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Observed Quasi-steady Kinetics of Yeast Cell Growth and Ethanol Formation under Very High Gravity Fermentation Condition

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Abstract Using a general *Saccharomyces cerevisiae* as a model strain, continuous ethanol fermentation was carried out in a stirred tank bioreactor with a working volume of 1,500 mL. Three different gravity media containing glucose of 120, 200 and 280 g/L, respectively, supplemented with 5 g/L yeast extract and 3 g/L peptone, were fed into the fermentor at different dilution rates. Although complete steady states developed for low gravity medium containing 120 g/L glucose, quasi-steady states and oscillations of the fermented parameters, including residual glucose, ethanol and biomass were observed when high gravity medium containing 200 g/L glucose and very high gravity medium containing 280 g/L glucose were fed at the designated dilution rate of 0.027 h^{-1}. The observed quasi-steady states that incorporated these steady states, quasi-steady states and oscillations were proposed as these oscillations were of relatively short periods of time and their averages fluctuated up and down almost symmetrically. The continuous kinetic models that combined both the substrate and product inhibitions were developed and correlated for these observed quasi-steady states.

Keywords: *Saccharomyces cerevisiae*, continuous ethanol fermentation, quasi-steady states, oscillations, observed quasi-steady states, kinetics

INTRODUCTION

Kinetic information is essential for process design and optimization. The kinetics of ethanol fermentation, one of the oldest forms of fermentation, were investigated as early as half a century ago [1,2]. The energy crisis resulting from the Libyan, and later Arab, oil embargoes in 1970s stimulated research and technology development on bioethanol, an alternative to petroleum-based liquid fuels. This research was especially focused on the kinetics and process optimization of bioethanol, in order to decrease its production cost [3-14]. However, as the energy crisis alleviated thereafter, and together with great frustration faced due to poor economic competitiveness with petroleum-based liquid fuels, the continuation of this research was severely threatened and almost interrupted.

At the beginning of this new millennium, the potential shortage of petroleum oil supply and exacerbation of environmental deterioration resulting from over-consumptions of petroleum-based liquid fuels were widely recognized, making both governments and industries begin to re-evaluate the benefit of bioethanol, which is not only renewable, but also environmentally friendly. Moreover, the achievements of modern biotechnology continuously contribute the reduction of bioethanol production cost. For example, Genencor International, Inc. successfully made cellulase for biomass conversion and bioethanol production decreased in cost 10-folds within a period of 38 months from April, 2000 to June, 2003 under financial support from the Department of Energy of the United States. Bioethanol began to appear favorable again worldwide. The output of bioethanol in the United States has increased from 1.3 billion gallons in 2000 to 3.4 billion gallons in 2004 (data from American Coalition for Ethanol). The Chinese government also initiated the National Bioethanol Program in 2001, and three sizable bioethanol production plants were approved thereafter, adding a total production capacity of over 1 million tons per year. One of these was put into operation at the end of 2003, the other two are currently under construction.

Although the long-term objective for bioethanol production is to use much cheaper lignocellulosic biomass instead of grain materials, reductions in energy consumption are also potential benefits, especially for those developing countries where energy cost is much higher than in developed countries. Ethanol, one of the strongest inhibitory products, exerts its inhibition on both yeast cell growth and ethanol formation, which makes the fermented broth contain relatively low ethanol concentration at present, normally no more than 12% (v/v) in industrial

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productions. This not only makes ethanol purification by distillation highly energy-intensive, but also produces more waste distillage that needs to be further treated by multi-stage evaporation technology. Therefore, very high gravity (VHG) fermentation technologies that use media that contain total sugar over 25% (w/v) [15] and aim at achieving ethanol concentrations over 15% (v/v) have garnered great attentions [16-18]. For VHG fermentations, the kinetic behaviors of yeast cells are predicted to be quite different from those reported before, in which lower gravity media were used and steady states or chemostat conditions were maintained [2,3]. When cascade fermentation systems are applied for VHG fermentations, different conditions will present inside different fermentors. These unavoidably affect yeast cell growth and ethanol fermentation kinetic behaviors. For example, the residual sugar inside those fermentors ahead is the highest and the strongest substrate inhibition presents, whereas the highest ethanol inside those rear fermentors exerts the strongest product inhibition, and both substrate and ethanol inhibition probably occur inside those middle fermentors. Moreover, the quasi-steady states, even oscillatory behaviors of the fermented parameters, including residual sugar, ethanol and biomass may occur at some conditions and exert their influence on kinetic behaviors. These unusual experimental phenomena, as far as we know, were never reported, except in our recent work and Borzani's research [19-21].

