Original article (accepted manuscript): Improving the cost effectiveness of enhanced green fluorescent protein production using recombinant *Escherichia coli* BL21 (DE3): Decreasing the expression inducer concentration

Improving the cost-effectiveness of Enhanced Green Fluorescent Protein production using recombinant *Escherichia coli* BL21 (DE3): decreasing the expression inducer concentration

Short Title: Improving Green Fluorescent Protein production

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Abstract: Green Fluorescent Protein (GFP) is a globular protein used as biosensor and biomarker in medical and industrial fields. However, due to the expensive production costs of expressing proteins using high-cost inducers like isopropyl-β-D-1-thiogalactopyranoside (IPTG), the number of GFP applications are still scarce. This work studied the production of Enhanced GFP (EGFP) using *Escherichia coli* BL21 (DE3) [pLysS; pET28(a)], aiming to increase its yield and reduce costs. Firstly, the influence of agitation rate, induction time and concentration of IPTG in the production of EGFP were evaluated, but only the first two parameters were significant. Subsequently, aiming to reduce costs related to the use of inducer, the IPTG concentration (0.005, 0.010 and 0.025 mM) was decreased and, interestingly, the production levels were maintained or increased. These results show that a proper choice of production conditions, particularly through the decrease of inducer concentration, is effective to reduce the upstream production costs and guarantee high EGFP expression.

Keywords: biomarker; production; *Escherichia coli*; green fluorescent protein; inducer protein expression; IPTG.

Abbreviations: Absorbance Unities (AU), Enhanced Green Fluorescent Protein (EGFP), *Escherichia coli* (*E. coli*), Green Fluorescent Protein (GFP), isopropyl-β-D-1-thiogalactopyranoside (IPTG), Luria Bertani broth (LB), Optical Density at 600 nm (OD_{600nm}), Revolutions per minute (rpm).

1. Introduction

Biotechnology and recombinant DNA technology allowed the production of several biomolecules with industrial and medical relevance [1, 2]. Among these, the Green Fluorescent Protein (GFP), originally isolated from jellyfish *Aequorea victoria*, stood out for its great use as biosensor and biomarker, with many applications in the pharmaceutical and industrial fields [3, 4]. GFP presents a chromophore in its structure that confers fluorescence at an excitation wavelength from 395 to 489 nm and emission from 504 to 511 nm, depending on the GFP variant [3, 4], which allows this protein to be easily detectable and quantifiable [5]. GFP has a relatively low toxicity, present intense and natural fluorescence and a large pH and temperature stability range [5]. Furthermore, by a proper manipulation of its structure, its fluorescence intensity and spectra can be changed, adjusting its use for a wide array of purposes [6], from biological applications as a fusion protein (to quantify cells, cellular components and reactions), for whole-organism visualization and even as a transcriptional probe to monitor environmental variables such as pH, oxygen, temperature and nutrient availability [7]. Even considering the advantages of GFP when used as biosensor and biomarker, due to its high costs, the number of possible applications for this protein is still very limited. Nowadays, the costs of GFP are still very high, with commercial GFP reaching values above US$ 2,000.00 per mg in January 2019 in companies like BioVision and MyBioSource, which restrict its use to research and small-scale purposes. The development of other GFP variants (like Enhanced GFP, EGFP, and ultraviolet visible GFP, GFPuv) and recombinant microorganisms capable of expressing GFP overcame some of the drawbacks related with its production, like the inefficient folding of the protein close to 37° C [3, 4]; however, most procedures for the expression of recombinant proteins still heavily relies on the use of expensive inducers like isopropyl-β-D-1-thiogalactopyranoside (IPTG) [8]. Thus, studies to increase and improve the production of GFP or to present alternatives to the use of elevated concentrations of expensive inducers and substrates are still essential to favor the large-scale use of this promising protein.

An improvement of the upstream process, therefore, seems to be one of the most interesting strategies to obtain GFP at affordable costs. In fact, the use of recombinant DNA technology allied with the development of enhanced upstream processes using microbial sources has been a recurrent alternative for an effective production of high quantities of proteins at lower prices [9-11]. Through the isolation and cloning of the gene responsible for the expression of GFP, the protein was successfully produced by several recombinant organisms, such as *Escherichia coli* (*E. coli*) [12], *Aspergillus niger* [13] and *Lactobacillus casei* [14]. Among these microbial systems, *E. coli* is the most frequently used for the production of recombinant bioproducts, particularly because it exhibits fast growth in a relatively cheap and simple culture medium, while still generating high yields of the target biomolecule [9, 15]. Briefly, the expression of heterologous proteins in *E. coli* can be achieved by the insertion of a gene in a plasmidial vector under the transcriptional control of a promoter, that guarantees the capacity to "activate" the expression of a foreign gene with the variation of an environmental factor, as for example, the presence of an expression inducer [16]. To prevent leaky expression (expression of the heterologous proteins even when the activation stimuli are not in place), another plasmid can be inserted to guarantee the regulation of the protein production [17]. It is recommended to limit the expression of recombinant proteins either to control the concentration of toxic biomolecules or to maintain the distinction among the cycles of cellular proliferation and production of heterologous proteins [18]. Therefore, considering that the insertion of plasmids in bacteria can have various effects in its metabolism and growth, testing the combination of host cell and plasmids is always required to enhance production [17] Although *E. coli* can reach high cell density and high concentrations of the target products, cellular growth, and protein expression can be impaired by oxygen limitation and accumulation of acetic acid [19].

The expression of recombinant proteins in *E. coli* can be carried out using several inducers depending on the promoter used. For *lac* promoter, for example, can be used IPTG, lactose or galactose [8]. Stress conditions can be used to improve the extraction of recombinant proteins in *E. coli*, such as the presence of glucose, tryptophan, or phosphate starvation, increase or decrease of temperature, presence or absence of oxygen, osmolarity and pH.The choice of the most suitable inducer is defined by considering factors such as toxicity of the target protein, inducer costs, induction aptitude, among others. For example, toxic proteins usually require highly repressible promoters to reduce detrimental impacts to the host cell; however, this in turn can result in low yields [8]. IPTG is a strong chemical inducer [20], offering an advantage over lactose in the expression of innocuous proteins. Additionally, even though lactose has some advantages over IPTG (particularly lower costs and toxicity), it can generate unwanted auto-induction [21]. On the other hand, induction by changing processual conditions are usually considered more economically attractive than chemical induction using IPTG or lactose, however, the stress can cause several unwanted responses, as for example: heat-shock; concomitant upregulation of proteases [8]; degradation of the target protein; increase of the fermented contaminants increasing the complexity of downstream processing (the most expensive stage of protein manufacturing budget [22]). Therefore, a proper balance between cost and benefit regarding the choice of inducer (chemical or processual) is of upmost importance. For example, production processes using lactose usually requires large amounts of inducer, and thus, the induction using low concentration of IPTG can be far more convenient at industrial processing. It is evident that there is no perfect inducer for all circumstances, and IPTG remains the most used inducer for protein expression [23-25].

