Advanced Journal of Microbiology Research Vol. 2004
Available online at http://internationalscholarsjournals.org/journal/ajmr
© 2004 International Scholars Journals

Full Length Research Paper

Expression of green fluorescent protein (GFPuv) in *Escherichia coli* DH5-α, under different growth conditions

Thereza Christina Vessoni Penna*[1], Marina Ishii[1], Luciana Cambricoli de Souza [1], Olivia Cholewa[2]

[1]Department of Biochemical and Pharmaceutical Technology, School of Pharmaceutical Science, University of São Paulo, SP, Brazil.

[2]Molecular Probes, Inc. Eugene, Or, USA. 97402. Email: olivia.cholewa@probes.com.

Accepted 25 November 2003

The recombinant green fluorescent protein (GFPuv) was expressed by transformed cells of *Escherichia coli* DH5-α grown in LB/amp broth at 37°C, for 8 h and 24 h. To evaluate the effectiveness of different parameters to improve the expression of GFPuv by *E. coli*, four variable culturing conditions were set up for assays by a fractional factorial (2^{4-1}) design at two levels: (i) the effect of storing (24-48 h) the seeded broth at 4°C prior to incubation at 37°C; (ii) the effect of agitation speed (100-200 rpm); (iii) the final concentration (0.05-0.5 mM) of IPTG (isopropyl–β-D-thiogalactopyranoside) and (iv) the addition of IPTG at set cell densities (OD_{660} 0.01-0.8). GFPuv was extracted from cells by the three phase partitioning method (TPP) and further purified with a methyl HIC column. The cultures grown at 37°C/24 h provided the highest yields of GFPuv under the conditions: (i) pre-storage at 4°C/24 h; (ii) agitation speed at 100 rpm; (iii) 0.5 mM IPTG and (iv) IPTG addition at OD_{660}~0.01. On the other hand, at 37°C/ 8 h, GFPuv expression was dependent upon agitation of broth cultures at 200 rpm and the IPTG addition at the beginning of the growth exponential phase.

Key words: Green fluorescent protein (GFPuv), *Escherichia coli* DH5-α, growth kinetic parameters, expressed GFPuv kinetic parameters, three phase partitioning extraction (TPP).

INTRODUCTION

Green fluorescent protein (GFP) is a 27-29 kDa protein that provides a unique environment for three residues in its primary sequence to act as a fluorophore (Chalfie and Kain, 1998). The protein must be in the proper conformation, but once properly formed, it requires no further modification or co-factor to provide fluorescence.

Recombinant GFPuv was developed by introducing point mutations that replaced three amino acid codons in the native GFP DNA sequence (Chalfie et al., 1994, Crameri et al., 1996). GFPuv is expressed two to three times faster in *E. coli* and exhibits eighteen-fold greater fluorescence intensity than native GFP . With the excitation/emission maxima of 395/509 nm (Chalfie and Kain, 1998, Chalfie et al., 1994), the fluorescence characteristics of GFPuv can be quantified for *in vivo* and *in vitro* studies by a variety of techniques using fluorescence microscopy, flow cytometry and spectrofluorometry.

*Corresponding Author. E-mail: tcvpenna@usp.br. Phone: 0055-11-30913710. Fax: 0055-11-38156386.

The purpose of this study was to investigate the effects of various culture conditions upon the expression of GFPuv in transformed *E.coli*. To evaluate the effectiveness of different parameters to improve the expression of GFPuv by *E. coli*, nine culture conditions were set up by a fractional factorial (2^{4-1}) design at two levels: (i) the effect of storing the starting culture in LB/amp broth at 4 ^{O}C for 24 h, 36 h and 48 h ("storage") prior to incubation at 37°C; (ii) the effect of rotary speed (100, 150 and 200 rpm); (iii) the effect of the addition of IPTG at set cell densities; (iv) the concentration of IPTG with final concentrations of 0.05, 0.275 and 0.5 mM; (v) incubation of the culture at 37°C a period of 8 h or 24 h.

