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A Mathematical Model of Ethanol Fermentation from Cheese Whey

II. Simulation and Comparison with Experimental Data

CHEN-JEN WANG AND RAKESH K. BAJPAI*

Department of Chemical Engineering, University of Missouri-Columbia, Columbia, MO 65211

ABSTRACT

A cybernetic model for microbial growth on mixed substrates, was used to simulate the anaerobic fermentation of cheese whey and multiple sugars in semisynthetic media by *Kluyveromyces marxianus* CBS 397. The model simulations quite successfully predicted the observed behavior in batch and during transients in continuous operation, in single-substrate systems as well as in media involving multiple substrates, and in semisynthetic and reconstituted cheese whey solutions. The results of simulations and their comparison with the experimental data are presented.

Index Entries: Cybernetic model; batch culture; chemostat; transient; simulations.

INTRODUCTION

In Part I of this two-part article, the cybernetic approach of microbial metabolism in presence of multiple substrates was extended to include the product formation observed in anaerobic fermentations. Therein, the cybernetic variable controlling the cell-maintenance function was also modified considering the crucial energy-supplying function of product formation in achieving the cybernetic goal. In this article, the applicability of the extended model has been tested with experimental results under diverse operating conditions.

Some of the experimental data dealing with fermentations of cheese whey by *Kluyveromyces marxianus* CBS 397 have already been presented elsewhere *(1)*, Additional experimental data with single or mixed carbon

*Author to whom all correspondence and reprint requests should be addressed.

Table 1
Composition of semisynthetic medium

Nutrient	Composition, g/L
Sugar	5–20
Yeast extract	4.0
$(NH_4)_2SO_4$	2.038
KH_2PO_4	0.334
$MgSO_4 \cdot 7H_2O$	0.122
$FeSO_4 \cdot 7H_2O$	0.012
$CaCl_2 \cdot 2H_2O$	0.007

substrates (glucose and/or lactose) were collected through specifically designed fermentations in order to identify the parameters of the model. These experiments were conducted in a semisynthetic medium. In this work, the influence of galactose on ethanol fermentations from cheese whey has been disregarded, since it is not significant compared to that of glucose *(1)*.

MATERIALS AND METHODS

Experimental conditions for fermentations of semisynthetic media were kept the same as those reported before *(1)* except for the composition of supplemental nutrients as shown in Table 1. This composition was made to ensure the growth conditions without limitation of nitrogen source.

Analytical methods were also the same as previously described *(1)*. Samples containing <0.3 g/L glucose or lactose were analyzed by the Nelson-Somagyi method *(2)*. In a mixture of glucose and lactose at low concentrations, glucose content was analyzed by a glucose analyzer (Yellow Springs Instrument, Yellow Springs, OH), and lactose concentration was determined by subtracting glucose concentration from the total sugar concentration obtained from the Nelson-Somagyi method.

For determination of cell concentration, 5-mL samples were vacuum-filtered on preweighed 0.2-μm cellulose-acetate membrane filter (Gelman Sciences, Ann Arbor, MI). The solids were washed once with 5 mL distilled water and dried along with the filter in a microwave oven for 5 min at full power (700 W). The solids were cooled in a desiccator for 10 min before weighing.

MATHEMATICAL MODEL

The mathematical model has been described in detail in the previous article, Part I.

RESULTS AND DISCUSSIONS

Simulation of Single-Substrate System

Several batch fermentations were conducted with low concentrations of glucose or lactose in the medium. In lactose media, glucose and galactose were not detected at any time. These batch fermentations were simulated by the extended cybernetic model (Eqs. [19–22] with D = 0 and $i = j = 1$ in Part I) using kinetic parameters obtained from continuous-culture data. The kinetic parameters have been listed in Table 2 of Part I (previous article). Michelson's (3) method was used for the solution of differential equations.

Since the initial concentration of the "key enzyme" significantly influences the duration of lag phase in batch cultures, a successful simulation is determined by the consistent use of the same enzyme level. A low specific enzyme level leads to a longer lag period, whereas a high level reduces it. The specific enzyme concentration depends on culture history. Since the inocula for all the semisynthetic media were prepared with a glucose medium, it is reasonable to set the initial levels at 90% of the maximum specific enzyme activity for fermentations on glucose and at 10% for those on lactose. These hypothetical level have been found to give the best simulation results in this work and in the other study (4). The maximum specific activity of enzyme, e^{max}, was calculated as

