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PRODUCTION OF THERMALLY INDUCED RECOMBINANT PROTEINS RELATIVE TO CELL BIOMASS IS INFLUENCED BY CELL DENSITY IN *Escherichia coli* BATCH CULTURES

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SUMMARY Fed-batch cultures of recombinant *Escherichia coli* were studied regarding their ability to permit CI857^{ts}-controlled gene expression at different growth phases. Cells, growing at 50% pO_2 and 28°C in a bioreactor, were transferred to a shaker flask at 42°C and the product yield was analysed after a short incubation time. Within the batch period, the synthesis of recombinant β-galactosidase relative to the increase of biomass during the induction period was enhanced in an biomass-dependent fashion. Whereas in young cells biomass production dominates over recombinant protein production, the available energetic resources of aged cells seem to be employed more efficiently for CI857-controlled biosynthesis of recombinant proteins than of cell material. A model to describe this 'altruistic' behaviour of old cultures is here proposed.

INTRODUCTION

Many procariotic expression vectors have been dessigned, developed and studied in order to permit a thigh regulation of gene expression during production of recombinant proteins in *E. coli*. Among them, those based on the strong bacteriophage λ lytic promoters (p_R or p_L) and its temperature sensitive CI857 repressor have been extensively employed. This system has been extremely useful at laboratory scale to obtain small amounts of proteins for research purposes, and its application to large-volume processes has also been considered (Caulcott and Rhodes, 1986). The temperature is an excellent controller of gene

expression (Villaverde et al., 1993) and some very elegant, detailed analysis indicate that the yield of a recombinant product can be largely improved by adopting an appropriate temperature profile during induction (Hortacsu and Ryu, 1990, and references therein). However, there is a general lack of data referring to the best growth phase of a batch culture in which induction produces a maximal yield. In continuous cultures, low dilution rates seem to enhance production, but this common observation has not a direct applicability in other experimental situations. In general, it is asumed that for a batch culture, an early exponential phase is appropriate to carry out the temperature shift.

In this work, we have explored the product yield obtained in batch cultures induced at different phases, and a general model to explain the increase of both recombinant proteins and biomass during induction is proposed. By means of two parameters with presumable biological significance, the model describes how the distribution of biosynthetic resources in the recombinant cells is influenced by cell density.

MATERIAL AND METHODS

Plasmids, bacterial strains and growth conditions Plasmid pJCO46 is similar to pJLACZ (Benito et al., 1993) but contains some additional restriction sites at the 5' end of the *lacZ* gene (Vila et al., accepted for publication). The expression of *lacZ* is controlled by both lambda p_L and p_R promoters placed in tandem (Schauder et al, 1987). Experiments were done with *E. coli* MC1061 strain (Sambrook et al, 1989) harbouring pJCO46. Cultures from a 1.5 ml glicerolate were reactivated overnight in LB medium, growth in CAM9 (Maniatis et al, 1982) plus 2 g/l glucose, 100 μg/ml ampicillin and 50 μg/ml streptomycin at 28°C and 250 rpm in 100 ml flasks, and further used to inoculate the final volume of fresh medium at 1:30 dilution.

Batch and fed-batch culture conditions Fed-batch cultures were performed in a 2-liter Braun Biotech Biostat B bioreactor equiped with a gas mixing station. The culture media has been previosly described (Mori et al., 1979). Glucose and antibiotics were added at the concentrations stated above. pH was kept to 7.0 and pO2 to 50% by both air flow and stirring control loops. The initial volume was 1 l, and variable volumes of glucose, salts and oligoelements solutions were added when the μ was observed to decrease.

Thermal induction Samples of about 10 ml of cells growing at 28°C were transferred to a 50 ml flask and incubated at 42°C in a water bath at 200 rpm. The inducing temperature was achieved almost immediatly.

Analysis of biomass and β-galactosidase Optical density at 550 nm of diluted samples was used as a measure of biomass. β-Galactosidase was analyzed by the Miller's procedure (Miller, 1972), but cell lysis of culture samples in Z buffer was mediated by chloroform treatment.

RESULTS AND DISCUSSION

Production of β-galactosidase and biomass during temperature induction of fed-batch cultures Every 30 minuts, cell samples from the culture growing at 28°C in the bioreactor were transferred to a shaking bath prewarmed at 42°C. After one hour of induction, β-galactosidase activity as well as other parameters were analyzed in each of these samples. This short period of induction was choosen to reduce at maximum putative degradation of the enzyme, thus allowing us to estimate recombinant protein concentration by its enzymatic activity. Results of a representative experiment are sumarized in Table 1.

