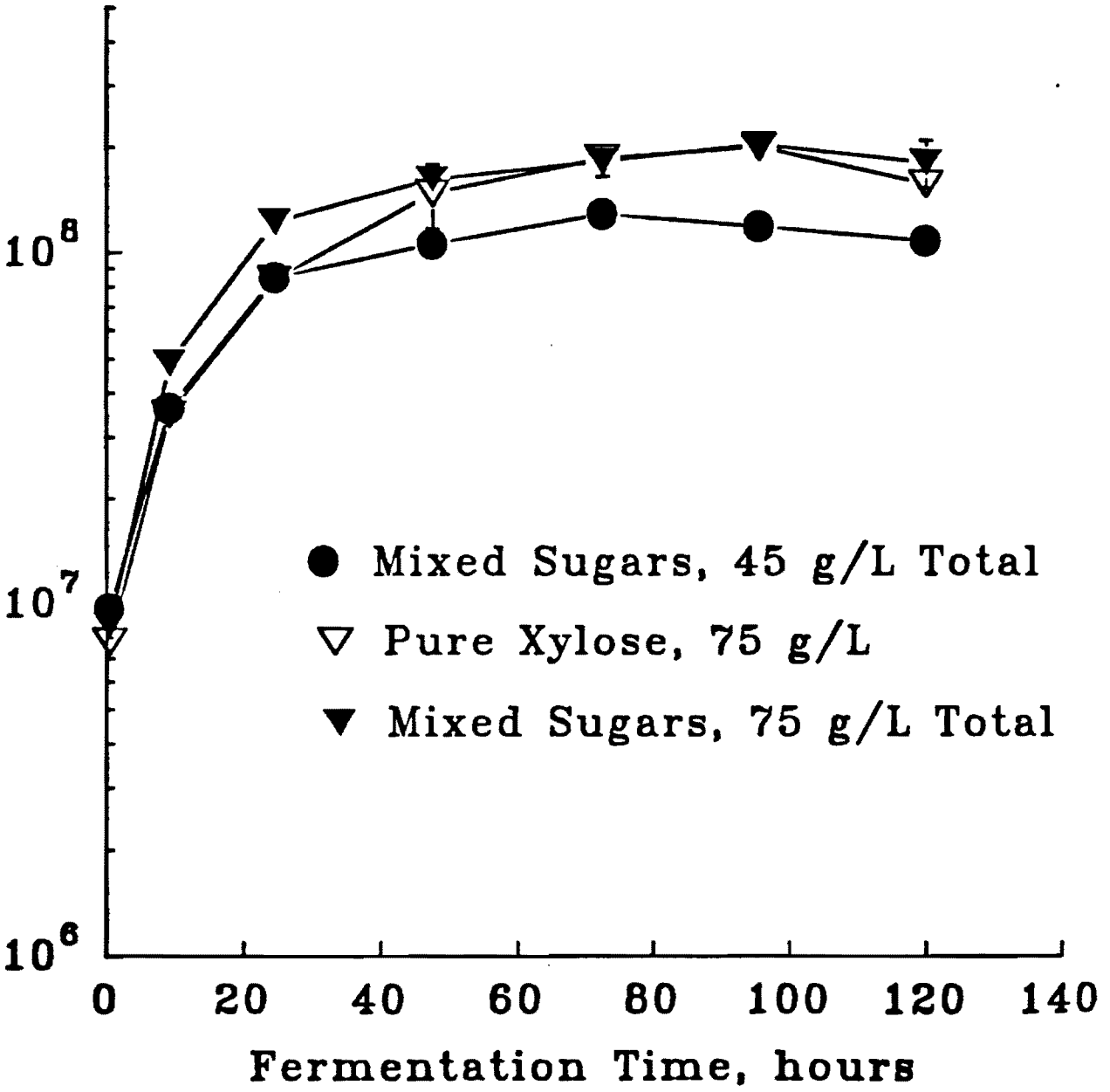


Figure 35 - Change in Cell Viability During D-Xylose and Mixed Sugar Fermentations.

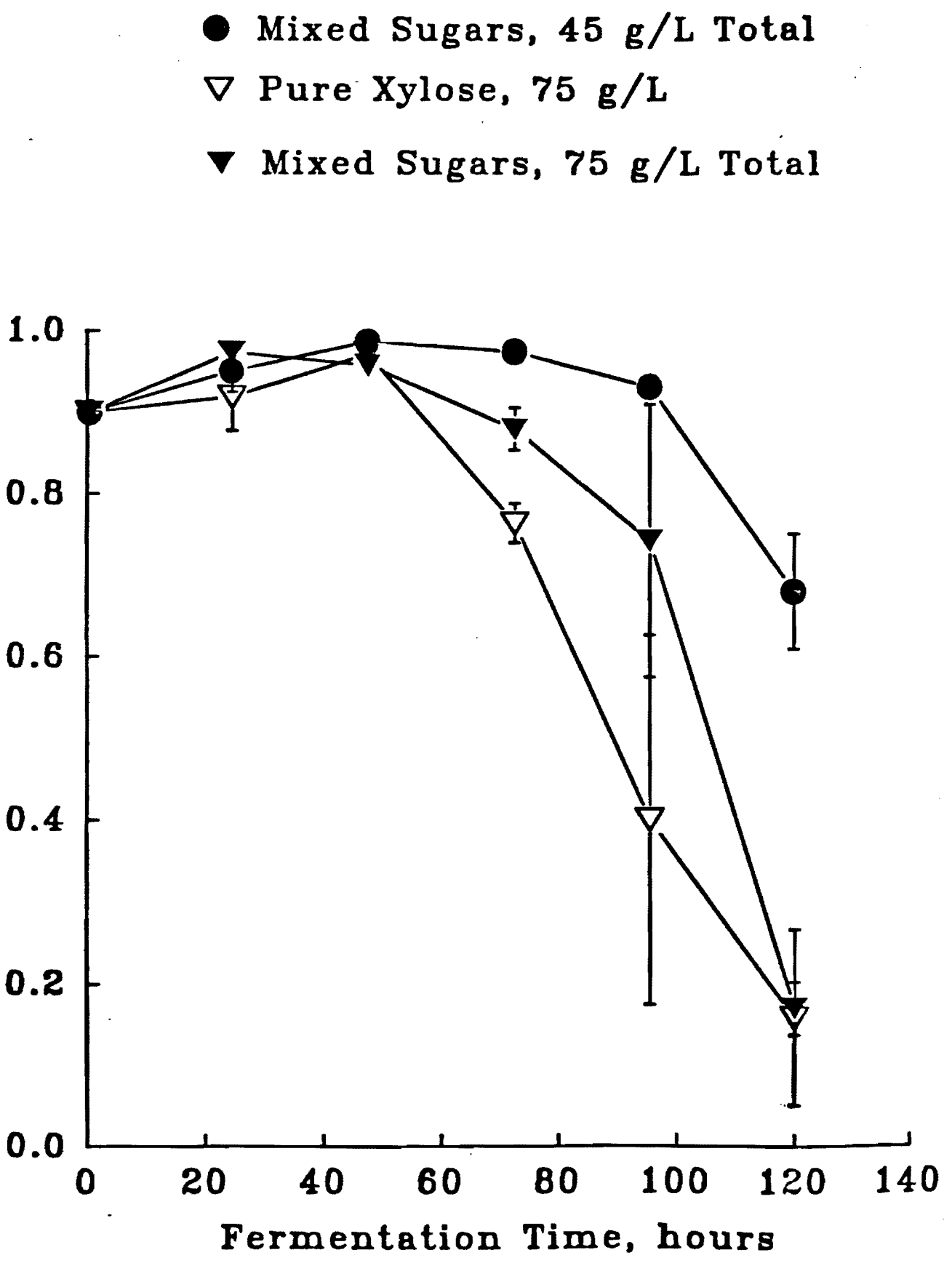


Figure 36 - Simultaneous Utilization of Glucose and Xylose in a Batch Reactor with 50% Glucose and D-Xylose (40 g/L Total) at Time Zero.

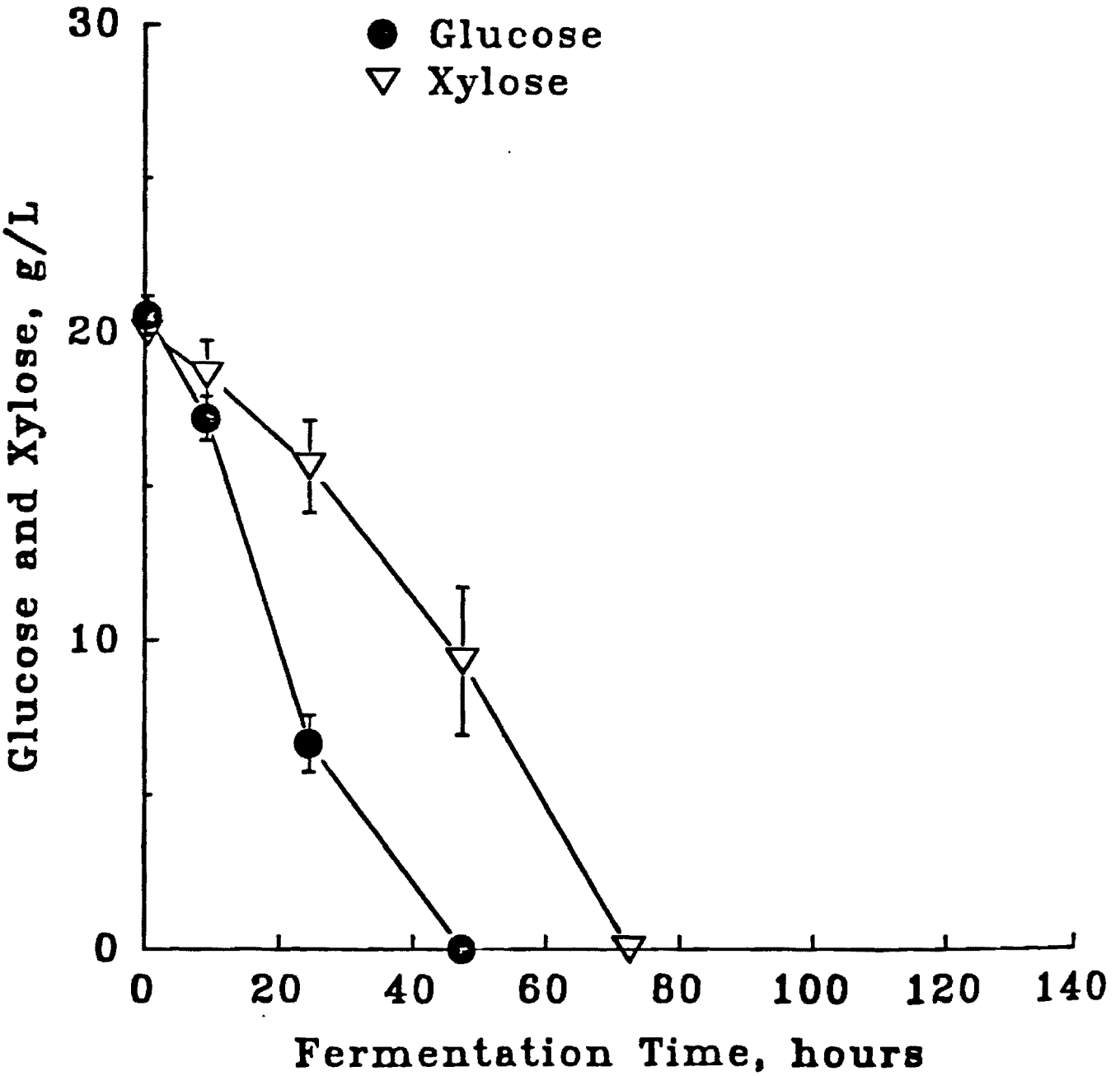


Figure 37 - Simultaneous Utilization of Glucose and Xylose in a Batch Reactor with 50% Glucose and D-Xylose (76 g/L Total) at Time Zero.

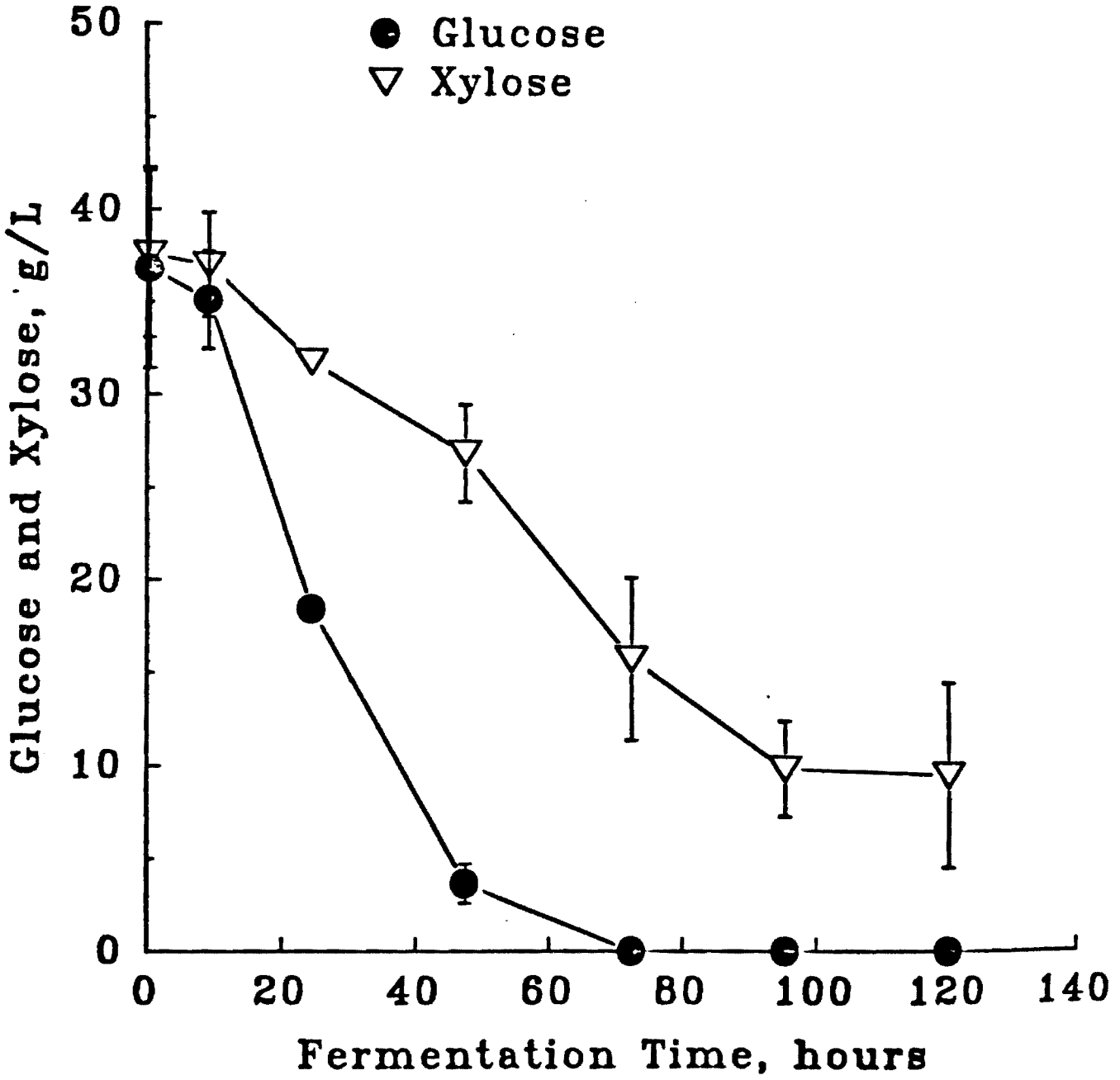


Figure 38 - Effect of Glucose Level on D-Xylose Uptake in a Mixed Sugar Fermentation.

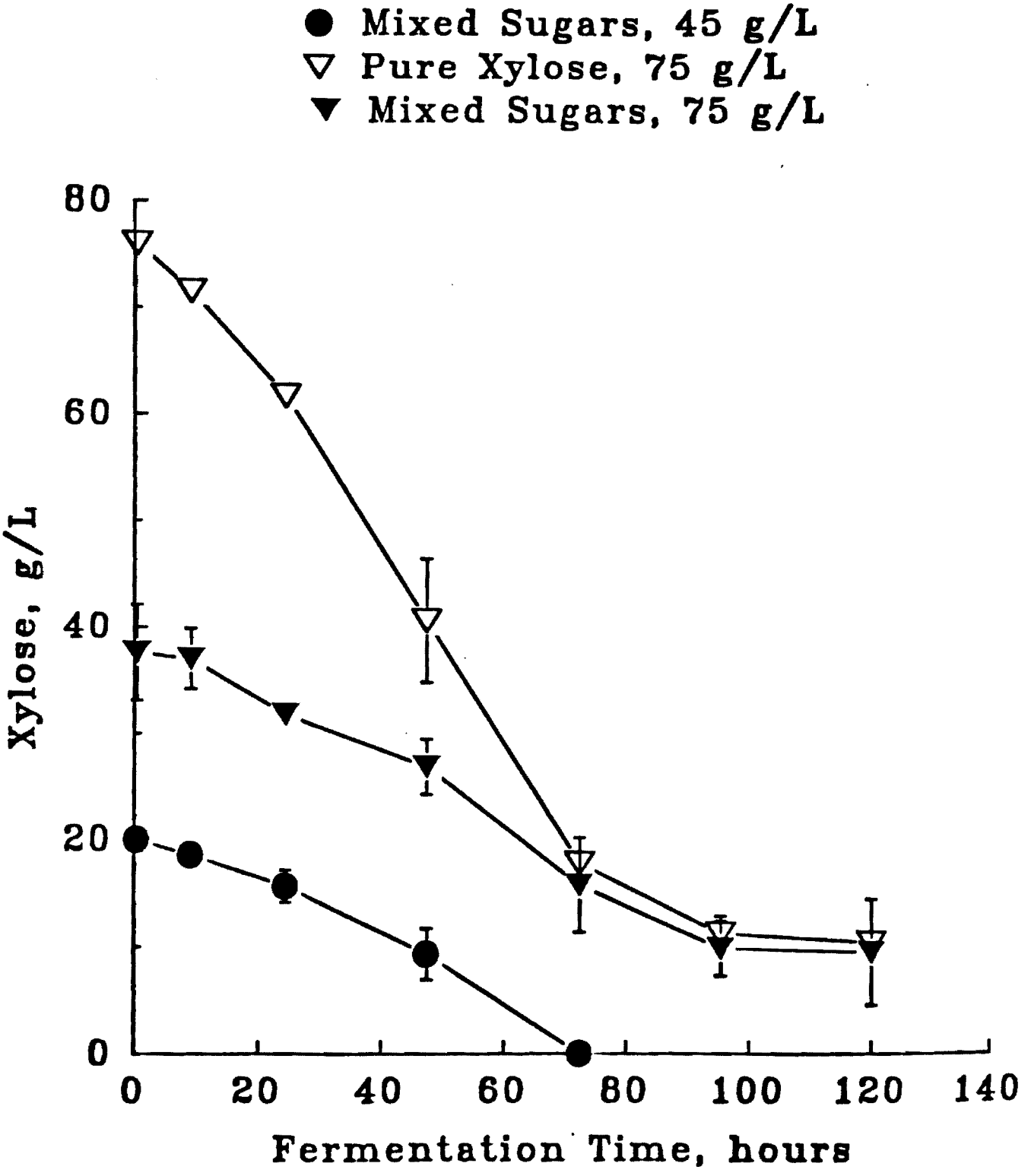


Figure 39 - Ethanol Production in Mixed Sugar and Pure D-Xylose Fermentations on a Defined Medium.

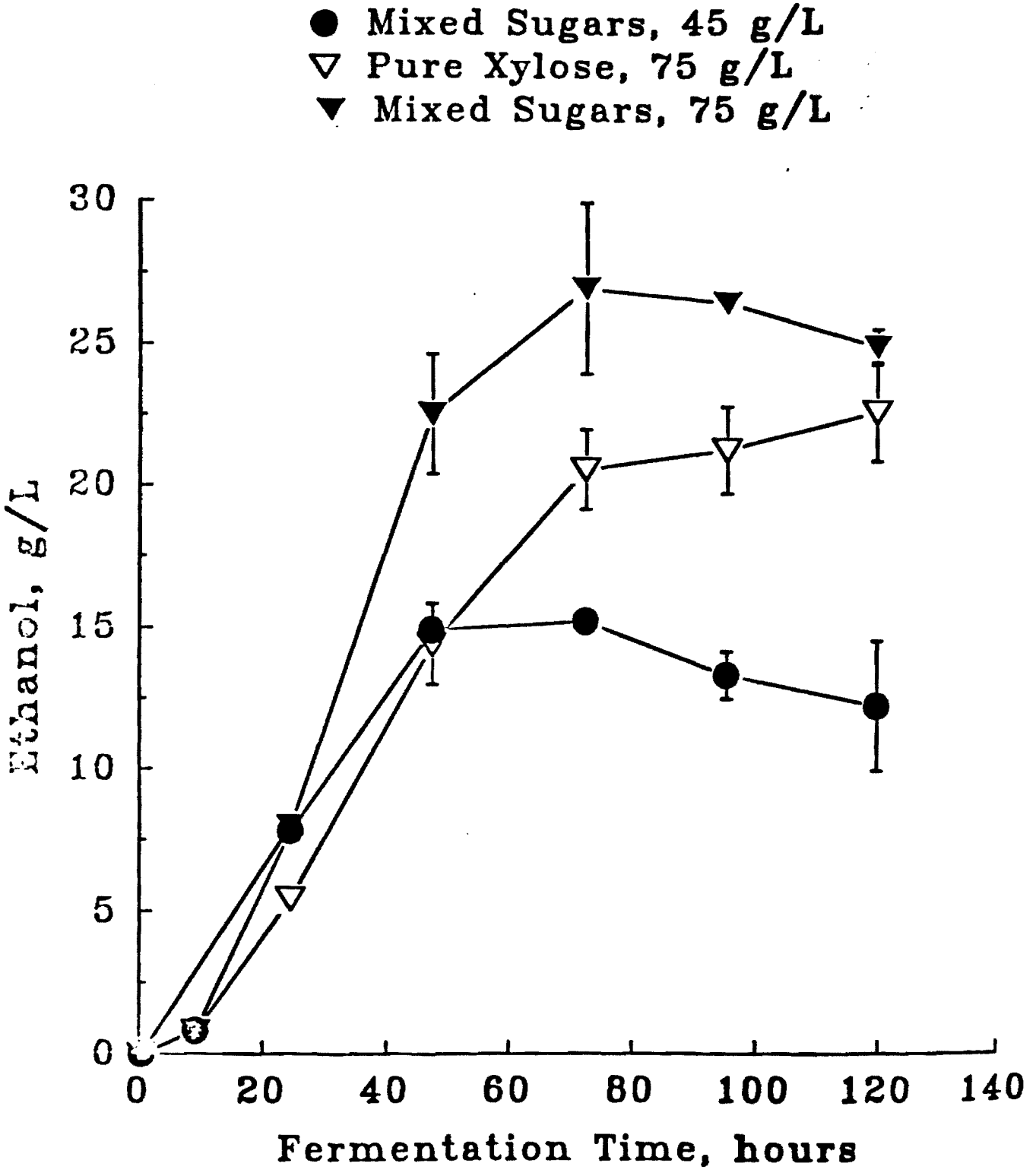
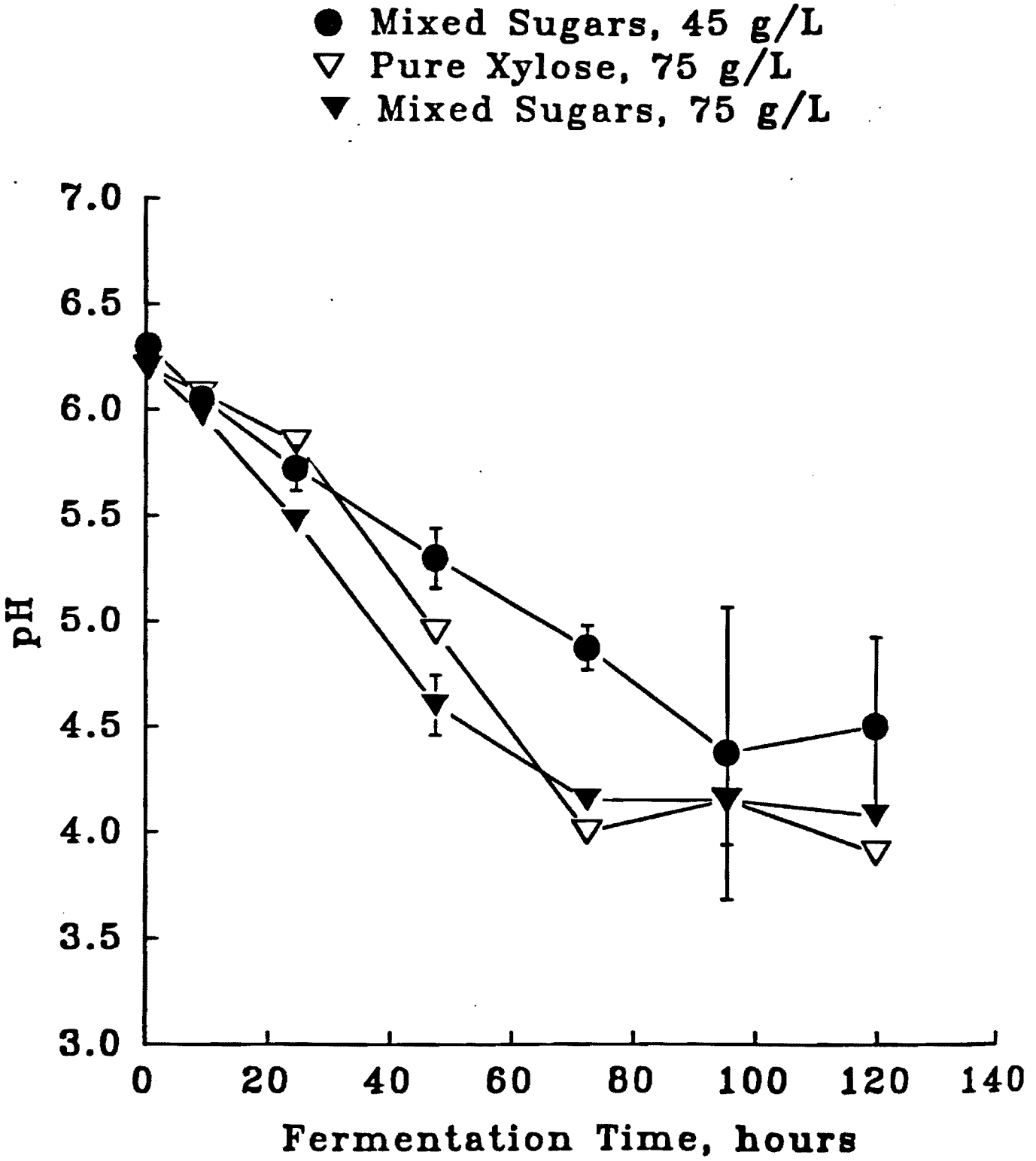


Figure 40 - Change in pH During Mixed Sugar and Pure D-Xylose Fermentations on a Defined Medium.



