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Continuous Fermentation for the Production of Citric Acid from Glucose

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ABSTRACT

Citric acid is finding new areas of use each year and the demand for the acid is constantly increasing. Being a bulk chemical, the continuous production of citric acid would be advantageous. The paper presents the results from ammonia limited batch and continuous fermentations using the yeast strain *Saccharomycopsis* (*Candida*) *lipolytica* (NRRL Y-7576). Mathematical models were developed for growth and glucose utilization in batch and continuous culture. Cell and acid yields appeared to be almost the same in batch and continuous culture. The specific production rates were found to be constant, equal to 0.053 g/g h, in the batch fermentations but varied in the continuous experiments from 0 to 0.11 g/g h depending on the fermentation conditions. Continuous production in a single stage CSTR was studied for over 1,000 hours without shutdown.

Index Entries: Citric acid; *Saccharomycopsis lipolytica; Candida lipolytica;* continuous production; stirred-tank reactor.

NOMENCLATURE

[CIT]	Citric acid concentration (anhydrous)	g/L
D	Dilution rate	h^{-1}
[GLU]	Glucose concentration	g/L
[ISO]	Isocitric acid concentration	g/L
K	Constant in growth equations	h^{-1}
[NH_3]	Ammonia concentration	g/L
t	Time	h

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X	Dry weight cell concentration	g/L
[YE]	Yeast extract concentration	g/L
$Y_{A/G}$	Actual acid yield on glucose	g/g
$Y_{N/Y}$	Ammonia equivalent of yeast extract	g/g
$Y_{X/G}$	Actual cell yield on glucose	g/g
$Y_{X/N}$	Overall cell yield on ammonia	g/g
$Y'_{X/N}$	Cell yield on ammonia at beginning of batch	g/g
μ	Specific growth rate of cells	h^{-1}
ν	Specific production rate of acids	g/g h

Subscripts

o	Initial concentration in batch
f	Feed concentration in continuous

Superscripts

c	Continuous

INTRODUCTION

Citric acid is one of the most commonly used acids in the food, beverage, and pharmaceutical industries, mainly because of its low toxicity and ease of assimilation. It has also found a niche in the detergent industry, where its salt, trisodium citrate, is replacing tetrapotassium pyrophosphate as a surfactant (*1*). The world production of citric acid and its salts was about 300–350 thousand tons in 1982 (*2*).

The classic method for the production of citric acid is by batch fermentation using strains of the fungus *Aspergillus niger*. The two main producers of citric acid in the US are Charles Pfizer, Inc. and Miles Laboratories, Inc. Pfizer, the first US commercial producer, has developed a batch surface culture process (*2*), and Miles Laboratories has adapted a submerged culture fermentation process (*3*).

Batch fermentation processes have several advantages over continuous processes, such as easy control of microbial contaminants and increased product quality per batch. However, for large scale production, batch reactors require high capital investments since the volumetric efficiency of the reactor is low, based on total processing time. The batch process also requires extensive labor. A continuous system (CSTR) does not have these disadvantages, can give a more consistent product, and provides a steady rate of crude product to be processed in the recovery system.

In employing a continuous fermentation process, the microorganism used must be rigid enough to withstand shear while still being easily pumped. The fungus *A. niger* generally yields multicellular growth and develops mycelia that can easily cause clogging problems in a continuous system. The yeast *Saccharomycopsis* (*Candida*) *lipolyticia* does not form mycelia, has shown good stability, and gives high citric acid yields on glucose (*4*). However, one disadvantage in using yeasts is that isocitric acid is also produced in parallel with citric acid.

Several continuous processes using yeast have been reported in the literature. Pfizer (*3,5*) patented a single-stage continuous process with *Candida lipolytica* grown on an *n*-paraffin mixture for a total period of 304 hours. After an initial batch stage, continuous feed was started and effluent withdrawn. A multistage process using three fermentors has also been patented (*3,6*) using *C. oleophila* with yields up to 148% by weight reported. A German patent (*7*) claimed a continuous process using molasses as a substrate with strains of *C. guilliermondii* as the organism. This continuous fermentation consisted of a batch startup period followed by continuous feed after nearly complete conversion of sugars in the batch stage. The overall productivity for a fermentation lasting 189 h (including 47 h at the batch stage) was 0.35 g/L h. One of the few reported results (*8,9*) in the open literature of a successful continuous fermentation used *C. lipolytica* in a single-stage CSTR in order to study the metabolic pathway for growth on different substrates. The highest volumetric productivity reported was 0.2 g/L h (*8*).

Citric acid is generally considered to be a secondary metabolite, produced only in nutrient-limited cultures where growth has ceased. However, some of the examples shown above indicate that growth and production are somewhat coupled. Yeasts such as *S. lipolytica* have a well-developed TCA-cycle and are known to overproduce citric acid when subjected to ammonia limitation (*8,10,11*). The phenomena is easily seen in a batch fermentation, where the ammonia concentration is closely followed. After ammonia exhaustion the citric and isocitric acids accumulate in the broth.

Being a bulk-chemical, citric acid production in a continuous operation, if possible, would prove advantageous. However, the lack of reliable data for true continuous fermentations makes it hard to evaluate and compare batch and continuous systems. Kinetic parameters for growth and production are vital information for any process design and scaleup. This type of data for yeast growing on glucose is not readily available in the literature, mainly because it was long thought that citric acid could not be produced in a continuous fermentation.

The purpose of this paper is to compare citric acid production and growth for the yeast *S. lipolytica* in batch and single-stage CSTR culture. Models are developed and compared for predicting the specific growth rate and product yields for the strain in batch and continuous culture.

MATERIALS AND METHODS

Organism and Media

Saccharomycopsis lipolytica, NRRL Y-7576, was obtained from Northern Regional Research Center, Peoria, IL. Stock cultures were kept on slants (YM-broth, 1.5% agar) at 4°C and transferred every 6 mo.

Table 1
Media Composition for the Fermentations, in g/L

Glucose	30–150
Yeast extract (Difco)	0.05 (batch), 2.0 (CSTR)
NH_4Cl	0.3–1.3
KH_2PO_4	0.3 (0.45 in one CSTR exp.)
$MgSO_4 \cdot 7\ H_2O$	0.2 (0.30 in one CSTR exp.)
Thiamine-HCl	0.0001 (0.00015 in one CSTR exp.)

The fermentation media for batch and continuous fermentation studies is shown in Table 1. When the time for autoclaving was greater than 30 min and the NH_4Cl concentration was over 1.0 g/L, the NH_4Cl was sterilized separately from the rest of the media. The pH was adjusted to 5.5 before autoclaving using H_2SO_4 and/or NaOH. The dissolved oxygen was kept above 50% air saturation by mixing oxygen and air in the sparger. NaOH was used to control the pH at 5.5 in all fermentations.

Equipment

A Braun Instruments Company Biostat-M, with a working volume of 750–1500 mL, was used in carrying out both batch and continuous fermentations. The fermentor was equipped with electrodes for measuring pH and dissolved oxygen. The Biostat-M also had controls for pH, temperature, agitation, and foam. The pH was controlled using 5 *N* NaOH and DF 10P MOD 11 antifoam (Mazer Chemicals, Inc.) was used to control foam at a tolerable level. For the continuous fermentations a Masterflex pump (Cole Palmer) with two pump-heads for different size tubing diameters was used for controlling feed and effluent. A schematic of the fermentor can be seen in Fig. 1.