MATERIALS AND METHODS

Transformation

Escherichia coli DH5-α were transformed with *pGFPuv* (Clontech, CA, USA), by the standard calcium chloride method (Sambrook et al., 1989), following the procedure described by Vessoni Penna and Ishii, 2002. The recombinant GFPuv is under tight control of the *lacZ* protein β-galactosidase promoter/repressor that allows induction and high expression using isopropyl-β-D-thiogalacto-pyranoside *(*Chalfie and Kain, 1998*,* Chalfie et al., 1994, Crameri et al., 1996*)*. The vector includes the *bla* gene for ampicillin selection. The transformed cells were stored at -75ºC in Luria Bertani broth ("LB") supplemented with 100 ∝g/mL ampicillin and 50% glycerol.

Inoculum

A 24 h culture of transformed *E. coli* (LB/amp broth; 37°C/100 rpm) was transferred on to the surface of LB/amp/IPTG agar and incubated (37°C/24 h). Illuminating the agar surface with a hand-held long UV lamp (λ = 360 to 395 nm), isolated green fluorescent colonies were picked and transferred to 25 mL LB/amp broth in 250 mL Erlenmeyer flasks ("starter cultures"), which were incubated at 37°C/100 rpm. The inoculum of 1.0 mL was transferred to each of 32 Erlenmeyers (250 mL) containing 25 mL of LB/amp. As part of the experimental design, the inoculated broth in Erlenmeyers were stored at 4°C for 24 h, 36 h or 48 h prior to being incubated at 37°C on a rotary incubator.

Induction by IPTG addition

The expression cultures were incubated (37°C) until the broth cultures attained a set OD_{660} for the addition of IPTG (Table 2). Expression cultures from corresponding flasks (two flasks/hour) were assayed every hour for cell density: (*i*) OD_{660} with LB/amp broth in the reference cell; (*ii*) dried biomass related to GFPuv expression (∝g GFPuv/mg DCW; DCW = dried cellular weight) from cells retained on the surface of a 0.22 ∝m membrane (Millipore, SP, Brazil) and dried at 105°C for 3 h to attain a consistent weight; After IPTG addition, the GFPuv expression was monitored every hour by UV light at λ = 395 nm.

Extraction of GFPuv by three-phase-partitioning extraction (TPP) and HIC purification

GFPuv was extracted by following previous methodologies (Vessoni Penna and Ishii, 2002; Vessoni Penna et al., 2003; Denninson and Lovrient, 1997). The expression cultures were centrifuged (1000xg/30 min/4°C). The pellets were observed under UV light (395 nm), resuspended into cold extraction buffer ("XE": 25 mM Tris-HCl, pH 8 with 1.0 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulphonylfluoride) and subjected to direct extraction by the TPP method. To each 450 ∝L of cell suspension, 300 ∝L of 4 M $(NH_4)_2SO_4$ (1.6 M final concentration) and 750 ∝L of *t*-butanol (final ratio 1:1 aqueous phase:butanol) were added. The mixtures were vortexed for 2 min, centrifuged at 6,000xg/10 min. The three phases formed were collected. After the *t*-butanol upper phase and the white interfacial precipitate were removed, another equal volume of *t*-butanol was mixed with the lower aqueous phase and centrifuged. The upper phase of the system was discarded. The interfacial green phase was collected and dissolved in 1 mL XE buffer. Purification of the extracts was made through methyl support hydrophobic interaction chromatography (HIC) by eluting 1 mL portions GFPuv with the storage buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8). Purified GFPuv was stored at 4°C until to be used.

Fluorescence assay

The fluorescence intensity of GFPuv of the eluted samples was measured using a spectrofluorometer with an excitation filter of 395 nm and an emission filter of 509 nm. The fluorescence intensity of the samples was compared to a standard curve (1) (GFPuv ∝g/mL = [(fluorescence intensity) + 33.078]/38.943; R^2= 0.997) and the concentration was expressed in ∝g GFPuv/ mL. The standard curve was prepared using known amounts (between 1.32 ∝g/mL and 10.00 ∝g/mL) of purified recombinant GFPuv (Clontech) diluted in the same buffer solution (10 mM Tris-HCl, 10 mM EDTA, pH 8.0).