Besides the choice of a suitable host, expression microbial system and inducer, the improvement of cultivation cultures remains the most interesting approach to attain high producing yields. There are many variables that can impact bacterial cultivation, as for example, nutritional composition of culture medium, pH, cellular density of the inoculum, agitation rate, aeration and oxygenation of culture, induction time, the concentration of inducer, temperature and total time of cultivation [12, 25, 26]. Previous studies to enhance the expression of GFP evaluated the effect of different parameters in its production, with some presenting a more expressive role than others. Pérez-Arellano and Pérez-Martínez studied the variables pH, temperature and agitation rate, showing that the latest exerted the greatest influence in GFPuv production using *Lactobacillus casei* ATCC 393 [pLZ15-], achieving a maximum of 1.3 µg of GFP per mg of total protein from which only 55% was fluorescent [14]. Penna *et al.* 2004 evaluated the influence of IPTG concentration, time of storage of the seeded broth, agitation rate and cellular density at the time of induction in the expression of GFPuv using *E. coli* DH5-α, observing that only the concentration of IPTG did not impact GFPuv production, which was up to 33.68 $mg.L^{-1}$ in the most favorable condition [27]. Chew *et al.* 2012 tested how the initial medium pH, the concentration of inducer, inoculum density, agitation rate, temperature and induction time impact the production of EGFP using *E. coli* BL21 (DE3) with plasmid RSETEGFP. The authors identified that agitation rate, temperature and induction time were the most significant parameters, achieving a maximum of 241 $mg.L^{-1}$ of EGFP in optimal conditions for these variables (agitation rate of 206 rpm, temperature of 31 °C and time of induction when optical density at 600 nm was 1.04) [12]. All previous studies reported in the literature have shown a strong influence of agitation over GFP production, indicating this is a key element to improve the process. Additionally, both Penna *et al.* 2004 and Chew *et al.* 2012 highlighted that different concentration of inducer did not alter GFP production; however, no further studies were performed to reduce the IPTG concentrations and to identify the minimum concentrations of inducer required for GFP expression.

Considering that the improvement of agitation rate can potentially allow an enhanced bacterial growth and GFP synthesis, while lower concentrations of an inducer can help reducing production

costs, herein, the production of the most applied GFP variant, EGFP [28, 29], using the bacteria *E. coli* BL21 (DE3) [pLysS; pET28 (a)] was thoroughly studied. In this work, the influence of agitation rate, induction time and concentration of inducer IPTG over EGFP production was determined, aiming both to increase EGFP production yields and improve the sustainability of the processes with the reduction of IPTG concentration, consequently leading to the decrease of related production costs.

2. Materials and methods

2.1. Material

The culture medium Luria Bertani broth (LB) from Difco®, the inducer IPTG and the antibiotics kanamycin and chloramphenicol were acquired from Sigma Aldrich®. Tris-HCl buffer was prepared with 50 mM of tris(hydroxymethyl)aminomethane (Synth, > 99%) in water with pH adjustment to 7.0 and 8.0 using hydrochloric acid 37% (HCl, Neon). Milli-Q water (ultrapure water of Type 1) was used in the preparation of all solutions and experiments.

2.2. Microorganism and inoculum preparation

The EGFP gene was inserted into the pET28(a) plasmid through the restriction sites NcoI and BamHI, and therefore, placed under control of the strong T7 promoter and a strong ribosome binding site (RBS). *E. coli* BL21 (DE3) [pLysS] was transformed with the resulting plasmid pET28(a)-EGFP (**Figure 1**). To validate the heterologous expression system used in this study, the sequencing of plasmid DNA was executed using the equipment ABI PRISM® 3130 Genetic Analyzer, with the sequences evaluated in the Sequence Scanner Software 2.0 and compared in the software SerialCloner 2.6.1.

The microorganism was maintained frozen at −80°C with glycerol in a cryotube. The inoculum was prepared transferring a small amount of microorganism (retired with a platinum handle) to 100 mL of LB broth containing the antibiotics kanamycin and chloramphenicol (both at 50 $mg.L^{-1}$) in 500 mL Erlenmeyer flasks during 12 h at 30°C in an orbital shaker (TE-421 from Technal, Brazil). The agitation rate varied in each experiment (100, 150, 200 or 250 revolutions per minute - rpm), as detailed in the next sections. Following, the inoculum was added in 500 mL Erlenmeyer flasks containing 100 mL of the culture media to achieve an initial optical density at 600 nm (OD_{600nm}) of 0.05 absorbance unity (AU). For the EGFP production, a set of experiments described in the next sections were performed, but the following conditions were kept for all the experiments: the culture media had the initial pH adjusted to 7.0 and were autoclaved at 121°C for 15 min; the microbial growths of the inoculums were carried out at 30°C; IPTG was used as inducer. The cells growth was determined by OD_{600nm} as detailed in **section 2.5**. All assays were performed in triplicate with the respective blanks, and the respective standard deviations were determined.

2.3. Bacterial growth curves

Firstly, to determine the beginning, middle and end of the exponential growth phase of the strain *E. coli* BL21 (DE3) [pLysS; pET28(a)], the corresponding bacterial growth curves were obtained under different agitation rates (100, 150, 200 and 250 rpm). The curves were determined through the sampling at each 2 h during 24 h of culture, and then, the respective induction time for each agitation rate properly defined.

2.4. EGFP production

The study of recombinant EGFP production was carried out evaluating the following three parameters: *i*) agitation rate; *ii*) concentration of inducer (IPTG); *iii*) time of induction. Firstly, the production of EGFP was evaluated at four agitation rates (100, 150, 200 and 250 rpm) inducting the protein production with 0.500 mM of IPTG at the middle of the exponential growth phase of *E. coli* BL21 (DE3) [pLysS; pET28 (a)]. Samples were collected before the induction, and 1, 2, 3, 4, 6, 8, 10 e 12 h after the induction, and the corresponding OD_{600nm} and EGFP concentration ($mg.L^{-1}$) determined as detailed in the **section 2.5.**

From the initial set of experiments, 150 rpm was defined as the ideal agitation rate for the EGFP production (see **section 3.2**) and kept constant for the second set of experiments, where the influence of the IPTG concentration and time of induction were evaluated. Similar to the first set of experiments (samples recording and analysis), the influence of both parameters on the protein production was carried out, testing three concentrations of IPTG (0.250, 0.500 and 0.750 mM) and three different induction times (induction after 2, 6 and 10 h of cultivation, the beginning, middle and end of the exponential growth phase, respectively).

