JOURNAL OF FERMENTATION AND BIOENGINEERING
Vol. 79, No. 1, 54–58. 1995

Use of Chemostat Data for Modelling Extracellular-Inulinase Production by *Kluyveromyces marxianus* in a High-Cell-Density Fed-Batch Process

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Received 18 June 1994/Accepted 4 November 1994

Production of extracellular inulinase by low-cell-density (2 kg dry weight·m^{-3}) sucrose-limited chemostat cultures of *Kluyveromyces marxianus* obeyed saturated kinetics at dilution rates ranging from 0.02 to 0.5 h^{-1}. A non-structured Monod-type equation, describing the relation between specific growth rate and specific extracellular-inulinase production rate, was used to fit experimental data. This equation was subsequently incorporated in a model for the production of biomass and extracellular inulinase in a high-cell-density (>100 kg dry weight·m^{-3}) fed-batch culture of *K. marxianus* grown on sucrose. The model adequately described biomass production in the fed-batch culture. However, the production of extracellular inulinase in the fed-batch process was slightly higher than predicted by the model. This observation may be related to differences in growth conditions between in the chemostat and fed-batch cultures.

[**Key words:** modelling, chemostat, extracellular inulinase, *Kluyveromyces marxianus*, fed-batch process]

Fed-batch fermentation is commonly employed for large-scale industrial processes involving bacteria, filamentous fungi and yeasts (1). Volumetric productivity (kg product·m^{-3}·h^{-1}) is a often a key factor in determining the economic viability of such processes. One way to improve volumetric productivity is the application of high biomass densities.

The development of single-cell protein processes has given a strong impetus to the development of high-cell-density processes for the cultivation of microorganisms (Wegner, E. H., US patent 4414329, 1981). A novel field in which high-cell-density fermentation is required is the production of heterologous proteins (2). Currently, processes for the cultivation of yeasts at biomass concentrations exceeding 100 kg·m^{-3} have been described for a number of yeasts, including *Hansenula polymorpha* (3), *Pichia pastoris* (4) and *Saccharomyces cerevisiae* (5).

To avoid problems arising from limited cooling and oxygen-transfer capacities of industrial bioreactors, high-cell-density fed-batch cultivations are characteristically performed at a low and often decreasing specific growth rate. For the modelling of such processes and to achieve maximum productivity within the constraints imposed by limited oxygen- and heat-transfer capacity of industrial bioreactors, it is essential to know the relation between the specific growth rate (μ) and the specific rate of product formation (q_p) (6, 7). Chemostat cultivation is a commonly used and powerful tool to study the growth-rate dependency of microbial processes. Under steady-state conditions, the specific rate of product formation (q_p) follows from Eq. 1.

$$q_p = D^* C_p^* C_x^{-1} \quad (1)$$

However, there are few studies in which data obtained in laboratory chemostat cultures have actually been used to predict growth and product yields in high-cell-density fed-batch processes.

The aim of the present paper was to assess the applicability of chemostat data for the modelling of growth and production of the extracellular enzyme inulinase (β-2,1-D-fructanfructanohydrolase; EC 3.2.1.7) by *Kluyveromyces marxianus* in a 100-liter-scale fed-batch process, at cell densities exceeding 100 kg·m^{-3}. To this end, chemostat data were used for the formulation of an empirical relation between growth rate and product formation. Growth and product formation predicted by a model based on the chemostat data were compared with those observed in an actual high-cell-density fed-batch process, taking into account the non-negligible volume occupied by cells at high biomass densities.

MATERIALS AND METHODS

Microorganism and maintenance *K. marxianus* var. *marxianus* CBS 6556 was obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures (CBS), Delft, The Netherlands and maintained on YEPD-agar slopes. YEPD contained per liter of demineralized water: yeast extract (Difco, MI, USA), 10 g; Bacto-peptone (Difco), 20 g; and glucose, 20 g.