$$e^{max} = [(\alpha + a)/(\mu^{max} + \beta)] \quad (1)$$

Figures 1A–1D show the experimental and the computed results of simulation. Profiles of the concentration of key enzymes are not shown, since no corresponding experimental measurements were obtained. Generally, the batch experimental data are in good agreement with model predictions with parameters estimated from continuous-culture data. Evidently, the cybernetic model can be used to simulate batch fermentations satisfactorily as well as the continuous data (*see* Part I). Experimental observations of batch fermentations with lactose generally lasted longer than those with glucose (*see* Fig. 1A–D). The simulations also predicted a similar behavior. When the inoculum was initially grown on glucose and the cells were transferred to a medium containing lactose only, these require a longer period to synthesize enough enzymes to utilize lactose effectively. This lag period is marked by a very slow growth at the beginning of batch cultures. As shown in the Figs. 1A-D, ethanol production profiles follow the same trend as those of cell growth.

Transients in chemostat were studied by changing feed sugar concentrations at constant dilution rate. Both shift-up and shift-down experiments were conducted, and the concentrations of cell mass, sugar, and product (ethanol) were monitored during shift from one steady state to another. These transient results are plotted in Figs. 2A–D as discrete points. The time in abscissa with negative values indicates the original steady state before a transient experiment was started. For fermentations of glucose,

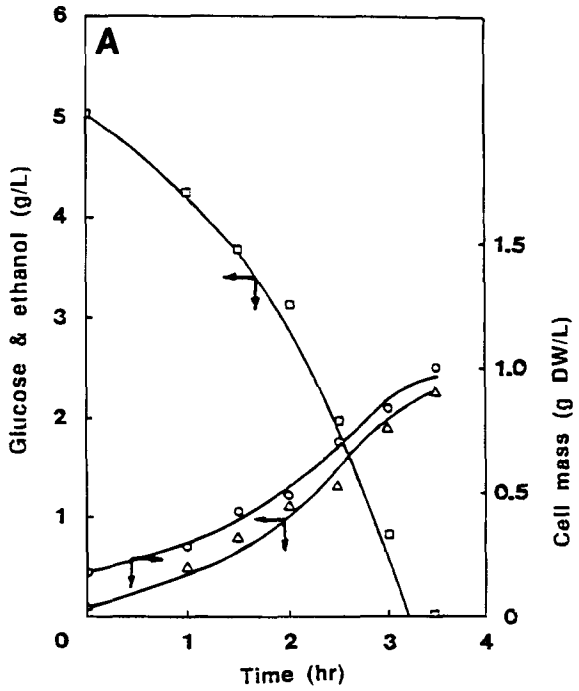

Fig. 1A. Batch fermentation of 5 g/L glucose. Symbols: □, glucose; ○, cell mass; △, ethanol. Solid lines represent simulation results.

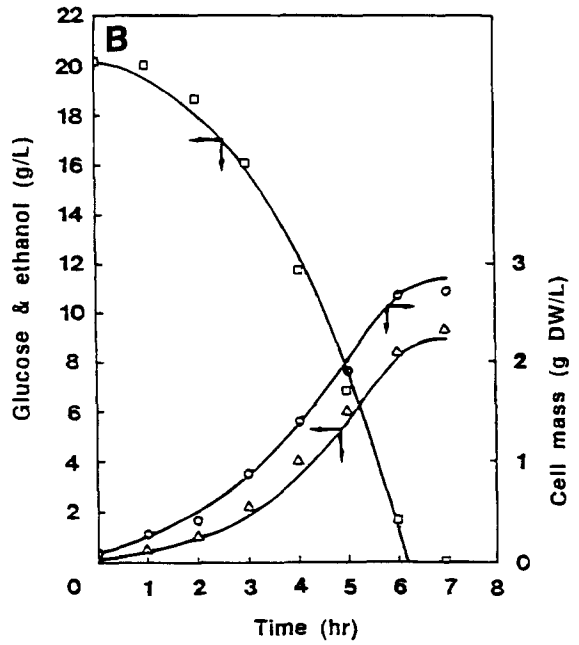

Fig. 1B. Batch fermentation of 20 g/L glucose. Symbols: □, glucose; ○, cell mass; △, ethanol. Solid lines represent simulation results.