In the last set of experiments, the minimal concentration of IPTG required to produce EGFP was determined. For this purpose, *E. coli* BL21 cells were cultivated at 150 rpm with induction after 6 of cultivation at 4 different concentrations of IPTG, 0.005, 0.010, 0.025 and 0.250 mM. To evaluate if the IPTG addition was required for EGFP production, *E. coli* BL21 cells were cultivated under similar conditions but without the addition of IPTG. For this last set of experiments, samples were taken every 2 h during 11 h of cultivation, and the respective values of OD_{600nm} and EGFP concentration ($mg.L^{-1}$) measured as described in the **section 2.5**.

2.5. Analytical methods

Bacterial culture growth was determined by measuring the optical density at 600 nm of absorbance (OD_{600nm}) using a spectrophotometer UltrospecTM 2100 pro UV/Vis (GE Healthcare®).

The EGFP production was assessed by analyzing the *E. coli* culture fluorescence in the excitation wavelength at 395 nm and emission at 510 nm, using a multimode plate reader (EnSpire® Multimode Plate Reader, PerkinElmer®). For that purpose, after the cultivation, the cells were harvested by centrifugation at 5000 x*g* during 20 min at 4°C, and then the supernatant was discarded and the *pellet* resuspended in buffer Tris-HCl 50 mM, pH 8.0. The fluorescence of the cells was measured and the concentration of EGFP ($mg.L^{-1}$) determined according to a calibration curve previously established using a commercial standard [EGFP Quantification Kit K815-100 (BioVision®)].

3. Results and discussion

3.1. Bacterial growth curves of *E. coli* BL21 (DE3) [pLysS; pET28(a)]

Initially, to find the best growth conditions of *E. coli* BL21 (DE3) [pLysS; pET28(a)] cells, the bacterial cultivation was evaluated at 30°C under four different agitation rates, 100, 150, 200 e 250 rpm, as depicted in **Figure 2**. All the experiments were initiated with OD_{600nm} of, approximately, 0.05 AU, to guarantee a similar number of cells at the beginning of the cultivation.

As shown in **Figure 2**, a similar growth pattern was observed for all agitation rates, with a short lag phase until the first 2 h of cultivation and stationary phase beginning after 8 – 9 h. The highest cell

growth (approximately 4.2 AU) was obtained with the incubation at 250 rpm. In this condition, *E. coli* growth rates reduced with the decrease of agitation rate (namely, at 150 and 200 rpm, the OD_{600nm} of the cultures was around 3.4 AU, while at 100 rpm it reached only 2.8 AU).

The analysis of each bacterial growth curve allowed the determination of the beginning, middle, and end of the exponential (log) growth phase of *E. coli* BL21 (DE3). The beginning of the log phase occurred 2 h after inoculation for all conditions studied. On the other hand, the end of the exponential phase at 100 and 150 rpm was achieved after 12 h of incubation, but at higher agitation rates (200 and 250 rpm), the end of the exponential phase occurred after 11 h of growth. Previously, Robichon *et al.* 2012 [30] evaluated the *E. coli* BL21 (DE3) growth at 30ºC using LB medium, obtaining OD_{600nm} values of, approximately, 5 AU after 8h of incubation, as well as short lag phase (up to 1 h of growth). Larentis *et al.* 2014 [31] attained OD_{600nm} values close to 4.5 AU for *E. coli* cellular growths carried out at 28°C and 37°C, with agitation at 200 rpm. The authors have noticed that the cellular growth rate decreased at 28°C, with no increase in lag and log phases; although the bacterial growth rates were diminished, the maximum OD_{600nm} values were almost the same that the ones for the cultivation at 37°C.

As expected, the maximum bacterial growth values of the present study are relatively close to previously published data in the subject, and particularly for the cultivation at 250 rpm. In batch and aerobic bacterial cultivations, as happens for *E. coli*, the bacterial growth rate is directly dependent on the amount of dissolved oxygen available in the culture [32]. Thus, as the solubility of oxygen in aqueous medium is low (7 $mg.L^{-1}$ at 30°C in distilled water) [33], when using an orbital shaker for the cell incubation, the agitation speed is a key parameter to provide and control the amount of dissolved oxygen available for the cellular respiratory activity. Limitations in oxygen can trigger more than 200 genes in a metabolic response to adapt to the unfavorable conditions, justifying the significant impact of this parameter in the cellular growth [17]. Therefore, by proving more oxygen with the higher agitation rates, this metabolic change is prevented and the most adequate conditions for bacterial aerobic growth are guaranteed. Meier *et al.* 2016 [34] studied the impact of agitation rate (100-450 rpm) on the maximum oxygen transfer capacity of different media compositions for the *E. coli* cellular growth. The authors found that the maximum oxygen transfer capacity rose almost linearly with an increasing shaking frequency for all media (inclusive LB medium), where high agitation rates elevated the amount of oxygen available to the microorganism, allowing high cell division.

3.2. Influence of agitation rate on the production of EGFP

From the *E. coli* growth curves, the middle of the exponential (log) phase was determined for each agitation rate, and the following studies to produce EGFP with induction with IPTG (0.500 mM) were performed. The middle of the log phase was chosen for the induction of EGFP production because it corresponds to the minimal cell doubling period, where most bacteria are alive and healthy, resulting in the ideal condition to induce the production of the target-protein. Hence, the microbial cultures incubated at 100 and 150 rpm were induced 6 h after inoculation, while for the 200 and 250 rpm agitation rates, the IPTG was added after 5 h and 30 min of cultivation. The results of bacterial growth curves (with or without induction) and EGFP production are presented in **Figs. 3A** and **3B**, respectively.

From **Figure 3A**, independently of the agitation rate, it is observed that the induction of EGFP production by IPTG (0.500 mM) did not affect negatively the bacterial growth since the *E. coli* cells kept growing and exhibited an, even more, accentuated growth than the cultivation controls without IPTG. This effect was even clearer in the experiments presented in **Figure 5**, which will be discussed

in **section 3.4**. Again, the lowest bacterial growth was obtained at 100 rpm (OD_{600nm} values of 3.8 AU with induction and 2.7 AU without induction), confirming the results obtained in the previous section. The increase of the shaking frequency led to the increase in *E. coli* growth rate for both sets of cultivations (with or without induction), but again, OD_{600nm} values were superior when IPTG was added as an inducer. The presence of IPTG allowed the expression of EGFP and the growth of the microorganism probably because the amount of IPTG employed did not completely modify the metabolite route for the protein expression.

