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Heterologous protein production in *Escherichia coli* biofilms: A non-conventional form of high cell density cultivation

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ABSTRACT

Escherichia coli is one of the favourite hosts for recombinant protein production and is recognized as an excellent model for biofilm studies. High cell density cultures (HCDC) of this bacterium enable attractive volumetric production yields and cells growing in biofilms share some of the challenges of conventional high cell density planktonic cultures.

This work assesses the production potential of *E. coli* JM109(DE3) biofilm cells expressing a model protein, the enhanced green fluorescent protein (eGFP), from a recombinant plasmid. A control strain harbouring the same plasmid backbone but lacking the *eGFP* gene was used to assess the impact of heterologous protein production on biofilm formation. Results show that specific eGFP production from biofilm cells was about 30 fold higher than in planktonic state. Moreover, eGFP-expressing cells had enhanced biofilm formation compared to control cells. Volumetric production values were 2 fold higher than those previously reported with the same protein and are within the range of what can be obtained by conventional HCDC in the production of soluble proteins. Although the cellular density that was obtained was lower than in conventional HCDC (0.5 fold), this novel system reached good production values which are likely to be improved after optimization of culture conditions.

1. Introduction

The Gram-negative bacterium *Escherichia coli* is a preferred host for the production of recombinant proteins [1,2] due to its fast growth at high cell densities, simple nutrient requirements, well-known genetics and the availability of a large number of cloning vectors and mutant host strains [3]. This bacterium has the ability to accumulate many recombinant proteins to at least 20% of the total cell protein [4] and, in some cases, to translocate them from the cytoplasm to the periplasm [5]. *E. coli* cultivation in high cell density cultures (HCDC) presents many advantages such as reduced culture volume, enhanced downstream processing and lower production costs [6]. Despite these advantages, there are still many challenges that have to be addressed in HCDC and these include insufficient oxygen transfer, specific culture medium requirements, reduced mixing efficiency in the reactor, accumulation of carbon dioxide which decreases growth rates and increased acetate production [5,6]. Recombinant protein production by biofilm cells shares some of the challenges of conventional HCDC, namely in diffusion of nutrients and oxygen through the biofilm and also in the accumulation of toxic waste products [7,8].

Recombinant protein expression in *E. coli* biofilms was pioneered by Huang et al. [9–11] who have studied the production of β-galactosidase in *E. coli* DH5α carrying a plasmid containing the *tac* promoter. Later, O'Connell et al. [12] have described the first system for high level heterologous protein production in *E. coli* biofilm cells using a pUC-based vector for the expression of enhanced green fluorescent protein (eGFP). Despite the enormous potential of this expressing system, heterologous protein production from *E. coli* biofilm cells remains largely unexplored.

The expression of heterologous proteins in *E. coli* is commonly accomplished by inserting the gene of interest into a multicopy plasmid under the transcriptional control of either a constitutive or a regulatable promoter [1]. It is well documented that plasmids impose a metabolic burden on the host cell, as cellular resources must be used for their replication as well for the expression of plasmid-encoded genes [13,14]. In planktonic cells, this added metabolic burden decreases cellular growth rates [15,16] and biomass yields [17], particularly when the plasmid vector is used to direct production of a recombinant protein [14,18–20]. This metabolic burden also promotes segregational and structural plasmid instability [21] and several metabolic changes in the host cell [22,23] which may, in turn, affect the yield and activity of the product protein. In contrast to planktonic cells, the presence of

