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CONDITIONS FOR A CONTINUOUS PRODUCTION OF TRANSIENT MICROBIAL PRODUCTS IN A TWO-STAGE FERMENTATION SYSTEM

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SUMMARY: The optimal residence time in the inducing reactor of a continuous two-stage system has been studied in order to maximize the yield in such a process. The attention has been focused on the case in which the product (or one of its forms) appears in the culture only transiently after the induction. Whereas in some cases the two-stage system is able to improve the yield and to allow a continuous product concentration in the outgoing stream, in others, and depending on how the product is accumulated in the induced culture, no optimal residence time can be found, and a higher productivity will be achieved by using the one-stage continuous strategy or a batch fermentation.

Introduction. Microbial cells are able to produce substances after a change in the physical or chemical parameters in the media. Apart from the natural compounds, whose synthesis is controlled by fine regulatory mechanisms, recombinant proteins are some times transiently obtained after induction in heterologous systems such as *Escherichia coli*, at least in the expected form. The reasons for those non-continuous productions include toxicity of the inducer or the product on the host cells, cell death or decrease in protein synthesis rate, degradation of recombinant proteins by cellular proteases, plasmid instability and others. To improve productivity, two-stage fermentation strategies were developed in recent years, and they have been used both for natural (Berry et al., 1990; Lee et al., 1992) and recombinant (Lee et al., 1988) products. Recombinant plasmid stability and other parameters of the cultures have been independently analyzed in these conditions (Siegel and Dewey, 1985; Hortacsu and Ryu, 1990). In a previous work (Cubarsi et al., 1991), we proposed a model to predict the product concentration in the inducing reactor during such a bioprocess, using data about the ability of each single fraction of the culture in accumulating the product after the induction. In the present work, we have determined the optimal residence time in the inducing reactor. Results obtained show that not all the transient products can be better obtained in the two-stage system. However, for substances detected during a very critical period, an optimal residence time can be found and a continuous product concentration could be maintained in the outlet flow by using the two-stage strategy.

Fermentation system. Two reactors are connected in series. In the first one, the cells are growing in a steady state. A constant output flow Q enters to a second perfectly mixed inducing reactor of a defined volume V, in which conditions for induction are kept constant. The residence time in this second vessel is $\theta=V/Q$. At the age T from the entering of the first culture unit in the second reactor, the culture induced during a period of time between t and $t+dt$ can be expressed as previously described (Cubarsi et al., 1991),

$$N_T(t) = Q \exp(-\frac{t}{\theta}), \text{ if } t < T-\theta$$
$$N_T(t) = Q \exp(-\frac{T-\theta}{\theta}), \text{ if } t \geq T-\theta \qquad (1)$$

To find out the optimal residence time regarding productivity, we propose to use experimental data about evolution of product concentration in a continuous culture in a single-stage, which has been induced after reaching the steady-state. The strain, media, dilution rate and the other physical and chemical parameters must be the same that those which are going to be established in the two-stage system. If the product is only detected during a very short period, even data from an induced batch culture could be tentatively considered. This kinetics of product concentration versus induction time is a reflect of the ability of each culture volume unit to accumulate the product under inducing conditions. All the variables tending to increase and decrease the amounts of product become synthesized in the kinetics, and are represented by means of the function $p(t)$.

The outflow from the inducing reactor contents the desired product with a concentration $P(T)$, at the age T. This value is the product accumulated by the culture in the second reactor at the age T:

$$P(T) = \frac{1}{V} \int_0^T p(t)\, N_T(t)\, dt \qquad (2)$$

A further production in a two-stage system, will depend on the shape of the kinetics, which is specific for each strain and conditions, and on the residence time in the second reactor. Thus, only the residence time have to be modified in order to obtain the maximum yield. Furthermore, the general features of the kinetics in a one-stage process with a single reactor can be characterized from three different periods after the induction (Figure 1):

1) Up to the time a, when there is no detectable product.

2) Between the times a and b, with a non constant product concentration.

3) From the time b, where the product concentration remains nearly constant at $p(b)=m$.

In some experimental situations it would be possible an absolute reduction of the product concentration. In this case the value of m is null, and we call it *type-I* kinetics. On the other hand, for non toxic and resistant substances, the product concentration can follow a monotonic increasing curve, with a trend to the asymptotic value m. Then we say that it is a *type-III* kinetics. In other cases, the kinetics of the product concentration can show an intermediate behavior, with a first increasing period followed by further decrease. We name this situation *type-II* kinetics.

