J. Microbiol. Biotechnol. (2006), **16**(7), 1026–1031

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Production of a Fusion Protein Containing the Antigenic Domain 1 of Human Cytomegalovirus Glycoprotein B

SOUSA, FANI, SUSANA FERREIRA, JOÃO QUEIROZ, AND FERNANDA DOMINGUES*

Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6201-001 Covilhã, Portugal

Received: September 5, 2005
Accepted: November 25, 2005

Abstract The optimization of the production of a fusion protein containing the antigenic domain 1 (AD-1) is of a great importance, considering its use in diagnostic tests. The fusion protein is produced by the fermentation of a recombinant strain of *Escherichia coli* containing the plasmid Mbg58, which expresses the AD-1 (aa 484–650) of human cytomegalovirus glycoprotein B as a fusion protein together with aa 1–375 of β-galactosidase. An important characteristic of promoters (*lac* and derivatives) used in recombinant protein production in *E. coli* is their inducibility. Induction by IPTG is widely used for basic research; however, its use in large-scale production is undesirable because of its high cost and toxicity. In this work, studies using different inducers and carbon sources for the production of a fusion protein containing the AD-1 were performed. The results showed that lactose could be used as an inducer in the fermentation process for the production of this protein, and that expression levels could exceed those achieved with IPTG. The use of lactose for protein expression in *E. coli* should be extremely useful for the inexpensive, large-scale production of heterologous proteins in *E. coli*. Addition of sucrose to the fermentation medium improved the yield of recombinant protein, whereas addition of fructose or trehalose decreased the yield.

Key words: Recombinant *Escherichia coli*, antigenic domain 1, HCMV, lactose, IPTG

Infection with human cytomegalovirus (HCMV), a beta herpesvirus, continues to be a significant cause of morbidity and long-term sequelae in infants infected *in utero* following maternal infection. In addition, HCMV is a major infectious complication in immunosuppressed individuals, such as transplant recipients and patients suffering from AIDS [15].

The complex biology of HCMV necessarily begins with an initial interaction between the envelope of the infectious virion and the host cell. Glycoprotein B (gB) is the major antigen on the envelope of HCMV for the induction of neutralizing antibodies. The region between aa 552 and 635 of HCMV gB (termed antigenic domain 1, AD-1) has been identified as the immunodominant target for the humoral immune response following natural infection. In fact, nearly 100% of the infected persons who are seropositive for gB have antibodies against AD-1 [3, 15, 17].

Screening methods for detection of neutralizing antibodies have not been used because they are costly and labor intensive, and thus far are not feasible for use on a large scale [11]. For the development of reliable and inexpensive serodiagnostic tests, the AD-1 of HCMV glycoprotein gp58, which are known to bind neutralizing antibodies, was expressed in prokaryotic systems.

Among many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive, and has been widely used in basic research studies as a host strain for the overproduction of proteins from cloned genes [1, 2]. The *lac* promoter-derived expression systems are generally used for the production of heterologous proteins in *E. coli* [18], and one of the most commonly used strategies is its induction with the nonmetabolizable analog of lactose, isopropyl-β-D-thiogalactopyranoside (IPTG) [2, 19]. In spite of its higher cost and toxicity towards humans when compared with lactose, only a small number of works can be found in the literature that describe the use of the latter as the inducer of foreign gene expression, probably because of the great difficulty in establishing ideal induction conditions in this case, as lactose serves simultaneously as inducer and as a carbon and energy source [8, 13].

The economical feasibility of a heterologous protein production process depends both on the attainment of high cell concentration and on high levels of the target protein in the biomass. Bioprocess-based approaches for the

*Corresponding author
Phone: 351-275-319-730; Fax: 351-275-319-730;
E-mail: fdomingues@ubi.pt

optimization of specific levels of the target protein include manipulation of induction time, manipulation of inducer availability, and control of the specific growth rate prior to induction. In addition to these approaches, the increase of target protein stability has also been reported as a way to enhance the intracellular content of target protein [10].

