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Lysine Production in Continuous Culture

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ABSTRACT

Lysine is an essential amino acid that is widely used as a feed additive. Many animal feeds are deficient in lysine, so the lysine, as well as other amino acids, are added to these feeds to supply an adequate diet. Lysine is also used in pharmaceuticals as a diet supplement.

Lysine is produced commercially in batch culture. Owing to the economic importance and the relatively high annual production of lysine, however, improvements in the process could be made by producing lysine in continuous culture. This paper presents the results obtained from the continuous production of extracellular lysine by direct fermentation by a strain of *Brevibacterium lactofermentum*. These results are compared to batch fermentation results by the same organism.

Index Entries: Lysine; *Brevibacterium lactofermentum*; continuous production; amino acids.

NOMENCLATURE

ABS	absorbance at 540 nm or 580 nm	
D	dilution rate	h^{-1}
F	feed rate	$1/h^{-1}$
P	product concentration	g/L
S	substrate concentration	g/L
$[Soytone]_o$	Initial Soytone concentration	g/L
t	time	hr
t_{lag}	lag phase time	hr
V	reactor volume	L
X	cell concentration	g/L
X_o	initial cell concentration	g/L

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$Y_{x/s}$	apparent cell yield on glucose	g cells/g glucose
$Y_{p/s}$	apparent lysine yield on glucose	g lysine/g glucose
$Y_{p/x}$	lysine produced per cell	g lysine/g cell
μ	specific growth rate	h^{-1}
μm	maximum specific growth rate	h^{-1}

INTRODUCTION

Fermentation as a method for the industrial production of amino acids began in 1957 when Kinoshita discovered that large quantities of glutamic acid could be accumulated in the culture broths of certain bacteria (*1*). After the discovery and successful utilization of glutamic acid producing bacteria, researchers began searching for organisms capable of producing sufficient quantities of other amino acids in their culture broths.

The discovery and successful mutation of several organisms capable of producing significant quantities of other amino acids led to a rapid expansion of the amino acid industry during the 1970s. Growth rates of 7–10% per year for this industry are still expected (*2,3*). Also, with the increased need for plant proteins as food sources throughout the world, there will be an increasing demand for many of the essential amino acids, such as lysine, to improve the protein quality of these food sources.

The industrial fermentation of amino acids is typically a batch process. Most of the amino acids are produced in relatively small volume. However, a few of the amino acids such as glutamic acid and lysine are produced in large enough quantities to consider continuous fermentation processes. Continuous production could improve the process economics by lowering capital costs and operating expenses.

The purpose of this paper is to study the potential of producing lysine from glucose in a continuous fermentation process using the organism *Brevibacterium lactofermentum*. Experimental results are shown and models are presented for batch and continuous fermentation experiments.

EQUIPMENT AND PROCEDURES

Equipment

The equipment used for both the batch and continuous operations was essentially the same. The seed culture was prepared in a 1-L vessel made from a 1-L nongraduated Pyrex cylinder fitted with a septum 2 in. from the bottom for sampling, pH adjustment, and antifoam addition. The cylinder was stoppered with a number four rubber stopper with 2 holes. A fritted tube was placed into 1 of the holes for sparging air into the culture. The second hole was used as an exhaust gas vent.

The fermentation vessel used for all of the batch and continuous experiments was a Biostat M chemostat manufactured by B. Braun Instru-

Table 1
Typical Culture Medium

Nutrient	Concentration
Glucose	50. g/L
Soybean hydrolyzate [Soytone]$_0$	20. g/L
$(NH_4)_2SO_4$	25. g/L
$MgSO_4$	0.4 g/L
KH_2PO_4	1.0 g/L
Mg^{2+}	2 ppm
Fe^{2+}	2 ppm
Biotin	50. μg/L
Thiamine HCl	50. μg/L
Water	1. L

ments. The fermenter was equipped with a pH probe and pH control, an oxygen probe, temperature probe and temperature control, pumps for acid, base, and antifoam addition, and a nutrient dosing pump for continuous feeding. Agitation in the fermentor was controlled between 0–2000 rpm. The fermentation vessel had a minimum working volume of 750 mL and a maximum working volume of 1400 mL. When the apparatus was set up for continuous operation, a Masterflex pump was used as an exit pump to maintain a constant liquid level in the fermentor.

Experimental Procedures

Organism

Brevibacterium lactofermentum, ATCC 21798, was used in all studies. The organism was stored on slant cultures in the refrigerator. The slant medium, designated as PY 1%, consisted of 1% peptone, 1% yeast extract, 0.5% NaCl, and 2% agar in distilled water.

Medium

The composition of the medium used in preparing the seed culture and in batch and continuous reactor studies is shown in Table 1. Modifications to this basic medium were made in studying the effects of various nutrients. The pH of the medium in batch and continuous culture was maintained at 7.2 by the addition of 3 *N* KOH.

Fermentation Operation

The agitation rate during batch and continuous operation was adjusted to keep the oxygen level above the 50% saturated value. When the agitation rate became greater than 1500 rpm a mixture of 50% air and 50% pure oxygen was sparged in place of air alone. This allowed the agitation rate to be reduced.

The following variables were studied in batch culture to determine their effects on growth and production: glucose, soybean hydrolyzate (Soytone), $(NH_4)_2SO_4$, $MgSO_4$, KH_2PO_4, $MnSO_4$, $FeSO_4$, biotin, and thiamine · HCl. The soybean hydrolyzate (Soytone) was an enzymatic hydrolyzate of soybean meal produced by DIFCO. Variables studied in continuous culture were glucose and Soytone concentrations over a range of dilution rates from 0.03 to 0.5 h^{-1}. The fermentation broth was sampled routinely for cell, glucose, and lysine concentrations.

Analytical Methods

Optical density was used to measure the cell concentration in the culture. The absorbance of the culture was read using a Bausch & Lomb Spectronic 21 spectrophotometer at a wavelength of 580 nm. The cell concentration was obtained by comparing the absorbance with a standard curve of cell concentration as a function of absorbance. The culture from the experiments was diluted an appropriate amount to keep the absorbance in the range of the standard curve.

Glucose concentration was measured using dinitrosalicylic acid (DNS) by following the reducing sugar concentration as outlined by Clark (*4*). High pressure liquid chromatography was used to measure the lysine concentration in each of the samples. The method was that of Bidlingmeyer (*5*), developed at Waters Associates. The instrument used was a Varian Vista 5500 Liquid chromatograph connected to a Varian 9090 autosampler and a Varian DS604 data station. The column for lysine analysis was a Waters PICO-Tag column and the solvents used were: (1) Solvent A, an aqueous buffer of 0.14 *M* sodium acetate, 0.5 mL/L triethylamine (TEA) adjusted to pH 6.35 with glacial acetic acid, and (2) solvent B, 60% acetonitrile (UV grade) in water. Both solvents were filtered and degassed. The solvent gradient that was run for the separation consisted of 10% B increasing to 51% B in 10 min. After this, a washing step was programmed to 100% B to clean the column.

RESULTS AND DISCUSSION

Batch Fermentation Results

The fermentation results reported in patents for producing lysine were all conducted using high levels of soybean hydrolyzate (*6–10*), usually around 15 g/L. This was done to supply any required amino acids for the organisms that are typically auxotrophic mutants. The first series of experiments in this research were conducted to determine what level of soybean hydrolyzate (Soytone) was necessary for adequate growth and lysine production by *Brevibacterium lactofermentum*. Figs. 1, 2, and 3 are the results of fermentations using 10, 15, and 20 g/L of Soytone, respectively.

