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Optimizing Recombinant Protein Production in *Escherichia coli* biofilms

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by

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"Não sou nada.

Nunca serei nada.

Não posso querer ser nada.

À parte isso, tenho em mim todos os sonhos do mundo."

Fernando Pessoa

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Abstract

In the last years, the growth of the biotechnological industry led to improvements in recombinant processes. Despite the production of a large number of proteins at a commercial scale in suspended cells, it is known that the production of recombinant proteins in planktonic cells has an associated metabolic burden on the host cell that can affect plasmid stability and protein yield. Bacterial biofilms have recently been used in the production of added-value compounds since they present many advantages over suspended cells, such as higher cell density, protection against hostile conditions, and greater plasmid stability. Since previous research of our group has demonstrated that interesting production values could be achieved for recombinant enhanced Green Fluorescent Protein (eGFP) production in *Escherichia coli* biofilms, the main goal of this thesis was to optimize this production strategy in order to determine the maximum specific and volumetric productivities that can be attained with the biofilm reactor. For that, the influence of different cultivation conditions (nutrient medium composition and hydrodynamic conditions) and the induction regime (induction time and inducer concentration) in the expression of the heterologous protein on *E. coli* biofilms were investigated.

The production of the model recombinant protein eGFP in *E. coli* planktonic and biofilm cells was evaluated by using a novel single-cell methodology that combines the common epifluorescence microscopy with an image analysis tool. The results showed that, in steady-state, the percentage of planktonic cells expressing the protein oscillated around 5%, whereas for biofilm, the eGFP-expressing cells represented on average 21% of the total cell population. Furthermore, the average specific eGFP production from biofilm cells was 10-fold higher than planktonic cells. Additionally, the plasmid maintenance was assessed for the first time and showed that biofilms presented a higher frequency of plasmid-containing bacteria than planktonic cells (0.90 *versus* 0.33).

Biofilm formation and the level of heterologous protein expression are affected by several factors, including the composition of the culture medium and hydrodynamic conditions. The impact of medium composition was evaluated by growing *E. coli* cells in

two different culture media, Lysogenic Broth (LB) and M9ZB, in a flow cell system. Although biofilm development was favoured in M9ZB medium, the number of eGFP-expressing cells was higher in LB for both planktonic and sessile states (2-fold and 7-fold, respectively). In addition, plasmid stability was evaluated by determining the plasmid copy number (PCN) in both types of cells by high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC). The results showed that PCN in biofilm cells was slightly higher when using LB medium, and higher plasmid stability was observed in biofilms compared to their planktonic counterparts. Regarding hydrodynamic conditions, planktonic and biofilm cells were grown at two different flow rates (255 and 128 L h^{-1}, originating shear stress values of 0.3 and 0.1 Pa) in a recirculating flow cell system for 7 days. It was observed that doubling the flow rate seemed to have no impact on biofilm formation. However, the percentage of eGFP-expressing cells was, on average, 52% higher at the highest shear stress.

The use of inducible expression systems is an important variable to consider when trying to increase the overall specific protein production. The effect of chemical induction by isopropyl-β-D-1-thiogalactopyranoside (IPTG) on eGFP production in biofilms was evaluated by testing different IPTG concentrations (0.1 and 0.5 mM) and induction times (days 3 and 5). Induction at day 5 using both IPTG concentrations increased the overall eGFP levels compared to non-induced biofilms and early induction on day 3. It was also suggested that eGFP production was not related to gene dosage, and that induction promoted plasmid retention. The results obtained in this work are important to define the operating conditions to be used in further studies focused on the production of other heterologous proteins in *E. coli* biofilms.

Keywords: Recombinant protein production; biofilm; *Escherichia coli*; enhanced Green Fluorescent Protein; plasmid; flow cell; nutrient medium composition; hydrodynamics; chemical induction.

Resumo

Nos últimos anos, o crescimento da indústria biotecnológica tem levado à melhoria dos processos recombinantes. Apesar da produção de um grande número de proteínas à escala comercial usando culturas em suspensão, sabe-se que a produção de proteínas recombinantes em células planctónicas está associada a um fardo metabólico no hospedeiro que pode afetar a estabilidade do plasmídeo e os níveis de expressão da proteína. Os biofilmes bacterianos têm sido usados recentemente na produção de compostos de valor acrescentado devido às vantagens que apresentam comparativamente às células em suspensão, nomeadamente elevada densidade celular, proteção em ambientes hostis, e maior estabilidade do plasmídeo. Uma vez que, anteriormente, o nosso grupo demonstrou que valores de produção interessantes podem ser obtidos em biofilmes de *Escherichia coli* para a proteína verde fluorescente melhorada (em inglês, "enhanced Green Fluorescent Protein" ou eGPF), o principal objetivo desta tese foi otimizar a estratégia de produção desta proteína com vista a atingir valores de produção específica e volumétrica máximos em reator de biofilme. Para isso, foi investigada a influência de diferentes condições de cultivo (composição do meio de cultura e condições hidrodinâmicas) e do regime de indução (tempo de indução e concentração de indutor) na expressão da proteína heteróloga em biofilmes de *E. coli*.

A produção da proteína recombinante modelo eGFP em células planctónicas e do biofilme de *E. coli* foi avaliada usando uma nova metodologia à escala celular, que combina microscopia de epifluorescência com ferramentas de análise de imagem. Os resultados mostraram que, em estado estacionário, a percentagem de células planctónicas a expressar a proteína oscilou em torno dos 5%, enquanto no biofilme a percentagem de células a expressar eGFP representou em média 21% da população total de células. Além do mais, a média da produção específica de eGFP nas células do biofilme foi 10 vezes maior do que a das células planctónicas. Adicionalmente, a capacidade de as células manterem o plasmídeo for avaliada pela primeira vez, tendo-se mostrado que as células do biofilme apresentam uma alta frequência de bactérias com plasmídeo (0.9).

Resumo

A formação de biofilme e a expressão de uma proteína heteróloga são afetadas por vários fatores, incluindo a composição do meio de cultura e as condições hidrodinâmicas. O impacto da composição do meio foi avaliado pelo crescimento de células de *E. coli* num sistema de célula de fluxo usando dois meios de cultura diferentes, o meio lisogénico (em inglês, "Lysogeny Broth" ou LB) e o meio M9ZB. Embora o desenvolvimento do biofilme tenha sido favorecido no meio M9ZB, o número de células a expressar eGFP foi maior em LB para ambos os estados, planctónico e séssil (2 e 7 vezes superior, respetivamente). Além disso, a estabilidade do plasmídeo foi avaliada pela determinação do número de cópias de plasmídeo (em inglês, "plasmid copy number" ou PCN) em ambos os tipos de células por cromatografia líquida de alta eficiência (em inglês, "high-performance liquid chromatography" ou HPLC) baseada na cromatografia de interação hidrofóbica (em inglês, "hydrophobic interaction chromatography" ou HIC). Os resultados mostraram que o PCN nas células do biofilme foi ligeiramente maior quando se usou o meio LB, e uma maior estabilidade do plasmídeo foi observada em biofilme comparativamente ao estado planctónico. Em relação às condições hidrodinâmicas, as células planctónicas e do biofilme foram crescidas na célula de fluxo durante 7 dias a dois caudais diferentes (255 e 128 L h^{-1}) correspondendo a valores de tensão de corte de 0.3 e 0.1 Pa. A duplicação de caudal pareceu não afetar a formação do biofilme. Contudo, a percentagem de células a expressar eGFP foi em média 52% maior à tensão de corte maior.

O uso de sistemas de expressão induzíveis é uma importante variável a considerar na tentativa de aumentar a produção específica da proteína. O efeito da indução química pelo isopropil-β-D-1-tiogalactopiranosido (em inglês, "isopropyl-β-D-1-thiogalactopyranoside" ou IPTG) na produção de eGFP em biofilmes foi avaliada através de diferentes concentrações de IPTG (0.1 e 0.5 mM) e diferentes tempos de indução (dias 3 e 5). A indução ao dia 5 com ambas as concentrações de IPTG aumentou os níveis globais de eGFP comparativamente aos biofilmes não induzidos e aos biofilmes induzidos ao dia 3. Observou-se também que a produção de eGFP não está relacionada com a dosagem génica, e que a indução promoveu a retenção do plasmídeo. Os resultados obtidos neste trabalho são importantes para definir as condições de operação a serem usadas em estudos futuros de produção de outras proteínas heterólogas em biofilmes de *E. coli*.

Palavras-chave: Produção de proteína recombinante; biofilme; *Escherichia coli*; proteína verde fluorescente melhorada; plasmídeo; célula de fluxo; composição nutricional do meio; hidrodinâmica; indução química.

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Nomenclature

Abbreviations

cAMP	Cyclic adenosine monophosphate
CDase	Cyclomaltodextrinase
CFD	Computational fluid dynamics
CFU	Colony forming units
CGTase	Cyclodextrin glucanotransferase
CLSM	Confocal laser scanning microscopy
DAPI	4',6-Diamidino-2-Phenylindole
DM	Diluted medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
eGFP	Enhanced green fluorescent protein
EMEA	European medicines agency
EPS	Extracellular polymeric substances
FDA	Food and drug administration
GFP	Green fluorescent protein
GOSs	Galacto-oligosaccharides
GRAS	Generally regarded as safe
HCDC	High cell density culture
hG-CSF	Human granulocyte colony-stimulating factor
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB	Lysogenic broth
LPS	Lipopolysaccharides
MRD	Modified robbins device
mRNA	Messenger ribonucleic acid
OCT	Optical coherence tomography
OD	Optical density
PCA	Plate count agar
PCN	Plasmid copy number
PPFC	Parallel-plate flow cell
PVC	Polyvinyl chloride
RFB	Rotating fibrous bed
RNAP	RNA polymerase
SB	Super broth
SD	Standard deviation

SDS	Sodium dodecyl sulfate
TB	Terrific broth
TY	Tryptone yeast
UV	Ultraviolet

Symbols

$\boldsymbol{f}$	Darcy friction factor (dimensionless)
$\boldsymbol{\rho}$	Density of water at 30 °C (kg m^{-3})
$\boldsymbol{U}$	Linear flow velocity (m s^{-1})
$\boldsymbol{D_h}$	Hydraulic equivalent diameter of the semicircular flow cell (cm)
$\boldsymbol{\mu}$	Dynamic viscosity of water at 30 °C (kg (m^{-3} s^{-1}))
$\boldsymbol{\tau_w}$	Wall shear stress (Pa)
$\boldsymbol{Re}$	Reynolds number (dimensionless)

1 General overview

1.1 Relevance and motivation

Recombinant proteins are used in different biotechnological industries [1]. With the development of recombinant protein methodology, it became possible to clone genes encoding proteins from different origins and express them in several organisms at much higher levels than those naturally found [1, 2], leaving behind the necessity of huge amounts of animal and plant tissues or volumes of biological fluids [3]. Since the approval of recombinant human insulin for the treatment of diabetes by the US Food and Drug Administration (FDA) in the early '80s [4], hundreds of recombinant proteins have been licensed by the FDA and European Medicines Agency (EMA) to be used in the pharmaceutical industry [5].

Recombinant protein production has been mainly performed using suspended cultures. To obtain recombinant proteins, the gene encoding the protein is often isolated and cloned into an expression vector that is later introduced into the chosen host [1, 3]. The insertion of the gene of interest into a multicopy plasmid may result in high recombinant protein expression [1]. Nevertheless, this may impose a metabolic burden on the host cell due to the energy and metabolites needed for the replication of plasmid DNA and synthesis of recombinant protein [1, 6]. In planktonic cells, this added metabolic burden may decrease cellular growth rates and biomass yield, besides affecting the yield and activity of the desired protein [6]. In order to overcome these problems, a strategy based on the use of biofilm systems has been studied. Microbial biofilms are communities of microorganisms, single or multispecies, attached to surfaces that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) [7-9]. Biofilms are usually known for their negative effects on health and industrial sectors as they can cause diseases, equipment corrosion, local clogging, heat transfer resistance and product contamination in food processing environments [7, 10]. However, biofilms have been beneficial use in wastewater treatment and bioremediation processes [11], and are being tested for the production of several added-value compounds, such as alcohols, organic

acids, antibiotics, enzymes, and polysaccharides [12-14]. The biological organization of biofilms provides them with many advantages over suspended cells, including high cell density and protection against hostile conditions [15], which suggests that biofilms have great potential for heterologous protein production [16].

Biofilm establishment and their development is a dynamic and complex process, which is strongly influenced by various factors at the cellular, surface [17] and environmental levels [18, 19]. Furthermore, it has been described that the presence of expression vectors in the sessile cells increases biofilm formation and leads to higher production of recombinant proteins compared to planktonic cells [20-23]. However, it has been shown that the expression of recombinant proteins in biofilm cells is highly heterogeneous [24], which may be caused by inefficient penetration of nutrients or oxygen.

Recombinant protein expression in biofilm was pioneered by Huang et al.[25-27], who have produced the enzyme β-galactosidase in *Escherichia coli* (*E. coli)* biofilm cells. This bacterium offers some advantages over others expression systems since it has fast growth at high cell densities, well-characterized genetics, a large number of cloning vectors is available and grows with inexpensive nutrients [28, 29]. More recently, preliminary findings of our research group have shown that relatively high specific productions were attained with enhanced green fluorescence protein (eGFP) from biofilm cells [16, 24]. In this sense, the optimization of this production platform may have a significant impact on recombinant protein production in different biotechnology industries.

1.2 Aims and outlines of this thesis

The main aim of this thesis was to optimize the production strategy in *E. coli* biofilms by increasing both the specific and volumetric production of the eGFP as a model protein. For that, the influence of environmental factors, such as medium composition and hydrodynamic conditions, as well as the effect of a chemical inducer on the expression of the heterologous protein on *E. coli* biofilms were evaluated.

In addition to the present chapter, this thesis is structured into additional 6 chapters, as follows:

Chapter 2 provides a brief literature review describing state of the art on biofilm development and the main factors affecting it. Then the main decision steps needed for recombinant protein production in *E. coli* are discussed. Particular emphasis is given to the advantages and concerns of using biofilms for the production of recombinant proteins as well as the different biofilm systems that have been described for this purpose.

Chapter 3 evaluates the production of eGFP as a model recombinant protein from *E. coli* JM109(DE3) biofilm cells compared with their planktonic counterparts. Comparison between planktonic and biofilm cells was explored using two different techniques, fluorometry and epifluorescence microscopy. Additionally, the plasmid maintenance in both planktonic and sessile cells was also determined.

Chapter 4 assesses the impact of different nutrient media compositions on biofilm development, plasmid stability, and eGFP expression in *E. coli* biofilm and planktonic cells. For that, *E. coli* JM109(DE3) cells transformed with the pFM23 plasmid for eGFP expression were grown in a flow cell system with two different media, Lysogenic Broth (LB) and M9ZB. This chapter also presents a chromatographic method for plasmid isolation enabling the determination of the plasmid copy number (PCN) in both planktonic and biofilm cells over time.

In **Chapter 5** the influence of hydrodynamic conditions on *E. coli* biofilm formation and eGFP production was assessed. Two flow conditions 255 and 128 L h^{-1} (corresponding to Re of 4600 and 2300, respectively) were tested.

Chapter 6 determines the optimal isopropyl-β-D-1-thiogalactopyranoside (IPTG) induction conditions to achieve the maximum eGFP production in *E. coli* biofilms. For this purpose, two different IPTG concentrations, 0.1 and 0.5 mM, at two different days, 3 and 5 were tested.

Finally, **chapter 7** presents the main conclusions of this work and future research needs that will improve the knowledge about recombinant protein production using biofilm systems.

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2 Literature review

Part of this chapter is included in the publication:

Soares A, Azevedo A, Gomes LC, Mergulhão F. Recombinant protein expression in biofilms. AIMS microbiology (2019) **5(3):** 232

2.1 Biofilm definition and occurrence

It is understood that in nature, microorganisms are mostly found attached to surfaces rather than growing freely in bulk solution, the planktonic mode [1]. These communities of surface-attached microorganisms encased in a self-produced extracellular polymeric substances (EPS) matrix are called biofilms [2].

Since microbial biofilms are ubiquitous, it is logical that they have an impact on various contexts of interest such as environment, industrial, and biomedical [3-5]. For example, in aquatic environments, biofouling in ships increases the surface roughness, which results in speed reduction, leading to higher fuel consumption [6]. In the food industry, pathogenic bacteria are able to form biofilms in processing equipment, leading to contamination and spoilage of processed food and endangering consumers' health [7]. In the hospital, persistent infections are caused by bacterial colonization in medical devices, such as catheters or prostheses, or even directly in patient tissues [8, 9].

Biofilms are mostly known for their negative impact, however, bacterial biofilm can also have beneficial effects [10]. For example, in aquatic ecosystems, biofilms contribute to sustaining food channels as well as to nutrient recycling [11]. Also, biofilm formation by commensal microorganisms in teeth prevents pathogenic microorganisms from forming biofilm themselves [12]. In agriculture, biofilms play an important role in recycling nutrients and as biological control agents against soil-borne pathogens [13]. In addition, the use of biofilm-based biofertilizers can promote plant growth, improve soil quality and reduce the use of chemical fertilizers [14]. Biofertilizers can mobilize minerals from inaccessible forms, make atmospheric nitrogen available to plants, increase the organic carbon in the soil, and promote plant growth through the increase of growth hormone

production [15]. Since microorganisms living in biofilms display a higher tolerance to toxic environments, biofilms have been used as bioremediation agents. They convert hazardous substances (e.g. oil spills, persistent organic pollutants, pesticides, and pharmaceutical products, among others) into lesser or harmless compounds [16-18]. In addition, bacterial communities have also been employed to remove organic and inorganic contaminants in wastewater [19].

Besides biofilm reactor applications for wastewater treatment and bioremediation, they have been studied for the production of several added-value compounds, such as alcohols, organic acids, antibiotics, enzymes, and polysaccharides [20, 21]. The large-scale production of these compounds is mainly based on suspended cell fermentations, however, there is an increasing demand to obtain higher productivity and reduce production costs [21]. With properties like high biomass density, operational stability, and potential for long-term operation, biofilm reactors have been demonstrating their potential in the large-scale production of different added-value products [20, 22]. Therefore, biofilms have been engineered for various applications in industry, environment, and medicine (Table 2.1).

Table 2.1. Beneficial application of bacterial biofilms. Adapted from Muhammad et al. [23].

Biofilm context	Application	Purpose	Refs.
Environment	Biofertilizer/biocontrol	Plant growth promotion and protection against phytopathogens	24
	Bioremediation	Transformation of hazardous pollutants to harmless substances	25
Industry	Wastewater treatment	Contaminant removal from wastewater	26
	Microbial fuel cells	Electricity generation and biohydrogen production	27
	Anticorrosion	Metal corrosion inhibition	28
	Added-value products	Use of biofilm reactors to produce added-value compounds	29
Medicine	Human gut microbiome	Production of vitamins, degradation of toxic compounds, and conversion of complex sugar polymers into short-chain fatty acids	30

2.1.1 Biofilms formation process

In general, bacterial biofilm formation relies on a sequence of events involving initial cell adhesion to the surface, followed by microcolonies formation and polymer matrix production that results in mature biofilm development, and finally, dispersion of cells from the biofilm [2, 11] (Figure 2.1).

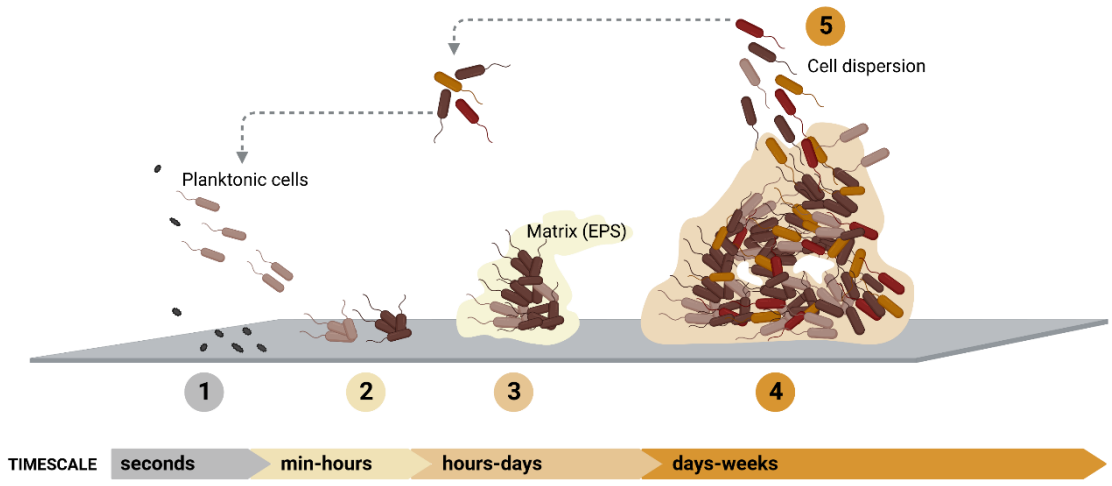


Figure 2.1. Schematic representation of biofilm development: (1) substratum pre-conditioning by ambient molecules; (2) microcolonies formation, initial reversible attachment leading to proliferation; (3) irreversible attachment that includes the production of an extracellular polymeric substances (EPS) matrix; (4) development of biofilm architecture and maturation, and (5) dispersion of cells from the biofilm into the surrounding environment. Adapted from Monroe et al. [31]

Initial exposure of the surface to the aqueous medium promotes the formation of an organic monolayer, the conditioning layer, that acts as a docking place for the reversible cell attachment [2, 23]. The molecules involved in the initial development of the biofilm start to consolidate the adhesion process through the formation of polymeric chains with the exopolymers on the surface of microorganisms or through the external filamentous appendages, such as flagella, pili and fimbriae, present in some bacteria such as *Escherichia coli* (*E. coli*) [32, 33]. At the end of this stage, the microorganisms are firmly attached to the surface and the subsequent adhesion of other cells is favoured [32]. Microbial cells replicate and grow into microcolonies involved in an EPS matrix, composed of nucleic acids, proteins, lipids, and other compounds [23, 34]. These structures

promote interaction between cells, creating a favourable environment for oxygen and nutrient diffusion into cells, exchange of genes, and quorum sensing [35]. Small colonies grow into mature biofilms, which have complex architectures with pedestal-like structures, water channels, and pores to enable the convective and diffusive transport of oxygen and nutrients into the biofilm [36]. A final stage that may occur in the life of a biofilm is the detachment and dispersion of cells from the biofilm to colonize a new niche [35]. Cell detachment can be due to various factors like nutrient limitations, fluid dynamics, secretory proteins, and catabolite repression [37].