Analytical Methods

The following analytical methods were used on a routine basis for substrate, product, and cell concentration measurements.

Glucose Assay

Glucose concentrations were determined using a DNS reducing sugar procedure, based upon the work of Summers (*12*). A Spectronic 21 (Milton Roy Co.), set at 540 nm, was used for measuring light absorption.

Cell Concentration

Cell concentrations were determined by turbidity measurement at 660 nm using the Spectronic 21. A standard curve for dry (48 h, 105°C) cell weight was prepared.

Ammonia Assay

Ammonia analyses were performed with a gas-sensing ammonia electrode, obtained from Orion Research, Inc.

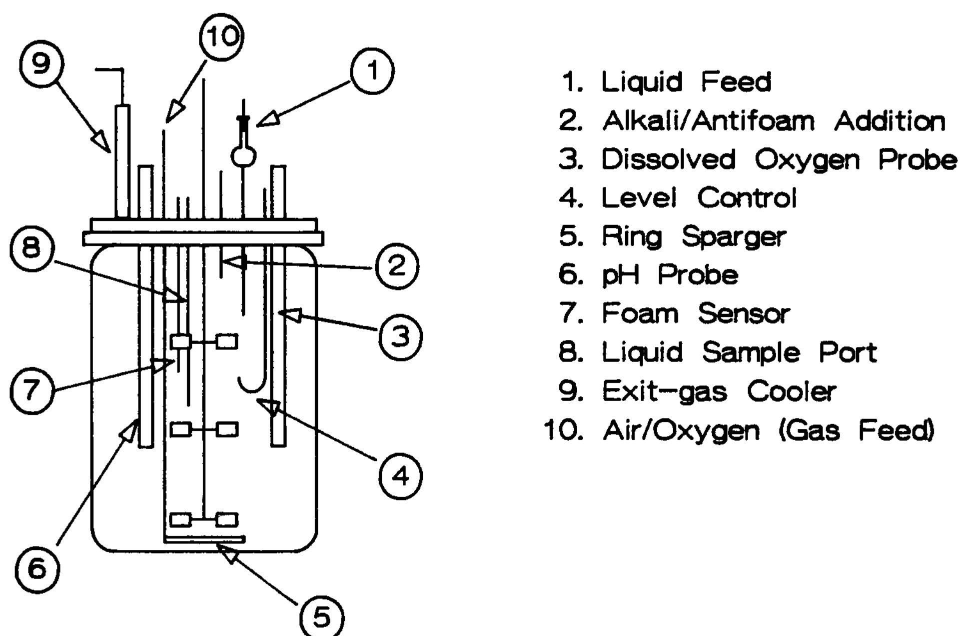

Fig. 1. Schematic of reaction system.

Citric and Isocitric Acid

Product concentrations were determined according to the methods of Marier and Boulet (*13*) and Stern (*14*). The specific enzymatic conversion of isocitrate to α-ketogluturate was measured with the Spectronic 21 at 340 nm. The colorimetric method for tricarboxylic acids served as a basis for the citric acid assay, using the Spectronic 21 at 420 nm. Citric acid concentrations were calculated by difference from the results of the two assays.

RESULTS AND DISCUSSION

Batch Results

The results from batch experiments with *S. lipolytica* showed the same type of behavior as reported by Briffaud et al. (*10*) and Behrens et al. (*11*).

1. Rapid growth, decreasing as the ammonia concentration dropped to low values;
2. Citric and isocitric acid accumulation initiated by ammonia exhaustion, followed by a period of acids production and increase in cell mass; and,
3. A stationary phase with continued acid production.

The results from a typical batch fermentation are shown in Figs. 2 and 3. The solid curves represent the results from the mathematical models developed for substrate, cell, and product concentrations in batch culture.

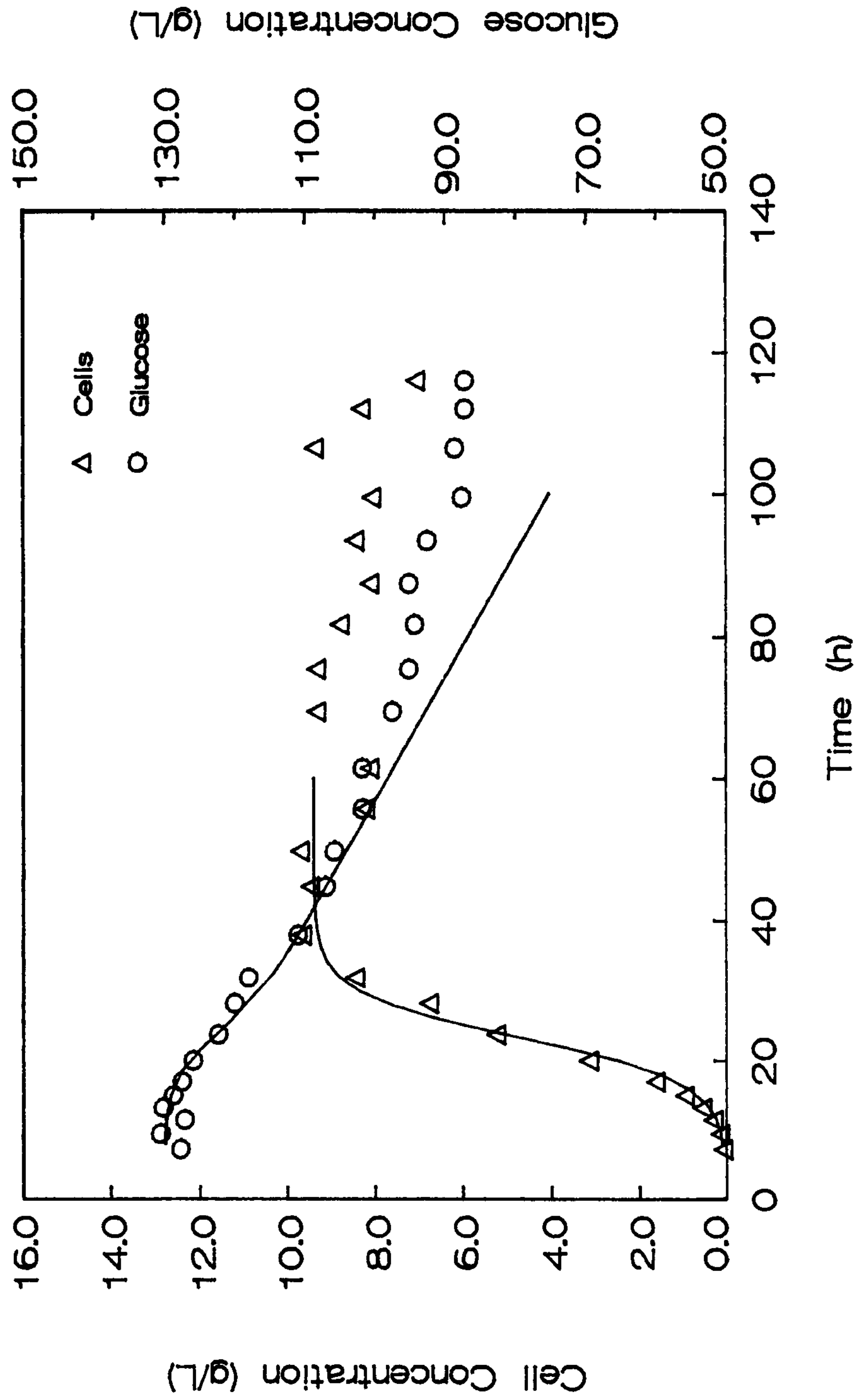

Fig. 2. Typical batch cell concentration and glucose consumption profiles.