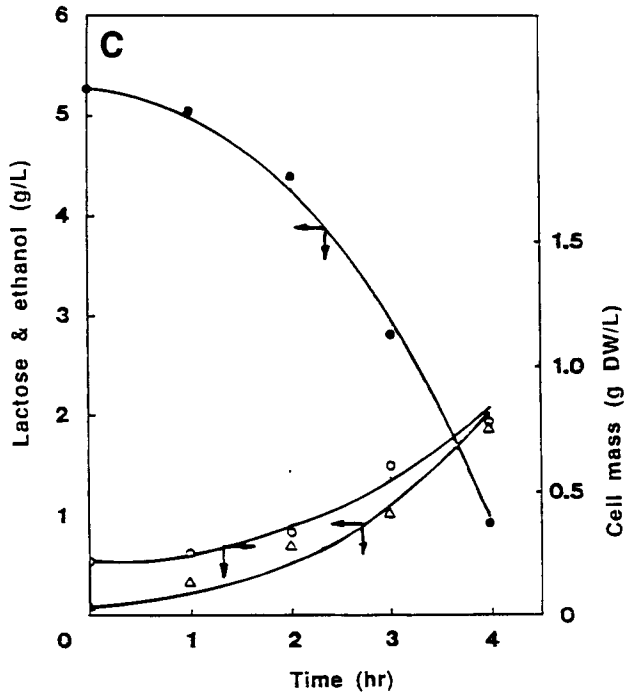

Fig. 1C. Batch fermentation of 5.3 g/L lactose. Symbols: ●, lactose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

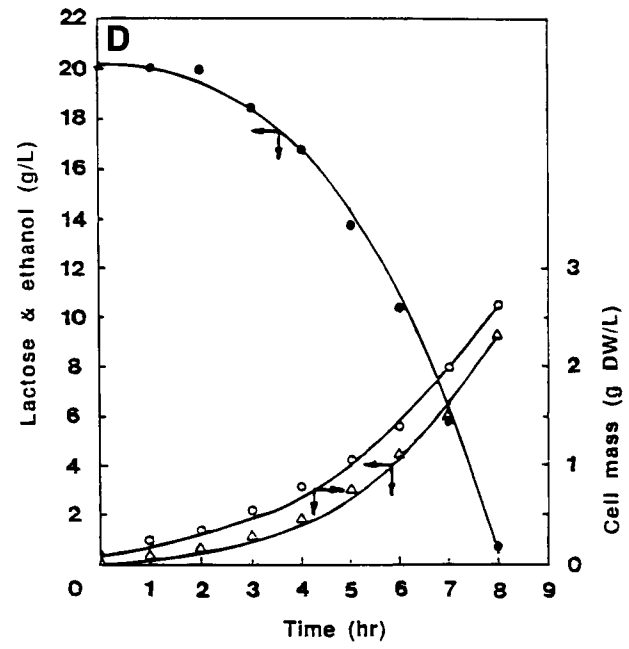

Fig. 1D. Batch fermentation of 20 g/L lactose. Symbols: ●, lactose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