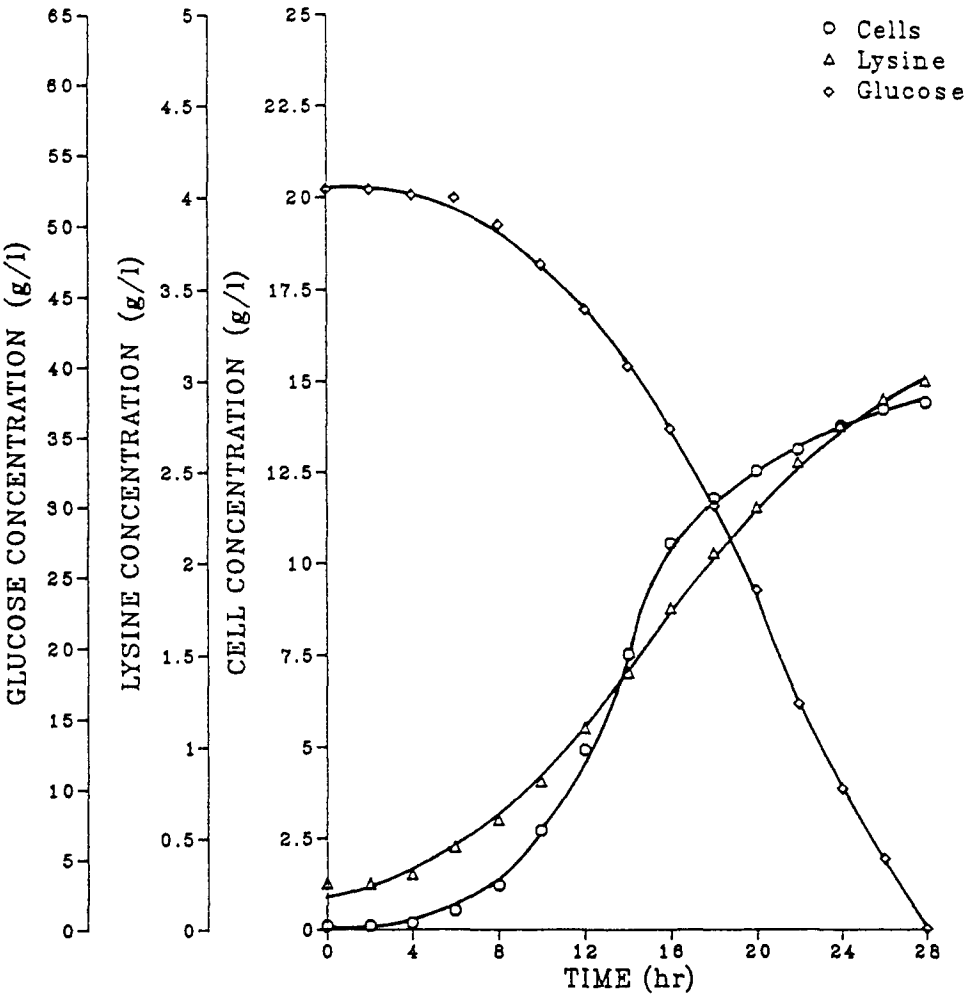

Fig. 1. Batch fermentation results for Soytone = 10.0 g/L.

The initial glucose concentration was held constant at 50 g/L in each of these fermentations. The remainder of the nutrients were present at the levels shown in Table 1.

In comparing the results obtained in Figs. 1-3 it is seen that, in general, the time for complete glucose conversion decreased with increasing Soytone concentrations, whereas the maximum cell and lysine concentrations increased with increasing initial Soytone concentration. As the initial Soytone concentration increased from 10 to 20 g/L, the time for complete utilization of the 50 g/L glucose decreased from 28 to 22 h. Over the same Soytone concentration range, the maximum cell concentration increased from 15 to 30 g/L, and the maximum lysine concentration increased from 2.9 to 4.6 g/L. It is apparent that glucose conversion, maximum cell concentration, and maximum lysine concentration are all affected by the initial Soytone concentration. Since the initial Soytone level was the only differ-

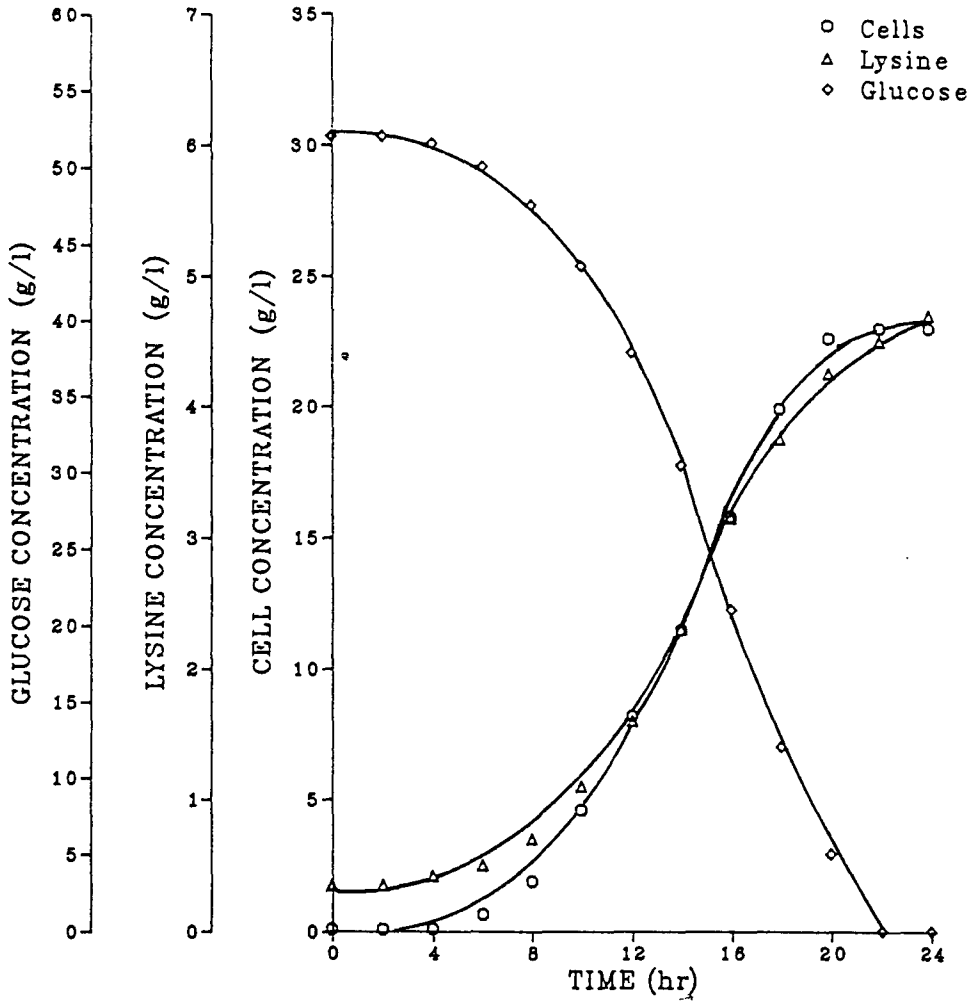

Fig. 2. Batch fermentation results for Soytone = 15.0 g/L.

ence in the fermentations shown in Figs. 1-3, it is apparent that some component in the Soytone was the limiting substrate in these fermentations. Further experimentation (data not shown) showed this component to be serine (*11*).

