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Optimization of Human Interferon Gamma Production in *Escherichia coli* by Response Surface Methodology

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ABSTRACT

The production of human interferon gamma (hIFN-γ) using a synthetic gene in *Escherichia coli* BL21-SI was optimized by response surface methodology (RSM) and a Box-Behnken design. The process variables studied were temperature, biomass concentration at induction time and the NaCl concentration as inducer. According to the Box-Behnken design, a second order response function was developed. The optimal expression conditions were a temperature of 32.6°C, induction biomass of 0.31 g/l and 0.3 M NaCl in minimal medium. The model prediction for the maximum hIFN-γ production was 77.3 mg/l, which corresponded satisfactorily with the experimental data. The hIFN-γ concentration attained under optimized conditions was 13-times higher than that obtained using the non-optimized conditions. We conclude that RSM is an effective method for the optimization of recombinant protein expression using synthetic genes in *E. coli.*

Keywords: *Escherichia coli* BL21-SI, minimum medium, therapeutic protein, synthetic gene.

INTRODUCTION

Human interferon gamma (hIFN-γ) is a cytokine involved in transcriptional control of a large number of immunological relevant genes. This glycoprotein is composed of 143 amino acid residues with a molecular mass of 20–25 kDa. The recombinant hIFN-γ produced in *Escherichia coli* has a molecular mass of 16.7 kDa and it is not glycosylated, however is physiologically active [1]. Clinical trials indicate that recombinant hIFN-γ has therapeutic efficacy on kidney cell carcinoma, colon cancer, osteopetrosis, chronic granulomatous disease and rheumatoid arthritis [2,3]. *E. coli* has many advantages as an expression system that include rapid growth, cheap cultivation, well-studied genetic background and high expression level in large-scale cultures. Thus, *E. coli* is widely used for heterologous protein expression [4–7]. However, the rapid degradation of mRNA from foreign cDNA causes poor expression in heterologous systems, which has been attributed to the difference of codon usage between eukaryotic organisms and those preferred by *E. coli* [8,9]. Codon optimization through synthetic gene design leads to improved heterologous expression, reducing translational errors [10]. On the other hand, heterologous protein production is strongly dependent on the composition of the culture medium and culture conditions such as temperature, inducer concentration, induction time and others [11,12]. Thus, optimization of fermentation conditions is further recommended before scale-up [13].

Response surface methodology (RSM) is a collection of statistical and mathematical techniques for designing experiments, building models, evaluating the effects of factors and the optimization of many chemical and biochemical processes [14,15]. In this work, we

optimized the culture conditions for the expression of a synthetic gene of hIFN-γ in *E. coli* BL21-SI using a minimal medium and the Box-Behnken design. The temperature, biomass concentration at induction time and optimal inducer concentration were determined by RSM.

MATERIALS AND METHODS

Plasmid and bacterial strain

The synthetic hIFN-γ gene was amplified using pCR4-516 (Entelechon, Regensburg, Germany) as template. The restriction sites *Nde*I and *Bam*HI were added using the forward primer 5´ CAT ATG CAG GAC CCA TAC GTG AAA 3´ and reverse primer 5´ GGA TCC TTA CTG ACT TGC 3´, respectively. The amplified product was cloned in pET12a (Novagen, Darmstadt, Germany) in the same restriction sites to obtain the expression vector pBAL0, then *E. coli* BL21-SI (GIBCO, Darmstadt, Germany) was transformed with pBAL0 and ampicillin was used for selection of transformants cells.

Culture Media

LBON agar [16] containing 100 μg/mL ampicillin was used routinely to grow *E. coli* BL21-SI/pBAL0. The minimal medium contained (per liter): 5 g glucose, 3.5 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 1.0 g $MgSO_4$, 40 μg thiamine and 100 mg ampicillin. The pH was adjusted to 7.0 prior to autoclaving for 15 min at 121°C; glucose and $MgCl_2$ solutions were autoclaved separately. For all experiments, pre-inocula were cultured overnight at 250

rpm and 37°C using the minimal medium plus 0.5% yeast extract (Difco, Franklin Lakes, NJ).