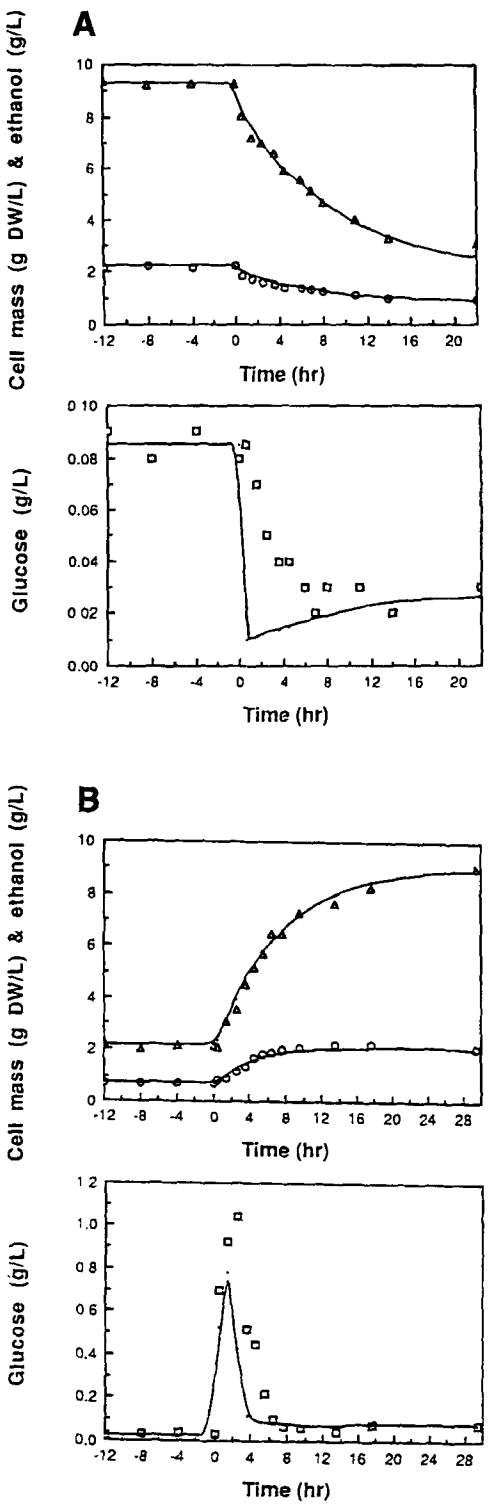

Fig. 2A. Transient response of continuous culture to the change in feed glucose concentration from 20–5 g/L at a dilution rate of 0.144 h^{-1}. **B.** Transient response of continuous culture to the change in feed glucose concentration from 5–20 g/L at a dilution rate of 0.144 h^{-1}. Symbols: □, glucose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

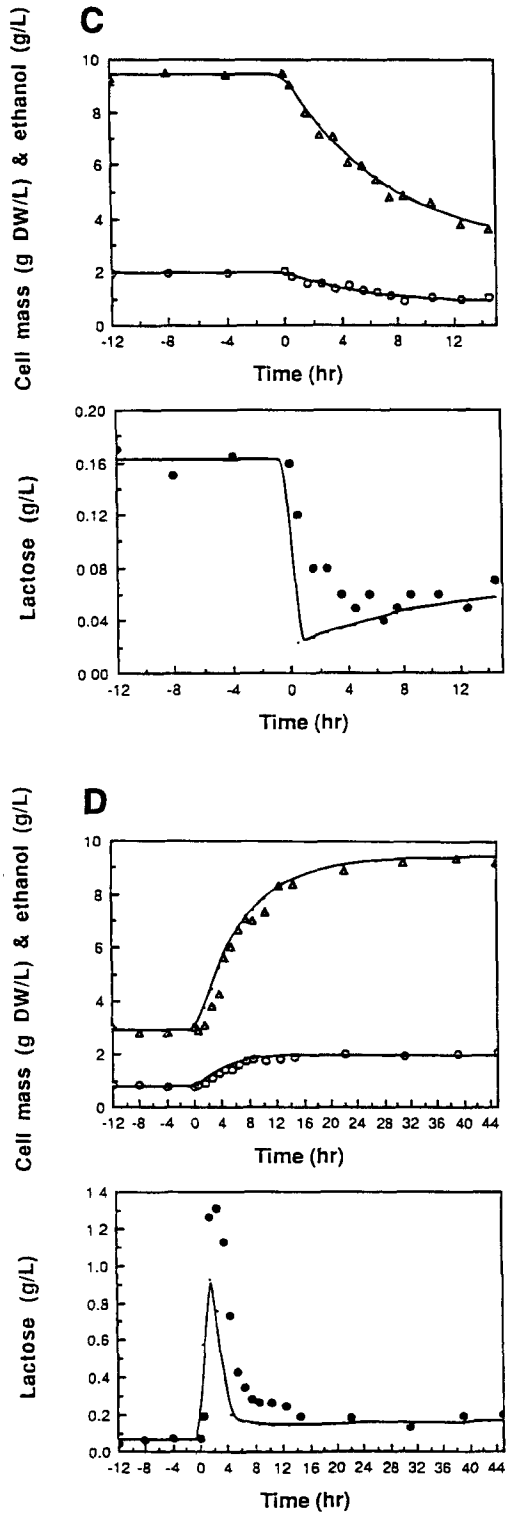

Fig. 2C. Transient response of continuous culture to the change in feed lactose concentration from 20–6.4 g/L at a dilution rate of 0.146 h^{-1}. D. Transient response of continuous culture to the change in feed lactose concentration from 6.4–20 g/L at a dilution rate of 0.146 h^{-1}. Symbols: ●, lactose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

the employed concentrations of sugar in feed media were 5 and 20 g/L, and the constant dilution rate was set at 0.144 h^{-1}. For lactose, these were 6.4 and 20 g/L along with a constant dilution rate of 0.146 h^{-1}.

Simulation of the transient response was conducted with the help of dynamic Eqs. (19)–(22) with $i = j = 1$ in the companion article (Part I) to yield a steady state at the beginning of the transient. The simulation results of cell mass, sugar, ethanol, and enzyme at steady state were used as initial conditions to simulate the transients, and the results are shown in Figs. 2A–D as solid lines.