The concept of observed quasi-steady states that would incorporate these steady states, quasi-steady states, and potential oscillations was proposed for VHG fermentations in paper presented here. The corresponding kinetic models for continuous yeast cell growth and ethanol formation were developed and correlated, which can be further used to establish dynamic models that are required for process controlling and optimization.

MATERIALS AND METHODS

Microorganism, Media and Fermentation Conditions

A pure culture of *Saccharomyces cerevisiae*, provided by Jana Otrubo (Department of Chemical Engineering, University of Waterloo, Canada), was selected as a model strain. Pre-culture of this yeast was carried out in 250-mL Erlenmeyer flasks containing 100 mL medium composed of (g/L) glucose, 30; yeast extract, 5; and peptone, 3. The rotary shaker speed and temperature were controlled at 150 rpm and 30°C, respectively. The overnight growth culture was used to inoculate the stirred tank bioreactor, a Bioflo fermenter (New Brunswick Scientific, New Brunswick, NJ, USA) with a working volume of 1,500 mL, which contained medium composed of (g/L) glucose, 120; yeast extract, 5; and peptone 3. After inoculation, batch culture was initiated until the glucose was exhausted. Continuous ethanol fermentation was then initiated by feeding media containing 120, 200 and 280 g/L glucose, respectively. The dilution rates were precisely controlled at designated set-points by adjusting the peristaltic pump attached to the fermentor, and by calibrating daily when samples were taken. Yeast extract (5 g/L) and peptone (3 g/L) were added into the media to supply necessary nutrients for yeast cell growth during ethanol fermentation.

Samples were taken to measure residual glucose, ethanol and biomass concentrations. At least 3 days were maintained for any designated dilution rates under steady states. When quasi-steady states and oscillations were observed, the system was maintained for longer times that depended on both the gravity of media and the dilution rates.

The pH was controlled at 4.5 by automatically adding of 1 N NaOH. The temperature was controlled at 30°C. The impeller speed was set at 300 rpm to guarantee the bioreactor to be an ideal continuous stirred tank bioreactor (CSTR). Because the dissolved oxygen in the media was expected to be very low after sterilization, by controlling airflow rate at 0.05 vvm, limited oxygen was supplied to stimulate yeast cell growth. This situation was similar to the seed or pre-fermentors in cascade systems for industry applications.

Several 5,000-mL flasks were used as medium reservoirs. The sterilization of the media was carried out by heating the flasks to 121°C for 10 min, then immediately cooling to room temperature, to avoid inhibitor production.

Analytical Methods

After the samples were diluted ten times with isopropanol, ethanol was analyzed by gas chromatography (HP 5890: Capillary column, solid phase: crossbond phenylmethyl polysiloxane, carrier gas: helium, 70°C isothermally. Injection temperature, 150°C. Flame iron detector temperature, 250°C. Peaksimple Data Handling System). Glucose was analyzed using enzymatic method (Sigma Glucose Diagnostic Kit, Catalog No. 115-A). The dry weight method was used to measure biomass concentrations. A 1 mL sample was centrifuged, washed 3 times by deionized water, dried at 85°C for 24 h, and weighed. Three parallel samples were simultaneously collected to guarantee the reliability of analytical results. 0.1% methylene blue water solution was used to qualitatively evaluate the viability of yeast cells after samples were diluted 10 times with deionized water. Contamination was checked through microscopic observations.