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plasmids was shown to increase biofilm formation and plasmid stability in many studies. However, most of these studies were performed with conjugative plasmids [24–30]. Although non-conjugative plasmids are commonly used for heterologous protein production in *E. coli*, information regarding their impact on biofilm formation remains scarce. It has been shown in flow conditions that when a plasmid (pTKW106 or pMJR1750) containing a mutated pMB1 origin was transformed into *E. coli* DH5α, the plasmid-bearing cells formed biofilms with a higher cell density than non-transformed cells [9,31]. Lim et al. [32] also revealed that upon transformation of *E. coli* O157:H7 with a 92 kb virulent and non-conjugative plasmid (pO157), biofilm formation and architecture were affected. Under smooth flow conditions, pO157 enabled biofilm development through increased production of extracellular polymeric substances (EPS) and generation of hyperadherent variants [32]. In a previous work, the effect of *E. coli* JM109(DE3) transformation with non-conjugative plasmids (pET28A and pUC8) on biofilm formation was assessed under turbulent flow conditions [33]. Plasmid-bearing cells formed biofilms with higher cell densities than non-transformed cells, which is an indication that biofilm cells may be a good platform for heterologous protein production, unless the high-level expression of the foreign gene is detrimental for biofilm formation.

One of the major concerns about HCDC is that the specific productivity of recombinant protein is often much lower than in flask culture [6,34]. The aim of this work was to evaluate the specific production level of biofilm cells when compared to planktonic cells and to assess if heterologous protein expression is detrimental for biofilm development.

2. Materials and methods

2.1. Bacterial strain, plasmids and culture conditions

E. coli JM109(DE3) from Promega (USA) was used because it is a well-characterized microorganism and recommended for protein expression with the pET system [35]. Moreover, this strain has shown good biofilm forming ability in turbulent flow conditions [33]. Its genotype is *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ$^-$, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^{q}ZΔM15], λ(DE3).

Competent *E. coli* cells were transformed by heat shock [36] with the control plasmid pET28A (Novagen, USA) or with the plasmid pFM23 for the intracellular production of eGFP, which was obtained by cloning the *eGFP* gene into the pET28A vector as previously described [37]. Transformants were selected on Lysogeny Broth (LB-Miller, Sigma, USA) agar supplemented with kanamycin (Eurobio, France).

Heterologous protein expression is obtained through the transcription of the *eGFP* gene, which is under the control of the T7 promoter. Transcription is made by the chromosomally encoded T7 RNA polymerase, which in turn is controlled by the *lac*UV5 inducible promoter [38]. Induction can be achieved by the addition of IPTG to the culture medium; however, as in the work performed by O'Connell et al. [12], no IPTG was added to avoid the possible decrease in protein production observed in cells containing multicopy plasmids [39] or cultivated in complex media [40].

Bacterial growth and reactor feeding was performed as described by Teodósio et al. [33]. The recirculating tank of 1 L (without mechanical agitation) was aerated using an air pump (flow rate 108 L h^{-1}) and continuously fed with 0.025 L h^{-1} of nutrient medium containing 0.55 g L^{-1} glucose, 0.25 g L^{-1} peptone, 0.125 g L^{-1} yeast extract and phosphate buffer (0.188 g L^{-1} KH_2PO_4 and 0.26 g L^{-1} Na_2HPO_4), pH 7.0. For maintenance of selective pressure, the antibiotic kanamycin was added to the growth and feeding media at a final concentration of 20 μg mL^{-1}.

2.2. Biofilm formation system and sampling

To assess the eGFP expression in both biofilm and planktonic cells, a biofilm flow cell reactor connected to a recirculating tank was used (see Supplementary materials) [33,41]. The flow cell consists of a semicircular Perspex duct (3.0 cm diameter and 1.2 m length) with apertures on its flat wall to fit removable coupons. Polyvinyl chloride (PVC) slides were glued onto coupons and the biofilms were formed on the upper faces that were in contact with the bacterial suspension circulating through the system [41]. *E. coli* cells containing the pET28A or pFM23 plasmid were grown by recirculating the bacterial suspension at 30 °C during 12 days under a turbulent flow with a Reynolds number (*Re*) of 4600 (average wall shear stress of approximately 0.3 Pa) [33,42]. The *Re* is here defined as

$$Re = \frac{\rho U D_h}{\mu} \quad (1)$$

where ρ and μ are the density and dynamic viscosity of the fluid, respectively, U is the average velocity in the flow cell, and D_h is the hydraulic diameter of the semicircular flow cell ($D_h = \pi D/(2 + \pi) = 1.8$ cm) of diameter D.