Statistical growth variables

The effects of the variables, interaction coefficients (95%), correlation matrix for estimated parameters, respective confidence intervals, significance levels ($p < 0.05$) and regression variance analysis (ANOVA) (Drapper and Smith, 1981) were calculated using the SGWIN program (Statgraphics Plus for Windows version 3, 1997 - Statistical Graphics Corporation, Rockville, MD). The four variables (x) considered in regression analysis were taken by dimensionless values over the same (-1) to (+1) range. The maximum (+1), the intermediate (0) and minimum (-1) codified ranges for each independent variable, shown in Table 2, were: x_1 = storage at 4°C (24 h, 36 h, 48 h); x_2 = agitation speed (100 rpm, 150 rpm and 200 rpm); x_3 = the effect of the addition of IPTG at set OD_{660} (0.01, 0.40 and 0.80); x_4 = the final concentrations of IPTG (0.05 mM, 0.275 mM and 0.5 mM); and x_5 = the incubation time at 37°C for 8 h (x_5 = - 1) and 24 h (x_5 = +1). The intermediate levels in code units were given by the equations: codified variable (x_n)= {[(effect studied) - (maximum level + minimum level)/2] / [(maximum level - minimum level)/2]}.

RESULTS AND DISCUSSION

Parameters of growth for *E. coli* and expression of GFPuv

The strain, *E. coli* DH-5α, achieved competence easily and has been shown to be transformed efficiently with pGFPuv, a circular DNA plasmid constructed from pUC (Chalfie et al., 1994). After incubation at 37°C for 8 h and

Table 1. Variables for *E. coli* growth and simultaneous expression of GFPuv: pre-inoculum cell density, inoculated medium storage (4^oC) before incubation (37^oC/100 rpm or no agitation), culture OD_{660} at IPTG addition. Kinetic parameters estimated: μ (h^{-1}) = exponential rate constant; g (h, min) = generation time; ∝p (h^{-1}) = specific product (GFPuv) rate (h^{-1}).

Growth conditions						Growth parameters				GFPuv parameters		
	Growth	Pre Inoculum	Incubation	Pre Storage	IPTG added	Growth Equation		$\propto_x$	g	GFPuv production Equation		$\propto_p$
Group		OD_{660}	h	4ºC/h	OD_{660}	$LnX=lnX_o+ux*t$ LnX=	R^2	h^{-1}	min	$lnP=lnP_o+up*t$ lnP	R^2	h^{-1}
1	1	≥ 1.4	24	-	1.02	- 0.0441 + 0.0738*t	0.95	0.95	43.77	1.1107 + 0.0696*t	0.67	0.07
1	2	≥ 1.4	24	-	0.78	- 0.2316 + 0.0886*t	0.88	0.88	47.25	1.259 + 0.0982*t	0.63	0.10
2 (A_1)	3	0.014	8	-	0.77	- 8.0293 + 1.5236*t	0.91	0.45	91.38	1.9083 + 0.4351*t	0.90	0.44
2 (A_2)	4	0.014	8	-	0.84	- 6.4724 + 1.2016*t	0.97	0.48	85.73			
2 (B_1)	5	0.014	8	24	0.023	- 9.163 + 1.5452*t	0.90	0.45	92.40	5.4979 + 0.0474*t	0.80	
2 (B_2)	6	0.014	8	24	0.044	- 8.0484 + 1.4397*t	0.93	0.46	89.42	2.4815 + 0.142*t	0.98	
3 (A_1)	7	0.016	8	24	0.015	- 8.462 + 1.2065*t	0.93	0.31	134.13	- 2.9801 + 0.7765*t	0.94	0.78
3 (A_2)	8	0.016	8	24	0.015	- 7.1397 + 1.0228*t	0.98	0.33	127.29	- 3.6671 + 0.9088*t	0.96	0.91
3 (B_1)	9	0.026	8	24	0.021	- 7.9173 + 1.2972*t	0.92	0.31	135.59	- 0.1049 + 0.5185*t	0.93	0.52
3 (B_2)	10	0.026	8	24	0.028	- 6.203 + 0.9946*t	0.93	0.31	134.13	- 2.9118 + 0.8898*t	0.95	0.89
4 (A)	11	0.0054	8	24	0.0098	- 6.6989 + 0.9658*t	0.99	0.25	168.00	- 2.1094 + 0.7097*t	0.91	0.71
4 (B)	12	0.0101	8	24	0.0091	- 7.1599 + 0.9635*t	0.98	0.24	169.71	- 2.9047 + 0.8914*t	0.92	0.89
5 (A)	13	0.0072	8	24	0.029	- 6.8642 + 1.0746*t	0.96	0.19	216.56	- 3.0169 + 1.0154*t	0.93	1.02
5 (B)	14	0.0072	8	24	0.032	- 6.2902 + 0.9821*t	0.94	0.19	221.17	0.4374 + 0.9269*t	0.97	0.93
5 (C)	15	0.0119	8	48	0.013	- 3.9696 + 0.8405*t	0.97	0.19	214.33	- 6.6989 + 0.9658*t	0.94	0.97