Theories and Mathematical Models

The Monod model is widely used to express the relationship between the specific growth rate and the limiting substrate concentration.

$$\mu = \mu_{max} \frac{S}{K_S + S} \quad (1)$$

This well-known Monod model is only applicable when the presence of toxic metabolic products plays no inhibitory role. For ethanol fermentation, especially for VHG

fermentations, ethanol produced will accumulate to such a level that yeast cell growth is significantly inhibited. In this case, Eq. (1) must be modified to include the influence of ethanol concentration, *i.e.*,

$$\mu = f(S, P) \tag{2}$$

Many previous studies have revealed that the growth of yeast cells was inhibited by ethanol in a noncompetitive manner similar to that of enzymatic reactions [2,4]. Therefore, only maximum specific growth rate was affected by ethanol concentration.

$$\mu = \mu_i \frac{S}{K_S + S} \tag{3}$$

The dependence of μ_i on the ethanol concentration can be correlated using a generalized nonlinear equation [22].

$$\mu_i = \mu_{max}(1 - \frac{P}{P_{max}})^{\alpha} \tag{4}$$

Substrate inhibition can be treated by introducing substrate inhibition constant into the corresponding kinetic expression [23,24].

$$\mu = \mu_{max} \frac{S}{K_S + S + S^2/K_I} \tag{5}$$

Combining Eqs. (3), (4) and (5), a model combining both substrate and product inhibitions can be established.

$$\mu = \mu_{max} \frac{S}{K_S + S + S^2 / K_I}(1 - \frac{P}{P_{max}})^{\alpha} \tag{6}$$

When the dilution rates are controlled at relatively lower levels during continuous cultures or fermentations, especially when low gravity media are used, limit substrate concentrations are normally undetectable and the specific growth rates predicted by Eq. (6) are much lower than experimentally measured. Therefore, Eq. (6) needs to be modified by adding an experimental parameter μ_0 that equals to the dilution rate at which limit substrate concentration is detected to be 0.

$$\mu = \mu_{max} \frac{S}{K_S + S + S^2 / K_I}(1 - \frac{P}{P_{max}})^{\alpha} + \mu_0 \tag{7}$$

Ethanol is a common primary metabolite of yeast cells under anaerobic conditions, and its production is tightly associated with yeast cell growth. Therefore, a similar kinetic model for ethanol formation can be developed.

$$\nu = Y_{E/X}\,\mu = Y_{E/X}[\mu_{max} \frac{S}{K_S + S + S^2 / K_I}(1 - \frac{P}{P_{max}})^{\alpha} + \mu_0] \tag{8}$$

As biomass concentration is generally expressed based on dry weight, viable cells cannot be quantitatively distinguished from viability-lost or died ones. This complicated biological phenomenon can be incorporated into the model parameters in Eqs. (7) and (8) by modifying model parameters, but the maximum ethanol concentrations for both yeast cell growth and ethanol formation should remain the same.

$$\mu^* = \mu^*_{max} \frac{S}{K_S^* + S + S^2 / K_I^*}(1 - \frac{P}{P_{max}})^{\alpha} + \mu_0 \tag{9}$$

and

$$\nu^* = \nu^*_{max} \frac{S}{K_S^{**} + S + S^2 / K_I^{**}}(1 - \frac{P}{P_{max}})^{\beta} + \nu_0 \tag{10}$$

For continuous suspension culture system, biomass mass balance gives:

$$\frac{dX}{dt} = \mu^* X - DX \tag{11}$$

Under steady or quasi-steady conditions in which the fluctuations of biomass concentrations are either within normal analytical errors or almost symmetrical up and down their average values, we have:

$$\frac{dX}{dt} \approx 0 \tag{11}$$

Therefore,

$$D = \mu^* \tag{12}$$

Mass balance for ethanol gives:

$$\frac{dP}{dt} = \nu^* X - DP \tag{13}$$

Under steady or quasi-steady conditions:

$$\frac{dP}{dt} \approx 0 \tag{14}$$

Therefore,

$$\nu^* = \frac{DP}{X} \tag{15}$$

The specific growth rate and ethanol production rate can be determined by controlling the medium dilution rate, measured biomass, and ethanol concentrations for