For both types of transient experiments, the observed concentrations of cell mass and ethanol were in good agreement with the model predictions. The model predicted net changes in sugar concentration rather remarkably; there were, however, some discrepancies between model predictions and the experimental observations of this variable. The model predicted sharp minima in sugar concentrations during shift-downs in sugar concentration; the observed changes in sugar concentration were smooth and without a minimum. During shift-up, both (the experimental observations and the model predictions) showed sharp maximum, but the magnitude of increase in sugar concentration was not predicted to be as large as observed in the experiments. Both the discrepancies suggest that the activities of "key enzyme" in model predictions were higher than the real levels. The concentrations of cell mass and ethanol in the model are not very sensitive to the precise enzyme levels and therefore, show a good agreement with the experimental observations. No effort was made to achieve a better fit. It should be noted that the parameters used in these simulations were estimated from steady-state experiments, and we aimed at using the same parameters for new experiments.

These delays and differences will not be observed in batch cultures and in steady-state continuous cultures. In batch cultures, the initial concentrations of key enzyme were assumed in order to obtain the best simulation results. The real process (or the time period) to reach these levels was ignored, although they are dependent on culture history. In steady-state continuous cultures, cells have ample time to synthesize enough enzymes to utilize sugar effectively for growth. The present model, involving enzyme synthesis, essentially introduces an inertia in cellular response to environmental changes. This structure improves the model performance during transients in continuous culture.

There have been few other attempts to model enzyme production under transient conditions. Imanaka *et al. (5)* have modeled α-galactosidase production during transient response using a model similar to ours. Small changes in dilution rate (0.140–0.142 h^{-1}) were used in shift-up experiments by these workers, and the response of cells to such a small change could be well described by the model. Incorporation of "more structures" in the form of the amount of RNA polymerase, its affinity to promoter *(6)*, synthesis and activation of ribosomes, and so forth *(7)*, may be required to predict the effect of large changes successfully in system behavior during transients.

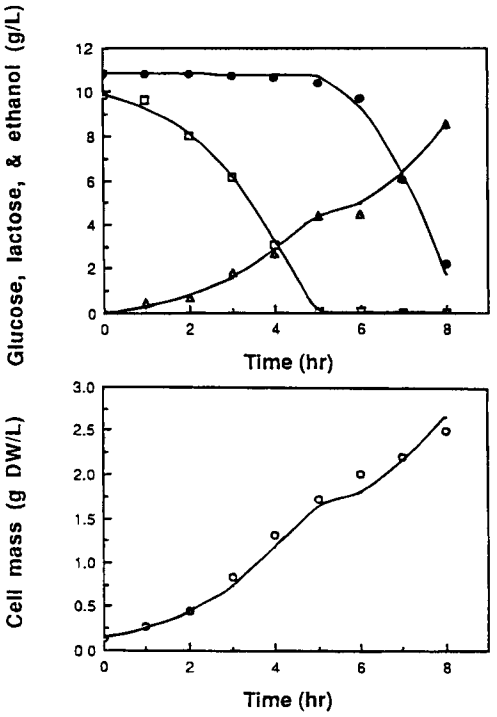

Fig. 3. Batch fermentation of a mixture of glucose (10 g/L) and lactose (10 g/L). Symbols: ●, lactose; □, glucose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

Simulation of Two-Substrate System

The usefulness of the cybernetic model is best realized in predicting the behavior of substrate utilization in a mixed-substrate environment. The kinetic parameters obtained from fermentations on single substrates can be used to simulate ethanol fermentation from a mixture of substrates. Therefore, batch and continuous cultures were conducted with a mixture of glucose and lactose.

A batch fermentation was conducted where glucose-grown cells were used to inoculate a nutrient solution containing 10 g/L glucose and 10 g/L lactose. The experimental results are plotted in Fig. 3. A sequential consumption of the two sugars was observed. Cell growth and ethanol production profiles showed an intermediate lag between two distinct phases. Presence of glucose catabolically repressed utilization of lactose. As glucose in the broth was nearly exhausted, consumption of lactose began. Equations (19)–(22) in the companion article (Part I) with D = 0 were solved to simulate the results of this batch experiment. The parameter values used were the ones established from pure substrate experiments (Table 2 of Part I). Since the inoculum was grown on glucose, initial concentrations of the key enzymes were set at 90% of the maximum for glucose and 10% for lac-

