Optimization Study of *Escherichia coli* TB1 Cell Disruption for Cytochrome b_5 Recovery in a Small-Scale Bead Mill

I. Belo,[†] J. A. L. Santos,[‡] J. M. S. Cabral,[‡] and M. Mota*,[†]

Departamento de Engenharia Biológica, Universidade Minho, Largo do Paço, 4709 Braga Codex, Portugal, and Laboratório de Engenharia Bioquímica, Instituto Superior Técnico, 1000 Lisboa, Portugal

The recovery of a recombinant intracellular protein, cytochrome b_5, from *Escherichia coli* TB1 cells was carried out by bead mill disintegration in a discontinuous small-scale instrument. This process was optimized by the use of experimental factorial design. Several parameters were studied: operating time, amount and size of beads, cellular suspension concentration, and presence of toluene and lysozyme. For the experimental conditions used, only the time of treatment and bead load had significant effects. The optimal values of these variables were found by applying the response surface methodology.

Introduction

Cellular disruption processes have been receiving special attention in recent years due to the increase in economically attractive intracellular products (such as enzymes and high-value proteins), as a result of the application of genetic engineering innovations (Baldwin and Robinson, 1993).

Although the developments in secretion techniques of those bioproducts seem very promising in some microorganisms like *Bacillus* (Donald, 1990), this is not always the case in the most commonly used host in DNA-recombinant techniques, the bacteria *Escherichia coli*, where the expressed proteins generally remain inside the cell.

Therefore, cellular disintegration constitutes the first stage in the protein isolation and its optimization is very important because any improvement or damage made in this step will benefit or invalidate, respectively, the subsequent stages of purification (Keshavarz *et al.*, 1987).

Many methods of protein release are available and have been recently reviewed (Garcia, 1993). There are several techniques (chemical, physical, or enzymatic) of cellular membrane permeabilization, enabling a selective release of the intracellular material (Felix, 1982). However, its implementation on a large scale presents technical and/or economical limitations. Intracellular product recovery at an industrial scale is still accomplished by mechanical methods, mainly by high-pressure homogenization and bead mill disintegration.

Disruption efficiency depends on a large number of factors according to the characteristics of the technique, equipment, type of cell, product of interest, etc. Optimization studies should be carried out whenever a new technique or cell is to be used or a new product released.

Studies on optimization of cell disruption in a bead mill have been carried out by several authors (Currie *et al.*, 1972; Limon-Lason *et al.*, 1979), but most of the work has been done on yeast cells. More recently, van Gaver and Huyghebaert (1990) investigated the influence of several variables, e.g., cell and bead concentration, rotation speed, and number of passes on the yeast disruption, using a sequencial experimental approach and applying the response surface methodology (RSM) thereafter.

On the other hand, Garrido *et al.* (1994) studied the disruption of a recombinant yeast by sequentially investigating the effect of each variable, e.g., yeast cell concentration, agitation speed, bead loading, feed flow rate, beads diameter, number of passes, and pH of the slurry, on protein release. The authors did not apply the response surface methodology to build the model equation for protein excretion.

In a previous work, the release of cytochrome b_5, a recombinant protein, from *E. coli* TB1 cells, by freeze−thawing and sonication, was studied (Santos *et al.*, 1995).

Cytochrome b_5 (cyt.b5) of the endoplasmic reticulum of mammalian liver is a well characterized, small haemoprotein of 13.6 kD which plays a central role in a variety of electron-transfer bioreactions (Karim *et al.*, 1993). Its successful gene cloning and expression in *E. coli* TB1 cells provides an excellent way for obtaining large amounts of purified protein for many biophysical and chemical studies (von Bodman *et al.*, 1986).

In this work, we have optimized the cyt.b5 release by bead mill disintegration. For this, we have applied the experimental factorial design, whereby a large number of variables can be screened and the important ones identified, with a relatively small number of experiments. This methodology has been recently used in biotechnology for many purposes (Sarra *et al.*, 1993; Rodrígues *et al.*, 1992; Garcia-Ochoa *et al.*, 1992). In this work, after the identification of the important variables, the response surface methodology was used to construct the empirical model for optimal cyt.b5 release.