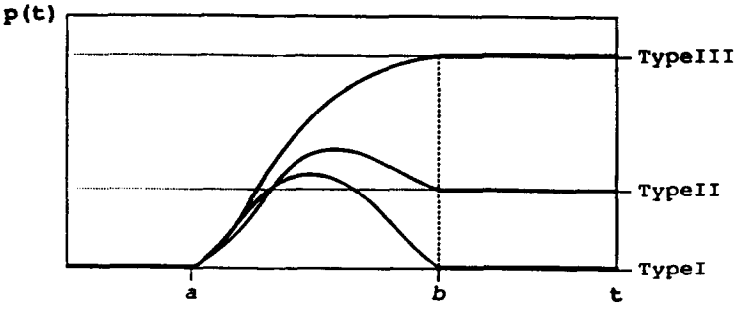

Figure 1: Three types of kinetics depending on the shape of the product concentration curve in a single stage production process.

Maximal product concentration in a two-stage system. For the foregoing types of kinetics, and when the age T is much greater than the residence time θ, the product concentration in the two-stage fermentation system, $P(T)$, becomes constant. The goal of this work is precisely to see whether it is possible to maximize this asymptotic production as a function of the residence time in the second reactor. For this reason, according to Eq.(1) and Eq.(2), we must find the maximum value of

$$L(\theta) \equiv lim_{T\to\infty}\, P(T) = \frac{1}{\theta}\int_0^{\infty} p(t)\, \exp(-\frac{t}{\theta})\, dt \qquad (3)$$

Nevertheless, taking into account the characteristic kinetic intervals mentioned in the previous section, the expression to maximize becomes the following one,

$$L(\theta) = \frac{1}{\theta}\int_a^{b} p(t)\, \exp(-\frac{t}{\theta})\, dt + m\, \exp(-\frac{b}{\theta}) \qquad (4)$$

If the shape of the function $p(t)$ were known, the maximum value of the asymptotic product concentration could be found in an analytical way. However, only some isolated experimental values of the one-stage kinetics are usually available, and diverse strategies based on numerical methods have to be improved. Nevertheless, our purpose is not to discuss the optimization methods, but to analyze which of the three types of kinetics lead to an optimal residence time:

a) Type-I kinetics. The product elimination is complete and the value m is null. Then the asymptotic production $L(\theta)$ vanishes for the residence time $\theta \to \infty$ and the optimal residence time θ_0 satisfies the condition $a < \theta_0 < b$. In other words, it is always possible to find a residence time that maximize the function $L(\theta)$. For this time, the concentration product of the kinetics $p(t)$ is not zero.

b) Type-II kinetics. There is a certain elimination of product with the value $m \neq 0$. Then there are two possibilities. First, an optimal value for $L(\theta)$ may exist, depending on the shape of the kinetics $p(t)$ and, in particular, on the value of m. Second, the product concentration may be an increasing function of the residence time. In both cases the asymptotic product concentration tends to m for the residence time $\theta \to \infty$. Hence, if an optimal residence time does exist, then the maximal product concentration must be greater than the value m.

c) Type-III kinetics. There is not a finite optimal residence time in the second reactor of the two-stage system, since the kinetics of the one-stage continuous production is a monotonic increasing function with the value $m \neq 0$. Thus the greater the residence time, the higher the product concentration. In this case it is not suitable to try a two-stage continuous fermentation.

Maximal product concentration in type-II kinetics. Let us see when is it possible to obtain an optimal asymptotic product concentration in the two-stages system, with cultures whose kinetics $p(t)$ shows a certain decrease of the yield after the induction. First, we compare the optimal residence times between a type-II kinetics with constant yield $m \neq 0$ from a time b, and the nearest type-I kinetics, which is just the same up to the time b, but after this time the product concentration falls to zero (Figure 2).

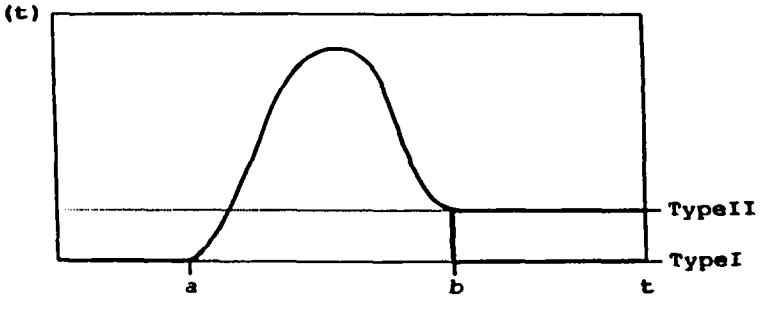

Figure 2: Type-I kinetics transformed into the nearest type-II kinetics.