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.

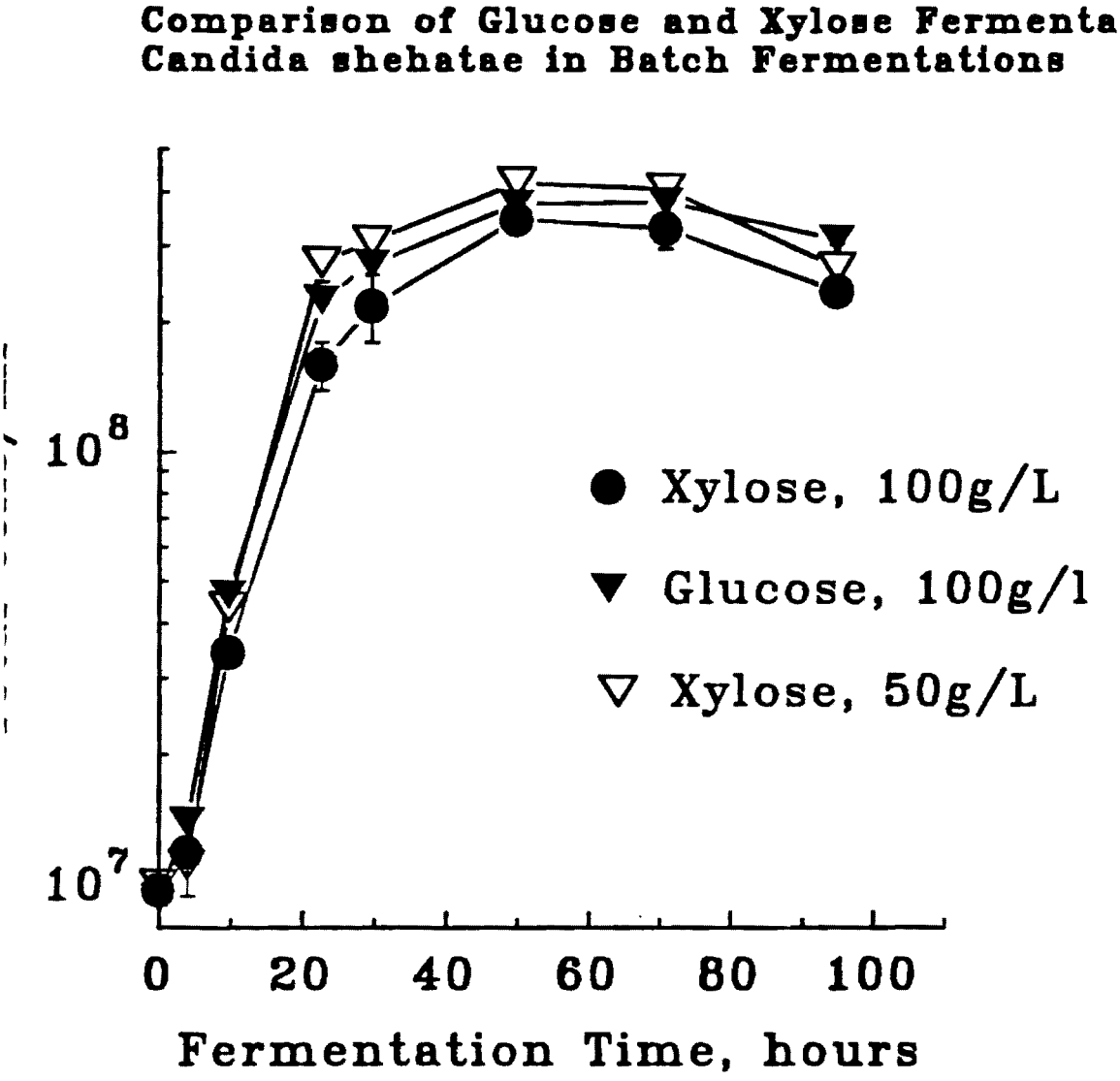


Figure 42 - Change Cell Viability as Measured by Methylene Blue Staining During Glucose and D-Xylose Fermentations by *Candida shehatae*.

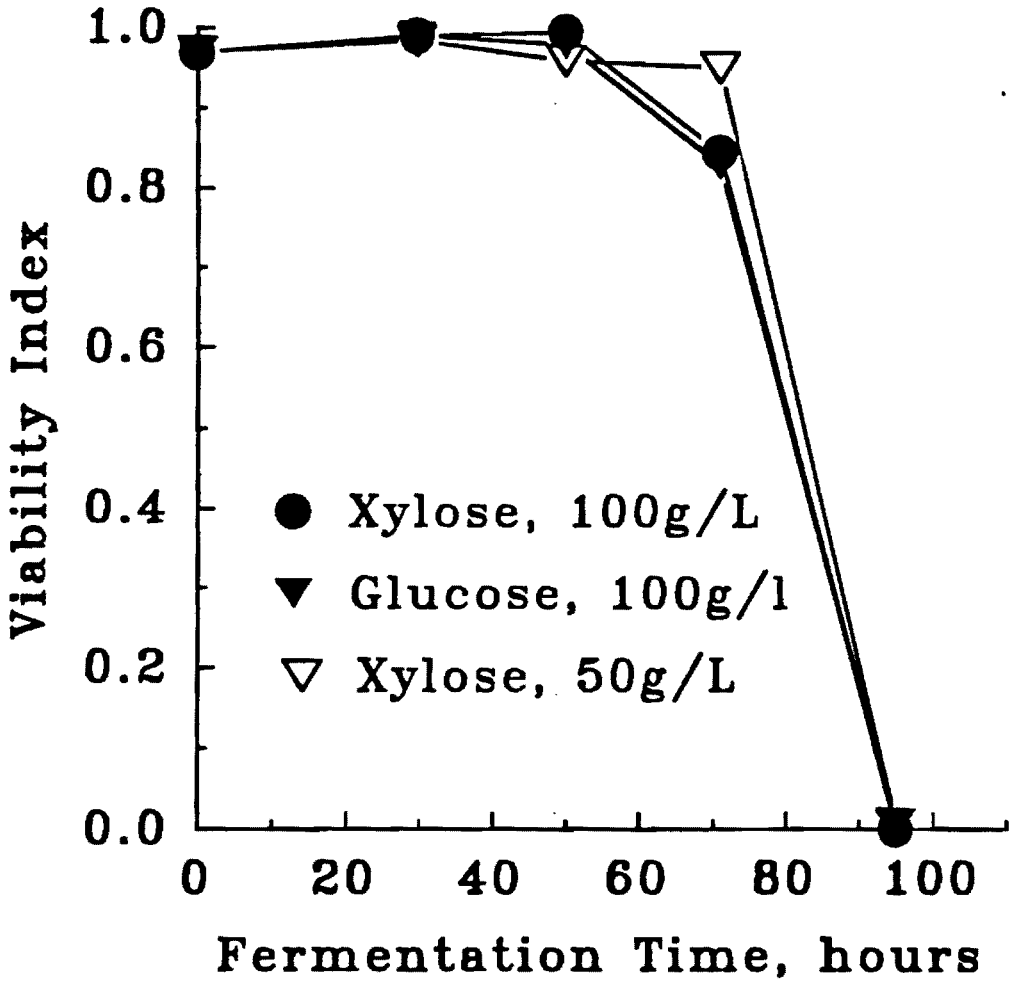
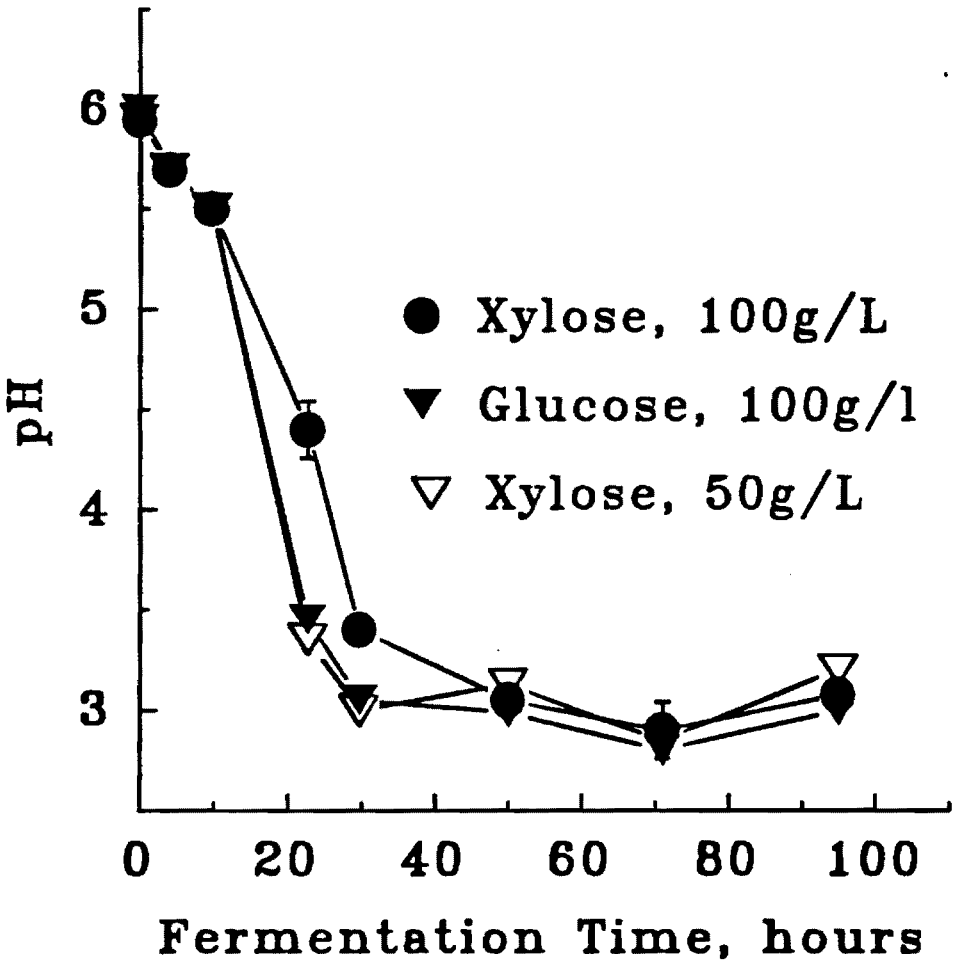


Figure 43 - Change in pH During Glucose and D-Xylose Fermentations by *Candida shehatae* in Shaker Flasks.



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.

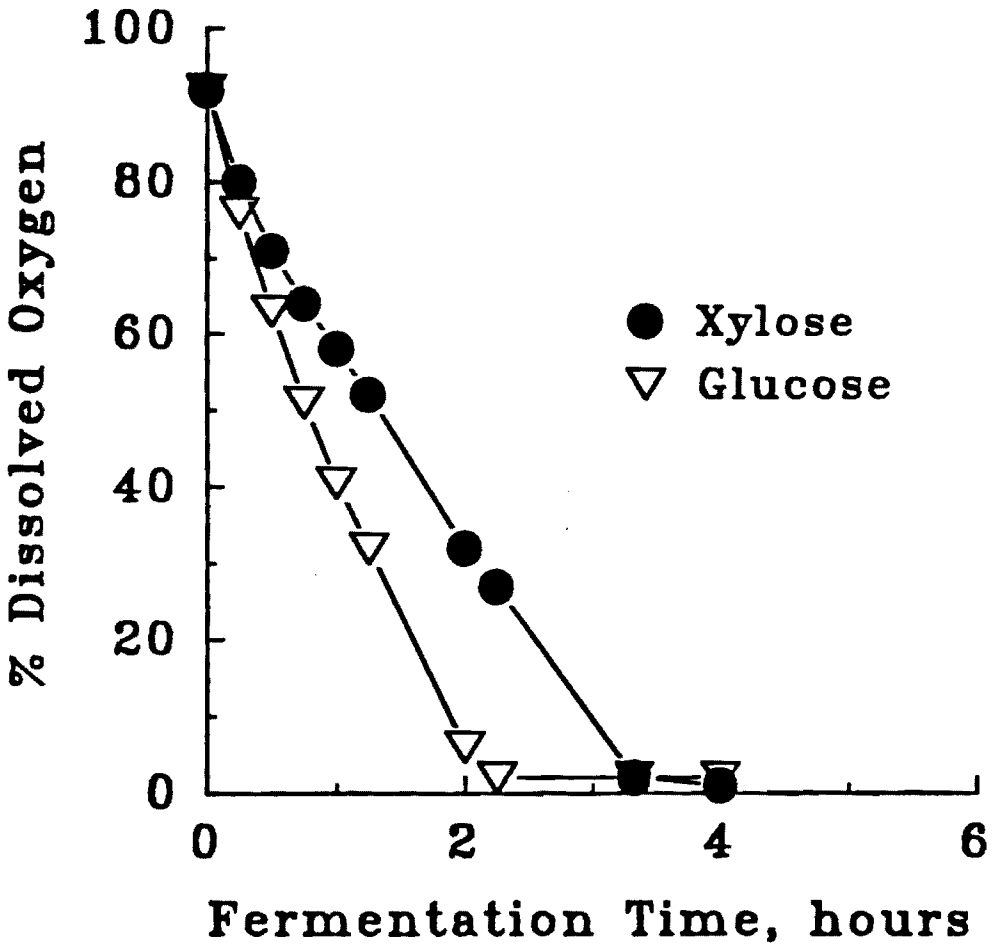


Figure 45 - Effect of Oxygen Limitation on the Growth of *Candida shehatae* on Glucose and D-Xylose.

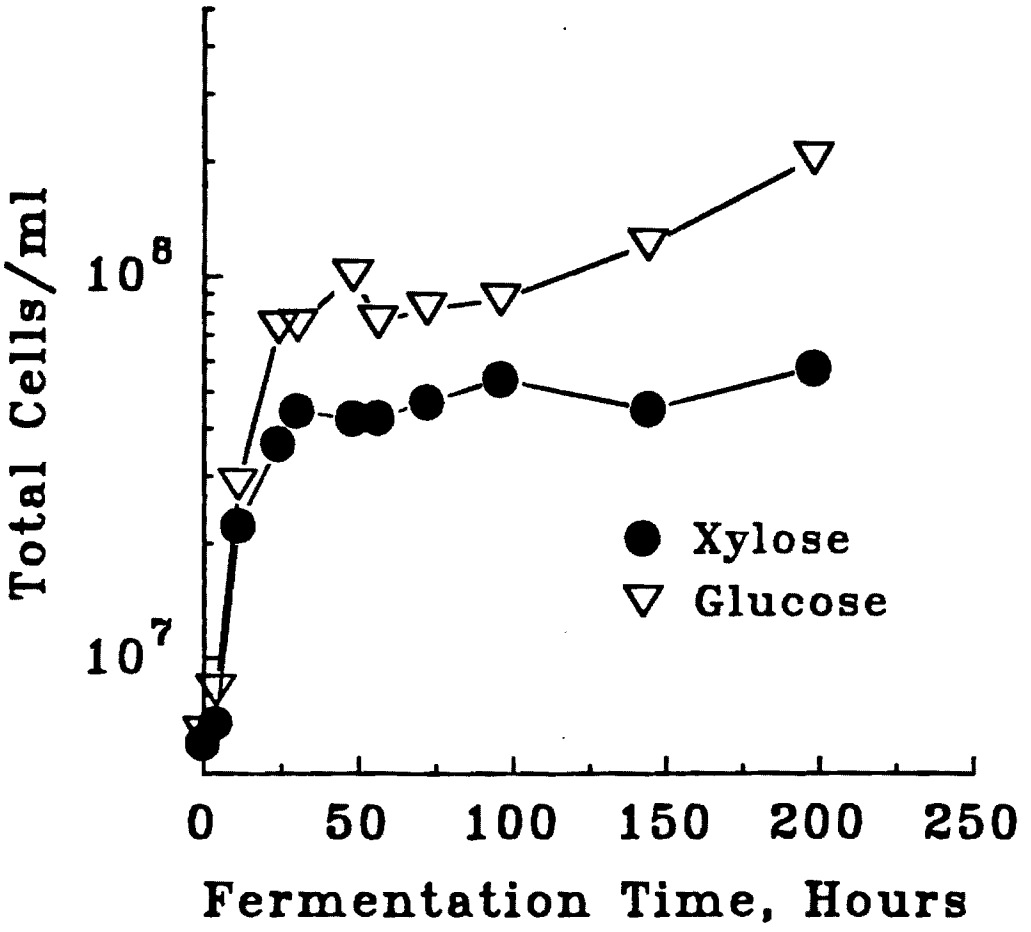
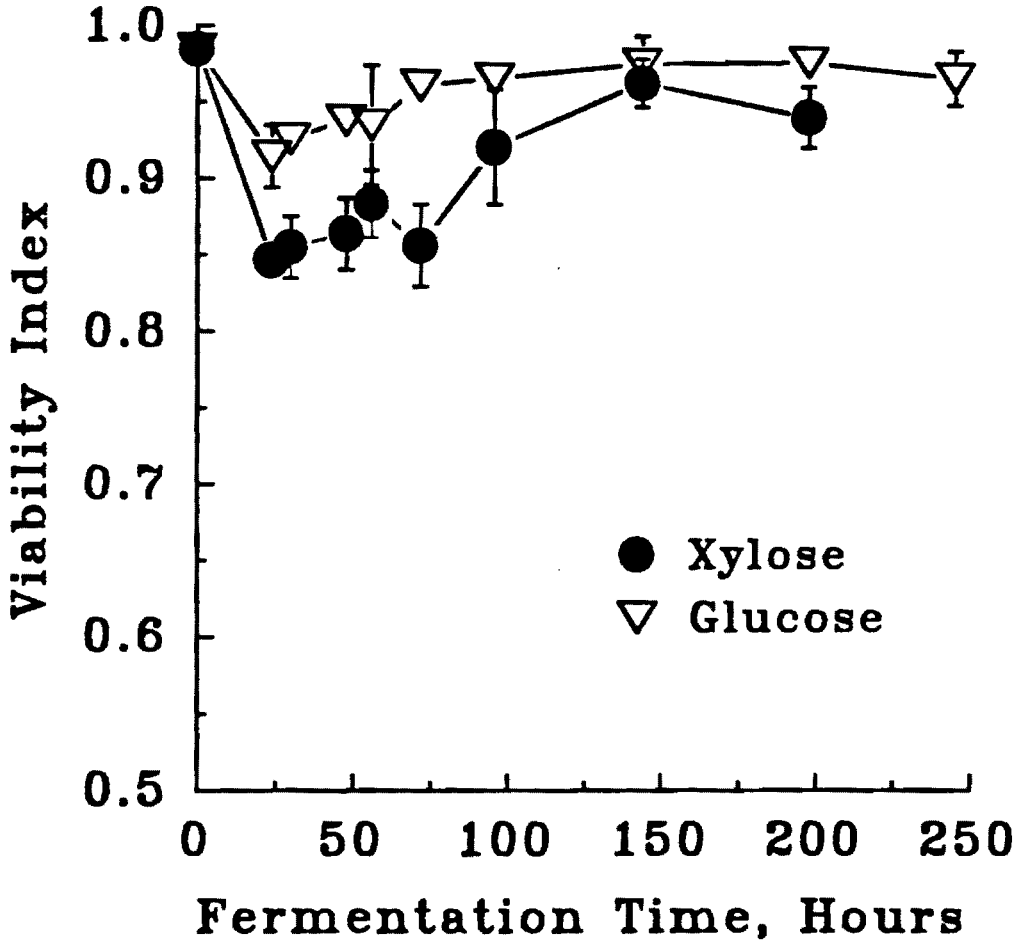


Figure 46 - Effect of Oxygen Limitation on the Viability of *Candida shehatae* on Glucose and D-Xylose.



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*.