Both cell growth and lysine production were found to be directly proportional to the initial Soytone concentration (data not shown). The cell yield on Soytone was 1.527 g cells/g Soytone, and the lysine yield from Soytone was 0.243 g lysine/g Soytone.

As is shown in Figs. 4 and 5 the cell yield on glucose, $Y_{x/s}$, and lysine yield on glucose, $Y_{p/s}$, regardless of Soytone concentration, were obtained by plotting cells and lysine produced as a function of the glucose consumed during the growth phase. The cell yield coefficient for all data is 0.48 g cells/g glucose. The lysine yield coefficient for all data is 0.09 g ly-

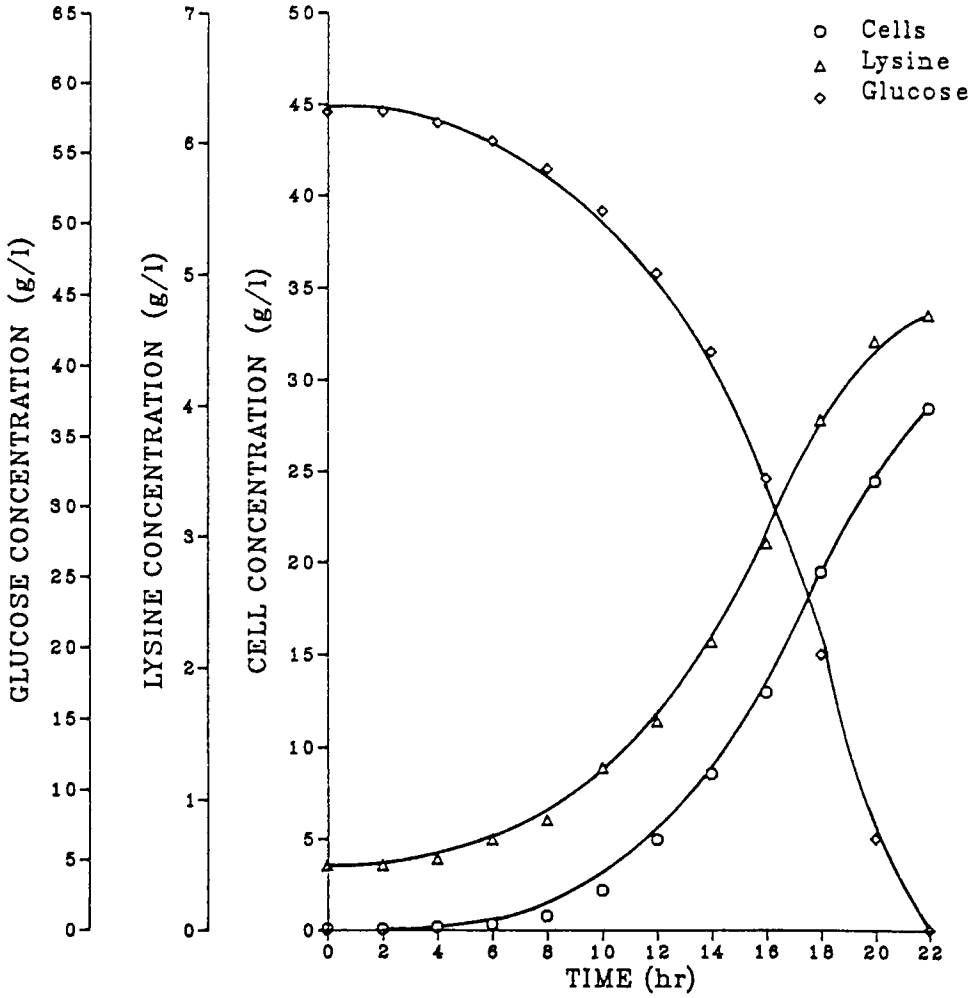

Fig. 3. Batch fermentation results for Soytone = 20.0 g/L.

sine/g glucose. The maximum specific growth rate, μm, was found by plotting ln (x) as a function of time and finding the initial slope. The value of μm was found to be 0.43 h^{-1}.

The effects of all other nutrients shown in Table 1 also were studied. However, these nutrients seemed to have little or no effect on the fermentation in terms of yields or rates with the exception of biotin. Biotin concentrations greater than 50 mg/L decreased the lysine produced per cell from 0.15 g lysine/g cell to 0.10 g lysine/g cell. This result could be caused by the fact that too much biotin can cause decreased permeability of the cell membrane (*12*). The decreased permeability could prevent the excretion of lysine and cause lower production of the extracellular lysine.

An empirical model was developed to describe the growth and production kinetics of *Brevibacterium lactofermentumn* in batch culture. Because

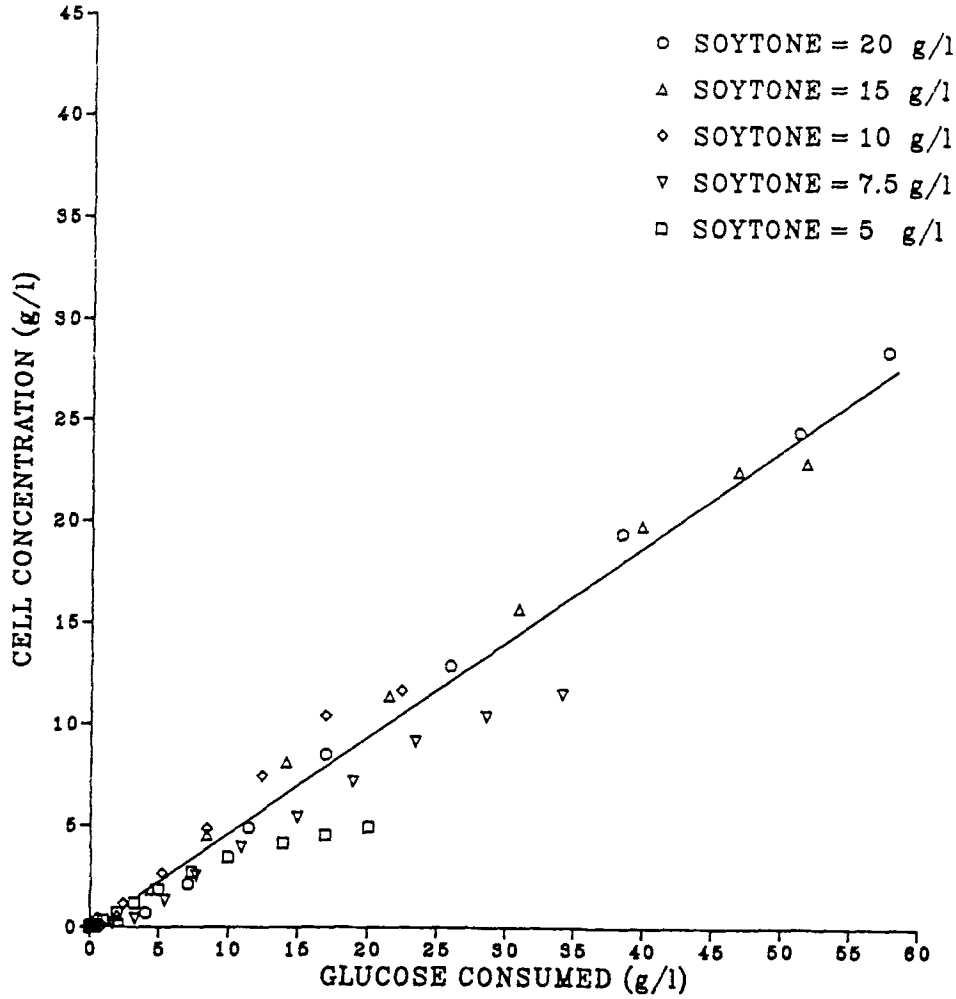

Fig. 4. Apparent cell yield (all data).