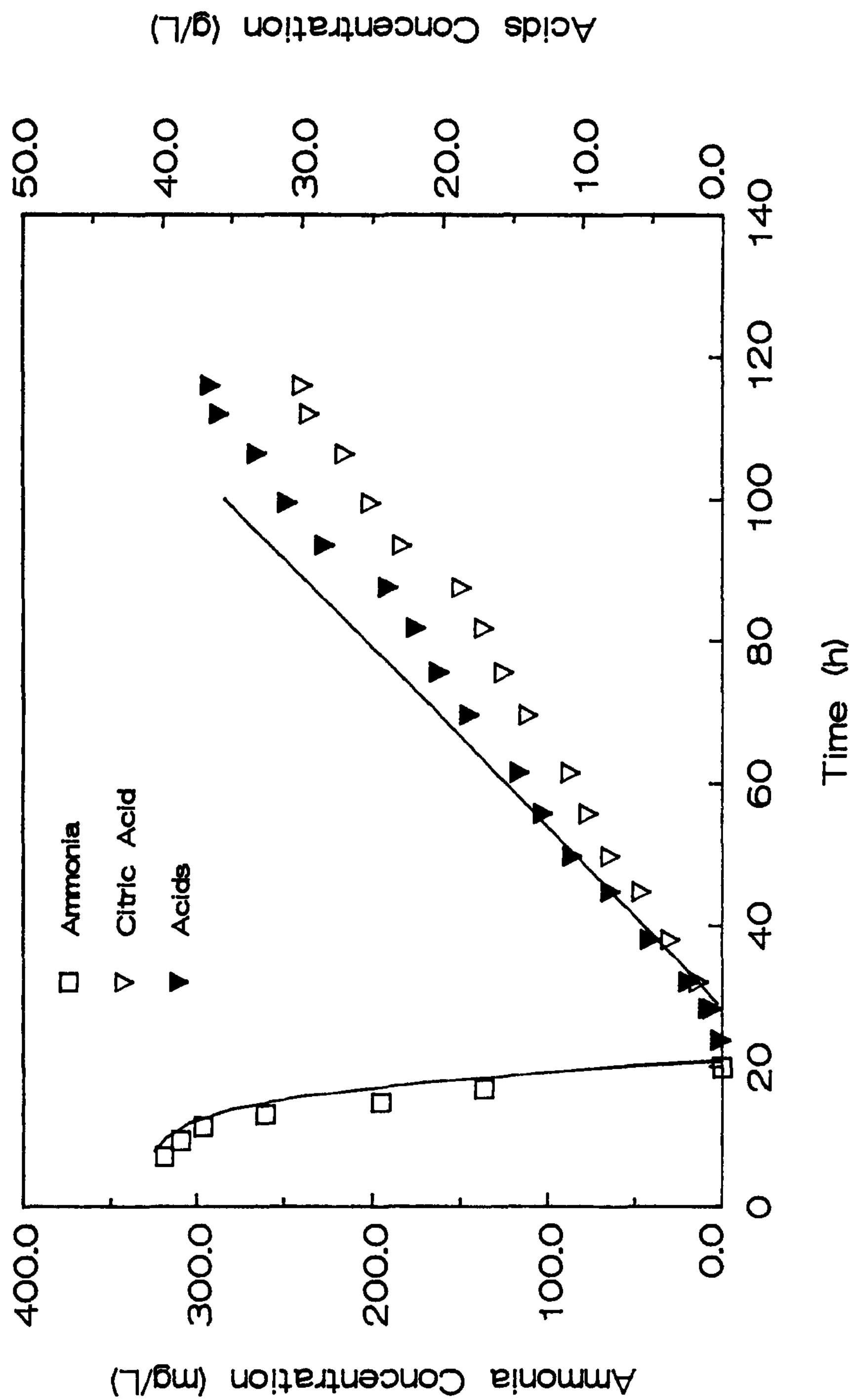

Fig. 3. Typical batch ammonia consumption and acid production profiles.

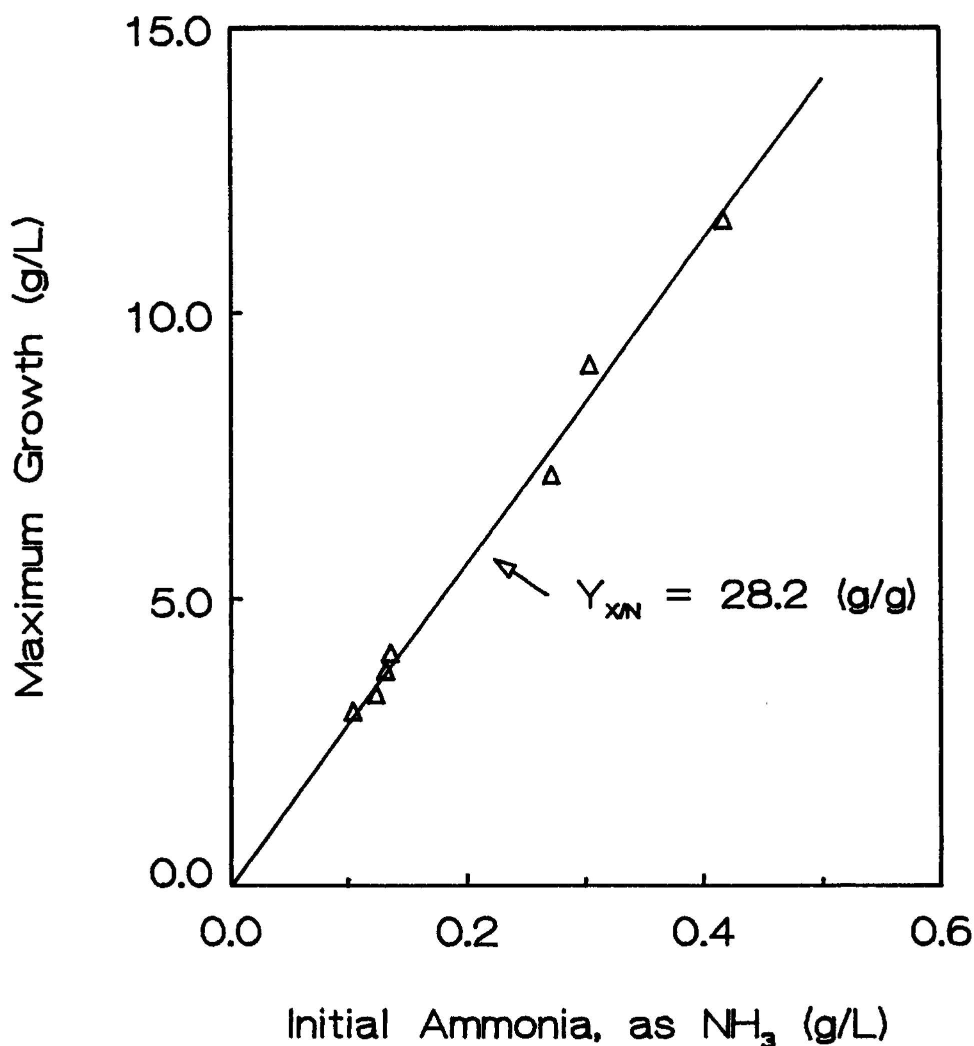

Fig. 4. Cell yield on ammonia.