Chemostat cultivation Aerobic, sucrose-limited chemostat cultivation was performed at 40°C in Applikon laboratory fermentors with a working volume of 1 *l*. The cultures were sparged with air (1 *l*·min^{-1}) and stirred at 800 rpm. The dissolved-oxygen concentration, measured with a polarographic electrode (Ingold), remained above 50% of air saturation. The culture pH was controlled at 4.5 by the automatic addition of 1 mol·*l*$^{-1}$ KOH. The mineral medium contained per liter of demineralized water: $(NH_4)_2SO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 3 g; EDTA, 15 mg; $ZnSO_4 \cdot 7H_2O$, 4.5 mg; $MnCl_2 \cdot 7H_2O$, 1 mg; $CoCl_2 \cdot 6H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.3 mg; $NaMoO_4 \cdot 2H_2O$, 0.4 mg; $CaCl_2 \cdot 2H_2O$, 4.5 mg; $FeSO_4 \cdot 7H_2O$, 3 mg; H_3BO_3, 1 mg; KI,

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0.1 mg; biotin, 0.1 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; silicon antifoam agent (BDH, Poole, UK), 0.15 ml. The medium was sterilized at 120°C for 20 min. Sucrose (CSM, Amsterdam, The Netherlands) was sterilized separately at 110°C for 20 min and added to a final concentration of $5\ kg \cdot m^{-3}$.

Determination of cell number and cell volume Samples from a sucrose-limited chemostat culture, grown at a dilution rate of $0.2\ h^{-1}$, were diluted in Isoton buffer (Coulter Electronics, Harpenden, England) and counted with a Casy 1 model TTC Cell Counter and analyzer system (Schärfe System, Reutlingen, Germany). For cell volume distribution assays, the apparatus was calibrated with 8.7 μm latex beads (Coulter Electronics). A spherical cell shape was assumed for cell-volume estimates.

Fed-batch fermentation Fed-batch cultivation was performed in an Applikon fermentor with a working volume of 100 *l*, at 40°C and at an impeller speed of 800 rpm. During fermentation, the dissolved-oxygen concentration in the fermentor was kept above 30% of air saturation by sparging with mixtures of air and oxygen. The pressure in the fermentor was adjusted manually up to a maximum of 2.5 bar. The culture pH was controlled at 4.5 by the automatic addition of $7.5\ mol \cdot l^{-1}$ NH_4OH. The medium feed was continuously monitored by following the weight of the fermentor and the reservoir vessels, all of which rested on electronic balances.

The fermentor, containing 30 *l* of two-fold concentrated mineral medium (see above), was steam-sterilized (45 min at 120°C). After inoculation, sucrose was added to give a concentration of $10\ g \cdot l^{-1}$. The culture was inoculated with cells from a sucrose-limited chemostat culture grown at a dilution rate of $0.10\ h^{-1}$. During batch growth, the aeration was automatically adjusted to keep the dissolved-oxygen concentration above 50% of air saturation. After depletion of sucrose, indicated by a steep rise of the dissolved-oxygen concentration and a drop in oxygen consumption and carbon-dioxide production, the fed-batch phase was initiated.

The medium feed used in the fed-batch phase contained per liter of demineralized water: $(NH_4)_2SO_4$, 10 g; $MgSO_4 \cdot 7H_2O$, 5 g; KH_2PO_4, 20 g; EDTA, 0.75 g; $ZnSO_4 \cdot 7H_2O$, 0.225 g; $MnCl_2 \cdot 7H_2O$, 0.05 g; $CoCl_2 \cdot 6H_2O$, 0.015 g; $CuSO_4 \cdot 5H_2O$, 0.015 g; $NaMoO_4 \cdot 2H_2O$, 0.02 g; $CaCl_2 \cdot 2H_2O$, 0.225 g; $FeSO_4 \cdot 7H_2O$, 0.15 g; H_3BO_3, 0.05 g; KI, 5 mg; calcium pantothenate, 0.1 g; nicotinic acid, 0.1 g; Struktol J673 antifoam agent (Struktol Co., Stow, USA), 0.13–0.33 ml (depending on the biomass concentration in the fermentor). Sucrose was present in the feed medium at a concentration of $500\ kg \cdot m^{-3}$. Medium components were dissolved in sterile demineralized water, but not autoclaved. Microscopic examination did not reveal contamination during the fed-batch cultivation. The medium was pumped into the reactor from 20-*l* reservoir vessels, using a controllable Watson-Marlow 503U pump (Watson-Marlow, Falmouth, UK) with flow rates ranging from 0.2 to 4.8 $l \cdot h^{-1}$. The growth rate was kept at $0.20\ h^{-1}$ until a dissolved-oxygen concentration of 30% air saturation could no longer be maintained. At this stage, the feed was kept constant. A personal computer with ONSPEC software (Heuristics, Sacramento, USA) was used to control the medium pump. The feed rate (F_s) required to maintain a constant specific growth rate was programmed according to Eq. 2.