For biofilm sampling, the system was stopped to allow coupon removal and carefully started again maintaining the same flow conditions. Day 1 corresponds to the start of the reactor system and the sampling was initiated on day 3 of the experiment.

2.3. Analytical methods

Photographs of coupons with wet biofilms formed after 3 and 7 days of growth were taken with a FinePix S1500 digital camera (Fujifilm Co. Ltd., Japan).

Biofilm wet weight was determined by weighing each coupon before the experiment and subtracting this value from the weight of the same coupon at each sampling time. Biofilm thickness was also determined using a digital micrometer [43], and afterwards the biofilm was resuspended and homogenized in 25 mL of 8.5 g L^{-1} NaCl solution to assess total cell number and culturability. Biofilm total cell counts were assessed by staining with 4′-6-diamidino-2-phenylindole (DAPI) as described by Gomes et al. [44]. Briefly, the biofilm suspension was filtered through a Nucleopore, Track-Etch Membrane (Whatman Int. Ltd., USA) black polycarbonate membrane (pore size 0.2 μm) and the cells were stained with 1 mL of DAPI reagent (0.5 mg L^{-1}) for 10 min in the dark. Bacterial observation and counting was performed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd., Switzerland). Cell numbers on each membrane were estimated from counts of a minimum of 20 fields of view and the final values were presented as log cell cm^{-2}.

For biofilm culturability, samples were diluted to an appropriate cell density to yield > 10 and < 300 colony forming units (CFU) per plate of solid growth medium (PCA; Merck, Portugal) supplemented with kanamycin (20 μg mL^{-1}). Colony enumeration was carried out after 24 h incubation at 30 °C and the final values were expressed as log CFU cm^{-2} of coupon area.

For planktonic cells, total cell number and culturability were assessed using the same methods as for biofilms. Results were presented as log CFU mL^{-1}.

Glucose concentration and consumption in the whole system were determined as an indicator of metabolic activity [43]. Glucose consumption was obtained by multiplying the difference between the glucose concentration entering the system and the glucose concentration in the tank by the feeding flow rate (0.025 L h^{-1}). The dissolved oxygen and pH of the recirculating culture were also monitored.

2.4. Heterologous protein expression

For *E. coli* cells with the pFM23 expression vector, eGFP was

analysed for both biofilm and planktonic cells. This soluble cytoplasmic protein was quantified as indicated in Mergulhão and Monteiro [45]. A sample volume corresponding to an equivalent OD_{610nm} = 1 was used to harvest the cells by centrifugation (3202*g* for 10 min). The pellet was resuspended in 100 μL of Buffer I (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and added to a 96-well microtiter plate (Orange Scientific, USA) already containing 100 μL of Buffer I. Fluorescence was measured using a microtiter plate reader (SpectraMax M2E, Molecular Devices, Inc., UK) with the excitation filter of 488 nm and the emission filter of 507 nm. In order to verify if the loss of recombinant protein by cell leakage was interfering with the GFP fluorescence that was measured, one assay was performed where readings were made prior to and after cell washing with Buffer I. The difference between these values was only 2%, indicating that the eGFP lost into the extracellular medium was not significant on this assay.

Calibration curves were constructed with purified eGFP standards (0–3.14 μg) mixed with 100 μL of washed JM109(DE3) cells harbouring the pET28A plasmid (OD_{610nm} = 1). Buffer I was added to a final volume of 200 μL prior to measuring fluorescence and final values were expressed in specific eGFP production (fg $cell^{-1}$).

2.5. Quantification of extracellular polymeric substances (EPS)

The content of the main EPS found in biofilms (proteins and polysaccharides) was assessed for both strains after 7 days of growth. Matrix proteins and polysaccharides from biofilms were separated from cells using a Dowex resin (50 × 8, Na^+ form, 20–50 mesh; Fluka Chemika, Switzerland) as described by Gomes et al. [44]. The biofilm was resuspended in 20 mL of extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7) and 2 g of Dowex resin per g of wet weight were added to the biofilm suspension. The extraction was performed at 400 rpm for 4 h at 4 °C, and ultimately the extracellular components (matrix) were separated from the cells through centrifugation.