It is known that several factors affect cell attachment and biofilm formation, including nutrient availability, surface properties, hydrodynamics, temperature, pH, and microbial cell properties [11, 38] (Table 2.2).

Table 2.2. Variables influencing cell attachment and biofilm formation. Adapted from Donlan [2] and Campoccia et al. [39]

Properties of the adhesion surface	Properties of the bulk fluid	Properties of the cell
Conditioning film	Nutrient availability	Gram-positive/Gram-negative
Roughness	Temperature	Bacterial shape
Hydrophobicity	pH	Genus/Species
Surface energy	Flow velocity	Extracellular appendages (e.g. fimbria, flagella, etc.)
	Fluid flow rate	Extracellular polymeric substances

2.1.2 Factors affecting biofilm formation

2.1.2.1 The effect of medium composition

The availability of nutrients in the surrounding medium has been proven to influence bacterial attachment and consequent biofilm development [40-43]. It has been reported that an increase in nutrient concentration is associated with an increase in microbial cell attachment [44-46]. Several studies have addressed the effect of nutritional content on biofilm growth [47, 48]. For example, in *Pseudomonas aeruginosa*, high nutrient concentrations promote biofilm formation [49]. Another study using *Pseudomonas putida* reported that biofilm increased with increasing nutrient levels up to a certain limit [50].

However, higher nutrient levels reduced biofilm accumulation as a result of higher cell detachment [50].

Regarding the effect of nutrient levels on *E. coli* biofilm formation, contradictory results have been reported. Some authors have demonstrated that higher glucose concentrations promote *E. coli* biofilm growth [51, 52]. Using a membrane system, Buhler et al. [51] reported that increasing the glucose concentration of the culture medium can promote the growth of *E. coli* biofilms. Similar results have been obtained by Moreira et al. [53] with *E. coli* JM109(DE3) biofilms formed in 96-microtiter plates. Additionally, Dewanti and Wong [54] observed that using a minimal salts medium (MSM) supplemented with glucose resulted in a stable biofilm with shorter bacterial cells and a thicker extracellular matrix compared to those formed in complex media. On the contrary, other studies referred that the addition of glucose to the media inhibit *E. coli* biofilm formation [55, 56]. Furthermore, this negative effect has also been observed in several species of *Enterobacteriaceae* family, such as *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Salmonella enterica* [56]. Jackson et al. [57] found that glucose addition in the inoculation period caused a significant decrease in *E. coli* biofilm formation as a result of the classical catabolic repression system of *E. coli*. Additionally, the authors found that this inhibitory glucose effect became progressively weaker since glucose addition after 24 h increased biofilm formation [57]. Regarding nitrogen content, *E. coli* biofilm formation on glass tubes was observed when Lysogenic Broth (LB) and a minimal medium supplemented with casamino acids were used, whereas no biofilm formation was observed when cells were grown in a minimal medium without casamino acids [58]. Oh and colleagues [59] reported that biofilms form faster and with a higher number of bacterial cells on glass surfaces using a low nitrogen medium.

2.1.2.2 The impact of hydrodynamics

Biofilms in different environments are exposed to various hydrodynamic conditions. It is known that flow conditions have a strong impact on cell adhesion since they will determine the shear stress on the surfaces where biofilm forms [60].

It has been observed that, in addition to the influence that hydrodynamics has on bacterial attachment, it is also one of the most important factors affecting biofilm

development [61, 62]. Higher flow velocity enables a higher external mass transfer, which means that more nutrients and oxygen are brought to the external layers of the biofilm, contributing to cell growth and exopolymer production [62]. However, when the external shear forces exceed the inner strength of the matrix, cell detachment may occur [60]. Higher shear stress usually results in thinner, denser, and stronger biofilms, while lower shear stress conditions result in biofilms with thick multilayer structures [2, 63]. Although higher flow velocity increases the supply of nutrients and oxygen, the biofilm density may reduce the diffusion/penetration of the substrate into the inner layers of the biofilm [64]. Besides the effect that shear stress has on the growth rate, biofilm structure and mass transfer, it has also been demonstrated that it influences the metabolic/genetic behaviour of the biofilm [63, 65, 66]. Studies in microbial fuel cells have shown that higher shear operation resulted in more viable cells with increased metabolic activity [67, 68]. In biofilm reactors used for the production of added-value compounds, hydrodynamic conditions have been used as an effective parameter to increase their productivity [69, 70]. A study conducted by Mokwatlo et al. [67] reported the use of bioreactors to evaluate the effect of shear forces on the biofilm of a succinic acid producer. The authors have shown that hydrodynamic conditions have an impact on the morphology, viability and metabolic activity of *Actinobacillus succinogenes* biofilms [67]. Results demonstrated that higher shear forces improved the metabolic activity of the biofilm, which resulted in 3-fold higher succinic acid productivity compared to low shear developed biofilms [67].

2.1.3 Biofilm platforms for *in vitro* studies

The increased interest in the mechanisms of biofilm formation led to the development of *in vitro* platforms for the study of biofilms under controlled conditions [71]. Several platforms are available such as the Modified Robbins device (MRD), flow chambers, microplates, Calgary Biofilm Device, Biofilm Ring Test, rotating biofilm devices, and microfluidic devices that are used for the study of cell adhesion and/or biofilm formation in controlled hydrodynamic conditions [72-74]. The focus of this thesis is on microtiter plates and flow cells, which are two of the most used platforms for *in vitro* biofilm studies.

2.1.3.1 12-well microtiter plates

Microtiter plates are one of the most used platforms for biofilm development screening studies [75]. They consist of plates with multiple wells arranged (6, 12, 24, 48, 96, and 384 wells) in a rectangular array with a 2:3 aspect ratio, and well volume can range from tens of microliters to a few millilitres, depending on the number of wells [76]. Depending on the format used, biofilms are formed on the bottom and wall of the plate well (most commonly a 96-well microtiter plate) or on coupons placed inside the wells (most commonly in a 6, 12, or 24-well plate) [73]. They can be operated in static or dynamic conditions since they can be placed in orbital incubators under controlled shaking conditions [53, 76]. These devices are easy to handle, requiring small reagent volumes and consequently having reduced cost [77]. Additionally, microtiter plates provide a large number of miniaturized reactors with identical geometry and fluid dynamics, which allows for studying biofilm formation at different conditions in a single run [71]. However, microtiter plates operate in batch mode with the intrinsic exhaustion of nutrients and accumulation of toxic metabolites, unless the fluid is regularly replaced. Furthermore, biofilm formation in this platform can be affected by sedimentation and detachment of the poorly attached cells during medium replacement and washing steps [72].

The 12-well microtiter plate (Figure 2.2) was selected to perform screening studies reported in this thesis since this format allows for long-term assays with uniform shear forces using coupons inside the wells (round surfaces with 1 cm diameter), and it is possible to analyse different conditions (surface materials, culture media or induction conditions) in a single run. The hydrodynamics inside the wells have been simulated in joint publications with our group using computational fluid dynamics (CFD) [78].



Figure 2.2. Illustrative photograph of 12-well microtiter plate.

2.1.3.2 Flow cell system

Flow cells have been one of the platforms of preference for biofilm studies for more than 30 years. Although they exist in a variety of shapes and sizes, flow cells can be divided into two types, those containing removable coupons, usually referred to as large-scale flow cells [79, 80], and those essentially built for real-time microscopic analysis, usually small-scale flow cells [81, 82]. Flow cell systems are mostly custom-made and are typically composed of pumps and tubes required to circulate the cellular suspension and the growth medium to a chamber containing the surfaces where microorganisms will adhere [80, 83].

Large flow cells are most suitable for the simulation of industrial biofilms since they contain a higher number of sampling ports that enable analysis in a long time-course assay and can be operated in high flow regimes [84]. Most of these cells are based on the initial Robbins device developed by Jim Robbins and Bill McCoy [85]. These MRD allow direct access to the coupon, although it must be removed for biofilm analysis. Additionally, this momentaneous flow stop may cause some loosening of the biofilm formed in the other coupons [78]. These flow cells can be operated either in laminar or turbulent flow; however, it is important to guarantee that the flow cell has an entry section long enough to allow the flow development before the sampling zone, making sure all coupons are subjected to the same shear conditions [86].

The flow cell system used to perform the studies reported in this thesis is represented in Figure 2.3 and was previously described by our group [80, 87]. This platform is composed of a recirculating tank (planktonic cells), a vertical flow cell reactor (where biofilms are formed), and centrifuge pumps that allow the circulation of the bacterial suspension. The flow cell reactor is a semi-circular Perspex duct (3.0 cm in diameter and 1.09 m in length) with 21 apertures on its flat wall to fit removable Perspex pieces (coupons) to which surfaces slides (2 x 1 cm) are glued. The flow cell hydrodynamics was fully characterized in joint publications with our group using CFD [80], which provides knowledge information regarding relevant flow characteristics (such as shear stress and shear strain rate) and guaranteeing that all sampling coupons are exposed to the same shear forces [86].

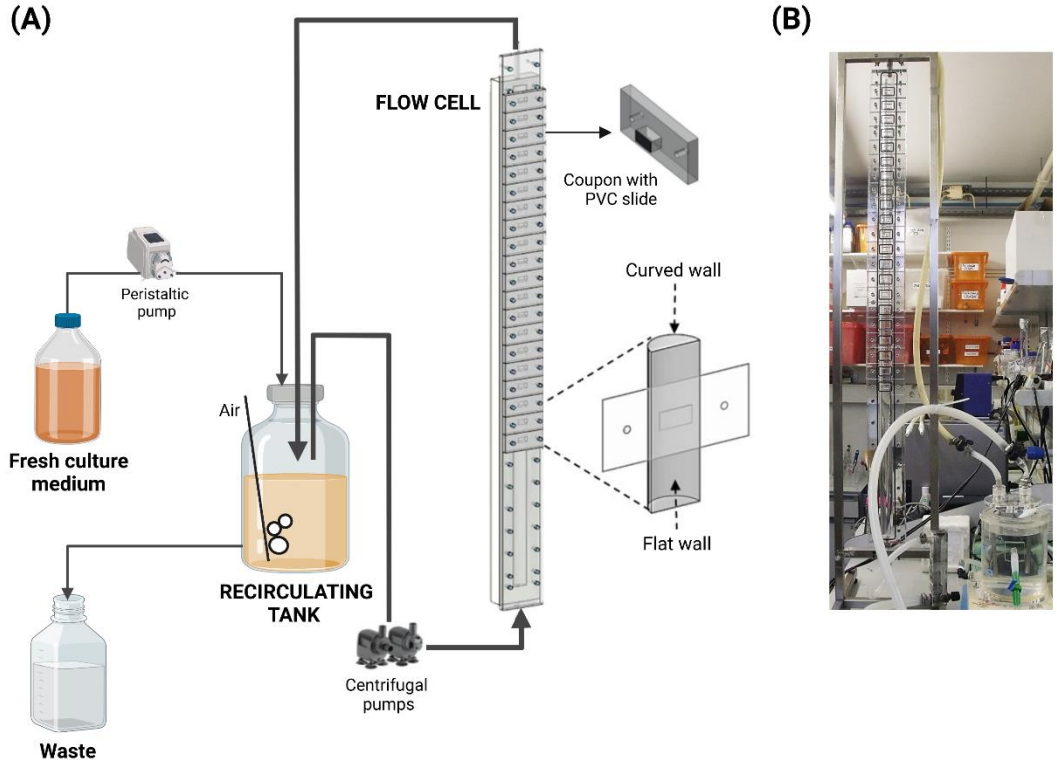


Figure 2.3. (**A**) Schematic representation and (**B**) photograph of the flow cell system [88].

2.2 Recombinant protein production

The development of recombinant deoxyribonucleic acid (DNA) technology in the early '70s [89] has revolutionized the strategies for protein production. Indeed, it became

possible to obtain genes from a variety of sources and express them in different host cells, resulting in the production of proteins called recombinant proteins [90, 91]. Recombinant proteins have emerged as an attractive and effective class of proteins to be used in the research field and different biotechnological industries due to the development of techniques capable of producing large amounts of these desired proteins [90].

Somatostatin was the first functional recombinant protein produced in 1977 using *E. coli* as a cell host [92]. In the early '80s, the US Food and Drug Administration (FDA) approved the clinical use of recombinant human insulin for the treatment of diabetes, being the first recombinant pharmaceutical to enter the market [93]. Since then, hundreds of recombinant proteins have been licensed by the FDA and European Medicines Agency (EMA) to be used in the pharmaceutical industry [94].

After a few decades, the relevance of recombinant proteins in research, clinical and industrial purposes has solidified. Nowadays, recombinant protein production benefits major sectors of the pharmaceutical and enzyme industries. Products of these industries have wide-ranging applications in fields such as medicine, diagnostics, food, feed, detergents, textiles, leather, paper, pulp, polymers, plastics, biofuels, and fine chemical industries [95, 96]. More than 150 recombinant proteins have been licensed by the FDA and EMA to be used as a pharmaceutical, with an estimated global market to be worth approximately €50–60 billion [97].

Although the process development is rarely straightforward and often demands an empirical approach, most commonly the production of recombinant protein originates from the same sequence of events, which includes an initial clone of the gene of interest in an expression vector, transforming it into a host cell, thereby allowing the biosynthesis of the protein, and recovery and purification of the protein [98, 99].

An important step in recombinant protein production is the choice of the ideal expression system. A myriad of organisms (or their derivatives) can be chosen as hosts for recombinant protein production. The list includes bacteria, yeast, filamentous fungi, mammalian, plant and insect cells, and transgenic organisms (plants and animals) [100]. Although there is a lot to choose from, the selection of the most appropriate expression host is often limited to a few options, given the intrinsic characteristics of the protein and

the process to be developed. For low-scale protein production (mainly for research purposes), intrinsic attributes of the protein are usually the most important parameters, but for large-scale production (industrial scale) other criteria are also relevant. A summary of the factors that influence the host selection for low and industrial scale can be found in Table 2.3.

In either production scale, the intrinsic characteristics of the protein are of the utmost importance. These include the requirement for post-translational modifications such as glycosylation, disulfide bond formation, phosphorylation, acetylation, acylation, carboxylation, and other modifications that can affect folding and activity [101]. Some of these modifications can be performed *in vitro,* namely using refolding strategies, but this is often not ideal. The production level is also very important in both cases because if a protein is inherently unstable in a given host, or if the host is for any reason incapable of expressing it in reasonable amounts, another system must be used [102]. This may be related to the codon bias of the gene (which can be easily solved), but also to the instability of the produced messenger ribonucleic acid (mRNA), the susceptibility to attack by host proteases (protease deficient hosts are sometimes available), the incompatibility with secretion, or the deficient folding and aggregation in a particular host or cellular compartment [102]. The technical expertise of the staff/researcher may also be an important factor. In the lab, prior experience with a certain system may introduce a certain bias to a particular host and in industrial settings, a small diversity of "cell factories" is often used in a given facility so that the staff becomes highly experienced in dealing with the peculiarities of each system.

The cell growth rate is important in both production scales, but it has a much larger impact on industrial production [96]. If the cell growth is slow, the bioreactor must be operated for a longer period, and this may affect the scheduling of the unit operations in a production plant. Since, in some cases, downstream processing equipment is shared by different production lines, an increased bioreactor operation time may impact the possibility to integrate the production of that particular protein in the industrial unit which may be producing several products. The time for setup and development is also important in both production scales and it concerns the time required to go from gene to product [96]. Since in industrial production protein products often require approval by

regulatory bodies and this approval process must follow the process development, this issue becomes critical in the industrial case and is somewhat less important in the lab scale [94]. At the host level, scalability means that the cells will be able to withstand very demanding cultivation conditions, particularly concerning the sensitivity to shear forces, tolerance to deficient oxygen mixing, and heat and mass transport limitations that are common in large-scale reactors [103]. Additionally, the possibility of performing fed-batch fermentation in High Cell Density Culture (HCDC) mode is also an advantage for some of the hosts [104].

For large-scale production and commercialization, issues like the safety component are also important. If possible, the organism should have a Generally Regarded As Safe (GRAS) status so that additional precautions are not necessary and even cheaper (e.g., non-explosion proof) equipment or handling procedures can be adopted [105]. The issues surrounding regulation are also critical as many protein products must be approved by regulatory bodies as mentioned before. The regulatory bodies will make sure that the protein product is safe, and it can provide the claimed effect before commercialization. Hosts with a very solid track record of approval are most likely to get faster and more favourable decisions from the regulatory bodies. If a protein product is under patent protection or even if the process of producing the desired protein is protected, the inventors may be entitled to royalties until the patent expires. Thus, it is important to guarantee that the product or the production process does not infringe on pre-existing patents [106]. The cost of goods is particularly important in large-scale production because of the amount of culture medium and also other components required during downstream or formulation [107]. Particular attention is given to the culture medium, which sometimes is very expensive due to the metabolic requirements of the host organism. However, in some cases, waste and by-products of other industries can be used as a cost reduction strategy.

Table 2.3. Factors that influence the selection of the most appropriate expression host for low and industrial-scale production.

Low-level Production	**Industrial Production**
Intrinsic protein properties	Intrinsic protein properties
Level of production	Level of production
Technical expertise	Technical expertise
Cell growth	Cell growth
Time for setup and development	Time for setup and development
Scalability potential	Scalability potential
	Safety component
	Regulatory issues
	Patenting issues
	Cost of goods

2.2.1 *E. coli* as a host for recombinant protein production

The Gram-negative bacterium *E. coli* has been the most intensively used workhorse for recombinant protein production since the '80s [92, 108]. The profound knowledge that has accumulated about this organism and the associated genetic methodologies still make it a highly versatile host capable of adapting to different production requirements [109]. *E. coli* grows fast (doubling times of 20 min can be achieved) in non-expensive culture media [99, 110, 111], and the fermentation is easy to scale up. In this host, there is no unintended glycosylation in non-engineered strains, no viral contamination risk (with viruses that can affect humans), and it has a very solid track record in product approvals by the FDA. Additionally, some strains have a GRAS status, they can accumulate recombinant proteins at more than 20% of the total cellular protein content, a very well-established fermentation know-how is available, and it is amenable to HCDC [111]. Although there are many available expression strains, some of them have been specifically manipulated to be used with particular expression systems [99].

Protein production can be targeted to different locations (cytoplasm, periplasm, inner/outer membrane, or culture medium) with a significant impact on downstream processing [112]. Although extracellular production can greatly reduce the complexity of the production process, the low secretion capacity of *E. coli* has always been considered

a major drawback. Different techniques can be used to circumvent this limitation such as the engineering of dedicated secretion systems existing in pathogenic strains, the use of native translocation mechanisms eventually using carrier-proteins, cell envelope mutants, and the co-expression of lysis-promoting proteins [112, 113].

The inability to perform glycosylation and several other post-translational modifications have also been a hallmark of *E. coli*. Although it was initially thought that bacteria were not capable of producing glycosylated proteins, the discovery of an N-linked glycosylation system in *Campylobacter jejuni* changed this perception. This system was transferred to *E. coli* [114] and the production of glycosylated proteins at a scale of 5 L was possible [115]. There are still several significant challenges to be overcome in the production of glycosylated proteins in *E. coli*. Not only sometimes glycosylation is incomplete, but also its efficiency can be very low, and heterogeneity can be found if more than one glycosylation site is present. Additionally, the production of glycosylated proteins was shown to negatively affect cell growth [113]. Since most of the eukaryotic proteins are N-terminally acetylated, this modification is very important in recombinant protein production in prokaryotic systems as acetylation has been shown to affect several cellular processes. Also, phosphorylation is very important for the activity of many recombinant proteins and it was shown that either by co-expressing chaperone proteins or by performing gene fusions, it was possible to obtain some acetylated and phosphorylated proteins in *E. coli* [113].