Table 1 Cell growth and β-galactosidase production in a fed-batch MC1061/pJCO46 culture

	Before induction			After induction			
	AGE (hours)[a]	OD_1	μ_1 [b]	OD_2	μ_2	U/ml	$U/mlOD_2$
	0	0.25	ND	0.50	0.69	1,480	2,960
	0.5	0.28	0.22	0.61	0.78	2,272	3,725
	1	0.36	0.50	0.73	0.70	2,467	3,380
	1.5	0.45	0.44	0.92	0.71	3.797	4,128
	2	0.56	0.43	1.02	0.60	4,383	4,297
	2.5	0.71	0.47	1.26	0.57	4,484	3.559
	3	1.00	0.68	1.44	0.36	4,036	2,803
▸	3.5	1.40	0.67	1.72	0.20	3,314	1,927
	4	1.68	0.36	2.20	0.27	5,907	2.685
▸	4.5	2.20	0.90	2.70	0.20	4,706	1,743
	5	2.80	0.48	3.50	0.22	4,865	1,390
▸	5.5	4.10	0.76	4.50	0.09	5,449	1,211
	6	4.90	0.35	5.20	0.06	4,134	795
	6.5	5.80	0.33	6.40	0.01	5,600	875
▸	7	7.00	0.37	8.01	0.13	5,302	662
▸	22.5	38	0.11	35	0[c]	ND	ND
	23	39	0.05	31	0	589	19
	23.5	41	0.10	36	0	468	13
	24	39	0	36	0	ND	ND

[a] Time after the inoculum. Arrows indicate the sample after which the culture was fed.

[b] Average growth rates before induction (μ_1) were calculated using the OD_1 of the present sample and that from the previous one, and those during induction (μ_2) using OD_1 and OD_2 of the same sample.

[c] Negative values of μ are not considered.

ND: not determined or not detected.

Cell counting done throughout the experiment revealed the absence of plasmid-free cells even at high cell densities (not shown). The analysis of these data permits to compare optical density just before induction (OD_1) with biomass

evolution during the inducing period and both the absolute and relative product concentration. In Figure 1, it is shown a plain representation of these relationships. The first addition of substrate during growth at 28°C has a clear influence on biomas increase during induction (panel A) and on recombinant protein concentration (panels B and C). The two further ones only have a detectable influence on biomass. On the other hand, the amount of recombinant protein corrected by optical density after induction (U/ml OD_2) has a maximum when the culture was induced at a OD_1 of 0.5 units.

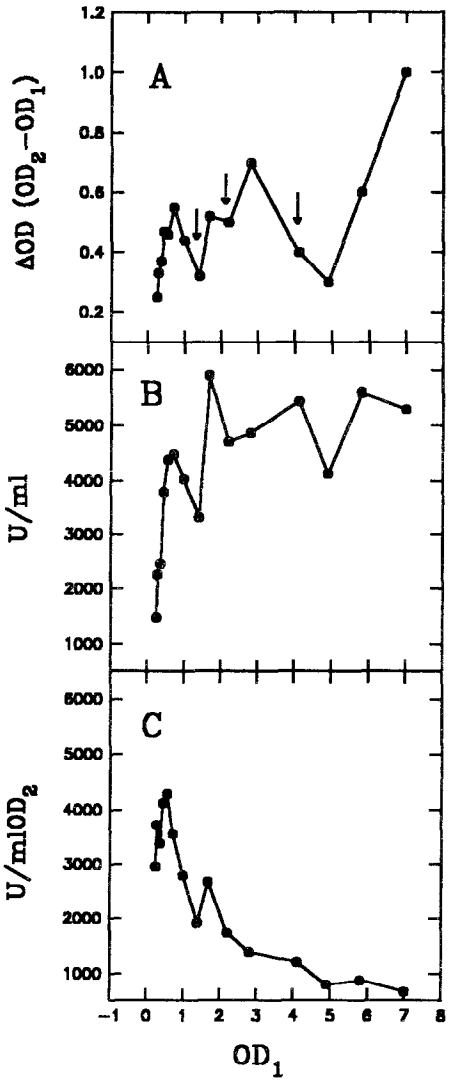

Figure 1 Biomass increase during induction (A), β-galactosidase activity (B) and the same parameter corrected by OD_2 (C) 1 hour after induction. Only the samples in which an increase in biomass is detected during induction have been considered. Arrows indicate the moments of addition of nutrients.