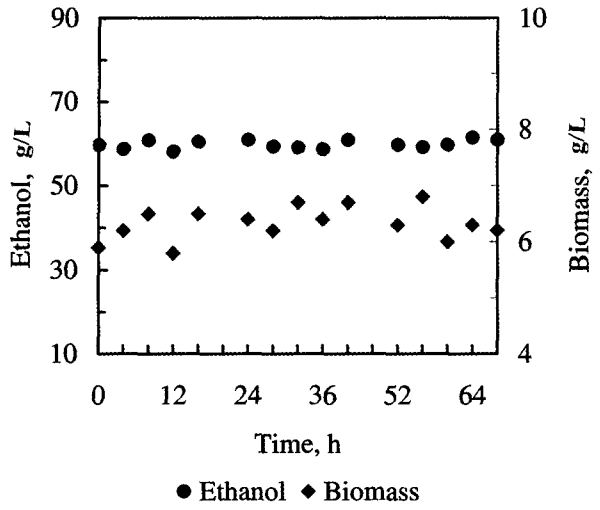

Fig. 1. Fermentation parameters versus time (S_0 = 120 g/L, D = 0.027 h^{-1}).

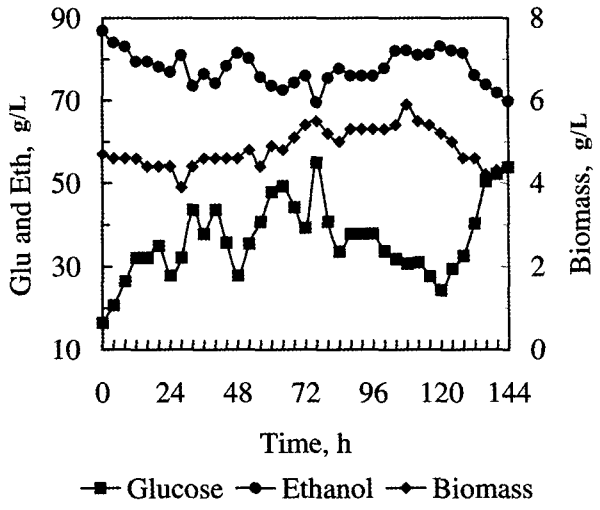

Fig. 2. Fermentation parameters versus time (S_0 = 200 g/L, D = 0.027 h^{-1}).

continuous culture system.

RESULTS AND DISCUSSION

Steady States, Quasi-steady States, Oscillations and Observed Quasi-steady States

After the system experienced the transitional stage from its inoculation, the residual glucose, ethanol and biomass concentrations were monitored at an interval of 4 h when the dilution rate was controlled at 0.027 h^{-1} for the media containing 120, 200 and 280 g/L glucose, respectively, in order to investigate the system states. The results are illustrated in Figs. 1~3.

When lower gravity medium containing 120 g/L glucose was fed into the bioreactor, no residual glucose was detected in the broth. About 58.0 g/L ethanol and 6.3 g/L biomass were produced. In this situation, no substrate inhibition was exerted and ethanol inhibition was minor. The ethanol and biomass were observed to be at their steady states, as their fluctuations were small (between ± 3% and ± 8% of their average levels, within the normal analytical errors). However, when the glucose concentration in the medium increased to 200 g/L, the residual glucose concentration in the broth still maintained a relatively lower level, but fluctuated, with its minimum and maximum of 24.3 and 55.0 g/L or −33.4% and +50.6%, respectively, based on an average value of 36.5 g/L. This level of residual glucose still did not significantly exert its inhibition [25], but ethanol concentration increased to an average value of 80.5 g/L and exerted significant inhibition on yeast cells. Similar fluctuations were also observed for ethanol and biomass, as illustrated in Fig. 2. Two fluctuation profiles presented. First, the residual glucose, ethanol and biomass fluctuated within relatively narrow ranges and experienced longer times. The quasi-steady states developed at this

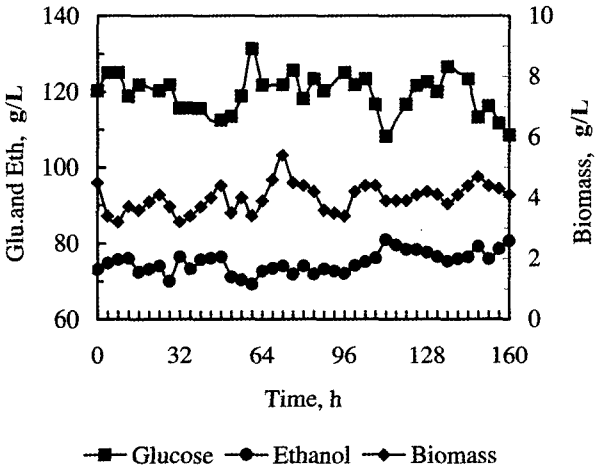

Fig. 3. Fermentation parameters versus time (S_0 = 280 g/L, D = 0.027 h^{-1}).