Materials and Methods

Microorganism. The bacteria *E. coli* TB1, genetically modified with the plasmid pUC13 (von Bodman *et al.*, 1986), was used. The vector contains the cyt.b5 expression gene and confers ampicillin resistance to the microorganism.

Culture Conditions. *E. coli* TB1 cells were grown in a 2 L batch fermenter (BIOLAB, B. Braun) with a medium composed of 1% (W/V) tryptone, 0.5% yeast extract, 0.5% sodium chloride, and 1% glucose and supplemented with 100 μg/mL ampicillin. Fermentations were carried out for 30 h at 37 °C, with 0.6 vvm of aeration, and at a pH of 7.0 adjusted automatically with the addition of 4 N NaOH and 4 N HCl.

[†] Universidade Minho.
[‡] Instituto Superior Técnico.
* To whom correspondence should be addressed.

8756-7938/96/3012-0201$12.00/0

Table 1. Experimental Matrix 2^{6-2}: Factor Levels for Each Treatment and Respective Results of Cyt.b5 Yield

treatment[a]	time (A)	cell concentration (B)	bead load (C)	bead size (D)	toluene (E)	lysozyme (F)	$Y_{cyt.b5}$ (mg of cyt.b5/gDCW)
F	−	−	−	−	−	+	3.34
AE	+	−	−	−	+	−	2.04
BE	−	+	−	−	+	−	1.89
ABF	+	+	−	−	−	+	3.72
CE	−	−	+	−	+	−	1.96
ACF	+	−	+	−	−	+	5.39
BCF	−	+	+	−	−	+	3.78
ABCE	+	+	+	−	+	−	2.11
D	−	−	−	+	−	−	0.58
ADEF	+	−	−	+	+	+	1.89
BDEF	−	+	−	+	+	+	1.10
ABD	+	+	−	+	−	−	2.53
CDEF	−	−	+	+	+	+	1.64
ACD	+	−	+	+	−	−	5.09
BCD	−	+	+	+	−	−	1.50
ABCDEF	+	+	+	+	+	+	5.38
level −	2 min	10 gDCW/L	10 g	0.15 mm	0	0	
level +	10 min	20 gDCW/L	20 g	0.5 mm	10%(v/v)	0.7 mg/mL	

[a] The letters attributed to each treatment refer to the factors which are at the highest level.

After the fermentation, cells were harvested by centrifugation at 4 °C and 9000 rpm and resuspended in phosphate buffer (pH = 7.0, 20 mM) for the disruption assays. The cells appeared pink due to the cyt.5 accumulation.

Bead Mill Disintegration. A discontinuous laboratory Edmund Bühler Vibrogen VI4 was used. This consists in a vibratory chamber which holds up stainless steel or Teflon tubes where the cellular suspension and glass beads are shaked with a fixed frequency of 75 Hz. The chamber also serves as a cooling jacket. In all of the assays the temperature was kept below 25 °C. The experiments were performed in tubes of 20 mL of capacity with 10 mL of cellular suspension. For the assays with toluene, 1 mL of pro analysis reagent was used, and for assays with lysozyme, 700 mL of a 10 mg/mL freshly prepared solution was added to the suspension.

After disruption cellular debris and beads were removed by centrifugation at 4 °C and 12 000 rpm for 45 min.

Analytical Methods. Cellular concentration (g of dry cell weight (gDCW)/L) was evaluated by the optical density measured at 600 nm. Total soluble protein was obtained by Bradford's method (Bradford, 1976). Quantification of cyt.b5 produced was achieved by an absorbance measure at 410 nm and by using the Lambert−Beer law with a known extinction coefficient (e = 130 mM^{-1} cm^{-1}) (von Bodman *et al.*, 1986).

Results and Discussion

In order to identify important variables such as operation time (factor A), cellular concentration (factor B), beads load (factor C), and bead size (factor D) for the cyt.b5 release and to investigate the possible synergistic action of the solvent toluene (factor E) and the enzyme lysozyme (factor F) with the mechanical disruption, we have used a fractional factorial design of experiments 2^{6-2} (Goupy, 1988). Table 1 shows the treatments executed, which were chosen by confounding the following interactions: I = ABCDF = ABCE = DEF.