Influence of cell density on production of *β*-galactosidase in the batch period Only the first 8 samples among those depicted in Table 1, taken before the first feding, were considered for further analysis. The evolution of β-galactosidase during induction can be wiewed according the following equation,

$$\frac{d\beta}{dOD} = a + b \cdot OD \quad (1)$$

that, after integration can be rewritted as follows

$$\beta = \beta_1 + a\,(OD_2 - OD_1) + \frac{b}{2} \cdot (OD_2^2 - OD_1^2) \quad (2)$$

By defining $\Delta\beta=\beta_2-\beta_1$, $\Delta OD=OD_2-OD_1$ and $OD_m=(OD_2+OD_1)/2$, then

$$\frac{\Delta\beta}{\Delta OD} = a + b \cdot OD_m \qquad (3)$$

Note than in general $\beta_1=0$ and therefore, $\Delta\beta=\beta_1$. The values of parameters *a* and *b* for the experiment shown in Table 1 have been ajusted by least squares method from equation (2) and are 5,347 and 3,327 respectively. The parameter *a* could be interpreted as a summary of all the factors that coordinately influence the capability of the cells to produce β-galactosidase, mainly the strenght of the promoters and the gene dosage, and it would be a specific property of the expression vector for a given inducing condition. On the other hand, the meaning of *b* is a more intriguing point. A first analysis indicates that if $b=0$, the increase of both product and biomass during induction occurs in parallel irrespective of the OD_1, and that in all the cases the ratio between the energy that cells destinate to the synthesis of biomass and of β-galactosidase is constant. For values of $b\neq0$, this ratio is influenced by the average OD_m during induction (and therefore by OD_1). In our case, with a $b>0$, the balance between production of biomass and β-galactosidase at the suboptimal growth conditions provided by a shaker flask, is displaced towards the recombinant product when OD_1 increases. The 'altruism' represented by a positive value of *b* would indicate that the ability to produce recombinant protein in absence of growth increases whith the average cell density of the culture. A participation of proteolytic activities in the value of *b* (apart from the biosynthetic ones) cannot be discarted. From both *a* and *b*, a prediction of the ratio between production of the enzyme and biomass was done for the batch period, and compared to experimental data, showing a good concordance (Figure 2).

Results presented in this work show that in the used defined medium, the optimal OD for a maximal production yield is about 0.5 units, reaching about 4,700U/mlOD_2. This value cannot be enhanced by further addition of nutrients, whereas these additions do increase the total enzymatic activity to a constant value of almost 6,000U/ml. No influences of μ before or during induction on production have been evidenced. On the other hand, two parameters describing

the biosynthetic power of the expression system and the influence of cell biomass on the distribution of cell energy have been proposed. A deeper analysis of the models represented by the above equations could provide mechanisms to improve CI857^{ts}-controlled recombinant gene expression in batch cultures.

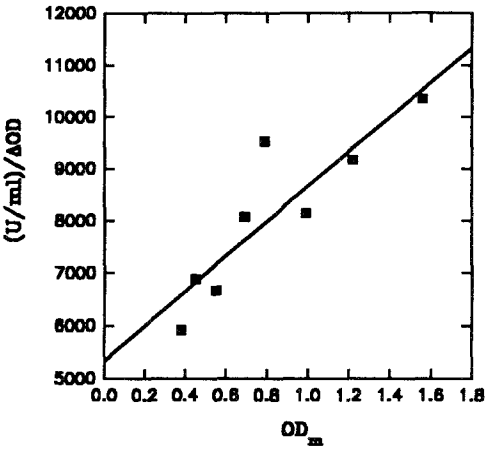

Figure 2 Relative increase of β-galactosidase during induction in the batch period of the experiment depicted in Table 1 (■). The expected behaviour according to equation (3) is also shown.

Notation

a= Ratio between β-galactosidase and biomass production (U/ml/units).
b= Variation of a depending on the OD during the induction (U/ml/units^2).
β_1= β-galactosidase activity before induction (U/ml).
β_2= β-galactosidase activity one hour after induction (U/ml).
OD_1= Optical density before induction (units).
OD_2=Optical density one hour after induction (units).
OD_m= Average optical density during the induction period (units).
μ_1= Growth rate of non induced cultures (h^{-1}).
μ_2= Growth rate of induced cultures (h^{-1}).

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