In this work, the effect of different process conditions on the production of the fusion protein containing the AD-1 domain was investigated. The type of inducer (lactose or IPTG), the growth at the beginning of induction, the carbon source availability, cell concentration during induction experiments with *Escherichia coli* containing the plasmid Mbg58, and their influence on parameters such as growth and target protein content are discussed. As sucrose and trehalose were shown to stabilize proteins, their influences in cellular growth and fusion protein production were also examined.

MATERIALS AND METHODS

Bacterial Strains and Plasmid

Recombinant *E. coli* W3110 harboring pMbg58 was a gift from Dr. M. Mach (Germany). This strain was used for the production of a fusion protein, which is the antigenic domain 1 of glycoprotein B of human cytomegalovirus fused to the truncated β-galactosidase.

Production and Recovery of Fusion Protein

Luria-Bertani (LB) medium [1% (w/v) of peptone, 0.5% (w/v) of yeast extract, 1% (w/v) of NaCl] supplemented with 100 μg/ml of ampicillin was used as preculture and culture media. All bacterial growths were performed in flasks of 4-fold greater volume than the culture volume and agitated at 250 rev/min to provide adequate aeration.

To reduce variations in expression levels, a preculture in a 100-ml Erlenmeyer flask containing 25 ml of LB medium was prepared to initiate all experiments. This preculture medium was inoculated with a single colony of *E. coli* (that was grown overnight on LB agar plates) and cultivated at 37°C on a rotary shaker until OD_{600} was approximately 2.6. Two ml from the preculture was then used to inoculate culture medium. In all culture experiments, cells were induced when the optical density at 600 nm (OD_{600}) reached 0.8–1, by adding either lactose or IPTG (0.75 or 1 mM final concentration, respectively). Cells were cultivated for another 4 h and then were harvested by centrifugation at 20,000 ×*g* for 15 min at 4°C.

Cell lysis and fusion protein recovery were performed according to what had been developed by Ferreira *et al.* [7]. The pellet resulting from the last extraction with Triton X-100/EDTA was resuspended twice in 5 ml of 8 M urea at 4°C and stirred overnight. The suspension was centrifuged at 18,000 ×*g* for 10 min and the supernant was reserved for further analysis.

Analytical Methods

Protein samples were analyzed by electrophoresis on SDS-PAGE gels containing 12.5% (w/v) polyacrylamide. A 30-min gel staining with Coomassie Brilliant Blue R-250 solution was performed in order to visualize fusion protein as a major band close to the 66 kDa protein marker.

Quantitative measurements of β-galactosidase activity in liquid cultures were done using the β-galactosidase reporter gene activity detection kit (Sigma), based on Miller's method. Cell lysis was carried out by toluene/deoxycholate solution. To 1 ml samples, 20 μl of toluene and 20 μl of 1% sodium deoxycholate were added, and the mixture was shaken for over 5 min to allow cell lysis. The β-galactosidase activity measurements were performed according to the manufacturer's instructions. Activity was calculated as $A_{420}\times0.8/4.6\times15$, where 0.8 is the final volume of the assay in ml, 15 is the incubation time, and 4.6 is the molar extinction of 1 mM.

RESULTS AND DISCUSSION

The inducible system of the plasmid pMbg58 contains the *lac* promoter, which is commonly induced by IPTG. Owing to the toxicity and high cost of IPTG [5, 8] the possibility of using another inducer such as lactose for the production of the fusion protein containing the AD-1 was investigated. Different cultures, each including both a growth and a induction phase, were performed with *E. coli* strain W3110 in shake flasks at 37°C and 250 rev/min. The induction phase is considered here as the period between the first inducer addition into the medium and the end of the cultivation.