Protein concentrations were determined for each strain using the Bicinchoninic Acid Protein Assay Kit – BCA™ Protein Assay Kit (Thermo Fisher Scientific, USA) and the polysaccharide concentrations by the phenol-sulphuric acid method of DuBois et al. [46]. Protein and polysaccharide assays were performed using biofilm suspensions before EPS extraction (total constituents), and with cells (cellular constituents) and EPS (matrix constituents) after extraction. The final values were calculated taking into account the biofilm dry weight assessed as described by Simões et al. [47].

2.6. Statistical analysis

Results originated from averages of triplicate data sets obtained in independent experiments for each strain. Average standard deviations (SDs) on the triplicate sets were calculated for all analysed parameters. For biofilm formation (Fig. 1), the following averages were obtained: SD < 17% for glucose consumption, SD < 29% for biofilm wet weight, SD < 28% for biofilm thickness, SD < 9% for planktonic and biofilm culturability, and SD < 5% for planktonic and biofilm total cell counting. Regarding the fluorescence readings for eGFP quantitation (Fig. 3), SD < 16% and SD < 10% were obtained for planktonic and biofilm cells, respectively.

In order to ascertain the statistical significance, paired *t*-test analysis was performed based on a confidence level of 90% (differences reported as significant for P values < 0.1 and marked with *) and 95% (differences reported as significant for P values < 0.05 and marked with $^{*}_{*}$).

3. Results

In order to assess if eGFP production was affecting biofilm development, the biofilm forming capacity of *E. coli* JM109(DE3) cells containing the pFM23 plasmid was compared to a strain harbouring the same plasmid backbone (pET28A) but devoid of the *eGFP* gene (Figs. 1–3).

Fig. 1(A) shows the glucose consumption profiles in the whole flow system (sessile plus planktonic cells). Higher glucose consumption values (on average 13%) were obtained for the eGFP-expressing strain, although statistically significant differences were only obtained in 4 experimental points (P < 0.05 and P < 0.1). Additionally, glucose consumption increased over time for both strains until day 9. Thereafter, steady state was reached for the eGFP-expressing strain and a slight decrease was observed for *E. coli* cells bearing the pET28A plasmid.

Biofilm wet weight values (Fig. 1(B)) were generally higher for the eGFP-expressing strain with statistically significant differences found mostly in the steady state (at days 8, 9 and 11, P < 0.05).

Planktonic culturability (Fig. 1(C)) showed relatively little difference between the two strains. However, while the culturability of planktonic cells containing pET28A remained practically constant over time (around 7.9 log CFU mL^{-1}), culturability of the eGFP-expressing strain increased during the first half of the experiment before declining again after day 10. When comparing biofilm culturability, similar values were also obtained for both strains (Fig. 1(D)).

The number of planktonic total cells (Fig. 1(E)) was higher for the eGFP-expressing cells (on average 62%) with statistically significant differences at days 4 and 5 (P < 0.1) and 9 and 12 (P < 0.05). The total planktonic cell number seemed to stabilize from day 6 onwards. The number of biofilm total cells (Fig. 1(F)) was higher for the eGFP-expressing strain (on average 93%) for most of the experimental time (statistically significant differences were confirmed for all days, P < 0.05, with the exception of days 4 and 12), following the same trend of biofilm wet weight values (Fig. 1(B)).

Biofilm thickness (Fig. 1(G)) was higher for the eGFP-expressing strain following the tendency of the biofilm wet weight curve (Fig. 1(B)) with statistically significant differences in most data points (P < 0.05). Considering the biofilm thickness in steady state, the cellular density of the biofilms producing eGFP was estimated to be 3.8×10^{10} cells mL^{-1}. Taking into account the average dry weight of the *E. coli* cell [48], this corresponds to an approximate cellular concentration of 10.8 g L^{-1}.