The response of interest was the cyt.b5 yield (Y cyt.b5) expressed as mg of cyt.b5/gDCW of cell.

The treatments were executed in a random order, and three assays with all the factors at the average value (treatment "0") were performed to estimate the experimental error variance.

The absorbance at 410 nm of the supernatant of a disrupted wild type of *E. coli* (without cyt.b5) was assayed. It gave a background noise which corresponded to about 0.5 mg of cyt.b5/gDCW. This value is on the same order of magnitude of experimental error obtained for the assays with *E. coli* TB1. Therefore, it did not interfere with the results obtained in this work.

The estimates of main effects and second-order interactions (the high-order interactions were neglected) were calculated by the following equation:

$$E = \frac{1}{N/2}\left[\sum Y_{(\text{level}+)} - \sum Y_{(\text{level}-)}\right] \qquad (1)$$

where N is the number of treatments and E is the effect or interaction.

The variance estimate of the error (V_E) was obtained by combining the variance of the treatments for the treatment "0" and the sum of squares (SQ) of the nonsignificative interactions and applying the following equation (Box *et al.*, 1978):

$$V_E = \frac{4}{N}S^2 \qquad (2)$$

where S^2 is the combined variance.

By comparing the experimental error with the effects and interactions by variance analysis (Table 2), we can conclude that only the cellular suspension concentration has a nonsignificative effect (P > 10%). This is in accordance with the results obtained by Limon-Lason *et al.* (1979) and Garrido *et al.*(1994) for *S. cerevisiae* cell disruption in a continuous bead mill. The bead size and toluene have significative but negative effects. These results were expected for bead size because cytochrome b_5 is a cytoplasmic protein and smaller beads are more adequate for the total disruption of bacteria cells (Schütte and Kula, 1990). Thus, the release of cyt.b5 is greater for a smaller diameter of beads. For toluene, the result was not expected but it can be explained by the appearance of a two-phase suspension in the assays where toluene was used. Probably some cyt.b5 remaining in the toluene phase has not been quantified. This problem may be avoided by reducing the concentration of toluene used (De Smet *et al.*, 1978).

Time and bead load presented positive effects on the protein release, and the presence of lysozyme seemed to have the same action. However, as the lysozyme effect was confounded with a second-order interaction (f = de), more experiments were needed.

Table 2. Results of the 2^{6-2} Design: Values of Factor Effects and Second-Order Interactions Estimates

factor effects and interactions	estimate	SQ	df[a]	F[b]	P[c]
A	1.55	9.560	1	111.2	<1%
B	0.01	0.001	1	0.01	>10%
C	1.22	5.934	1	69.0	<1%
D = EF	−0.56	1.272	1	1.48	<1%
E = DF	−0.99	3.928	1	45.7	<1%
F = DE	1.07	4.562	1	53.0	<1%
AB = CE	−0.18	0.124	1		
AC = BE	0.73	2.114	1	24.6	<1%
AD	0.97	3.764	1	43.8	<1%
AE = BC	−0.34	0.457	1		
AF	0.09	0.030	1		
BD	0.02	0.407	1		
BF	0.02	0.706	1		
error		0.688	2		
combined		2.415	7		
V_E		0.086	7		

[a] Degrees of freedom. [b] Fisher's F statistical parameter. [c] Confidence level.

Table 3. Factorial Design 2^3: Factor Levels for Each Treatment and Respective Results of Cyt.b5 Yield

treatment	time (A)	beads (B)	lysozyme (F)	$Y_{cyt.b5}$ (mg of cyt.b5/gDCW)
(1)	−	−	−	11.63
A	+	−	−	14.03
B	−	+	−	14.36
AB	+	+	−	14.69
F	−	−	+	12.48
AF	+	−	+	14.63
BF	−	+	+	14.52
ABF	+	+	+	14.24
level −	2 min	10 g	0	
level +	10 min	20 g	0.7 mg/mL	