As noted in Fig. 2, the glucose concentration decreased steadily with time over the entire fermentation. The cell concentration, on the other hand, reached a maximum cell concentration level after 40 h of fermentation. Figure 3 shows that citric acid was produced only after the ammonia was depleted from the medium. A maximum citric acid concentration of 30 g/L was obtained at the end of this experiment. Higher levels of citric acid can be obtained by allowing the fermentation to proceed further.

In measuring the effect of ammonia concentration on growth, the maximum cell concentration was found to be linearly dependent on the initial ammonia concentration in the medium. The overall cell yield, $Y_{X/N}$, was found to be 28.2 g of cells (dry wt)/g of ammonia (as NH_3), as noted in Fig. 4. During non-ammonia limited growth, an average yield, $Y'_{X/N}$ of 9.2 g cells/g NH_3 was found. It is important to realize the difference be-

tween the two yields. The overall yield, $Y_{X/N}$, refers to the maximum cell concentration obtained from a known amount of ammonia. $Y'_{X/N}$, on the other hand, is equal to $dX/d[NH_3]$ during the initial phase of the batch fermentation when ammonia is still present in the medium. $Y_{X/N}$ was found to be approximately three times higher than $Y'_{X/N}$, indicating a cell increase of about 200% after ammonia was depleted from the medium.

The growth of *S. lipolytica* in batch culture was modeled using a logistic curve, shown in Eq. (1) below. The simplicity of this equation is also one of its drawbacks (*15,16*), since no term is included for substrate concentration. However, since the limiting substrate, ammonia, was not present in the medium during the most part of the cell growth, other empirical equations which relate growth to a limiting substrate were impossible to use, unless intracellular measurements are made.

$$dX / dt = K X (1 - X / X_{max}) \tag{1}$$

Equation (1) implies that growth is proportional to the product of cell concentration and an inhibition term, which is the fraction remaining in reaching the maximum cell concentration. With ammonia limited growth, the maximum cell concentration depends upon the initial concentration of ammonia and the number of cells present at startup. Equation (1) fits the data well in all of the experiments where the limiting nutrient was ammonia. Figure 5 shows a plot of specific growth rate, $1/X\ dX/dt$, as a function of $1-X/X_{max}$, where X_{max} was estimated as:

$$X_{max} = Y_{X/N} ([NH_3]_o + X_o/Y'_{X/N}) \tag{2}$$

The correlation coefficient for the data was 0.92, indicating that the model of Eq. (1) fits the data very well.

The specific production rate in batch culture was found to be constant and equal to 0.053 g/g h. Figure 6 shows a plot of the total acids produced as a function of the time integrated cell concentration at various ammonia concentrations. As noted, a single straight line was obtained, yielding the specific production rate. A specific production rate of 0.05 g/g h was also found by Briffaud and Engasser (*10*) as the maximum specific production rate, when studying the effect of oxygen on production. The ratio between citric acid and isocitric acid varied during the production phase from 3 to 6.5 g citric acid/g isocitric acid. The ratio increased steadily, favoring citric acid as production proceeded.

The glucose consumption rate decreased from a maximum during the early part of the fermentation to a constant specific consumption rate during acid production. The consumption rate can thus be described by the equation

$$- d[GLU] / dt = 1 / Y_{X/G}\, dX / dt + 1 / Y_{A/G}\, d([CIT] + [ISO]) / dt \tag{3}$$

Equation (3) may be integrated to yield the following expression.

$$[GLU]_o - [GLU] = 1 / Y_{X/G} (X - X_o) + 1 / Y_{A/G} ([CIT] + [ISO]) \tag{4}$$

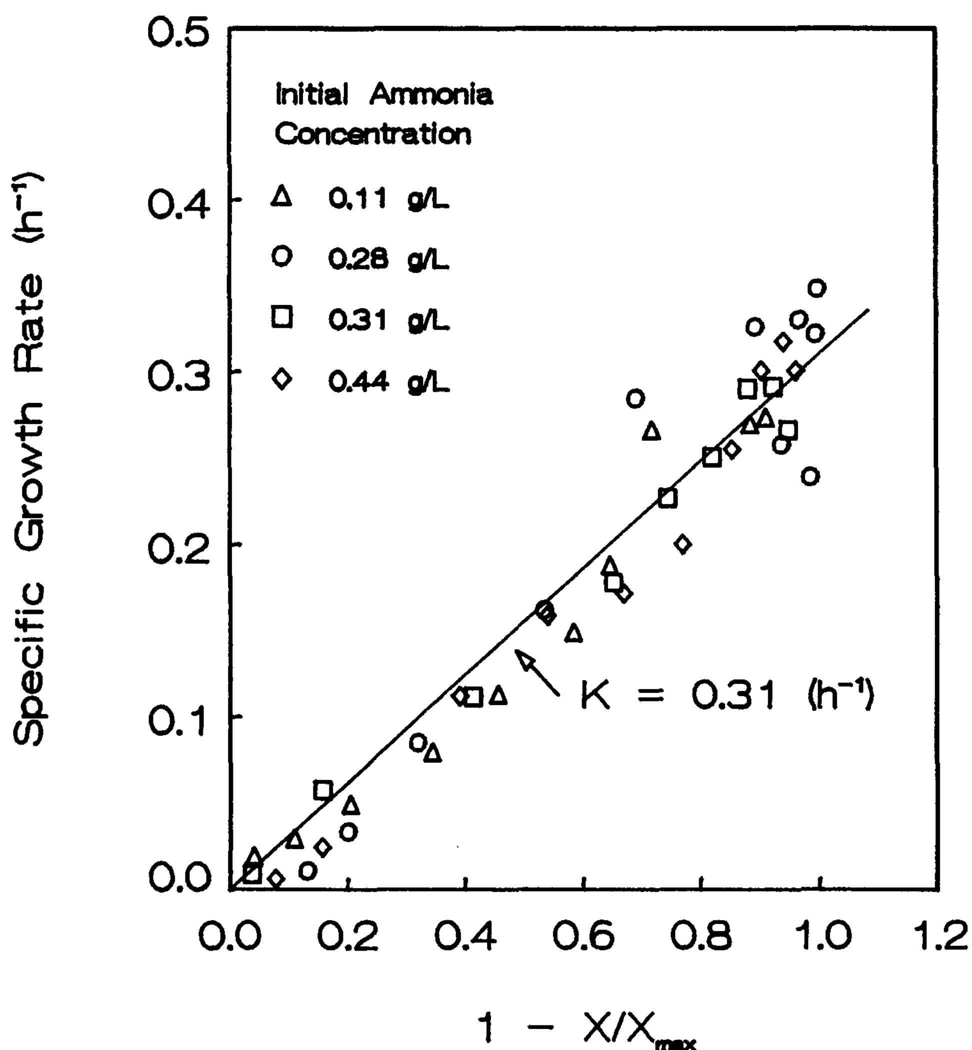

Fig. 5. Testing the batch model of Eq. (1) for growth.