Growth conditions

Cultures were generated in 500 mL flasks containing 100 mL of minimal medium and shaken at 250 rpm. Each flask was inoculated with 3 mL of pre-inoculum to provide an initial optical density at 600 nm (OD_{600nm}) of 0.2. Temperature of incubation, biomass concentration at induction time and NaCl concentration for each experiment were defined according to the Box-Behnken design described below.

Box-Behnken design and surface response methodology

The Box-Behnken design [17] was selected to study the effect of culture conditions on the production of hINF-γ in *E. coli* BL21-SI/pBAL0. The design evaluates three independent variables, each one at three levels with three replicates at the center points (Table 1), to fit a polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^{\,2} + \beta_{22} X_2^{\,2} + \beta_{33} X_3^{\,2} \quad (1)$$

Where Y is the production of hIFN-γ, X_1, X_2 and X_3 are the independent variables for temperature, biomass concentration at induction time and NaCl concentration, respectively. β_0 is the intercept term; β_1, β_2, and β_3 are linear coefficients; β_{12}, β_{13}, and β_{23} are interactive coefficients; and β_{11}, β_{22}, and β_{33} are quadratic coefficients. The model was evaluated with

significance, good fit and R^2 values. Eq. 1 was used to build surfaces graphs for the model. The analysis of RSM, analysis of variance (ANOVA) and the optimal conditions were performed using Statgraphics Plus v5.0 software (Statistical Graphics, Herndon, VA, USA). The significant effects on dependent variables were determined by *t*-test with a probability value (*P*-value) smaller than 0.05.

Analytical procedures

Cell growth was monitored at OD_{600nm} (Varian Cary BIO-50; Varian, Palo Alto, CA, USA) and converted to dry cell weight (DCW) by a calibration curve. Total protein concentration was analyzed by Lowry method [18] using bovine serum albumin (BioRad, Hercules, CA, USA) as standard. Expression of hIFN-γ was determined by 15% w/v sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19] and visualized with 0.1% (w/v) Coomassie Brilliant Blue R250 (BioRad). For immunoblotting, proteins were transferred from the gel onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) using a Semi-Dry Transblot (BioRad). The primary antibody was polyclonal rabbit anti-hIFN-γ (PreproTech, Rocky Hill, NJ, USA) and the secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (BioRad). Color was developed with *p*-nitro blue tetrazolium and sodium-5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Amersham Biosciences). Analysis of nitrocellulose membranes was carried out using a photo-documenter Gel-Doc 2000 (BioRad) and the Quantity One™ v4.5 software (BioRad). Recombinant hIFN-γ (PreproTech) was used as standard. The hIFN-γ was quantified by an enzyme-linked immunosorbant assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Design of the synthetic hIFN-γ gene

The native hIFN-γ gene has 16 codons reported as “rare”, which cause expression problems in *E. coli* [9,20,21]. These codons are: 1 AGG and 2 AGA for arginine; 2 ATA for isoleucine; 2 CCA for proline; 3 AGT, 2 TCA and 1 TCC for serine; GGG and GGA for glycine and 1 ACA for threonine. The rare codons were replaced for those reported with more usability by *E. coli* (Table 2). The resultant synthetic hIFN-γ gene consisted in 432 bp and had 74% homology with respect to the wild-type hIFN-γ gene sequence. Sixty-three other codons with low usability by *E. coli* were also modified in one or two basis to produce codons with more frequency of use. Codon bias is one of the most important factors in the expression of eukaryotic genes in prokaryotic systems [22,23]. Therefore, many authors have proposed the use of synthetic genes to increase translational efficiency [24–29]. The optimization of the synthetic hIFN-γ gene eliminated the presence of 16 rare codons, which cause translational errors [21,30]. However, the changes did not generate codons with maximum usage in *E. coli*. This can be explained because the full optimization of the gene could be detrimental for high protein expression, due to metabolic overload in the bacteria [10]. The functionality of the synthetic hIFN-γ gene was demonstrated by its expression in *E. coli* BL21-SI.