Table 4. Results from the 2^3 Design: Values of Factor Effects and Interactions Estimates

factor effects and interactions	estimates	SQ	F	P (1,4 df)
A	1.147	2.632	49.7	<1%
B	1.259	3.168	59.8	<1%
F	0.290	0.168	3.2	>10%
AB	−1.123	2.524	47.6	<1%
AF	−0.433	0.375	7.1	>5% 10%
BF	−0.216	0.093	1.8	>10%
ABF	−0.089	0.016	0.3	>10%
S^2 (4 df)		0.106		
V_E		0.053		

To clarify the enzyme effect and to follow an optimization procedure, we have executed a complete factorial design 2^3 (Table 3) involving the three factors with positive significant effects in previous experiments.

The treatments were carried out with cells from a new batch fermentation with a cellular suspension concentration of 10.4 gDCW/L. Glass beads of 0.15 mm diameter were used.

From the results in Table 3, the effects and interactions were estimated (Table 4). A new estimate of the experimental error was obtained at the average point of the experimental domain.

It must be noted that the yield for cyt.b5 is higher in this case because a new stock culture was used for the inoculation. The conclusions, however, are not affected, as this set of experiments was independent from the previous ones.

As we can see in Table 4, the enzyme addition did not improve the process of cyt.b5 release. The importance of operating time and bead load was confirmed as well as the existence of a significant interaction between these two variables. This result suggests that an optimum is near the chosen experimental domain. In this region, we can assume the response surface to be described by the following second-order polynomial model (Philippe, 1967):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{12} x_1 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 \quad (3)$$

where x_1 and x_2 are the coded variables for each factor, assuming the values −1, 0, or 1 as the factor is at its lowest, average, or highest level, respectively. These variables are defined by eq 4, where v_i is the value of

$$x_i = \frac{v_i - v_{oi}}{\lambda_i} \quad (4)$$

factor i at normal units, v_{oi} is the factor i average value, and λ_i is the step for each factor. Table 5 presents the values for the present case. β_i, β_{ii}, and β_{ij} are the model parameters which were estimated by the results of the 3^2 factorial design in Table 6 (Philippe, 1967).

Table 5. Values for the Determination of the Coded Variables

	v_{oi}	λ_i
x_1 (time) (min)	6	4
x_2 (bead load) (g)	15	5

Table 6. 3^2 Factorial Design

	treatment								
	1[a]	2[b]	3[a]	4[b]	5[c]	6[b]	7[a]	8[b]	9[a]
x_1	−1	0	1	−1	0	1	−1	0	1
x_2	−1	−1	−1	0	0	0	1	1	1
$Y_{cyt.b5}$ mg of cyt.b5/g DCW	12.0 6	14.7 5	14.3 3	13.6 7	14.5 1	15.6 1	14.4 4	14.4	14.4 6

[a] The yields were obtained by reorganizing the previous experiments in a 2^2 design. [b] Supplementary treatments executed with the same cell suspension as the previous ones. [c] Treatment at the central point.

The number of treatments in Table 6 was sufficient to calculate the six parameter estimates and to test for lack of fit, which was not significant at a 10% level.

$$Y_{cyt.b5} = 14.93 + 0.71x_1 + 0.36x_2 - 0.56x_1x_2 - 0.48x_1^2 - 0.54x_2^2 \quad (5)$$

It can be predicted from the response surface obtained (Figure 1) that a maximum exists for factor values close to the high level for the assayed time and to the central level for bead load.

The optimum location can be obtained by doing

$$\left.\frac{\partial Y}{\partial x_1}\right|_{x_2} = \left.\frac{\partial Y}{\partial x_2}\right|_{x_1} = 0 \quad (6)$$

Applying this expression to the model equation (eq 5) produces the values $x_1 = 0.78$ and $x_2 = -0.07$. By eq 4 this correspond to 9 min of operating time and 14.65 g of bead load. At these variable values the cyt.b5 yield predicted is 15.19 mg of cyt.b5/gDCW. To validate this result an experiment was performed with the same variable values which gave an experimental yield of 15.24 mg of cyt.b5/g DCW. The two yields, theoretical and experimental, differ only by 0.3%, demonstrating that the model adjusts very well to the experimental points.

