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A MODEL FOR CONTINUOUS PRODUCTION OF THERMALLY INDUCED RECOMBINANT PROTEINS

R. Cubarsí[(1)], A. Benito, M. Vidal, J. Cairó and A. Villaverde*
Institut de Biologia Fonamental and Departament de Genètica i Microbiologia; Universitat Autònoma de Barcelona; Bellaterra; E-08193, Barcelona; SPAIN and (1) Departament de Matemàtica Aplicada i Telemàtica; Universitat Politècnica de Catalunya; Barcelona, SPAIN.

Summary: General conditions for continuous expression of heterologous genes from P_L promoter in two fermenters connected in series have been established. The induction time of the bacterial cells is calculated as a function of the retention time in the inducing reactor. Using this model, it is possible to adapt fermentation parameters to the particular behaviour of any specific recombinant clone.

INTRODUCTION

The strong P_L promoter of bacteriophage lambda is one of the most useful genetic elements for the controlled expression of heterologous recombinant proteins in *Escherichia coli* (de Boer and Shepard, 1983). If the host strain or the vector contains the *c*I857 gene, encoding a thermosensible repressor of P_L (Casadaban, 1980), the production of the desired proteins can be carried out by thermal induction of the culture (Rossenberg *et al.*, 1983). Under non inducing conditions, transcription from P_L is repressed. This fact allows to maintain stable plasmid clones for the production of toxic proteins. Thermal induction has also the advantage of being independent of the addition of inducing genotoxic substances such as nalidixic acid, which are required in CI^+ strains and have to be carefully removed during the downstream processing of the products.

In strains that retain the recombinant proteins inside the cell, the maximum yield is reached transiently during a period of time after the temperature shift. The duration of this period is specific for each particular clone, and depends on the toxicity of the product, its degradation rate, the ability of the host strain to grow at 42°C, and probably other undefined variables. The production of such proteins is usually done by batch fermentation procedures (Okita *et al.*, 1989 and references therein).

In this work we have developed a fermentation model for the continuous expression of P_L-controlled genes in a *c*I857 strain using two reactors in series. A computer simulation based on this model predicts an efficient and constant production of recombinant proteins.

MODEL DESCRIPTION

The system consists of two fermenters connected in series. In the first one, at 30°C, the culture is maintained in a steady-state, with the cells at the optimal metabolic conditions. The flow is to be adapted to the particular conditions of the strain. In the second reactor at 42°C, cells are thermally induced during their residence. The volume of this reactor needs to be established, according to the strain-specific optimal induction time as described below. Our purpose was to determine the variation of the induction time of each elementary volume unit of the culture during the fermentation. This information allows to assess the system by predicting the product yield in a continuous fermentation of a strain in which the induction time is a critical factor.

The model focuses on the parameters of the inducing reactor and is based on several assumptions:

1) The second, inducing, fermenter is considered as "perfectly mixed", and product and cell concentration in the outlet are identical to its concentration in and throughout the fermentation broth (Bailey and Ollis, 1986).
2) Each unit volume of the incoming non-induced culture is considered an individual source of the desired product, irrespective of the cell concentration and of the number of cell divisions occurring during the induction.
3) The cells reach the temperature of 42°C immediately after entering the second fermenter. Thus, the induction time is considered to be equal to the residence time.

When the steady-state is reached in the first reactor, the second one begins to be filled. In circumstances in which it were advantageous to alter the input rate of the culture during the fermentation, the flow would be

$$F(t) = \frac{dv(t)}{dt} \tag{1}$$

If V is the volume and θ the retention time in the inducing reactor, the following relationship is satisfied,

$$V = v(\theta) = \int_0^{\theta} F(t)\, dt \tag{2}$$

and the average induction time $\tau(t)$, when the induced broth starts to flow from the full reactor, is

$$\tau(\theta) = \frac{1}{V} \int_0^{\theta} t\, F(t)\, dt \tag{3}$$

Thereafter, in time t after the retention time, the variation of the average induction time caused by the entering of an elemental volume of culture dv will be

$$\tau+d\tau = \frac{dv\ dt + (V-dv)(\tau+dt)}{V} \tag{4}$$

By simplifying the foregoing equation and taking into account Eq.(1), we get the following linear differential equation,

$$\frac{d\tau}{dt} + \frac{F(t)}{V}\tau - 1 = 0 \tag{5}$$

whose solution, by integration, is

$$\tau(t) = e^{-\frac{v(t)}{V}} \left[\int e^{\frac{v(t)}{V}} dt + C \right] \tag{6}$$

where C is a constant to be calculated from Eq.(3).