24 h, no lag phase was exhibited. During the exponential phase, *E. coli* divides at a constant rate and the increase in the number of cells was shown to be directly proportional to GFPuv expression after induction with IPTG at set culture OD_{660} conditions (Figure 1). The exponential growth rate (∝, h^{-1}) for the bacteria is proportional to the number of viable cells (X) at the starting time (X_o at $t_o = 0$) and after a time of incubation (X at t, h), observed as first order kinetics, which can be represented by the following relation: $\ln X= \ln X_o + \propto_x{*}t$ (1). The generation time (g, min) necessary to double the number of cells ($X= 2X_o$), or 'doubling time', is given by the relation $g = 0.693/ \propto_x$ (Miguel et al., 2003). With the expression of GFPuv (P_o, P) observed to be directly proportional to the cell growth (X_o, X) and showed the same logarithmic kinetic order of increase per unit of incubation time (t), as shown in Figure 1, this relation was represented by $\ln P= \ln P_o + \propto_p{*}t$ (1), where $\propto_p$ (h^{-1}) is the exponential expression rate constant for GFPuv. The kinetic parameters (Table 1) were calculated for growth cultures ("G", from G1 to G15).

For group #1, the exponential growth rate ∝ (h^{-1}) and generation time (g, min) varied ~7%. The exponential rate for the expression of GFPuv was between $\propto_p = 0.07\ h^{-1}$ (G1) and $\propto_p = 0.10\ h^{-1}$ (G2), around 10- (G1) to 14-times (G2) less than the specific rates for the *E. coli* cells. After 24 h incubation at 37^oC (without shaking) the cultures exhibited no lag phase, 4 to 5 h of exponential growth phase and the remaining stationary phase interval was accompanied by the expression of GFPuv (Figure 1).

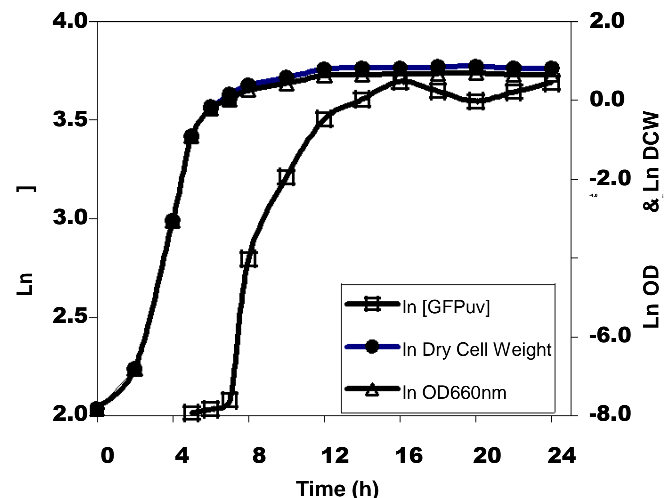

Figure 1. Data of cell densities (OD_{660}) and dry cell weight (DCW) correlated with the amount of GFPuv (∝g GFPuv /mL) expressed during 24h *E. coli* growth, as measured by fluorescence intensity of extracted GFPuv.

For group #2, the inoculated media for G3 and G4 were directly incubated for 8 h at 37^oC/100 rpm with IPTG added at the end of the logarithmic phase; for G5 and G6, the inoculated media were stored refrigerated (4^oC/24 h) before incubation (37^oC/100 rpm/8 h) and IPTG was added at the intermediate logarithmic phase.

Table 2. Concentrations of extracted GFPuv (µg GFPuv/ mL) and effects of expression (µg GFPuv/mg DCW**)** derived from *E.coli* cells cultivated at set conditions for standard periods of 8 h (x_5 = -1) and 24 h (x_5 = +1) incubation at 37°C. Where: x_1 = storage at 4°C for 24 h (x_1=-1), 36 h (x_1=0), 48 h (x_1=+1); x_2 = agitation speed for 100 rpm (x_2=-1), 150 rpm (x_2=0), 200 rpm (x_2=+1); x_3 = the effect of the addition of IPTG at set OD_{660}= 0.01 (x_3=-1), 0.40 (x_3=0) and 0.80 (x_3=+1) and x_4 = the final concentrations of IPTG= 0.05 mM (x_4=-1), 0.275 mM (x_4=0) and 0.5 mM (x_4=+1).