The two yields on glucose, $Y_{X/G}$ and $Y_{A/G}$, were found through multiple regression analysis to be equal to 0.67 and 0.87, respectively. Figure 7 demonstrates the validity of Eq. (4), showing that a slope of essentially unity was obtained when plotting glucose uptake as a function of estimated consumption. In Fig. 7, the points correspond to smoothed data for different initial ammonia concentrations. Any decrease in cell mass during the final part of the fermentation was neglected. Eqs. (1), (2), and (3) together with the two following equations for ammonia uptake and total acid production constitute a differential equation system that describes the time events in a batch citric acid fermentation.

$$- d [NH_3] / dt = 1 / Y'_{X/N} \, dX / dt \qquad Y'_{X/N} = 9.2 \text{ g/g} \qquad (5)$$

and

$$d [CIT + ISO] / dt = \nu X \qquad \nu = 0.053 \text{ g/g h} \qquad (6)$$

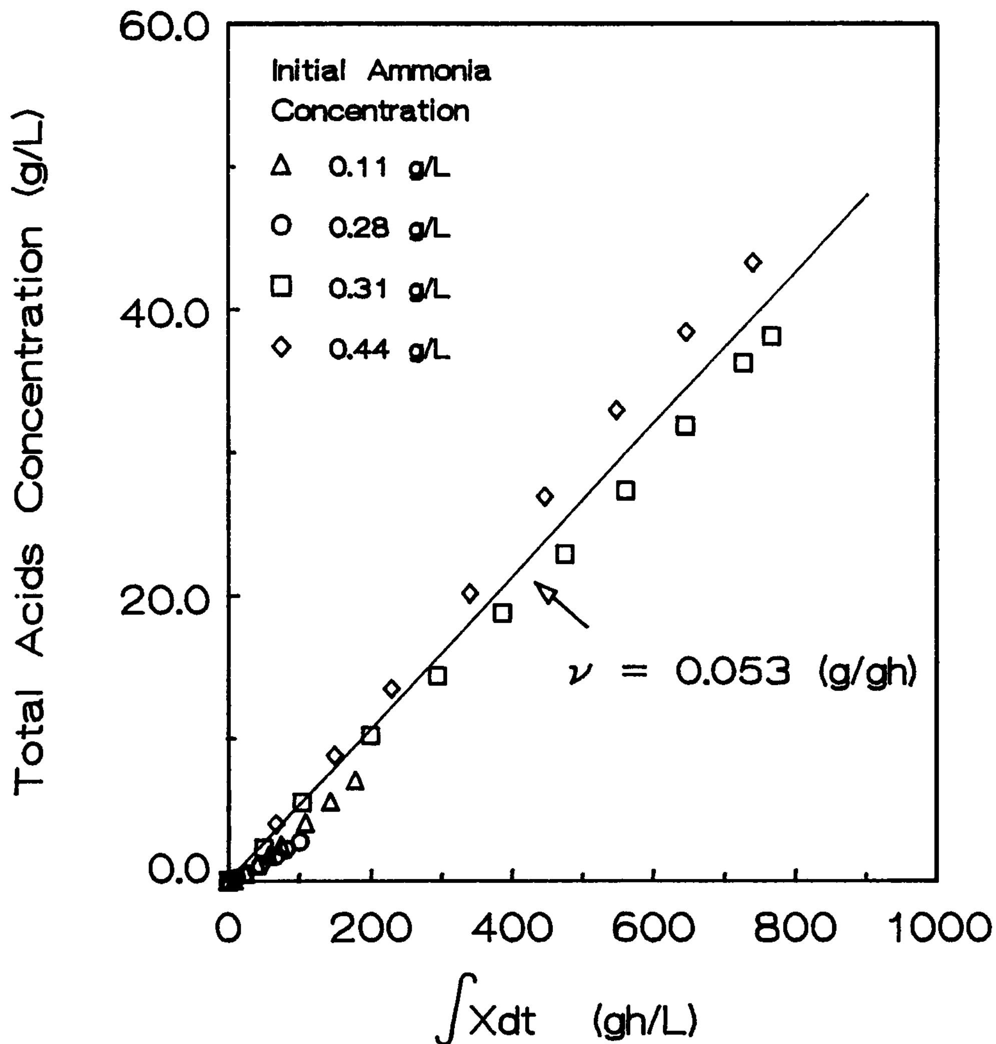

Fig. 6. Determination of specific production rate in batch culture.

As a final check the differential equation system was solved numerically and curves were generated predicting cell, ammonia, glucose, and total acid concentrations. The solid curves in Figs. 2 and 3 correspond to the numerical solutions. A short lag phase for Eq. 6, between ammonia exhaustion and citric acid production was also accounted for when solving the equations. A similar time period after ammonia exhaustion and before citric acid production was found by Behrens et al. (*11*).

CONTINUOUS FERMENTATION RESULTS

Prior to this work, continuous fermentations for citric acid production using yeast had only been run for short time periods. Continuous