To analyze the influence of cultivation time on bacterial growth and fusion protein production, *E. coli* strain W3110 was grown until OD_{600} was approximately 1.0. At this point, the culture was induced by addition of IPTG or lactose, and the synthesis of fusion proteins was allowed to continue for 4 to 6 h. Electrophoresis analysis showed that it is not advantageous to prolong cells cultivation for more than 4 h after induction, since the fusion protein production did not increase after this period. It is highly likely that decrease of available nutrients in the fermentation medium leads cells to produce preferentially homolog proteins, in detriment of heterologous proteins (results not shown). Therefore, the total amount of fusion protein was analyzed 4 h after the addition of the inducer in all the experiments performed in this work.

To evaluate the effect of cell density at the time of induction on production of fusion protein, cells were induced with lactose at different OD_{600} values; in three pulses of

inducer at OD_{600} 0.5, 1.0, and 1.5, with the aim of avoiding inhibitory effects that could eventually occur because of a high residual concentration of lactose in the medium. The best results for fusion protein production were achieved at OD_{600} value of 1; the cells were induced at that OD_{600} value in all the experiments performed in this work.

In order to compare the induction by lactose and IPTG, different cultivations were done. The time profiles for cell density, obtained by measuring OD_{600} of samples periodically taken, are shown in Fig. 1A. By comparing the growth curves, it is apparent that lactose slightly increased cell density, compared with IPTG. However, fusion protein recovery and electrophoresis gel analysis showed a much more intense band corresponding to the target protein under lactose induction, when compared with the band attained under IPTG induction (Fig. 1C). Western blot analysis, with specific antibody, confirmed that the visualized band in SDS-PAGE, corresponding to lactose induction, was the target protein (data not shown).

A previous work showed higher values of heterologous proteins production under IPTG induction than the use of lactose [10]. The difference between these results could probably be related to the utilization of glucose under IPTG induction in these assays.

In order to analyze the effect of lactose and IPTG induction on the expression of fusion protein, we followed the β-galactosidase activity throughout fermentation. As shown in Fig. 1B, an increase of β-galactosidase activity was always observed after induction (about 2 h) for lactose and IPTG induction. However, β-galactosidase activity was higher under IPTG induction during the first two hours after induction, whilst the β-galactosidase levels found in crude extracts at the end of fermentation were higher under lactose induction. The reduced β-galactosidase activity under lactose induction at the beginning of induction, compared with the use of IPTG, is probably due to the fact that the latter can directly bind to few *lac*-repressor molecules present in *E. coli*, after having entered the cells by either simple diffusion or active transport [14]. In the case of lactose induction, however, a few steps are needed for repressor binding, eventually delaying the onset of the induction process: first, the cells have to synthesize lactose permease for lactose transport into the cells, and then lactose has to be converted to allolactose by β-galactosidase, and finally, allolactose binds to the repressor.

Considering the facts that the synthesis of intracellular proteases was more intense under IPTG than under lactose induction, a crucial issue for protein stability during downstream processing [10], i.e., that IPTG causes damage on cell metabolism [5] and that IPTG is more expensive and toxic than lactose towards humans, our results suggest that lactose is a more appropriate inducer of gene expression for the production of this fusion protein containing the AD-1 domain of human cytomegalovirus glycoprotein B.

Since the present results indicate that lactose was an efficient inducer of fusion protein expression, conditions that could increase this expression were studied, namely different lactose concentrations. The higher levels of fusion protein production were obtained with 0.75 mM concentration. Higher lactose concentrations did not increase recombinant protein yields, indicating that the system was fully induced at the latter concentration. Although this optimum lactose concentration is much lower than the one used in other expression systems in *E. coli*, 7 and 28 mM [19], it is in accordance with Khlebnicov and Keasling [9], who reported that 1 mM lactose was enough to fully turn on the *lac* operon in a wild-type *E. coli* culture. It is apparent that each strain of *E. coli* responds differently to its environment, and that the growth characteristics of each strain differ greatly under similar fermentations conditions [12], suggesting that the concentration of inducer used for the production of recombinant proteins should be optimized for each