Cyt.b5 release achieved at these optimal operating conditions was about 84% of the release obtained with

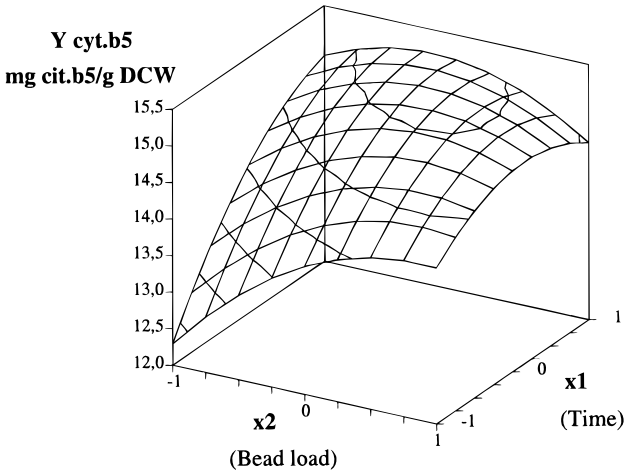

Figure 1. Theoretical response surface; predicted cyt.b5 yield as a function of operation time and bead load.

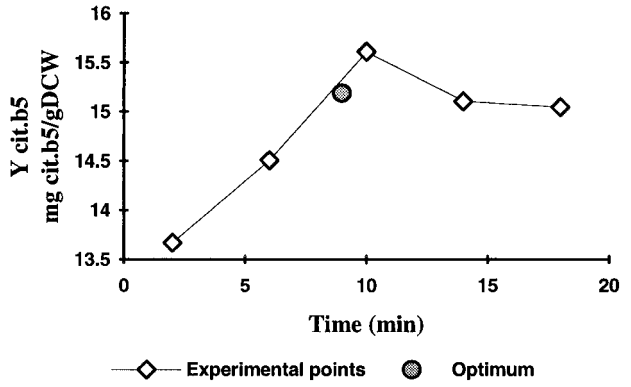

Figure 2. Time operation effect on cyt.b5 release.

ultrasound treatment (Santos *et al.*, 1995). Therefore, we tested higher values of operation time of bead mill disintegration to search for a new optimum. As we can see in Figure 2, an increase in time from 10 to 20 min did not significantly improve the process, which means that the optimum found leads to the maximum cyt.b5 release possible in the bead mill and with the operating conditions used. Changing some operating variables, for instance, increasing the bead concentration, might produce a higher protein release.

Conclusions

The mathematical methodology (factorial design of experiements and response surface methodology) used in this study of *E.* coli TB1 cell disruption in a small-scale bead mill proved to be successful for the prediction and modeling of cytochrome b_5 release.

In our case, the cell concentration was shown to be of no importance which was also the conclusion of other work done with yeasts (Limon-Lason *et al.*, 1979; Garrido *et al.*, 1994).

By studying the effect of other pertinent variables, viz. stress frequency and feeding flow rate, this same methodology may also be applied on a larger scale.

Acknowledgment

The authors acknowledge JNICT for financial support of this work (Project STRDA/C/BIO/379/92).

Literature Cited

Baldwin, C. V.; Robinson, C. W. Enhanced Disruption of *Candida utilis* Using Enzymatic Pretreatment and High-Pressure Homogenization. *Biotechnol. Bioeng.* **1994**, *43*, 46−56.

Box, G. E. P.; Hunter, W. G.; Hunter, J. S. *Statistics for Experimenters. An Introduction to Design, Data Analysis and Model Building*; John Wiley & Sons, Inc.: New York, 1978.

Bradford, M. A Rapide and Sensitive Method for the Quantification of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *J. Biochem.* **1976**, *72*, 248−254.

Currie, J. A.; Dunnill, P.; Lilly, M. D. Release of Protein from Baker's Yeast (*Saccharomyces cerevisiae*) by Disruption in an Industrial Agitator Mill. *Biotechnol. Bioeng.* **1972**, *14*, 725−736.