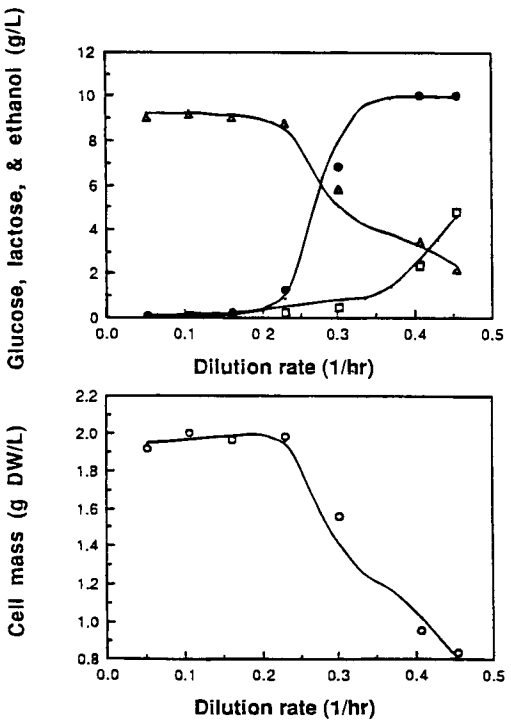

Fig. 4. Chemostat fermentation of a mixture of glucose (10 g/L) and lactose (10 g/L). Symbols: ●, lactose; □, glucose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

tose as described before. The results of simulation are also shown in Fig. 3 as solid lines. The model also predicted a diauxic growth on glucose and lactose. Glucose, which supported faster growth rate, as expected, was consumed first, followed by the utilization of lactose.

Continuous-culture studies involving ethanol production from mixed substrates were also conducted. The feed solution consisted of a mixture of glucose (10 g/L) and lactose (10 g/L). Steady-state values of X, P, and S were measured at different dilution rates, and the data are presented in Fig. 4. Simulation of the continuous culture on mixed substrates was conducted with the help of model Eqs. (19)–(22) presented in Part I. For simulation of continuous cultures, the initial concentrations of variables are not critical, although they influence the time to reach steady states. Parameter values used in the simulation were again those for single sugars listed in Table 2 of Part I. The steady-state results were obtained by simulating the dynamic equations for long periods (about 500 h) until steady state was achieved.

The model satisfactorily predicted fermenter performance in the presence of a mixture of sugars. In contrast to the sequential uptake in batch fermentation, a simultaneous uptake of both sugars was observed at dilution rates below 0.4 h^{-1}. Above this value, only glucose was consumed.

This switch in sugar uptake pattern was also predicted by the cybernetic model utilizing the information obtained from single sugar experiments.

Simulation of Fermentations from Sugars in Cheese Whey

In fermentations of cheese whey, monosaccharides may be present as initial contaminants or as additives to enhance supply of carbon substrates. The presence of mixed sugars can affect the fermentation patterns. Cybernetic models offer a potential tool to simulate and predict these effects. The applicability of the cybernetic approach to such mixed-substrate fermentations was demonstrated through the following experiments and simulations.

In these fermentations, no nutrient supplementation was done. Therefore, the growth characteristics may be different from those on glucose and lactose presented earlier. Therefore, new estimates of certain parameters (μ^e, $Y_{X/S}$, $Y_{P/S}$, and ϕ_M^e) were made from experimental data. The rest of the parameters were kept the same as those identified previously in Table 2 (accompanying article—Part I).

Figures 5A and B show batch fermentation data for two different concentrations of cheese whey powder (CWP). In these experiments, monosaccharides were either not observed or were very low in concentrations (not shown). Therefore, the fermentation may be considered as one having only single substrate (lactose). The experimental results, reported elsewhere *(1)*, for 100 g/L CWP were used to obtain new estimates of growth parameters on lactose in CWP. The inoculum media for fermentations involving cheese whey were prepared from cheese whey *(1)*. Therefore, the initial enzyme levels for glucose and lactose were set at 10 and 90%, respectively, of the maximum enzyme level in parameter estimation and in the following simulation. The estimated parameters along with all the others are tabulated in the second column of Table 2.

Any nutritional variation appears to affect only the maximum specific growth rate, maintenance, and yield parameters, as shown by a good match between predictions (solid lines) and experimental observations, shown in Fig. 5A. The same set of parameters were used to simulate the fermentations with 25 g/L CWP. Results of this simulation are also presented in Fig. 5B. Again, an excellent match is observed for 25 g/L CWP experiment.

To verify the applicability of cybernetic models to the complex system of glucose-supplemented cheese whey, batch fermentations were carried out with a mixture of cheese whey and glucose. In these experiments, 100 g/L CWP solutions were spiked with 10 and 20 g/L glucose, respectively. No additional nutrient supplement was provided. The experimental results are shown in Figs. 6A and B.