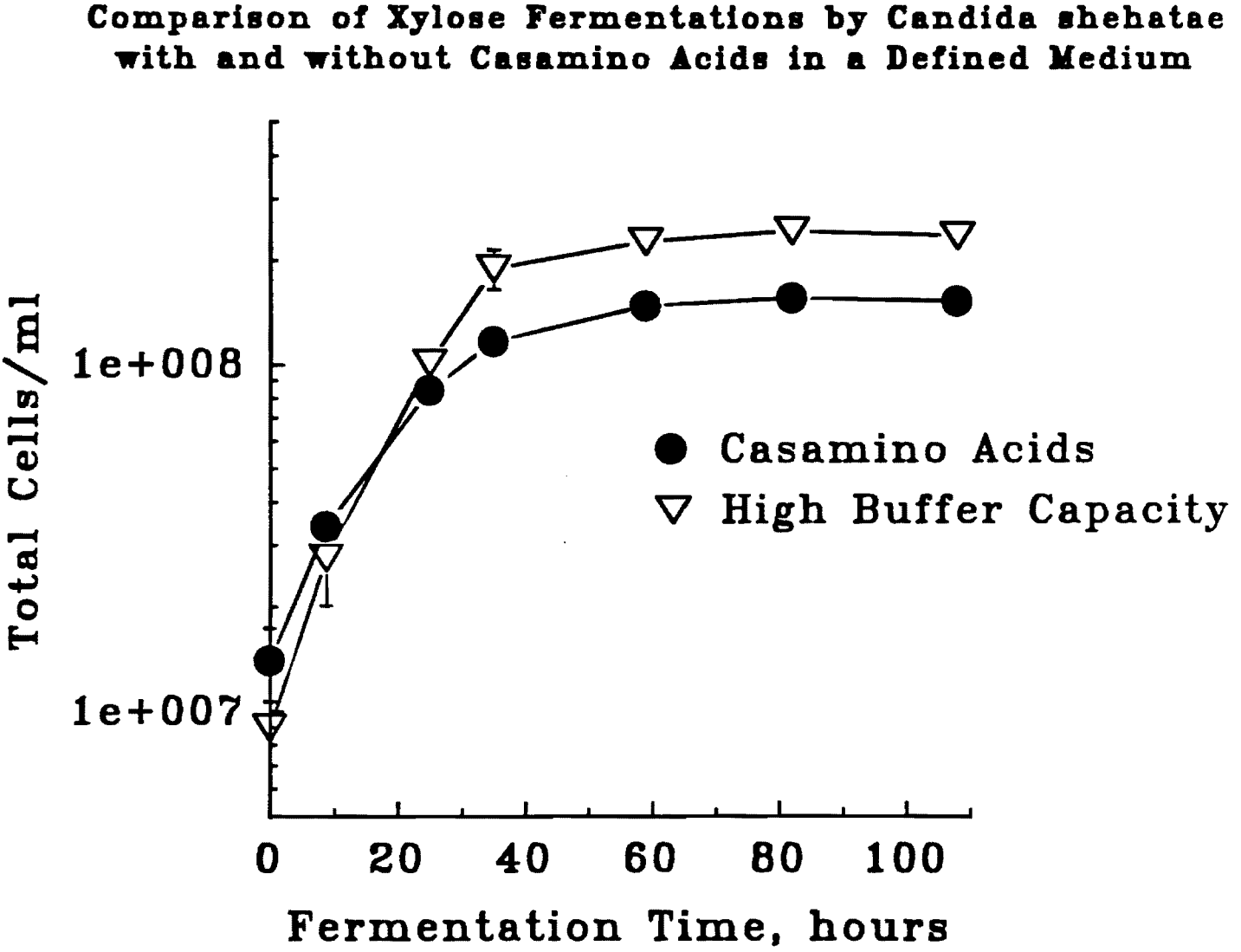


Figure 48 - Effect of Casamino Acids on D-Xylose Uptake.

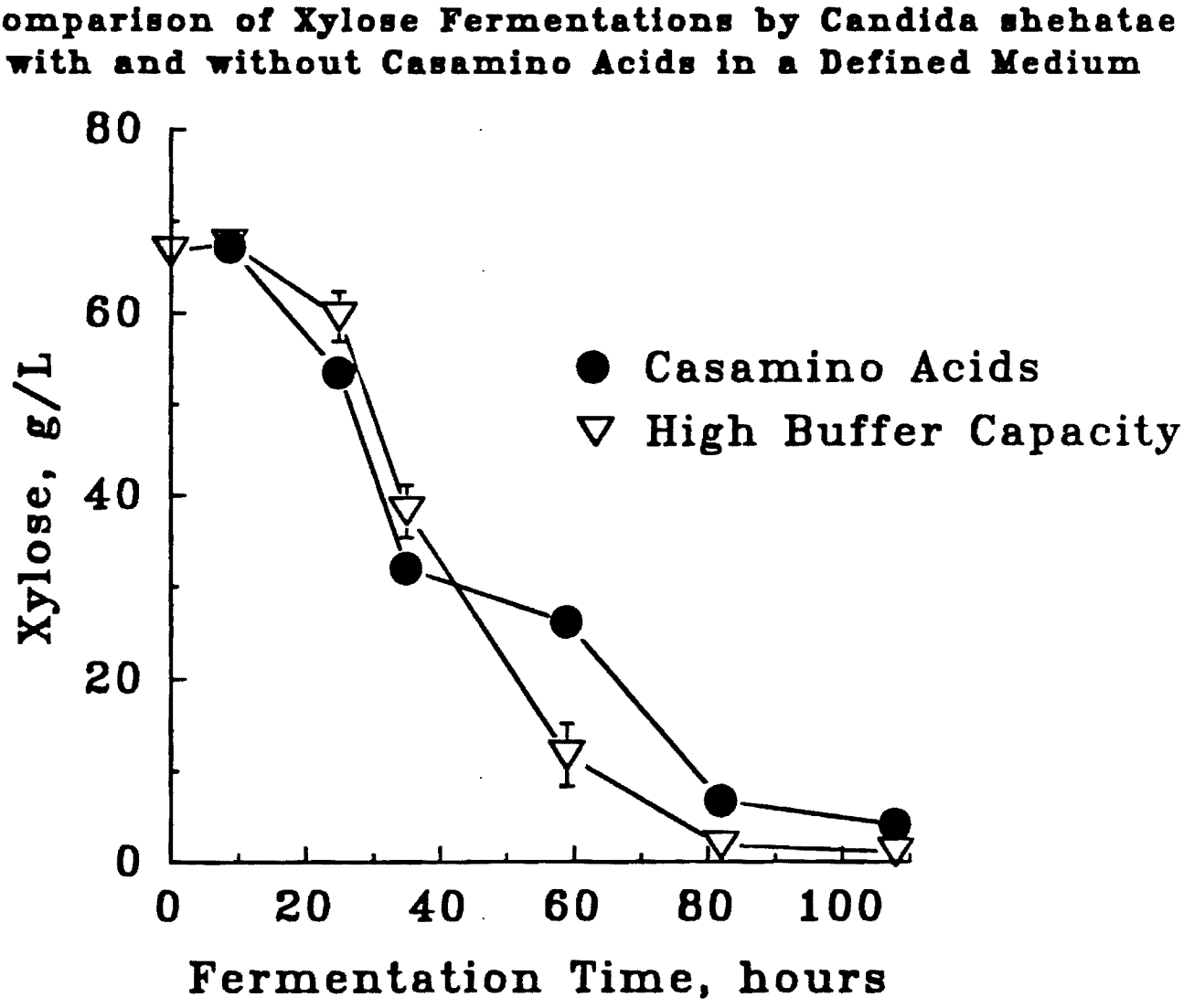


Figure 49 - Effect of Casamino Acids on Xylitol Formation.

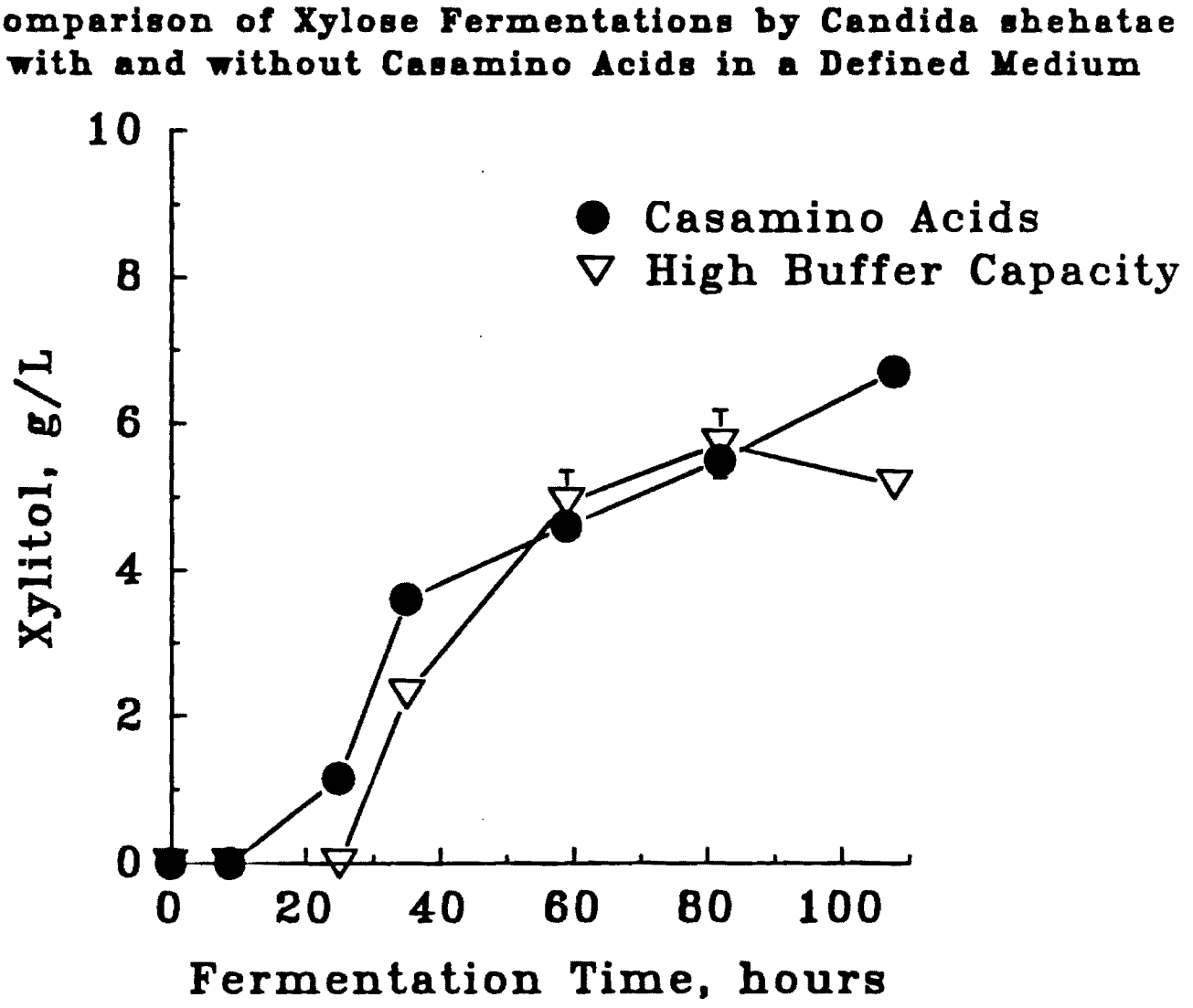


Figure 50 - Effect of Casamino Acids on Cell Viability.

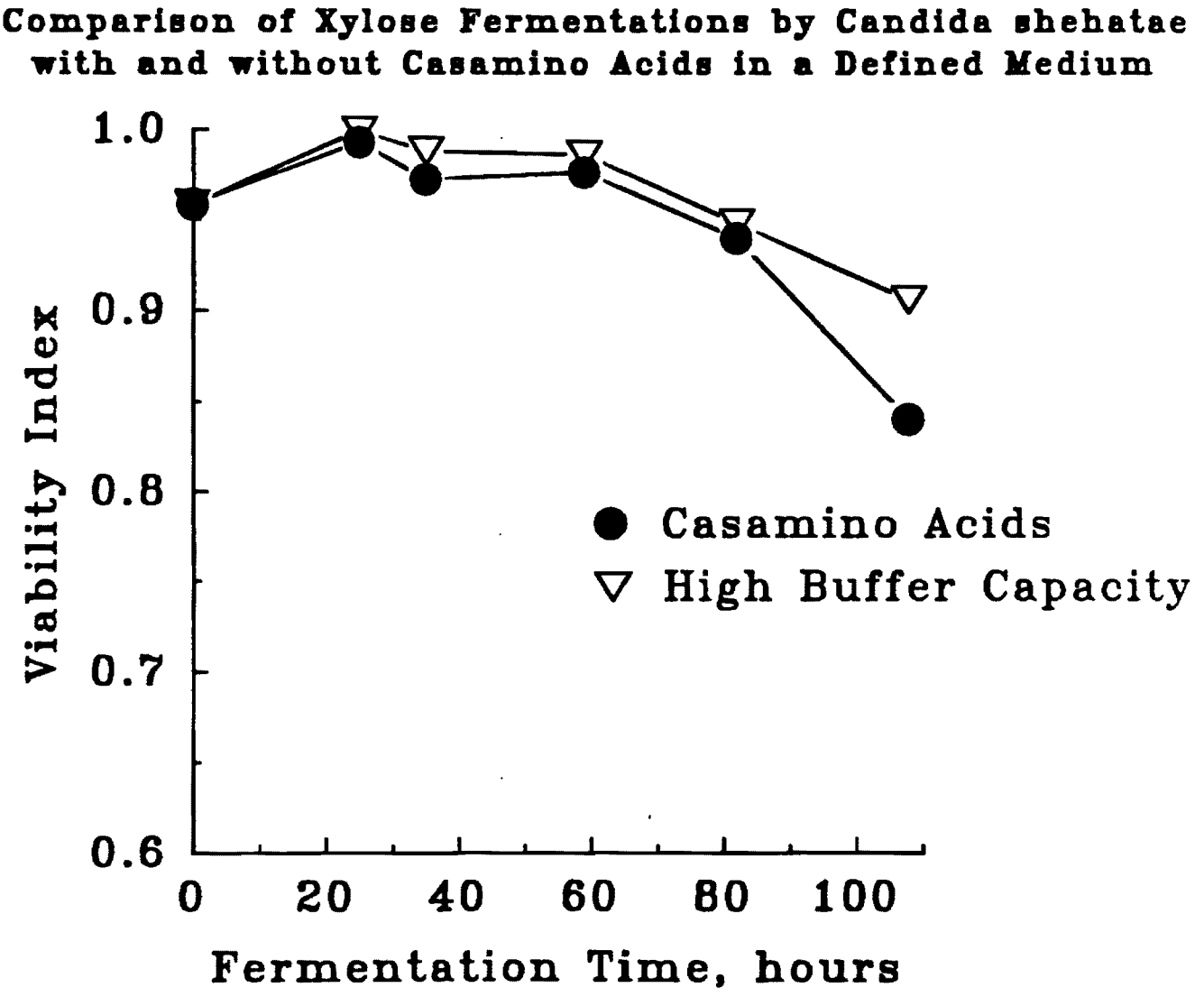


Figure 51 - pH Change In Defined Medium with Casamino Acids and a High Buffering Capacity (10 g/L K_2HPO_4 and 10 g/L of KH_2PO_4).

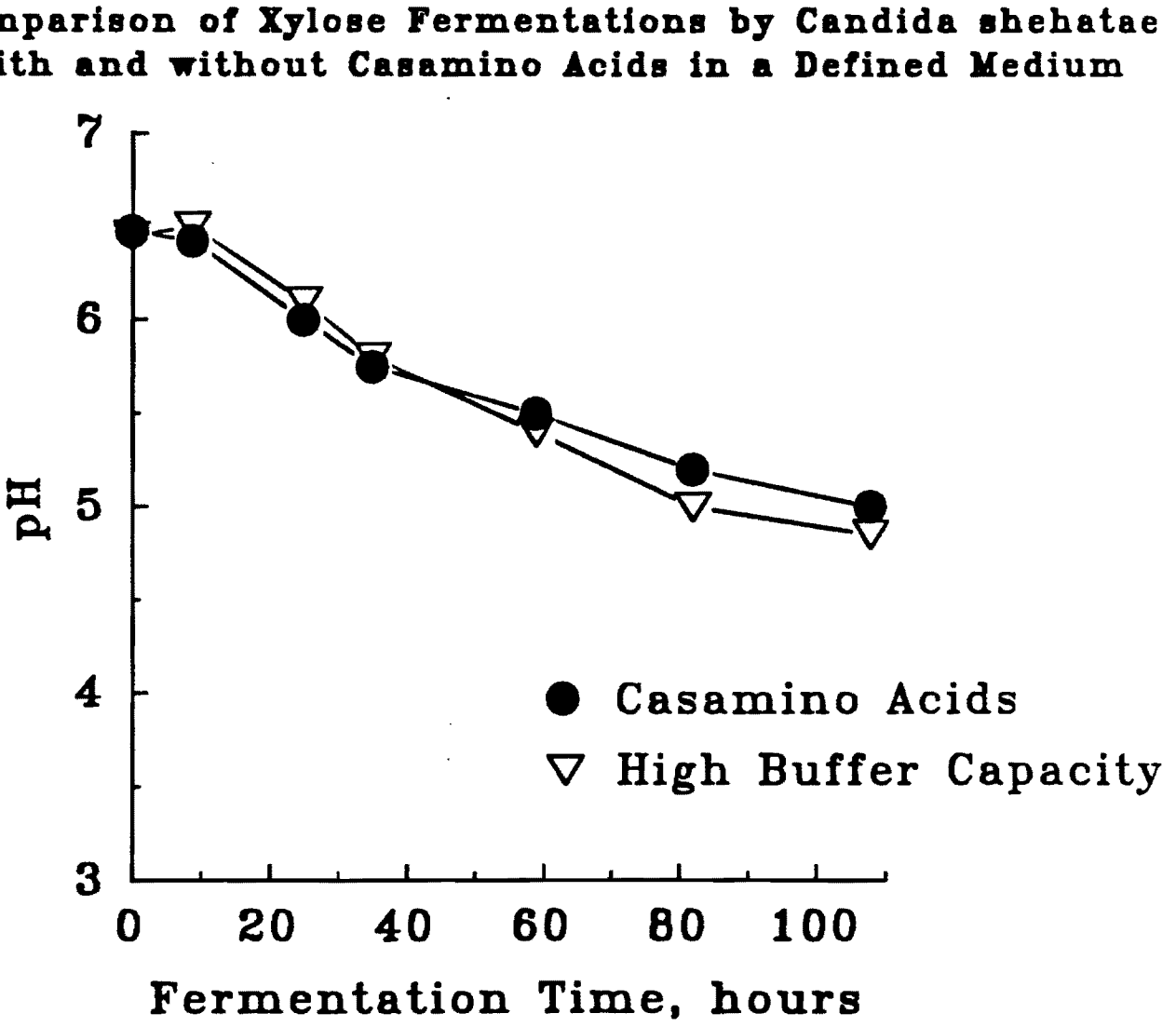


Figure 52 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and 5 g/L KH_2PO_4) on Cell Growth.

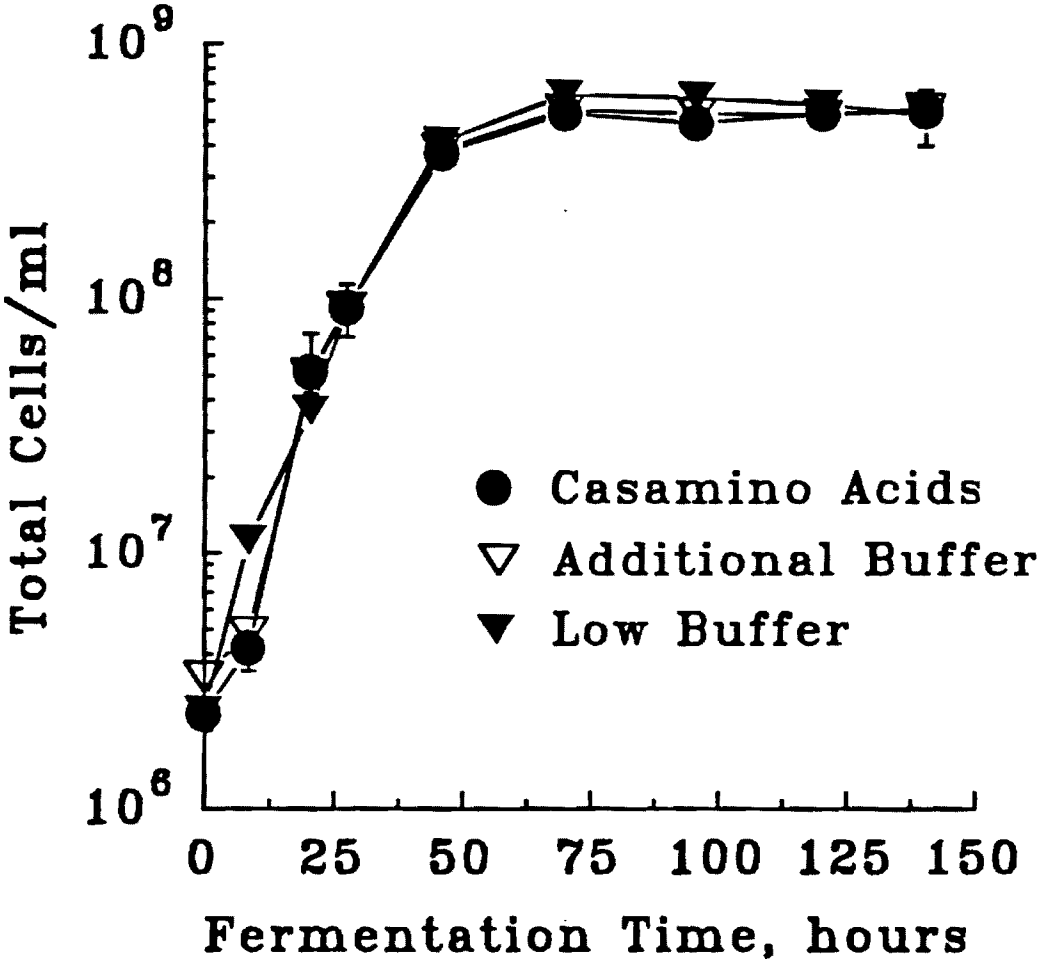


Figure 53 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and KH_2PO_4) on Cell Viability.

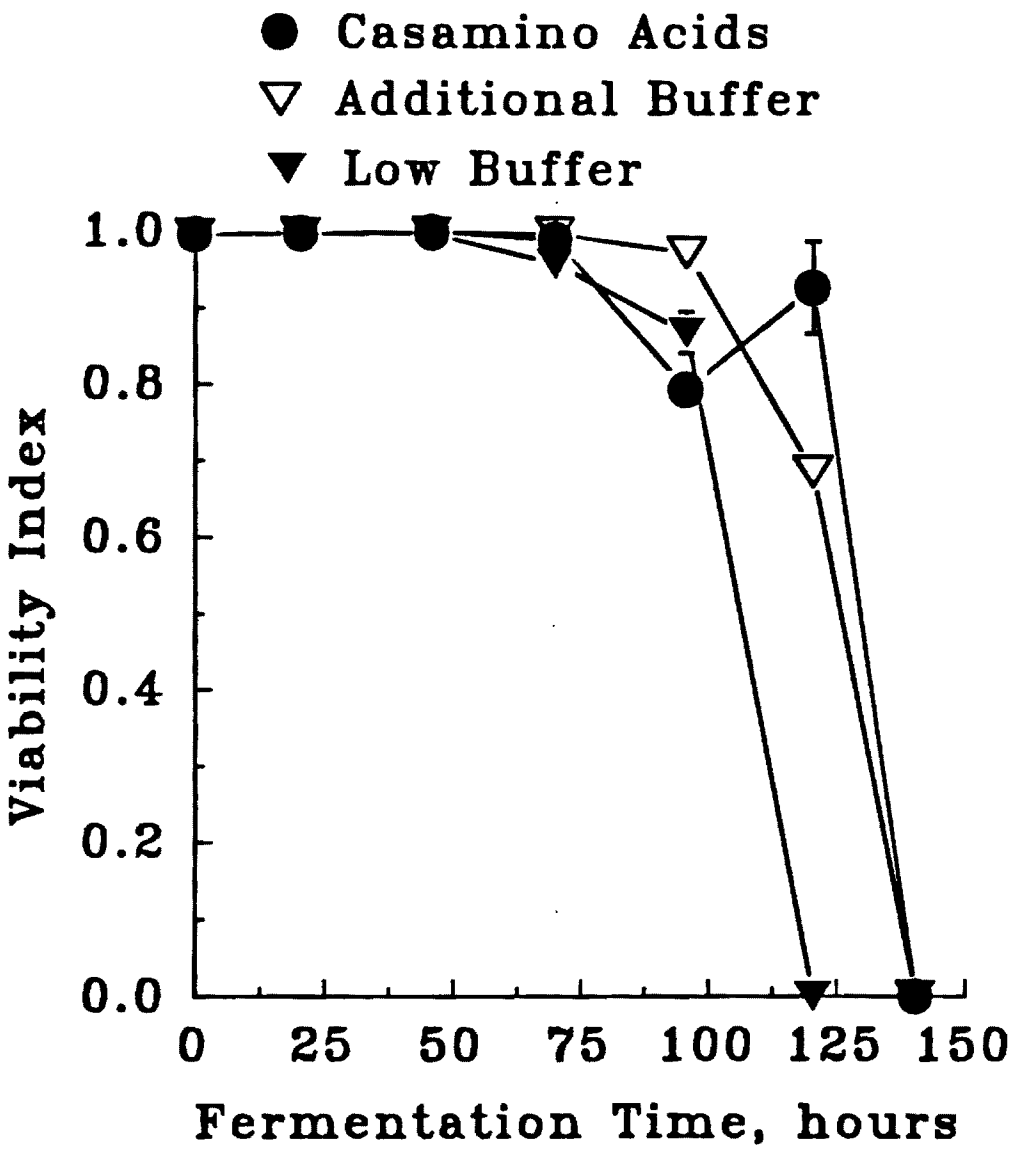
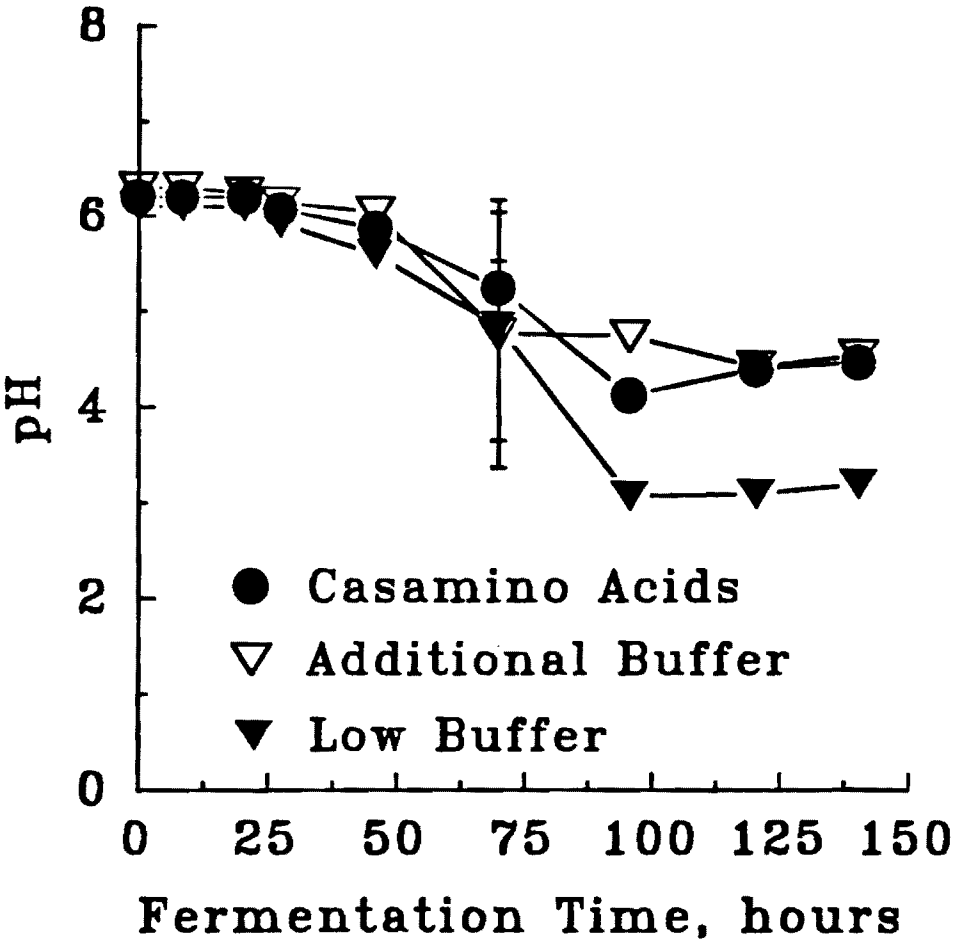


Figure 54 - Effect of Low Buffering Capacity (5 g/L of K_2HPO_4 and KH_2PO_4) on pH During D-Xylose Fermentations.