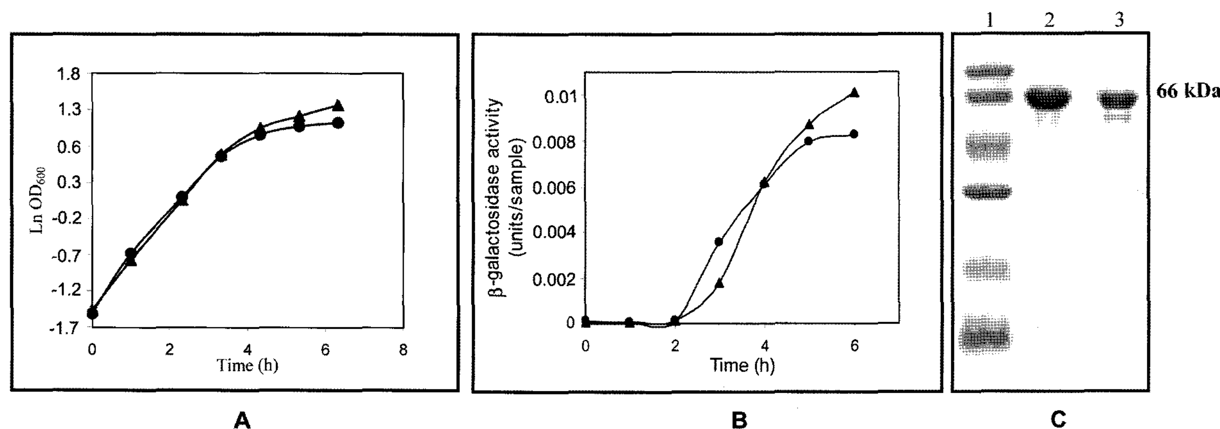

Fig. 1. Effects of lactose induction (▲) and IPTG induction (●) on *E. coli* growth (**A**), β-galactosidase activity (**B**), and fusion protein recovery SDS-PAGE electrophoresis gel (**C**).

Lane 1, Molecular weight marker; lane 2, Lactose induction; lane 3, IPTG induction. Molecular weight of fusion protein 66 kDa.

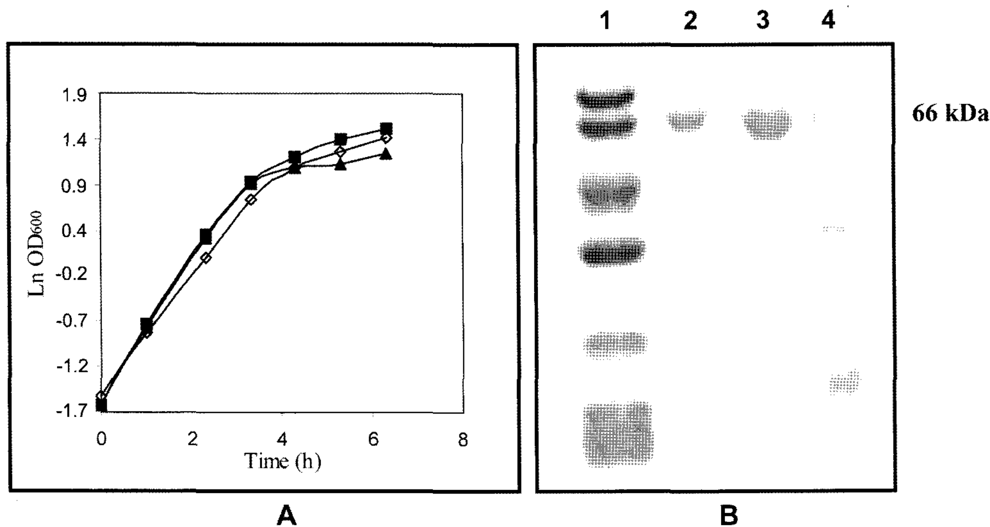

Fig. 2. Effects of glucose concentration on *E. coli* growth and fusion protein recovery under lactose induction.
A. Cell growth curves: medium without glucose (◇); medium with 1.33 mM glucose (■); medium with 100 mM glucose (▲). **B.** Electrophoresis gel SDS-PAGE of protein recovered from each culture: Lane 1, Molecular weight marker; lane 2, without glucose; lane 3, 1.33 mM glucose; lane 4, 100 mM glucose.

heterologous protein expressed in *E. coli.* These results, therefore, indicate that lactose concentration plays an important role in the induction process.