Since these fermentations were also not supplemented with nutrient, the growth parameters for glucose can be different from those in Table 2 of Part I. Therefore, one of the experiments (Fig. 6A) was used to estimate μ^e, $Y_{X/S}$, and ϕ_M^e on glucose. The other parameters for glucose and the

Table 2
Kinetic Parameters

Parameter		Glucose[a]	CWP[b]
μ^e		180	93
μ^{max}	(h^{-1})	0.414	0.281
K_{SX}	(g/L)	0.055	0.094
K_{IX}	(g/L)	10,000	370
K_{P1}	(g/L)	7.8	12.1
K_{P2}	(g^2/L^2)	330	330
v^e		688	506
v^{max}	(h^{-1})	1.531	1.531
K_{SP}	(g/L)	0.05	0.088
K_{IP}	(g/L)	10,000	250
K_P	(g/L)	24.9	33.0
ϕ_M^e		2.3	20.0
ϕ_M^{max}	(h^{-1})	0.005	0.06
K_{SM}	(g/L)	10^{-6}	10^{-6}
$Y_{X/S}$	(g/g)	0.532	0.433
$Y_{P/S}$	(g/g)	0.510	0.445

μ^e, v^e, and ϕ_M^e are in units of (g dry wt/[hr./u enzyme activity]).

[a]estimated using data from batch culture on 100 g/L CWP plus 10 g/L glucose.

[b]Estimated using data from batch culture on 100 g/L CWP.

parameters for CWP were kept the same as those in Table 2 of Part I. These estimated parameters are also listed in Table 2 of Part I and used to simulate the experiment of a mixture of 100 g/L CWP and 20 g/L glucose. The predictions are presented in Fig. 6B as solid lines, and an excellent agreement is obtained with experimental data.

Since the maximum rate of substrate consumption for maintenance is relatively low compared to the values of μ^{max} and v^{max}, it is difficult to verify the applicability of Eq. (18) in Part I solely from the results shown in figures. Verification of this equation requires further experimentation under conditions of low growth rates.

CONCLUSIONS

Experimental results from anaerobic fermentations of sugars in semi-synthetic media as well as those from cheese whey (2) have been used in simulation. The extended cybernetic approach can satisfactorily model the

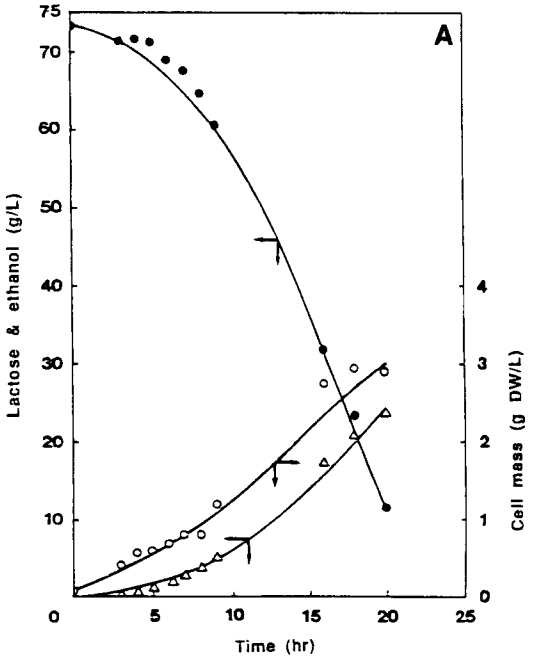

Fig. 5A. Batch fermentation of 100 g/L CWP. Symbols: ●, lactose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

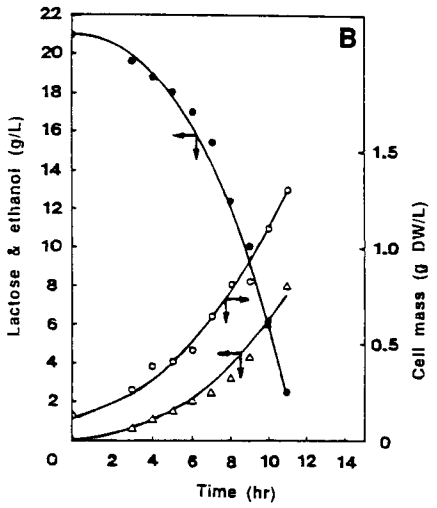

Fig. 5B. Batch fermentation of 25 g/L CWP. Symbols: ●, lactose; ○, cell mass; Δ, ethanol. Solid lines represent simulation results.