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*.

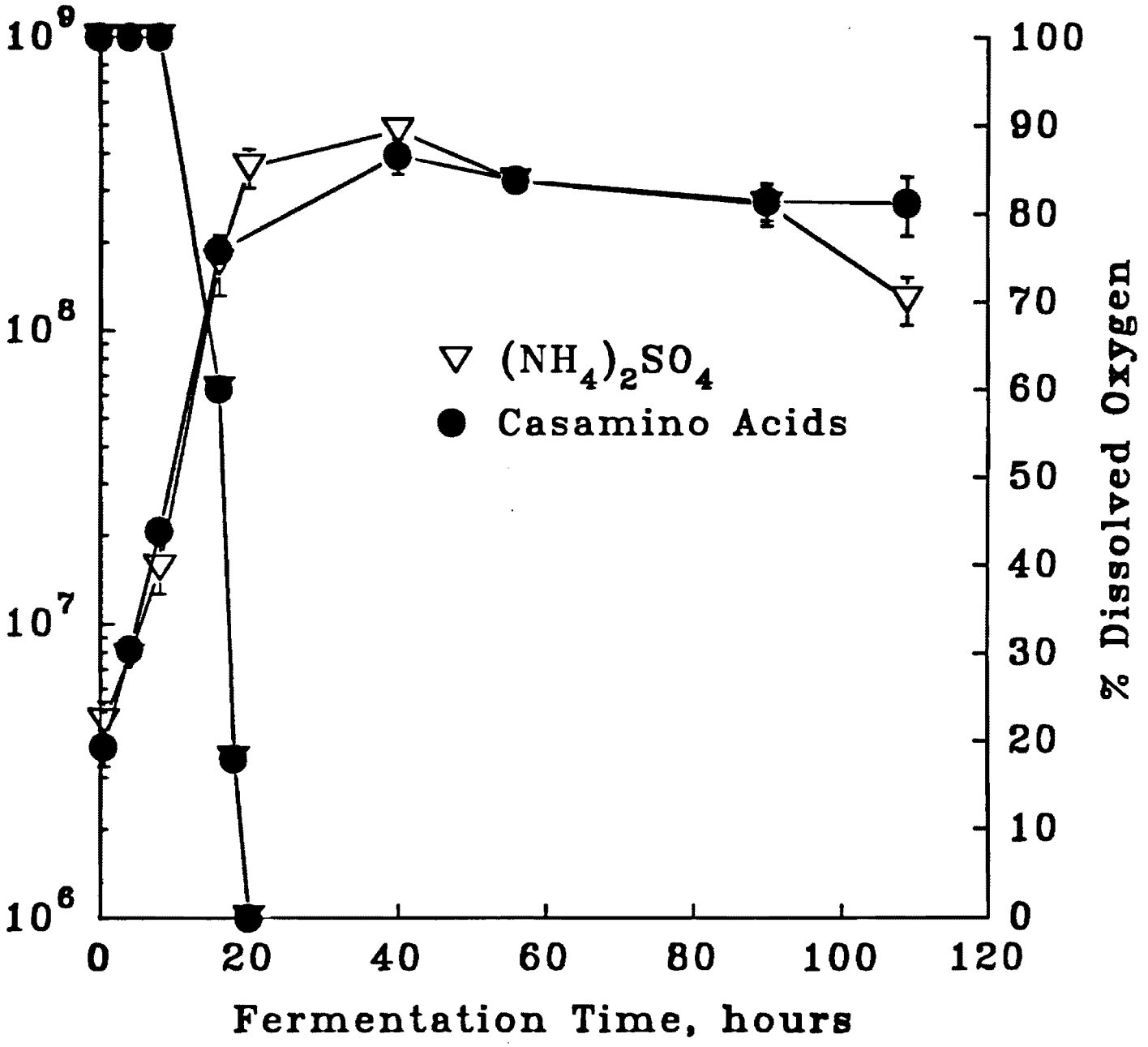


Figure 56 - D-Xylose Levels During a Fed-Batch Fermentation.

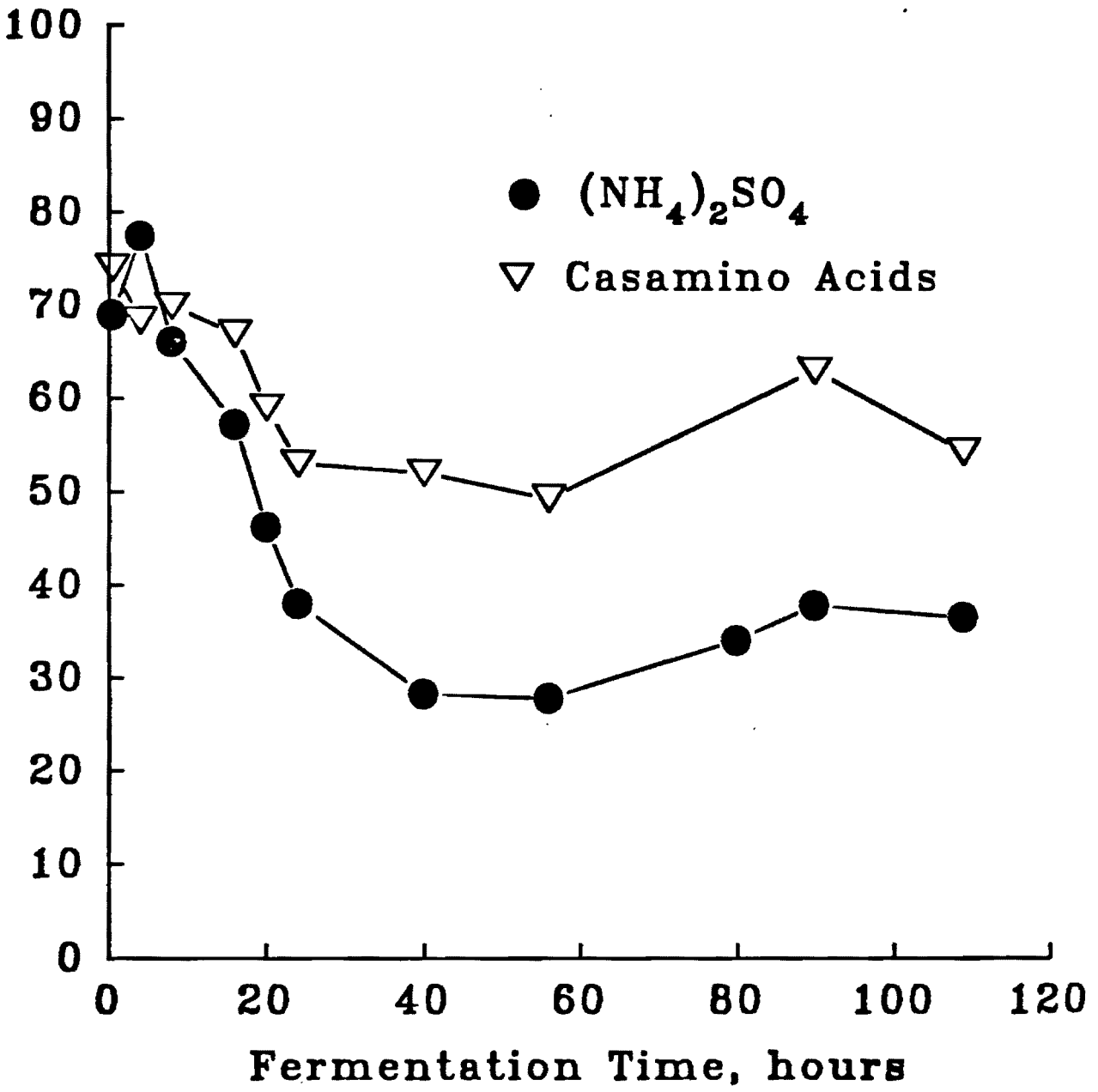


Figure 57 - Ethanol and Xylitol Production in Fed-Batch Fermentation with $(NH_4)_2SO_4$ as the Nitrogen Source.

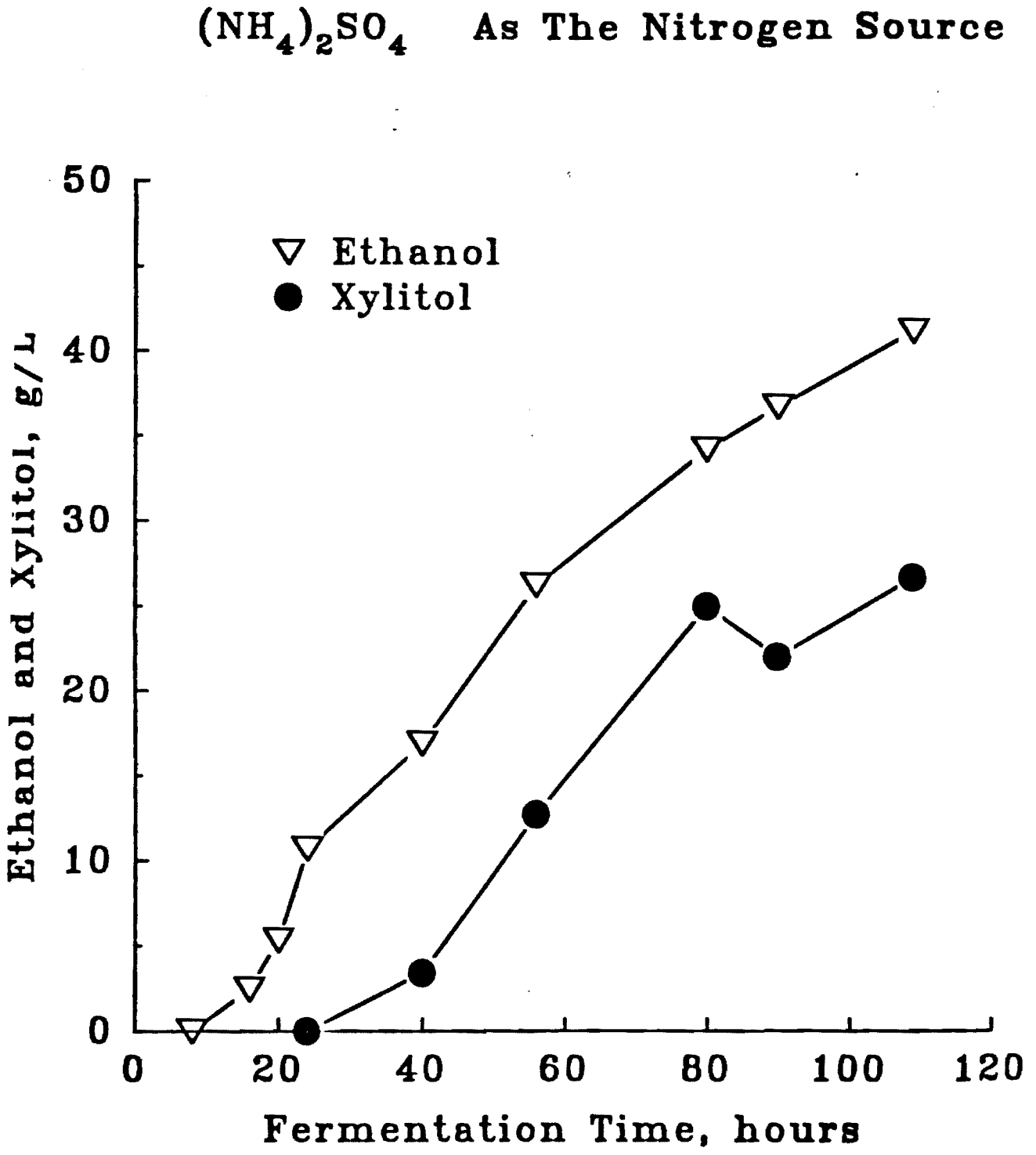


Figure 58 - Ethanol and Xylitol Production in Fed-Batch Fermentation with Casamino Acids as the Nitrogen Source.

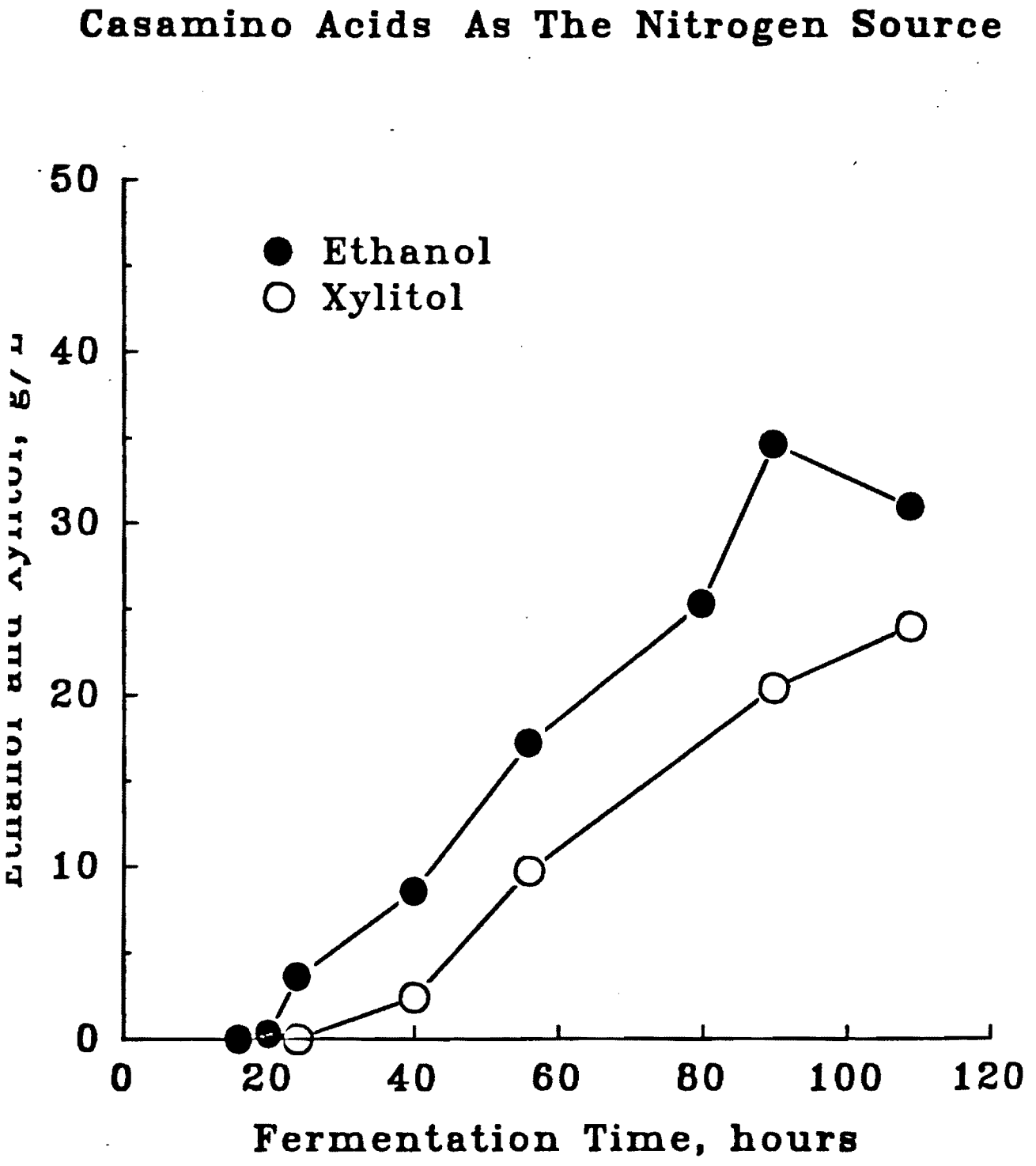


Figure 59 - Effect of $(NH_4)_2SO_4$ as the Nitrogen Source on Cell Viability.

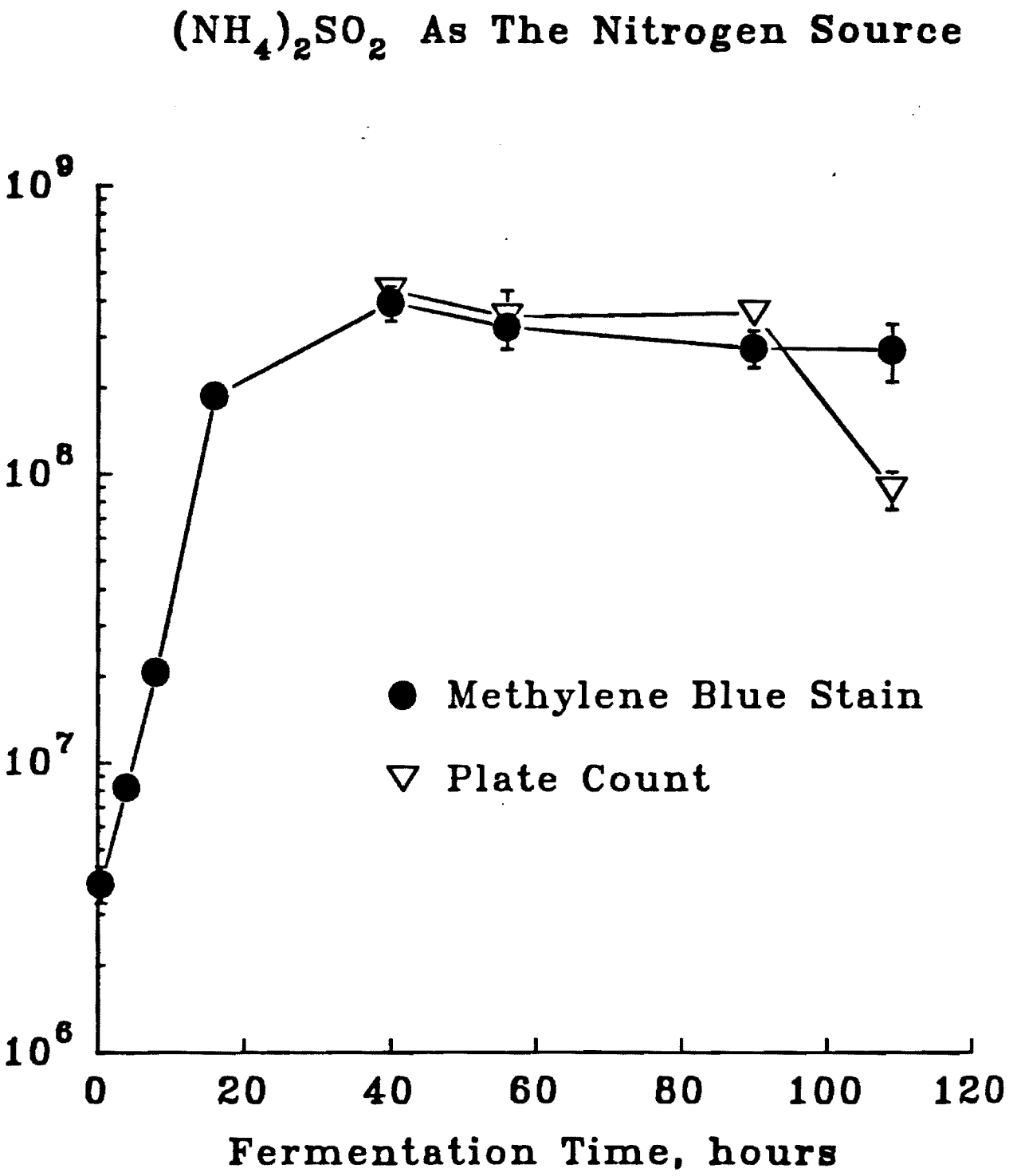
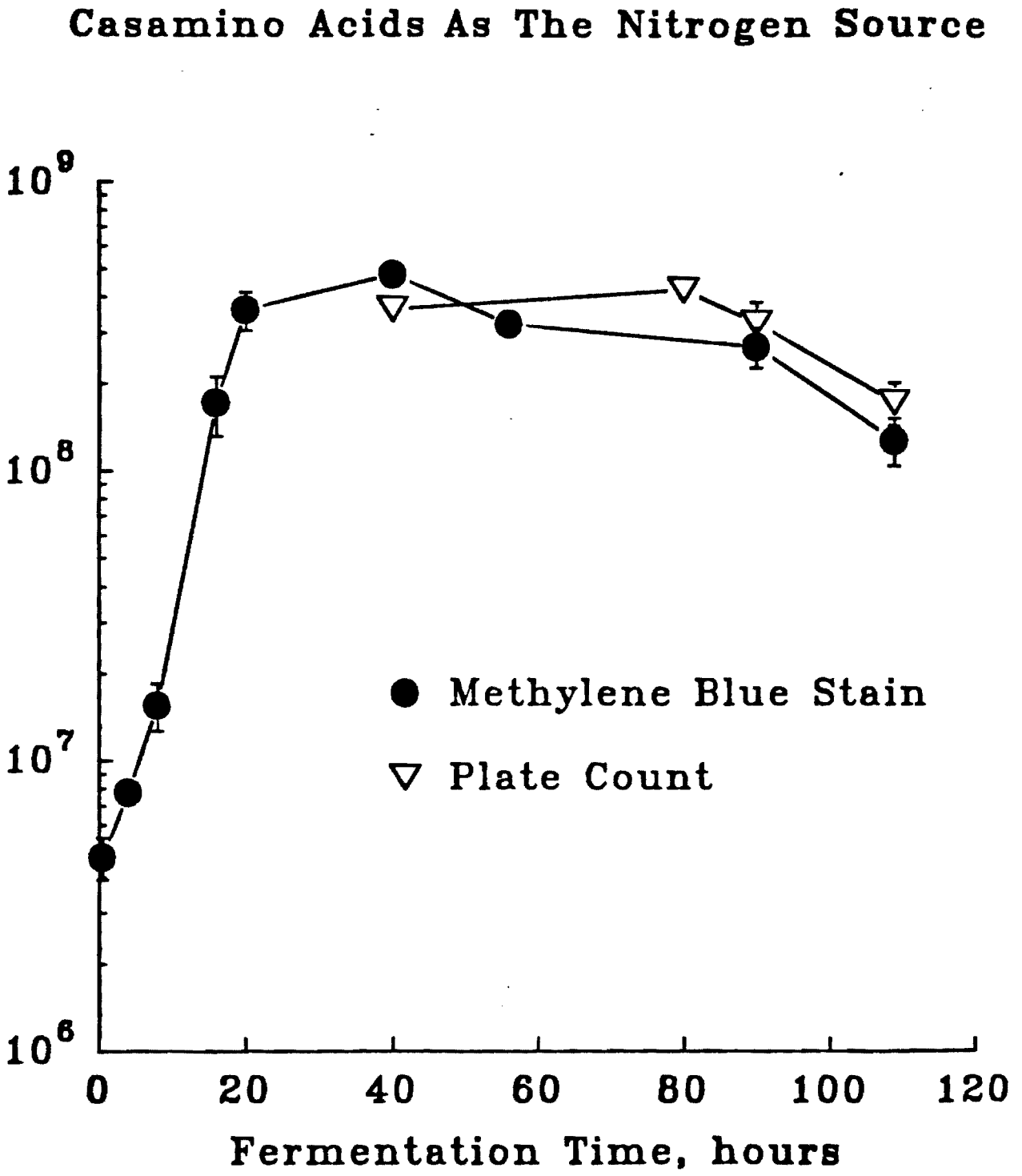


Figure 60 - Effect of Casamino Acids as the Nitrogen Source on Cell Viability.