De Smet, M. J.; Kingma, J.; Witholt, B. The Effect of Toluene on the Structure and Permeability of the Outer and Cytoplasmic Membranes of *Escherichia coli*. *Biochim. Biophys. Acta* **1978**, *506*, 64−80.

Donald, T. M.; Mao, J. Protein Secretion Systems in Microbial and Mammalian Cells. In *Separations Process in Biotechnology*; Asenjo, J. A., Ed.; Marcel Dekker: Inc.: New York, 1990; pp 67−94.

Félix, H. Permeabilized Cells. *Anal. Biochem.* **1982**, *120*, 211−234.

Garcia, F. A. P. Cell Wall Disruption. In *Recovery Process for Biological Materials*; Kennedy, J. F., Cabral, J. M. S., Eds.; J. Wiley: New York, 1993; pp 47−66.

Garcia-Ochoa, F.; Santos V. E.; Fritsch, A. P. Nutritional Study of *Xanthomonas campestris* in Xanthan Gum Production by Factorial Design of Experiments. *Enzyme Microb. Technol.* **1992**, *14*, 991−996.

Garrido, F.; Banerjee, U. C.; Chisti, Y.; Moo-Young, M. Disruption of a Recombinant Yeast for the Release of β-galactosidase. *Bioseparation* **1994**, *4*, 319−328.

Goupy, J. *La Méthode des Plans d'Experiences. Optimization du Choix des Experiences & de l'Interprétation des Résultats*, BORDAS, Paris, 1988.

Karim, A.; Kaderbhai, N.; Evans, A.; Harding, V.; Kaderbhai, M. A. Efficient Bacterial Export of a Eukaryotic Cytoplasmic Cytochrome. *Bio/Technology* **1993**, *11*, 612−618.

Keshavarz, E.; Hoare, M.; Dunnill, P. Biochemical Engineering Aspects of Cell Disruption. In *Separations for Biotechnology*; Vernall, M. S., Hudson, M. J., Eds.; Ellis Horwood, Ltd.: Chichester, U.K., 1987; pp 62−79.

Limon-Lason, J.; Hoare, M.; Orsborn, C. B.; Doyle, D. J.; Dunnill, P. Reactor Properties of a High-Speed Bead Mill for Microbial Cell Rupture. *Biotechnol. Bioeng.* **1979**, *21*, 745−774.

Philippe, J. *Les Méthodes Statistiques en Pharmacie et en Chimie (applications à la recherche à la production et au contrôle) [Statistical Method in Pharmacy and Chemistry (Applications to Research, Production, and Control)*]; Masson: Paris, 1967.

Rodríguez, M.; Güereca, L.; Valle, F.; Quintero, R.; López-Munguía, A. Penicillin Acylase Extraction by Osmotic Shock. *Process Biochem.* **1992**, *27*, 217−223.

Santos, J. L.; Belo, I.; Mota, M; Cabral, J. M. S. Freeze/Thawing and Sonication of *Escherichia coli* TB1 Cells for Cytochrome b5 Recovery. *Bioseparation* **1996**, in press.

Sarra, M.; Redin, I.; Ochin, F.; Godia, F.; Casas, C. Application of Factorial Design to the Optimization of Medium Composition in Batch Cultures of *Streptomyces lividans* TK21 Producing a Hybrid Antibiotic. *Biotechnol. Lett.* **1993**, *15* (6), 559−564.

Schütte, H.; Kula, M. Bead Mill Disruption. In *Separations Process in Biotechnolology*; Asenjo, J. A., Ed.; Marcel Dekker, Inc.: New York, 1990; pp 108−141.

van Gaver, D.; Huyghebaert, A. Optimization of a Yeast Cell Disruption with a Newly Designed Bead Mill. *Enzyme Microb. Technol.* **1990**, *13*, 665−671.

von Bodman, S. B.; Schuler, M. A.; Jollie, D. R.; Sligar, S. G. Synthesis, Bacterial Expression, and Mutagenesis of the Gene Coding for Mammalian Cytochrome b5. *Biochemistry* **1986**, *33*, 9443−9447.

Accepted December 18, 1995.[⊗]

BP950085L

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.