Other drawback of using *E. coli* for recombinant protein synthesis is the possibility of acetate accumulation, which has a toxic effect, although this can be controlled by the oxygen level and careful choice of the expression strain [116]. Additionally, many proteins are produced as insoluble inclusion bodies, which are inactive and require refolding [117]. Although it has been argued that recombinant protein production in inclusion bodies decreases the deleterious effects associated with protein toxicity (as the protein is not active), it must be remembered that high-level recombinant protein production is always toxic to the cells due to the metabolic burden associated with the expenditure of energy, amino acids, and other precursor molecules. In order to obtain an active protein from these inclusion bodies, it is often necessary to remove them from the cells, the proteins must be solubilized by denaturants that cause protein unfolding, and disulfide bonds

must be broken using reducing agents. Refolding then proceeds by removing the denaturant and reducing agent followed by renaturation with oxidation agents [95]. A further disadvantage of *E. coli* is the accumulation of lipopolysaccharides (LPS) requiring additional purification steps as some of these substances are pyrogenic in humans [118]. The relative advantages and disadvantages of *E. coli* are summarized in Table 2.4.

Table 2.4. Advantages and disadvantages of *E. coli* in recombinant protein production.

Advantages	**Disadvantages**
Proven track record in FDA approvals	Proteins with disulfide bonds are difficult to express
Many available expression systems	Production of glycosylated proteins is challenging
Simple, well-understood genetics	Phosphorylated or acetylated proteins are difficult to produce
Inexpensive culture media	Proteins produced with endotoxins
Fermentation is easy to scale up	Acetate formation resulting in cell toxicity
No unintended glycosylation	Proteins produced as inclusion bodies
No viral or prion contamination risk	Inactive proteins require refolding
Ease of handling, GRAS status	Protein stability can be low due to proteolytic degradation
Fast proliferation	Protein secretion is challenging
High expression levels	
Very well-established fermentation know-how	
Amenable to HCDC	
Protein can be directed to different cellular locations	

Recombinant protein production in *E. coli* often starts by inserting the gene encoding the desired protein into a multiple cloning site of an expression vector under the control of a promoter that regulates the expression [111]. The expression vector is then transformed into an *E. coli* strain that is capable of recombinant protein production, and the transformants are grown in liquid culture. At a specific stage of growth, protein production may be induced by adding an inducer that will activate the promoter on the expression vector. After the recombinant gene is expressed, the protein of interest can be recovered and purified [90]. The successful recombinant protein production in *E. coli* depends on several factors, including expression vector design, host strain, and cultivation conditions (Table 2.5) [119]. Among these factors, the expression vector design,

medium composition and induction conditions (inducer concentration and point of induction) will be addressed in this thesis.

Table 2.5. Variables affecting recombinant protein production in *E. coli.* Adapted from Overton et al. [90]

Expression vector design	Host strain	Cultivation conditions
Selection marker	Cell physiology	Medium (carbon and nitrogen sources)
Origin of replication	Growth rate	Temperature
Promoter		Inducer concentration
Tags (affinity, solubilization, and targeting)		Point of induction
		Shaking conditions

2.2.2 Plasmids as vectors for recombinant protein production

Many expression systems in research and the biotechnological industry use plasmids as vectors for the production of recombinant proteins. Plasmids are defined as extrachromosomal, double-stranded circular DNA molecules that carry their genetic elements that affect both transcriptional and translational steps of protein production [111, 120]. The genetic elements that compose an expression plasmid are the origin of replication, promoter, ribosome binding site, start codon, transcriptional terminator, and selective marker [90, 99, 121] (Figure 2.4).

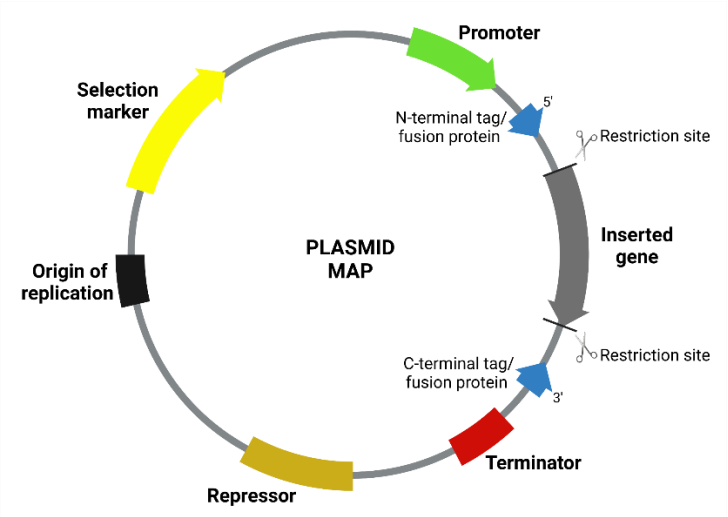


Figure 2.4. General features of a plasmid vector for recombinant protein expression in *E. coli*. The essential architecture of an expression vector includes: (1) an origin of replication, (2) a selection

marker (3) a promoter, (4) a coding sequence for the target protein and (5) a transcription terminator. Adapted from Ahmad et al. [122]

The origin of replication affects the plasmid copy number per cell and medium copy number plasmids (e.g. pBR322, 15-50 copies per cell) and high copy number plasmids (e.g. pUC, 500-700 copies per cell) [123] are often used. High-copy number plasmids usually generate high gene dosage but do not always guarantee a higher protein yield [124]. This could be related to the limited transcription and translation capacity of the host cell [125]. For example, if the bottleneck is at the translational level, high-copy plasmids only lead to a larger population of mRNA transcripts which results in an additional metabolic burden to the host without any improvement in protein synthesis [126]. Furthermore, high-copy plasmids put an extra metabolic burden on the host cell due to the energy and metabolites needed for the replication of plasmid DNA and synthesis of recombinant proteins [90, 127]. In planktonic cells, this added metabolic burden may decrease the cellular growth rates and biomass yield, besides affecting the yield and activity of the desired protein [127]. Additionally, lower copy number plasmids present some advantages over high copy plasmids, such as tight control of gene expression, the ability to replicate large pieces of DNA, and lower metabolic burden on the host strain [128].

Plasmid maintenance on the host cell is ensured by including a selection marker, usually an antibiotic-resistance gene [122]. Therefore, under antibiotic selective pressure, only cells harbouring the plasmid survive [129]. Among the most used antibiotic for maintaining selection pressure are ampicillin, kanamycin, tetracycline, and chloramphenicol [130]. At the lab scale, the use of antibiotics as a selection marker is the most common strategy since it is simple, cost-effective, and convenient [129]. However, there is a potential loss of selective pressure as a result of antibiotic degradation [130, 131]. For instance, the ampicillin gene product, β-lactamase enzyme, frequently leaks the outer membrane where degrades ampicillin [132]. Another problem arises from the increased metabolic burden due to metabolites and energy necessary to express these antibiotic resistance genes [133, 134]. For instance, genes associated with the resistance to kanamycin, chloramphenicol, and ampicillin are usually expressed at high levels which results in fewer resources available for plasmid production [135]. Indeed, it has been shown

that markers are produced at levels far exceeding those necessary for plasmid maintenance and selection, reaching levels of 20% of the total cellular protein in case of high copy plasmids [136, 137].

The selection of the appropriated promoter will influence the yield of the recombinant protein since it plays a key role in the transcription regulation of the heterologous gene. An effective promoter for recombinant protein expression in *E. coli* should have certain characteristics: (1) must be strong enough to allow the accumulation of the protein equal or greater than 10-30% of the total cellular protein; (2) should exhibit a minimal level of basal transcriptional activity avoiding unwanted protein expression before the induction; (3) must have a stringent regulation which is essential for the synthesis of proteins that are detrimental to the host, and (4) should be simple and inexpensive to induce [119, 121]. Different promoters (*tac*, *trc*, pBAD, among others), which can be thermal or chemical inducible, have been used for large-scale recombinant protein production in *E. coli* [124]. The isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible promoters such as *lac* promoter, the *lac-trp* hybrid promoter, and *tac* and *trc* promoters are widely used in laboratories [121]. The main drawback of these IPTG-inducible promoters is leaky expression under non-induction conditions and thus are not suitable if the target-gene product is toxic to the cell. The T7 promoter, more tightly regulated and IPTG-inducible, is found in pET systems, which are the most popular system for achieving high level expression of recombinant proteins [138, 139].

2.2.2.1 pET expression system

Several expression systems and promoters have been developed for the expression of heterologous proteins [123, 140, 141]. The pET system (Figure 2.5), based on the T7 RNA polymerase (RNAP), is the most widely used expression system in *E. coli* [142, 143]. Indeed, it was reported that an efficient expression of the desired protein can achieve 50% of the total protein content [123, 144]. In this system, the gene of interest is cloned behind the T7 promoter, which is only transcribed by the RNA polymerase of the bacteriophage T7. This highly active polymerase is incorporated into the bacterial chromosome at the DE3 locus in a prophage (λDE3) under the transcriptional control of the lacUV5 promoter [143]. This promoter can be inducible by lactose or its non-hydrolysable analog IPTG [145]. In the

absence of IPTG, the *lac* promoter is bound to the lac repressor, lacI, which inhibits transcription initiation from the T7 RNAP. After the addition of IPTG, lacI is released from the lac promoter region, and the T7 RNAP gene is transcribed and translated. The T7 RNAP is then able to activate transcription from the T7 promoter located on the pET plasmid [143].

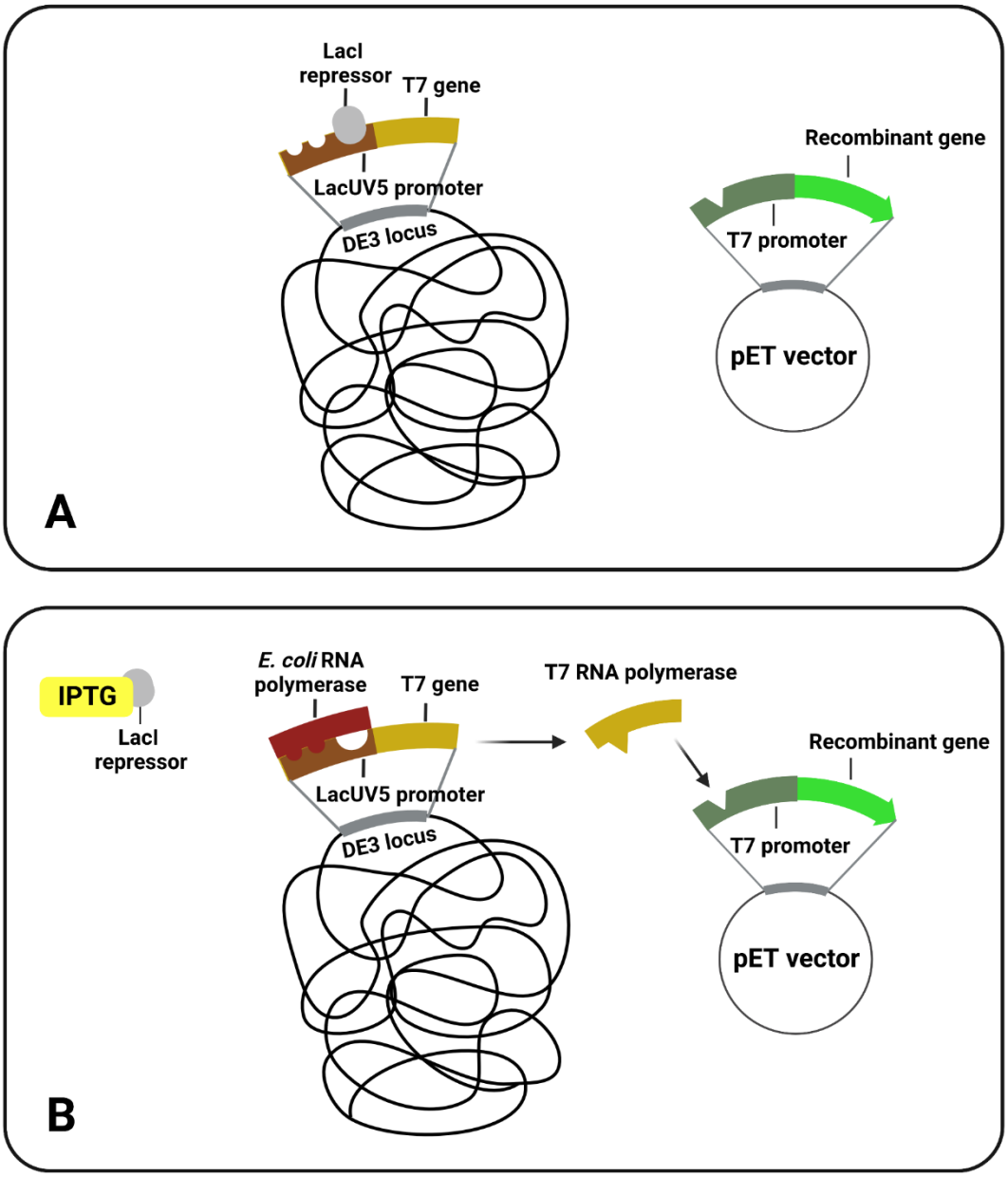


Figure 2.5. The pET system. The recombinant gene of interest is carried on a plasmid vector under the control of T7 promoter, which cannot be activated by the native *E. coli* RNA polymerase. The T7 RNA polymerase (RNAP) enzyme is required for transcription, which is encoded on a portion of the host genome known as the DE3 locus. (**A**) Expression of the T7 RNAP is regulated by the lacUV5 promoter, which is repressed by the lac repressor, lacI. (**B**) To express the recombinant gene, IPTG is added to the culture medium, which is a ligand for the lacI. When bound to IPTG,

lacI empties the lacUV5 promoter, enabling *E. coli* RNAP to transcribe the T7 gene encoding the T7 RNAP. The T7 RNAP is then able to activate the T7 promoter on the expression vector and transcribe the recombinant gene. Adapted from Overton et al. [90]

Even in the absence of IPTG, "leaky" expression of the target protein can be obtained, resulting from the uninduced expression of T7 RNAP from the lacUV5 promotor [90, 130]. This could be a problem when the recombinant protein being generated is toxic to the host cell [146]. However, basal expression can be controlled by transforming the host strain with pLysS or pLysE vectors to express T7 lysozyme, which is a natural repressor of T7 RNAP [142]. Additionally, a reduction of basal expression can be achieved by adding glucose to the growth medium [138].

2.2.3 Factors enhancing recombinant protein yield in *E. coli*

Different strategies have been explored to achieve maximum yields of recombinant protein *in E. coli*, including (1) changing the expression vector, (2) optimizing the culture parameters (medium, pH and temperature) and induction conditions (inducer concentration and point of induction) of the recombinant strain, (3) co-expressing other genes like chaperones, and (4) adjusting the synthesis of stress-related sigma factors and/or deleting specific gene sequences such as proteases, which may increase both the quantity and quality of the desired protein [97, 111, 129, 147-149]. Nonetheless, the optimization of culture conditions remains one of the first parameters to investigate since it is easy, cheap and has been proven to have an impact on protein yield [150-152].

It is proven that culture medium composition has an impact on many factors, such as cell growth and protein production [153]. Several studies have demonstrated that the increased concentration of salts, peptone, and yeast can increase the concentration of recombinant protein [154, 155]. Two types of media can be used in producing recombinant proteins: (1) complex media, which contain components whose exact chemical composition is unknown, such as meat protein and yeast hydrolysates, and (2) chemically defined media whose exact chemical composition is defined according to bacterial requirements [90]. Complex media are more frequently used since they give more

consistent yields, allow easier process control and simplify downstream recovery of the target protein [103]. Complex media, such as LB, Terrific Broth (TB), Super Broth (SB), and 2YT, have been used for recombinant protein production for many years [103, 151, 156, 157]. They contain yeast extract and tryptone that are known to provide biosynthetic precursors, such as amino acids and vitamins that support protein expression in *E. coli* [154, 158]. Furthermore, yeast extract can also be responsible for reducing cellular stress, namely the production of proteases, during the synthesis of recombinant proteins [159]. Additionally, Matsui et al. [160] found that the L-glutamic acid, a component of yeast extract, was effective in improving the production of tryptophan synthase (TSase) as well as plasmid stability. However, Vincentelli et al. [157] reported that the composition of the media (SB, 2YT, or TB) had a minimal impact on protein solubility. The overall results showed that the protein solubility per cell was the same, and the higher the biomass, the more protein was produced [157]. High cell densities attained with TB and SB medium are due to the fact that these rich media contain glycerol, resulting in higher protein concentration than other complex media used for recombinant protein expression in *E. coli* [103]. On the contrary, Bhuvanesh et al [161] reported that the use of LB medium resulted in higher recombinant abundant larval transcript (ALT)-2 protein concentration, although high cell density was achieved in TB. These results were related with the fact that specific growth rate can influence plasmid copy number and protein production [161].

The use of inducible expression systems allows the separation of the growth and production phases, and consequently, the increase of protein production by variation of inducer concentration and induction time [162]. Upon induction, the growth rate and metabolic activity of the host cell are often affected [163]. Although low inducer concentration may result in an inefficient induction, high inducer concentration does not necessarily lead to maximum protein expression [135, 164]. Indeed, excess inducer concentration can increase the metabolic burden of the host, resulting in reduced cell growth and protein production [165, 166]. Ramirez et al. [167] observed that IPTG concentration between 0-1 mM did not affect *E. coli* growth rate. In another study, Wood and Peretti [168] analysed the effect of different IPTG concentrations (between 0.01 and 7.5 mM) on the expression of β-galactosidase mRNA. The authors demonstrated that the synthesis rate of β-galactosidase mRNA increased linearly with IPTG concentration up to 1 mM.

Although the level of β-galactosidase continued to increase for higher IPTG concentrations, the β-galactosidase activity assay showed that only 37% resulted in active protein [168]. Furthermore, high inducer concentration might also lead to the accumulation of target protein in inclusion bodies [169, 170]. Additionally, the IPTG concentration can have an impact on the solubility and yield of the recombinant protein. Turner et al. [171] demonstrated that the recombinant cyclomaltodextrinase (CDase) was soluble and active when induced with 0.05 mM IPTG, whereas double IPTG concentration (0.1 mM) resulted in insoluble and inactive protein. Therefore, although the most common IPTG concentration used is between 0.05 and 1.0 mM, the optimal inducer concentration depends on the host and the target protein.

Besides the inducer concentration, induction time is also an important variable to consider. High cell densities for protein production can be achieved at the late-logarithmic or stationary phase, but at this point, the metabolic state of the cells can be unfavourable and the presence of stress-related proteins like proteases can reduce the yield of the protein [133]. The physiological state of the cells at the time of induction can affect the metabolic response and efficiency to produce the recombinant protein [133]. For this, most of the studies have reported the induction at the mid-logarithmic growth phase, since at this point, cells can provide sufficient levels of energy and metabolic precursors for recombinant biosynthesis [172, 173]. However, for strains whose growth and viability are drastically reduced upon induction, protein production can be favoured by induction at the late-logarithmic or stationary phase [174, 175]. In contrast, Ramirez et al. [167] observed that the addition of IPTG at the early log phase favoured the production of secreted acylase in *E. coli* under the control of the *lac* promoter. The induction at the early log phase could maximize overall foreign protein yield in cases where protein production is low and/or does not significantly influence bacterial cell growth [176].

2.3 Recombinant protein production in biofilms

2.3.1 Suspended cells vs. biofilm reactors

Biofilm systems have many advantages when compared with suspended growth systems, especially in their higher biomass density and operation stability. Biofilm reactors are able to retain 5 to 10 times more biomass per unit volume of reactor [22], thus increasing the production rates and reducing the risk of cell washout when operating at high dilution rates during continuous fermentation. Furthermore, biofilms provide a protective environment to the cells as the extracellular matrix protects them against extreme conditions of pH and temperature, contaminations, hydraulic shock, antibiotics, and toxic substances [22]. Another advantage of biofilm cultures is that cells can be easily separated from the liquid by sedimentation or filtration, resulting in more efficient downstream processes [177].

Recombinant protein production in biofilms was first described 25 years ago by Huang et al. [178-180], who showed that the maximum β-galactosidase concentration obtained in *E. coli* DH5α suspended cells was higher than that obtained in biofilm cells (0.47 vs. 0.12 pg cell^{-1}) [179]. On the other hand, the probability of plasmid loss in a biofilm culture was greater than that observed in suspended batch cultures [179]. These results suggested that the production of EPS by biofilm cells may compete with plasmid maintenance/replication and expression of a heterologous plasmid-encoded protein for metabolic intermediates and energy sources, a competition that suspended cells do not experience [179]. About 15 years later, O'Connell and colleagues [181] contradicted this previous work by demonstrating that the *E. coli* biofilm environment enhanced the heterologous protein production when compared to planktonic cells. This study was carried out with *E. coli* ATCC 33456 pEGFP, a strain that formed a robust biofilm and harboured a high copy number modified pUC vector encoding enhanced green fluorescent protein (eGFP). Additionally, cultivation of *E. coli* as a biofilm was found to have a beneficial effect on high copy number plasmid maintenance compared to chemostats [181], which increased the gene dosage and may have contributed to the fact that 90% or more of biofilm cells produced significant levels of eGFP after 6 days, even in the absence of selective pressure. The reason for the enhanced plasmid maintenance

in biofilms is that sessile cells tend to grow more slowly than their planktonic counterparts [182], leading to fewer cell divisions and consequently less plasmid partitioning. With infrequent cell division, less energy is directed toward replication, reducing the metabolic burden of plasmid maintenance on the cell. More recently, our research group assessed the potential of *E. coli* JM109(DE3) biofilms in the expression of the model protein eGFP from a pET-based vector [87, 183-185]. It was found that the specific protein production from *E. coli* biofilm cells was about 30-fold higher than in the planktonic state (5.8 vs. 0.18 fg cell^{-1}) [183]. When LB was tested, which is a common culture medium used for recombinant protein expression with the pET system, the difference between the specific eGFP production of biofilms and suspended cells decreased to 10 times (12 and 1.2 fg cell^{-1} in biofilm and cell suspension, respectively) [184, 185]. Furthermore, the percentage of planktonic eGFP-expressing cells oscillated around 5%, whereas for biofilms eGFP-expressing cells represented 21% of the total cell population [185]. The higher productivity of *E. coli* biofilms is probably related to their higher potential in maintaining the plasmid within the cells. In fact, in planktonic cells, the frequency of plasmid-containing bacteria was on average 0.33, while in the biofilm this parameter rose to approximately 0.90 [185].