Variables for standard 8 h incubation (x_5 = -1)							**Concentration**	**Specific productivity**
[1]**Assay**	**Storage**	**RPM**	**OD_{660} IPTG**	**IPTG (mM)**	**Incubation (h)**	**DCW mg/mL**	**∝g GFPuv / mL** mean± CI	**∝g GFPuv/mg DCW** mean±CI
	x_1	x_2	X_3	X_4	x_5			
1	-1	-1	-0.99	-1	-1	0.24	1.16±-0.11	4.85±0.47
2	1	-1	-0.98	1	-1	0.43	1.42±0.19	3.18±0.51
3	1	-1	-0.98	1	-1	0.43	1.68±0.22	3.91±0.52
4	1	-1	-0.98	1	-1	0.47	1.16±0.07	2.44±0.16
5	1	1	-0.98	-1	-1	1.76	15.24±3.28	9.20±1.54
6	-1	1	0.99	-1	-1	1.68	3.98±0.57	2.36±0.34
7	-1	1	0.99	-1	-1	1.68	3.98±0.57	2.36±0.34

Variables for standard 24 h culture incubation (x_5 = +1)							**Concentration**	**Specific productivity**
[1]**Assay**	**Storage**	**RPM**	**OD_{660} IPTG**	**IPTG (mM)**	**Incubation (h)**	**DCW mg/mL**	**∝g GFPuv / mL** mean± [3]CI	**∝g GFPuv/ mg DCW** mean±CI
	x_1	x_2	x_3	x_4	x_5			
1	-1	-1	-0.98	-1	1	1.98	33.54±1.07	16.97±0.54
2	-1	-1	-0.99	-1	1	2.03	30.92±1.57	15.24±0.77
3	1	-1	-1.00	1	1	2.14	25.64±1.84	12.00±0.86
4	1	-1	-0.99	1	1	2.19	29.12±1.80	13.30±0.82
5	1	1	-0.99	-1	1	2.06	14.54±0.82	7.05±0.40
6	1	1	-0.99	-1	1	2.12	16.32±1.11	7.68±0.52
7	-1	1	-0.99	1	1	2.16	22.73±1.29	10.52±0.60
8	-1	1	-0.98	1	1	1.95	23.46±1.30	10.86±0.56
9	1	-1	0.12	-1	1	2.05	19.24±2.20	9.38±1.08
10	1	-1	0.31	-1	1	2.09	21.76±0.58	10.43±0.28
11	1	-1	-0.35	-1	1	2.14	22.20±1.38	10.28±0.65
12	1	-1	-0.096	-1	1	2.16	17.32± 0.44	8.03±0.20
13	-1	-1	-0.56	1	1	1.98	31.98±2.02	16.19±1.02
14	-1	-1	-0.53	1	1	2.02	30.64±1.46	15.17±0.73
15	-1	-1	0.37	1	1	2.07	24.78±1.88	11.97±0.91
16	-1	1	1.00	-1	1	2.15	11.47±0.82	5.33±0.38
17	-1	1	0.69	-1	1	2.09	9.84±2.74	4.71±1.31
18	-1	1	0.73	-1	1	2.15	7.26±0.47	3.38±0.22
19	1	1	0.75	1	1	2.22	9.57±0.51	4.30±0.23
20	1	1	0.026	1	1	2.10	9.67±0.33	4.61±0.23
21	0	0	0.49	0	1	2.41	12.76±0.91	5.29±0.38
22	0	0	0.13	0	1	2.19	20.16±1.45	9.19±0.66
23	0	0	0.013	0	1	2.31	18.71±1.00	8.11±0.43

[1]Assay= the order in which the experiments were carried out.

[2]CI = Confidence Interval, $p< 0.05$; n= 10 observations

Table 3. Fitted models for µg GFPuv/ mL adjusted from the polynomial model (µg GFPuv/ mL = 10.28 - 1.13 x_1 - 5.65 x_3 + 8.28 x_5 - 1.76 x_1x_5 - 5.38 x_2x_5 + 1.21 x_4x_5,) for the factors: x_1 = storage at 4 ^{O}C; x_2 = rotary speed (rpm); x_3 = addition of IPTG at OD; x_4 = final concentration of IPTG (mM); with incubation set at 37°C for 8 h (x_5 = -1).