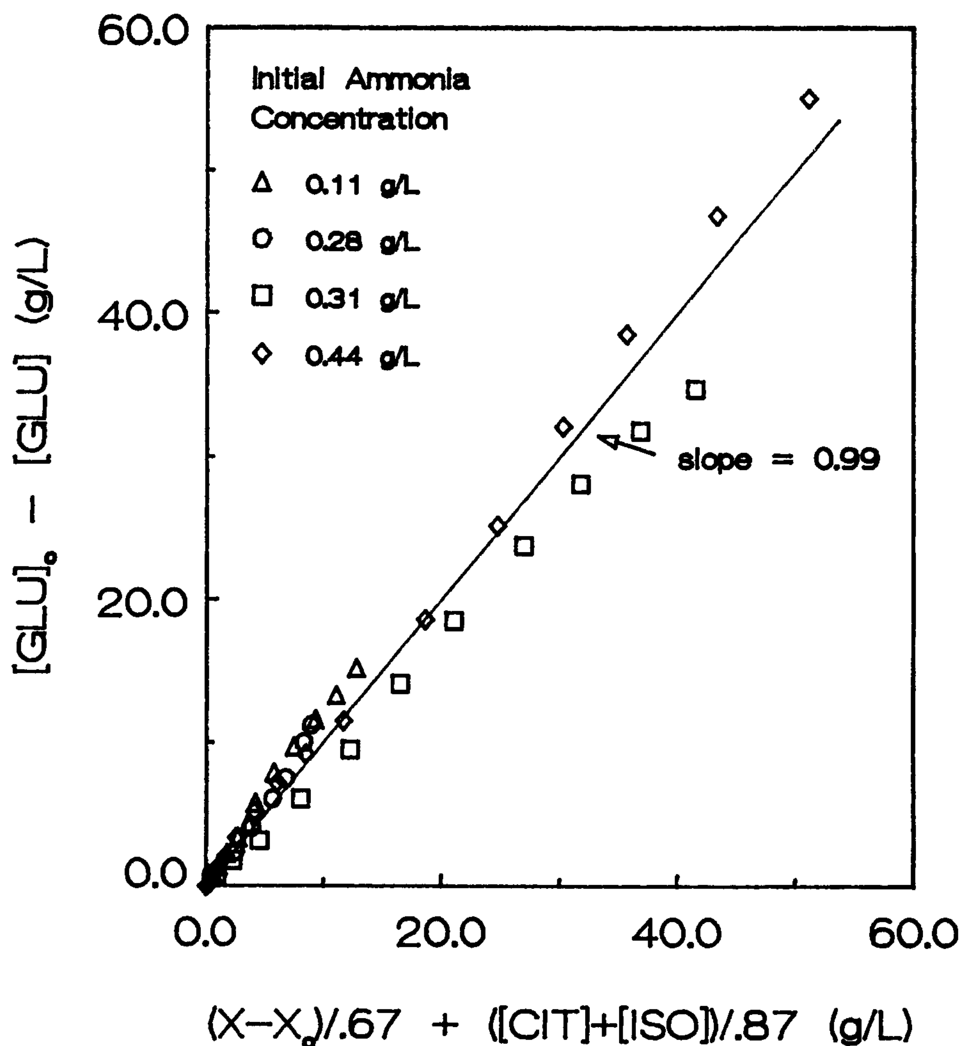

Fig. 7. Testing the batch model of Eq. (4) for glucose uptake.

runs for over 1000 h were carried out in this experimental study. Since the batch fermentation results clearly showed that growth and production were dependent upon the ammonia concentration, continuous fermentations were planned and conducted with different concentrations of ammonia in the feed and variable dilution rates. The batch model predicts that citric acid should be produced when the dilution rate is less than a dilution rate where the rate of ammonia input from the feed exceeds the consumption rate by the cells. Equation (1) may be rearranged noting that $\mu = D$ at steady-state in a continuous stirred-tank fermentor:

$$D = K^c (1 - X / X_{max}) \tag{7}$$

where X_{max} is a function of the ammonia and yeast extract concentrations in the feed. Solving for X, Eq. 7 becomes

$$X = X_{max}(1 - D / K^c) \quad (8)$$

which predicts a linear relationship between cell concentration and dilution rate.

Experiments were conducted in a single-stage continuous vessel, with the results shown in Figs. 8 and 9. Figure 8 shows typical cell concentration profiles for different ammonia concentrations in the feed. Figure 9 shows the corresponding acid profiles. As noted in Fig. 8, a linear relationship was indeed obtained in the region studied. However, since yeast extract was added to the continuous feed in concentrations up to 2.0 g/L, this contribution to cell mass must be accounted for when evaluating X_{max}. Therefore, the following expression was used for X_{max}

$$X_{max} = Y^c_{X/N}([NH_3]_f + [YE]_f Y^c_{N/Y}) \quad (9)$$

where $Y^c_{N/Y}$ corresponds to the ammonia equivalent (as NH_3) of yeast extract. The solids lines in Fig. 8 were determined using Eqs. (8) and (9). The values for K^c and $Y^c_{N/Y}$ were obtained using nonlinear regression analysis as demonstrated in Fig. 10. $Y^c_{X/N}$ was given a value of 28.2 g/g as was found in batch experiments. The latter value of K^c (0.23 h^{-1}) was much lower than in batch culture. This can be explained by considering that the collected data for continuous fermentation only compares to the action during the batch fermentation where growth and production occurred simultaneously. Data for the batch culture in this region would indicate a K value of 0.24 h^{-1}, which is close to the one found in continuous fermentation.

As noted in Fig. 9, the production of acids in continuous culture was not directly comparable to batch culture results. The specific production rate was found to be constant during the batch fermentations, but it changed in continuous culture. It is possible that the nitrogen content per cell was one of the factors affecting the production. For most of the production phase in batch culture (stationary growth phase), the nitrogen content per cell remained constant. In the continuous fermentation this value changed, since for a fixed nutrient composition in the feed, different cell concentrations can be obtained at various dilution rates. The ammonia to cell ratio was therefore different at each steady-state and may have caused the cells to work under different environments.

The glucose consumption rates in continuous culture were studied, comparing well with batch fermentation results. Figure 11 shows the actual glucose uptake rate as a function of the predicted consumption rate calculated using cell and acid yields found in batch studies. The line in the figure corresponds to expected values from batch culture (*see* Eq. (4)). A rigorous multiple regression analysis of the continuous fermentation data gave a cell yield of 0.67 g cell/g glucose and a product yield of 0.61 g acids/g glucose. These results are close to the yields predicted in batch culture.

