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Screening of Variables Influencing the Production of HPV E7 Oncoproteins by Recombinant *Escherichia coli*

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Abstract: E7 proteins are major oncoproteins of Human Papilloma Viruses (HPVs) which play a key role in virus-associated cervical carcinogenesis. Aiming at future optimization of the production of these proteins by a recombinant strain of *Escherichia coli*, this preliminary study has been addressed to the influence of the main process variables on their expression in three culture media, specifically LB, TB and LBG media. A 2^{5-2} experimental design utilizing multiple factorial levels was employed to identify single factors and interactions exerting significant impacts on protein expression. Temperature, agitation intensity, IPTG and kanamycin concentrations and inoculum size were selected as the five independent variables, while the specific growth rate of the microorganism and the concentration of insoluble proteins as well as those of soluble proteins in two successive extracts were selected as the four responses. The highest specific growth rate (1.06 h^{-1}) was obtained during run B7 performed in the TB medium using 40°C, 170 rpm, 0.25 mM IPTG, 40 μg mL^{-1} kanamycin and OD_{600} = 0.6, while the maximum total protein production was achieved during run C9 (5.60 mg mL^{-1}) performed in the LBG medium at 37°C, 140 rpm, 0.5 mM IPTG, 30 μg mL^{-1} kanamycin and OD_{600} = 0.5. The statistical approach used in this study revealed to be a powerful tool for the optimization of protein expression.

Key words: Temperature, agitation intensity, IPTG, kanamycin, inoculum

INTRODUCTION

The high-risk human papillomavirus is represented by types 16 and 18 (HPV-16 and -18), which modify the cellular cycle and lead to the development of cervical cancer (Zur Hausen, 2002). When an HPV virus infects a cell, it can be eliminated, remain dormant, produce a clinical or subclinical active infection (non-integrated viral DNA-episomal) or even integrate its genome into that of the immature host cell, thus hindering cell differentiation and maturation. Although the transformed cell ceases to produce the virus, it still contains viral DNA (Tyring, 2000; Zur Hausen, 2002; Bagarelli and Oliani, 2004).

When a gene is activated, the cell responds by synthesizing the coded protein (Weinberg, 1996). The E7 protein gene of the high-risk HPV codes for an oncoprotein that can indefinitely replicate epithelial human cells (Wang *et al*., 2003). It has been demonstrate that this oncoprotein is capable of modifying the expression of the proteins regulating the cell cycle, the most studied of which is the retinoblastoma protein (pRB) (Helt and Galloway, 2003). Linking of the E7 gene coded

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protein to pRB removes the negative control of the cell cycle, leading to a continuous proliferation of the infected cells (Munger and Howley, 2002; Wang *et al*., 2003; De-Boer *et al*., 2004). To obtain an expression system of recombinant proteins in *Escherichia coli*, an adequate combination of the plasmid and microbial strain is generally required (Sørensen and Mortensen, 2005a, b).

The large-scale production of recombinant proteins using different systems of expression has become important for several applications, including the productions of enzymes, hormones and growth factors (Clementschitsch *et al*., 2005). Fast to obtain, recombinant proteins are necessary to produce new therapeutically-active compounds, such as antigenic vaccines, interferon-γ, interleucin-7, insulin-dependent growth factor and human leptin, among others (Cho *et al*., 2005; Choi *et al*., 2006).

Cervical carcinoma is reported to be the second most common cause of cancer among women worldwide (Monk and Tewari, 2007). About 19, 260 new cases were expected in Brazil in 2006, which amounted to 20 cases per 100 thousand women, constituting of course a great public health concern (Guarisi *et al*., 2004). Poor socioeconomic and cultural standards, precocious sexual activity, multiple partners and smoking are among the factors predisposing for this neoplasia (Miller *et al*., 2000). The largest world-wide incidence of cervical cancer, 83.2 cases per 100,000 women, occurred in the city of Recife, situated in Northeastern Brazil (Lorenzato *et al*., 2001). The number of affected women is approximately the sum of all cases in Cali (Colombia) and in the whole Costa Rica; developing vaccines against the high-risk HPVs found in the majority of cervical cancers would be a great advance in epidemiology, owing to the morbidity and mortality associated with this type of cancer (Lorenzato *et al*., 2000).