In addition to *E. coli*, another bacterium - *Bacillus subtilis* - was studied for recombinant protein production in biofilms. The biofilm was reported as a new fermentation technique wherein the iturin A concentration almost doubled that obtained in the submerged culture [186]. This result was explained by the fact that biofilm cells remain in their metabolically active state for a longer period of time and maximize nutrient utilization [186].

The comparative analysis of classical fermentation and biofilm reactors for the production of recombinant proteins was also performed for the filamentous microorganisms *Aspergillus niger* (*A. niger*) and *Aspergillus oryzae* (*A. oryzae*) [187, 188]. Talabardon and Yang [188] cultured a recombinant strain of *A. niger* containing the gene coding for glucoamylase-green fluorescent protein (GLA-GFP) fusion protein on cotton cloth in two different biofilm reactor configurations. They revealed that the biofilms formed in these conditions produced 10-fold more fusion protein than free-living pellets [188]. Zune et al. [187] also showed that the fluorescence signal measured from the same fusion

protein produced in *A. oryzae* biofilm reactors was two times higher than that obtained in a tank operating at a low stirrer rate. In the case of filamentous microorganisms, the immobilisation in biofilm reactors leads to decreased medium viscosity and consequently to an enhanced nutrient and oxygen transfer [189, 190], which in turn may increase the product yield. At the same time, the internal structure of fungal biofilms comprises channels in the hyphal layers that allow fluid circulation and promote a better mass transfer in comparison with the more compact structure of the pellets found in submerged culture [191]. Another reason for the higher recombinant protein levels found in *Aspergillus* biofilm cultures is the low protease secretion [192, 193].

Although biofilm reactors show several advantages for recombinant protein production over planktonic cultures, there are some limitations, particularly in the diffusion of substrates to the bacterial cells. Usually, bacterial biofilms contain multiple layers of cells and their thickness may vary from a few to many μm. For the cells to be active in the production of added-value compounds, nutrients and substrate must diffuse/penetrate the inner layers of the biofilm. However, it is known that sometimes the nutrients and substrate are used up by the outer cell layers before they reach the innermost layers [87, 194]. According to previous work from our research group [87], eGFP-producing biofilms are highly heterogeneous, with the cells actively producing the recombinant protein restricted to the top layer of the biofilm. This is probably due to the lack of oxygen within the biofilm, which is necessary for eGFP maturation, and/or mass transfer limitation of nutrients. Hence, the operating conditions should be optimized in order to obtain a porous biofilm, thus facilitating the access of the bottom layer to oxygen and fresh nutrients, which could then be shifted to a productive state [87, 194, 195]. Nevertheless, some authors have shown that diffusion limitations are not always harmful to recombinant protein expression in biofilms [179, 196]. Mass transfer limitations on nutrients may create lower cell growth rates inside the biofilm, thereby contributing to increase plasmid stability [179, 196].

An additional drawback of recombinant protein production in biofilm cultures may come from the toxicity of the protein, which arises when it performs an unnecessary and detrimental function in the host cell [99]. Another aspect to consider is the location of the recombinant protein since the biofilm approach is more appropriate if the protein is

released from the biofilm and can be captured afterward from the culture medium [29]. If the recombinant protein is located intracellularly, a "milking" process must be established to operate the reactor in a continuous mode. This is a challenging task, in particular with regard to process engineering and the economics of the process.

The Strengths, Weaknesses, Opportunities and Threats (SWOT) analysis of the application of biofilm reactors in recombinant protein production is presented in Figure 2.6.

Strengths	**Weaknesses**
High biomass density Operational stability High production rates Protective environment against extreme conditions Can be operated for longer periods Ease of biomass-liquid separation Plasmid stability Plasmid copy number maintained or increased	Limited transfer of oxygen and nutrients Control of biofilm thickness is hard Process scalability Structural or segregational instability of the plasmid under different culture conditions
Opportunities	**Threats**
Biofilm cultures offer high reaction rates and are economical, so they can be employed to decrease production costs of various recombinant products on a large scale Considerable knowledge of the design and operation of biofilm reactors is available since they are being used in a number of full-scale applications for industrial and wastewater treatment Most of the microorganisms applied in biotechnology can be grown attached to a surface The stable maintenance of high copy number vectors by biofilms can benefit metabolic engineering processes (e.g. rational design of multispecies biofilms for recombinant protein production)	Competition with established production processes using suspended cultures Additional downstream challenges for separating the cells from the surface (intracellular proteins)

Figure 2.6. SWOT analysis of biofilm cultures for recombinant protein production.

2.3.2 Application of biofilms in recombinant protein production

Biofilms have been successfully used in the production of added-value chemicals, including organic acids, enzymes, polysaccharides, antimicrobial compounds, alcohols, solvents, and other products [10, 197, 198]. This production often resorts to biofilm reactors based on cell adsorption to a support [194, 199], or cells immobilised/entrapped in a polymer matrix [200]. Adsorption uses the natural ability of bacterial cells to adhere to a support, while entrapment consists of active cell immobilisation in a polymer matrix. This last one does not undergo an adhesion step and consequently, the changes in gene expression that normally follow adhesion are absent [200].

Despite the popularity of the cell immobilisation technique in the production of added-value chemicals, the use of this technique on recombinant protein production remains scarce and at the laboratory scale. Table 2.6 lists the recombinant proteins that have been produced on cell immobilised systems in the last 40 years.

Table 2.6. Overview of the published work on the production of recombinant proteins using immobilised cells.

Recombinant Protein	Host	Cultivation Conditions	Production Values	Refs.
Rat proinsulin	*Bacillus subtilis*	Stirred tank reactor 80 h LB medium	7-10 ng mL^{-1}	[201]
β-lactamase	*E. coli* C600(pBR322)	Hollow-fiber membrane bioreactor 23 days LB medium	23 U mL^{-1} h^{-1}	[202]
	E. coli RB791	Glass vertical fluidized bed reactor 120 days 37°C	390 U L^{-1} h^{-1}	[203]
	E. coli JM103	Immobilization on K-carrageenan gel bead M9P medium 37°C	75 U mL^{-1} F^{-1}	[204]
GM-CSF	*Saccharomyces cerevisiae* XV2181 (pRADH2)	Fibrous-bed bioreactor Rich (nonselective) medium 15 days 30°C	3.5 mg L^{-1} h^{-1}	[205]
Glucoamylase	*Saccharomyces cerevisiae*	Airlift bioreactor Immobilization on cotton cloth sheets YPG medium	70 U L^{-1}	[206]

Recombinant Protein	Host	Cultivation Conditions	Production Values	Refs.
		30°C		
	Saccharomyces cerevisiae C468	Airlift-driven fibrous-bed immobilized cell bioreactor Immobilization on cotton cloth	161 U L^{-1}	[207]
Anticogulant protein PCGFX	CHO8-1 mammalian cells	Opticell bioreactor Immobilization into a ceramic core 40–50 days 36.8°C	1.0 mg day^{-1}	[208]
β-galactosidase	*Spodoptera frugiperda9 (Sf9) insect cells*	Shake-flasks and stirred-tank bioreactor 27°C	30.8 U $(10^6$ cells$)^{-1}$ and 178 U $(10^6$ cells$)^{-1}$ for shake-flasks and stirred-tank bioreactor, respectively	[209, 210]
Exo-β-glucanase	*Saccharomyces cerevisiae*	Immobilization in calcium alginate gel	214 U mL^{-1}	[211]
GFP (part of the GLA-GFP fusion protein)	*Aspergillus niger*	Static and RFB reactor Immobilization on cotton cloth attached to a stainless-steel cylinder Modified Vogel's medium 25°C	0.78 g L^{-1}	[188]
	Aspergillus oryzae	Reactor based on a stainless-steel structured packing 30°C	n.a.	[187]
hEGF	*E. coli* JM101	Three-phase fluidized reactor Immobilization on polyurethane foam particles	245 mg L^{-1}	[212]

Recombinant Protein	Host	Cultivation Conditions	Production Values	Refs.
		32°C		
CGTase	*E. coli*	Immobilization on cotton	6.05 U mL^{-1} h^{-1}	69
	E. coli	Hollow fiber membrane	20.32 U mL^{-1} h^{-1}	213, 214
hLF	*Saccharomyces cerevisiae*	Immobilization by macroporous encapsulation 30°C YP medium	18.6 mg L^{-1} and 15.3 mg L^{-1} for constitutive and induced expression systems, respectively	215
HSA	*Pichia pastoris*	Magnetic immobilization	1 mg mL^{-1}	216-218

Abbreviations: n.a. - not available; LB – lysogeny broth; M9P - phosphate-enriched minimal medium; YPG - Yeast Extract–Peptone–Glycerol Medium; GM-CSF - murine granulocyte-macrophage colony-stimulating factor; GLA-GFP – glucoamylase-green fluorescent protein; hEGF - human epidermal growth factor; CGTase - cyclodextrin glucanotransferase; hLF - human lactoferrin; HSA - human serum albumin; RFB - rotating fibrous bed.

The first reports using immobilised cells for the production of recombinant rat proinsulin and β-lactamase appeared in the early 80s [201-204]. Mosbach et al. [201] used gel-entrapped cultures of *Bacillus subtilis* BS273 in a stirred tank reactor to produce rat proinsulin. The authors reported a continuous operation for 80 h and tested three different support materials for cell entrapment: alginate, polyacrylamide and agarose. The amount of pro-insulin produced was constant in a range of 7-10 ng mL^{-1}. The authors presented the immobilisation cell technique as a valuable alternative for protein production, once it was possible to maintain the production for a long period while controlling the cell growth [201]. As for β-lactamase, Inloes et al. [202] used a hollow-fiber membrane bioreactor with *E. coli* C600(pBR322) immobilised within the macroporous matrix. The β-lactamase productivity remained at high and relatively stable levels for more than three weeks of continuous operation in the bioreactor and was 100 times higher when compared with shaker-flask cultures [202]. Georgious and colleagues [203] also investigated β-lactamase production on several continuous immobilised cell reactors for 120 days. The highest β-lactamase productivity was achieved in a glass vertical fluidized bed reactor where production levels of at least 390 units L^{-1} h^{-1} could be sustained over the course of the experiment, and maximum production of 10,000 units L^{-1} h^{-1} was attained [203]. Moreover, Ryan and Parulekar [204] immobilised a recombinant *E. coli* JM103[pUC8] strain in a K-carrageenan gel to investigate the efficacy of cell entrapment for improving plasmid stability, and synthesis and excretion of the plasmid-encoded product (β-lactamase). The results showed much higher β-lactamase activity, especially in the extracellular liquid (about 6.5 times higher), and much longer retention of plasmid-bearing cells (improved plasmid stability) in immobilised cell cultures [204, 219].

Murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was produced by recombinant yeast *Saccharomyces cerevisiae* immobilised in a fibrous-bed bioreactor [205]. The feasibility of using the fibrous-bed bioreactor to achieve stable, long-term GM-CSF production was evaluated. The best GM-CSF productivity of 3.5 mg L^{-1} h^{-1} was reached in the fibrous-bed bioreactor fed with a rich (nonselective, pH 6.7) medium at a dilution rate of 0.16 h^{-1}. Furthermore, GM-CSF production was stable over a period of 2 weeks, and at the end of this period, the cells immobilised in the fibrous matrix still had a high fraction of 26% of plasmid-carrying cells [205].

In addition to GM-CSF, *Saccharomyces cerevisiae* was employed in two other studies for the production of glucoamylase [206, 207]. Zhang et al. [206] developed an immobilised-cell-film airlift bioreactor by employing cotton cloth sheets to study the effect of cell immobilisation on glucoamylase productivity and production stability. As a result, the biofilm reactor reached a concentration of glucoamylase of 70 units L^{-1} (about 60% higher than that of the corresponding free-cell system) after 36 h and remained constant for about 30 h [206]. A similar bioreactor system was used by Kilonzo et al. [207], which obtained a high glucoamylase activity of 161 U $L^{-1,}$ corresponding to a volumetric productivity of 3.5 U L^{-1} h^{-1} for a cell density of ~85 g L^{-1}. On the other side, the glucoamylase productivity of the suspended cell was half of the immobilised fermentation [207]. These results agree with the findings of Chau and colleagues [211], which obtained an improvement in plasmid stability (up to 50%) and exo-β-glucanase expression (up to 57%) from immobilised recombinant *Saccharomyces cerevisiae* in calcium alginate gel when compared with the suspended cells.

More recently, Ho et al. [215] produced human lactoferrin (hLF) using *Saccharomyces cerevisiae* immobilised in microporous encapsulation. A total soluble active hLF of 18.6 mg L^{-1} and 15.3 mg L^{-1} was obtained from constitutive and induced protein expression, respectively, with about 9-fold increase in expression levels from 2.0 mg L^{-1} previously reported in *S. cerevisiae* [220]. Additionally, the immobilised cells were active for 3 repeated-batch cycles [215].

Sugiura and Kakuzaki [208] performed a set of experiments to study the relationship between productivity and some metabolic parameters, such as glucose consumption rate (GCR), lactate production rate (LPR), and oxygen consumption rate (OCR), in order to improve the recombinant protein productivity. They used an Opticell bioreactor with CHO8-1 mammalian cell cultures producing recombinant anticoagulant protein PCGFX (protein C fused with gla domain of factor X). The mean productivity of recombinant PCGFX was about 1.0 mg day^{-1}, and a significant correlation between average OCR and LPR was found. Thus, the authors concluded that enough oxygen provision results in high lactate production and consequently an increase in the production of the target protein [208].

Yamaji et al. [209, 210] performed stepped studies on the production of β-galactosidase using baculovirus-infected insect cells immobilised in reticulated polyvinyl formal (PVF) resin of porous biomass support particles (BSP). Firstly, the effect of immobilisation on recombinant protein production was investigated in shake flasks [210]. High specific β-galactosidase productivity was achieved on immobilised cells within the BSPs, comparable to the maximum productivity in shake-flask cultures of non-immobilised cells [210]. Later, the authors optimized the growth conditions by using a two-step culture of cells immobilised within BSPs in shake flasks and a 2.5 L stirred-tank bioreactor [209]. An initial cell growth to a density over 10^7 cells cm^{-3}-BSP with the medium of 10% fetal bovine serum (FBS)-supplemented TNM-FH and a replacement with serum-free TNM-FH after post-infection day 1 showed to be the best conditions to achieve the highest β-galactosidase yield [209].

Talabardon and Yang [188] used a recombinant *A. niger* strain containing a gene encoding the glucoamylase-GFP (GLA-GFP) fusion protein to study the GFP and glucoamylase protein production in suspension and immobilized biofilm cells. They used different culture systems: free-cell cultures in conventional stirred-tank bioreactors grown in pellet form and cells immobilised in cotton cloth grown in a mycelial form in a rotating fibrous bed (RFB) to study the influence of the fungal morphology in the expression of the fusion protein. These authors found that the amount of glucoamylase and GFP produced by the immobilised cells of *A. niger* was 0.8 g L^{-1}, about 10 times higher than in suspended cells. Later, Zune et al. [187] used a tank with a metal-structured packing as a fungal biofilm reactor for the production of the GLA-GFP fusion protein by *A. oryzae*. The fluorescence measurements indicated that protein production in the biofilm reactor was similar to that of suspended cell cultures under higher shear stress conditions. However, the western blot analysis revealed that the band corresponding to the fusion protein in submerged conditions was thicker than that under biofilm conditions. This result could be explained by the operating conditions in the submerged reactor, namely the acid pH, which could promote biomass leakage and affect the quality of the protein. Based on the results obtained in the shake flasks, the authors investigated the production of the fusion protein in two different configurations of the biofilm bioreactor, one with the full immersion of the fungal biofilm with the liquid medium

and the other with the periodic immersion of the biofilm by the liquid in recirculation on the metal packing [187]. Although both biofilm reactors reached satisfactory protein productivities, a 2-D electrophoresis analysis indicated that the quality of the fusion protein was higher in the fully immersed bioreactor [187].

Chen et al. [212] described the application of porous polyurethane foam (PUF) particles as a supporter matrix to immobilise the recombinant *E. coli* for human epidermal growth factor (hEGF) production. The hEGF productivity of immobilised-culture was 186 mg L^{-1}, about 1.5 times of free-culture. Additionally, hEGF productivity was improved (245 mg L^{-1}) when produced in immobilized cultures in a three-phase fluidized reactor [212].

The production of cyclodextrin glycosyltransferase (CGTase) by recombinant *E. coli* immobilised has also been reported [69, 213, 214]. First, Kriaa et al. [69] used a recombinant *E. coli* immobilised on cotton (pre-treated with glutaraldehyde solution in order to strengthen the attachment of the microbial cells to the support) under several culture conditions (agitation rate, and carbon and nitrogen sources). The CGTase production (145.11 U mL^{-1}) at the optimal cultivation conditions (agitation of 200 rpm, yeast extract concentration of 20 g L^{-1}, wheat starch concentration of 10 g L^{-1}, and Hy-soy concentration of 2.5 g L^{-1}) was 3.6 and 23 fold higher than those obtained by the use of the initial conditions (39.77 U mL^{-1}) and free cells (6.37 U mL^{-1}), respectively [69]. More recently, Man and colleagues [213, 214] used a recombinant *E. coli* immobilised by adsorption and entrapment in a porous hollow fiber membrane for expressing CGTase. First, the authors investigated the effect of different polymers of hollow fiber membranes and culture conditions on enzyme excretion, cell lyses, and plasmid stability. CGTase excretion of 339.72 U mL^{-1} with a 100% increase in plasmid stability and 28-60% reduction in cell lysis was achieved in cells immobilised on the hollow fiber membrane composed of polyvinylidene fluoride (PVDF). The CGTase excretion obtained in immobilised cells was 4 times higher than in free cells (84.60 U mL^{-1}) [214]. Subsequently, they optimized the post-induction time, agitation rate, and pH conditions, and found that the optimal conditions were 24 h, 200 rpm, and pH 9, respectively. The results showed an increase of 2.8–4.6-fold in CGTase excretion and 323–464% in plasmid stability, and a reduction of 16–95% in cell lysis compared with free cells [213].

Tagizadeh and colleagues reported a few studies on the production of human serum albumin in biofilm reactors, studying the effect of magnetic immobilisation with nanoparticles (MNPs) coated with amino propyl triethoxy silane (APTES) on the growth of recombinant *Pichia pastoris* and the effect on human serum albumin (HAS) production and structure [216-218]. Magnetic immobilisation increases the metabolic activity of the cells with improved productivity of HSA by about 40% when cells were immobilized with 0.5 mg mL^{-1} of nanoparticles [216, 218].

On the other hand, biofilm can simply be started by inoculating cells and then allowing them to naturally adhere to the surface. These biofilms obtained by adhesion to carrier surfaces may provide distinct characteristics for the production of valuable compounds such as recombinant proteins [181, 183]. Despite the potential of this expressing system, recombinant protein production using biofilms has been scarcely investigated during the last decades. To the best of our knowledge, there are only six studies from independent groups on this topic (Table 2.7).

Table 2.7. Overview of the published work on the production of recombinant proteins in biofilms.

Recombinant Protein	Host	Cultivation Conditions	Production values	Refs.
β-galactosidase	*E. coli* DH5α	PPFC Glass slides 37°C Supplemented M9 minimal medium	0.08-0.12 pg cell^{-1}	178, 179, 221
eGFP	*E. coli* ATCC 33456	PPFC Cover glass 37°C LB medium Laminar flow	0.01-0.16 g L^{-1}	181
	E. coli JM109(DE3)	Flow cell system PVC surfaces 30°C DM and LB media Turbulent flow	5.8 fg cell^{-1} and 0.22 g L^{-1} for DM supplemented with 20 μg mL^{-1} kanamycin 12 fg cell^{-1} for LB supplemented with 20 μg mL^{-1} kanamycin	87, 183-185
D-Amino acid oxidase	*Acetobacter xylinum*	Static and shaken culture in shaker flasks 30°C HSG4 medium	1.2 U g^{-1} (static) 2.1 U g^{-1} (shaking)	222
Iturin A	*Bacillus subtilis*	24-well plates 28°C	0.6 g L^{-1}	186

Recombinant Protein	Host	Cultivation Conditions	Production values	Refs.
		LB medium		
mCherry, EgTrp and EgA31 (part of the TasA-mCherry, TasA-EgTrp and TasA-EgA31 fusion proteins, respectively)	*Bacillus subtilis*	Well plates with 22 mm^2 surface area 30°C MSgg medium	n.a.	[223]

Notes: n.a. - not available. *Abbreviations:* PPFC – parallel-plate flow cell; LB – lysogeny broth; DM – diluted medium; eGFP – enhanced green fluorescent protein; TasA-EgTrp – TasA-tropomyosin peptide; TasA-EgA31 – TasA-paramyosin peptide; PVC – polyvinyl chloride.