the limiting substrate was a component of Soytone and could not be measured, a model to describe the growth and production was developed that requires only the initial amount of Soytone added. The model was developed by noting the existence of a relationship between the initial amount of Soytone per cell, $[Soytone]_o/X$ and the specific growth rate, μ. Therefore, by plotting $[Soytone]_o/X$ as a function of μ the following empirical model was obtained

$$3.60\,\mu + 0.65 = [Soytone]_o/X \quad (1)$$

or

$$3.60\,dX/dt + 0.65X - [Soytone]_o = 0$$

By using the boundary conditions of $X = X_o$ at $t = t_{lag}$, the fermentation can be modeled. After solving for the cell concentration, the amount of sub-

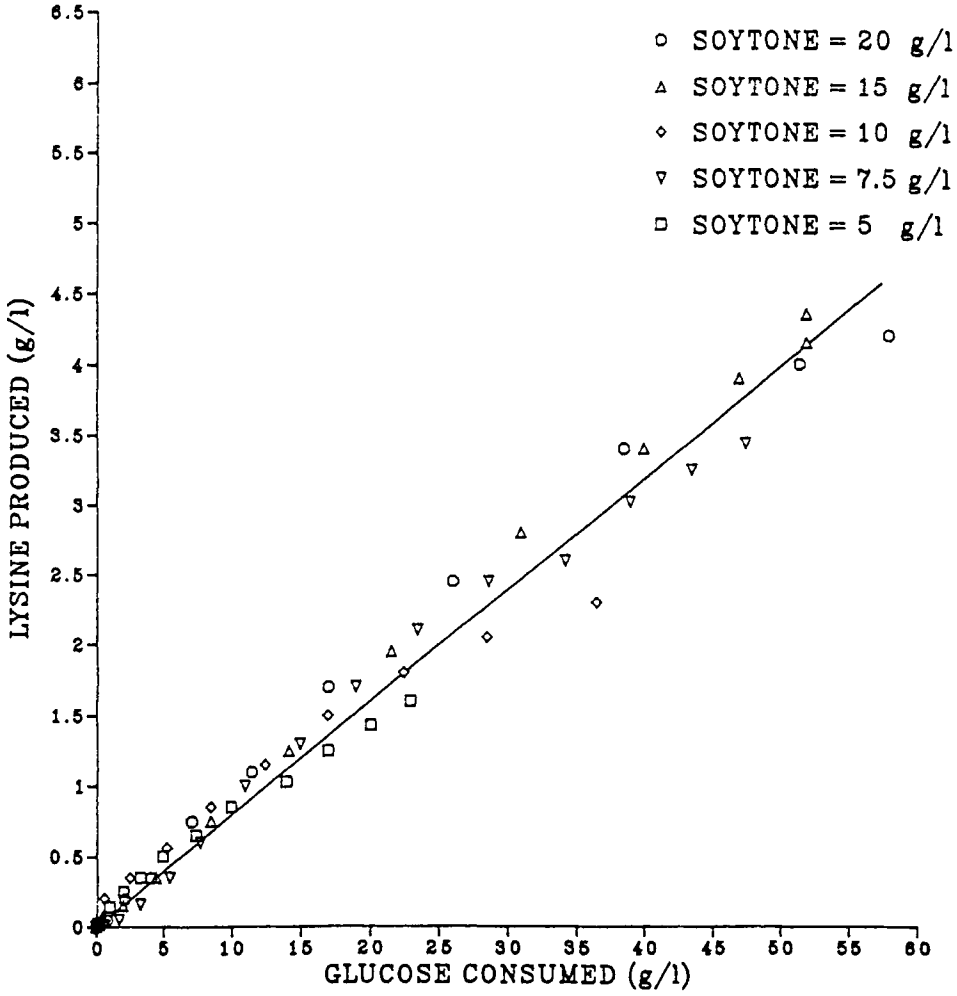

Fig. 5. Apparent lysine yield (all data).

strate consumed and the amount of product formed can be obtained from the apparent yield of cells on glucose $Y_{x/s}=0.48$, and the apparent yield of lysine on glucose, $Y_{p/s}=0.09$, or lysine per cell $Y_{s/x}=0.15$.

$$\text{Glucose consumed} = (X - X_o)/Y_{x/s} \quad (2)$$

$$\text{Lysine produced} = (\text{glucose consumed})\, Y_{p/s} \quad (3)$$

$$\text{or Lysine produced} = (X - X_o)/Y_{p/x} \quad (4)$$

By using Eq. (1-4) the batch fermentations can be modeled completely. Figure 6 is a comparison of the results of the model with the experimental results for initial Soytone concentrations of 20 g/L and 10 g/L. The model is shown as continuous lines. As can be seen from this figure, the model fits the data quite well.