Analyzing **Figure 3B,** it is possible to see that EGFP production is also dependent on the agitation rate, but with a different tendency than that one observed for cellular growth. For bacterial growth, the increase of the shaking frequency led to a proportional increase in OD_{600nm}, but for EGFP concentration, it increased in the following order: 150 > 200 ~ 250 > 100 rpm. Higher agitation rates in flask cultures promoted higher oxygenation of the medium, which in turn supports higher growth rates and higher cell density. With higher living cell counts in the culture, there is a higher production of total protein if no other limiting factor exists. But EGFP fluorescence is not only limited by protein synthesis. Once synthesized, EGFP must also be correctly folded in the *E. coli* cytoplasm. However, the high growth rates promoted by higher agitation increases the gene expression rate and can possibly lead to misfolding of some EGPF proteins. This can explain why the higher cell densities in cultures kept at 200 and 250 rpm did not result in a proportional increase in EGFP fluorescent compared to the ones kept at 150 rpm. But folding is not the final step for the EGFP be able to present fluorescence. Once folded, EGFP undergoes a fourth maturation process for the chromophore formation, which is a rate-limiting oxidation. Thereby, the low availability of oxygen to cells (caused by the low agitation rate at 100 rpm) probably delayed or even partially prevented the last step of EGFP maturation [35, 36].

After 12 h of induction with IPTG, the production of EGFP ranged from 127 to 193 $mg.L^{-1}$, from the lowest (100 rpm) to the highest (150 rpm) agitation rate. This is an interesting result since it demonstrates that a slight and simple modification in the shaking frequency (only 50 rpm) is enough to enhance the target-protein production. Comparing the data from **Figs. 3A** and **3B**, it is possible to see that the highest cellular growths do not always result in high production yields of the target-protein, since, herein, the best condition to produce EGFP (150 rpm) had only the third-best bacterial growth. In general, the production of EGFP is associated with the amount of viable bacterial cells in culture [25, 37], but, similarly to the results observed in our study, some previous reports highlighted that higher cellular density not always result in an increase in the production of some biomolecules with bacterial fermentation [38, 39]. Previously, Chew *et al.* 2012 [12], optimized the EGFP production by adjusting the agitation rate for the cultivation of *E. coli* BL21 (DE3) cells, demonstrating that the transfer of mass, heat, and oxygen distribution in the culture can be improved, which led to high cellular growth rates and boosted EGFP production. However, the authors noted that under excessive shaking frequencies, the bacterial cells growth can be negatively affected because of the stress caused by the high shear rates. Additionally, it is also important to note that low agitation rates can reduce the cellular metabolism, favoring the correct envelopment of recombinant proteins by the heterologous expression system [40], which can explain why cultivation at intermediate shaking frequency (150 rpm) had the highest EGFP productivity.

The gathered results elucidated the relevance of controlling the agitation rates in the production of EGFP, *i.e.*, by adjusting the aeration of the fermentative process, However, in the literature, several other variables such as temperature, pH, concentration of inducer, time of induction and inoculum cell density were already described as relevant in the production of recombinant proteins using *E. coli* heterologous expression systems [18, 27]. Therefore, considering the high cost of IPTG, the most

expensive raw material of the fermentative process, its influence in EGFP production was further studied, particularly, evaluating the effect of IPTG concentration and time of induction.

3.3. Influence of IPTG concentration and induction time on the production of EGFP

After defining the best agitation rate (150 rpm) to produce the recombinant EGFP, the influence of three IPTG concentrations (0.250, 0.500 and 0.750 mM of IPTG) and the induction time were evaluated, assessing the effect of the addition of IPTG in the beginning (2 h), middle (6 h) and end (10 h) of the exponential (log) phase. The results of both the cellular growth and EGFP production are depicted in **Figure 4**.

Figs. 4A, 4B and **4C** showed that, independent of the time of the induction, the different IPTG concentrations did not alter the kinetics of bacterial growth. However, the time of induction had a severe impact in the OD_{600nm} values, particularly when the inducer was added at the beginning of the log phase (low cellular density). Previously, Omoya *et al.* 2004 [41] highlighted that the addition of IPTG when the cellular density is low inhibited the production of the target protein. Similarly, herein, the metabolic stress imposed to the *E. coli* cells to produce the recombinant proteins impaired the kinetics of bacterial growth, leading to reduced OD_{600nm} values (approximately, 1.5 AU) after 12 h of induction. On the opposite, the induction in the middle or end of the exponential phase led to similar or even higher cellular growth rates than the cultivation without induction. These results are in close agreement with those from the first set of experiments (**Figure 3A**), suggesting a positive synergy among *E. coli* strain BL21 (DE3) and the two plasmids pLysS and pET28.

The effect of the activation of plasmids on bacterial growth and protein expression is dependent on many variables, like host cell, plasmids characteristics and the target recombinant protein, and different combinations can lead to various outcomes regarding the metabolism and cellular growth of the bacteria [17]. The association of *E. coli* BL21 (DE3) and pLysS is already extensively reported in literature [17, 42, 43], usually providing stringency and consistent expression [44]. Successful integrations of pET28 plasmid and *E. coli* BL21 (DE3) were also previously reported in the literature [45, 46]. Therefore, both plasmids in this study, at least individually, have a good compatibility with the *E. coli* as a host. Nevertheless, the positive synergy between the host and plasmids can be affected, as shown in **Figure 4A,** if an earlier induction is carried out, causing deleterious impacts in the cellular growth. Additionally, EGFP is extensively used as a biomarker in cells and is generally regarded as innocuous [47], which can partially explain why the high protein concentrations did not negatively impacted the bacterial growth. Hence, it seems that the effect of induction on cellular growth is not only dependent of the intrinsic characteristics of the host cells, plasmid and target protein, but also from the cultivation conditions, such as time of induction and agitation rate, which can lead to positive or negative outcomes.

Regarding the influence of both IPTG concentration and time of induction on EGFP production, similarly to the bacterial growth, only the second parameter had a strong impact in expression (**Figs. 4D, 4E** and **4F**). Thus, adding the inducer at the beginning of the exponential growth is unfavorable for EGFP productivity, allowing a maximum concentration of EGFP of only 65 $mg.L^{-1}$. On the other hand, the induction in the middle or at the end of the log phase enhanced EGFP production, achieving EGFP concentration values up to 210 $mg.L^{-1}$ and 314 $mg.L^{-1}$, respectively. Regarding the induction time, the results are fairly in accordance with previous studies using *E. coli* BL21 (DE3), pET plasmid and IPTG induction for the expression of their proteins. For example, Su *et al.* 2015 identified an increase in lipase production with induction in the middle or end of the exponential phase [48]. Ko *et al.* 2009 observed a considerable increase in xylanase production by delaying the induction time [49].

Peng *et al.* 2004 reported that the best production of Human β-defensin-2 occurred with induction at middle stage of exponential growth, followed by intermediate results at the end of exponential phase and worst results from inducing in the beginning of the exponential phase.[50] Additionally, in a study using a different *E. coli* variant (*E. coli* BLR (DE3)), Khushoo *et al.* 2005 found best expression of asparaginase when induction occurred at an intermediate $OD_{600\ nm}$ value [51]. The present study reinforces that defining an appropriate induction time can affect not only bacterial growth, but the target-protein production. This is a very simple procedure that does not involve any extra costs and can lead to significant improvements in the production of recombinant proteins.