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 x 10^8 total cells/ml compared to 1.5 x 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.

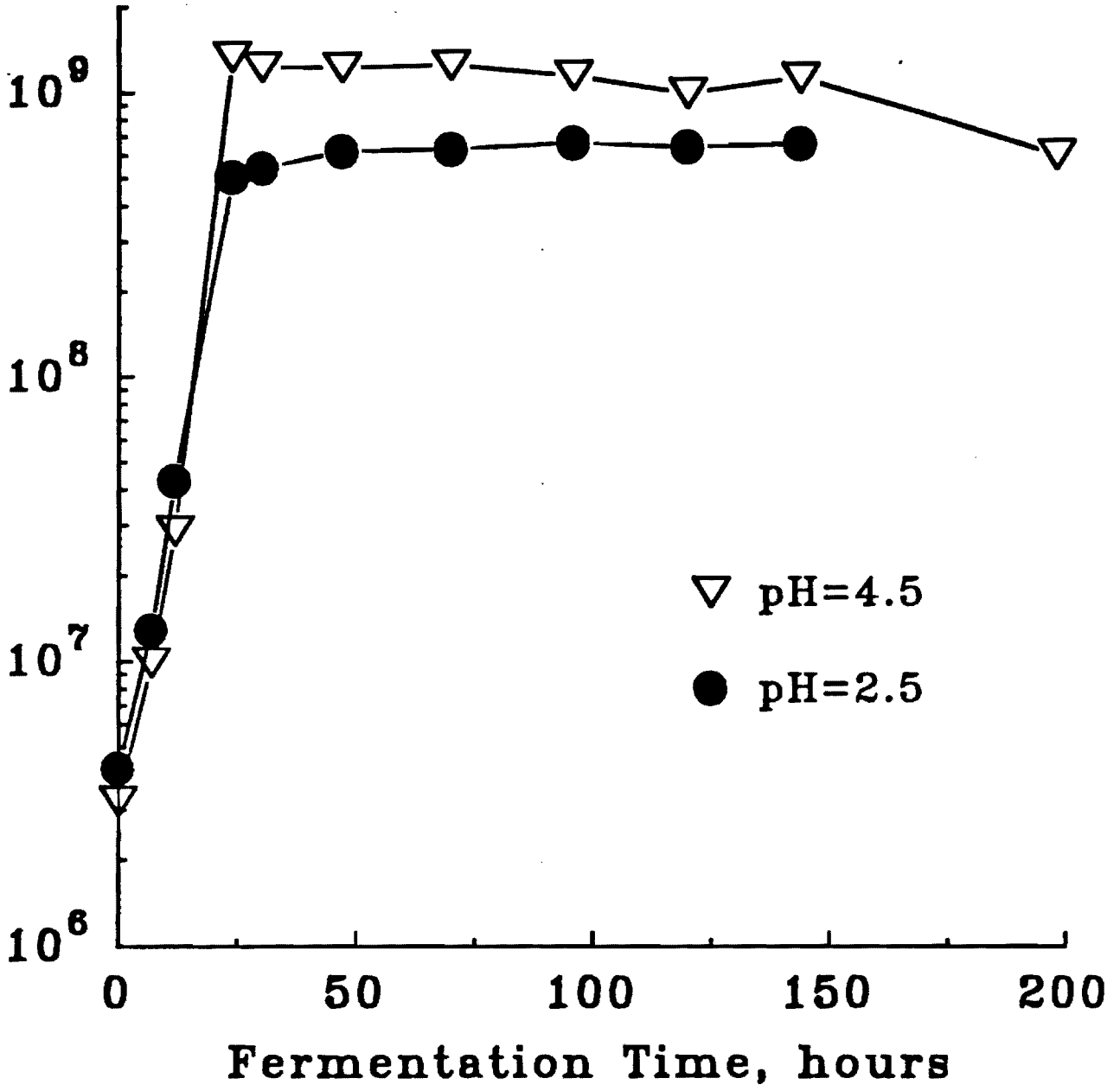


Figure 62 - pH Levels in Fed-Batch D-Xylose Fermentation by *Candida shehatae*.

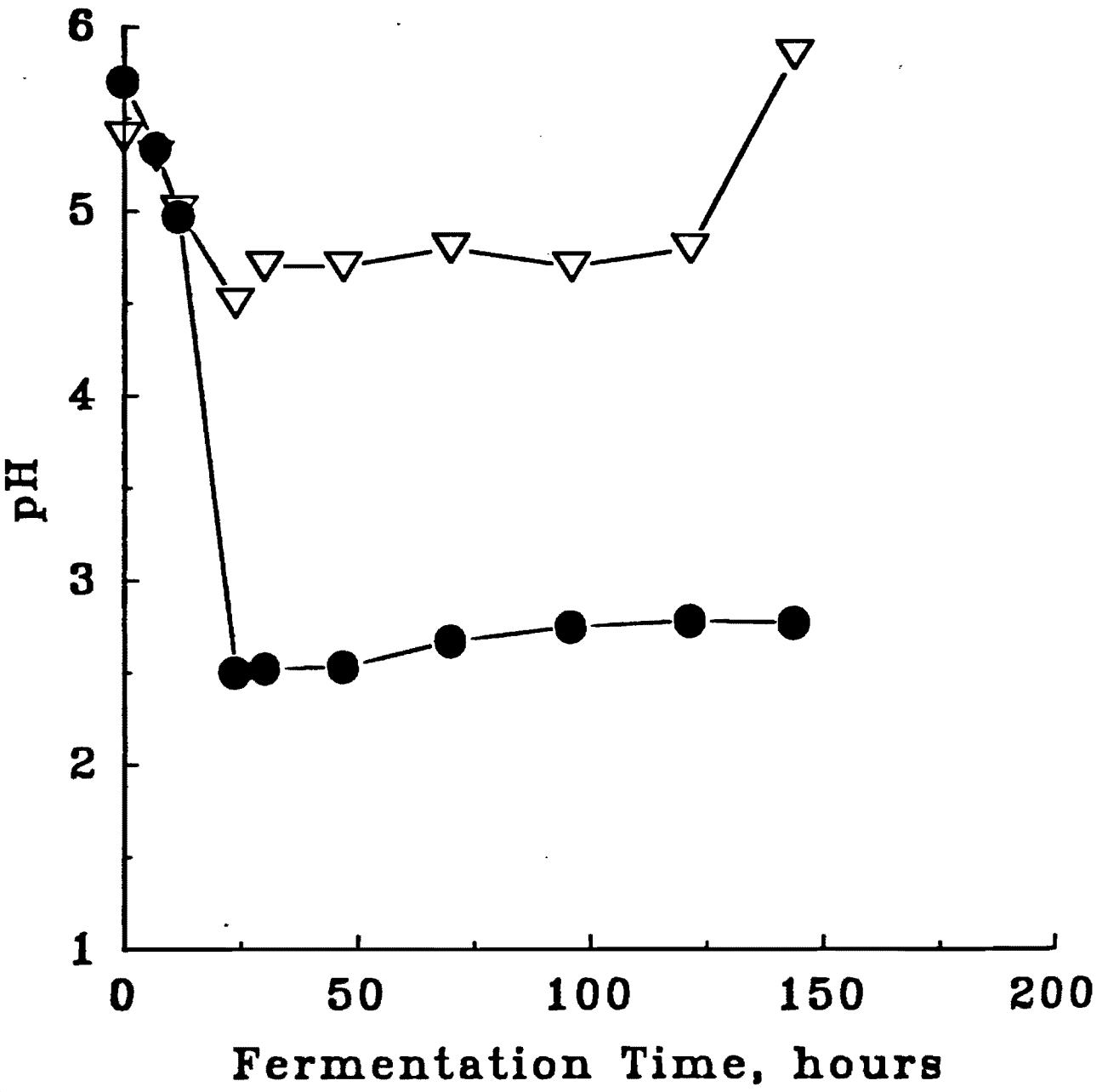


Figure 63 - Effect of pH on D-Xylose Utilization During a Fed-Batch Fermentation.

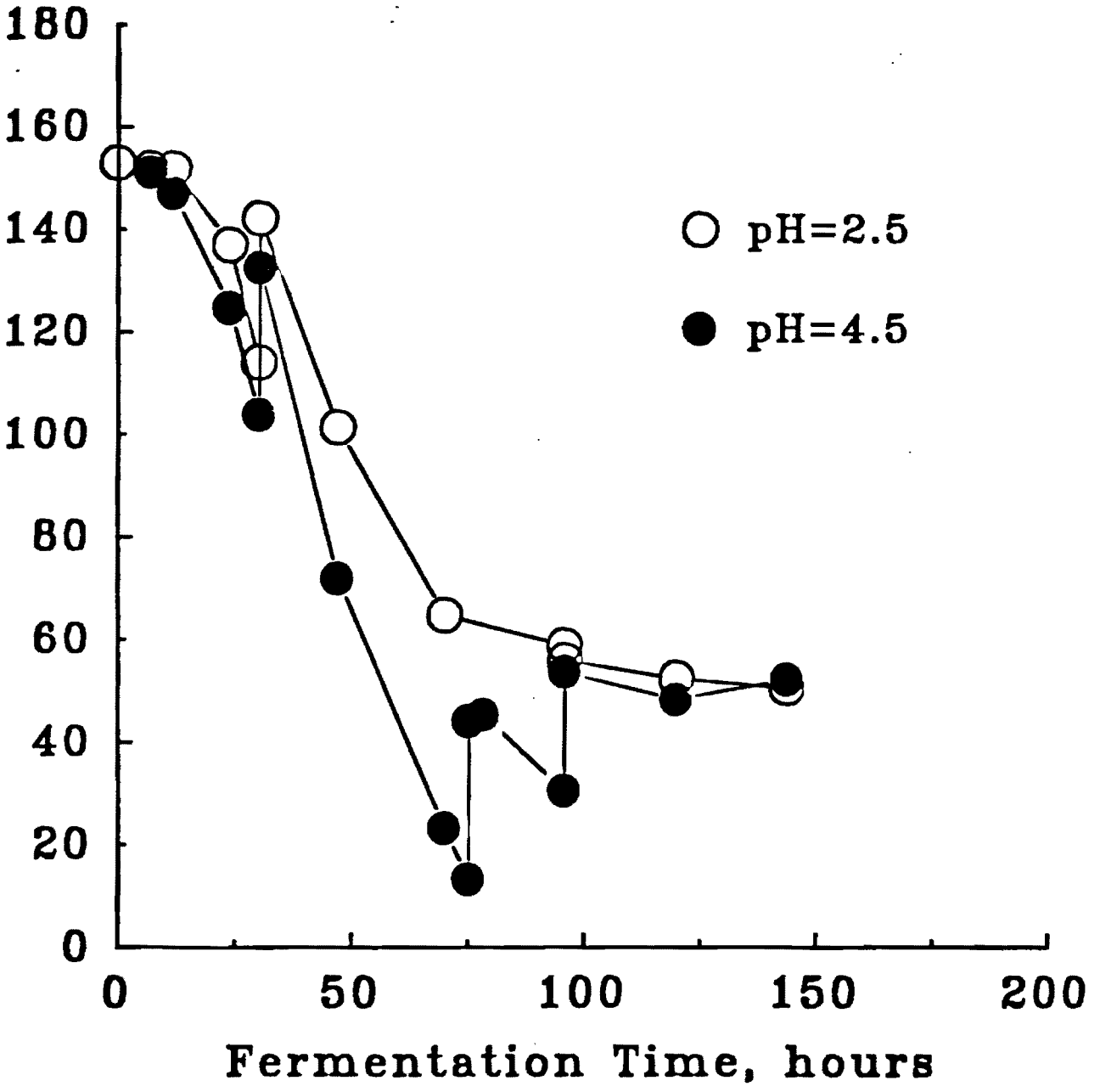


Figure 64 - Effect of pH on Cell Viability During a Fed-Batch Fermentation.

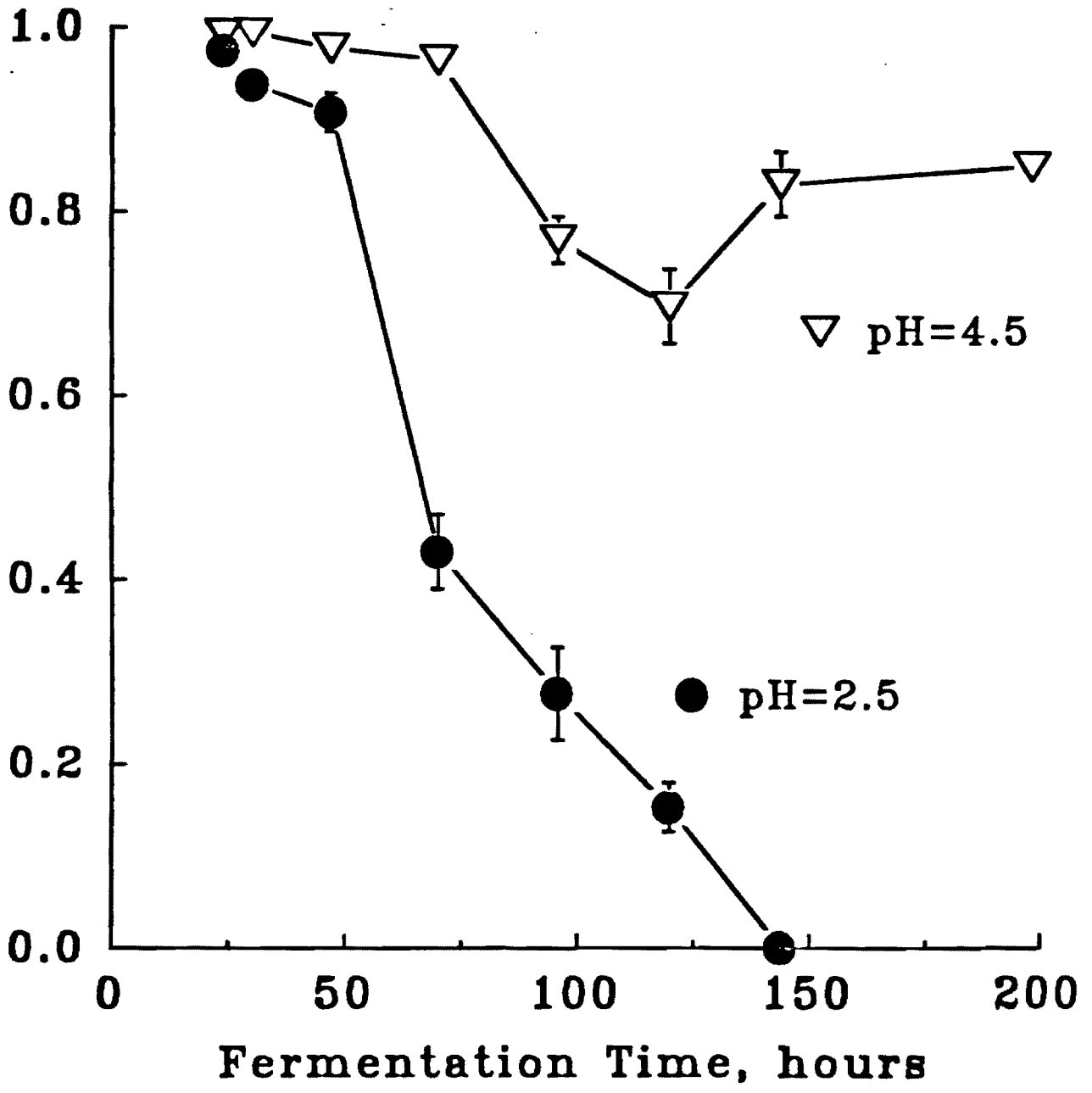
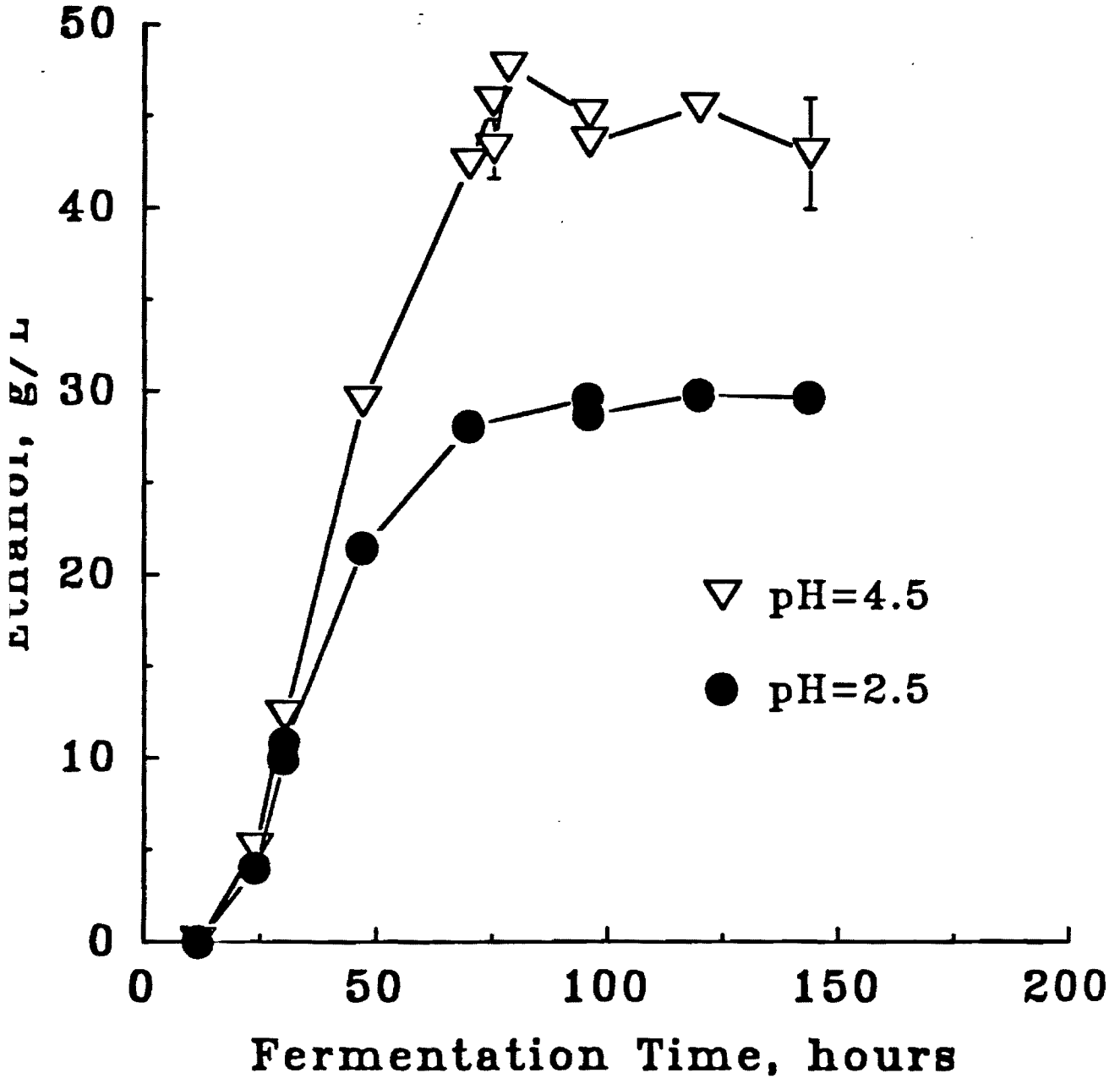


Figure 65 - Effect of pH on Ethanol Production in a Fed-Batch Fermentation.



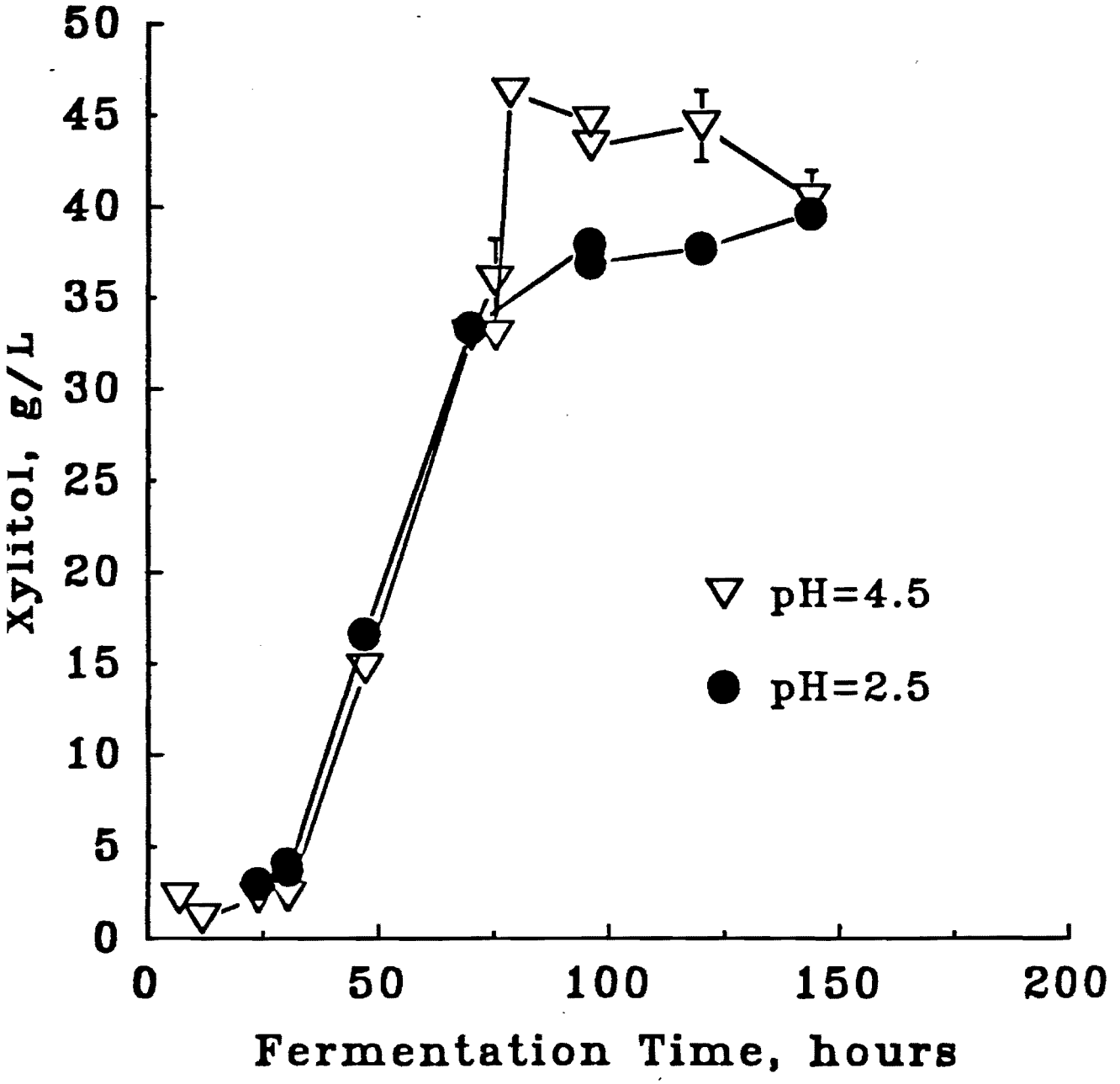
(grams of ethanol produce per gram of xylose consumed under anaerobic conditions) was 0.28 for a pH of 4.5 compared to 0.21 for a pH of 2.5. Also, more xylitol was produced at a pH of 4.5 compared to a pH of 2.5 (Figure 66).