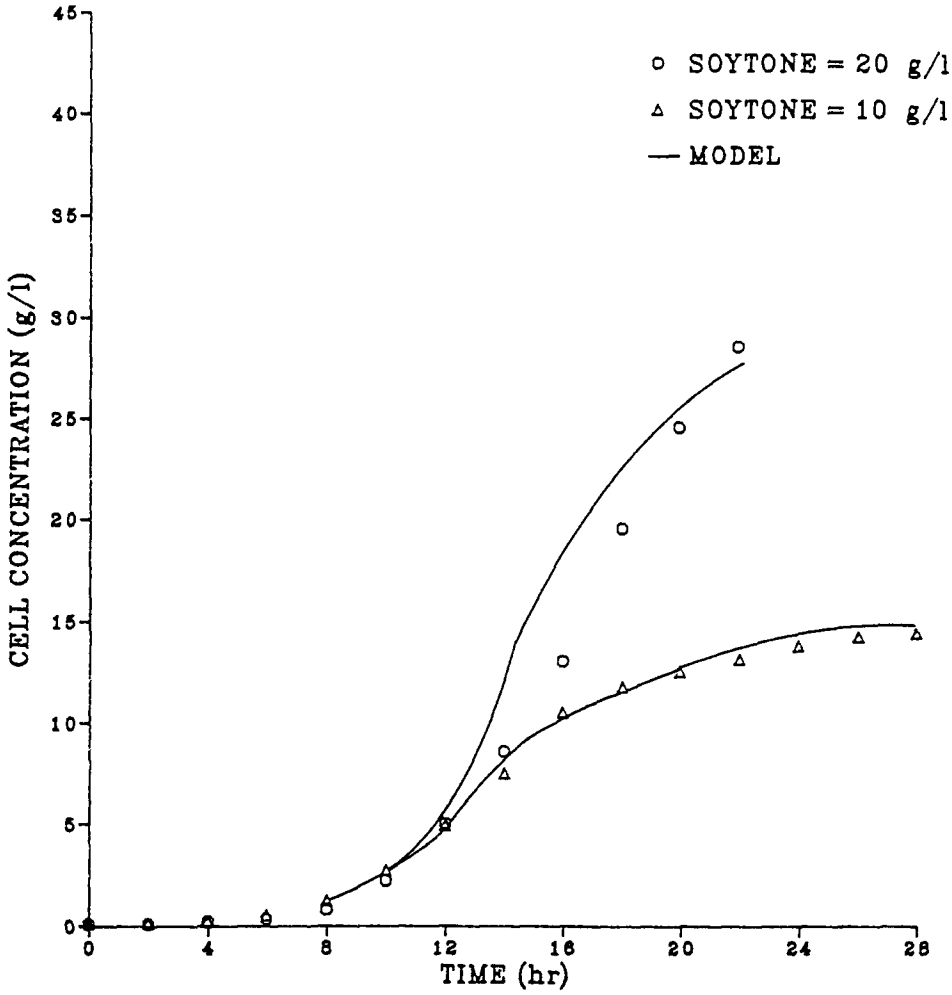

Fig. 6. Comparison of batch model with experimental data.

Continuous Culture Results

Soytone Studies

The results of the batch fermentation studies indicated that the most important component of the medium was Soytone and that the maximum cell concentration was proportional to the initial Soytone concentration. In order to check the effect of Soytone in continuous fermentations, feeds were prepared containing 10, 15, and 20 g/L Soytone. The glucose concentration in the feed was 100 g/L in all cases except at the lowest dilution rate with the 20 g/L Soytone feed. In this case a feed containing 200 g/L glucose was prepared so that glucose would not become limiting. The remainder of the medium was identical to the medium present in Table 1.

The results of the continuous fermentation are shown in Figs. 7-9 for the 20, 15, and 10 g/L Soytone feed concentrations. In these figures, the

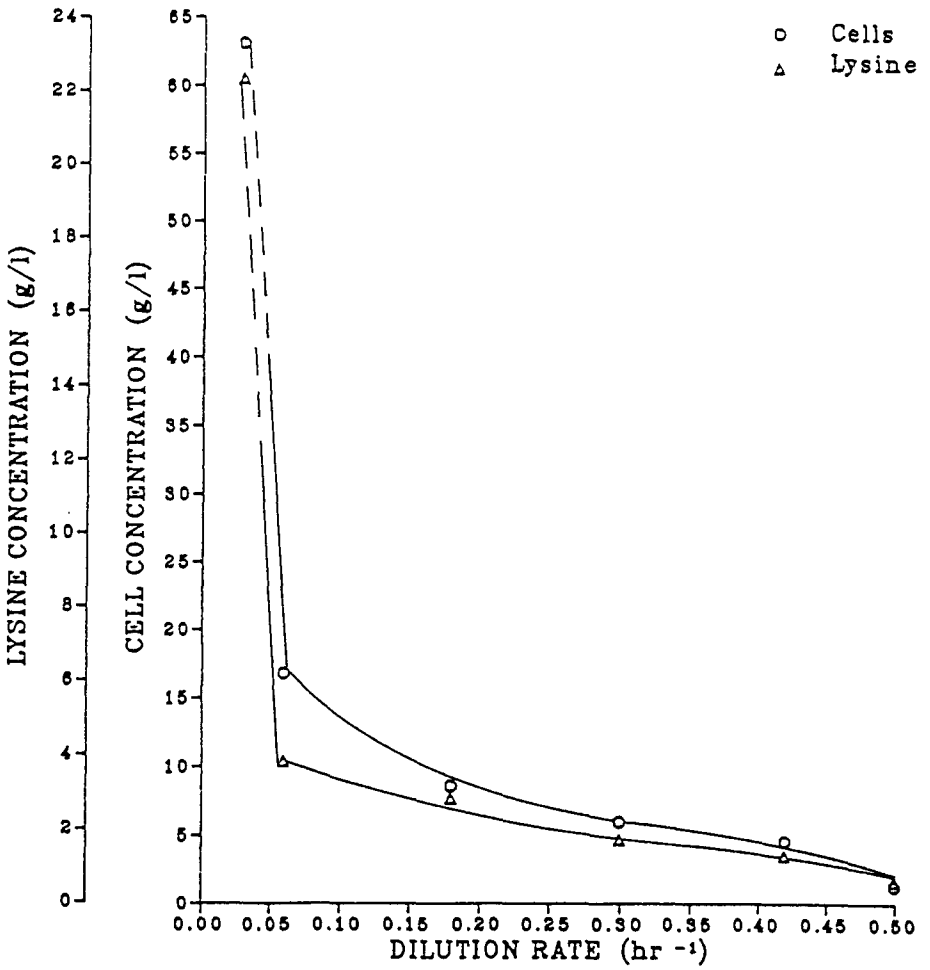

Fig. 7. Steady-state results of continuous fermentation, Soytone = 20 g/L.

steady-state cell concentrations and product concentrations at each dilution rate, D, are presented, where

$$D = F/V \tag{5}$$

In this equation, F is the volumetric feed rate to the fermentor in L/h, and V is the liquid volume in the fermentor in liters.

The solid curves in Fig. 7 indicate the concentrations of cells and lysine obtained when the glucose concentration in the feed was 100 g/L. The dashed lines indicate the levels obtained when the glucose concentration of the feed was 200 g/L.

As noted in the figures, the cell concentration increased with Soytone concentration, reaching 63 g/L with a 20 g/L Soytone concentration at a dilution rate of 0.03 h^{-1}. Cell washout was obtained at all Soytone concentrations at approximately 0.52 h^{-1}. The lysine concentrations also increased with Soytone concentration, reaching 22 g/L with 20 g/L Soytone at a dilution rate of 0.03 h^{-1}.