Fig. 2 shows photographs of wet biofilms formed by both strains in the flow cell reactor. It is evident from the images that the eGFP-producing strain showed greater biofilm formation than the pET28A-bearing strain, confirming the wet weight and thickness results presented on Fig. 1(B) and (G), respectively. After 7 days of growth, biofilms formed by the eGFP-producing strain appeared more homogenous and slimy, while those formed by the pET28A strain were more scattered on the PVC surface.

Fig. 3 presents the eGFP expression of planktonic and biofilm cells harbouring the pFM23 plasmid. The specific production of biofilm cells was very high on the first sampling day when biofilm formation had barely started. As the biofilm started to grow, the specific production by these cells decreased, attaining a constant value around 5.8 fg $cell^{-1}$ at steady state (from day 7 onwards). Heterologous protein production by planktonic cells remained constant at about 0.18 fg $cell^{-1}$ until the end of the experiment. Thus, the biofilm environment enhanced specific heterologous protein expression about 30 fold when compared to planktonic cells in steady state (P < 0.05). The values of dissolved oxygen in the GFP-producing culture varied between 5 and 7 mg L^{-1} throughout the experiment, whereas the pH remained practically constant at 7.1 ± 0.3, suggesting that acetate accumulation was not significant.

The exopolymeric matrix of biofilms formed by both strains was extracted and quantified in terms of protein and polysaccharide content (Table 1). Although biofilms formed by the eGFP-producing strain had higher total protein content, the mass percentage of proteins localized