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 ± 0.533 x 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 $\pm$ 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 $\pm$ 3.3 g/L), but its utilization was incomplete, and both ethanol

Figure 67 - Effect of Added Ethanol on Cell Growth.

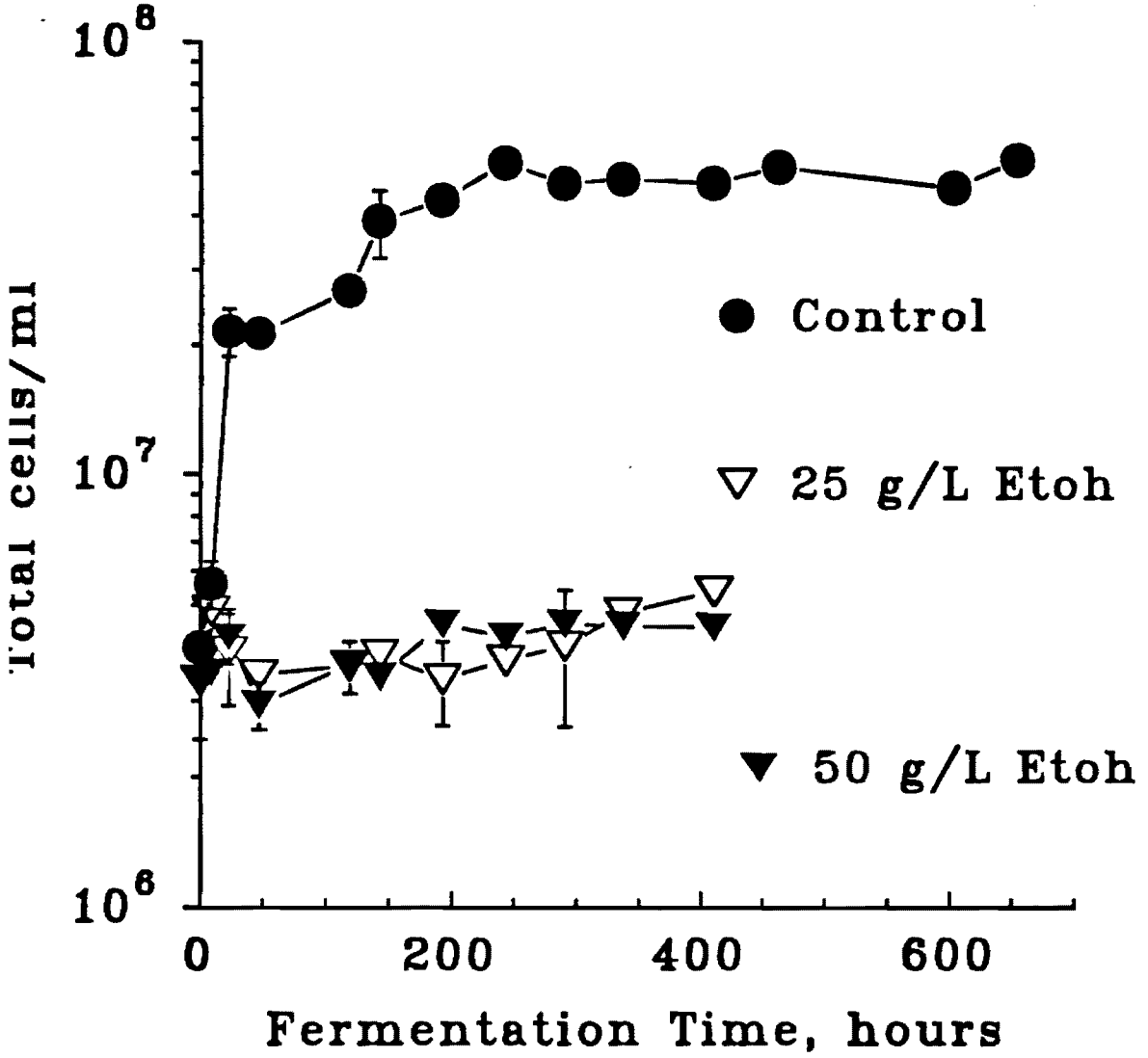


Table 6. Effect of added ethanol on oxygen-limited D-xylose fermentations by *Candida shehatae*.

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.

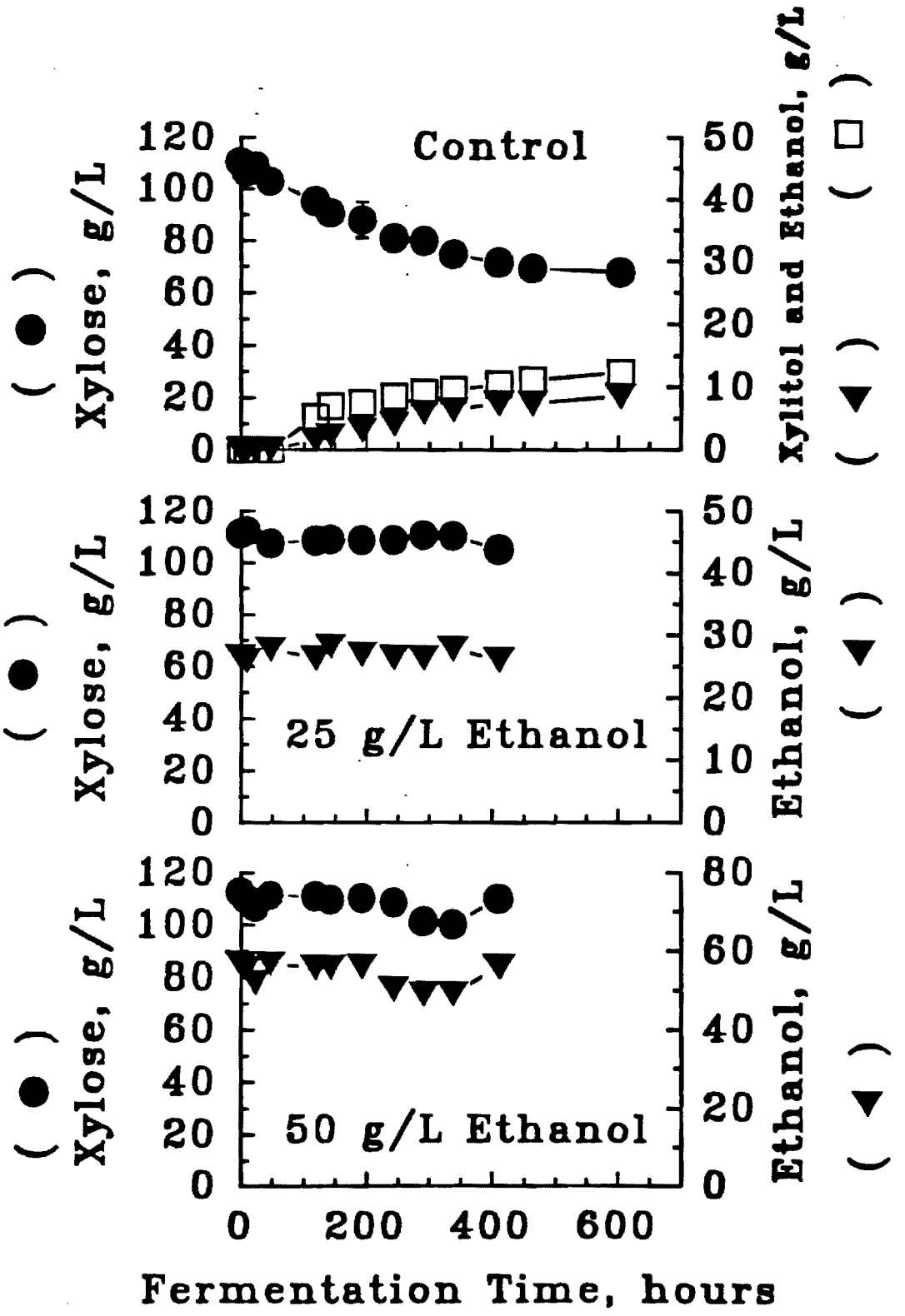
$Y_{x/s}$: Xylitol yield from D-xylose; grams of xylitol produced per gram of consumed D-xylose.

(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.

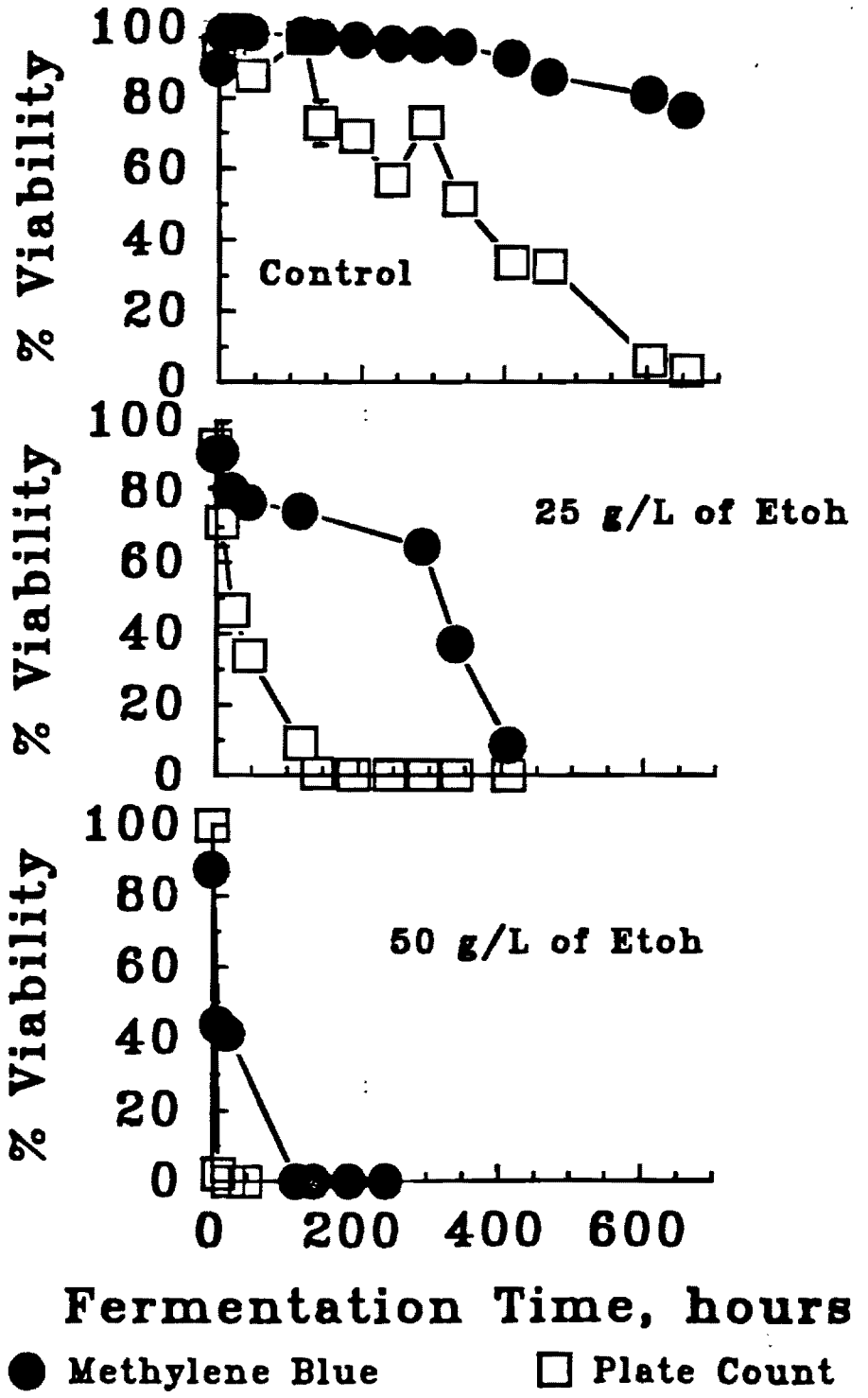


Figure 70 - Effect of Added Ethanol on Cell Volume Distribution for Cells Exposed to 50 g/L of Ethanol at Time Zero.

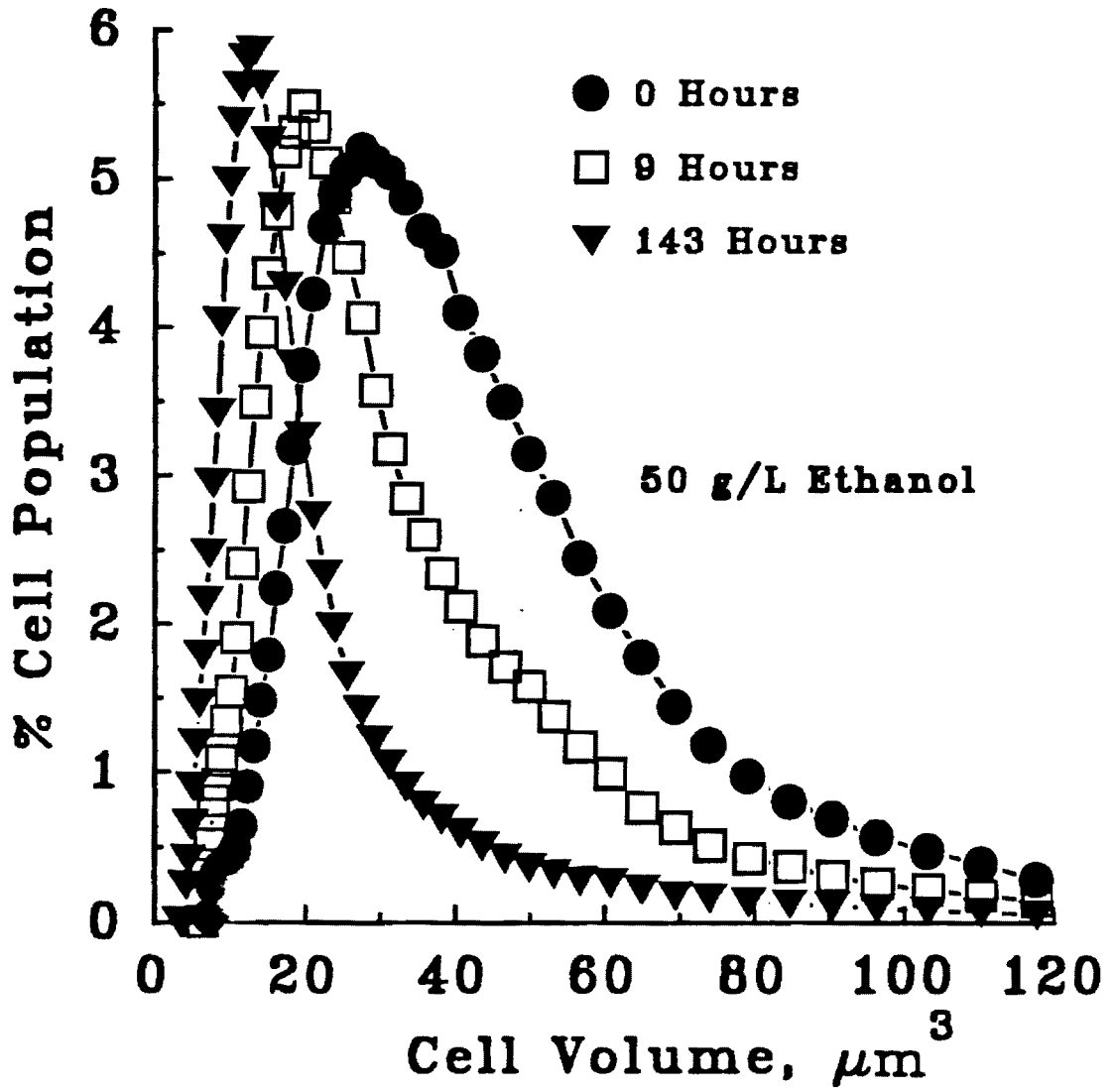
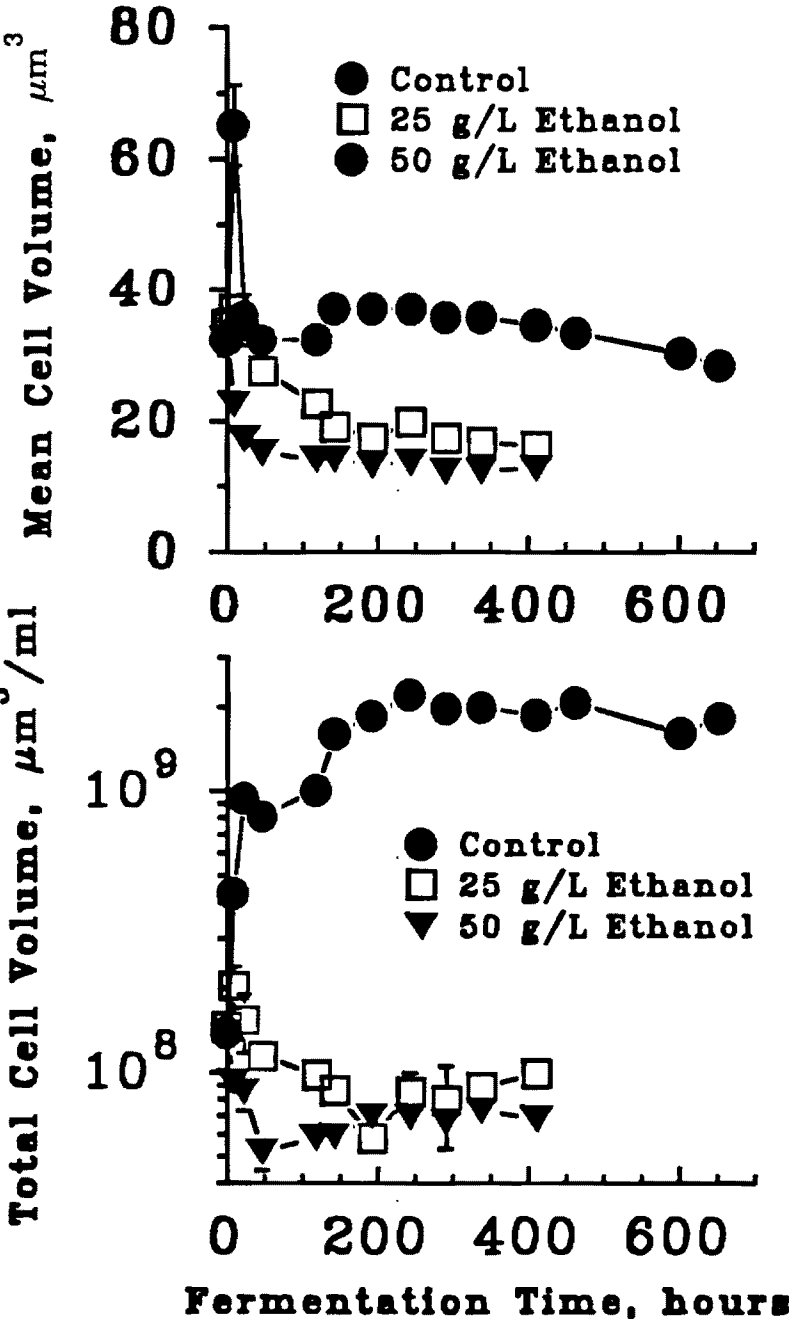


Figure 71 - Effect of Added Ethanol on Average Cell Volume and Total Cell Volume.



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 (1×10^7 cells/ml). The reactor

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

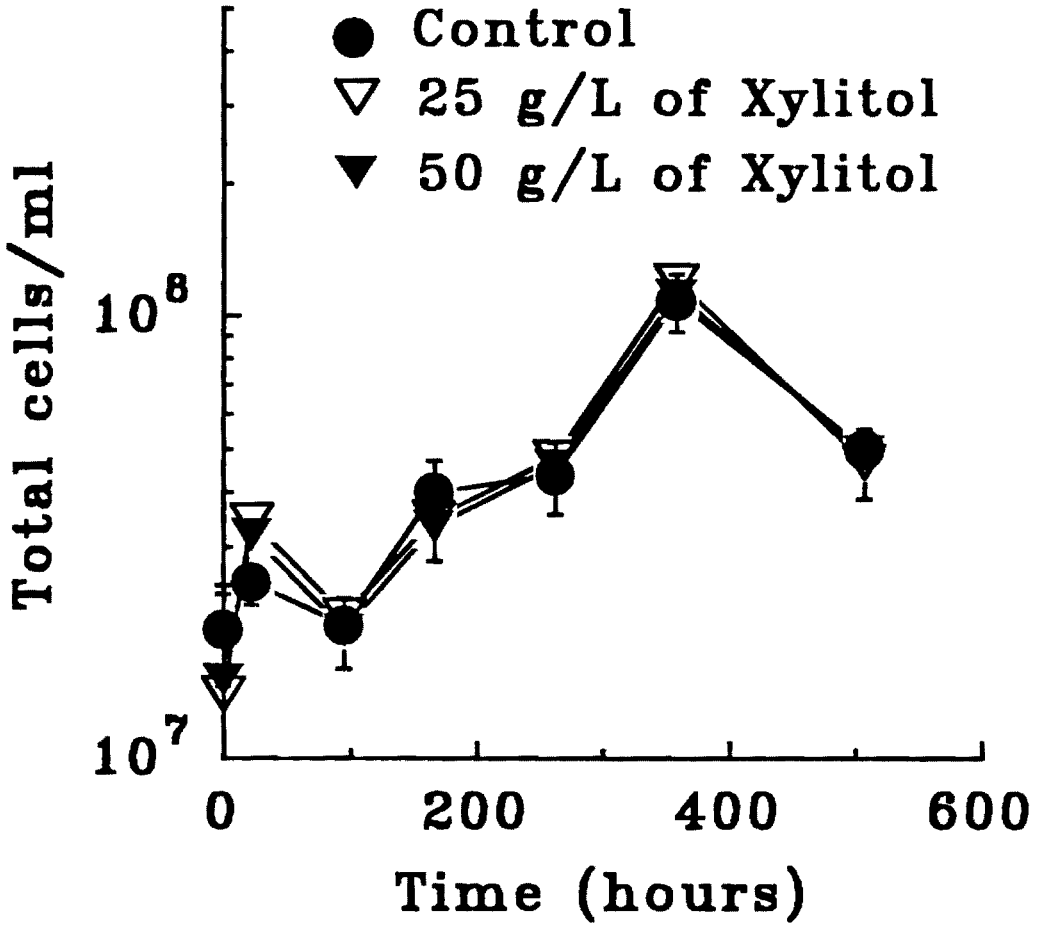


Figure 73 - Effect of Added Xylitol on the Cell Viability (as Measured by Methylene Blue Staining) of *Candida shehatae* under Anaerobic Conditions.