stage. Larger fluctuations then appeared, but were maintained for relatively short times, both the maximum and minimum of these fluctuations were observed at this stage. The glucose concentration in the medium continuously increased to 280 g/L, the average ethanol concentration in the broth decreased slightly, to 74.3 g/L, but the average residual glucose increased to 117.2 g/L, and began to exert substrate inhibition. The similar fluctuations observed in Fig. 2 still existed, as illustrated in Fig. 3.

In order to further investigate these fluctuations, the continuation using the medium containing 280 g/L glucose was extended to as long as two months and sampled daily. All of the operating parameters, including temperature, pH value, dilution rate and medium composition were precisely controlled during the running. The results, as illustrated in Fig. 4, demonstrated that these fluctuations were sustained and seemed to be periodic and

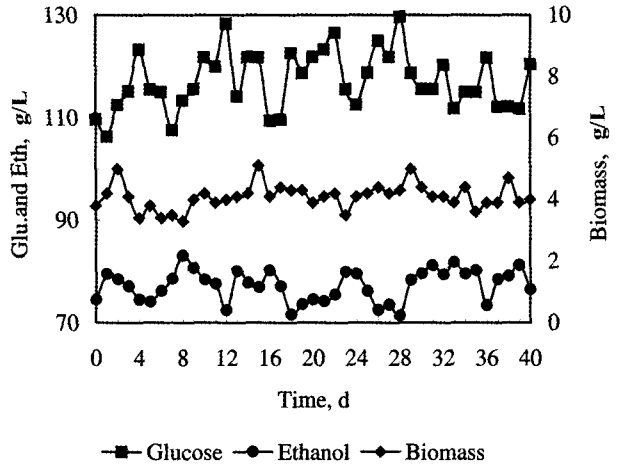

Fig. 4. Sustained quasi-steady state and oscillatory behaviors (S_0 = 280 g/L, D = 0.027 h^{-1}).

symmetrical up and down their average values, which can be characterized by oscillations. Therefore, the concept of observed quasi-steady states that incorporated steady states, quasi-steady states and oscillations was proposed.

When the dilution rates were changed, the steady states were maintained for the continuous fermentation using the medium containing 120 g/L glucose, and the quasi-steady states still existed for the continuous fermentations using the media containing glucose of 200 and 280 g/L. However, the oscillations observed at the dilution rate of 0.027 h^{-1} seemed to be attenuated to some extent (data unpublished). The mechanism(s) behind these usual experimental phenomena needs to be further explored.

Kinetics of Observed Quasi-steady States

The kinetic data for these observed quasi-steady states were illustrated in Table 1.

When $S \le K_S^*$ for growth and $S \le 2K_S^{**}$ for ethanol formation, the model parameters, μ_0 and ν_0, were experimentally obtained to be 0.03 and 0.262 h^{-1}, respectively. Beyond these criteria, these parameters were negligible.

The other model parameters in Eqs. (9) and (10) were evaluated with numerical calculation method (iterative least square method) and illustrated in Table 2.

The maximum ethanol concentration for both yeast cell growth and ethanol fermentation was 167.0 g/L, much higher than those reported before [6,7,25], but very near to the upper limit of 23% (*v*) [26]. The parameters K_S^* and K_S^{**} were also higher than previously reported [13], which means the substrate affinity under VHG conditions is quite poor. K_I^* and K_I^{**} are relatively lower compared with S^2, which indicates substrate inhibition cannot be neglected when substrate concentrations are relatively high, especially for yeast cell growth.