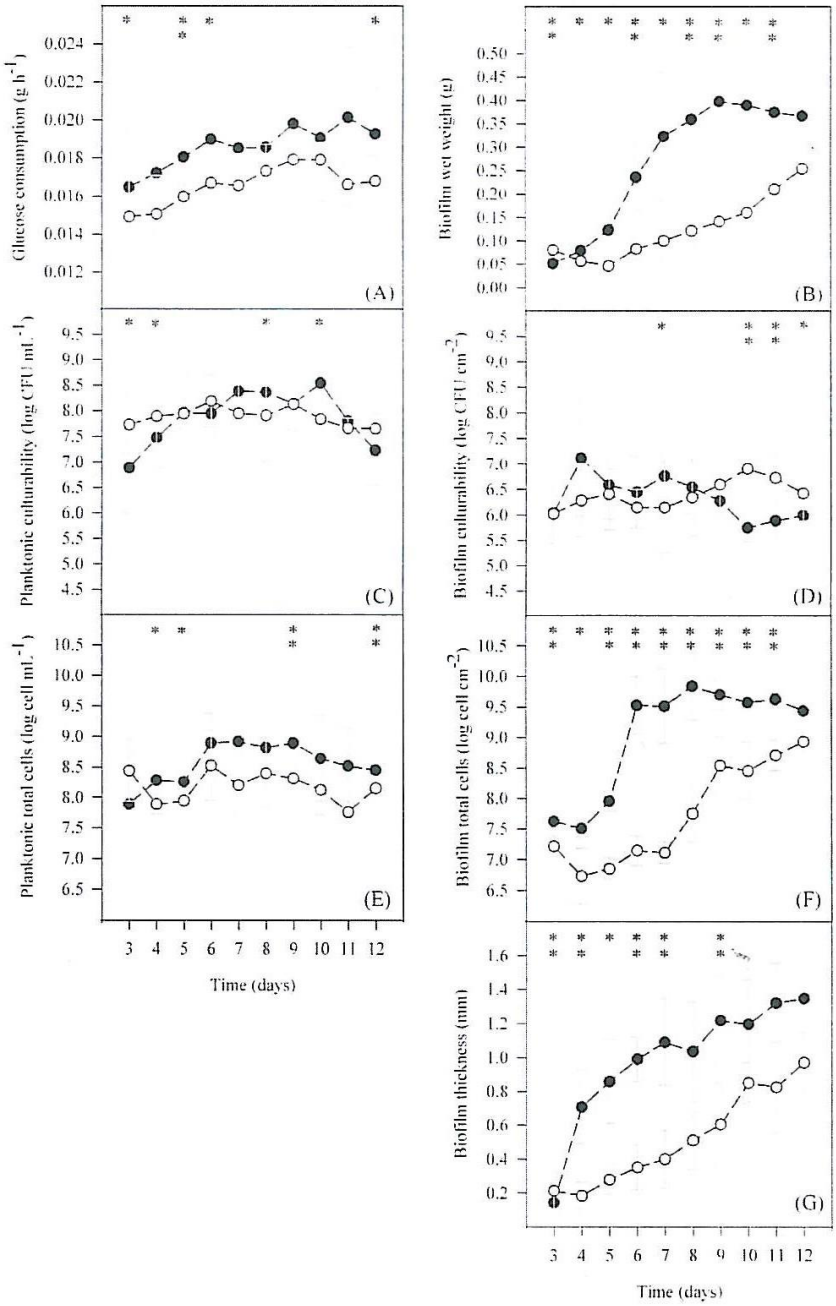

Fig. 1. Time-course evolution of planktonic and biofilm assayed parameters: (A) glucose consumption, (B) biofilm wet weight, (C) planktonic culturability, (D) biofilm culturability, (E) planktonic total cells, (F) biofilm total cells, (G) biofilm thickness. *E. coli* JM109(DE3) + pFM23 (-●-), *E. coli* JM109(DE3) + pET28A (-○-). The means ± SDs for three independent experiments are illustrated. The following average SDs were obtained: SD < 17% for glucose consumption, SD < 29% for biofilm wet weight, SD < 28% for biofilm thickness, SD < 9% for planktonic and biofilm culturability, and SD < 5% for planktonic and biofilm total cell counting. Statistical analysis corresponding to each time point is represented with * for a confidence level greater than 90% ($P < 0.1$) and with $\overset{*}{*}$ for a confidence level greater than 95% ($P < 0.05$).

at the matrix was similar (28% for the eGFP-producing strain and 25% for the pET28A-bearing strain). The eGFP-producing strain also produced biofilms with more polysaccharides, however the percentage of polysaccharides in the matrix of this biofilm was only 18% when compared to the 53% of the pET28A-bearing strain.

4. Discussion

The main goal of this work was to compare the specific production of a model heterologous protein by planktonic and biofilm cells. Additionally, it was important to verify if heterologous protein expression was not detrimental to biofilm development.

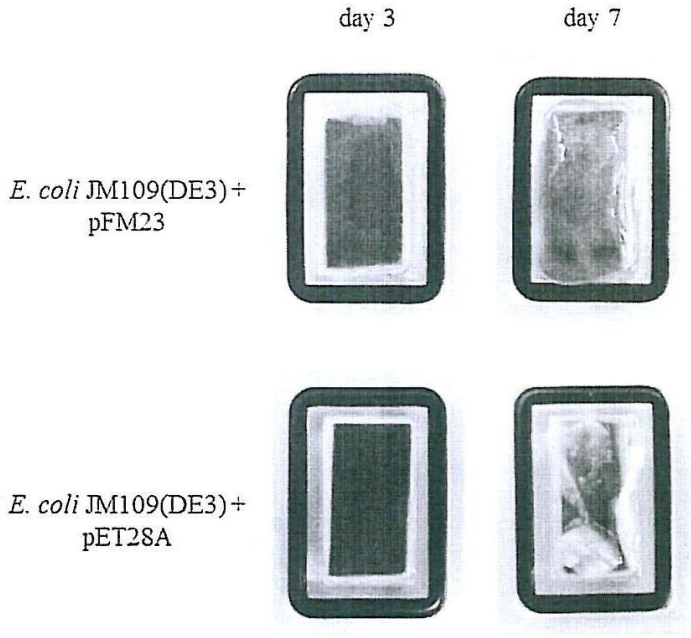

Fig. 2. Photographs of coupons with wet biofilms formed by *E. coli* JM109(DE3) + pFM23 and *E. coli* JM109(DE3) + pET28A on PVC slides after 3 and 7 days of growth. Photographs were taken with a FinePix S1500 digital camera (Fujifilm Co. Ltd., Japan).