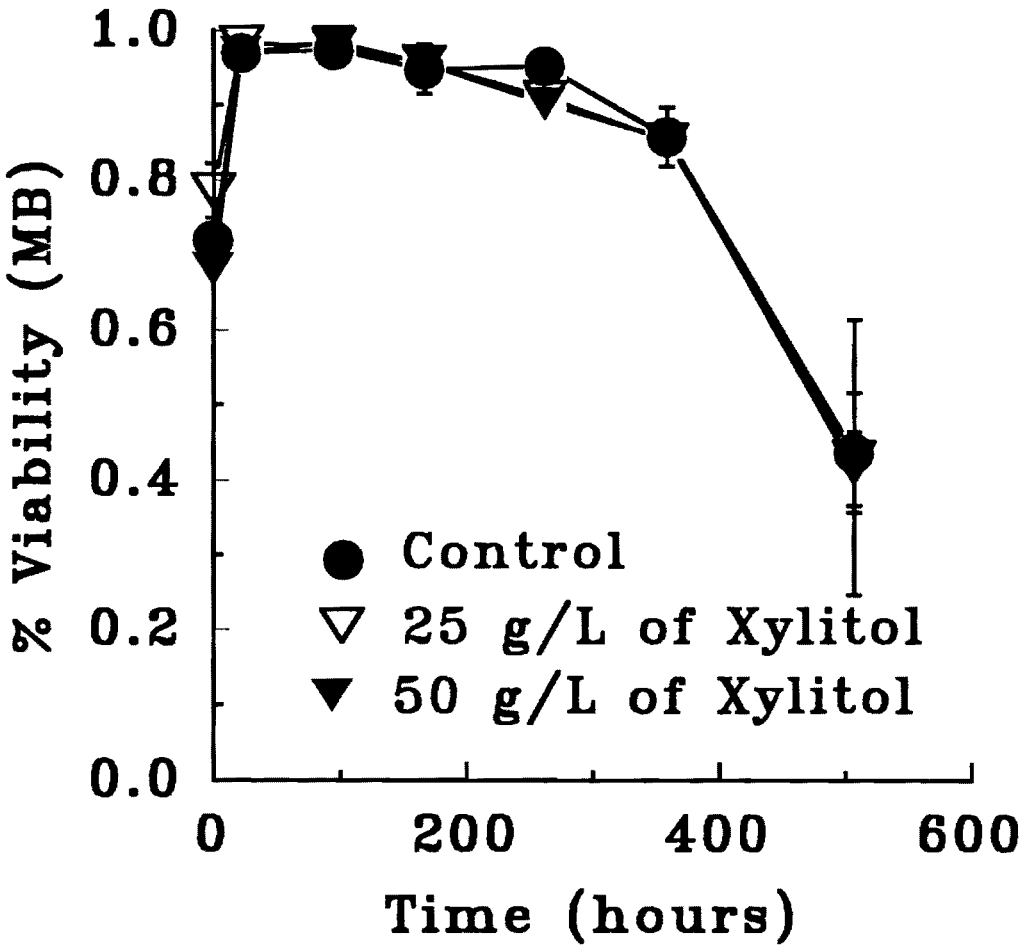
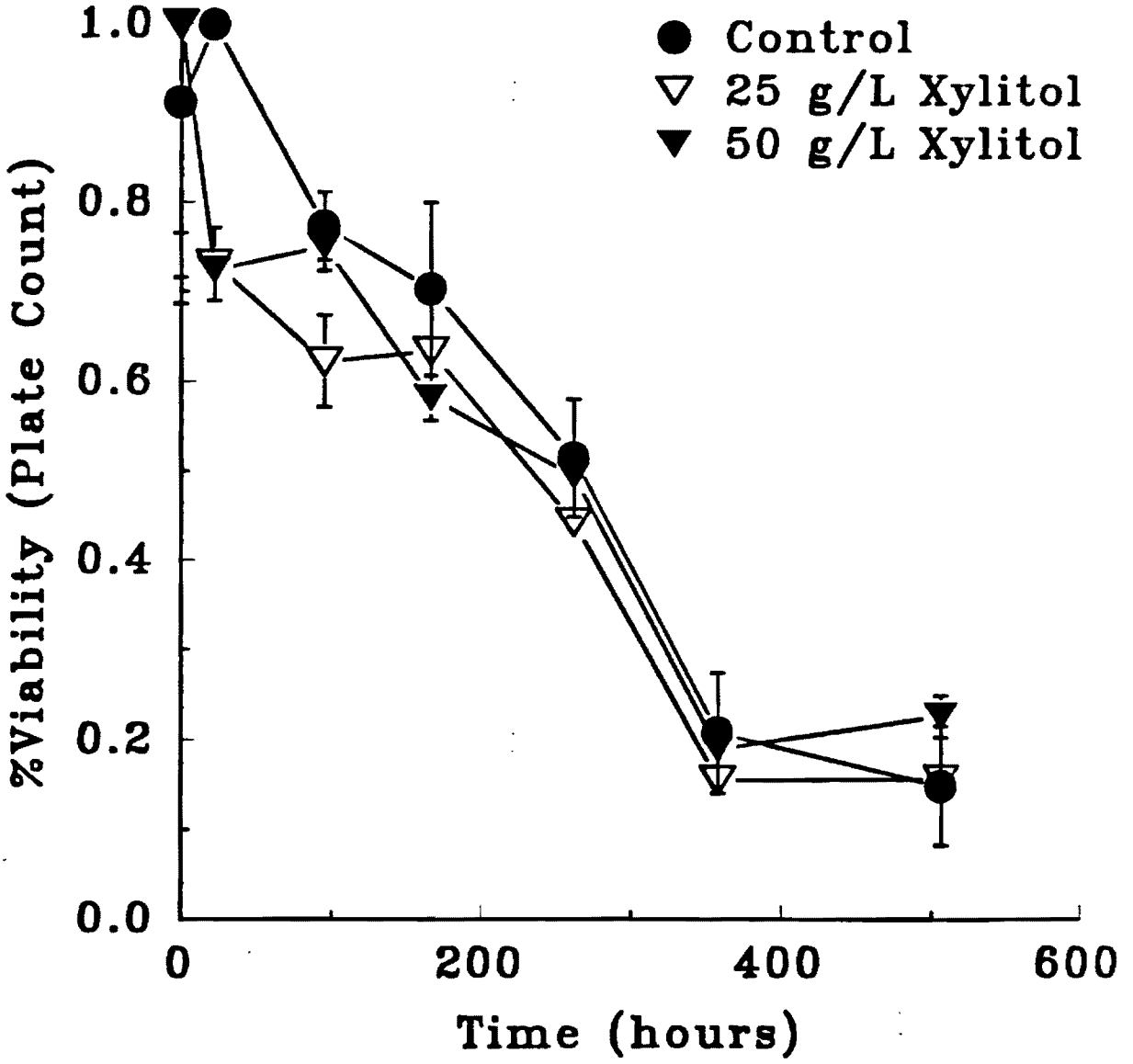


Figure 74 - Effect of Added Xylitol on Cell Viability as Measured by Plate Counts.



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*.

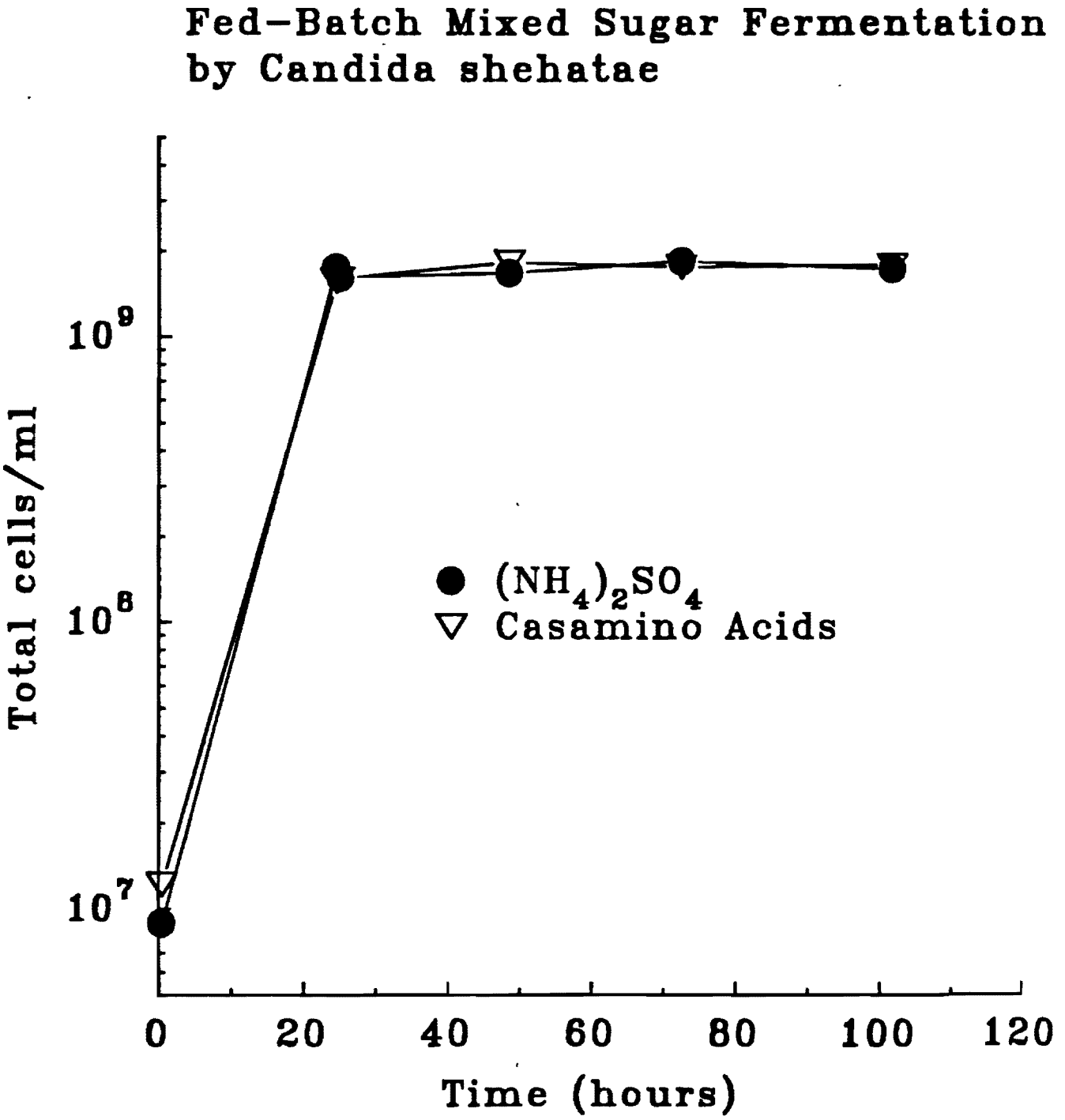


Figure 76 - Xylose Utilization and Ethanol and Xylitol Production in a Mixed Sugar Fermentation with $(NH_4)_2SO_4$ as the Nitrogen Source.

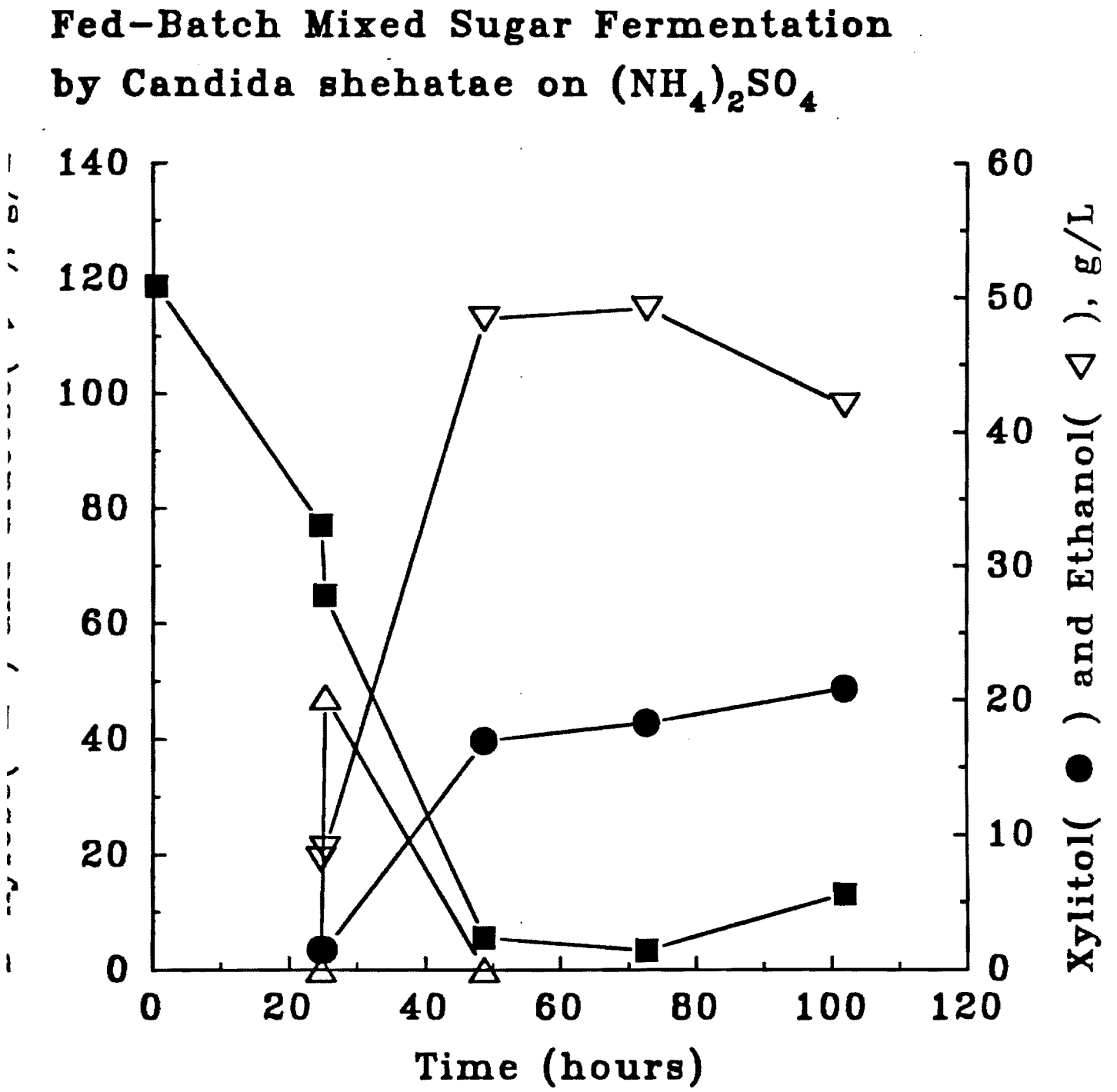


Figure 77 - Xylose Utilization and Ethanol and Xylitol Production in a Mixed Sugar Fermentation with $(NH_4)_2SO_4$ and Casamino Acids as the Nitrogen Source.

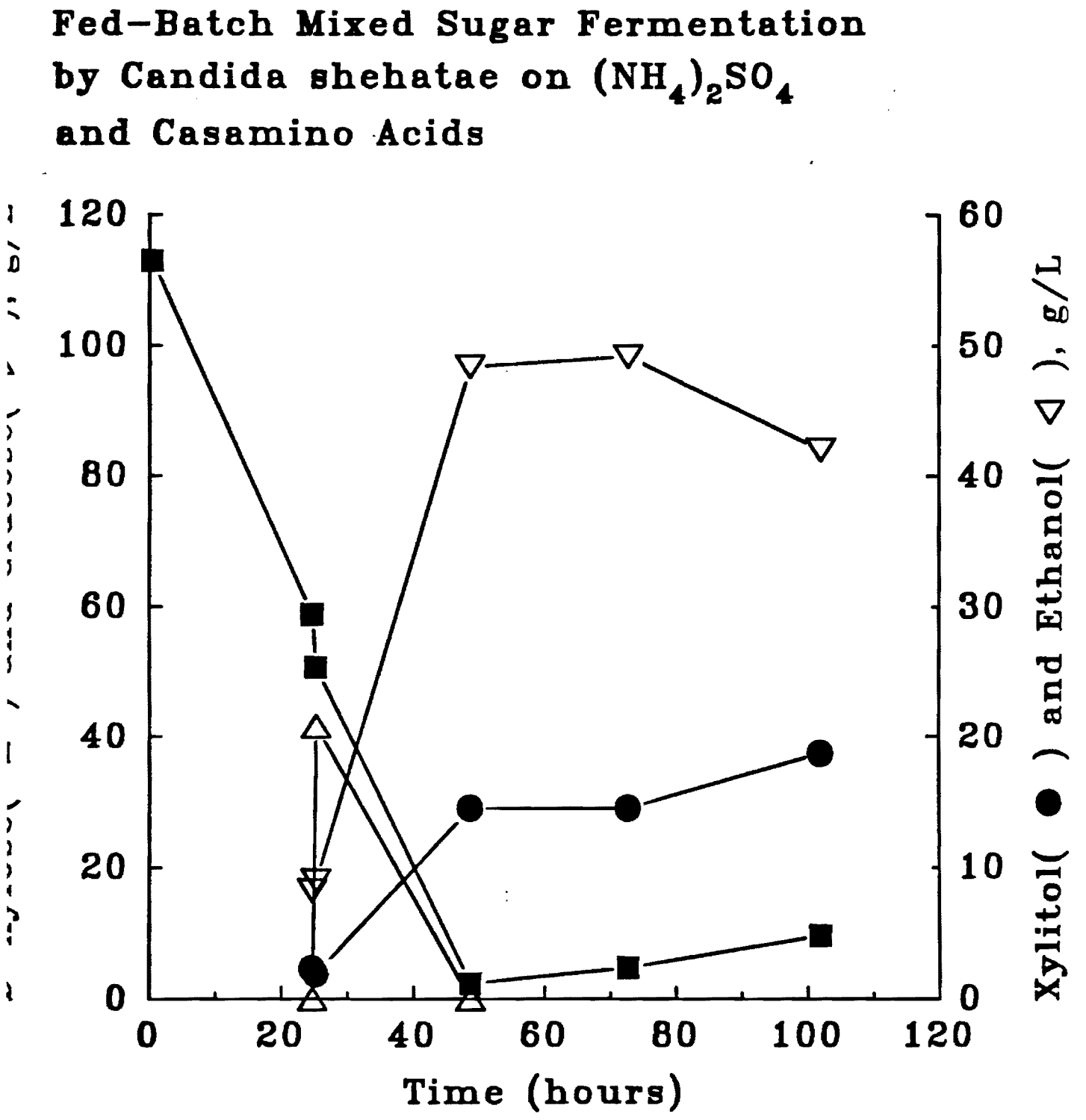
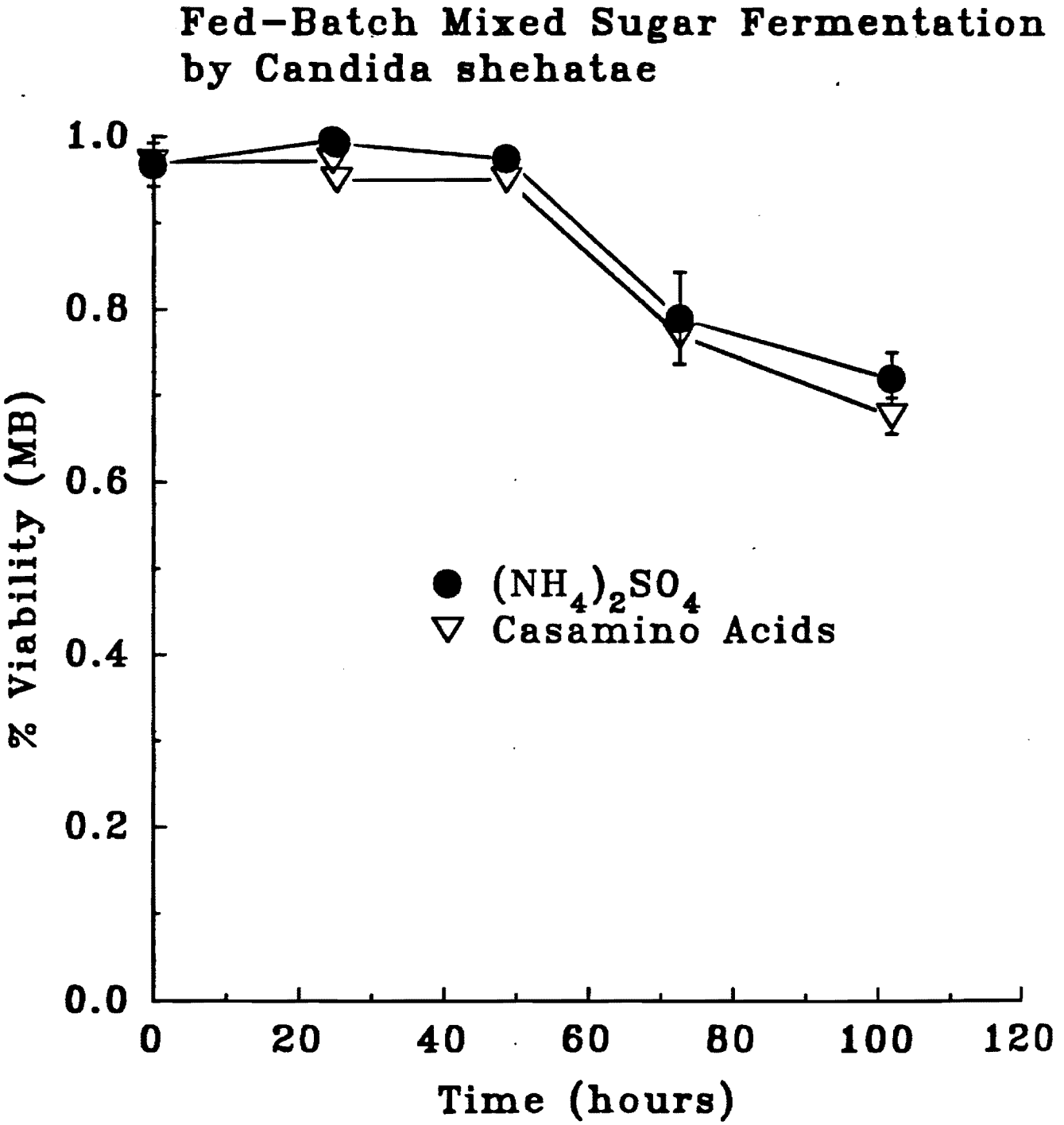


Figure 78 - Effect of Casamino Acids on Cell Viability in a Mixed Sugar Fed-Batch Fermentation.



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).