Cultures and hIFN-γ production

In the first experiments, the production of hIFN-γ was tested in triplicate in LBON at temperature of 37°C, 0.3 g/L of biomass and 0.3 M NaCl as inducer, as previously

suggested for the BL21-SI expression system [31]. The maximum biomass concentration was 2.96±0.02 g/L at 7 h of culture and the maximum concentration of hIFN-γ attained was 5.6±0.2 mg/L at 12 h (Fig. 1a). Interestingly, using the minimal medium under the same culture conditions, maximum hIFN-γ concentration was 6-times higher than the attained in LBON, but the maximum biomass concentration was only 0.88±0.02 g/L (Fig. 1b). To improve cell growth, we tested the use of the minimal medium plus 0.5% yeast extract (Fig. 1c). The biomass concentration increased to 1.39±0.02 g/L but only 12.9±0.3 mg/L of hIFN-γ was attained. These results led us to forego the use of yeast extract in subsequent experiments. Previous reports have shown that supplementation of culture media with yeast extract or aminoacids negatively affects the production of recombinant proteins [29,32,33].

Optimization by RSM

The production of hIFN-γ varied in the range of 8.1–78.0 mg/l for the treatments 15 and 7 (Table 3). The analysis of variance for the adjusted model showed that the production of hIFN-γ was significantly affected by the linear and quadratic coefficients and their interaction, except the linear term of the temperature (Table 4). The mathematical model representing the production of hIFN-γ as a function of the evaluated variables in the experimental region studied is expressed by the following equation:

$$Y = -2242.6 + 134.417\,X_1 + 303.222\,X_2 + 572.537\,X_3 + 1.52778\,X_1X_2 - 2.37037\,X_1X_3 + 76.6667\,X_2X_3 - 2.06111\,X_1^2 - 598.437\,X_2^2 - 880.556\,X_3^2 \quad (2)$$

where Y is the hIFN-γ concentration, and X_1, X_2 and X_3 are temperature, biomass concentration and NaCl concentration, respectively. The standard error was 1.04091 and the R^2 value was 0.999 (Table 4). These values indicate a good fit between the model and the experimental data indicating that the treatment was highly significant. The contour plots analysis showed that the central values of the factors were optimal for the production of hIFN-γ (Fig. 2). According to the mathematical model, the maximum hIFN-γ concentration of 77.3 mg/L is attained at temperature of 32.6^{o}C, induction biomass of 0.31 g/L and 0.3 M NaCl (Fig. 2). To verify the predicted results, additional experiments were performed using these optimized conditions. The maximum biomass attained was 0.90±0.02 g/L and the hIFN-γ concentration at 14 h was 78.3±0.2 mg/L (Fig. 3). This result agrees with the predicted value by the model proposed. Culture conditions such as inducer concentration, induction time and temperature affect both the cell growth and the protein production [14,29,34–36]. Zhang *et al*. [37] produced 70 mg/L of hIFN-γ using the native *hIFN-γ* gene in *E. coli* and using a complex medium. Dobrovolsky *et al*. [38] reported a maximum production of 0.02 mg/L of hIFN-γ expressed in murine mammary glands. Khalilzadeh *et al*. [1] reported a maximum hIFN-γ yield of 0.35 g hIFN-γ/g_{DCW} in fed-batch cultures using *E. coli* BL21(DE3), which is four-times higher than that attained presently (0.087 g hIFN-γ/g_{DCW}). Thus, fed-batch cultures using the *E. coli* BL21-SI/pBAL are further recommended to increase the hIFN-γ yield.