TABLE 1. Values of key parameters used for modelling of growth and extracellular-inulinase production in high-cell-density fed-batch cultures of *K. marxianus* CBS 6556

Parameter	Symbol	Default value	Reference
Initial volume	V_0	$35.2 \cdot 10^{-3}\ m^3$	
Initial biomass concentration	C_{x0}	$4.5\ kg \cdot m^3$	
Substrate requirement for maintance	m_s	$0.024\ kg \cdot kg^{-1} \cdot h^{-1}$	(13)
Sucrose concentration in feed	C_{si}	$500\ kg \cdot m^{-3}$	
Specific cell volume	V_{spec}	$2.15 \cdot 10^{-3}\ m^3 \cdot kg^{-1}$	This study
Biomass yield on sugar	Y_{sx}	$0.40\ kg \cdot kg^{-1}$	(9)
Specific rate of product formation	q_p	Eq. 2	
Linear feed	F_{lin}	$4.77 \cdot 10^{-3}\ m^3 \cdot h^{-1}$	

$$F_s = \left\{\left(\frac{\mu}{Y_{sx}}\right) + m_s\right\} * \left\{\frac{V_0 * C_{x0}}{C_{si}}\right\} * e^{(\mu * t)} \qquad (2)$$

Symbols and values of process parameters are given in Table 1. A detailed description of the development of this fed-batch process will be published elsewhere (8).

Inulinase assay Extracellular-inulinase activity was assayed in culture supernatants as described by Rouwenhorst *et al.* (9). Enzyme activities were converted to amounts of inulinase protein by using a specific activity of pure supernatant inulinase of 1,500 U (mg inulinase)$^{-1}$ (10).

Stability of supernatant inulinase At the end of the fed-batch process, a culture sample was centrifuged (10 min at 10,000 *g*). Sodium azide (0.04%) was added to the supernatant, which was subsequently filter-sterilized (0.2 μm Acrodisc, Gelman) and incubated at 40°C. Over a period of 100 h, samples were aseptically withdrawn for assaying inulinase activity.

Computer-assisted simulations Numerical solutions of models for fed-batch fermentation were calculated using the simulation program PSI/C (Boza, Pijnacker, The Netherlands).

RESULTS

Effect of growth rate on inulinase production by sucrose-limited chemostat cultures Independent of the growth conditions, approximately 50% of the total inulinase content of *K. marxianus* cultures is excreted into the growth medium (9, 11). Measurement of intracellular and cell-wall-associated inulinase was not included in the present study. Previous work on inulinase production by *K. marxianus* CBS 6556 in chemostat cultures has indicated that the highest inulinase production occurred in sucrose-limited chemostat cultures (9). Because sucrose, which is also the major carbon source in molasses, is highly soluble and cheap, it is an attractive feedstock for large-scale fermentations. It was therefore decided to use sucrose as a carbon and energy source in all experiments.