Induced culture density function

At a time $T > \theta$ during the fermentation (after the complete filling of the inducing reactor), it is possible to know the volume of culture $N_T(t)$ that has been induced during a time from t to $t+dt$. At an instant $x \in (\theta,T)$, the same volume of culture $F(x)$ enters and emerges simultaneously. Thus, during the interval of time from x to $x+dx$ and for any time $t < x$, the volume $N_x(t)$ will turn into the new volume $N_{x+dx}(t+dt)$, induced during the subsequent lapse of time, excluding the proportional fraction of the volume that has left the reactor during this lapse of time. Therefore, the diminution of the volume $N_x(t)$ in the reactor will be

$$N_{x+dx}(t+dt) - N_x(t) = - N_x(t) \frac{F(x)\,dx}{V} \tag{7}$$

Moreover, since this evolution is done being $dx=dt$, that is, by keeping constant the difference $s=x-t$, we can rewrite Eq.(7) depending only on the variable x:

$$dN_x = N_{x+dx}(x+dx-s) - N_x(x-s) = - N_x(x-s) \frac{F(x)\,dx}{V} \tag{8}$$

In order to solve this equation, we must distinguish two cases: (a) For $s > \theta$ then $t < T-\theta$ is deduced. In this case we consider a certain volume of culture $N_T(t)$ that entered after the complete filling of the inducing reactor. Thus, the integration of Eq.(8) from $x=T-t$ to $x=T$ with an initial volume $F(T-t)$ gives us the following solution:

$$N_T(t) = F(T-t)\, e^{-\frac{v(T)-v(T-t)}{V}} \quad ;\ t < T-\theta \tag{9}$$

In this case, the induced culture density function is proportional to a decreasing exponential function depending on the relative volume that has entered during a period

of time t just before the time T.

(b) For $s \leq \theta$ then $t \geq T\text{-}\theta$ is deduced. In this other case we consider a volume of culture $N_T(t)$ that was already present before the complete filling of the inducing reactor. Thus, Eq.(8) has to be integrated from $x=\theta$ to $x=T$ with an initial volume $F(T\text{-}t)$:

$$N_T(t) = F(T\text{-}t)\ e^{-\frac{v(T)\ -\ V}{V}} \quad ;\ t \geq T\text{-}\theta \tag{10}$$

In this case, the induced culture density function is proportional to a decreasing exponential factor depending on the relative volume that has entered after the filling of the inducing reactor.

Induced culture distribution function

At a time $T > \theta$, the volume of culture $M_T(t)$ induced up to time t is

$$M_T(t) = \int_0^t N_T(u)\ du \tag{11}$$

They are again two possibilities: (a) For $t < T\text{-}\theta$, obviously we have

$$M_T(t) = \int_0^t F(T\text{-}u)\ e^{-\frac{v(T)\ -\ v(T\text{-}u)}{V}}\ du = V\left(1\ -\ e^{-\frac{v(T)\ -\ v(T\text{-}t)}{V}}\right) \tag{12}$$

(b) For $t \geq T\text{-}\theta$, in the integration of Eq.(11), we must use Eq.(9) for $u < T\text{-}\theta$ and Eq. (10) for $T\text{-}\theta \leq u \leq T$. Thus,

$$M_T(t) = \int_0^{T\text{-}\theta} F(T\text{-}u)\ e^{-\frac{v(T)\ -\ v(T\text{-}u)}{V}}\ du + \int_{T\text{-}\theta}^t F(T\text{-}u)\ e^{-\frac{v(T)\text{-}V}{V}}\ du =$$

$$= V\ -\ v(T\text{-}t)\ e^{-\frac{v(T)\text{-}V}{V}} \tag{13}$$

Note that $M_T(T) = V$.