The expression of recombinant proteins in biofilms was first reported by Huang et al. [178, 179, 221], who have studied the production of β-galactosidase in *E. coli* DH5α cells carrying a plasmid containing the *tac* promoter. These authors have studied the plasmid retention and expression in both suspended and biofilm cells growing in a parallel-plate flow cell (PPFC). During the course of the experiments, they found that cell accumulation rates decreased with increasing induction levels for both suspended and biofilm cells [179]. Furthermore, the production of β-galactosidase in both types of *E. coli* cells increased with induction (no basal expression was detected). Three different IPTG concentrations (0.17, 0.34 and 0.51 mM) were tested to determine the optimal inducer concentration. In biofilm cells, different IPTG concentrations did not affect the maximum β-galactosidase concentration obtained 24 h after induction (about 0.1 pg $cell^{-1}$) [179]. Huang et al. [221] also determined that the concentration of nitrogen and carbon in the growth medium affected biofilm formation and plasmid stability. When the carbon/nitrogen ratio on the nutrient supply increased, the biofilm cell density decreased, while the probability of plasmid loss increased [221]. Additionally, it was shown that the synthesis rates of β-galactosidase mRNA were higher at higher expression levels, increasing 4-fold under 0.17 mM IPTG, and almost 12-fold under 0.34 and 0.51 mM IPTG. However, the β-galactosidase production did not increase at the same proportion of mRNA synthesis rate. It is possible that the synthesised mRNA was much less stable at high expression levels [178].

In 2007, O'Connell et al. [181] described the first system for high-level heterologous protein production in *E. coli* biofilm cells. These authors used an *E. coli* strain containing the plasmid pEGFP to investigate the production of eGFP in a chemostat (planktonic cells) and a PPFC reactor (biofilm cells). They detected different populations of sessile cells: strongly producing cells (capable of producing 0.16 g L^{-1} of eGFP), moderately producing cells (capable of producing 0.01 g L^{-1} of eGFP), and non-producing cells. In contrast with Huang et al. [178, 179], the results of this work indicated that biofilm environment enhanced both plasmid maintenance and heterologous protein concentration when compared to planktonic cells [181]. This study also found that the addition of low antibiotic concentrations to biofilm populations increased these beneficial effects, enabling them to obtain 60% of strongly producing cells [181].

More recently, our research group used *E. coli* JM109(DE3) cells transformed with the pFM23 plasmid to evaluate the production of eGFP in biofilm cells [87, 184, 185]. The experiments were performed in a flow cell system, which includes a recirculating tank where planktonic cells grow and a vertical flow cell for biofilm formation. To assess the impact of heterologous protein production on biofilm formation, a control strain harboring the pET28A plasmid (i.e. the same plasmid backbone but lacking the *eGFP* gene) was used. Results show that biofilm formation was enhanced for the eGFP-producing strain when compared to the control strain [183]. The specific eGFP production of biofilm cells was around 5.8 fg cell^{-1} in steady state, even before any optimization of the cultivation conditions. Moreover, the cellular density of biofilms was around 11 g L^{-1}, which corresponds to an eGFP volumetric productivity of 0.22 g L^{-1} [183]. Interestingly, the specific and volumetric productivities obtained with this system are within the range of what can be achieved by conventional HCDC in the production of soluble proteins [224-226]. Selective pressure is often necessary to ensure plasmid stability, which is achieved by adding an antibiotic resistance gene to the plasmid. However, it is known that the expression of this gene may increase the metabolic burden in the host and affect the production of the target protein [127]. The nutrient composition of the culture medium is another important parameter in biofilm formation [41, 43, 227] and recombinant protein production [228]. *E. coli* biofilms were formed under exposure to 20 and 30 μg mL^{-1} of kanamycin in two different culture media, a diluted medium (DM) and LB [184]. The diluted medium (mainly composed of 0.55 g L^{-1} glucose, 0.25 g L^{-1} peptone, and 0.125 g L^{-1} yeast extract) was described as a good medium for biofilm formation [43], whereas LB is frequently used for the expression of recombinant proteins and provides abundant carbon and nitrogen (10 g L^{-1} tryptone and 5 g L^{-1} yeast extract) [176]. This study reported a 2-fold increase in the eGFP production of biofilms developed in LB when compared to what was obtained in DM [184]. Regarding the effect of antibiotic concentration, the increase from 20 to 30 μg mL^{-1} did not affect the amount of recombinant protein produced by the biofilm [184]. Thus, among the tested conditions, LB supplemented with 20 μg mL^{-1} of kanamycin seemed to be the most advantageous medium to obtain the highest specific eGFP production in *E. coli* biofilms [184]. Our research group also examined the eGFP expression during biofilm development at the single-cell scale, showing that the biofilm population

became increasingly heterogeneous over the course of the experiment [87]. Three different types of biofilms were observed: one with a homogeneous population (between days 3 and 5), a second with a moderately homogeneous population (between days 6 and 8), and the third with a strongly heterogeneous population (between days 9 and 11). These results are consistent with those obtained by O'Connell et al. [181], who studied the dynamics of protein fluorescence during biofilm development. Moreover, by confocal microscopic analysis (Figure 2.7), we also showed that the majority of the eGFP-expressing cells were located in the liquid interface of the biofilm. It is known that the spatial organization of biofilms can affect the diffusion of oxygen and nutrients, resulting in heterogeneous populations in terms of metabolic and phenotypic behaviours [64, 229]. Lenz et al. [230] observed a spatially non-uniform pattern of green fluorescent protein (GFP) expression in *Pseudomonas aeruginosa* biofilms. These authors demonstrated that the amount of GFP mRNA was higher in the external layers of the biofilm, which is associated with the zone of active GFP fluorescence visualized at the top of the biofilm [230]. This is probably related with the higher access of sessile cells to the oxygen required to the final stage of protein folding [231].

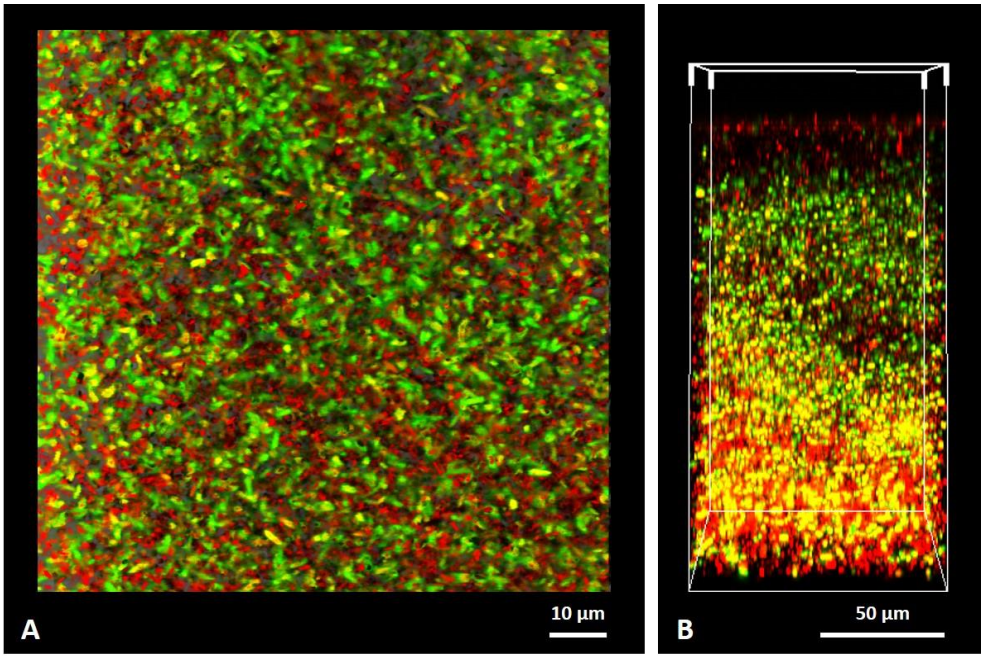


Figure 2.7. Spatial heterogeneity of an 11-day-old biofilm formed by *E. coli* JM109(DE3) expressing the recombinant protein eGFP: (**A**) top view and (**B**) three-dimensional view of the confocal laser

scanning microscopy (CLSM) images. The eGFP-expressing cells are labelled in green and the non-expressing cells are countermarked in red with Syto61.

Acetobacter xylinum is a Gram-negative bacterium that produces a cellulase pellicle during its growth. Like biofilm, *A. xylinum* cells are immobilized by simultaneously producing cellulose nanofibers in the pellicle. Setyawati et al. [222] studied the effect of the self-immobilized capability of *A. xylinum* to achieve a desired and stable biotransformation. They used a recombinant *A. xylinum* that expressed the enzyme d-Amino acid oxidase (DAAO) of *Rhodosporidium toruloides*. The results showed that the recombinant *A. xylinum* successfully produced DAAO as well as capable of self-immobilization by cellulose nanofibers in both the static and shaken culture. Furthermore, the self-immobilized cells prepared in shaken culture expressed a higher DAAO activity than in static culture (2.1 U g^{-1} versus 1.2 U g^{-1}), and the maximum DAAO expression was obtained when glycerol was used as a carbon source. Although the DAAO activity from the self-immobilized cells (in both static and shaken conditions) was much lower than in cell crude extract, it provided benefits of improved thermal stability, operational stability, and easy retrieval for repeated use [222].

The production of recombinant proteins in biofilms of the Gram-positive bacterium *Bacillus subtilis* has also been studied [186, 223]. Rahman et al. [186] used a transformant strain of *B. subtilis* 168, containing wild *sfp*, the *itu* operon, and *degQ* for the production of the lipopeptide iturin A. These authors reported an iturin A concentration in the biofilm of approximately 0.6 g L^{-1} in steady-state conditions, which is higher than the concentration of 0.4 g L^{-1} obtained in the cell suspension [186]. Later on, Vogt et al. [223] developed a strategy based on the TasA protein for the display of a heterologous protein within the *B. subtilis* biofilm. They engineered a fusion protein with TasA and the red fluorescent protein mCherry, and showed that this fusion protein was abundant and homogeneously distributed within the biofilm. TasA is one of the crucial matrix proteins responsible for biofilm formation and is dependent on the tapA-sipW-tasA operon [232]. The same authors also produced fusion proteins of TasA with *Echinococus granulosus* antigenic peptides, paramyosin, and tropomyosin [223]. The results showed that the antigens were expressed and could be efficiently located in the biofilm matrix. Additionally, it was demonstrated that the spores produced by the recombinant strain were physically and morphologically

identical to the wild-type strain, which could be an excellent strategy to preserve the integrity of heterologous proteins and facilitate their transport to the desired location [223].

2.3.3 Examples of recombinant proteins produced in biofilm cultures

2.3.3.1 Enhanced Green Fluorescent Protein (eGFP)

Since its discovery in 1962 [233], the GFP from the jellyfish *Aequorea Victoria* has become one of the most important and powerful tool in cell biology [234, 235]. Its intrinsic fluorescence, without the requirement of any additional co-factors or substrates, allows the monitoring of any target protein in the cell [236]. The fluorescence is the result of the covalent rearrangement of continuous amino acids, namely Ser65, Tyr66 and Gly67, which cyclase after the protein folds in the presence of oxygen to form a chromophore [236]. This chromophore is positioned and consequently protected at the center of a large β-barrel formed by 11 β-strands [234]. GFP has been widely used in biological and biotechnological fields, namely as a biomarker, a *tag* reporter, and a component in biosensors, in real-time imaging, and in bioprocess applications [237]. Although extensively used, GFP has several drawbacks that reduced its effectiveness as a tool for cell imaging, such as (1) slow maturation rate, (2) poor folding efficacy and fluorescence intensity at physiologically relevant temperatures like 37°C, and (3) coexistence of neutral and phenolate forms of the chromophore, resulting in two separated excitation peaks [238]. To overcome these problems, an engineered version of GFP was created, the enhanced GFP (eGFP), with two mutations (F64L and S65T) that improve the quantum yield of fluorescence. The extensive use of GFP increases the demand for pure GFP. However, a major drawback is its high cost, ranging from around USD 350 to 600 per 0.1 mg [239]. Recombinant protein technology can be used for large-scale GFP production.

This protein was used in the present work as a model for the evaluation of heterologous protein production in *E. coli* biofilms. Properties like non-toxicity, stability, and capability to be expressed at high levels in a soluble way make eGFP an excellent protein model [240]. Furthermore, eGFP exhibits fluorescence that allows the use of standard

fluorometric methods, which are highly sensitive, specific, simple, and have low instrumental costs when compared to other techniques, for monitoring the amount of target protein produced by the cells [241].

2.3.3.2 β-galactosidase

Enzymes are natural, environmentally safe, and non-toxic products which make them an important part of the food industry [242]. Furthermore, they also have the ability to perform under mild conditions of temperature, pressure, and pH [243]. With the development of recombinant protein methodology, it became possible to clone genes encoding enzymes from microbes and express them at levels hundreds of times higher than those produced naturally [242].

The enzyme β-galactosidase, most commonly known as lactase, is one of the most important enzymes used in food processing, which catalyses the hydrolysis of lactose into glucose and galactose [244]. These enzymes have long been used in the dairy industry to improve the digestibility, solubility, sweetness, and flavour of lactose [243, 244]. β-galactosidase is a relevant enzyme in food processing with applications in the production of lactose-free products suitable for lactose-intolerant people. A significant percentage of the global adult population (almost 75%) is affected by lactose intolerance [244]. They are unable to digest lactose due to a genetic deficiency in the β-galactosidase [244]. The global market for lactose-hydrolysed products was estimated at USD 6.7 billion in 2015 and the market is expected to keep on growing [245].

Furthermore, some enzyme cocktails containing β-galactosidase are known to have several benefits in the microflora of the intestine, by the formation of galacto-oligosaccharides (GOSs) during lactose hydrolysis which favours the growth of intestinal bacterial microflora [246]. GOSs are increasingly finding application in functional foods, being used as probiotics [247]. They are non-digestible food ingredients with health benefits in the microflora of the intestine [248].

β-galactosidase can be naturally synthesized by several organisms, including microorganisms, and plant and animal cells [249, 250]. However, some of these sources present low growth and productivity, and are usually not GRAS [249]. Recombinant protein

technology can be used to express and optimize the production of interesting β-galactosidases from the most diverse sources, expanding the range of potential applications for β-galactosidases and their economically effective utilization in industrial processes [249]. Furthermore, recombinant systems can also be essential for generating improved versions of the enzymes [245, 249].

2.4 References

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3 Comparing the recombinant protein production potential of planktonic and biofilm cells

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Abstract

Recombinant protein production in bacterial cells is commonly performed using planktonic cultures. However, the natural state for many bacteria is living in communities attached to surfaces forming biofilms. In this work, a flow cell system was used to compare the production of a model recombinant protein (enhanced green fluorescent protein, eGFP) between planktonic and biofilm cells. The fluorometric analysis revealed that when the system was in steady state, the average specific eGFP production from *Escherichia coli* biofilm cells was 10-fold higher than in planktonic cells. Additionally, epifluorescence microscopy was used to determine the percentage of eGFP-expressing cells in both planktonic and biofilm populations. In steady state, the percentage of planktonic-expressing cells oscillated around 5%, whereas for biofilms eGFP-expressing cells represented on average 21% of the total cell population. Therefore, the combination of fluorometric and microscopy data allowed us to conclude that *E. coli* biofilm cells can have a higher recombinant protein production capacity when compared to their planktonic counterparts.

Keywords: green fluorescent protein; *Escherichia coli*; biofilm; planktonic; flow cell reactor

3.1 Introduction

Recombinant proteins are currently used in different biotechnological industries and are produced in large amounts in bioreactors [1]. An important step in recombinant protein production is to choose the ideal expression system and factors like protein quality, functionality, production speed and yield should be considered [2]. *Escherichia coli* has been widely used for the production of recombinant proteins due to its fast growth at high cell densities, minimal nutrient requirements, well-characterized genetics and the availability of a large number of cloning vectors [2, 3]. In the early 1980s, the Food and Drug Administration (FDA) approved the first recombinant insulin produced in *E. coli* [4] and since then this bacterium is one of the organisms of choice for the production of several commercial recombinant proteins [5].

Most of the research on the expression of recombinant proteins has been based on planktonic bacteria grown in liquid cultures. However, the natural state for many bacteria is to live in communities of sessile cells forming biofilms [6]. Biofilms are communities of surface-attached microorganisms encased in a self-produced extracellular matrix [7]. Such biological organization provides distinct characteristics to bacteria comparing to their planktonic counterparts [8]. For instance, local environmental conditions arising as a result of mass transport limitations, intercellular signalling and other phenomena, may induce biofilm cells to modulate expression of genes differently than in suspended populations [9]. The expression of recombinant proteins in *E. coli* is commonly accomplished by inserting the gene of interest into a multicopy plasmid [10] that imposes a metabolic burden on the host cell [11]. In planktonic cells, this added metabolic burden may decrease cellular growth rates and biomass yields [11], particularly when the plasmid vector is used to the direct production of a recombinant protein [12]. Conjugative [13-17] and non-conjugative plasmids [18-21] were shown to increase biofilm formation. For example, our research group has previously shown that the presence of the non-conjugative plasmid pET28A in *E. coli* JM109(DE3) cells increased biofilm formation when compared to a non-transformed strain [21].

In 2007, O'Connell et al. [22] examined the production of eGFP in a chemostat with planktonic cells and in a parallel plate flow cell (PPFC) reactor with biofilm cells. This was

the first experiment showing a high-level production of an heterologous protein in *E. coli* biofilms. Although the number of studies on the effect of recombinant protein expression on sessile cells is still scarce, it has been suggested that the biofilm environment benefits recombinant protein production [21, 23].

The aim of this work was to compare the production of a model recombinant protein (enhanced green fluorescent protein, eGFP) between *E. coli* planktonic and biofilm cells. In the present study, the comparison between planktonic and biofilm cells was explored using a novel single-cell methodology which combines the common epifluorescence microscopy with an image analysis tool. Additionally, the plasmid maintenance in both planktonic and sessile cells was also assessed for the first time.

3.2 Materials and Methods

3.2.1 Bacterial Strain

The *E. coli* strain JM109(DE3) from Promega (USA) was transformed with the plasmid pFM23 (constructed from pET28A vector, Novagen, USA) for the cytoplasmic production of eGFP [24]. This plasmid contains a kanamycin resistance gene and a pMB1 origin of replication (medium-copy number replicon), and uses the T7 promoter for transcription of the foreign gene, which can be induced by lactose or its non-hydrolysable analogue isopropyl-β-D-1-thiogalactopyranoside (IPTG).

3.2.2 Flow Cell System and Experimental Conditions

A reactor system comprising a recirculating tank (planktonic cells), a vertical flow cell reactor (where biofilms are formed), and peristaltic and centrifuge pumps that allow the circulation of the bacterial suspension was operated as described by Gomes et al. [25]. The flow cell reactor is a semi-circular Perspex duct with apertures on its flat wall to fit removable Perspex pieces (coupons) to which polyvinyl chloride (PVC) slides were glued. The biofilms were developed on the upper faces of the PVC slides that were in contact with the bacterial suspension circulating through the system.

E. coli cells were grown by recirculating the bacterial suspension at 30 °C for 11 days under turbulent flow (Reynolds number of 4600) [25]. The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with lysogeny medium (LB-Miller, Sigma, USA) supplemented with 20 μg mL^{-1} kanamycin at a flow rate of 0.025 L h^{-1} [25].

3.2.3 Biofilm and Planktonic Monitoring

The system was stopped daily, and a coupon was carefully removed from the flow cell for biofilm sampling. The biofilm wet weight and thickness were firstly determined by comparing the coupon weight prior to the start of the experiment and on the sampling day, and by using a digital micrometer [26], respectively. Then, the biofilm was resuspended in 25 mL of sterile saline solution (8.5 g L^{-1} NaCl) for total and viable cell quantification and eGFP analysis.

Biofilm total (viable plus non-viable) and viable cell counts were determined using the Live/Dead® BacLight™ bacterial viability kit (Syto9/propidium iodide; Invitrogen Life Technologies, Alfagene, Portugal) as fully described in Gomes et al. [25]. Briefly, bacterial cells from the biofilm suspension were filtered through a Nucleopore Track-Etch Membrane of black polycarbonate (pore size of 0.22 μm; Whatman Ltd, UK), stained for 10 min in the dark and observed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Switzerland). The image processing software ImageJ v1.48 (NHI, USA) was used to estimate the number of viable and non-viable cells on each membrane from counts of a minimum of 15 fields of view. The final values of biofilm total cells were expressed as log cells cm^{-2} of coupon area and the percentage of biofilm viability on each day was calculated by dividing the viable cell counts by the total cell number.

The eGFP expression in biofilm cells was assessed by both epifluorescence microscopy [25] and fluorometry [24]. For the microscopic method, the biofilm suspension was filtered and observed using a Leica DM LB2 epifluorescence microscope. Fifteen fields of view were photographed for each sample in order to estimate the number of eGFP-expressing cells [25]. The percentage of eGFP-expressing cells in the biofilm was then

calculated by dividing the number of eGFP-expressing cells by the total number of cells. The specific eGFP production in biofilms was quantified by the fluorometric method fully described by Mergulhão and Monteiro [24]. Briefly, a volume of biofilm detached cells corresponding to an equivalent optical density (OD) of 1 at 610 nm was centrifuged. The pellet was resuspended in 100 μl of Buffer I (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and added to 100 μl of Buffer I in a 96-well microtiter plate (Orange Scientific, Belgium). Fluorescence was measured in a microplate reader (SpectraMax M2E, Molecular Devices, Inc., UK) with a 488 nm-excitation filter and a 507 nm-emission filter. Calibration curves were constructed with purified eGFP (0 to 3.14 μg) and the final values were presented as specific eGFP production (fg $cell^{-1}$). An $OD_{610\ nm}$ of 1 corresponds to a cellular concentration of 7.6×10^8 cells mL^{-1}.