In sucrose-limited chemostat cultures of *K. marxianus*, the specific rate of enzyme production q_p has been reported to depend strongly on the specific growth rate (9) and, at specific growth rates below $0.5\ h^{-1}$, appeared to follow saturation kinetics (Fig. 1). Even if detailed knowledge on the regulation of inulinase synthesis were available, a structured model would probably have to involve a large number of parameters and equations,

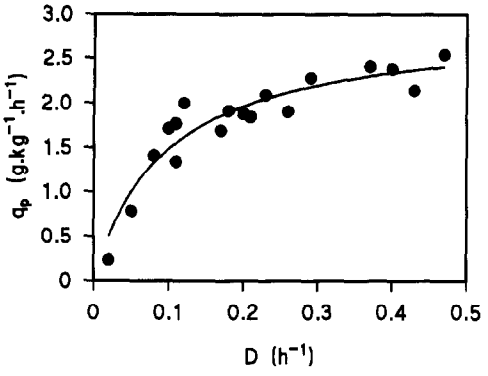

FIG. 1. Relationship between dilution rate (specific growth rate) and specific rate of extracellular-inulinase production by aerobic, sucrose-limited chemostat cultures of *K. marxianus* CBS 6556 [data recalculated from (9)]. Growth conditions: T=40°C, pH 5, C_{si}=5 kg sucrose·m^{-3}. Inulinase production rates (g inulinase·[kg dry weight]$^{-1}$·h^{-1}) were calculated from enzyme activities using a specific activity of pure inulinase of 1,500 U·(mg inulinase)$^{-1}$ (10). The line drawn through the data points is the best fit to a Michaelis-Menten-type satured-production-kinetics equation, calculated with the computer program Fig. P (Fig. P Software Co., Durham, USA).

thereby complicating its use for process optimization. Furthermore, the regulation of the *INU1* gene (12, 13) has not yet been studied in detail at the molecular level. An empirical approach was therefore followed to describe the kinetics of extracellular-inulinase production. The extracellular-inulinase-production rates observed in sucrose-limited chemostat cultures grown at dilution rates between 0.02 h^{-1} and 0.50 h^{-1} (9) were fitted with a Michaelis-Menten-type equation (Fig. 1). This resulted in the empirical description of q_p presented in Eq. 3:

$$q_p = 2.89\left(\frac{\mu}{0.095+\mu}\right) \tag{3}$$

Equation 3 was subsequently used to predict extracellular inulinase production in a high-cell-density fed-batch process.

The specific volume (V_{spec}) of *K. marxianus* was measured in cells sampled from a sucrose-limited chemostat culture grown at a dilution rate of 0.20 h^{-1}. V_{spec} of these cells was 2.15×10^{-3} m^3·(kg biomass)$^{-1}$, implying that at a biomass concentration of 100 kg·m^{-3}, over 20% of the culture volume will be occupied by the yeast cells. When production of an extracellular enzyme is studied in high-cell-density cultures, the volume occupied by biomass clearly cannot be neglected.

Stability of supernatant inulinase The product concentration in a fed-batch process is the net result of product formation, dilution and degradation. For modelling of the overall process, it is essential to know the kinetics of product degradation. Therefore, the rate of extracellular-inulinase degradation was investigated under process conditions. Cell-free supernatant, harvested at the end of a high-cell density fed-batch fermentation showed no loss of activity after incubation for 100 h at 40°C (data not shown). Since the fed-batch fermentations took less than one third of this time, absolute stability of inulinase was assumed.

Modelling of extracellular-inulinase production during fed-batch cultivation Growth and production of extracellular inulinase by *K. marxianus* during growth in fed-batch cultures were modelled by a series of standard equations, which are briefly described below.

The change of the biomass concentration in the fermentor is the net result of growth and the dilution caused by the medium feed. Thus, the increase of the amount of biomass can be described by Eq. 4.

$$r_x = \left(\mu - \frac{F_s}{V}\right)*C_x*V \tag{4}$$

During the exponential feed phase, the specific growth rate was kept constant at 0.20 h^{-1} by manipulating the medium flow rate according to Eq. 2 (see methods section). When this growth rate could no longer be maintained due to the limited oxygen-transfer capacity of the reactor, the culture was switched to a constant feed. A constant feed rate will result in a lower specific rate of substrate consumption and, consequently, in a decrease of the specific growth rate. This effect is augmented by the relatively high energy requirement for maintenance at low specific growth rates. The effect of the feed rate on the specific growth rate is described by Eq. 5.