Despite attaining the highest EGFP production yields with the induction at the end of the exponential phase, in general, late induction for the expression of recombinant proteins can entail some drawbacks in an industrial process. It can stimulate the synthesis and activity of proteases, impacting the productivity and increasing the contaminants present in the sample [37]. This is particularly relevant for the manufacturing of commercial proteins, because it can impairs and increase the costs of the following downstream purification stages. Previously, Carvalho [52] highlighted that a more selective synthesis of Enhanced Yellow Fluorescent Protein using *E. coli* M15 cells (at 27.5ºC with 40% of concentration of dissolved oxygen) is favored with the induction in the middle of the exponential phase, producing 0.410 g of the protein *per* g of biomass. Additionally, it is important to note that the concentration of EGFP in the present set of experiments was only monitored until 12 h after induction, and, as shown in **Figure 4E**, the stabilization of EGFP concentration was not attained in the culture induced in the middle of the log phase (6 h after start of culture). This suggests that with longer cultivation periods, the EGFP productivity for the culture induced at 6 h could be similar or even superior as the one induced at 10 h.

The influence of three IPTG concentrations (0.250, 0500 and 0.750 mM), depicted in **Figs. 4D, 4E** and **4F**, clearly indicates that this parameter is insignificant to produce EGFP, since the productivity yields were almost the same for the three induction times. Collins *et al.* 2013 also showed a negligible impact of IPTG concentration in the expression of silk-elastin-like protein in *E. coli* BL21(DE3) with pET plasmid using shake-flasks [53]. Interestingly, the authors have even observed an increase of silk-elastin-like protein with the increase of agitation rate, *i.e.* from 100 to 250 rpm. Considering the high-cost of IPTG, this outcome is quite relevant, since by decreasing IPTG while maintaining EGFP yields, EGFP production costs can be reduced. Thus, this can help overcome one of the most limiting factors of the IPTG-producing systems for the large scale production of recombinant proteins. Previously, Chew *et al.* 2012 [12], also reported that the IPTG concentration had a low impact in the production of the EGFP, while temperature, agitation rate and time of induction had relevant influence in EGFP yields; however, no further studies to determine the minimum amount of IPTG were yet performed.

3.4. Determination of the minimal concentration of IPTG required for induction of EGFP production

Considering that even the lowest concentration of IPTG (0.250 mM) had no relevant impact in neither bacterial growth nor EGFP production, as the main goal of this work, the last assays focused in the determination of the minimal concentration of the IPTG required for the induction of EGFP while maintaining high production yields. Thus, to determine the lowest IPTG concentration that the *E. coli* BL21 (DE3) [pLysS; pET28(a)] cells needed to produce EGFP, new bacterial cultures were induced with the lowest IPTG concentration of the previous set of experiments (0.250 mM) and three other lower concentrations of IPTG, namely, 0.005, 0.010 and 0.025 mM, which correspond to a 50-, 25- and 10-fold decrease. All the cultures were induced after 6 h of cultivation (middle of log phase) and

the bacterial growth and EGFP production were compared with a control culture without induction, as shown in **Figure 5.**

The results in **Figure 5A** show that, as before, different IPTG concentrations had no effect on the cellular growth of *E. coli* BL21 (DE3) [pLysS; pET28(a)], with all four concentrations leading a similar growth pattern. However, it should be pointed that after the addition of IPTG (> 6 h) an increase in the OD_{600nm} values was observed, in comparison with the culture without inducer. The positive effect of GPF production on culture density has been also shown in **Figure 2** in **section 3.1**.

Interestingly, as shown in **Figure 5B**, there was no expressive variation in EGFP production for the different IPTG concentrations. The concentration of EGFP 5 h after induction ranged from 148 ± 8.58 to 192.43 ± 1.55 $mg.L^{-1}$, for the inductions with 0.250 and 0.005 mM of IPTG, respectively. Surprisingly, the lowest concentration of IPTG (0.005 mM, the lowest we could assess with accuracy in our laboratory) not only did not reduced EGFP production but increased it. However, in the absence of inducer, there was only a residual production of EGFP (32.93 ± 5.45 $mg.L^{-1}$). This seems to indicate that although IPGT is required for EGFP production, the concentrations necessary are extremely low, with higher concentrations even impairing GFP expression.

The plasmid pET28(a) codes for EGFP (under control of the T7 promoter) and the regulator *Lac*I, while pLysS codes for the T7 lysozyme that suppresses basal expression from the T7 promoter [18, 54, 55]. Our sequencing results confirmed that both plasmids are correctly present in our engineered *E. coli* strain. Indeed, results presented in **Figure 5B** show that IPTG is strictly required for induction of EGFP production and that the control exerted by the *Lac*I combined with the T7 lysozyme is very tight. More importantly, it is shown that the system is very sensitive to IPTG, achieving full induction at a concentration as low as 5 µM.

Due to the excellent EGFP productivity levels obtained with very low IPTG concentration, we believe that the use of *E. coli* IPTG-inductive-based heterologous systems is the most feasible for the GFP production at industrial scale. In fact, it was even possible to achieve high production of GFP yields than by using the most common IPTG concentrations (usually suggested to be between 0.4 and 1.0 mM) for induction of pET plasmids [56].

Comparing these results with established literature regarding GFP production, depending on the conditions, we achieved a considerable increase in GFP expression. For example, Penna *et al*. used *E. coli* DH5-α cells and were able to produced only up to33.68 $mg.L^{-1}$ of recombinant GFP (GFPuv) [27], at least one order of magnitude lower than the gathered results in this study. However, comparing with previous works that used the same strain to produced Enhanced Green Fluorescent Protein (EGFP), the results are considerably similar. Chew *et al.* 2012 was able to produce up to 241 $mg.L^{-1}$ of EGFP using *E. coli* BL21 (DE3) with plasmid RSETEGFP [12], confirming that the *E. coli* BL21 (DE3) is the most adequate heterologous expression host for GFP production.

This study confirms it is possible to maintain high EGFP production yields using very low quantities of IPTG as an inducer. Thus, considering the high cost of this inducer (for example, in the USA, Sigma Aldrich® sells IPTG for U$ 68.00 per g), the results of this work support effectively the industrial use of IPTG-induction systems. For example, considering only the cost of IPTG, if the most reported concentration of 0.500 mM of inducer was used to produce a recombinant protein, U$ 810 would be required to produce 100 L of broth. Therefore, considering that the production levels are maintained at quite low IPTG concentration, by reducing the concentration from 0.500 to 0.005 mM (as achieved in this study), the minimal budget necessary to produce the same 100 L would fall to U$

8. This is only an example to elucidate how massive is the relevance of the inducer concentration in the final cost of recombinant proteins production and to demonstrate that sometimes, instead of changing the inductive systems, a simple screening of inducer concentration is sufficient to allow a feasible and economic industrial production of a recombinant protein.