As is well known, high levels of recombinant protein expression in *E. coli* result from the action-and possible interaction-of several factors. According to Saraswar *et al*. (1999), these effects can be divided into genetic factors (number of plasmid copies, transcription terminator, promoter length) and environmental factors (specific growth rate, pH, temperature, culture media composition and type of inducer). The yield and solubility of recombinant proteins are highly dependent on the specific protein sequence, as well as on the vector, host cell and culture conditions adopted. For optimal efficiency in the protein production, various combinations of these parameters should be simultaneously screened to determine the conditions that yield the best protein, for example, choosing five different expression clones for each protein of interest (Stevens, 2000). The recent proliferation of expression options has increased the number of variables to be examined in any given protein expression experiment (Sørensen and Mortensen, 2005a).

The present study is a preliminary attempt to investigate the combined effects of five different process variables, specifically the temperature, agitation intensity, IPTG and kanamycin concentrations and inoculum size, on the production of the HPV virus E7 protein by recombinant *E. coli*. The experiments were planned and analyzed using a two-level fractional statistical design, augmented with a central point and four response variables, namely the specific growth rate, the amount of insoluble proteins and the concentrations of soluble proteins in two different extracts.

MATERIALS AND METHODS

Construction of the expression vector: The E7 protein coding sequence was amplified by PCR (forward primer 5' GCT AGC ATG CAT GGA GAT ACA CCT ACA TTG C 3′ and reverse primer 5' AAG CTT TTA TGG TTT CTG AGA ACA GAT GGG GC 3') with the high-fidelity enzyme Platinum Taq polymerase (Invitrogen, São Paulo, Brazil). The forward and reverse primers included small forms of the restriction enzymes NdeI and HindIII, respectively. The resulting fragment, of 314 bp, was first inserted into the vector pGEM T-Easy (Promega, São Paulo, Brazil). The origin vector, pGEM-E7, was digested with the NdeI and HindIII enzymes and the free fragment was purified from agarose gel and inserted into the vector pET28a (Novagen, Madison, WI), previously treated with the same enzymes. Once the transformed forms had been analyzed, the kanamycin-resistant pET28a-E7 vector was amplified, extracted and transformed into *Escherichia coli* BL21(DE3) pLysS, used to express the E7 protein linked to the histidine tail in the amino-terminal region.

Inoculum preparation: The inoculum was obtained from a submerged culture of recombinant *E. coli* grown for 18 h (OD_{600} = 0.5) in 50 mL Erlenmeyer flasks containing 20 mL of fresh LB medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) plus 30 μg mL^{-1} kanamycin, placed on an orbital shaker at 140 rpm and 37°C.

Production of the E7 recombinant protein: The submerged cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL of different media, according to the levels combinations shown in Table 1. The selected media were the LB medium, the TB (Terrific broth) medium containing 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 0.4% v/v glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4 and

Table 1: Actual and coded factor levels of the 2^{5-2} experimental design used for HPV E7 recombinant protein production

Factors	Levels		
	Lower (-1)	Central (0)	Higher (+1)
Temperature (°C)	34.00	37.00	40.00
Shaking rate (rpm)	110.00	140.00	170.00
IPTG concentration (mM)	0.25	0.50	0.75
Kanamycin concentration (μg mL^{-1})	20.00	30.00	40.00
Inoculum size (OD_{600})	0.40	0.50	0.60

the LBG medium made up of the LB medium plus 10 g L^{-1} glucose. Aliquots of 2 mL were withdrawn after 2 h (t_0-no induction) in each experiment, after which they were immediately induced by addition of IPTG (isopropyl-β-D-thiogalacto-pyranoside). After an induction time of 2 h (t_2-induction), 2 mL aliquots were again withdrawn from the media, centrifuged and frozen for later extraction.