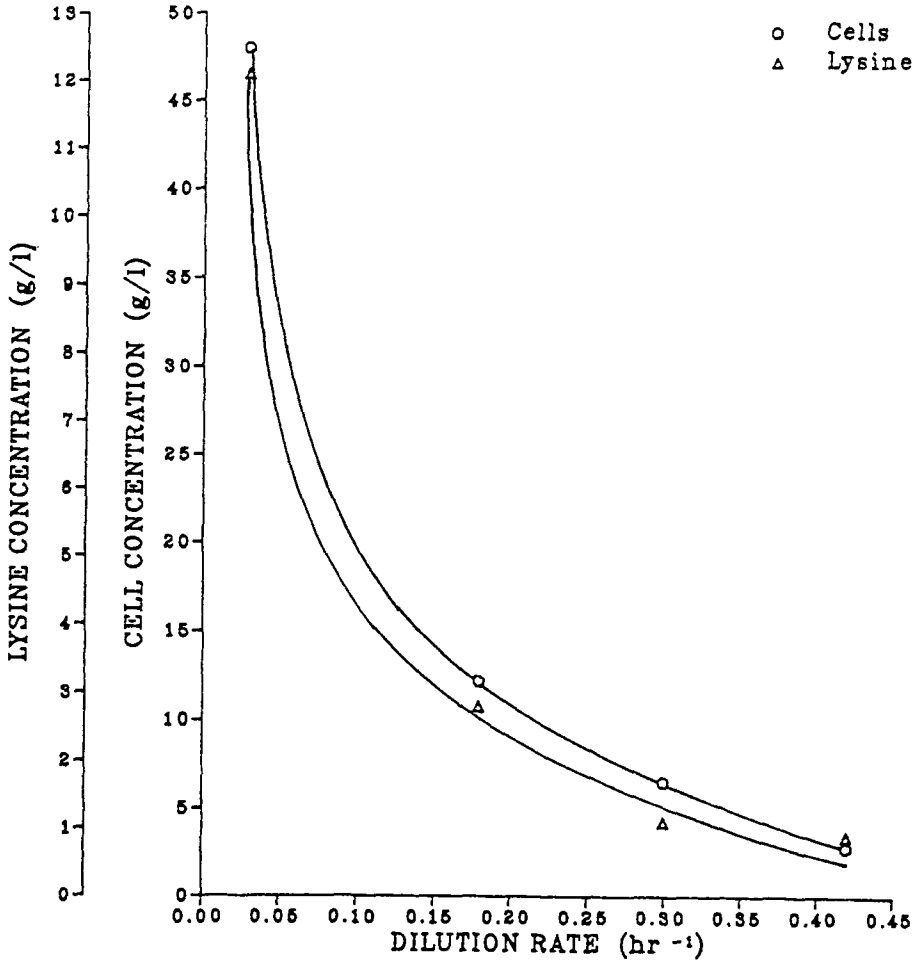

Fig. 8. Steady-state results of continuous fermentation, Soytone = 15 g/L.

The apparent cell yields and apparent lysine yields were determined in the same manner as in the batch studies. The value of $Y_{x/s}$ obtained in continuous culture was 0.47 g cells/g glucose, which compares very well with the value of $Y_{x/s}$ of 0.48 g cells/g glucose in the batch studies. The value of $Y_{p/s}$ was found to be 0.13 g lysine/g glucose in continuous culture compared to 0.09 g lysine/g glucose found previously in batch culture. The apparent lysine yield in continuous culture was 44% greater than that found in the batch studies.

In addition to the lysine yield from glucose being greater in the continuous studies than in batch culture, the cell yield on Soytone showed similar results. At dilution rates above 0.05 h^{-1} the continuous fermentation operated essentially the same as predicted from the batch data. It was expected that as the dilution rate decreased to zero, the maximum cell density would approach the cell density that was obtained in batch studies with identical medium conditions. However, this result did not occur. As the dilution rate was decreased to 0.03 h^{-1}, the cell concentra-

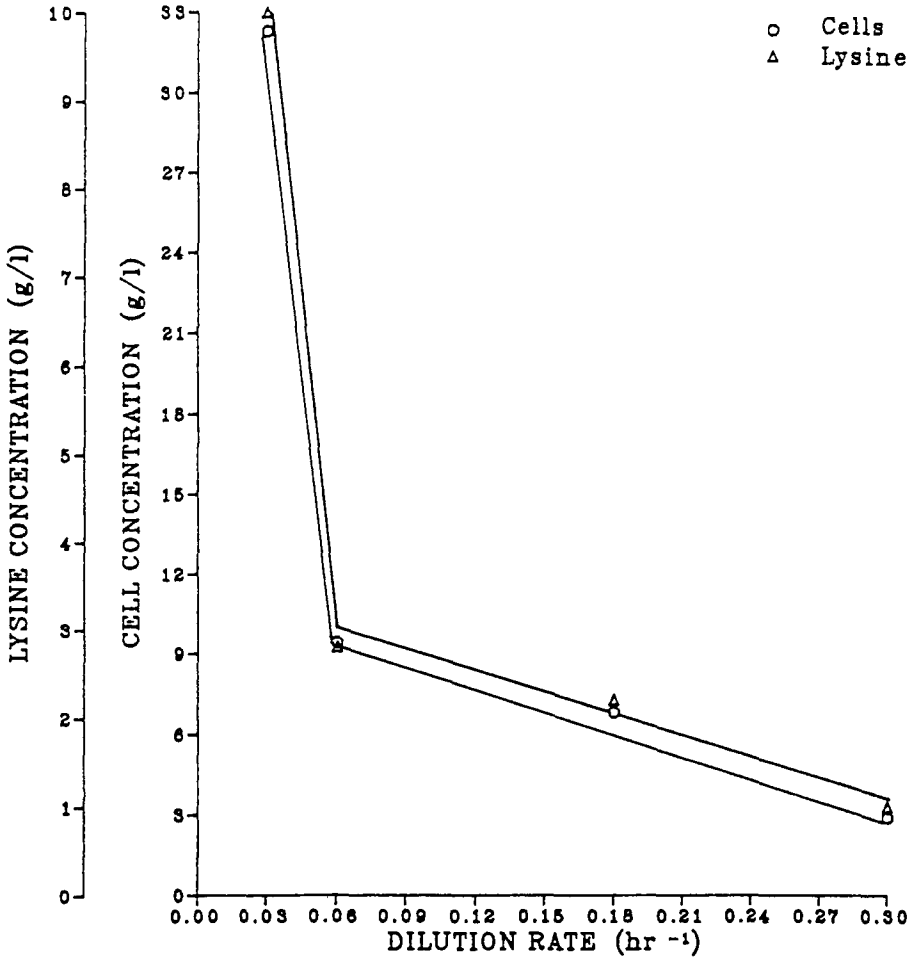

Fig. 9. Steady-state results of continuous fermentation, Soytone = 10 g/L.

tion and the lysine concentration both increased sharply, as was shown in Figs. 7-9. If the cell yield on Soytone is calculated at this point in the continuous fermentation a value of 3.07 g cells/g, Soytone is obtained. This yield is double the value of 1.53 obtained in the batch studies. As a result of this increased cell yield on Soytone, the lysine yield on Soytone also increased to 1.3 g lysine/g Soytone, as compared to 0.243 g lysine/g Soytone in batch culture.

One possible explanation for the increased yields at the low dilution rates is that the cells may have adapted to an environment where they were starved for a limiting nutrient. In continuous culture all of the cells were in the same stage of growth. In batch culture, on the other hand, they were in various stages of growth at any given time during the fermentation. When the medium became low in some essential nutrient in batch culture the cells went into stationary growth phase. In continuous culture, because all of the cells were in the same stage of growth, they may have been able to adapt and divide again using any excess nutrient