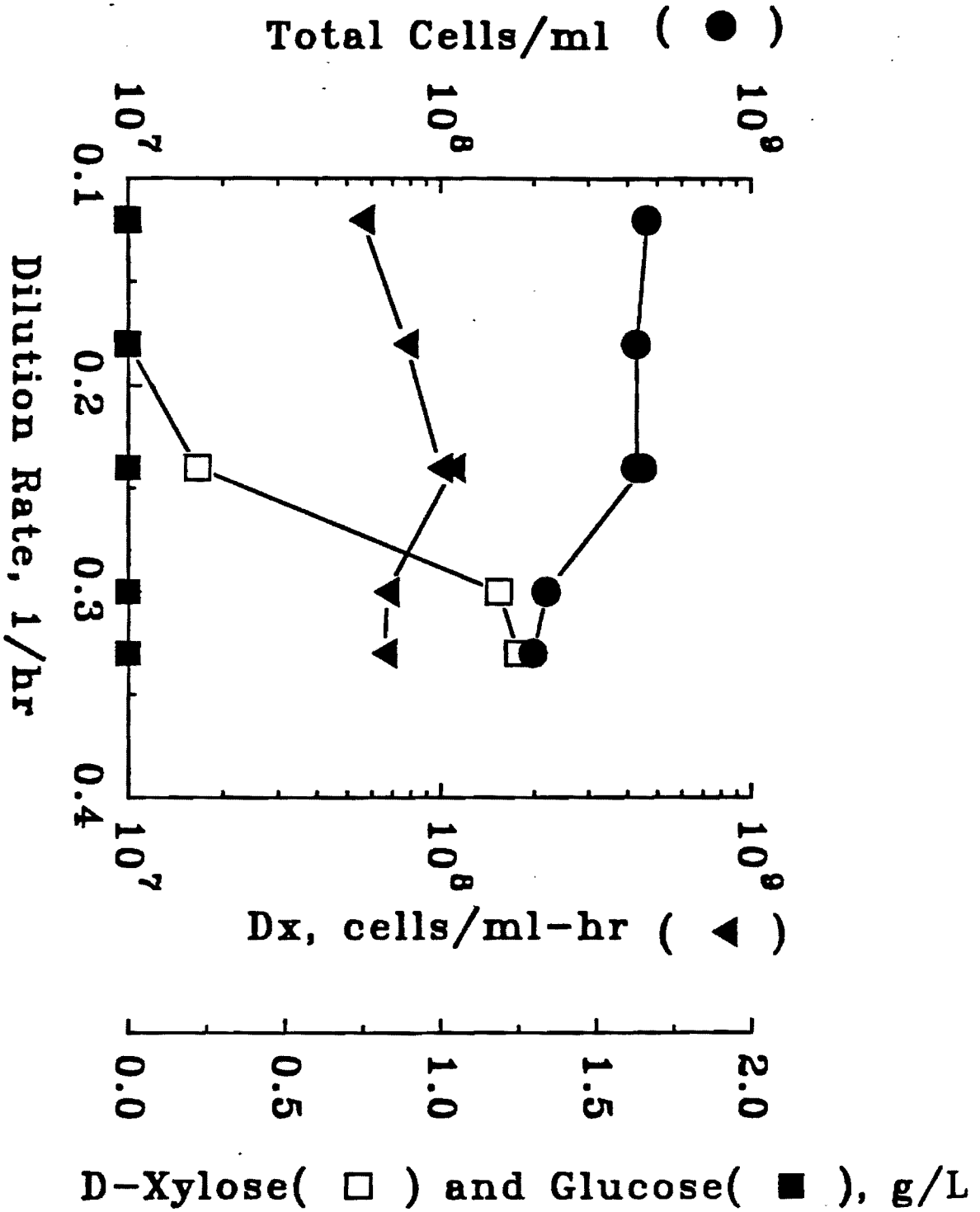
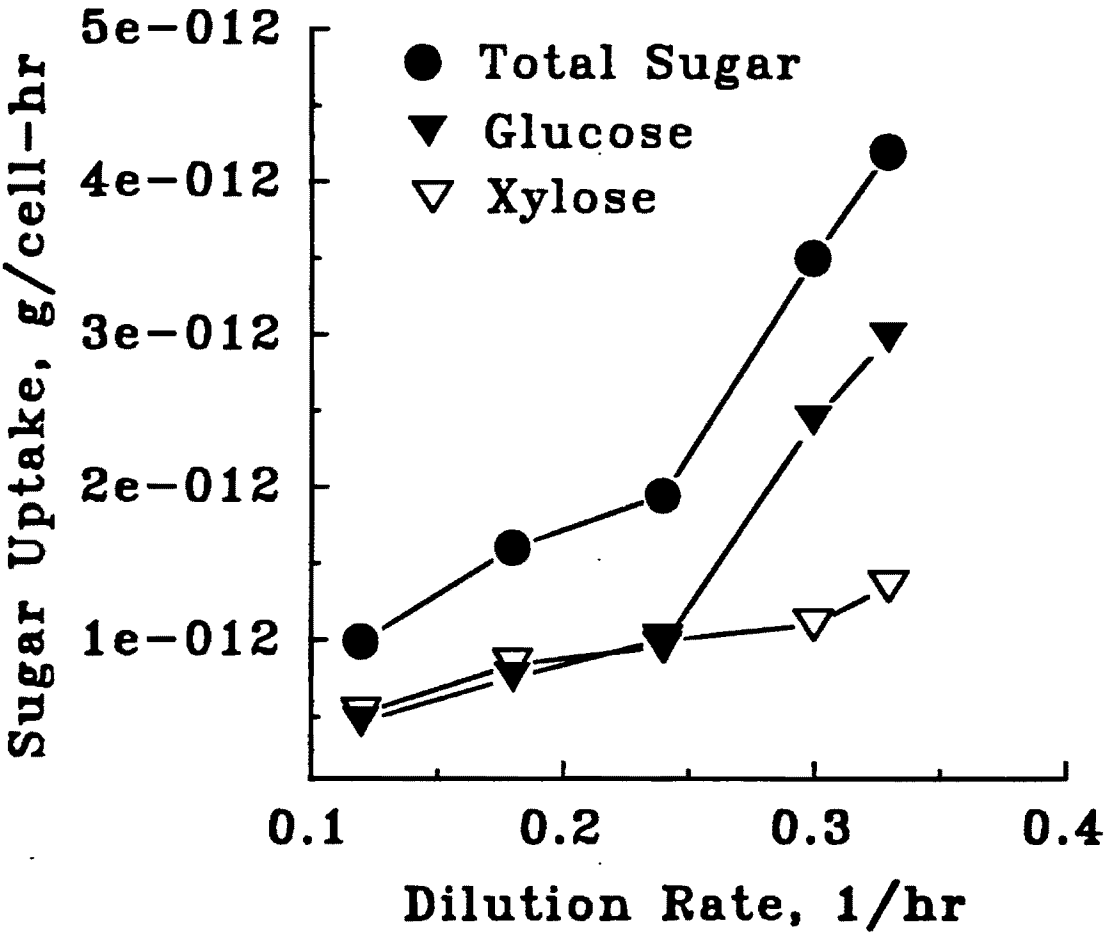


Figure 80 - Approximate Rate (g consumed per cell per hour) of Xylose, Glucose and Total Sugar Utilization in a Continuous Aerobic Fermentation of Mixed Sugar Stream.



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

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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 x 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 x 10^8 cells/ml. This is much lower than the 1 x 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 $7x10^{-12}$ for the undefined medium and $6x10^{-12}$ 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.

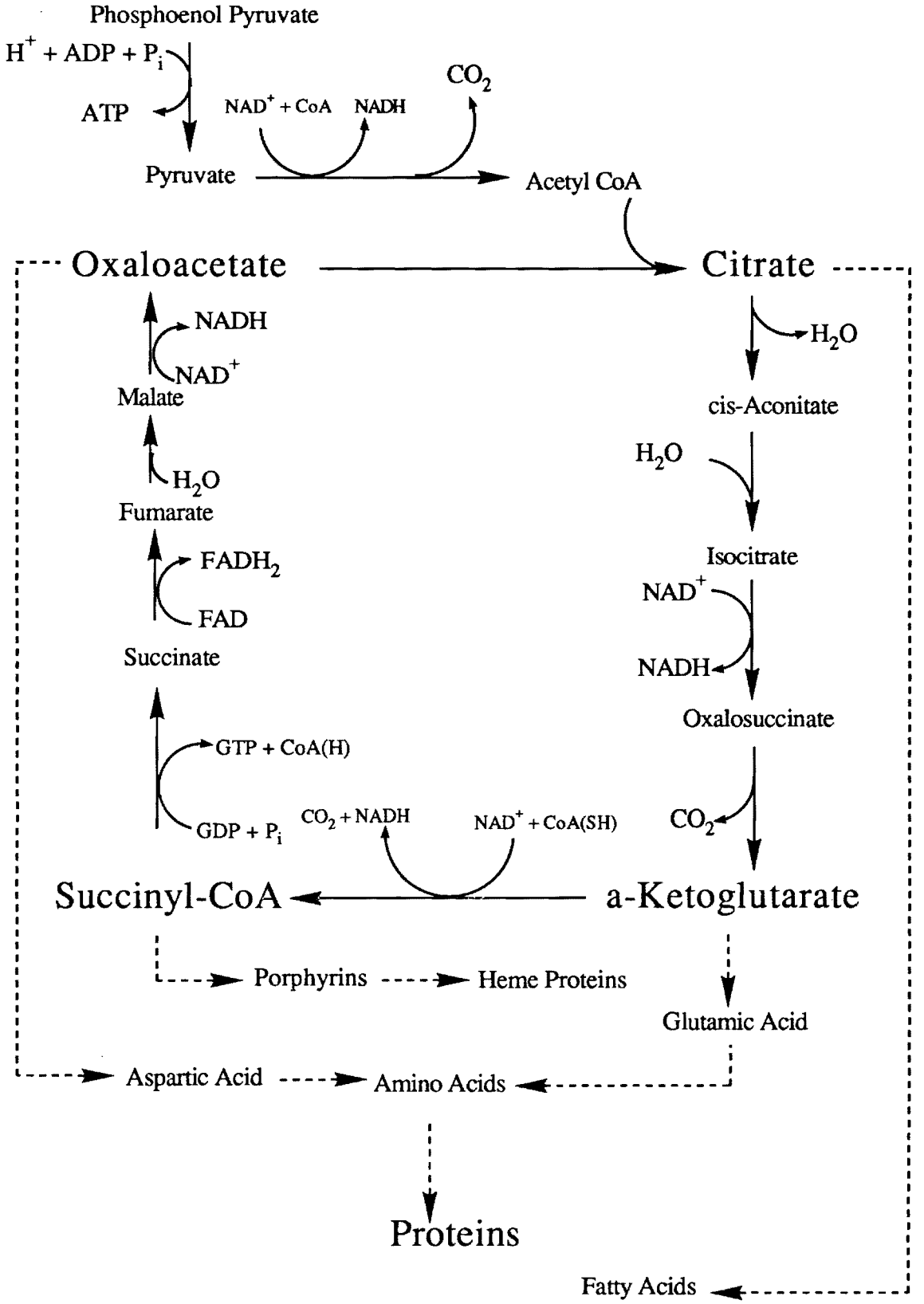

Figure 1 -- Pathway showing the TCA (Tri-Carboxylic Acid) cycle under aerobic conditions.

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.

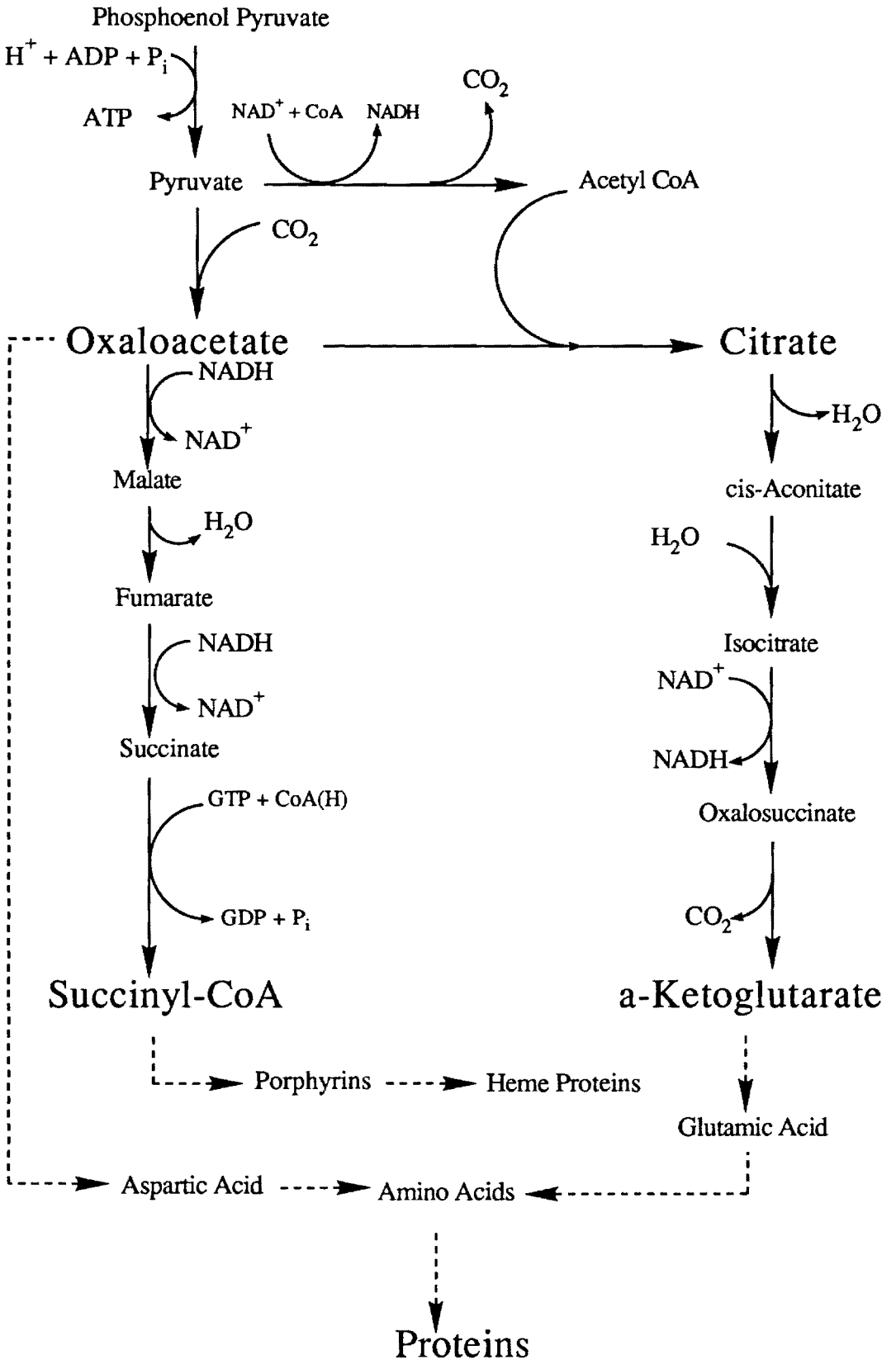

Figure 2 -- Generic TCA pathway for facultative microbes under anaerobic conditions.

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).

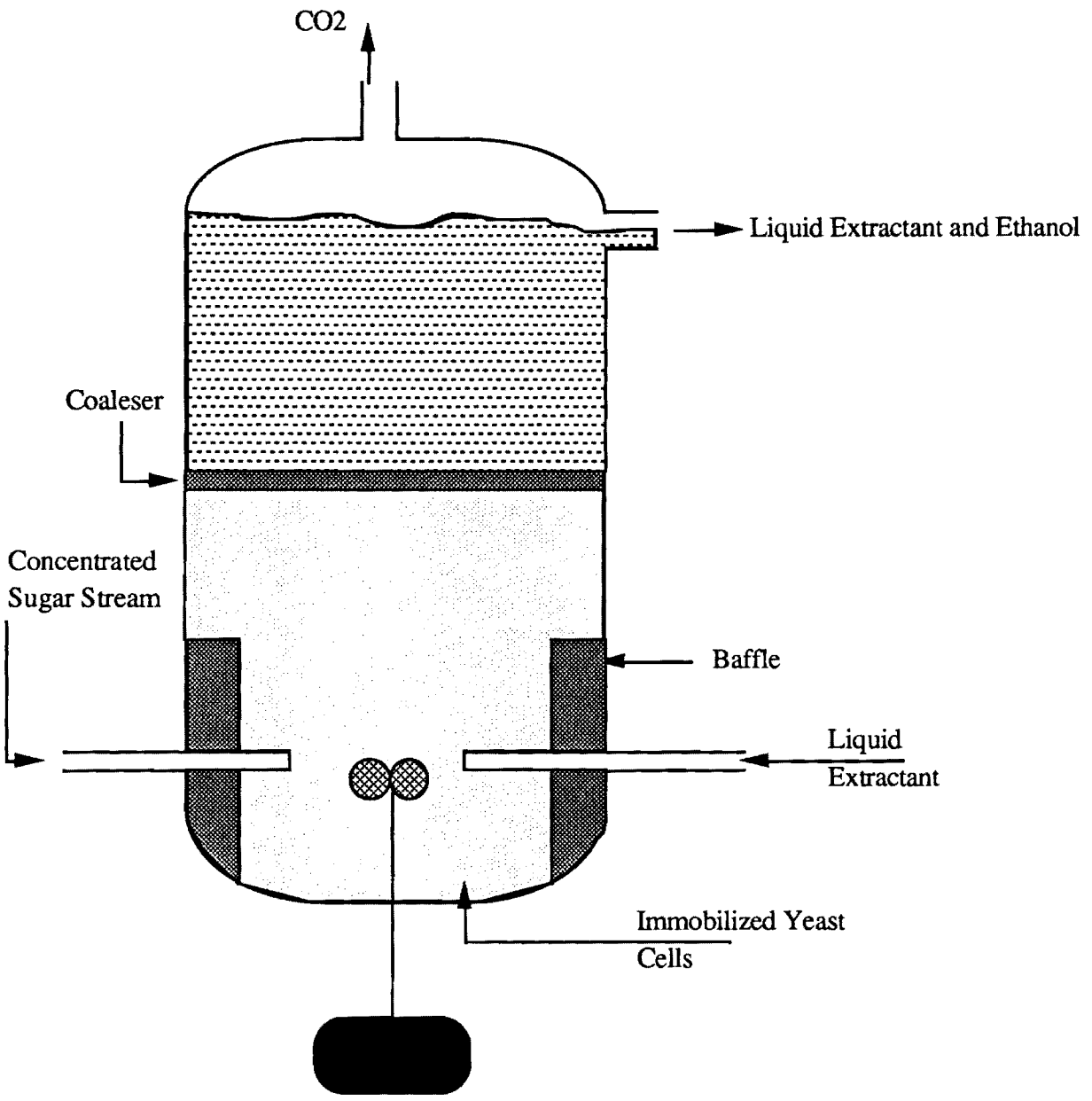

Figure 3 -- Drawing of Extractive Fermentor. This drawing was designed only to portray the characteristics of an Extractive Fermentor. The actual design of an industrial Extractive Fermentor could be quite different.

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.

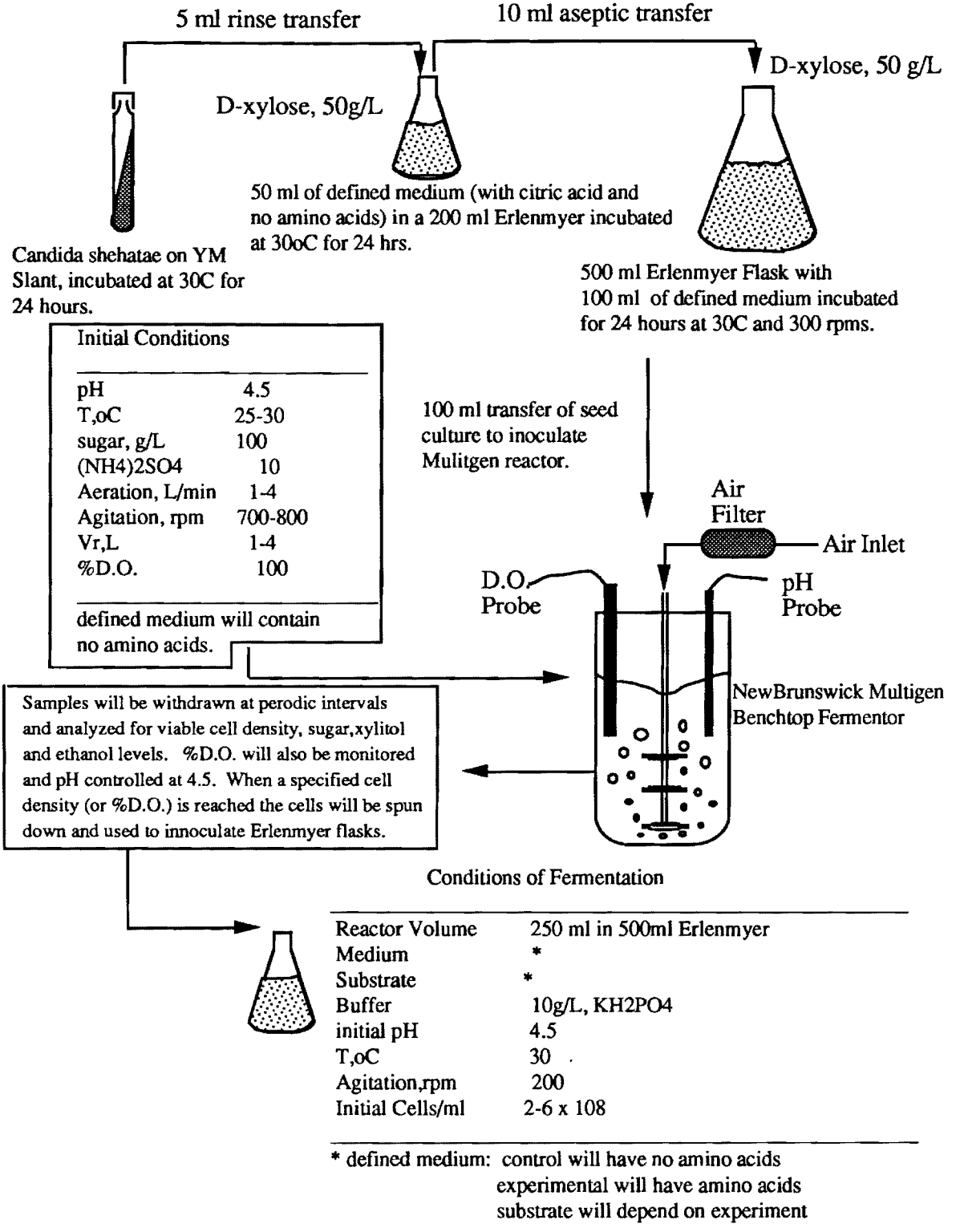

Figure 4 -- Flowsheet for *Candida shehatae* fementations with amino acids in the defined medium.

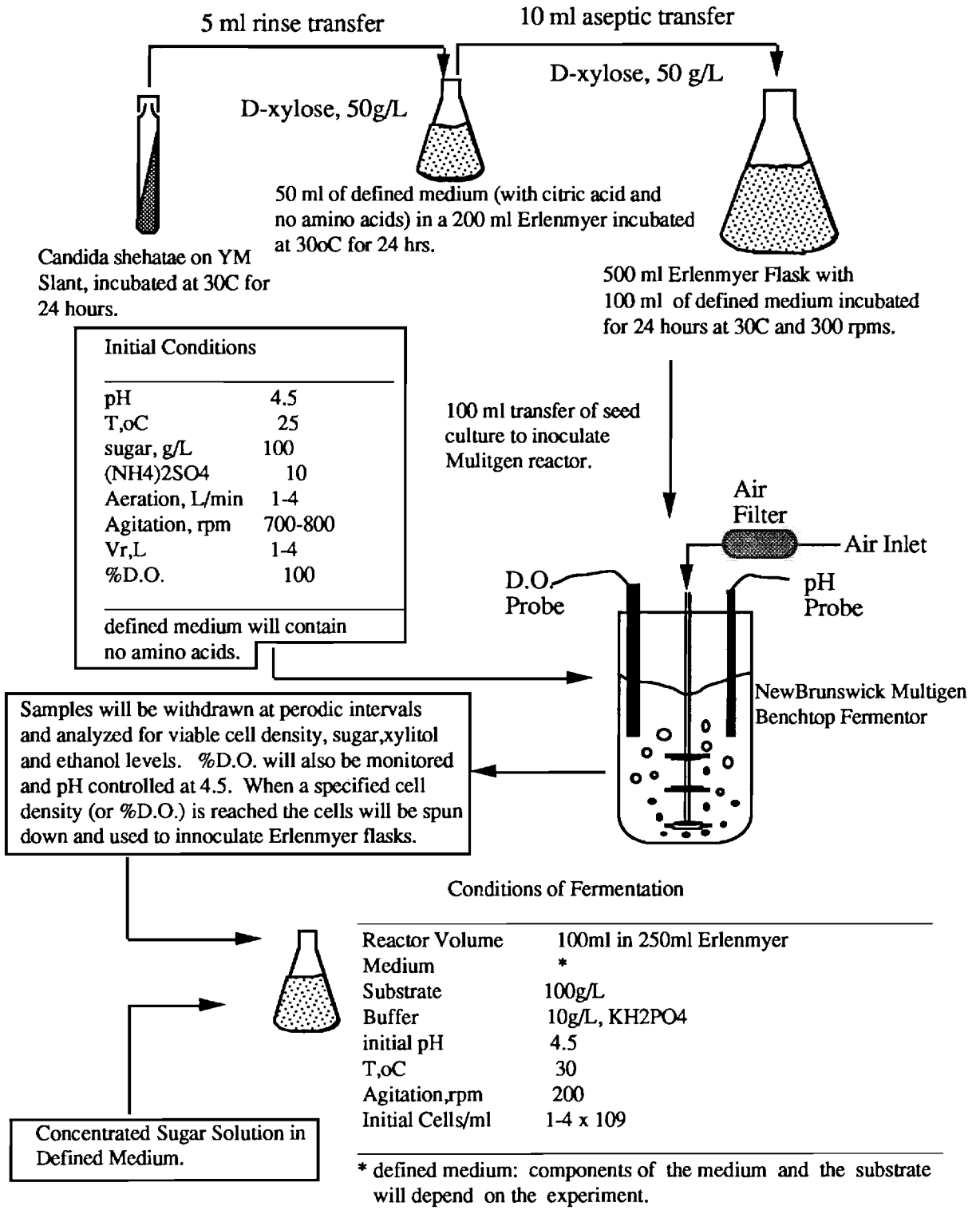

Figure 5 -- Flowsheet for rapid fed-batch fermentations in a defined medium.