For monitoring the planktonic cells, a sample was taken from the recirculating tank to first assess the $OD_{610\ nm}$ and the dissolved oxygen (OX 4100 H, Fisher, Germany). Total and viable cell counts, eGFP-expressing cell counts and specific eGFP production were also assessed as previously described for the biofilm cells. Results were presented as log cell mL^{-1} and fg $cell^{-1}$ for total and viable cells, and eGFP production, respectively.

3.2.4 Measurement of Plasmid Maintenance

Planktonic and biofilm sample suspensions were suitably diluted with saline solution and spread on both selective (supplemented with 20 μg mL^{-1} kanamycin) and non-selective agar plates (Plate Count Agar, Merck, Lisboa, Portugal) to form between 10 and 300 colony forming units (CFU) per plate. Colony enumeration was carried out after 24 h incubation at 30°C. The number of CFU on selective plates corresponds to the number of culturable cells carrying the plasmid pFM23, whereas the CFU detected on non-selective plates are equivalent to the total number of culturable cells, i.e., plasmid-bearing cells plus plasmid-free cells (see Supplementary Material, Figure S 3.1). The maintenance of plasmid pFM23 in both planktonic and biofilm cells was determined as the ratio between the number of plasmid-bearing cells and the total number of culturable cells.

3.2.5 Statistical Analysis

The results presented in Figure 3.1 and Figure S 3.1 originated from averages of triplicate sets obtained in independent experiments. Standard deviations (SDs) on the triplicate sets were calculated for each day and parameter. For planktonic growth (Figure 3.1A and 3.1B), the following SD averages were obtained: SD < 16% for $OD_{610\ nm}$ and dissolved oxygen, SD < 4% for total cell counts and SD < 5% for planktonic viability. Concerning biofilm formation (Figure 3.1D and 3.1E), the following SD averages were obtained: SD < 23% for biofilm wet weight, SD < 20% for biofilm thickness, SD < 3% for biofilm total cells and SD < 4% for biofilm viability. Regarding the eGFP quantification (Figure 3.1C and 3.1F) SD < 19% for planktonic eGFP production, SD < 4% for planktonic eGFP-expressing cells, SD < 8% for biofilm eGFP production and SD < 4% for biofilm eGFP-expressing cells were obtained.

Paired t-test analysis was performed based on a confidence level of 95% (differences reported as significant for P values < 0.05).

3.3 Results

In this work, two fluorescence-based techniques (fluorometry and epifluorescence microscopy) were used with the aim of comparing the eGFP production in planktonic and biofilm populations grown in a flow cell system.

Planktonic growth was monitored during 11 days by measuring the $OD_{610\ nm}$ and dissolved oxygen in the recirculating tank (Figure 3.1A) and by quantifying the total cell number and cell viability (Figure 3.1B). The $OD_{610\ nm}$ increased until day 4, which is in agreement with the corresponding decrease in the oxygen concentration in the recirculating culture over the same experimental period. Between days 4 and 6, the $OD_{610\ nm}$ decreased and then stabilized from day 6 onwards. The same stabilization was observed in the oxygen consumption profile with dissolved oxygen levels around 2 mg L^{-1}, thus it can be concluded that the flow cell system was in steady state between days 6 and 11. Looking at Figure 3.1B, the total number of planktonic cells remained practically constant over the experiment. However, the percentage of viability varied significantly

(Figure 3.1B) due to oscillations on the number of viable cells during the experimental time (data not shown). Between days 4 and 6, a strong decrease in planktonic viability (from 92% to 42%) was observed. But from day 8 onwards the planktonic viability increased significantly, reaching almost 100% at the end of the experiment.

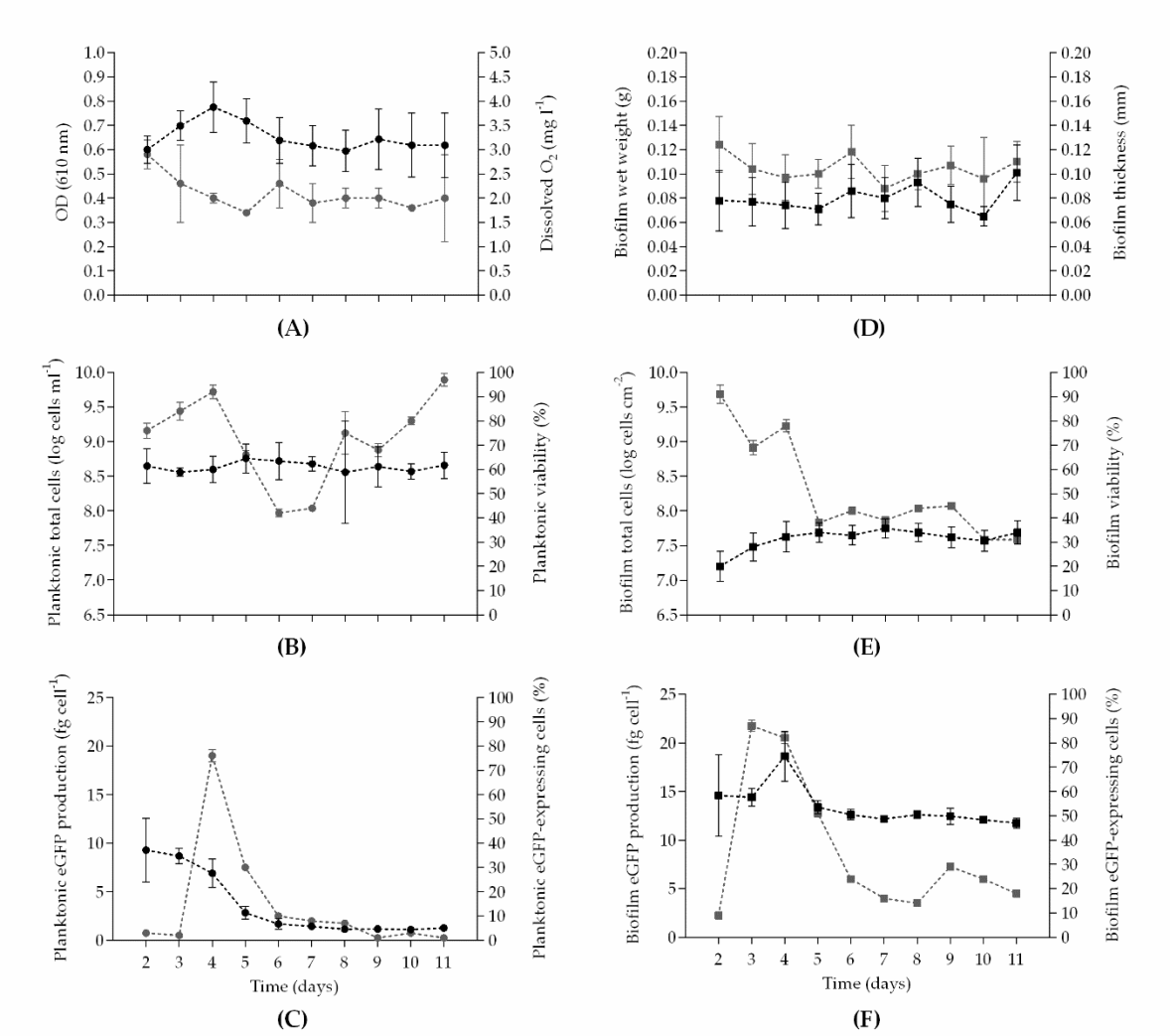


Figure 3.1. Time-course evaluation of planktonic and biofilm parameters: (**A**) $OD_{610\ nm}$ and dissolved oxygen in the recirculating tank, (**B**) planktonic total cells and planktonic viability, (**C**) planktonic eGFP production and percentage of planktonic eGFP-expressing cells, (**D**) biofilm wet weight and thickness, (**E**) biofilm total cells and biofilm viability, (**F**) biofilm eGFP production and percentage of biofilm eGFP-expressing cells. Black circles (●) and squares (■) on the left y-axis, and grey circles (●) and squares (■) on the right y-axis. The means ± SDs for three independent experiments are illustrated.

Regarding the biofilm growth, it was monitored by determining the wet weight and thickness (Figure 3.1D), and by quantifying the total cell number and cell viability (Figure 3.1E) as performed for planktonic cells. Biofilm wet weight remained practically constant

during the experimental time, but a slight increase was observed in the last day of the experiment. The evolution of biofilm thickness was similar to the wet weight with average values around 0.104 mm. Figure 3.1E shows that the total number of biofilm cells increased slightly between days 2 and 5, remaining constant until the end of the experiment. Concerning biofilm viability (Figure 3.1E), it is possible to observe a marked reduction in cell viability until day 5 (38%) that resulted in viability percentages of on average 39% until day 11. Note that lower viability percentages were found for biofilms (Figure 3.1E) when compared to planktonic population (Figure 3.1B).

By analysing the specific eGFP production (Figures 3.1C and F), it can be seen that sessile cells produced more recombinant protein than those grown in planktonic state. The biofilm environment enhanced the specific eGFP expression about 10-fold when compared to planktonic cells in steady state (statistically significant differences were confirmed for all experimental days, $p < 0.05$, except on day 2). Additionally, epifluorescence microscopy was used to determine the percentage of eGFP-expressing cells in both planktonic (Figure 3.1C) and biofilm populations (Figure 3.1F). The percentage of eGFP-expressing cells in planktonic state started to increase at day 3, reaching a maximum value of 76% at day 4 and then decreasing abruptly to values around 5% (Figure 3.1C). The equivalent biofilm curve (Figure 3.1F) also shows an initial increase in the percentage of expressing cells (with a maximum value of 87% between days 3 and 4), followed by a strong reduction (of approximately 58%) until day 6. In steady state, the percentage of planktonic expressing cells oscillated around 5%, whereas for biofilms, eGFP-expressing cells represented on average 21% of total cell population. Combining this information with the fluorimetric data, it is possible to conclude that *E. coli* biofilm cells can have a higher recombinant production capacity when compared to their planktonic counterparts under the tested conditions. This may be related with the highest potential of *E. coli* biofilms in maintaining the plasmid pFM23 within cells. In fact, in planktonic cells, for which the total cell number was constant over the course of the experiment (Figure 3.1B), the frequency of plasmid-containing bacteria was on average 0.33, while in the biofilm this parameter rose to approximately 0.90 in steady state (from day 5 onwards, Figure S 3.1).

3.4 **Discussion**

In this study, the production of a model recombinant protein (eGFP) in *E. coli* planktonic and biofilm cells was evaluated by fluorometry (a bulk method) and epifluorescence microscopy (a single-cell method).

We found that the specific eGFP production was lower in planktonic cells and this may have contributed to the higher viability of these cells when compared to biofilm cells. It is well documented that cells bearing a plasmid may suffer from a metabolic burden as cellular resources are being used for its replication and for the expression of plasmid-encoded genes [11, 27, 28]. In particular, a decreased cellular viability of plasmid-bearing cells and an increased protease activity are some stress signals suffered by these cells [29, 30].

The expression of recombinant proteins in *E. coli* biofilms was first reported by Huang et al. [18, 19, 31], who have studied the production of β-galactosidase in a chemostat and in a parallel plate flow. These authors found that the recombinant protein was successfully produced in biofilm cells, although at low level than in planktonic cells [18, 31]. In contrast, our results show that the *E. coli* biofilm environment enhanced eGFP production when compared to planktonic cells. Furthermore, biofilms presented a higher frequency of plasmid-bearing cells than the planktonic population. These results corroborate those obtained in a previous study of O'Connell et al. [22] who produced eGFP in a chemostat (planktonic cells) and in a PPFC reactor (biofilms). The results of their work indicated that the biofilm environment enhanced both plasmid maintenance and cellular eGFP concentrations when compared to planktonic cells. Continuous biofilm cultures for recombinant protein production can be more beneficial for retention of plasmid-bearing cells than chemostats [22] since cells in biofilms tend to grow more slowly than their planktonic counterparts [32], leading to fewer divisions and correspondingly less plasmid segregation. Other authors have also shown that the plasmid loss is more significant in planktonic populations [33-35]. More recently, our research group found that the volumetric productivity of the biofilms developed in the flow cell system is already within the range that can be obtained by conventional high cell density cultures, even before optimization of cultivation conditions [23].

During recombinant protein production, it is essential to monitor the amount of target protein produced in order to find the best processing conditions. The use of a fluorescent protein as a model recombinant protein enables bulk and single-cell quantification. Bulk production can be assessed by fluorometry, which is a high sensitive, specific and simple method with low instrumental costs when compared to other techniques, like flow cytometry, that are also used to follow recombinant protein expression [36, 37]. On the other hand, single-cell analysis by epifluorescence microscopy can provide information about the heterogeneity level of protein expression within a cell population [38]. In this work, the combination of these two techniques showed to be very useful in monitoring recombinant protein production in both planktonic and sessile cells.

3.5 Conclusions

This study revealed that the specific recombinant protein production and the percentage of eGFP-expressing cells were higher in *E. coli* biofilms than in cell suspension, which was associated with the highest potential of biofilms in retaining the plasmid.

When combined with a bulk method such as fluorimetry, epifluorescence microscopy and the corresponding image analysis is an innovative tool for determining important production parameters in both planktonic and sessile cells, namely the number of cells expressing the recombinant protein within a bacterial population. The methodology presented here is a simple, fast and low-cost way of obtaining information about the heterogeneity level of protein expression within a cell population and it can be further used to find the best processing conditions for recombinant protein production in biofilm reactors.

3.6 References

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3.7 **Supplementary material**

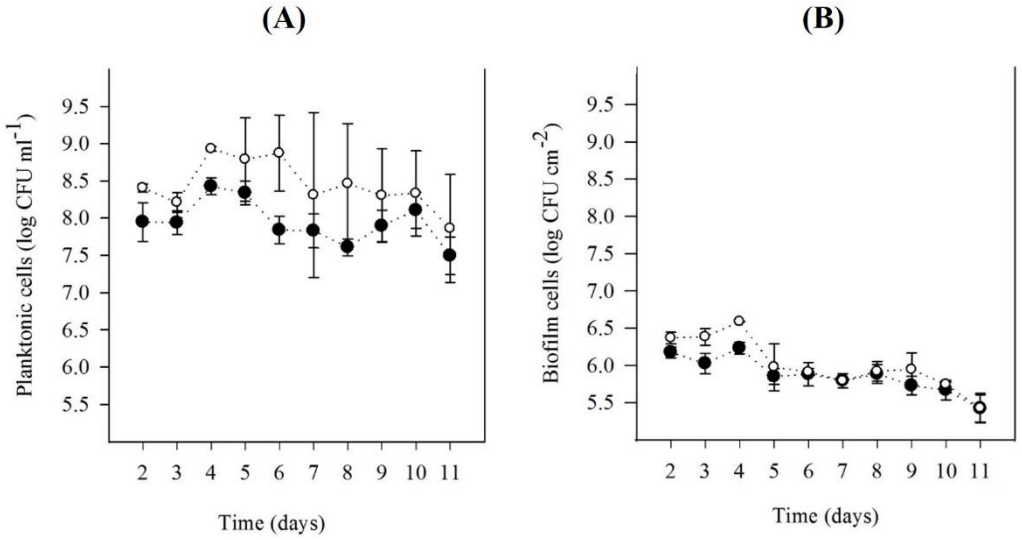


Figure S 3.1. Planktonic (**A**) and biofilm (**B**) population dynamics of plasmid-bearing cells (●) and total culturable cells (plasmid-bearing cells plus plasmid-free cells) (○). The means ± SDs for three independent experiments are illustrated.

4 The influence of nutrient medium composition on *Escherichia coli* biofilm development and heterologous protein expression

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Abstract

In the present study, the effect of different nutrient media on the development of *Escherichia coli* biofilms and the production of a heterologous protein were examined. *E. coli* JM109(DE3) cells transformed with pFM23 plasmid carrying the gene for enhanced green fluorescent protein (eGFP) expression were used. Cells were grown in two different culture media, Lysogenic Broth (LB) and M9ZB, in a flow cell system for 10 days. Epifluorescence microscopy, fluorimetry, and a high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC) were used to assess bacterial growth, plasmid copy number (PCN), and eGFP production in both planktonic and biofilm cells. The results showed that biofilm development was favoured in M9ZB medium when compared with LB. However, the number of eGFP-expressing cells was higher in LB for both planktonic and sessile states (2-fold and 7-fold, respectively). In addition, the PCN in biofilm cells was slightly higher when using LB medium (on average, 29 plasmids per cell versus 20 plasmids per cell in M9ZB), and higher plasmid stability was observed in biofilms formed in LB compared to their planktonic counterparts. Hence, *E. coli* biofilms grown in LB enhanced both plasmid

stability and capacity to produce the model heterologous protein when compared to M9ZB.

Keywords: biofilm; flow cell system; *Escherichia coli*; green fluorescent protein; culture medium; plasmid copy number

4.1 Introduction

Escherichia coli has been one of the most used organisms for heterologous protein production due to its inexpensive and fast high-cell density cultivation, well-known genetics, and large number of cloning vectors [1-3]. In addition to the host strain, factors such as the expression vector, cultivation conditions (medium composition, temperature, pH, and induction conditions), and purification strategies are essential for the successful production of heterologous proteins [4].

E. coli plasmids have been the preferred expression vectors because of the high gene dosage that can be attained and the capability to control the levels of gene expression through inducible promoters [5]. Although a high plasmid copy number (PCN) may ensure a high gene dosage, this does not always mean a high level of target heterologous protein [5, 6]. Studies in suspension cell cultures have demonstrated that high plasmid copy number imposes a metabolic burden on the host cell, which reduces the bacterial growth rate and favours plasmid instability, thereby decreasing the overall protein yield [7, 8].

The use of biofilm cells in recombinant protein production has been scarcely studied, but some promising results have been obtained by our research group in the last few years [6, 9-11]. The biological organization of biofilms provide them with many advantages over cells grown in suspension cultures, including high cell density, protection against hostile conditions, and operation stability, particularly when biofilms are used in biotechnological processes for the production of value-added compounds [12]. In addition, the presence of plasmids has been shown to stimulate biofilm formation and lead to a higher production of recombinant proteins compared to planktonic cells [10-15]. Our group has demonstrated that cells expressing eGFP (enhanced green fluorescent protein) formed more biofilm than non-expressing cells [10]. Moreover, *E. coli* biofilms were able to

produce eGFP at much higher level (30-fold) than their planktonic counterparts [10]. Furthermore, it has been shown that biofilm cultures have a beneficial effect on the maintenance of high and medium copy number plasmids [6, 16]. O'Connell et al. [16] reported that continuous biofilm cultures of *E. coli* ATCC 33456 containing the pEGFP plasmid (a pUC family vector) maintained a high PCN over extended periods, while in chemostat, a significant plasmid loss was observed. More recently, our research group showed that although the PCN was higher in planktonic cells, a significant PCN reduction (around 89%) was observed in planktonic cells after 5 days of flow cell operation, while no plasmid loss was observed in *E. coli* JM109(DE3) biofilms [6].

Different strategies have been explored to achieve maximum yields of heterologous proteins in suspended cell reactors, including by (1) changing the expression vector and/or the host, (2) optimizing the culture parameters (medium, pH and temperature) and induction conditions (inducer concentration and temperature) of the recombinant strain, (3) co-expressing other genes such as chaperones, and (4) adjusting the synthesis of stress-related sigma factors and/or deleting specific gene sequences such as proteases, which may promote both the quantity and quality of the desired protein [2, 3, 5, 17-19]. Nonetheless, the investigation of productive biofilms remains scarce. One of the parameters that can affect both biofilm formation and the level of heterologous protein expression is the culture medium composition. Several reports have demonstrated that the nutritional medium content affects biofilm formation [11, 20-22]. Some authors have shown that an increase in nutrient levels promotes biofilm formation [12, 13]. However, contradictory results showing that high nutrient concentration inhibits biofilm formation were also reported [14, 15]. Regarding the effect of nutrient medium composition on heterologous protein production, most of the studies were performed in planktonic conditions [23-25]. In these conditions, it has been demonstrated that the increased concentration of some salts, peptone, and yeast extract can increase the production of the desired protein [26, 27]. Furthermore, Matsui et al. [28] showed that the use of tryptone and yeast extract can increase plasmid stability. However, it is known that the high-level expression of heterologous protein in suspended cells often promotes a metabolic burden in the host cell that can affect plasmid stability and consequently the protein yield [29].

The main aim of this study was to determine the impact of different nutrient media compositions on biofilm development, plasmid stability, and eGFP expression in *E. coli* biofilm and planktonic cells. For that, *E. coli* JM109(DE3) cells transformed with the pFM23 plasmid for eGFP expression were used. Biofilm formation in two growth media, Lysogenic Broth (LB) and M9ZB, was carried out in a flow cell system for 10 days. LB medium is a rich medium that is frequently used for *E. coli* growth and the expression of heterologous proteins in *E. coli* [30]. M9ZB medium is the combination of M9 and ZB media, which are a minimal growth medium used for bacterial cultures and a medium recommended for the preparation of competent *E. coli* cells, respectively. M9ZB is also used for the cultivation of *E. coli* recombinant strains [31]. As far as we know, this is the first time that the M9ZB medium has been tested for biofilm formation and heterologous protein expression in sessile cells. On the other hand, this work enriches the group's previous studies [6, 11] with the assessment of the population of planktonic and biofilm cells that produce the protein of interest by a single-cell analysis, and the evaluation of plasmid stability by determining the PCN in both types of cells by a chromatographic method.