Substituting Eqs. (9) and (10) with these model parameters gives:

Table 1. Experimental data of continuous ethanol fermentations[a]

S_0 = 120 g/L, steady states						
μ^*_{exp} (h^{-1})	0.027	0.050	0.083	0.100	0.116	0.140
μ^*_{cal} (h^{-1})	0.030	0.032	0.082	0.097	0.115	0.142
S (g/L)	0	0.14	17.3	20.2	32.5	44.7
P (g/L)	58.2	52.2	44.3	40.8	38.4	32.5
X (g/L)	6.33	7.43	4.96	4.73	4.63	4.23
ν^*_{exp} (h^{-1})	0.262	0.351	0.741	0.862	0.962	1.075
ν^*_{cal} (h^{-1})	0.262	0.268	0.730	0.787	0.934	1.051
S_0 = 200 g/L, quasi-steady states						
μ^*_{exp} (h^{-1})	0.027	0.040	0.067	0.100	0.120	0.146
μ^*_{cal} (h^{-1})	0.022	0.041	0.062	0.102	0.113	0.130
S (g/L)	36.5	61.4	86.8	110.7	118.8	129.0
P (g/L)	82.5	68.3	56.8	41.3	37.3	32.1
X (g/L)	4.0	4.8	4.6	4.1	4.4	4.0
ν^*_{exp} (h^{-1})	0.500	0.575	0.832	1.020	1.017	1.172
ν^*_{cal} (h^{-1})	0.556	0.631	0.751	0.867	0.911	0.951
S_0 = 280 g/L, quasi-steady states						
μ^*_{exp} (h^{-1})	0.018	0.027	0.053	0.080	0.106	
μ^*_{cal} (h^{-1})	0.021	0.028	0.041	0.079	0.114	
S (g/L)	96.8	117.2	143.9	175.3	213.2	
P (g/L)	82.5	76.0	65.2	45.2	31.5	
X (g/L)	3.7	4.0	4.3	3.6	3.5	
ν^*_{exp} (h^{-1})	–	–	0.803	1.004	0.954	
ν^*_{cal} (h^{-1})	–	–	0.718	0.856	0.939	

[a] μ^*_{exp} and ν^*_{exp} are measured according to Eqs. (12) and (15), respectively. Ethanol concentrations have been modified by considering exhaust gas stripping loss.

For growth

$$\mu^* = \frac{0.463S}{9.893 + S + S^2/296.2}\left(1 - \frac{P}{167.0}\right)^4 + 0.030 \quad S \le K_S^* \quad (16)$$

and

$$\mu^* = \frac{0.463S}{9.893 + S + S^2/296.2}\left(1 - \frac{P}{167.0}\right)^4 \quad S > K_S^* \quad (17)$$

For fermentation:

$$\nu^* = \frac{1.56S}{23.4 + S + S^2/2020.8}\left(1 - \frac{P}{167.0}\right) + 0.262 \quad S \le 2K_S^{**} \quad (18)$$

and

$$\nu^* = \frac{1.56S}{23.4 + S + S^2/2020.8}\left(1 - \frac{P}{167.0}\right) \quad S > 2K_S^{**} \quad (19)$$

Table 2. Values of the kinetic parameters

μ_{max}^* (h^{-1})	K_S^* (g/L)	K_I^* (g/L)	α	P_{max} (g/L)
0.463	9.89	296.2	4	167.0
ν_{max}^*	K_S^{**} (g/L)	K_I^{**} (g/L)	β	
1.56	23.4	2020.8	1	

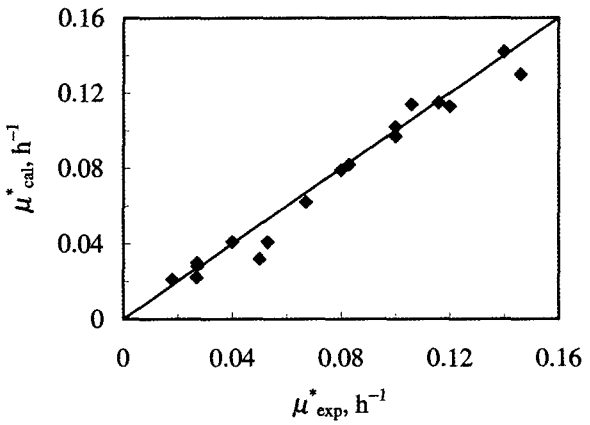

Fig. 5. Comparison of the experimental data with the model predicted for growth (R=0.9718).