$$\mu = \frac{(C_{si}*F_s - m_s*V*C_x)*Y_{sx}}{V*C_x} \tag{5}$$

During fed-batch cultivation, the biomass concentration and the culture volume increase according to Eqs. 6 and 7, respectively.

$$C_x = \frac{V_0}{V}*C_{x0} + \frac{1}{V}\int r_x dt \tag{6}$$

$$V = V_0 + \int F_s dt \tag{7}$$

The volume occupied by the biomass is the product of the biomass concentration, the culture volume and the specific volume of the biomass (m^{-3}·[kg dry weight]$^{-1}$; Eq. 8).

$$V_{cell} = (C_x*V*V_{spec}) \tag{8}$$

The rate of product formation by the culture is equal to the product of the biomass concentration, the specific rate of product formation and the culture volume (Eq. 9).

$$r_p = q_p*C_x*V \tag{9}$$

The product concentration in the fermentor is a function of the initial product concentration, the rate of product formation and the volume of the culture (Eq. 10). Finally, correction for the volume occupied by the cells gives the product concentration in the extracellular fluid (Eq. 11).

$$C_p = \frac{V_0}{V}*C_{p0} + \frac{1}{V}\int r_p dt \tag{10}$$

$$C_{p,\,ext} = \frac{C_p*V}{(V - V_{cell})} \tag{11}$$

Verification of the model The Eqs. 2–11 were numerically solved to predict growth and product formation in a sucrose-limited fed-batch fermentation of *K. marxianus*. After an exponential growth phase at μ=0.20 h^{-1}, the culture was switched to a constant feed, which was expected to cause a progressive decrease of the growth rate to below 0.07 h^{-1} (Fig. 2A). This decrease of the growth rate caused a decrease of q_p by approximately 50% in the sucrose-limited chemostat cultures

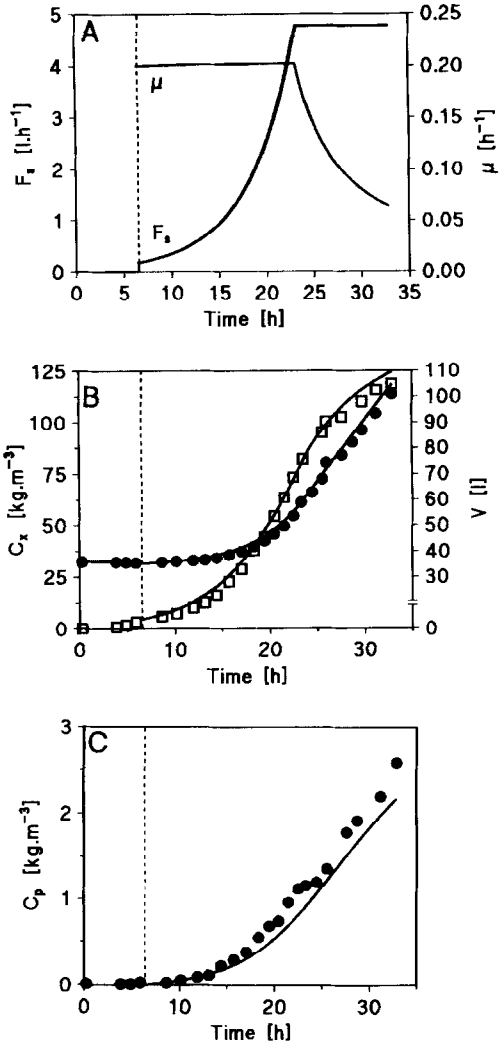

FIG. 2. Comparison of experimental data obtained in a high-cell-density fed-batch fermentation of *K. marxianus* CBS 6556 (symbols) with results predicted by numerical solution of the Eqs. 2–11 (solid lines). The exponential-feed phase was initiated after 6.3 h (dashed line). After 23 h, a constant feed of 4.77 $l \cdot h^{-1}$ was applied. Further process parameters are presented in Table 1. (A) Programmed medium feed (F_s) and specific growth rate (μ); (B) culture volume (V) and culture dry weight (C_x); (C) extracellular inulinase concentration (C_p).

(Fig. 1).