4.2 Materials and Methods

4.2.1 Bacterial Strain

The pFM23 plasmid (created from pET28A, Novagen, Madison, WI, USA) containing the eGFP gene was transformed by heat shock in *E. coli* JM109(DE3) (Promega, Madison, WI, USA) for the intracellular production of eGFP [32]. Transformants were selected on agar medium supplemented with 20 μg mL^{-1} kanamycin (Eurobio, Courtaboeuf, France).

4.2.2 Flow System

A flow cell system (Figure 4.1) containing a recirculating tank (planktonic cells) and a flow cell composed of removable coupons with polyvinyl chloride (PVC) slides for biofilm formation was operated as described by Gomes et al. [9]. Briefly, *E. coli* cells were

recirculated at 30 °C for 10 days under turbulent flow conditions (Reynolds number = 4600) [9], which are the hydrodynamic conditions previously used by the group [9, 10, 22, 33] and completely characterized by computational fluid dynamics (CFD) [34]. The tank was aerated and continuously fed with fresh medium at a flow rate of 0.025 L h^{-1} [9].

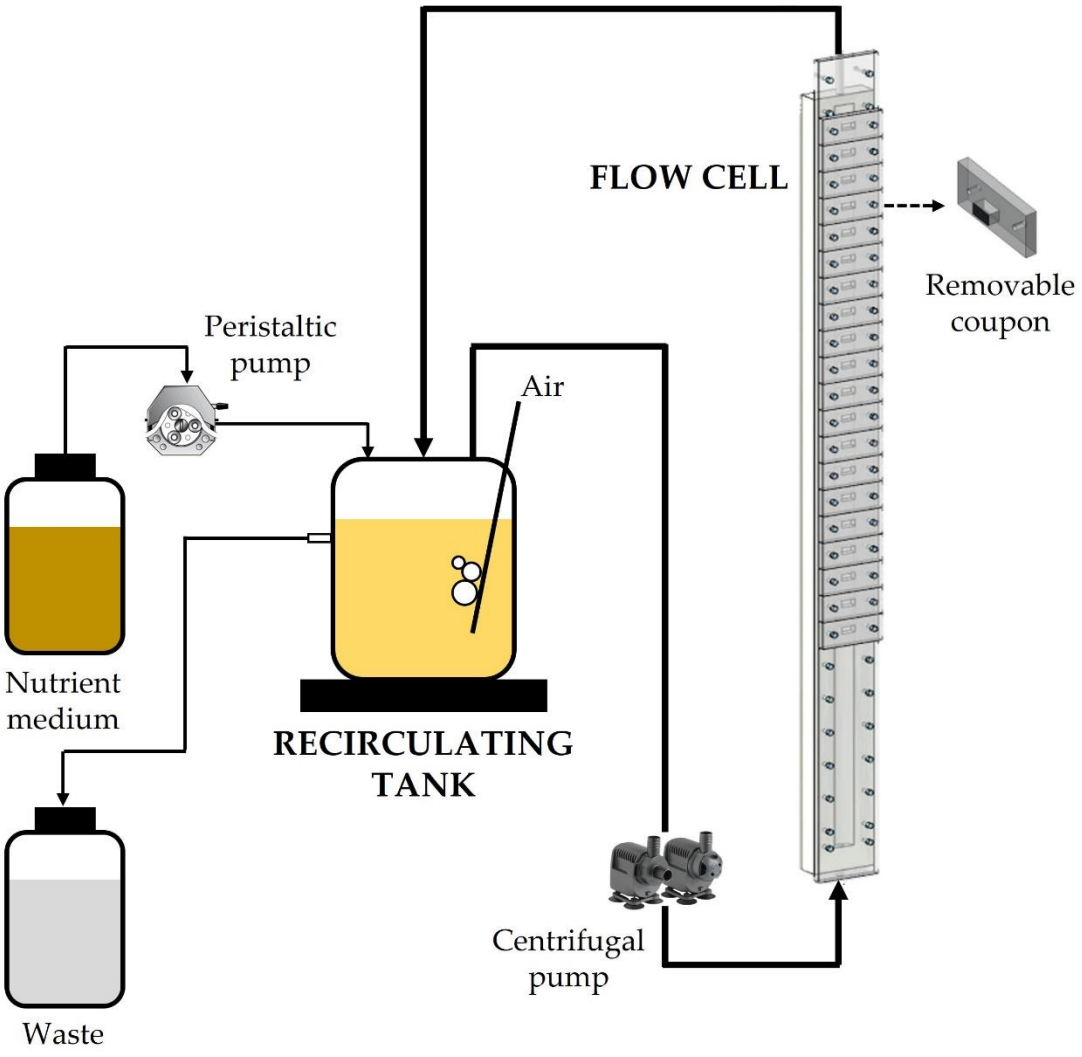


Figure 4.1. Schematic representation of the flow cell system (adapted from [3]).

To determine the effect of culture medium on biofilm formation and eGFP expression, two different media were tested: LB-Miller (Sigma, St. Louis, MO, USA) and M9ZB medium. According to the supplier, LB-Miller is made of 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl. The M9ZB medium was prepared as indicated by the Novagen pET System manual and Studier et al. [30, 35]. The mixture of 10 g L^{-1} N-Z-amine A and 5 g L^{-1} NaCl was first prepared. Then, 100 mL of 10× M9 salts solution (10 g L^{-1} NH_4Cl, 30 g L^{-1} KH_2PO_4, and 60 g L^{-1} $Na_2HPO_4{\cdot}7H_2O$), 1 mL of 1 M $MgSO_4$ solution, and 10 mL of

400 g L^{-1} glucose solution were added. All solutions were prepared and autoclaved separately. All compounds were purchased from Merck, Algés, Portugal. The comparison of carbon and nitrogen sources and amounts between media are presented in Table S 4.1 in Supplementary Material. Both liquid media were supplemented with 20 μg mL^{-1} kanamycin.

4.2.3 Biofilm and Planktonic Analysis

The system was stopped every day to allow biofilm sampling and restarted using the same flow rate. The biofilm thickness was first determined by using a digital micrometer [22]. Then, it was resuspended in 25 mL of 8.5 g L^{-1} NaCl solution for total and culturable cell quantification and eGFP and plasmid analysis. For biofilm culturability, samples were spread on solid growth medium (PCA; Merck, Algés, Portugal) supplemented with 20 μg mL^{-1} kanamycin, and colony enumeration was performed after incubation at 30 °C. Biofilm total cells were determined with the Live/Dead® BacLight™ bacterial viability kit (Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal) as described in Gomes et al. [9]. The final values of biofilm total and culturable cells were expressed as log cells cm^{-2} and log CFU cm^{-2}, respectively.

For planktonic fraction, a 10 mL sample was taken from the recirculating tank daily, and total and culturable cell numbers were assessed by the same methodology as biofilm cells. The final values of planktonic total and culturable cells were presented as log cells mL^{-1} and log CFU mL^{-1}, respectively.

4.2.4 Quantification of eGFP-Expressing Cells and Specific Protein Concentration

The number of eGFP-expressing cells in planktonic and biofilm cells was assessed by epifluorescence microscopy as indicated by Gomes et al. [9]. The percentage of eGFP-expressing cells was calculated by dividing the number of expressing cells by the total cell number.

The specific eGFP production was analysed by fluorimetry as fully described by Mergulhão and Monteiro [32]. The eGFP fluorescence was measured using a microplate reader (SpectraMax M2E, Molecular Devices, Inc., Berkshire, UK) with an excitation filter of 488 nm and an emission filter of 507 nm. Then, the eGFP concentration was calculated through a standard curve obtained from purified eGFP serially diluted (range of 0 to 0.31 μg μL^{-1}), and the final values were presented as specific eGFP production (fg $cell^{-1}$).

4.2.5 Plasmid Extraction and Quantification

Plasmid DNA was extracted by alkaline lysis as described by Alves et al. [36]. Briefly, bacterial pellets were resuspended in 100 μL of Solution I (50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, pH 8). Alkaline lysis was performed by adding 100 μL of Solution II (0.2 M NaOH, 1% (*w*/*v*) SDS) and incubating the mixture for 10 min at room temperature. Cellular debris, genomic DNA and proteins were precipitated by adding 100 μL of Solution III (5 M acetate/3 M potassium, pH 5) and incubating for 10 min in ice. The precipitate was removed by centrifuging twice at 20,000× *g* for 30 min at 4 °C (Centrifuge 5424, Eppendorf, Hamburg, Germany).

Plasmid quantification in biofilm and planktonic samples was performed according to the analytical high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC) as described by Diogo et al. [37]. A 4.6/100 mm HIC source 15 PHE PE column (Amersham Bioscience, Uppsala, Sweden) was connected to an HPLC system (Shimadzu, Nexera-i LC-2040C 3D, Duisburg, Germany) and equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl buffer, pH 8. Then, 50 μL of each sample was injected and eluted at 1 mL min^{-1}. After 1 min, the elution buffer was changed to 10 mM Tris–HCl without ammonium sulfate and maintained for 0.8 min in order to elute all bound species. Finally, the elution buffer was changed to the equilibration buffer and maintained for 8.2 min to re-equilibrate the column for the next run. The absorbance of the eluate was recorded at 260 nm. The plasmid concentration of the samples was calculated using calibration curves constructed with serial dilutions of plasmid DNA standards (ranging from 0.04 to 20 μg mL^{-1}). The calibration curves were established among the peak area appearing between 0.67 and 0.7 mL of elution volume

vs. the concentration of plasmid DNA standard [37]. Then, the plasmid copy number (PCN) was calculated using Equation (1) [38]:

$$\mathrm{PCN} = \frac{6.02 \times 10^{23}\ (\mathrm{copy\ mol^{-1}}) \times \mathrm{DNA\ amount\ (g)}}{\mathrm{DNA\ length\ (bp)} \times 660\ (\mathrm{g\ mol^{-1}\ bp^{-1}})} \quad (1)$$

where the size of pFM23 plasmid is 6053 bp.

4.2.6 Statistical Analysis

The results originated from averages of independent experiments for each culture condition (LB and M9ZB). All reported data are presented as mean ± standard deviation (SD) from at least three experiments with triplicates.

Paired *t*-test analysis was conducted to determine the differences between culture media on each experimental day. Statistically significant differences were reported for p values < 0.1 (corresponding to a confidence level of 90%) and <0.05 (corresponding to a confidence level of 95%).

4.3 Results

In this study, two different nutrient media were used with the aim of comparing plasmid stability and eGFP production in biofilm and planktonic populations growing in a flow cell system.

Planktonic fraction was monitored by quantifying the total and culturable cells (Figure 4.2A and C, respectively). Analysis of the planktonic total cells (Figure 4.2A) indicated that there were very few statistical differences in *E. coli* growth in both media (only one of the eight experimental points has $p < 0.05$). Despite the few statistical differences, from day 5 onwards, it seems to be a higher cell number of bacteria when growing in LB medium, with on average 29% more cells being present in LB than in M9ZB until the end of the experiment. Regarding the number of planktonic culturable cells (Figure 4.2C), similar behaviour was also observed for both media, but slightly higher

CFU mL^{-1} values were obtained in M9ZB in all experimental points (on average 62%) when compared to LB.

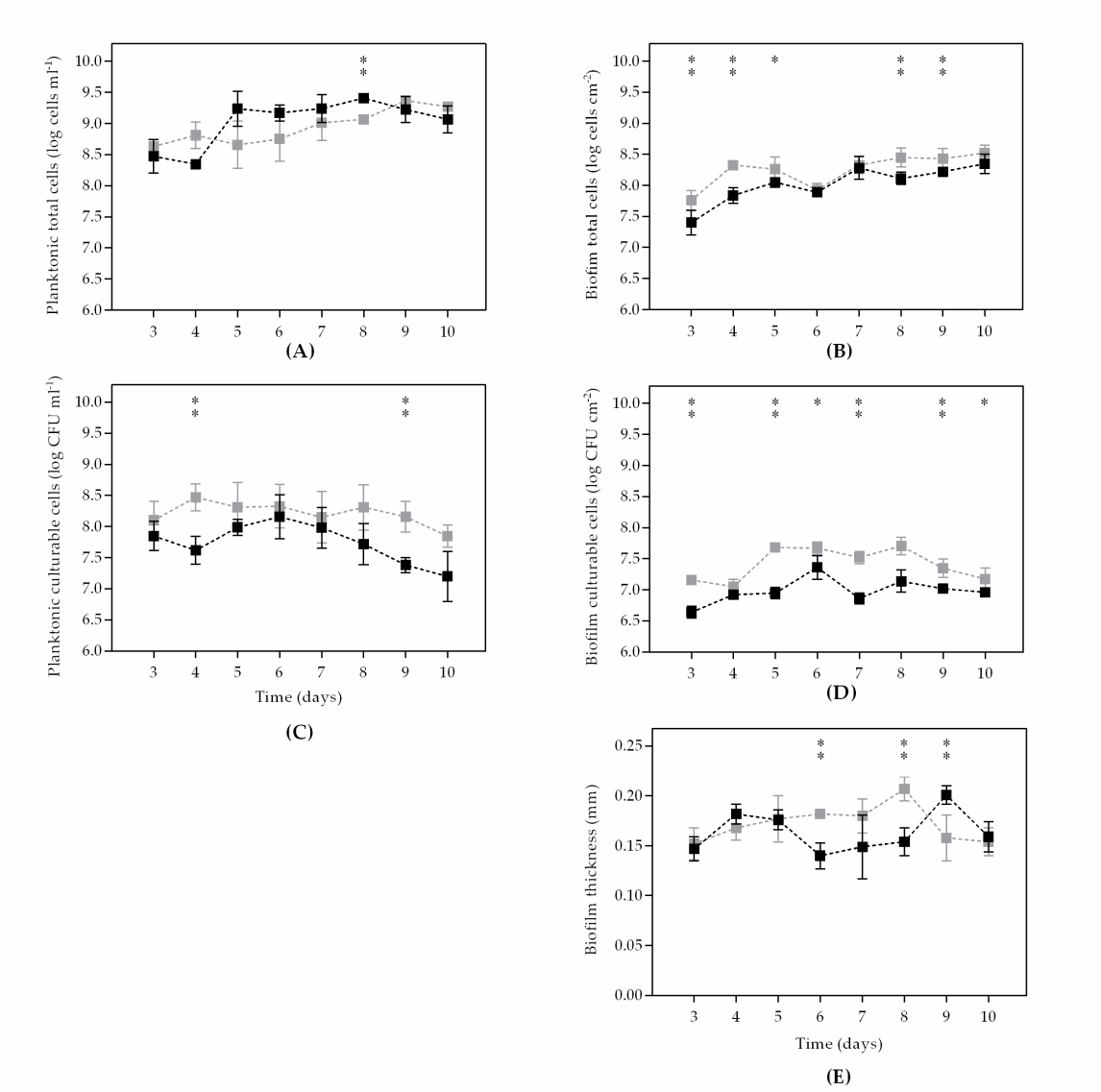


Figure 4.2. Planktonic and biofilm monitoring in LB (black squares) and M9ZB (gray squares): (**A**) planktonic total cells, (**B**) biofilm total cells, (**C**) planktonic culturable cells, (**D**) biofilm culturable cells, and (**E**) biofilm thickness. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 90% (an asterisk indicates $p < 0.1$) and 95% (a double asterisk indicates $p < 0.05$).

The impact of nutrient medium composition on biofilm development was assessed by quantifying total and culturable cells and determining the biofilm thickness (Figure 4.2B, D and E, respectively). Globally, the amount of biofilm total cells (Figure 4.2B) was

higher for M9ZB (on average 8.25 log cells cm^{-2}) than for LB (on average 8.0 log cells cm^{-2}) with statistically significant differences for five of the eight experimental points ($p < 0.1$). When comparing biofilm culturable cells (Figure 4.2D), higher values were also obtained for M9ZB (on average 65%) with statistical differences in most time points ($p < 0.1$ for days 6 and 10, and $p < 0.05$ for days 3, 5, 7 and 9). Biofilm thickness (Figure 4.2E) was similar between growth media until day 5, but thereafter, the biofilms developed in M9ZB were thicker than biofilms formed in LB (on average 176 μm vs. 161 μm, respectively), which is most likely a result of their higher cell density (Figure 4.2B,D).

Looking at the results of heterologous protein expression (Figure 4.3), the number of planktonic cells expressing eGFP relative to the number of total cells (Figure 4.3A) was slightly higher for LB medium in most experimental points. The number of eGFP-expressing cells in both media increased until day 6, when on average 27% of the total cells produced eGFP. Regarding the percentage of eGFP-expressing cells in biofilm (Figure 4.3B), it can be seen that in M9ZB, this parameter remained almost constant throughout the experiment (around 7%, with the maximum value of 13% on day 6), while in LB, a strong increase was observed until day 6 when 66% of the total cells expressed eGFP ($p < 0.05$). From day 6 onwards, the amount of expressing cells decreased about 20% and was nearly similar until the end of the experiment. Furthermore, in LB medium, the number of sessile cells expressing eGFP was about 54% higher than in *E. coli* suspensions, while in M9ZB, the number of expressing cells in biofilm was similar to its planktonic counterparts (Figure 4.3A,B).

The specific eGFP values (Figure 4.3C,D) showed that, for both planktonic and biofilm states, the cells grown in LB synthesized more protein than cells grown in M9ZB. On average, the eGFP production in LB medium was 8-fold and 3-fold higher for planktonic and biofilm cells, respectively, than in M9ZB. Additionally, in M9ZB, the eGFP levels remained practically constant over time for both planktonic and biofilm states (around 0.31 fg $cell^{-1}$ and 1.51 fg $cell^{-1}$, respectively), whereas in LB, the specific eGFP concentration increased until days 5 and 7 for planktonic and biofilm environments, respectively, reaching a maximum of 4.31 fg $cell^{-1}$ and 15.96 fg $cell^{-1}$, and then it started to decrease, reaching the steady state.

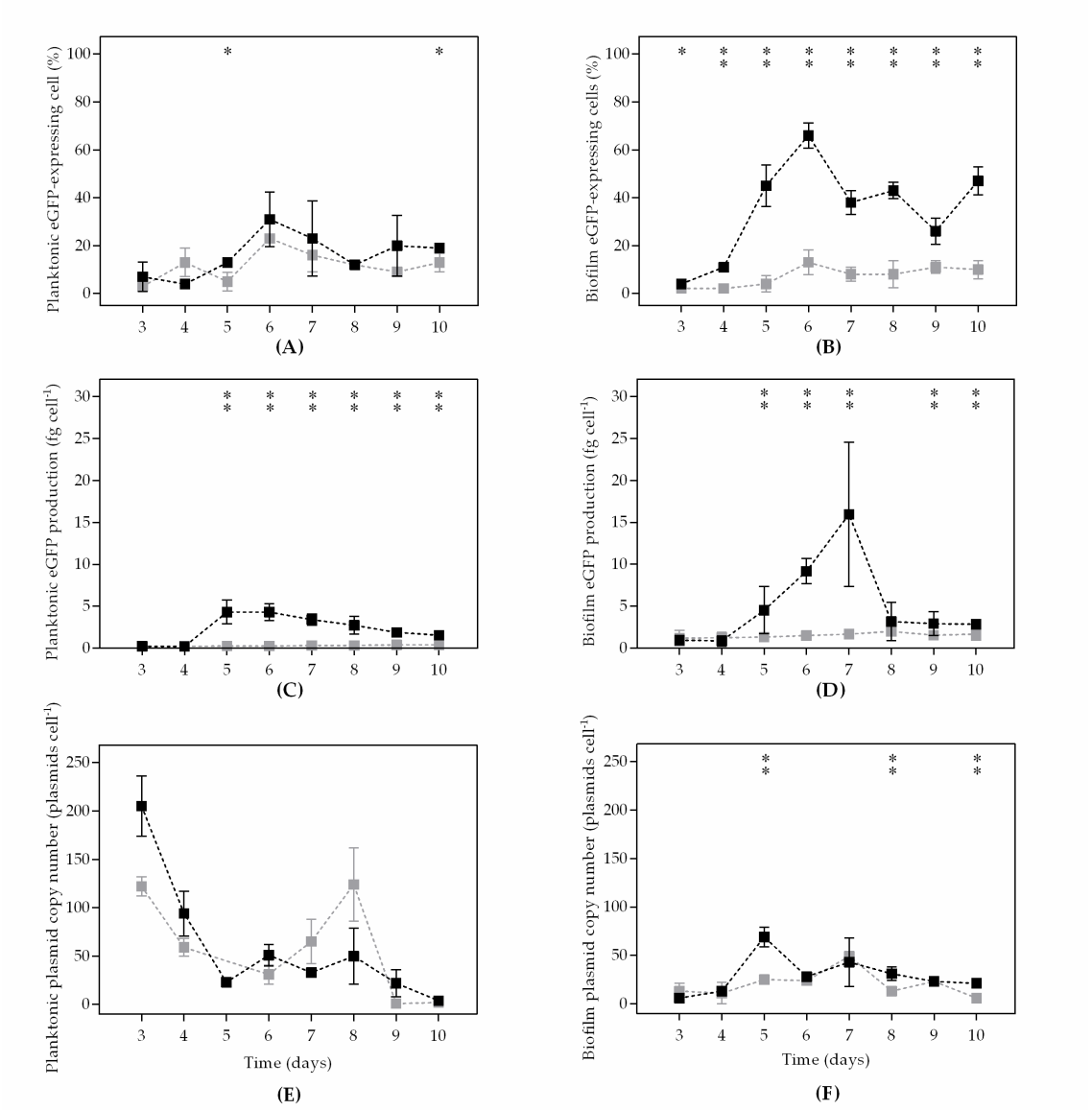


Figure 4.3. Planktonic and biofilm monitoring in LB (black squares) and M9ZB (gray squares): (**A**) planktonic eGFP-expression cells, (**B**) biofilm eGFP-expression cells, (**C**) planktonic eGFP production, (**D**) biofilm eGFP production, (**E**) planktonic plasmid copy number, (**F**) biofilm plasmid copy number. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 90% (an asterisk indicates $p < 0.1$) and 95% (a double asterisk indicates $p < 0.05$).