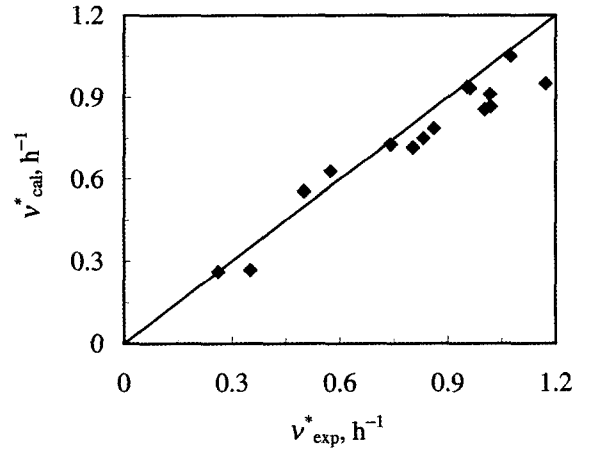

Fig. 6. Comparison of the experimental data with the model predicted for fermentation (R^2 = 0.9298).

The comparisons between the experimental data and the model predicted values are illustrated in Figs. 5 and 6. The high correlation coefficients (R^2 = 0.9718 for yeast cell growth and R^2 = 0.9298 for ethanol formation) demonstrate these models properly reveal the kinetic natures of *S. cerevisiae* under VHG medium conditions.

CONCLUSION AND SUGGESTIONS

The steady states observed under lower gravity medium conditions could not be maintained for continuous ethanol productions when HG or VHG media were fed. Quasi-steady states, and oscillations of fermented parameters, including residual sugar, ethanol and biomass occurred at some conditions, for example, the specific dilution rate and high/very high gravity media in our case. The observed quasi-steady states, which incorporated these steady states, quasi-steady states and oscillations, were proposed. The kinetic models previously developed, mostly based on batch cultures and ethanol fermentations, were proven to be unsuitable for continuous cultures and ethanol fermentations, especially when low gravity media were fed and the dilution rates were relatively lower. A modified kinetic model(s) was developed and the model parameters were correlated based on continuous cultures and ethanol fermentations in which different gravity media and dilution rates were designated. These kinetic model(s) can work as the basis for developing dynamic kinetic models in which quasi-steady states and oscillations of fermentation parameters will be correlated with time variable in order to control and optimize ethanol production processes.

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NOMENCLATURE

D	dilution rate, h^{-1}
K_S	intrinsic Monod constant for growth, g/L
K_I	intrinsic substrate inhibition constant for growth, g/L
K_S^*	observed Monod constant for growth, g/L
K_S^{**}	observed Monod constant for ethanol fermentation, g/L
K_I^*	observed substrate inhibition constant for growth, g/L
K_I^{**}	observed substrate inhibition constant for ethanol fermentation, g/L
P	ethanol concentration, g/L
P_{max}	maximum ethanol concentration for both growth and ethanol formation, g/L
S	residual glucose concentration, g/L
X	biomass concentration, g(d.w)/L
$Y_{E/X}$	ethanol yield coefficient from biomass
μ	intrinsic specific growth rate, h^{-1}
μ^*	observed specific growth rate, h^{-1}
μ^*_{cal}	predicted observed specific growth rate by Eqs. (16) and (17), h^{-1}
μ^*_{exp}	experimentally measured observed specific growth rate, h^{-1}
μ_i	intrinic maximum specific growth rate modified by ethanol inhibition, h^{-1}
μ_0	specific growth rate at lower dilution rates for lower gravity medium, h^{-1}
μ_{max}	intrinsic maximum specific growth rate, h^{-1}
μ^*_{max}	observed maximum specific growth rate, h^{-1}
ν	intrinsic specific ethanol production rate, h^{-1}
ν^*	observed specific ethanol production rate, h^{-1}

ν°_{cal} predicted observed specific ethanol production rate by Eqs. (18) and (19), h^{-1}
ν°_{exp} experimentally measutred observed specific ethanol production rate, h^{-1}
ν_0 specific ethanol production rate at lower dilution rates for lower gravity medium, h^{-1}
ν°_{max} observed maximum specific ethanol production rate, h^{-1}
α ethanol inhibition constant for growth
β ethanol inhibition for ethanol formation

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