The observed biomass concentration and culture volume closely followed the pattern predicted by the model (Fig. 2B). This was essential, because one of the aims of this experiment was to test the assumed relation between specific growth rate and enzyme production. Only during the final hours of the process, the measured biomass concentrations, which exceeded 115 $kg \cdot m^{-3}$, were slightly lower than predicted by numerical solution of the model (Fig. 2B). This may reflect an inaccuracy in the assumed maintenance-energy requirement, which had been derived from literature data (Table 1).

The amount of extracellular inulinase at the various stages of the fed-batch process was predicted on the basis of Eq. 3, derived empirically from data obtained with low-biomass-density (2 $kg \cdot m^{-3}$) chemostat cultures. From the results presented in Fig. 2C, it appears that the observed enzyme production was slightly higher than predicted by numerical solution of the Eqs. 2–11.

DISCUSSION

The present study clearly illustrates that chemostat cultivation can be used to obtain the key parameters needed for modelling of high-cell-density fed-batch processes. The unstructured model described by the Eqs. 2–11 adequately predicted biomass formation in a high-cell-density fed-batch fermentation of *K. marxianus* (Fig. 2). The observation that the concentration of extracellular inulinase was consistently about 20% higher than predicted (Fig. 2C) may be due to the different ionic composition of the medium used for fed-batch cultivation. This medium, although derived from the chemostat medium, was not extensively optimized. For example, the fed-batch medium contained rather high concentrations of EDTA, which may affect the distribution of inulinase over cell wall and extracellular medium (10). Other factors that affect cell structure, including the elevated pressure applied in the fed-batch fermentation, may also be of importance in this respect. Furthermore, refinement of models based on chemostat cultures will have to take into account the dynamic nature of fed-batch fermentations which, in contrast to chemostat cultures, cannot be considered to be in steady state. In particular the relaxation time of product formation in response to a decreasing growth rate is an important parameter in this respect (15). Transient-state experiments using chemostat cultures may provide quantitative data applicable in even more reliable models.

In principle, the strategy used in the present study can also be applied for modelling heterologous-protein production. However, the large number of generations involved in chemostat cultures requires that expression vectors used for such studies be extremely stable. Unfortunately, this is not the case for most episomal vectors currently available for the expression of heterologous proteins in yeasts (3). Techniques for the multi-copy integration of expression cassettes in the yeast genome combine a high copy number with a high mitotic stability (3, 16). Such vectors have been used to study the relationship between growth rate and specific product formation rates of heterologous α-galactosidase in the yeasts *S. cerevisiae* and *H. polymorpha* (17). When the simple model presented in this paper is adapted by including an empirical μ-versus-q_p relation and the relevant physiological parameters, it should be directly applicable to other wild-type and genetically engineered strains.

NOMENCLATURE

C_p : product concentration, kg product $\cdot m^{-3}$
$C_{p,ext}$: product concentration in extracellular medium, kg product $\cdot m^{-3}$
C_s : substrate concentration in reactor, kg substrate $\cdot m^{-3}$
C_{si} : substrate concentration in feed, kg substrate $\cdot m^{-3}$
C_x : biomass concentration, kg dry weight $\cdot m^{-3}$
D : dilution rate, h^{-1}
F_s : feed rate, $m^3 \cdot h^{-1}$
μ : specific growth rate, h^{-1}
m_s : substrate requirement for maintenance, kg substrate $\cdot$ (kg dry weight)$^{-1} \cdot h^{-1}$

q_p : specific rate of product formation, kg product·(kg dry weight)$^{-1}$·h^{-1}
r_p : rate of product formation, kg product·h^{-1}
r_x : rate of biomass production, kg dry weight·h^{-1}
V : culture volume, m^3
V_{cell} : volume occupied by biomass, m^3
V_{spec} : specific volume of biomass, m^3·(kg dry weight)$^{-1}$
V_0 : initial culture volume, m^3
Y_{sx} : biomass yield on substrate, kg dry weight·(kg substrate)$^{-1}$

ACKNOWLEDGMENT

This research was sponsored by the Dutch Ministry of Economic Affairs and by Unilever Research Vlaardingen.

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