A summary of the gathered results is presented in **Table 1**, listing the main effects of IPTG concentration, agitation and induction time over the bacterial growth (represented by OD_{600nm}) and EGFP production (indicated by EGFP concentration) by *E. coli* BL21 (DE3) [pLysS; pET28(a)] at 30°C. **Table 1** reports that changes in agitation rate and induction time significantly affected the production of EGFP by recombinant *E. coli* BL21 (DE3) [pLysS; pET28(a)], but IPTG concentration had no effect in the productivity. Although the best results for bacterial growth were obtained at an agitation rate of 250 rpm, the highest production of the protein was achieved with 150 rpm. Additionally, changing the time of induction also affected EGFP concentration, with best results obtained with induction in the middle or at the end of the log phase. Finally, it was also demonstrated that it is possible to maintain productivity levels of EGFP by *E. coli* BL21 (DE3) while using very low concentrations of inducer (0.005 mM).

This study allows to conclude that the decrease of IPTG concentration can be an effective alternative to reduce recombinant protein production costs of the upstream processing stages when IPTG-induction systems are used as heterologous systems, favoring the use of *E. coli* BL21 to commercially produce this important biomarker and biosensor protein.

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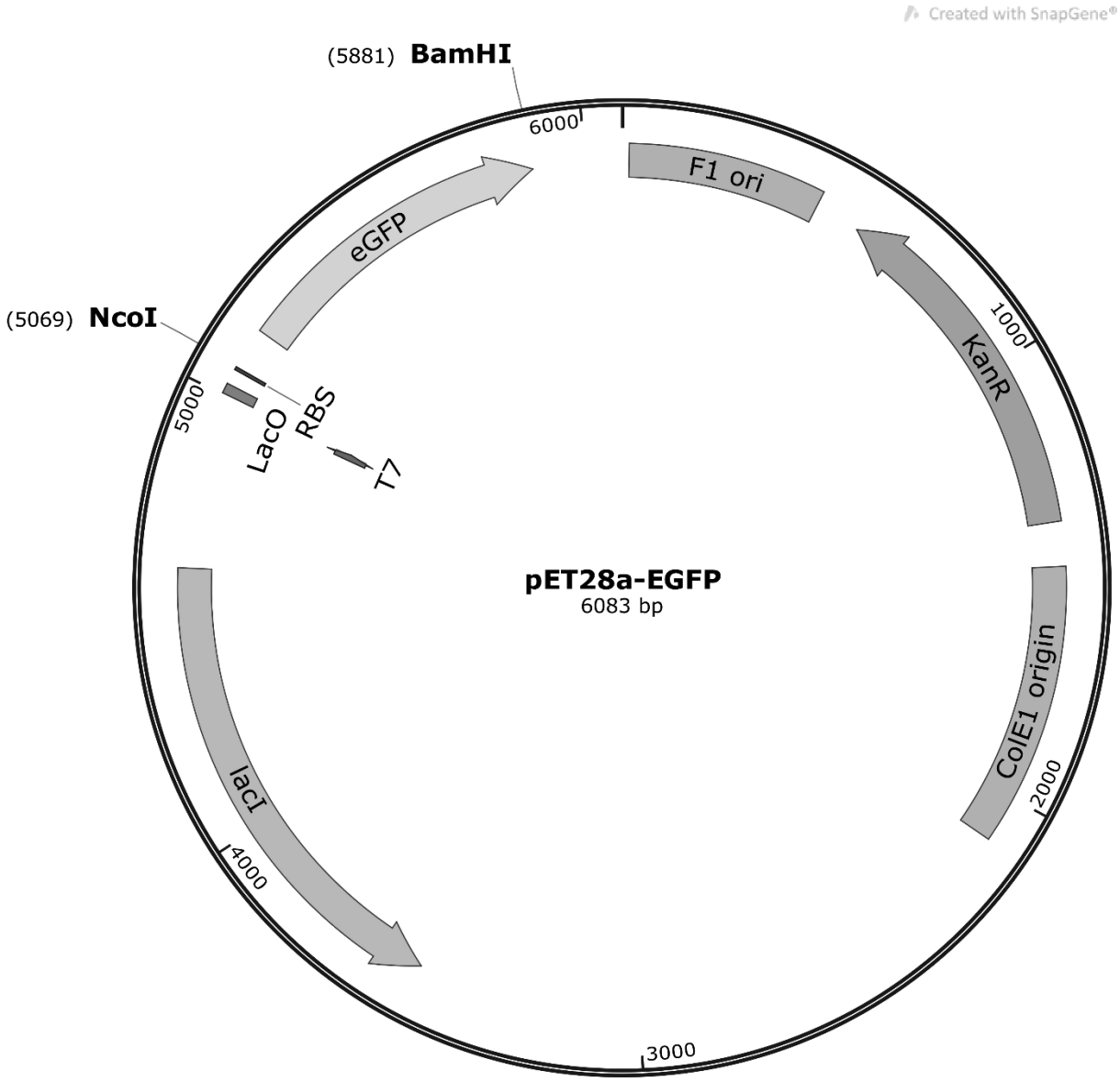
Tables

Table 1. Summary of the main effects of IPTG concentration ([IPTG], agitation rate and induction time over the bacterial growth (represented by optical density at 600 nm, OD_{600nm}) and EGFP production (indicated by EGFP concentration, [EGFP]) by *E. coli* BL21 (DE3) [pLysS; pET28(a)] at 30°C.