CONTINUOUS FERMENTATION WITH CONSTANT FLOW

We apply the model to a particular case in which the input flow is constant:

$$F(t) = Q \tag{14}$$

Then, the volume of the second reactor is, according to Eq.(2), $V=Q\theta$ and the average induction time when the first sample of induced culture emerges is, according to Eq.(3), $\tau(\theta)=\theta/2$. From Eqs.(1) and (14), by integration we obtain $v(t)=Qt$ and Eq.(6) becomes

$$\tau(t) = \theta\left[1\ -\ \frac{1}{2}\,e^{-\frac{t}{\theta}}\right] \tag{15}$$

Hence, the average induction time varies from the half retention time to the retention time in a exponential way. Moreover, for the density function corresponding to Eq.(9), in the first domain, we get the following decreasing exponential

$$N_T(t) = Q\ e^{-\frac{t}{\theta}} \ ;\ t < T\text{-}\theta \tag{16}$$

and for Eq.(10), in the second domain, we get the constant function

$$N_T(t) = Q\ e^{-\frac{T-\theta}{\theta}} \ ;\ t \geq T\text{-}\theta \tag{17}$$

which is a simple prolongation of Eq.(16). Finally, according to Eqs.(12) and (13), the distribution function is transformed into

$$M_T(t) = V\left(1 - e^{-\frac{t}{\theta}}\right) \ ;\ t < T\text{-}\theta \tag{18}$$

$$M_T(t) = V\left(1 - \frac{T-t}{\theta}\ e^{-\frac{T-\theta}{\theta}}\right) \ ;\ t \geq T\text{-}\theta \tag{19}$$

Thus, the induced culture distribution function is a continuous function with a first domain depending also on an exponential law and a second domain depending on a linear function of time.

Previous equations demonstrate that it is possible to set both average induction time and induced culture distribution, by modifying the volume of the inducing reactor or the input flow.

PRODUCT YIELD DURING A CONTINUOUS FERMENTATION

To test the usefulness of our two-stage system we have performed a numerical simulation based on the model described above. The simulation predicts the product yield at the outlet during a fermentation of an hypothetical CI857 bacterial strain, in which a gene of interest has been cloned downstream of the P_L promoter. From data obtained in a discontinuous culture experiment, we can estimate the units of protein p(t) that will be detected by processing one *ml* of culture at a time t during the fermentation. Hence, the total yield of protein $P(T)$ at time T will be calculated as

$$P(T) = \frac{1}{V}\int_0^T p(t)\ N_T(t)\ dt \tag{20}$$

We have defined a working case in which the period of detection of the product after the induction is very short (Table 1). Within this period, we have selected 100 *min* as retention time. If the flow to maintain the steady-state in the first reactor is 20 *ml/min*, then we will need a 2 *l* volume inducing reactor. The numerical integration

of Eq.(20) predicts a nearly constant yield of the protein from 150 *min* after filling the second reactor (Fig. 1). Thus, we conclude that our design based on two fermenters connected in series is a useful tool to continuously express proteins in a P_L-cI857 bacterial system, even when the recombinant strain exhibits a very critical induction time. The applicability of this two-stage design to the large-scale production of recombinant proteins needs to be further evaluated.

time *(min)*	$p(t)$ *(units/ml)*
$0 < t \leq 15$	0
$15 < t \leq 45$	0.50
$45 < t \leq 90$	0.75
$90 < t \leq 110$	1
$110 < t \leq 155$	0.75
$155 < t \leq 185$	0.50
$185 < t$	0

Table 1
Product yield after induction in a discontinuous culture.

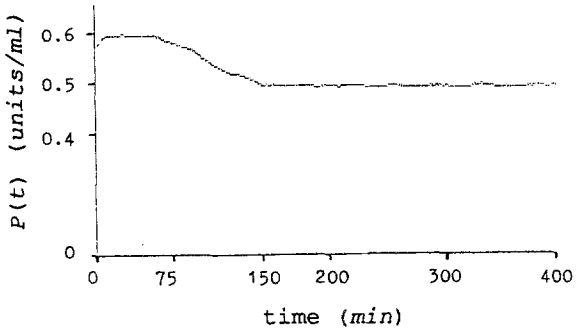

Figure 1
Product yield at the outlet in a continuous fermentation.

NOMENCLATURE

$F(t)$ =flow at time t [*ml/min*]
$M_T(t)$ =culture induced, at time T of fermentation, during a period up to t [*ml*]
$N_T(t)$ =culture induced, at time T of fermentation, during a period from t to $t+dt$ [*ml*]
$p(t)$ =product yield in a discontinuous culture [*units/ml*]
$P(t)$ =product yield at the outlet of the fermenter [*units/ml*]
$v(t)$ =volume of culture entered into the inducing reactor up to time t [*ml*]
V =volume of the inducing reactor [*ml*]
Greek letters:
θ =retention time in the inducing reactor [*min*]
$\tau(t)$ =average induction time at time t [*min*]

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