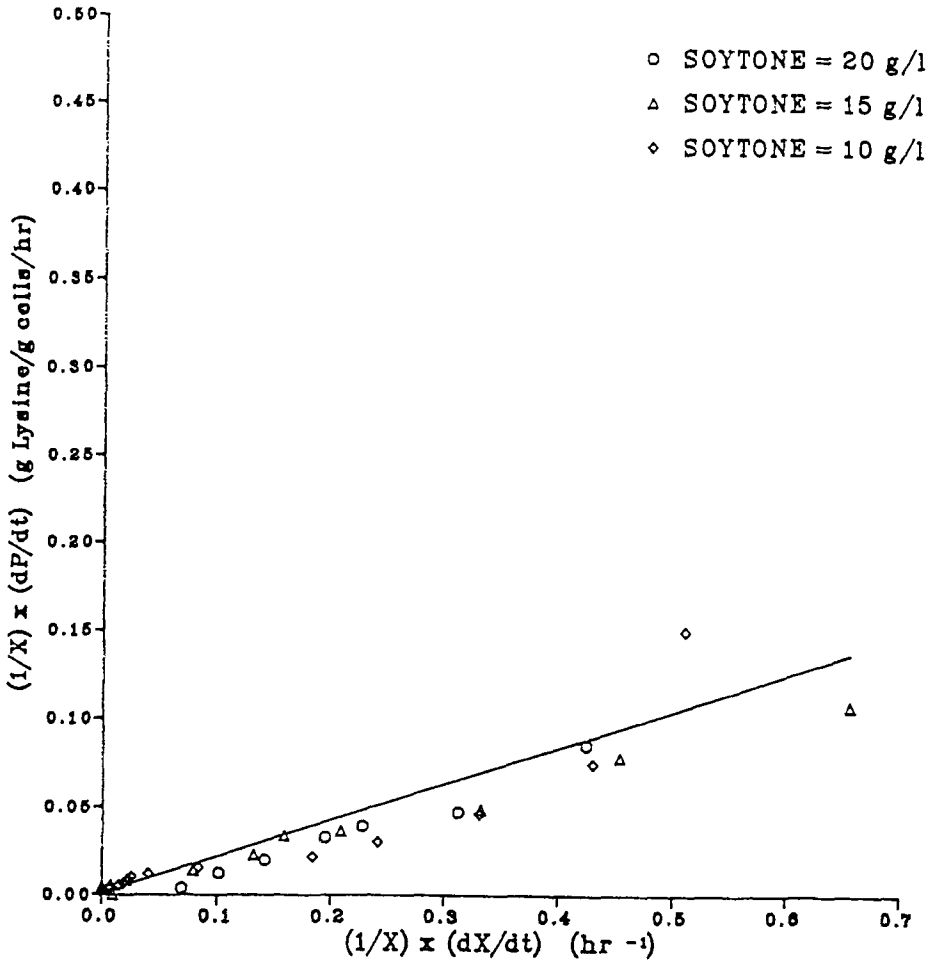

Fig. 10. Lysine productivities in batch culture.

they may have stored. This could explain the doubling of the cell yield as a final division of starved cells.

In addition to higher lysine yields in continuous culture, the maximum lysine concentration also increased. The maximum lysine concentration was 22 g/L at a dilution rate of 0.03 h^{-1} when 20 g/L Soytone was used. The increased lysine concentration certainly represents an advantage for the continuous culture process over batch processing.

The apparent cell yield on glucose, $Y_{x/s}$, in continuous culture was previously found to be 0.47 g cells/g glucose, which agreed with the value obtained in the batch studies.

In order to compare reactor productivities in batch and continuous culture a common basis must be chosen for both the batch culture and continuous culture. The basis chosen here is specific growth rate. Figure 10 is a plot of specific productivity as a function of the specific growth rate for the batch culture studies, where the Soytone concentration was the variable. Figure 11 is an analogous plot for the continuous culture studies.

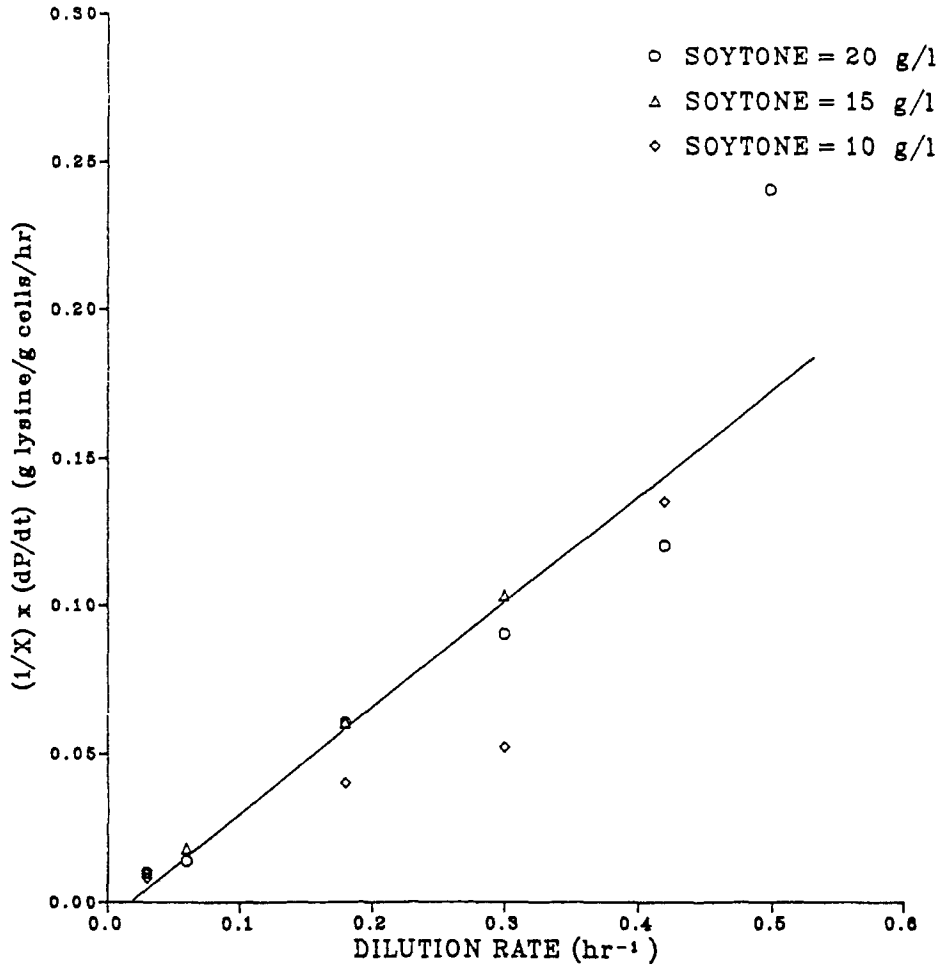

Fig. 11. Lysine productivities in continuous culture.

Both batch and continuous culture systems show a linear relationship between the specific productivity and specific growth rate with slopes of 0.162 g lysine/g cells in batch culture and 0.369 g lysine/g cells in continuous culture. Thus, the specific productivity in continuous culture is 128% higher than in batch culture.

In developing the kinetic models for continuous culture, it was assumed that the same equation form used for the batch system could be applied to the continuous culture system. With this in mind, Eq. (1) was rewritten as

$$A \cdot dX / dt - [\text{Soytone}]_o + BX = 0 \tag{6}$$

In continuous culture

$$dX / dt = D \cdot X \tag{7}$$

where D is the dilution rate. This allows Eq. (6) to be written as

$$A\,(D \cdot X) + BX - [\text{Soytone}]_o = 0 \quad \text{for } D > 0.03\ h^{-1} \tag{8}$$