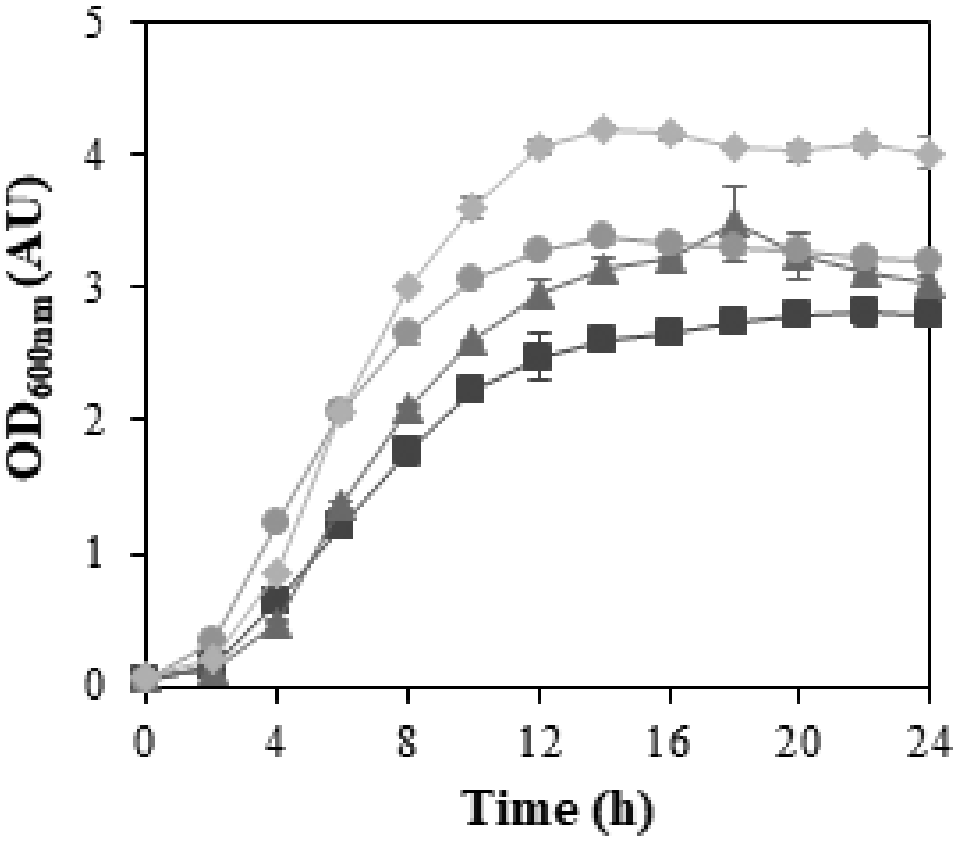
Parameter	**OD_{600nm}**	**[EGFP]**
[IPTG]	No impact or slight ↑OD_{600nm} with the addition of IPTG	No impact or slight ↑[EGFP] at lowest [IPTG]
Agitation	↑agitation = ↑OD_{600nm} ↓agitation = ↓OD_{600nm}	100 < 200 ≈ 250 < 150 rpm
Induction time		
Beginning of exponential phase	↓	↓
Middle of exponential phase	No impact or ↑OD_{600nm}	↑
End of exponential phase	No impact or ↑OD_{600nm}	↑

571 **Figures**



572

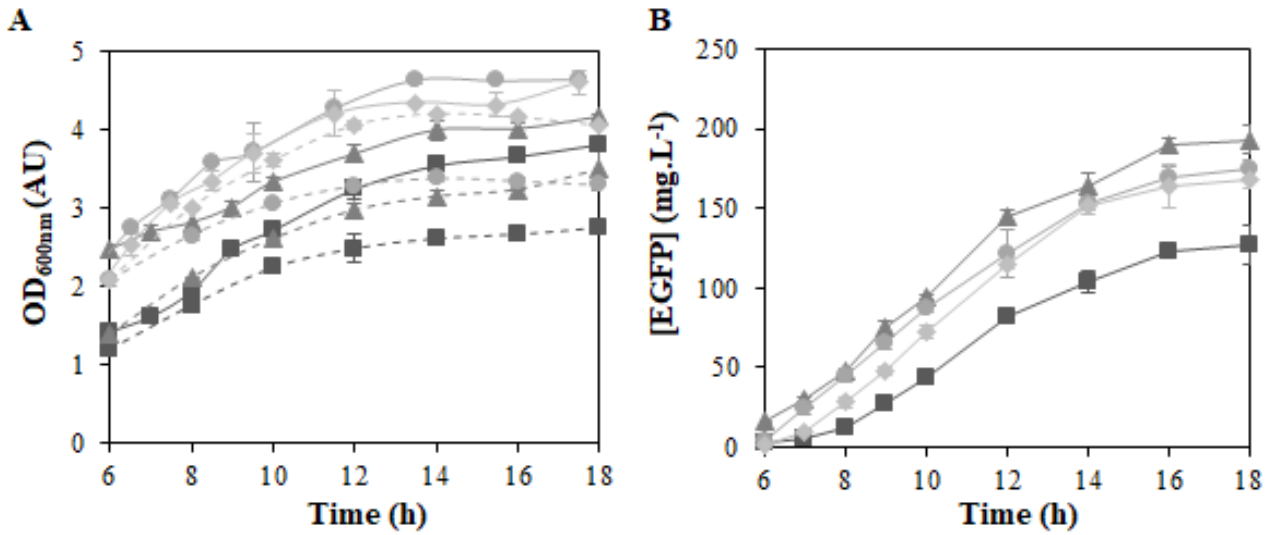
573 **Figure 1. Map of the pET28(a)-EGFP plasmid.** Gene coding for the enhanced green fluorescent
574 protein (EGFP) was inserted into the plasmid backbone through restriction sites *Nco*I and *BamH*I.
575 Therefore, EGFP was placed under control of the LacO regulated-T7 promoter and ribosome binding
576 site (RBS) originally present in the pET28(a). *KanR* codes for the kanamycin resistance gene; *lacI*
577 codes for the LacO binding repressor; F1 ori and ColE1 are origins of replication active in *E. coli*.
578 Figure created by the authors with the SnapGene program.