Factor Relation	Fitted models (µg GFPuv/ mL)	Extreme Conditions		µgGFPuv/ mL
1st Case: x_1 = +1; x_4 = +1; x_5 = -1		x_2 = +1	x_3= +1	1.15
	µg GFPuv/ mL= 1.41 + 5.38 x_2 – 5.65 x_3	x_2= -1	x_3= -1	1.68
		x_2= -1	x_3= +1	-9.61
		x_2= +1	x_3= -1	12.44
2nd Case: x_1 = -1; x_4 = -1; x_5 = -1		x_2= +1	x_3= +1	2.32
	µg GFPuv/ mL= 2.58 + 5.38 x_2 – 5.65 x_3	x_2= -1	x_3= -1	2.84
		x_2= -1	x_3= +1	-8.45
		x_2= +1	x_3= -1	13.61
3rd Case: x_1 = +1; x_4 = -1; x_5 = -1		x_2= +1	x_3= +1	3.58
	µg GFPuv/ mL= 3.84 + 5.39 x_2 – 5.65 x_3	x_2= -1	x_3= -1	4.10
		x_2= -1	x_3= +1	-7.19
		x_2= +1	x_3= -1	14.87
4th Case: x_1 = -1; x_4 = +1; x_5 = -1		x_2= +1	x_3= +1	-0.10
	µg GFPuv/ mL= 0.16 + 5.38 x_2 – 5.65 x_3	x_2= -1	x_3= -1	0.42
		x_2= -1	x_3= +1	-10.87
		x_2= +1	x_3= -1	11.19
5th Case: x_1 = 0; x_4 = 0; x_5 = -1		x_2= 0	x_3= 0	2.00
	µg GFPuv/ mL= 2.00 + 5.38 x_2 – 5.65 x_3	x_2= +1	x_3= +1	1.74
		x_2= -1	x_3= -1	2.26
		x_2= -1	x_3= +1	-9.03
		x_2= +1	x_3= -1	13.03

For group #3, growth cultures G7, G8, G9 and G10 were kept refrigerated (4°C/24 h) before incubation (37°C/100 rpm/8 h), and IPTG added after ~3 h of incubation (Figure 1). The influence of exponential cell growth rate on GFPuv expression rate was evident with these samples, the slower the bacterial growth ($\propto_x$= 0.32 h^{-1}, group #3), the greater the expression of GFPuv ($\propto_p$= 0.78±0.18 h^{-1}).

For groups #4 and #5, the starter inoculum consisted of 3 to 4 times lower cell densities inoculating growth resulted in similar exponential rates for cell growth and expression of GFPuv compared to those observed for growth #3.

Optimal conditions for the expression of GFPuv during 8 and 24 h incubation times

The ANOVA analysis interpreted the results by fitting a multiple linear regression model to describe the relationship between ∝g GFPuv/mL and the independent variables set (Table 1). The quadratic polynomial model derived for ∝g GFPuv/mL, as shown in the Appendix and main equation on Tables 3 and 4, for the independent variables: x_1, x_2, x_3, x_4 and x_5. Since the p-value in the ANOVA is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence levels.

The greatest improvement in the GFPuv expression for extreme culture conditions

After 8 h culture incubation at 37°C (x_5 = -1), the concentration of extracted GFPuv in the fitted equations (Table 3) is proportional to the positive coefficient related to rotary speed (x_2) and to the negative coefficient of the culture cell density (OD_{660}, x_3) at which IPTG was added. This observation is confirmed by the 3rd case with storage at 4°C for 48 h (x_1 = +1) and the final IPTG concentration of 0.05 mM (x_4 = -1). By the analysis of the extreme conditions: (i) the highest concentration of GFPuv extracted (14.87 ∝g GFPuv/mL) is related to 200 rpm (x_2 = +1) rotary speed and cell densities (OD_{660} = 0.01, x_3 = -1) upon the addition of IPTG; (ii) the worst condition, no expression of GFPuv, was evident with 100 rpm (x_2 = -1) rotary speed and addition of IPTG at OD_{660} of 0.8 (x_3 = +1); (iii) equivalent effect on the expression of ~4 ∝g GFPuv/mL is verified for both x_2 and x_3 at minimum levels (100 rpm and 0.01 OD_{660}) or at maximum levels (200 rpm