CONCLUSION

The use of minimal medium is shown to be most suitable for hIFN-γ production. This production is affected by the culture conditions. The hIFN-γ concentration attained under

optimized conditions was 13-times higher than the obtained using the non-optimized conditions, corroborating the usefulness of RSM in the protein expression. RSM is, thus, an effective method for the optimization of recombinant protein expression using synthetic genes in *E. coli.*

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REFERENCES

1. Khalilzadeh, R., S. Shojaosadati, A. Bahrami and N. Maghsoudi (2003) Over-expression of recombinant human interferon-gamma in high cell density fermentation of *Escherichia coli*. *Biotechnol Lett.* 25: 1989-1992.
2. Schroder, K., P. Hertzog, T. Ravasi and D. Hume (2004) Interferon-γ: an overview of signals, mechanisms and functions. *J Leukoc Biol.* 75: 163-189.
3. Whiteside, T. (2005) IFN-γ: Detection and prevention of release. *Curr Med Chem Anti Inflammatory & Anti Allergy Agents.* 4: 121-131.
4. Makrides, S. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev.* 60: 512–538.
5. Swartz, J. (2001) Advances in *Escherichia coli* production of therapeutic proteins. *Curr Opin Biotechnol.* 12: 195-201.

6. Schmidt, F. (2004) Recombinant expression systems in the pharmaceutical industry. *Appl Microbiol Biotechnol.* 65: 363–372.
7. Studier, F. (2005) Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif.* 41: 207-234.
8. Hannig, G. and S. Makrides (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Focus.* 16: 54-60.
9. Jana, S. and J. Deb (2005) Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Appl Microbiol Biotechnol.* 67: 289–298.
10. Wu, G., N. Bashir-Bello and S. Freeland (2006) The synthetic gene designer: A flexible web platform to explore sequence manipulation for heterologous expression. *Protein Expr Purif.* 47: 441-445.
11. Nikerel, I., E. Toksoy, B. Kirdar and R. Yildirim (2005) Optimizing medium composition for TaqI endonuclease production by recombinant *Escherichia coli* cells using response surface methodology. *Process Biochem.* 40: 1633-1639.
12. Zheng, S., K. Friehs, N. He, X. Deng, Q. Li, Z. He, C. Xu and Y. Lu (2007) Optimization of medium of components for plasmid production by recombinant *E. coli* DH5α PUK21CMVβ1.2. *Biotechnol Bioprocess Eng*. 12: 213-221.
13. Bird, P., S. Pak, D. Worrall, P. Stephen and S. Bottomley (2004) Production of recombinant serpins in *Escherichia coli*. *Methods.* 32: 169–176.
14. Ma, X., W. Zheung, T. Wang, D. Wei and Y. Ma (2006) Optimization and high-level expression of a functional GST-tagged rHTL-b in *Escherichia coli* and GM1 binding ability of purified rHLT-B. *J Microbiol.* 44: 293-300.
15. Baş, D. and İ. Boyacı (2007) Modeling and optimization I: Usability of response surface methodology. *J Food Eng.* 78: 836-845.

16. Donahue, R. and R. Bebee (1999) BL21-SI competent cells for protein expression in *E. coli*. *Focus*. 21: 49-51.
17. Box, G. and D. Behnken (1960) Some New Three-Level Designs for the Study of Quantitative Variables. *Technometrics.* 2: 455-475.
18. Lowry, O., N. Rosebrough, A. Farr and R. Randall (1951) Protein measurement with the folin phenol reagent. *J Biol Chem.* 193: 265-275.
19. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227: 680-685.
20. Wada, K., Y. Wada, F. Ishibashi, T. Gojobori and T. Ikemura (1992) Codon usage tabulated from the GenBank genetic sequence data. *Nucleic Acids Res.* 20 (Suppl): 2111-2118.
21. Kane, J. (1995) Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr Opin Biotechnol.* 6: 494-500.
22. Lithwick, G. and H. Margalit (2003) Hierarchy of sequence-dependent features associated with prokaryotic translation. *Genome Res.* 13: 2665–2673.
23. Gustafsson, C., S. Govindarajan and J. Minshull (2004) Codon bias and heterologous protein expression. *TRENDS Biotechnol.* 22: 346-353.
24. Deng, T. (1997) Bacterial expression and purification of biologically active mouse c-Fos proteins by selective codon optimization. *FEBS Lett.* 409: 269–272.
25. Hale, R. and G. Thompson (1998) Codon optimization of the gene encoding a domain from human Type 1 neurofibromin protein results in a threefold improvement in expression level in *Escherichia coli*. *Protein Expr Purif.* 12: 185–188.