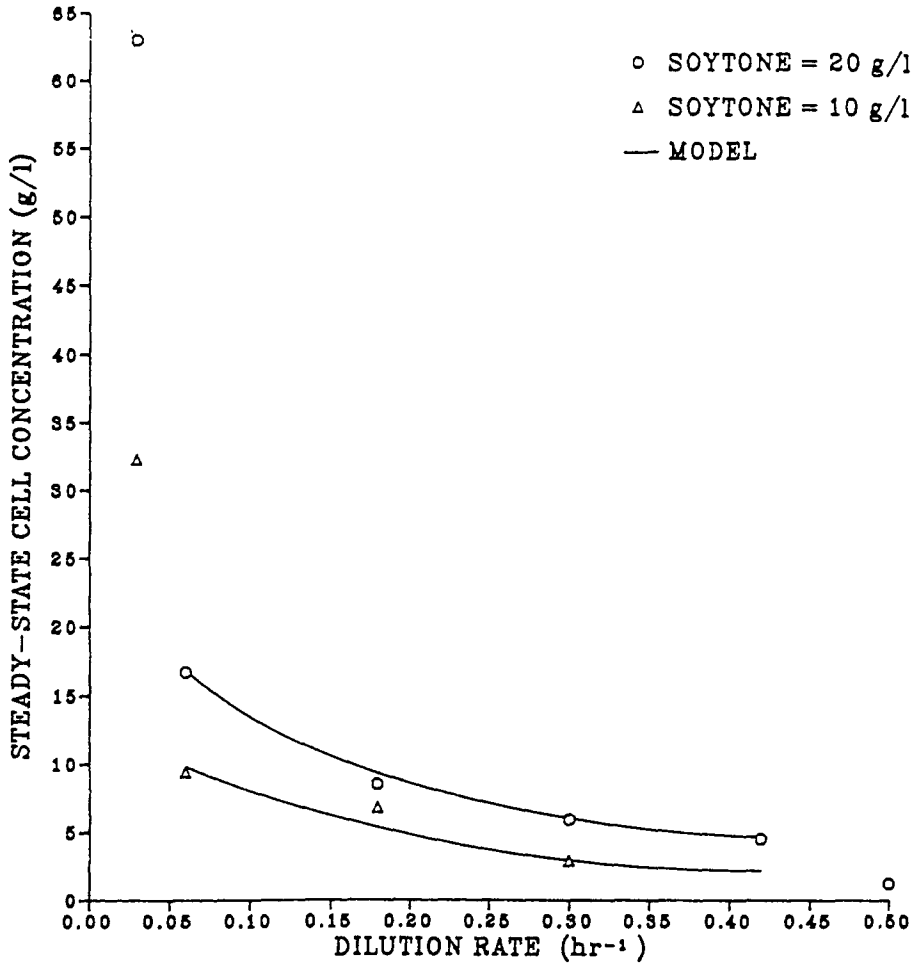

Fig. 12. Comparison of continuous model results with steady-state cell concentrations.

The parameters A and B were found from a least squares fit of $[\text{Soytone}]_o/X$ as a function of the dilution rate, D. The final model for the continuous culture system becomes

$$(8.98\,D + 0.65)\,X - [\text{Soytone}]_o = 0 \qquad (9)$$

The results of this model match the experimental data very closely above dilution rates of 0.03 h^{-1}. Below this dilution rate the model does not apply. A comparison of experimental and calculated steady state cell concentrations is shown in Fig. 12. The experimental and calculated product concentrations are shown in Fig. 13. The amount of glucose consumed and the amount of lysine produced were calculated as in Eqs. (2) and (3). The apparent cell yield on glucose in Eq. (2) is the same for batch and continuous culture. The apparent lysine yield on glucose, $Y_{p/s}$ in Eq. (3), however, is 0.09 g lysine/g glucose for batch studies and 0.13 g lysine/g glucose in the continuous studies. Thus, the apparent L-lysine yield on glucose in continuous culture is 44% higher than in batch culture.

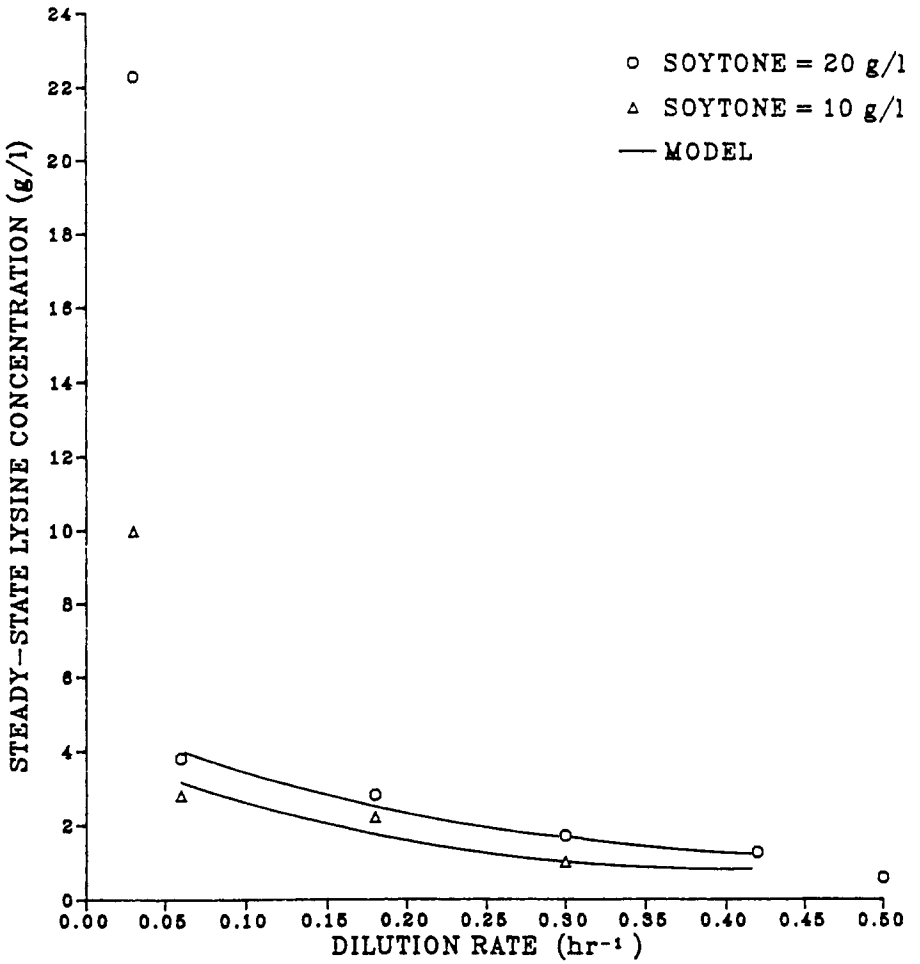

Fig. 13. Comparison of continuous model results with steady-state lysine concentrations.

At dilution rates of 0.03 h^{-1} and below the model will not predict accurately because, as discussed previously, the cell yields on Soytone change at dilution rates of 0.03 h^{-1}.

CONCLUSIONS

Brevibacterium lactofermentum ATCC 21798 was shown to produce good yields of lysine in continuous culture in a continuous stirred tank reactor. The maximum cell concentration and the maximum lysine concentration were found to be much higher in continuous culture as compared to batch, probably owing to the ability of the cells to adapt differently in the continuous culture system. The apparent cell yield on glucose in continuous culture was identical to the value in batch culture, $Y_{x/s} = 0.47$ g cells/g lysine. The apparent lysine yield, however, was higher in continuous culture,

$Y_{p/s} = 0.13$ g lysine/g glucose in continuous culture, compared to $Y_{p/s} = 0.09$ g lysine/g glucose in batch culture.

The initial Soytone concentration was found to affect the steady-state cell concentration and models were developed that can account for this. It was also found that overall reactor performance was better in continuous operation as compared to batch culture based on reactor productivities. The productivity was 128% higher in continuous culture when compared to batch culture.

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