Extraction of recombinant protein: Cell pellets obtained either before (t_0) or after induction (t_2) of the recombinant *E. coli* cultures were used for protein extraction. In the first extraction step, the pellets were resuspended in 8 mL of buffer A, containing 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5 mM phenylmethylsulphonylfluoride (PMSF) and 160 μL of 10 g L^{-1} lysozyme. This solution was kept at 0°C for 60 min. After sonication in a deep-well cup horn Vibra cell ultrasonicator (Branson Ultrasonics, Carrollton, TX) with 5×30 sec bursts, cells were centrifuged at 2000 g for 30 min and the supernatant, labeled Extract 1 (E1), was stored for subsequent determination. In a second extraction step, cell pellets were resuspended in 6 mL of buffer A spiked with 2% of Triton X-100 (buffer B) and sonication was carried out at 0°C with the above ultrasonicator with 3×30 sec bursts until the cells were lysed, after which the solution was centrifuged at 5500 g for 15 min. The new supernatant, labeled Extract 2 (E2), was stored for subsequent determination. Cell pellets were again resuspended in 100 μL of buffer A and then stored for later analysis.

Analytical methods: Protein concentration was measured according to the method of (Bradford, 1976). A calibration curve was made with bovine serum albumin (BSA) standards and absorbance was measured at 595 nm using a spectrophotometer Ultrospec 3000 pro UV/Visible (GE Healthcare, Life Sciences, Uppsala, Sweden).

Cell growth was followed by optical density (OD) measurements at 600 nm every 60 min intervals.

Electrophoresis was carried out by the method developed by Laemmli (1970), using sodium dodecylsulfate and 15% (m/v) polyacrylamide gel (SDS-PAGE), resulting in both soluble and insoluble E7 protein expression values. The molecular weight standards (SigmaMarker™ Low Range (M.W. 6,500-66,000) was provided by Sigma Aldrich (São Paulo, Brazil) and consisted of bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa) and α-lacto albumin (14 kDa).

Table 2: Time confounding patterns of 2^{5-2} experimental design used for HPV E7 recombinant protein production. Fourth-order interactions were omitted, because they were assumed to be negligible broth

Contrast	Order		
	1st	2nd	3rd
1-Temperature	2-4	3-5	
2-Shaking rate	1-4	3-4-5	
3-IPTG concentration	1-5	2-4-5	
4- Kanamycin concentration	1-2	2-3-5	
5-Inoculum size	1-3	2-3-4	
23-Interaction	4-5	1-2-5	1-3-4
25-Interaction	3-4	1-2-3	1-4-5

Experimental design and data analysis: Batch cultivations were carried out according to a 2^{5-2} fractional experimental design, consisting in a one-fourth fraction of the full two-level factorial design described by Bruns *et al.* (2006). In such a fraction the effects were confounded in groups of four and for this reason the calculated results were called contrasts, to distinguish them from the main effects themselves. Because fourth- and higher-order interactions are usually considered negligible, they were excluded from the confounding patterns. Table 2 shows all the significant contrasts and first-, second- and third-order interactions taken into consideration in this study. For instance, contrast 2, which was associated with the shaking rate, was actually the sum of the shaking rate main effect and two interactions: 2+1-4+3-4-5.

A central point, in which the five factors were fixed at their intermediate levels, was added to the two-level design for each medium. Four response variables were evaluated for each experimental run, specifically the specific growth rate, the amount of insoluble protein and the amounts of soluble proteins in two successive extracts. The results were analyzed with the statistica 6.0 software.

According to Pirt (1975) the mean specific growth rate (μ_m) was calculated by the equation:

$$\mu_m = \frac{\ln X - \ln X_0}{t}$$

where, X is the final biomass concentration, X_0 the initial biomass concentration and t the final time.

RESULTS AND DISCUSSION

The main experimental results collected under different experimental conditions are shown in Table 3 for the three cultivation media selected for this study.