Let $L_0(\theta)$ and $L_m(\theta)$ be the asymptotic product concentrations for a residence time θ in the type-I and type-II kinetics respectively (Figure 3), and let θ_0 and θ_m be the

corresponding optimal residence times, if they exist. Then the following inequalities must be satisfied

$$\theta_0 < \theta_m , \quad L_0(\theta_0) < L_m(\theta_0) < L_m(\theta_m) \tag{5}$$

These inequalities lead us to express a sufficient condition over the kinetics of the one-stage continuous fermentation, in order to prove the existence of an optimal value for the product concentration.

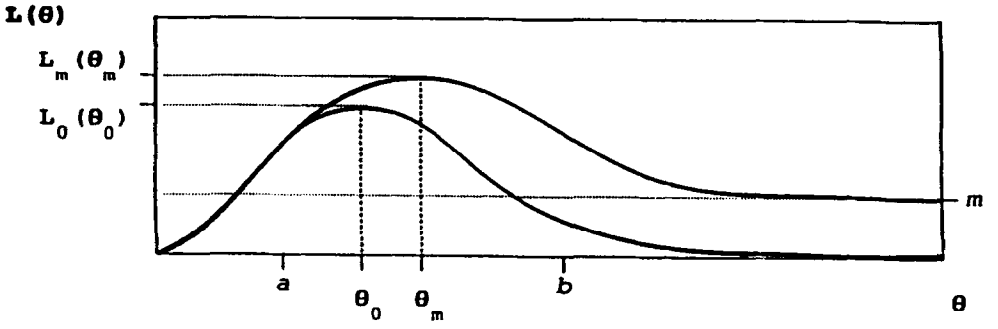

Figure 3: Optimal residence times in a two-stage process for the kinetics of the Figure 2.

Condition 1. Under the circumstances described above, if the following relationship is satisfied,

$$m < L_0(\theta_0) \left(1 - \exp(-\frac{b}{\theta_0}) \right)^{-1} \tag{6}$$

then, for type-II kinetics, an optimal residence time with maximal yield does also exist. Notice that the right hand member of Eq.(7) is easily calculated from the optimal residence time θ_0 of the nearest type-I kinetics, which can be always computable by using an adequated numerical method.

It is also possible to give a stronger sufficient condition. This new condition becomes an equivalence in the case of a great family of functions $L(\theta)$ which have a sole relative extreme (Figure 4).

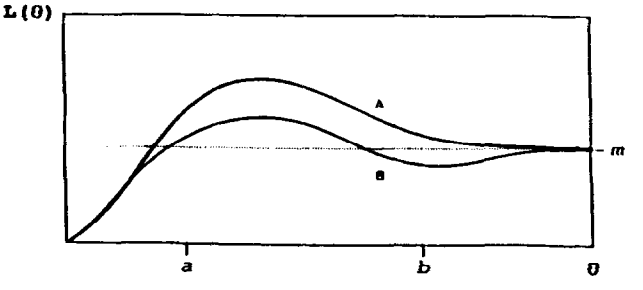

Figure 4: The asymptotic product concentration (A) becomes stable in a decreasing way from its maximum. The existence of the maximum is guaranteed by the second sufficient condition. For the curve (B) this is not true.

Condition 2. For type-II kinetics, if the function $L(\theta)$ reaches the asymptotical value m in a decreasing way when $\theta \to \infty$, then a finite residence time exists with maximal product concentration. In this case, the following relationship must be always fulfilled:

$$m < \frac{1}{b} \int_a^b p(t)\ dt \qquad (7)$$

Let us note that the right hand member of this inequality corresponds to the mean value of the kinetics in a single-stage continuous culture from zero up to the end of the non constant yield. This is a key value enough to asses a maximal asymptotic product concentration for a finite residence time.

The general applicability of our model in production processes has to be assessed by performing a reasonable number of experiments and comparing the obtained to the predicted results. Therefore, further modifications could have to be introduced to improve the reliability of the predictions in particular systems. However, in its present form, the model is a useful tool to decide the convenience of assaying a two-stage strategy for the obtention of a specific transient product (or one of its forms, such as intracelular protein when cell lysis occurs after induction, active enzymes or infectious viruses, etc.), by analyzing the evolution of the product yield in a one-stage continuous process.

Nomenclature

a = time when product appears in a single reactor [*min*].
b = time when product concentration becomes constant in a single reactor [*min*].
$L(\theta)$ = asymptotic product concentration in the two-stage system [*units/ml*].
m = constant product concentration $p(b)$ in a single reactor [*units/ml*].
$N_T(t)$ = volume of culture induced a time between t and $t+dt$ at the age T [*ml*].
$p(t)$ = product concentration in a one-stage continuous culture at time t [*units/ml*].
$P(T)$ = product concentration at the outlet of the fermenter at the age T [*units/ml*].
Q = constant flow [*ml/min*].
V = volume of the inducing reactor [*ml*].

Greek letters:
θ = residence time [*min*].

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