579

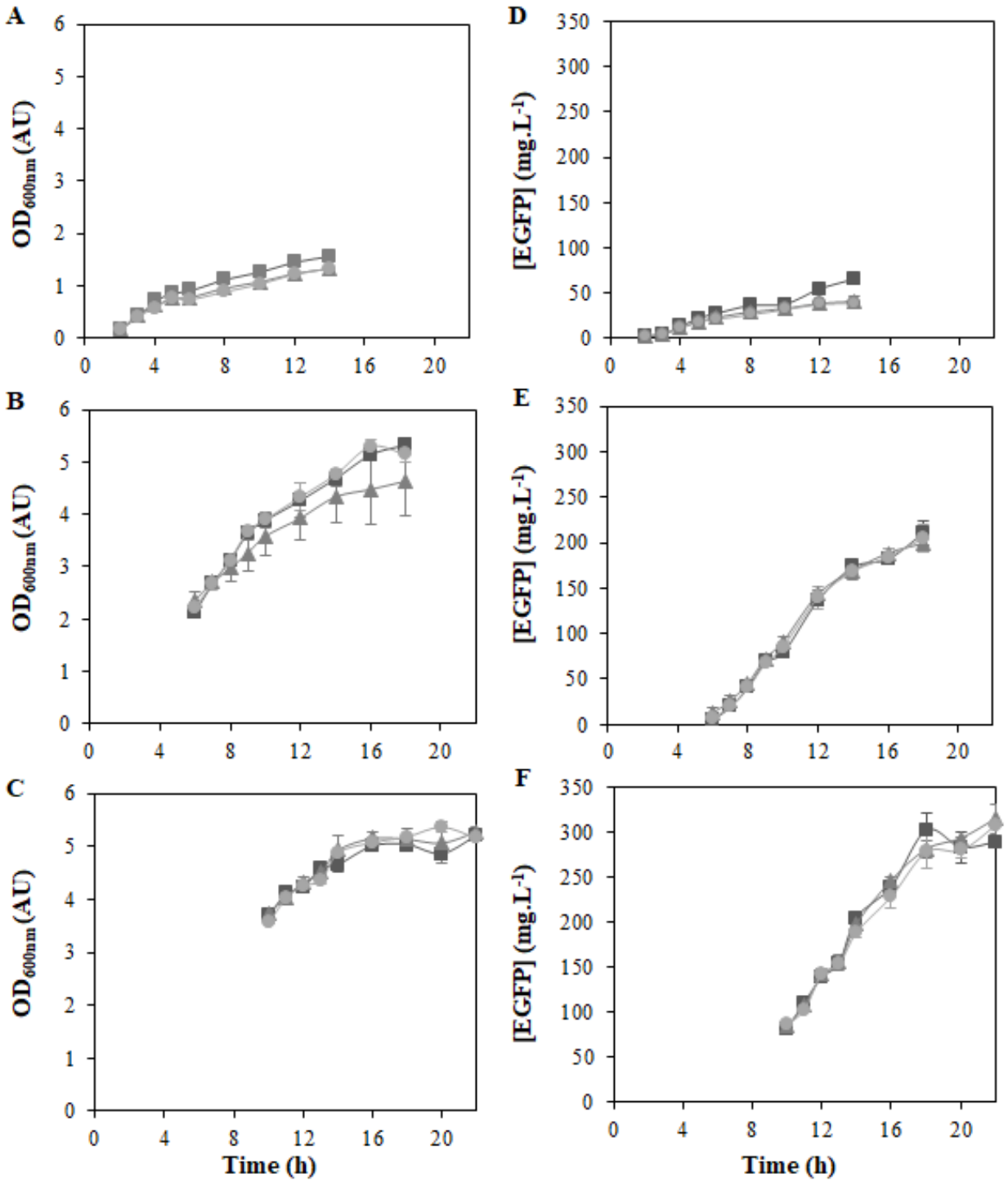
580 **Figure 2. Effect of agitation rate on bacterial growth.** Bacterial growth curves of *E. coli* BL21 (DE3)
581 [pLysS; pET28(a)] at 30°C under different agitation rates (rpm): (-■-) 100; (-▲-) 150; (-●-) 200; (-♦-)
582 250. The experiments correspond to the average of three independent assays with respective standard
583 deviations.

584



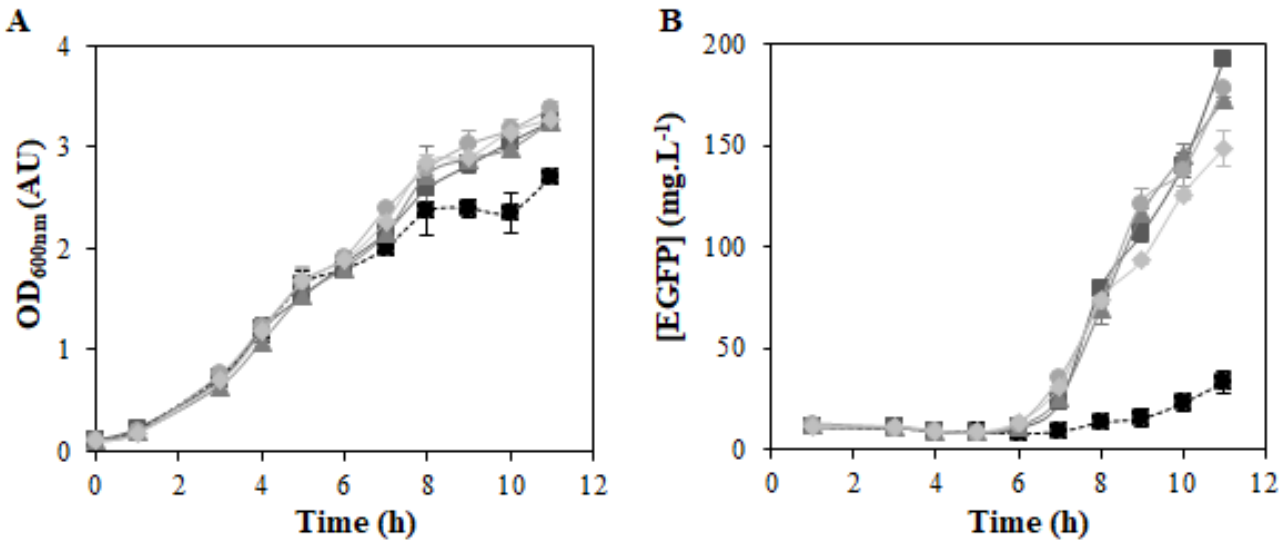
585

586 **Figure 3. Effect of agitation rate and IPTG induction in bacterial growth and EGFP production.**
587 **A)** Bacterial growth curves of *E. coli* BL21 (DE3) [pLysS; pET28(a)] at 30°C, with (continuous lines)
588 or without (broken lines) induction with 0.500 mM of IPTG and **B)** concentration of EGFP ($mg.L^{-1}$)
589 produced after induction with 0.500 mM of IPTG in the middle of the exponential phase, under
590 different agitation rates (rpm): (-■-) 100; (-▲-) 150; (-●-) 200; (-♦-) 250. The experiments correspond
591 to the average of three independent assays with respective standard deviations.



592

593 **Figure 4. Effect of IPTG concentration and time of induction on bacterial growth and EGFP**
594 **production. (A, B,** and **C)** Bacterial growth curves of *E. coli* BL21 (DE3) [pLysS; pET28] at 30°C
595 and 150 rpm and **(D, E** and **F)** concentration of EGFP ($mg.L^{-1}$) produced after induction with IPTG
596 (concentrations of (-■-) 0.250 mM, (-▲-) 0.500 mM and (-●-) 0.750 mM) and induction time of **A** and
597 **D)** 2 h, **B** and **E)** 6 h and **C** and **F)** 10 h.



598

599 **Figure 5. Study of minimal IPTG concentration required for EGFP production. A)** Bacterial
600 growth curves of *E. coli* BL21 (DE3) [pLysS; pET28] at 30°C and 150 rpm and **B)** concentration of
601 EGFP (mg.L^{-1}) produced after induction at 6 h with the following concentrations of IPTG (mM): (-■-)
602 0.005; (-▲-) 0.010; (-●-) 0.025; (-♦-) 0.250. For comparison, both parameters were also determined for
603 the cultivation without inducer, depicted by the broken line (-■-). The experiments correspond to the
604 average of three independent assays with respective standard deviations.