Table 3: Experimental results of the production of HPV E7 oncoproteins by recombinant *E. coli* under different experimental conditions

Run	Medium	Temperature (°C)	Shaking rate (rpm)	IPTG (mM)	Kanamycin (μg mL^{-1})	Inoculum size (DO_{600})	μ_m (h^{-1})	IP[a] (μg mL^{-1})	E1[b] (mg mL^{-1})	E2[c] (mg mL^{-1})	E1+E2[d] (mg mL^{-1})
A1	LB	34	110	0.25	40	0.4	0.43	0.84	0.00	0.19	0.19
A2	LB	34	110	0.75	40	0.6	0.26	1.50	0.13	0.96	1.09
A3	LB	34	170	0.25	20	0.4	0.64	0.82	0.06	0.34	0.40
A4	LB	34	170	0.75	20	0.6	0.40	0.64	0.11	0.29	0.40
A5	LB	40	110	0.25	20	0.6	0.54	0.16	0.02	0.36	0.38
A6	LB	40	110	0.75	20	0.4	0.47	0.92	0.05	0.05	0.10
A7	LB	40	170	0.25	40	0.6	0.77	1.70	0.06	0.62	0.68
A8	LB	40	170	0.75	40	0.4	0.66	1.60	0.06	0.04	0.10
A9[e]	LB	37	140	0.50	30	0.5	0.64	4.30	0.12	0.43	0.55
B1	TB	34	110	0.25	40	0.4	0.42	1.50	0.02	0.48	0.50
B2	TB	34	110	0.75	40	0.6	0.27	2.00	0.02	0.22	0.24
B3	TB	34	170	0.25	20	0.4	0.84	0.84	0.07	0.29	0.36
B4	TB	34	170	0.75	20	0.6	0.49	2.20	0.08	0.08	0.16
B5	TB	40	110	0.25	20	0.6	0.81	0.43	0.05	0.11	0.16
B6	TB	40	110	0.75	20	0.4	0.63	0.54	0.06	0.14	0.20
B7	TB	40	170	0.25	40	0.6	1.1	2.70	0.02	0.73	0.75
B8	TB	40	170	0.75	40	0.4	0.93	2.50	0.02	0.05	0.07
B9[e]	TB	37	140	0.50	30	0.5	0.91	5.30	0.09	0.43	0.52
C1	LBG	34	110	0.25	40	0.4	0.24	0.91	0.00	0.42	0.42
C2	LBG	34	110	0.75	40	0.6	0.34	0.96	0.02	0.53	0.55
C3	LBG	34	170	0.25	20	0.4	0.63	0.76	0.08	0.17	0.25
C4	LBG	34	170	0.75	20	0.6	0.55	1.50	0.05	0.23	0.28
C5	LBG	40	110	0.25	20	0.6	0.62	0.98	0.01	0.05	0.06
C6	LBG	40	110	0.75	20	0.4	0.53	0.42	0.01	0.01	0.02
C7	LBG	40	170	0.25	40	0.6	0.75	1.40	0.02	0.58	0.60
C8	LBG	40	170	0.75	40	0.4	0.66	1.20	0.02	0.21	0.23
C9[e]	LBG	37	140	0.50	30	0.5	0.72	5.60	0.11	0.47	0.58

[a]: Insoluble proteins; [b]: Soluble proteins (extract 1); [c]: Soluble proteins (extract 2); [d]: Total soluble proteins; [e]: Central point

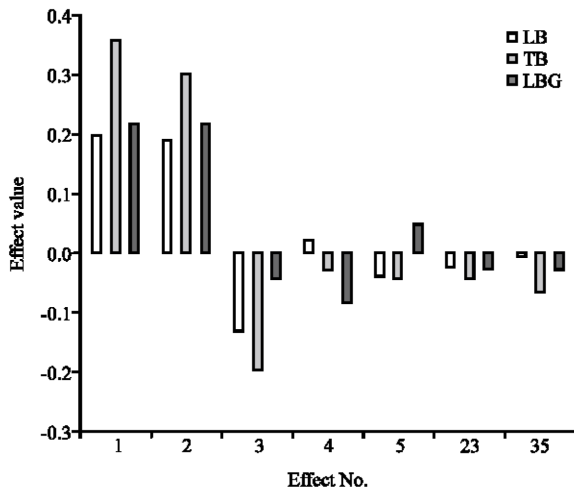

Fig. 1: Main effects of the five independent variables on the mean specific growth rate in the LB, TB and LBG culture media. Effects are shown in Table 2