26. Feng, L., W. Chan, S. Roderick and S. Cohen (2000) High-level expression and mutagenesis of recombinant human phosphatidylcholine transfer protein using a synthetic gene: evidence for a C-terminal membrane binding domain. *Biochem.* 39: 15399–15409.

27. Li, A., Z. Kato, H. Ohnishi, K. Hashimoto, E. Matsukuma, K. Omoya, Y. Yamamoto and N. Kondo (2003) Optimized gene synthesis and high expression of human interleukin-18. *Protein Expr Purif.* 32: 110-118.

28. Frelin, L., G. Ahlen, M. Alheim, O. Weiland, C. Barnfield, P. Liljestrom and M. Sallberg (2004) Codon optimization and mRNA amplification effectively enhances the immunogenicity of the hepatitis C virus nonstructural 3/4A gene. *Gene Ther.* 11: 522-533.

29. Paz-Maldonado, L., V. Balderas, E. Medina, A. Barba, J. Flores, L. Ordoñez and A. De León (2007) Optimization of culture conditions for a synthetic gene expression in *Escherichia coli* using response surface methodology: the case of human interferon beta. *Biomolec Eng*. 24: 217-222.

30. Niemitalo, O., A. Neubauer, U. Liebal, J. Myllyharju, A. Juffer and P. Neubauer (2005) Modelling of translation of human protein disulfide isomerase in *Escherichia coli*-A case study of gene optimization. *J Biotechnol.* 120: 11-24.

31. Bhandari, P. and J. Gowrishankar (1997) An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. *J Bacteriol.* 179: 4403-4406.

32. Ling, H. (2002) Physiology of *Escherichia coli* in batch and fed-batch cultures with special emphasis on amino acid and glucose metabolism. Doctoral thesis.

Department of Biotechnology, Royal Institute of Technology, Stockholm, Sweden. http://media.lib.kth.se:8080/dissengrefhit.asp?dissnr=3334.

33. Ignatova, Z., A. Mahsunah, M. Georgieva and V. Kasche (2003) Improvement of posttranslational bottlenecks in the production of penicillin amidase in recombinant *Escherichia coli* strains. *Appl Environ Microbiol.* 69:1237-1245.
34. Neubauer, P., K. Hofmann, O. Holst, B. Mattiansson and P. Kruschke (1992) Maximizing the expression of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose as inducer. *Appl Microbiol Biotechnol.* 36: 739-744.
35. Donovan, R., C. Robinson and B. Glick (1996) Optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. *J Ind Microbiol.* 16:145-154.
36. De León, A., H. Jiménez-Islas, M. González-Cuevas and A. Barba de la Rosa (2004) Analysis of the expression of the *Trichoderma harzianum* ech42 gene in two isogenic clones of *Escherichia coli* by response surface methodology. *Process Biochem.* 39: 2173-2178.
37. Zhang, Z., T. Tong, M. Belew, T. Petterson and J. Janson (1992) Production, purification and characterization of recombinant human interferon-γ. *J Chromatogr.* 604: 143–155.
38. Dobrovolsky. V., O. Lagutin, T. Vinogradova, I. Frolova, V. Kuznetsov and O. Larionov (1993) Human gamma-interferon expression in the mammary gland of transgenic mice. *FEBS Lett.* 319: 181-184.

Table 1. Values of independent variables and the levels used in the experimental design.

Symbol	Independent variable	Levels			Units
		-1	0	+1	
X_1	Temperature	28	32.5	37	ºC
X_2	Biomass	0.1	0.3	0.5	g/L
X_3	NaCl	0.15	0.3	0.45	M

Table 2. Rare codon contents in the hIFN-γ genes and their frequency of use by *E. coli*.