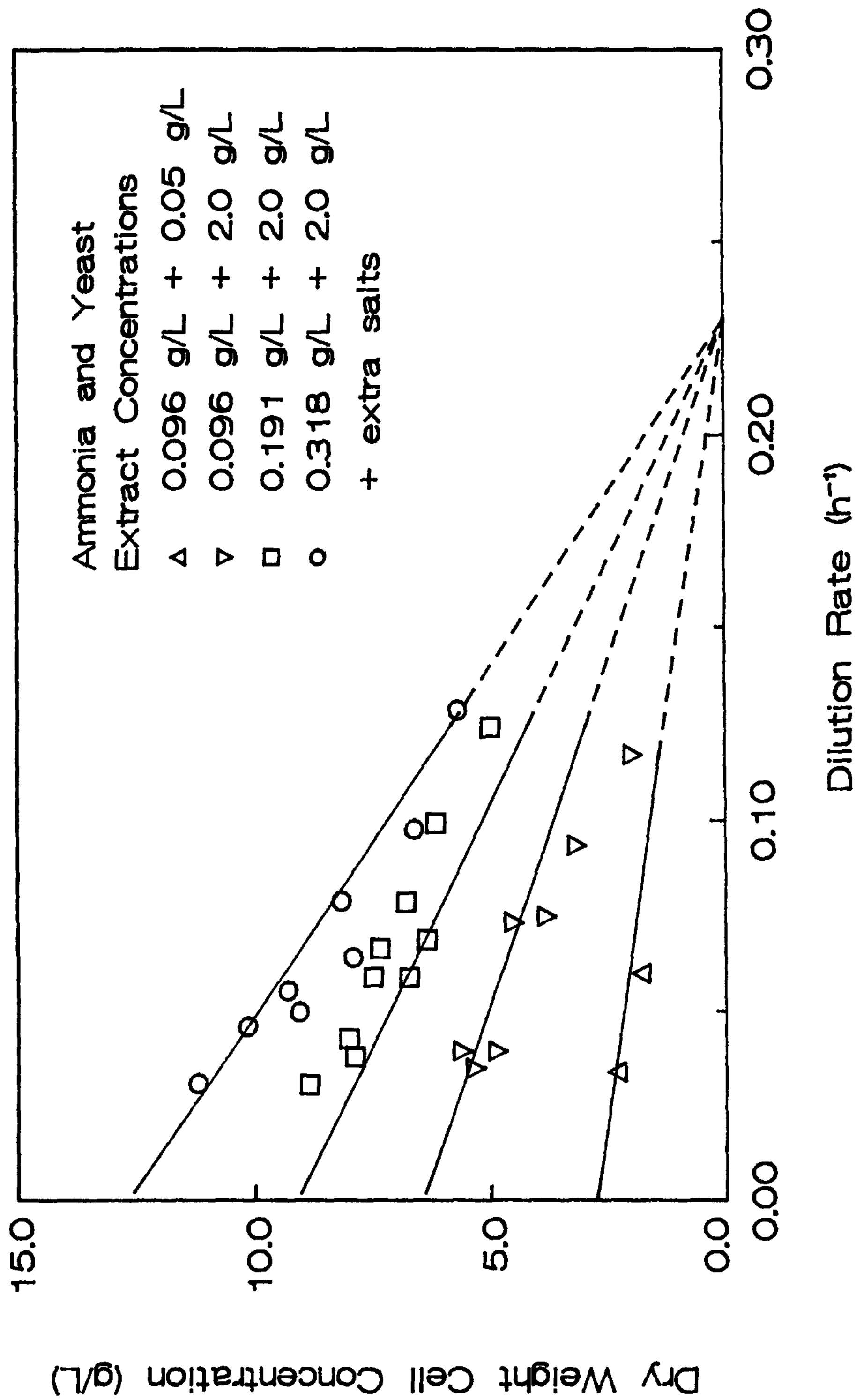

Fig. 8. Typical continuous cell concentration profiles.

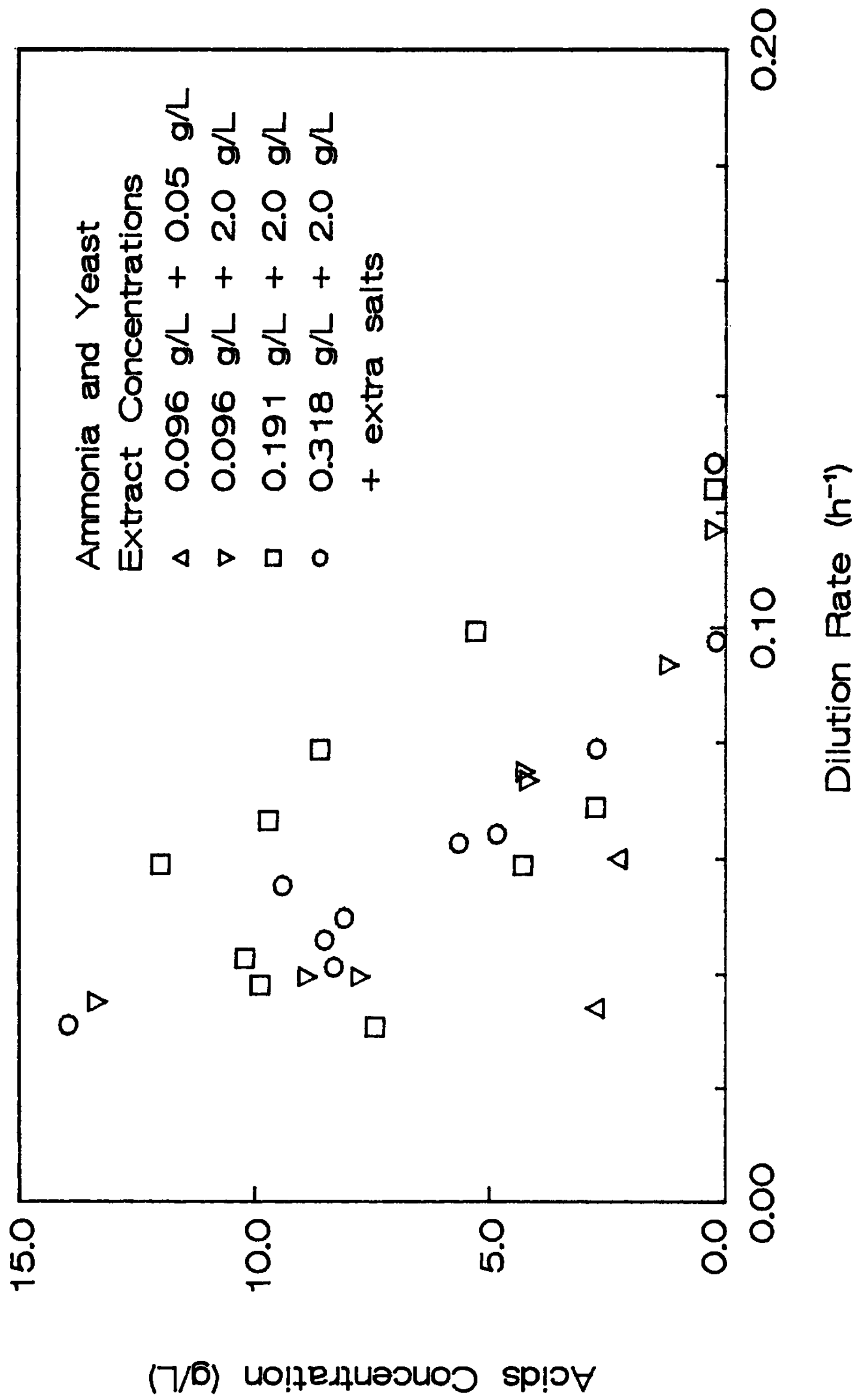

Fig. 9. Typical continuous acids concentration profiles.