Specific growth rate: Figure 1 is a bar plot of the contrasts/effects of the five independent variables on the mean specific growth rate (μ_m). The most significant effects were those exerted by temperature 1, shaking rate 2 and IPTG concentration 3. Although qualitatively similar in these media, these effects were stronger in the TB medium. In particular, the effects of temperature and shaking rate were positive, whereas that of IPTG concentration was negative. Cserjan-Puschmann *et al.* (1999) observed that the expression of the recombinant human superoxide dismutase in *E. coli* (HMS174 (DE3) pET11ahSOD) was affected by an increased cell growth rate, likely due to a loss in the efficiency of the transport systems of both the inducer (IPTG) and the substrates (tryptone and yeast extract) under these conditions.

As expected from these trends, the highest μ_m values (0.77, 1.06 and 0.75 h^{-1} in the LB, TB and LBG media, respectively) were actually obtained in all the three media at the highest temperature (40°C) and shaking rate (170 rpm) and the lowest IPTG level (0.25 mM) (runs A7, B7 and C7 in Table 3). These values are appreciably higher than those reported by Stevens (2000) for two recombinants expressing the human skin growth factor, i.e., *E. coli* JM101 [pWKW2] (0.48 h^{-1} at 32°C and 0.1 mM IPTG) and *E. coli* K-12 JM101 [lacUV5par8EGF] (0.67 h^{-1} at 34°C and 0.2 mM IPTG).

Other four runs carried out in the TB medium (B3, B5, B8 and B9) exhibited μ_m values higher than those obtained in the other two media, hence confirming its best

suitability for the cultivation of the selected microorganism.

As is well known, the growth rate is used in some production systems to control product formation with the aim of achieving the desired yield at the end of the culture, which in turn depends on the cultivation time, biomass concentration and specific product formation rate along the production path (Jenzsch *et al*., 2005). The specific growth rate is also an important factor in the expression of recombinant proteins in *E. coli*; therefore, a correlation between this kinetic parameter and the recombinant gene expression would be of great concern (Chaves *et al*., 1999; Saraswat *et al*., 1999). Although the present results suggest a positive correlation between this parameter and the level of E7 protein expression (r = 0.36), this was not significant at the 95% confidence level.

Proteins production: The effects of the selected independent variables on the total production of proteins (including the E7 protein) were less clear than those observed for μ_m. The most evident finding is that most of proteins were obtained in the insoluble form. For such an insoluble fraction (Fig. 2a) the largest contrasts were those associated to the main effects of the shaking rate 2 and the kanamycin concentration 4. Their positive values point out that increased levels of both factors should ensure higher protein expression. Larger inoculum size also appeared to increase this response in the TB and LBG media. However, these predictions only partially agree with the experimental results shown in Table 3. In fact, in all the three media, the highest values of this response were by far those obtained at the intermediate level combination corresponding to the central point (runs A9, B9 and C9). A comparison of the results obtained for the insoluble protein fraction in the three media shows that the use of TB and LBG media did not allow for any appreciable improvement with respect to the LB one; therefore, this last medium was selected for the subsequent experiments because of its simplicity and low cost. These results partly agree with the observations of Fernando *et al*. (1999), who found that about 70% of the E7 protein is just located within the cell in inclusion bodies and that the cultivation medium has a strong influence on its production.