Table 4. Fitted models for µg GFPuv/mL adjusted from the polynomial model (µg GFPuv/mL = 10.28 - 1.13 x_1 - 5.65 x_3 + 8.28 x_5 - 1.76 x_1x_5 - 5.38 x_2x_5 + 1.21 x_4x_5,) for the factors: x_1 = storage at 4°C; x_2 = rotary speed (rpm); x_3 = addition of IPTG at OD; x_4 = final concentration of IPTG (mM); with incubation set at 37°C for 24 h (x_5 = +1).

Factor Relation	Fitted Equation (µg GFPuv/ mL)	Extreme Conditions	µg GFPuv/ mL
1st Case: x_1 = +1; x_4 = +1; x_5 = +1		x_2= +1 x_3= +1	5.85
	µg GFPuv/ mL = 16.88 - 5.38 x_2 – 5.65 x_3	x_2= -1 x_3= -1	27.90
		x_2= -1 x_3= +1	16.61
		x_2= +1 x_3= -1	17.14
2nd Case: x_1 = -1; x_4 = -1; x_5 = +1		x_2= +1 x_3= +1	9.20
	µg GFPuv/ mL = 20.23 - 5.38 x_2 – 5.65 x_3	x_2= -1 x_3= -1	31.26
		x_2= -1 x_3= +1	19.97
		x_2= +1 x_3= -1	20.49
3rd Case: x_1 = +1; x_4 = -1; x_5 = +1		x_2= +1 x_3= +1	3.42
	µg GFPuv/ mL = 14.45 - 5.38 x_2 – 5.65 x_3	x_2= -1 x_3= -1	25.48
		x_2= -1 x_3= +1	14.19
		x_2= +1 x_3= -1	14.71
4th Case: x_1 = -1; x_4 = +1; x_5 = +1		x_2= +1 x_3= +1	11.62
	µg GFPuv/ mL= 22.65 - 5.38 x_2 – 5.65 x_3	x_2= -1 x_3= -1	33.68
		x_2= -1 x_3= +1	22.39
		x_2= +1 x_3= -1	22.92
5th Case: x_1 = 0; x_4 = 0; x_5 = +1	µg GFPuv/ mL= 18.55 - 5.38 x_2 – 5.65 x_3	x_2= 0 x_3= 0	18.55
		x_2= +1 x_3= +1	7.52
		x_2= -1 x_3= -1	29.58
		x_2= -1 x_3= +1	18.29
		x_2= +1 x_3= -1	18.81

and 0.8 OD_{660}).

After 24 h incubation at 37°C (x_5 = +1), setting the storage at 24 h at 4°C (x_1 = -1) and IPTG concentration of 0. 5 mM (x_4 = +1), we observe through the fitted equations of the 4th case (Table 3), that the influence of both negative coefficients for rotary speed (x_2) and cell OD_{660} density (x_3) on the expression of GFPuv, provided the highest yield of 33.68 ∝g GFPuv/mL by keeping their minimum levels of rotary speed (100 rpm, x_2 = -1) and OD_{660} cell density (0.01 OD_{660}, x_3 = -1). Otherwise, at maximum levels for both variables, 200 rpm (x_2 = +1) rotary speed and 0.8 OD $_{660}$ (x_3 = +1) cell densities upon the addition of IPTG to the culture, the expression of GFPuv by *E.coli* decreases three times to 11.62 ∝g GFPuv/mL.

Comparing the highest expression of GFPuv by *E.coli* after incubations at 8 h (x_5 = -1) and 24 h (x_5 = +1) at 37°C, we observed that although the GFPuv expression begins following the addition of IPTG (maximum and minimum concentrations), the rotary speed was shown to exhibit a remarkable influence on the increase in cell density at the minimum level of 0.01 OD_{660} at the beginning of the logarithmic phase, when the newly dividing cells are induced by IPTG to express GFPuv.