To evaluate advantageous conditions for the production of fusion protein, optimization experiments on shake flask were further performed with different combinations of the carbon sources. Therefore, different concentrations of glucose, 1.33 mM and 100 mM, were added as energy/carbon source to the culture medium under 0.75 mM lactose induction. Although the cell growth with glucose was equal to the values attained during growth without glucose (Fig. 2A), the synthesis of fusion protein occurred only when glucose concentration was low or was not included in the culture media (Fig. 2B). For 100 mM concentration, glucose repression seems to play an important role, inhibiting the production of fusion protein. In the medium with glucose or other carbon source that is readily available to the glycolytic pathway, cAMP concentration in the cell decreases, causing a reduction or even an ending of transcription of the structural genes in the *lac* operon, and resulting in catabolic repression. Moreover, Viitanen *et al.* [18] reported that glucose prevents lactose uptake by the

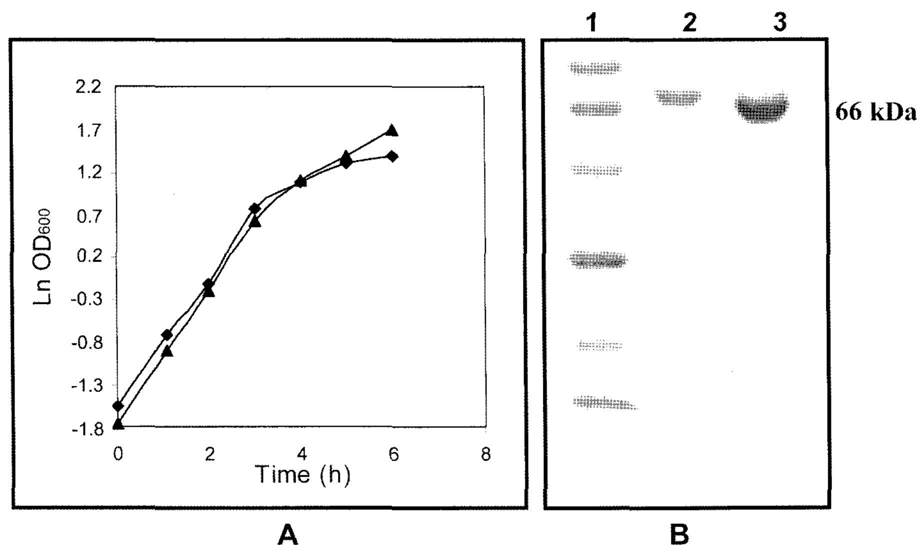

Fig. 3. Evaluation of *E. coli* growth and fusion protein recovery in experiments with lactose addition at the onset of cultivation and lactose induction.
A. Cell growth curves without lactose addition (◆), and with 100 mM lactose addition (▲). **B.** Gel electrophoresis SDS-PAGE of the resulting samples; lane 1, Molecular weight marker; lane 2, medium without lactose; lane 3, medium with 100 mM lactose.