As shown in Fig. 2b, the effects on the total soluble fraction of proteins were quite different. The algebraic signs of the largest effects suggest that the highest responses should be expected when using the highest kanamycin concentration 4 and inoculum size 5 combined with the lowest IPTG concentration 3. A similar result was observed by Swalley *et al*. (2006) witch used a low

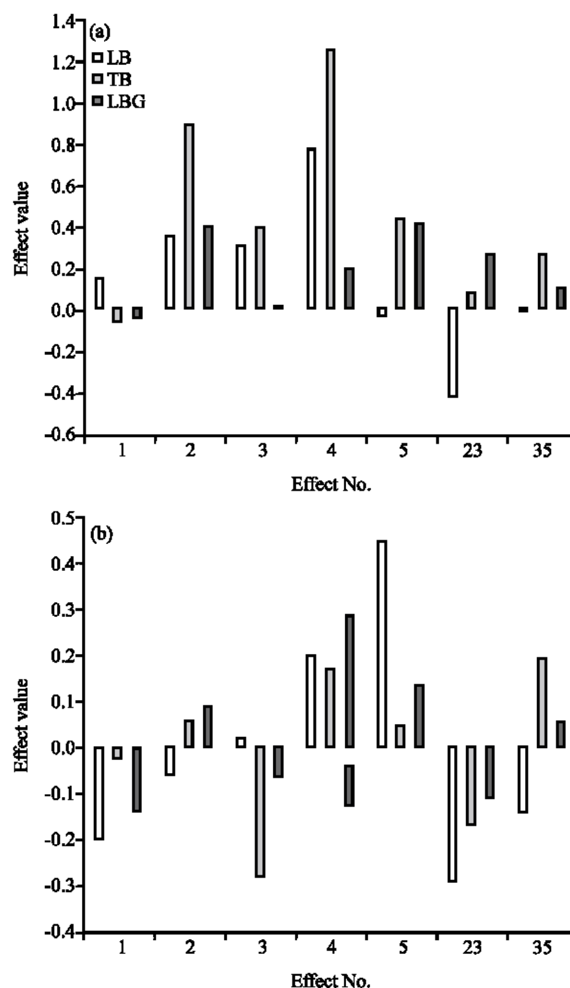

Fig. 2: Effects of the five independent variables on the recombinant protein production in the LB, TB and LBG culture media: (a) insoluble proteins; (b) total soluble proteins. Effects are shown in Table 2

concentration of IPTG in the induction of almost all of the proteins tested. Apart from the unexpected highest level of total soluble protein obtained in run A2, the conditions of runs A7, B7 and C7 actually ensured the second highest concentrations in all the three media (Table 3). Soluble recombinant protein preparations have often lower biological activity than the native ones, because they are not always present in the adequate conformation. In particular, Nominé *et al*. (2001) demonstrated that fusion of the E6 oncoprotein of the HPV with the MBP (*E. coli* maltose binding protein) formed many composite aggregates of folded MPB and misfolded E6 and that optimization of the conditions can lead to better income of the protein. Production of a recombinant protein in the inclusion body has several advantages, such as

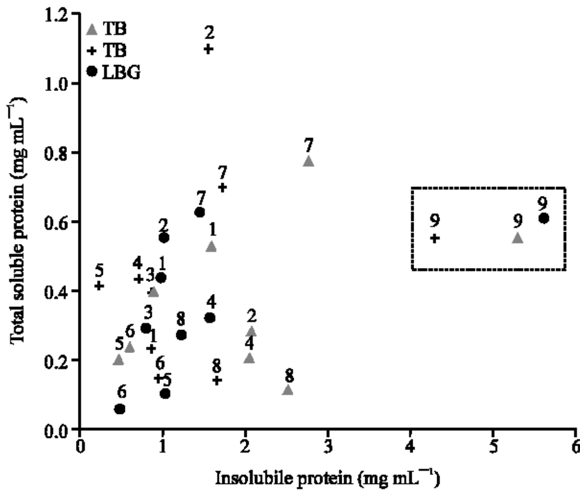

Fig. 3: Level of total soluble protein versus that of the insoluble fraction under all the experimental conditions tested. Runs are labeled according to the first column of Table 3. Panel gathers the central point runs