Another remarkable positive effect on GFPuv expression was the storage at 4°C for 24 h (x_1 = -1) and 48 h (x_1 = +1) of the starter culture and then 8 h (x_5 = -1) and 24 h (x_5 = +1) incubation at 37°C. The expression of the highest concentrations of GFPuv was shown to be independent of the time in storage. Refrigerated storage seems to increase the ability of the cells to multiply when incubated at 37°C: (i) at high speed (200 rpm, x_2 = +1) for a shorter incubation, 8 h (x_5 = -1), and (ii) at low speed (100 rpm, x_2 = -1) for a longer incubation, 24 h (x_5 = +1).

The refrigeration of cultures before incubation and the addition of IPTG in different stages of growth process did not interfere in the exponential rate constant but positively influenced the GFPuv expression which was dependent upon the culture agitation and incubation at 37°C.

The cultures grown at 37°C for 24 h incubation provided the highest yields of GFPuv, for the set conditions: (i) storage at 4°C for 24 h; (ii) rotary speed at 100 rpm; (iii) the concentration of IPTG between 0.05 mM and 0.5 mM; (iv) the addition of IPTG at OD_{660} = 0.01 corresponding to cell densities at the beginning of lag phase. Storage, rotary speed and the cell density at which the IPTG is added, showed greater influences on

GFPuv expression upon the *E. coli* cultures. The addition of IPTG in different stages of growth process did not interfere in the exponential rate constant.

ACKNOWLEDGEMENTS

Financial support for this work is provided by CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico and FAPESP–Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil. The authors are grateful for technical support of Irene A. Machoshvili.

APPENDIX:

Main polynomial model:
µg GFPuv/mL = 10.28 - 1.13 x_1 - 5.65 x_3 + 8.28 x_5 - 1.76 x_1x_5 - 5.38 x_2x_5 + 1.21 x_4x_5
Independent variables: x_1 = period (h) of storage at 4°C; x_2= rotary speed (rpm); x_3 = cell densities (OD_{660}) upon the addition of IPTG; x_4 = concentration of IPTG (mM) added, and x_5= culture incubation at 37°C, set at either 8 h or 24 h.
Statistical coefficients: $p < 0.01$ significance level; n = 170 observations; p = 7 coefficients
R^2 = 0.943 multiple determination for (n-1) = 169 degrees of freedom (d.f.)
R^2 = 0.941 (d.f. = p -1 = 6); Mean absolute error = 1.92467 (average value of the residuals)
SE = standard error = 2.35; (n -1=169 d.f.) deviation of the residuals from the estimate.
Model Student's $t_{(0.01/2)} > \pm 2.45$; Model F_{-ratio} (449.33)> $F_{critical}$ (0.99; p-1=6; n-1= 169; n-p=163) = 2.80

REFERENCES

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression, *Science* 263**,** 802-805.
Chalfie M, Kain S (1998), In: *Green Fluorescent Protein Properties, Applications and protocols*. Wiley-Liss, New York**,** 385p**.**
Crameri A, Whitehorn EA, Tate E, Stemmer WPC (1996), Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nat. Biotechnol.* 14, 315-319.
Denninson C, Lovrient R. (1997) Three phase partitioning: concentration and purification of proteins. *Protein Expression Purif.* 11, 149-161.
Drapper N, Smith, H (1981) Applied regression analysis. John Wiley, New York, 709p.
Sambrook J, Fritsch, EF, Maniatis T (1989), *Molecular cloning: a laboratory manual*, 2nd, Cold Spring Harbor Laboratory, New York, **1,** 1.74-1.84.
Miguel ASM, Martins das Neves LC, Vitolo M, Pessoa Jr. A (2003), Effect of flow rate pattern on glucose-6-phosphate dehydrogenase synthesis in fed-back culture of recombinant *Saccharomyces cerevisiae, Biotechnol. Prog*, 19, 320-324.
Vessoni Penna TC, Chiarini E, Machoshvili IA, Ishii M, Pessoa Jr. A (2002), Extraction of recombinant green fluorescent protein (GFPuv) from *Escherichia coli*. *Appl. Biochem. Biotechnol.* 98-100, 791-802*.*
Vessoni Penna TC, Ishii M (2002), *BMC Biotechnology,* http:/www.biomedcentral.com/ 1472-6750/2/7/qc, 2002, 2:7.
Vessoni Penna TC, Chiarini E, Pessoa Jr., A. (2003), Permeation Associated with Three-Phase-Partitioning Method on Release of Green Fluorescent Protein. *Applied Biochem. Biotechnol.*105-108, 481-491.