Amino acid	Native hIFNγ gene		Synthetic hIFNγ gene	
	Codon	Frequency per 1000 codons*	Codon	Frequency per 1000 codons*
Arg	AGG	1.4	CGT	21
Arg	AGA	2.1	CGG	5.6
Ile	ATA	4.1	ATC	25.3
Pro	CCA	8.2	CCG	23.3
Ser	AGT	7.2	AGC	16.2
Ser	TCA	6.8	AGC	16.2
Ser	TCC	9.4	AGC	16.2
Gly	GGG	9.7	GGT	24.9
Gly	GGA	7.0	GGT	24.9
Thr	ACA	6.5	ACG	14.6

*A cut-off of < 1% was used to arbitrarily define rare codons.

Table 3. Box-Behnken experimental design and results for the hIFN-γ production in minimal medium.

Treatment	X_1 Temperature (°C)	X_2 Biomass (g/L)	X_3 NaCl (M)	hIFN-γ (mg/L)	
				Experimental Data	Predicted Data
1	32.5	0.1	0.15	32.9	33.9
2	37.0	0.3	0.15	20.1	19.6
3	28.0	0.1	0.30	9.1	8.7
4	32.5	0.3	0.30	76.2	77.2
5	32.5	0.5	0.15	35.9	36.0
6	37.0	0.5	0.30	16.7	17.1
7	32.5	0.3	0.30	78.0	77.2
8	37.0	0.3	0.45	12.9	13.4
9	28.0	0.3	0.15	15.2	14.7
10	32.5	0.5	0.45	38.6	37.6
11	28.0	0.5	0.30	12.2	12.6
12	28.0	0.3	0.45	14.4	14.9
13	32.5	0.1	0.45	26.4	26.3
14	32.5	0.3	0.30	77.4	77.2
15	37.0	0.1	0.30	8.1	7.6

Table 4. Analysis of variance and coefficients for the adjusted model for the predicted hIFN-γ production in the minimal medium.

Source [a]	Polinomial coefficients	Sum of Squares	D.f.	Mean Square	*F*-ratio	*P*-value[b]
Constant	-2242.6					
X_1	134.417	5.95125	1	5.95125	5.49	0.0661
X_2	303.222	90.4512	1	90.4512	83.58	0.0003
X_3	572.537	17.405	1	17.405	16.06	0.0102
X_1^2	-2.06111	6432.07	1	6432.07	5936.38	0.0000
X_1X_2	1.52778	7.5625	1	7.5625	6.98	0.0459
X_1X_3	-2.37037	10.24	1	10.24	9.45	0.0277
X_2^2	-598.437	2115.71	1	2115.71	1952.66	0.0000
X_2X_3	76.6667	21.16	1	21.16	19.53	0.0069
X_3^2	-880.556	1449.36	1	1449.36	1337.67	0.0000
Total error		5.4175	5	1.0835		
Total (corr.)		9039.59	14			

[a] X_1: Temperature, X_2: Biomass conc., X_3: NaCl concentration, D.f.: degrees of freedom, *F*: Fisher test, *P*-value: probability distribution value. The correlation coefficient (R^2) was 0.999 and the standard error was 1.04091. [b]Statistically significant at 95% of probability level

Figure legends.

Figure 1. Batch cultures of *E. coli* BL21 SI/pBAL0 at non-optimized conditions in: a) LBON medium, b) minimal medium and c) in minimal medium plus 0.5% yeast extract. (○) biomass concentration (g/L) and (●) concentration of hIFN-γ (mg/L). Arrows indicate the induction time with 0.3 M NaCl. Data represent the average of three independent experiments.

Figure 2. Contour plot of hIFN-γ production by *E. coli* BL21 SI/pBAL0 in minimal medium: a) temperature *vs* biomass concentration at optimum NaCl concentration, b) temperature *vs* NaCl concentration at optimum constant biomass concentration and c) biomass concentration *vs* NaCl concentration at optimum constant temperature.

Figure 3. Batch culture *E. coli* BL21 SI/pBAL0, in minimal medium using optimized conditions, (○) biomass concentration (g/L) and (●) concentration of hIFN-γ (mg/L). Arrow indicates the induction time with 0.3 M NaCl. Data represent the average of three independent experiments.