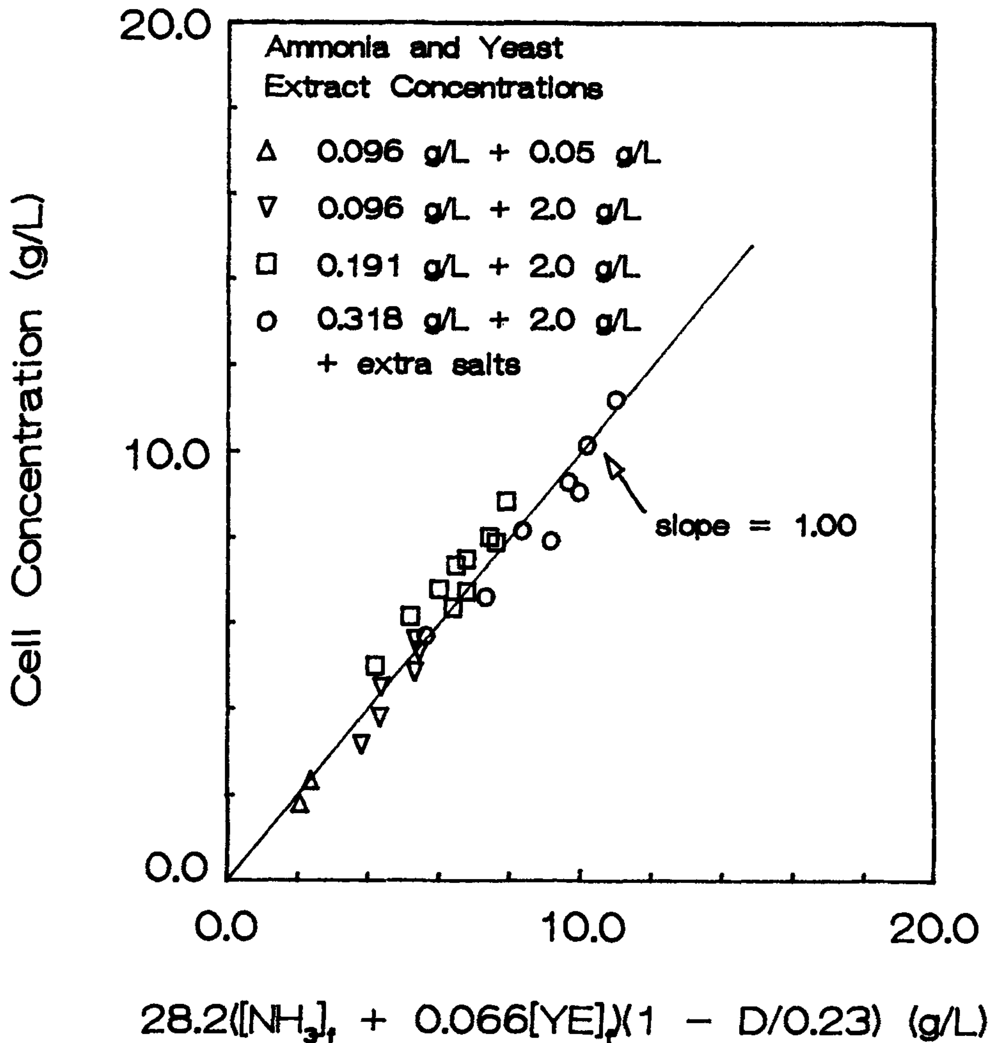

Fig. 10. Testing the continuous model of Eqs. (8) and (9) for growth.

Table 2 shows a summary of the results for the batch and continuous fermentations. The table also lists results for citric acid production by *S. lipolytica* from the literature. Specific production rates from the literature agree well with the results obtained in batch culture in the present work. Continuous specific production rates in the present work were as much as twice the values obtained in batch cultures.

The cell yields on ammonia were essentially the same in all studies reported. Cell yields on glucose in the present study, however, were nearly double the values presented in the literature. The yields of acids on glucose were comparable to the higher values presented in the literature.

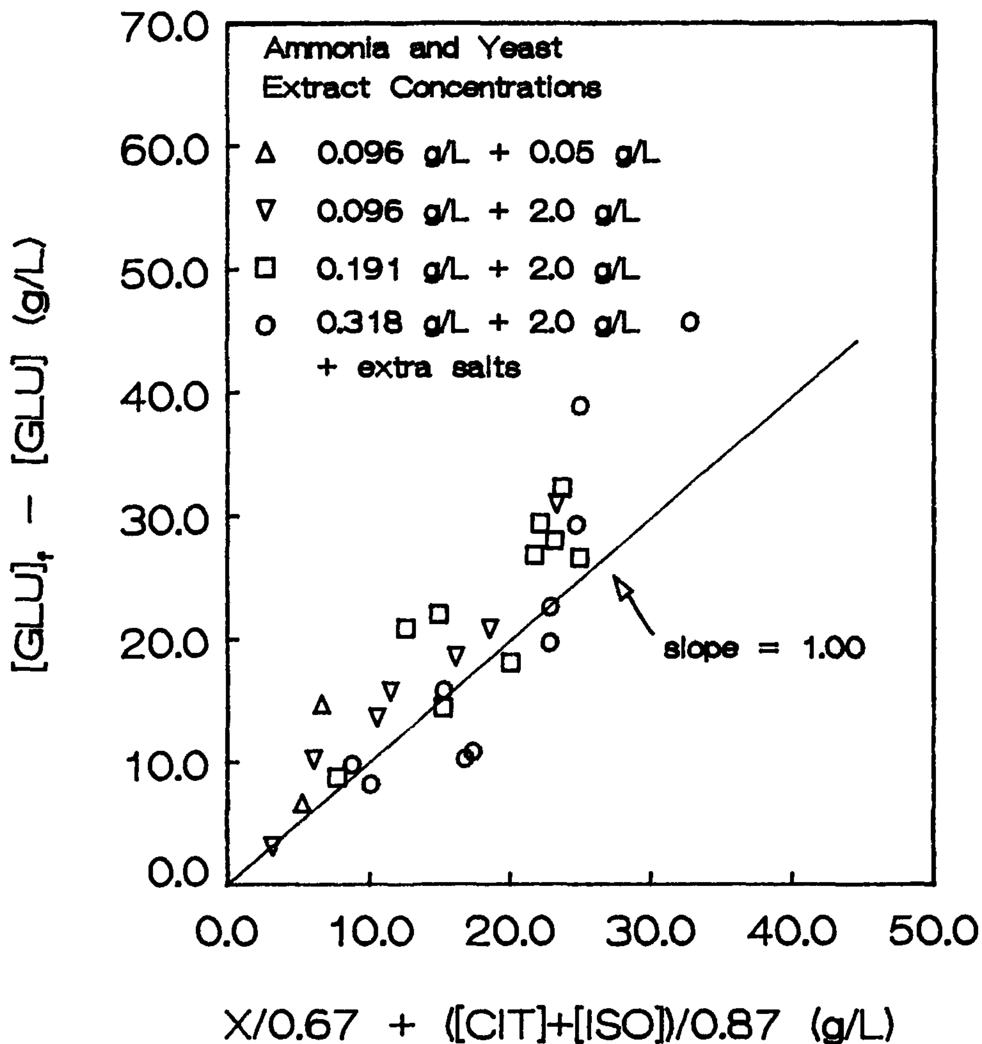

Fig. 11. Testing the batch model of Eq. (4) for glucose uptake in continuous culture.

CONCLUSIONS

Citric and isocitric acids were produced in batch and single-stage continuous culture under ammonia limitation. Batch fermentation results showed slightly overlapping regions of growth and production but, for the most part, the regions were separated. In continuous culture, production and growth occurred simultaneously because of the depletion of ammonia. Specific production rates of acids in batch culture were constant but varied in continuous culture depending on conditions. Continuous experi-

Table 2
Rates and Yields for Citric Acid Production by *S. lipolytica* from Glucose

	ν g/g h	$Y_{X/N}$ g/g	$Y_{X/G}$ g/g	$Y_{A/G}$ g/g
Batch, present work	0.053	28.2	0.67	0.87
Continuous, present work	0–0.11	28.2[a]	0.67	0.61
Enzminger et al. (*4*) Cell recycle	0.045			0.86
Enzminger et al. (*4*) Batch	0.040			0.63
Aiba et al. (*8*) CSTR	0.037		0.32	0.40
Maddox et al. (*17*) Batch	0.070			0.51
Maddox et al. (*17*) Gel immobilization	0.010			0.70
Briffaud et al. (*10*) Batch	0.041–0.05	22.9	0.31–0.42	0.75

[a] At zero dilution rate.

ments ran for over 1000 h, showing no apparent mutations of the yeast. A simple model to predict growth and production was used in batch culture and the same type of model applied for growth in continuous culture. Acid production in continuous culture was probably affected by the ammonia concentration per cell.

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