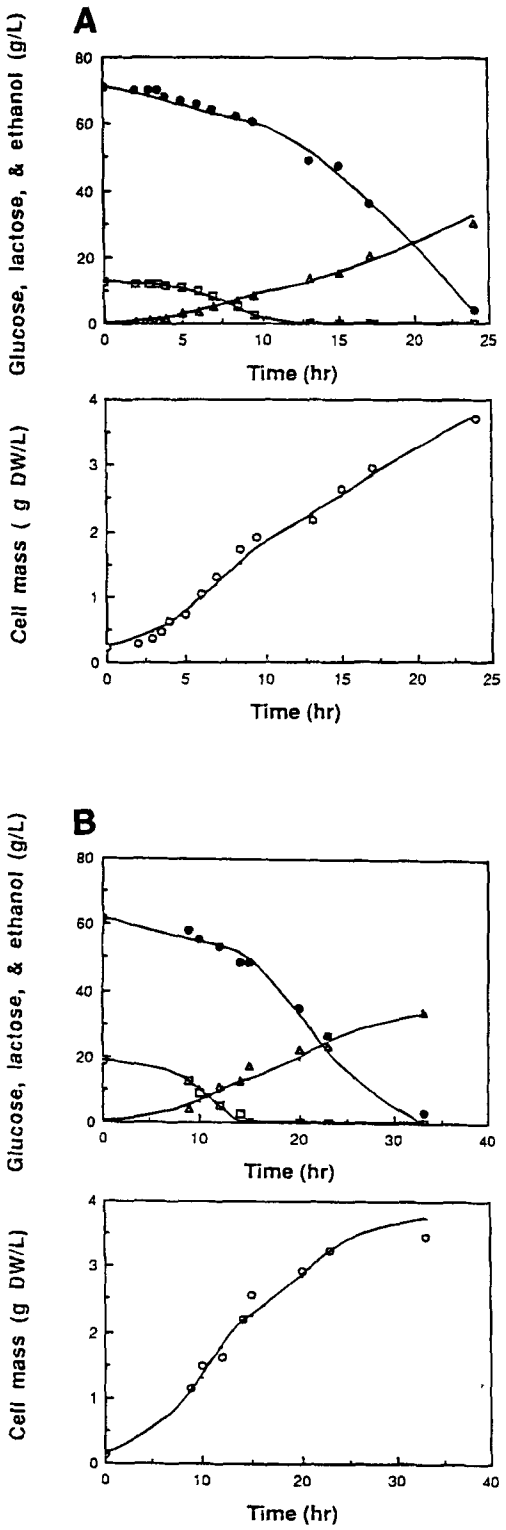

Fig. 6A. Batch fermentation of 100 g/L CWP and 10 g/L glucose. **B**. Batch fermentation of 100 g/L CWP and 20 g/L glucose. Symbols: ●, lactose; □, glucose; ○, cell mass; △, ethanol. Solid lines represent simulation results.

fermentations of cheese whey/semisynthetic media to ethanol under diverse experimental conditions. These include systems containing single substrates and multiple substrates.

NOMENCLATURE

a	basal enzyme production, unit of enzyme activity/g
D	dilution rate, L/h
e	key enzyme level, unit of enzyme activity/g
$K_{IX,i}$	substrate inhibition constant for cell growth, g/L
$K_{IP,i}$	substrate inhibition constant for product formation, g/L
K_P	product inhibition constant for product formation, g/L
$K_{P1,i}$	product inhibition constants for cell growth, g/L
$K_{P2,i}$	product inhibition constants for cell growth, g^2/L^2
$K_{SX,i}$	substrate saturation constant for cell growth, g/L
$K_{SP,i}$	substrate saturation constant for product formation, g/L
$K_{SM,i}$	substrate saturation constant for maintenance, g/L
P	ethanol concentration in broth, g/L
S	sugar concentration in broth, g/L
t	time, h
X	cell mass concentration in broth, g/L
α	enzyme synthesis constant
β	enzyme degradation constant
δ	cybernetic variable for inhibition/activation of enzyme activity
δ_M	cybernetic variable for uncoupling between catabolism and anabolism
ε	cybernetic variable for induction/repression of enzyme synthesis
μ^{max}	maximum specific rate of cell growth, 1/h
ν^{max}	maximum specific rate of product formation, 1/h
ϕ_M^{max}	maximum specific substrate consumption rate for maintenance, 1/h

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