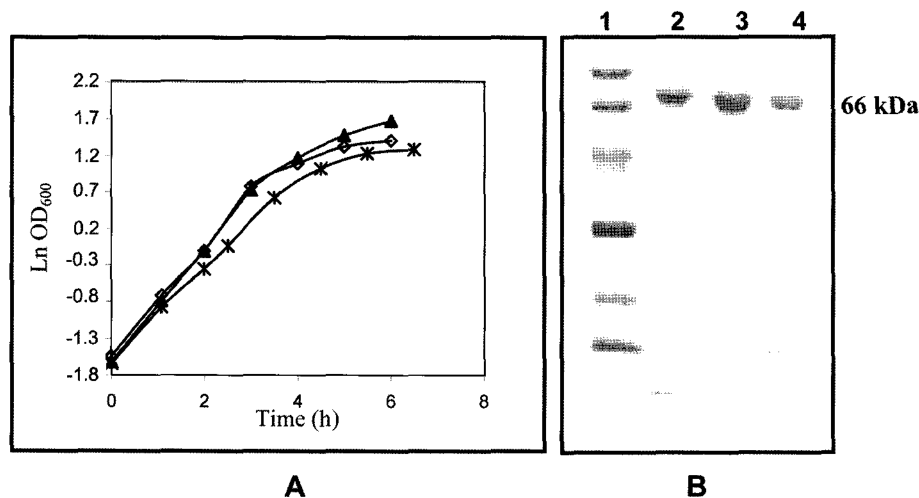

Fig. 4. Evaluation of *E. coli* growth and fusion protein recovery in fermentations using different disaccharides under 0.75 mM lactose induction.

A. Cell growth curves obtained by addition of different disaccharides to the medium: without disaccharides (◇); with 100 mM sucrose (*); with 100 mM trehalose (▲). **B**. Electrophoresis gel SDS-PAGE analysis of samples; lane 1, Molecular weight marker; lane 2, without disaccharides addition; lane 3, addition of 100 mM sucrose; lane 4, addition of 100 mM trehalose.

cell, resulting in lack of induction by lactose. The same results were obtained with fructose.

The results of β-galactosidase activity measured throughout the induction phase showed nearly undetectable levels even 4 h after induction, confirming that repression occurred when the cells were grown on 100 mM fructose or glucose. The addition of 1.33 mM glucose at the onset of fermentation showed an improvement in the synthesis of fusion protein, suggesting that the addition of this sugar could be useful, when kept low. In this case, β-galactosidase activity increased and 0.00857 Units/sample were obtained.

In order to maximize the production of the heterologous protein, the addition of 100 mM lactose at the onset of cultivation was assayed. Although no significant improvement in the growth was achieved, an increased titer of the target protein was obtained (Fig. 3). Thus, no correlation could be established between growth and fusion protein production. These results are in accordance with the result obtained by Kilikian *et al.* [10]; heterologous protein production decreased when biomass value increased. The benefits of addition of lactose before the induction phase can be explained by the reduction of different steps that are needed for repressor binding. Furthermore, since lactose is naturally cleaved to glucose and galactose before they can enter the catabolic routes, the availability of carbon source is controlled by cells, thus avoiding catabolic repression and providing a carbon source for maintenance purposes.

Another attempt was made to improve the yield of fusion protein by introducing sucrose or trehalose together with lactose. Sucrose and trehalose have been shown to stabilize proteins, in addition to being carbon substrates. Sucrose is a relatively cheap carbon and energy source, and trehalose can protect proteins and cellular membranes from inactivation or denaturation [6]. In order to examine their effect on cellular growth and fusion protein production, a series of cultivations with similar growth phases on LB medium and 100 mM each of either sucrose or trehalose were performed. As seen in Fig. 4A, sucrose addition led to growth reduction, compared with the other curves. However, synthesis of heterologous protein increased, as presented in Fig. 4B. Since the strain used is not able to metabolize sucrose, the improved production was probably due to the fact that sucrose could stabilize fusion protein, as discussed by Butler and Falke [4].

The influence of different inducers and carbon sources on the production of fusion protein, containing the AD-1 of the human cytomegalovirus glycoprotein B, was studied. The result indicates that lactose can be used for the induction of recombinant genes expression in this strain of *E. coli*. Moreover, the yield of the recombinant product was higher when induced with lactose than with IPTG. The highest levels of fusion protein production were obtained, when the culture was induced with 0.75 mM lactose at an OD_{600} of 1.

Lactose failed to induce fusion protein production in the presence of fructose or trehalose in the fermentation medium. In contrast, however, sucrose improved the production of fusion protein during induction with lactose.