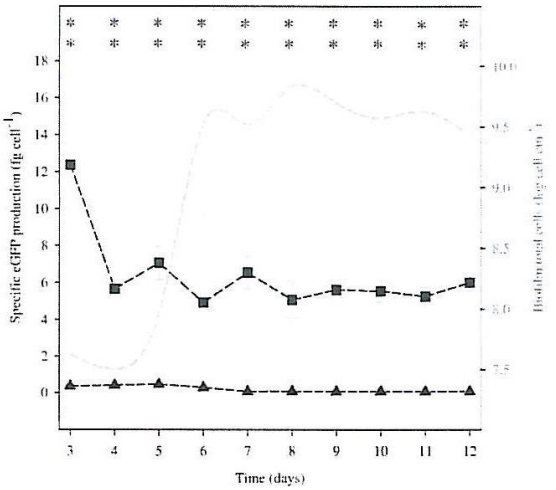

Fig. 3. Time-course evolution of specific eGFP production for *E. coli* JM109(DE3) + pFM23 in planktonic (-▲-) and biofilm cells (-■-). The dotted gray line represents the evolution of biofilm total cell number assessed by staining with DAPI. The means ± SDs for three independent experiments are illustrated. Average SD < 16% and SD < 10% were obtained for specific eGFP concentrations in planktonic and biofilm cells, respectively. Statistical analysis for the results of specific eGFP production is pointed as * * for a confidence level greater than 95% ($P < 0.05$).

Results showed that biofilm production was enhanced for the eGFP-producing strain (assayed by wet weight, thickness and total cell number) when compared to the strain harbouring the pET28A plasmid. It is commonly recognized that different types of plasmids, including conjugative and non-conjugative plasmids, can enhance biofilm production [9,24,26,32,33]. In the present study, the expression of genes for heterologous protein production amplified this effect. Higher glucose consumption values were detected on eGFP-producing cells and this observation is probably a consequence of the metabolic burden triggered by the expression of the heterologous protein. It is documented that the production of stress proteins, elevated respiration rates and high-energy requirements are induced by recombinant protein synthesis [19,49]. Since stress conditions can favour biofilm formation [50], the increased metabolic burden associated with recombinant protein expression may have stimulated biofilm formation by the eGFP-producing strain.

Table 1
Characteristics of the biofilm formed by *E. coli* JM109(DE3) + pFM23 and *E. coli* JM109(DE3) + pET28A after 7 days of growth.

Biofilm characteristics	*E. coli* JM109(DE3) + pFM23	*E. coli* JM109(DE3) + pET28A
Biofilm mass ($mg_{biofilm}$ cm^{-2})	8.00 ± 1.77	5.75 ± 1.06
Log cellular density (cells cm^{-2})	9.09 ± 0.740	7.08 ± 0.150
Total proteins (mg $g_{biofilm}^{-1}$)	97.4 ± 10.7	23.9 ± 5.52
Matrix proteins (mg $g_{biofilm}^{-1}$)	27.4 ± 2.01	6.09 ± 0.970
Total polysaccharides (mg $g_{biofilm}^{-1}$)	104 ± 19.2	24.6 ± 2.96
Matrix polysaccharides (mg $g_{biofilm}^{-1}$)	19.2 ± 1.68	13.1 ± 2.37

In a previous work [33], it has been shown that the presence of a non-conjugative plasmid in this *E. coli* strain can enhance biofilm formation. A selective pressure is often necessary to ensure plasmid stability within the cell population. This selection is often achieved by the expression of an antibiotic resistance gene [1]. In this work, both plasmids contain the aminoglycoside phosphotransferase gene which confers resistance to kanamycin [36]. It is known that the expression of these genes is a major cause for an increased metabolic burden [51] because these resistance proteins can represent up to 20% of total cellular protein [23,52], far exceeding the levels required for plasmid maintenance. The number of biofilm culturable cells was similar for