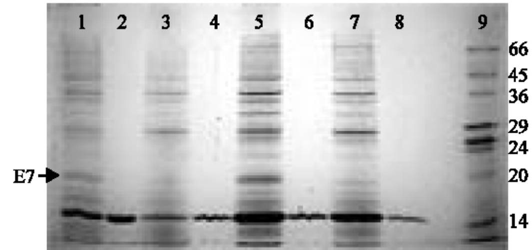

Fig. 4: SDS-PAGE analysis of the HPV E7 oncoproteins from recombinant *E. coli*. Lane 1, run C9 insoluble protein after induction; lane 2, run C9 soluble protein after induction; lane 3, run C9 insoluble protein before induction; lane 4, run C9 soluble protein before induction; lane 5, run B9 insoluble protein after induction; lane 6, run B9 insoluble protein before induction; lane 7, run B9 soluble protein after induction; lane 8, run B9 soluble protein before induction and lane 9, molecular weight standards (MW): bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and soybean trypsin inhibitor (20 kDa)

resistance to proteolytic degradation and simple primary recovery of the bodies. However, complex and costly processes are usually required for its final recovery from the bodies, during which the protein yield decreases significantly (Jeong and Lee, 1999). Since, the best combinations of levels obtained in this study for the soluble fraction of proteins were different from the those for the insoluble proteins, a suited compromise taking into account both responses should be found to exalt the overall E7 protein production.

These different responses of insoluble and soluble protein concentrations resulted in the absence of any clear relationship between them (Fig. 3), which means that both variables were strongly and differently influenced by either the cultivation conditions or the medium. Although the runs A2, A7, B7 and C7 showed the highest concentration of total soluble proteins, the conditions of the central point runs (A9, B9 and C9) (37°C, 140 rpm, 0.5 mM IPTG, 30 μg L^{-1} kanamycin and DO_{600} = 0.5) yielded comparable levels of this fraction and much higher levels of the insoluble one in all the three media; therefore, they were considered to be the most favorable conditions for possible industrial exploitation. In fact, the highest production of total proteins was in the form of inclusion bodies and the E7 protein constituted most of this fraction. This behavior was qualitatively similar to that observed by Jeong and Lee (1999), who studied the production of human recombinant leptin by *E. coli* BL21(DE3). According to Fiedler *et al*. (2004) the growth of *E. coli* below 37 °C has been often explored to minimize aggregation but without consistent, protein-irrespective results. The major drawback in *E. coli* cultivation at sub-optimal temperatures is, however, the decrease in biomass production which reduces the global process productivity.

Expression of soluble and insoluble forms of E7 protein: The 15% SDS-PAGE profiles of Fig. 4 confirms the successful expression of the recombinant E7 protein (~17 kDa) in both the soluble and insoluble fractions. The samples B9 and C9 were selected for these electrophoreses because they contained by far the highest total amounts of this protein. As expected, the soluble fraction of E7 protein exhibited a minor band in the gel of sample B9 electrophoresis (well 7), whereas it was practically absent in the C9 sample, likely because of its very low concentration. On the other hand, the insoluble form of protein E7 was identified and visualized as inclusion bodies in both samples (wells 1 and 5).

CONCLUSIONS

The high stability and productivity of the pET28a construct transformed into *Escherichia coli* BL21(DE3) pLysS is expected to make the production of E7 recombinant protein easier. Of the three culture media tested in this study, LB, TB and LBG, the second one

proved the most favorable for the production of HPV E7 oncoprotein by *E. coli*, yielding the highest specific growth rate.

The effects of five factors, specifically the temperature, agitation intensity, IPTG and kanamycin concentrations and inoculum size, were investigated through a 2^{5-2} experimental design adopting the specific growth rate, the concentration of insoluble proteins and those of soluble proteins in two successive extract as the responses. The combined effects of these variables influenced in different ways the selected responses. In particular, the highest production of total proteins took place in the form of inclusion bodies and the E7 recombinant protein constituted most of this fraction.

The production of soluble protein was smaller than that of the insoluble fraction, whose highest levels were obtained under the experimental conditions corresponding to the design's central point, irrespective of the medium used. In the best run, the IPTG concentration, which is of great concern owing to the high cost of this compound, resulted to be quite low and suitable for the industrial production.

Finally, the process described in this study is expected to reduce both the cost and the time of experimentation, while increasing the throughput. Nevertheless, further studies are required in order to optimize the E7 recombinant protein production for use in scale-up process.

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