Acknowledgments

This work was supported by Programa Operacional SAUDE XXI, from the Ministry of Health, Portugal. We

thank to Dr. Michael Mach from Universität Erlangen-Nürnberg, Germany, for the kind gift of recombinant *E. coli* W3110 harboring pMbg58.

REFERENCES

1. Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. *Curr. Opin. Biotechnol.* **10:** 411–421.
2. 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.
3. Britt, W. and M. Mach. 1996. Human Cytomegalovirus glycoproteins. *Intervirology* **39:** 401–412.
4. Butler, S. and J. Falke. 1996. Effects of protein stabilizing agents on thermal backbone motions: A disulfide trapping study. *Biochemistry* **35:** 10595–10600.
5. Donovan, R., C. Robinson, and B. R. 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.
6. Elbein, A. D., Y. T. Pan, I. Pastuszak, and D. Carrol. 2003. New insights on trehalose: A multifuntional molecule. *Glycobiology* **13:** 17R–27R.
7. Ferreira, S., F. Sousa, J. A. Queiroz, and F. C. Domingues. 2005. Improved recovery of a fusion protein containing the antigenic domain 1 of the human cytomegalovirus glycoprotein B. *Biotechnol. Lett.* **27:** 1241–1245.
8. Gombert, A. K. and B. V. Kilikian. 1998. Recombinant gene expression in *Escherichia coli* cultivation using lactose as inducer. *J. Biotechnol.* **60:** 47–54.
9. Khlebnicov, A. and J. Keasling. 2002. Effect of lac Y expression on homogeneity of induction from the P(tac) and P(trc) promoters by natural and synthetic inducers. *Biotech. Prog.* **18:** 672–674.
10. Kilikian, B. V., I. D. Suárez, C. W. Liria, and A. K. Gombert. 2000. Process strategies to improve heterologous protein production in *Escherichia coli* under lactose or IPTG induction. *Process Biochem.* **35:** 1019–1025.
11. Kropff, B., M. Landini, and M. Mach. 1993. An ELISA using recombinant proteins for the detection of neutralizing antibodies against human cytomegalovirus. *J. Med. Virol.* **39:** 187–195.
12. Luli, G. W. and W. R. Strohl. 1990. Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.* **56:** 1004–1011.
13. Menzella, H. G., E. A. Ceccarelli, and H. C. Gramajo. 2003. Novel *Escherichia coli* strain allows efficient recombinant protein production using lactose as inducer. *Biotechnol. Bioeng.* **82:** 809–817.
14. Miao, F. and D. S. Kompala. 1992. Overexpression of cloned genes using recombinant *Escherichia coli* regulated by a T7 promoter. I. Batch cultures and kinetic modelling. *Biotechnol. Bioeng.* **40:** 787–796.
15. Ohlin, M., V. Sundqvist, M. Mach, B. Wahren, and C. Borrebaeck. 1993. Fine specificity of the human immune response to the major neutralization epitopes expressed on cytomegalovirus gp58/116 (gB), as determined with human monoclonal antibodies. *J. Virol.* **67:** 703–710.
16. Speckner, A., D. Glykofrydes, M. Ohlin, and M. Mach. 1999. Antigenic domain 1 of the human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. *J. Gen. Virol.* **80:** 2183–2191.
17. Speckner, A., B. Kropff, S. Knor, and M. Mach. 2000. The antigenic domain 1 of the cytomegalovirus glycoprotein B contains an intramolecular disulphide bond. *J. Gen. Virol.* **81:** 2659–2663.
18. Viitanen, M., A. Vasala, P. Nevbaner, and T. Alatossava. 2003. Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli*. *Microb. Cell Fact.* **2:** 1–10.
19. Woyski, D. and J. R. Cupp-Vickery. 2001. Enhanced expression of cytochrome P450s from *lac*-based plasmids using lactose as the inducer. *Arch. Biochem. Biophys.* **388:** 276–280.