In general, the PCN (Figure 4.3E,F) was higher in planktonic cells for both culture media (on average 3-fold for M9ZB and 2-fold for LB). In planktonic cells (Figure 4.3E) grown in LB, a strong reduction in PCN (around 89%) was observed until day 5, and from then on, the PCN levels were kept between 4 and 51 plasmids per cell. On the other hand, in planktonic cells grown in M9ZB medium, following an initial decrease by 75% until

day 6, the PCN increased until day 8 to the values registered at the start of the experiment, decreasing to about 2 plasmids per cell in the next two days. In biofilm cells (Figure 4.3F), the PCN reached its maximum at days 5 and 7 in LB and M9ZB, respectively. Then, sessile cells in LB lost about 59% of plasmid content, maintaining values around 29 plasmids per cell until the end of the assay, while in M9ZB, this reduction was about 75% with cells keeping on average 14 plasmids inside them.

4.4 Discussion

Heterologous protein production using *E. coli* biofilms has shown to be advantageous for many reasons, including higher plasmid copy numbers [16] and higher level of protein production compared to their planktonic counterparts [10, 39]. However, the most important goal of heterologous protein production is to achieve high protein production at low cost. Several parameters, such as nutrient medium composition, temperature, hydrodynamic conditions, and surface properties can affect biofilm formation [11, 22, 40] and consequently influence the final protein yield.

In a previous study [11], our research group compared the effect of two culture media—a diluted medium (DM) and LB—and concluded that biofilms grown in LB produced more eGFP than those grown in DM. In this preliminary work from 2017, primacy was given to culture media typically used to promote biofilm growth such as DM [22, 33], while here, there was a concern in testing media recommended for the high expression of recombinant proteins in *E. coli* such as the M9ZB.

Planktonic results showed a higher number of culturable cells in M9ZB compared to LB medium. This could be related to the presence of glucose in M9ZB medium, which is a compound that is absent in the LB medium used. Previously, Tripathi et al. [41] also found that a culture of *E. coli* DH5α cells harbouring the recombinant plasmid pMAL-c2X achieved higher cell densities in media with high concentration of glucose, such as Tryptone Yeast (TY) medium, Terrific Broth (TB), and Super Broth (SB).

Biofilm formation was also favoured in M9ZB medium, as indicated by the higher values of both culturable and total cells when compared to LB. Several studies have shown that the nutritional content of the medium, namely carbon and energy substrates,

affects biofilm formation [42, 43]. Dewanti and Wong [44] observed that using a minimal salts medium (MSM) supplemented with glucose resulted in a stable biofilm with shorter bacterial cells and a thicker extracellular matrix. Buhler et al. [13] have also reported that increasing the glucose concentration of culture medium can promote the growth of *E. coli* nutrient-depleted biofilms.

Concerning heterologous protein production, a higher number of cells expressing eGFP and higher specific eGFP concentrations were found in LB for both planktonic and sessile cells. Complex media, such as LB, contain yeast extract and tryptone that are known to provide biosynthetic precursors, such as vitamins and amino acids, that support protein expression in *E. coli* [26, 27]. Furthermore, yeast extract can also be responsible for reducing the cellular stress (protease production) during the synthesis of recombinant proteins [45]. Similar to what was observed in the present work with LB, Goyal et al. [26] found that a synthetic medium containing yeast extract and tryptone resulted in higher Streptokinase protein concentration in *E. coli*, without increasing cell density [26].

The lower eGFP expression in M9ZB medium could be related to the presence of glucose in the medium by repressing the lac promoter [30, 46]. In the present work, a pET system was used, where the eGFP gene is transcribed by T7 RNA polymerase, which is regulated by the lacUV5 promoter [4]. It has been shown that glucose reduced the basal expression by reducing cyclic adenosine monophosphate (cAMP) levels, resulting in a low transcription from lacUV5 promoter [46]. Jevševar et al. [47] confirmed that glucose supplementation represses the expression of recombinant human granulocyte colony-stimulating factor (hG-CSF). On the other hand, it was revealed that the addition of yeast extract to the culture media increases the background expression from the lac promoter [48, 49].

Regardless of the culture medium used, there was an increase in the percentage of eGFP-expressing cells in both planktonic and biofilm between days 5 and 7. This phenomenon may be associated with the higher number of culturable cells also detected in the same period. It is expected that metabolically active cells such as the culturable cells are more likely to be eGFP-producing cells, as they have more biosynthetic precursors, energy, and other cellular components available for the synthesis of the foreign protein. In the particular case of biofilms grown in LB medium, the higher levels of expressing

cells on days 5–7 may also be related to the simultaneous decrease in biofilm thickness, since the transfer of oxygen for the maturation of the heterologous protein under study is facilitated in thinner biofilms [9].

Plasmid stability is an important factor in heterologous protein production [5]. In this work, high specific eGFP concentration was obtained in biofilm cells, although on average, higher copies of plasmid were found in planktonic cells. These results suggest that the gene dosage was not responsible for the expression of eGFP. The same phenomenon was observed by Gomes et al. [6], where biofilm cells had more eGFP concentration even though they presented 7-fold lower PCN than planktonic cells. In the present work, the low eGFP expression levels in planktonic cells for both media may be caused by the high metabolic burden linked to plasmid replication. This added metabolic burden usually causes structural and segregational plasmid instability as well as several metabolic changes in the host cell, which may reduce the yield and activity of the heterologous protein [29, 50].

Although a small number of plasmids were found in biofilm cells in LB, the biofilm environment seemed to be advantageous for plasmid retention, since the PCN decrease was more accentuated in suspended cells than in biofilms formed in the flow cell system (89% vs. 59% reduction). Similar to our results, O'Connell et al. [16] demonstrated that the *E. coli* cultivation as a biofilm has a positive effect on high copy number plasmid maintenance compared to chemostats. The reason for the higher plasmid maintenance in biofilms is that sessile cells tend to grow more slowly than planktonic cells [51], leading to fewer cell divisions and consequently less plasmid partitioning. Furthermore, it was reported that the use of yeast extract and tryptone as nitrogen sources may result in higher plasmid stability [28].

The results obtained in this optimization work are essential to define the operating conditions to be used in further studies focused on the synthesis of other heterologous proteins of different sizes and involved in synthetic pathways for the production of metabolites of high market value.

4.5 **Conclusions**

Biofilm cells have shown potential to be used as microbial cell factories, although the influence of environmental factors in the production of high added-value compounds has barely been explored. This study confirmed that heterologous protein production in *E. coli* biofilms is affected by nutrient conditions. Although the biofilm development was favoured in M9ZB medium when compared with LB, the number of eGFP-expressing cells and the eGFP yield were higher in LB, which is the medium with higher nitrogen content (yeast extract and tryptone). In addition, the PCN in biofilm cells was slightly higher when using LB. On the other hand, the use of M9ZB medium, which has glucose as carbon source, had an inhibitory effect on eGFP expression.

This work is a contribution to the study of operational conditions affecting heterologous protein production in biofilms systems in order to maximize protein production.

4.6 References

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4.7 Supplementary Material

Table S 4.1. Comparison of carbon and nitrogen sources and corresponding concentrations between M9ZB and LB media.

		M9ZB	**LB**
Carbon	Source	Glucose	Tryptone and yeast extract
	Concentration	1.6 g L^{-1} *	Residual **
		About 100% higher in M9ZB	
Nitrogen	Source	Amine and ammonium chloride	Tryptone and yeast extract
	Concentration	1.37 g L^{-1} *	1.59-1.99 g l^{-1} *
		16-45% higher in LB	

*based on suppliers; **based on Podpora et al. [1]

4.7.1 References

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5 Hydrodynamic effects on biofilm development and recombinant protein expression

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Abstract

Hydrodynamics play an important role in the rate of cell attachment and nutrient and oxygen transfer, which can affect biofilm development and the level of recombinant protein production. In the present study, the effects of different flow conditions on the development of *Escherichia coli* biofilms and the expression of a model recombinant protein (enhanced green fluorescent protein, eGFP) were examined. Planktonic and biofilm cells were grown at two different flow rates in a recirculating flow cell system for 7 days: 255 and 128 L h^{-1} (corresponding to a Reynolds number of 4600 and 2300, respectively). The fluorometric analysis showed that the specific eGFP production was higher in biofilms than in planktonic cells under both hydrodynamic conditions (3-fold higher for 255 L h^{-1} and 2-fold higher for 128 L h^{-1}). In the biofilm cells, the percentage of eGFP-expressing cells was on average 52% higher at a flow rate of 255 L h^{-1}. Furthermore, a higher plasmid copy number (PCN) was obtained for the highest flow rate for both planktonic (244 PCN $cell^{-1}$ versus 118 PCN $cell^{-1}$) and biofilm cells (43 PCN $cell^{-1}$ versus 29 PCN $cell^{-1}$). The results suggested that higher flow velocities promoted eGFP expression in *E. coli* biofilms.

Keywords: biofilm; flow cell system; *Escherichia coli*; green fluorescent protein; hydrodynamic conditions; plasmid copy number

5.1 Introduction

Recombinant DNA methodology is very important for biotechnological industries because by using this technology, it is possible to produce proteins at much higher levels than are found naturally [1, 2]. Although most heterologous proteins are produced on an industrial scale in suspended cells, it is known that the high-level production of heterologous proteins using planktonic cells has an associated metabolic burden on the host cell that can affect plasmid stability and protein yield [3]. Bacterial biofilms have recently received increased attention in terms of the production of added-value compounds since they present many advantages over their free and planktonic counterparts, such as a higher cell density, protection against hostile conditions, higher operation stability [4, 5], and greater plasmid stability [6, 7]. Biofilms are communities of surface-attached microorganisms that are encased in a self-produced matrix of extracellular polymeric substances (EPS) [8]. Bacterial biofilms can contain multiple cell layers and their thickness can vary from a few to many μm [8]. The biological organization of biofilms can promote different rates of growth and metabolic activity within the biofilm cells, which can be a consequence of different diffusion rates of oxygen and nutrients inside the biofilms [9]. Microbial adhesion and the subsequent biofilm development can be affected by several factors, such as nutrient levels, surface properties, and hydrodynamic conditions [10-13].

Hydrodynamics is one of the most important factors that affects biofilm development since it determines the shear stress on the surfaces on which the biofilms form and the mass transfer of nutrients, oxygen, and bacteria from the bulk medium to the biofilm [14-16]. A higher flow velocity enables a higher external mass transfer, which means that more nutrients and oxygen are brought to the surface of the biofilm [15]. Hydrodynamic conditions also influence biofilm formation and structure due to the shear forces, which can modulate microbial cell adhesion to and detachment from a given surface [17]. A higher shear stress usually results in thinner, denser, and stronger biofilm, while lower shear stress conditions result in biofilms with thick multilayer structures [8, 18]. Although a higher flow velocity increases the supply of nutrients and oxygen, the biofilm

density can reduce the diffusion or penetration of the substrate into the inner layers of the biofilm [19].

The successful production of recombinant proteins in bacterial cells depends on sufficient nutrients and oxygen supply. Although biofilm reactors have been shown to have several advantages over planktonic cultures for recombinant protein production, the diffusion of substrates into the sessile cells is still a significant limitation [5, 20]. It has also been shown that nutrients are mostly consumed by the outer cell layers before reaching the inner layers of the biofilm [19, 21]. Recently, our research group demonstrated that the eGFP expression in *E. coli* biofilms is highly heterogeneous, with the cells that express the protein being located mostly on the top of the biofilm [22]. This is probably due to limitations in the mass transfer of oxygen and nutrients from the bulk liquid to the biofilm. Nevertheless, Huang et al. [23] showed that diffusion limitations can have a beneficial effect on plasmid stability since they can lower the cell growth rates inside the biofilm. Recombinant protein production in biofilm cells has been mostly performed in static conditions or using low laminar flow rates (Reynolds numbers (Re) of 20 and 32) [6, 23-25]. Despite the lack of information on recombinant protein production in biofilms, it has been reported that agitation rate plays an important role in nutrient and oxygen diffusion within immobilised cells systems [26]. By using immobilised cells in sodium alginate for the production of recombinant aspartase, Singh and Yadav [27] demonstrated that a higher production rate (1234 U g^{-1} wet cells) was obtained with an agitation rate of 250 rpm compared to the lower agitation of 130 rpm (534 U g^{-1} wet cells). However, a high agitation rate (270 rpm) decreased the production to 931 U g^{-1} wet cells. Nevertheless, Man et al. [28] observed that an increased agitation rate negatively affected plasmid stability in the immobilised cells. However, the higher plasmid stability that was obtained at lower agitation rates was not associated with the increased excretion of the cyclodextrin glucanotransferase enzyme [28].

The aim of this work was to investigate the effects of flow rate variation on the development of *E. coli* biofilms and eGFP expression. For that, two flow conditions were tested: 255 and 128 L h^{-1} (corresponding to Re of 4600 and 2300, respectively). The flow cell system that was used in this study has been extensively characterized in terms of its hydrodynamics [15, 16, 29, 30]. Turbulent conditions (Re of 4600) have already been tested in

previous works using the same flow cell system for eGFP production [22, 31], but laminar (Re < 2300) and transient conditions (2300 < Re < 4000) could also be achieved with this system. The variations in this operational parameter are discussed here for the first time.

5.2 Materials and Methods

5.2.1 Bacterial Strain

The *E. coli* JM109(DE3) (Promega, Madison, WI, USA) was transformed by heat shock with the plasmid pFM23 (constructed from pET28A; Novagen, Madison, WI, USA), which contained the eGFP gene [32]. The transformants were selected on an agar medium that was supplemented with 20 μg mL^{-1} of kanamycin (Eurobio, Orsay, France).

5.2.2 Flow Cell System and Experimental Conditions

A flow cell system (Figure S 5.1 of Supplementary Material) composed of a vertical semicircular flow cell with removable coupons on which biofilms were formed and a recirculating tank (planktonic cells) was used, as described by Gomes et al. [22]. *E. coli* cells were grown by recirculating the bacterial suspension at 30 °C for 7 days at two different flow rates: 255 L h^{-1} (Re of 4600) and 128 L h^{-1} (Re of 2300). The Reynolds number was defined as (Equation 1):

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

where ρ is the density of water at 30 °C (kg m^{-3}), U is the linear flow velocity (m s^{-1}), D_h is the hydraulic equivalent diameter of the semicircular flow cell (D_h= $\pi D/(2+\pi)$ = 1.83 cm) of diameter D (m), and μ is the dynamic viscosity of water at 30 °C (kg (m^{-3} s^{-1})).

The average wall shear stress (τ_w) values for the two tested flow conditions (Re of 4600 and 2300) were estimated using the dimensionless Darcy friction factor (f), which was obtained from the work of Teodósio et al. [16], and applying Equation (2):

$$f = \frac{4\tau_w}{\rho U^2/2} \quad (2)$$

where ρ is the density of water at 30 °C (kg m^{-3}) and U is the linear flow velocity (m s^{-1}). A Re of 4600 and 2300 in the flow cell system corresponded to τ_w values of approximately 0.3 and 0.1 Pa, respectively.

The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with Terrific Broth (TB) medium, which was supplemented with 20 μg mL^{-1} of kanamycin, at a flow rate of 0.025 L h^{-1} [22]. In a recent screening test, our research group found that the eGFP production was higher using the TB medium compared to Lysogenic Broth (LB) (data not shown). The TB medium is composed of 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 2.31 g L^{-1} KH_2PO_4, 12.54 g L^{-1} K_2HPO_4 and 4 mL glycerol. All compounds were purchased from Merck, Algés, Portugal.

5.2.3 Biofilm and Planktonic Monitoring

For the biofilm sampling, the system was stopped every day for coupon removal and was then carefully restarted at the same flow rate [22]. The biofilm wet weight and thickness were firstly determined by subtracting the coupon weight on the sampling day from the weight prior to the start of the experiment and by using a digital micrometer, respectively [29]. Then, the biofilm was resuspended in 25 mL of 8.5 g L^{-1} NaCl solution for total, viable, and eGFP-expressing cells quantification and also for eGFP production and plasmid analysis. The biofilm total and viable cells were determined using the Live/Dead® BacLight™ bacterial viability kit (Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal), as fully described by Gomes et al. [22]. Briefly, each biofilm cell suspension was filtered through a Nucleopore Track-Etch Membrane of black polycarbonate (pore size 0.22 μm; Whatman Ltd., Banbury, UK), stained for 7 min in the dark, and then observed using an epifluorescence microscope (Leica DM LB2; Leica Microsystems Ltd., Heerbrugg, Switzerland) that was connected to a Leica DFC300 FX camera. ImageJ v1.48 software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the number of total and viable cells from the counts of a minimum of 15 fields of view per sample and the final values were expressed as log cells cm^{-2}.

For planktonic cells, a daily 10 mL sample was taken from the recirculating tank to assess the total and viable cell numbers and to perform eGFP and plasmid analysis. The

total and viable cell numbers were assessed using the same methodology as for the biofilm cells and the final values were presented as log cells mL^{-1}.

5.2.3.1 Assessment of eGFP-expressing Cells and Specific Protein Concentrations

The eGFP-expressing cells were observed and counted using epifluorescence microscopy with a Leica DM LB2 epifluorescence microscope, as described by Gomes et al. [22]. The final values were presented as log cells mL^{-1} and log cells cm^{-2} for planktonic and biofilm samples, respectively.

The specific eGFP production was analysed using the fluorometric method that was described by Mergulhão and Monteiro [32]. A microplate reader (SpectraMax M2E, Molecular Devices, Inc., Wokingham, UK) was used to measure the eGFP fluorescence using an excitation filter of 488 nm and an emission filter of 507 nm. The eGFP concentration was then calculated using a standard curve that was obtained from purified eGFP (ranging from 0 to 0.31 μg μL^{-1}), and presented as specific eGFP production (fg $cell^{-1}$).

5.2.3.2 Plasmid Extraction and Quantification

The plasmid DNA was extracted using alkaline lysis and quantified using the analytical high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC), as fully described by Soares et al. [7]. A 4.6/100 mm HIC source of 15 PHE PE columns (Amersham Bioscience, Uppsala, Sweden) that was connected to an HPLC system (Shimadzu, Nexera-i LC-2040C 3D, Duisburg, Germany) was used. The plasmid concentration was calculated using calibration curves that were constructed from the serial dilutions of the plasmid DNA standards (ranging from 0.04 to 20 μg mL^{-1}). Then, the corresponding plasmid copy number (PCN) was calculated using Equation (3) [33]:

$$\mathrm{PCN} = \frac{6.02 \times 10^{23}\ (\mathrm{copy\ mol^{-1}}) \times \mathrm{DNA\ amount\ (g)}}{\mathrm{DNA\ length\ (bp)} \times 660\ (\mathrm{g\ mol^{-1}\ bp^{-1}})} \quad (3)$$

where the size of plasmid pFM23 was 6053 bp.

5.2.4 Statistical Analysis

The results that are presented in Figures 5.1 and S 5.2 (Supplementary Material) were the averages of at least three independent experiments for each flow rate condition (255 and 128 L h^{-1}). All reported data are presented as the mean ± standard deviation (SD) from the three replicates of each independent experiment.

A paired *t*-test analysis using GraphPad Prism 6.01 software (San Diego, CA, USA) was performed based on a confidence level of 90% (differences are reported as significant for p values < 0.1) and a confidence level of 95% (differences are reported as significant for p values < 0.05).

5.3 Results

In this study, the effects of hydrodynamics on eGFP production and plasmid stability in *E. coli* planktonic and biofilm cells were assessed by testing two different flow rates: 255 L h^{-1} (Re of 4600) and 128 L h^{-1} (Re of 2300).

The number of plasmids per cell (PCN $cell^{-1}$; Figure 5.1A,B) was higher at the highest flow rate for both planktonic and biofilm cells (on average 2- and 1.5-fold higher, respectively). In planktonic cells (Figure 5.1A), PCN increased until day 4, on which it reached a maximum value of 377 PCN $cell^{-1}$ for the highest flow rate and 221 PCN $cell^{-1}$ for the lowest flow rate that was tested. After that PCN peak, a continuous decrease was observed until the end of the experiment. The same initial behaviour was observed in the biofilm cells (Figure 5.1B), with a maximum value of 113 PCN $cell^{-1}$ for the highest flow rate and 51 PCN $cell^{-1}$ for the lowest flow rate on day 4. From day 4 onwards, PCN decreased and reached a steady-state of 42 PCN $cell^{-1}$ for the highest Re and 38 PCN $cell^{-1}$ for the lowest Re.

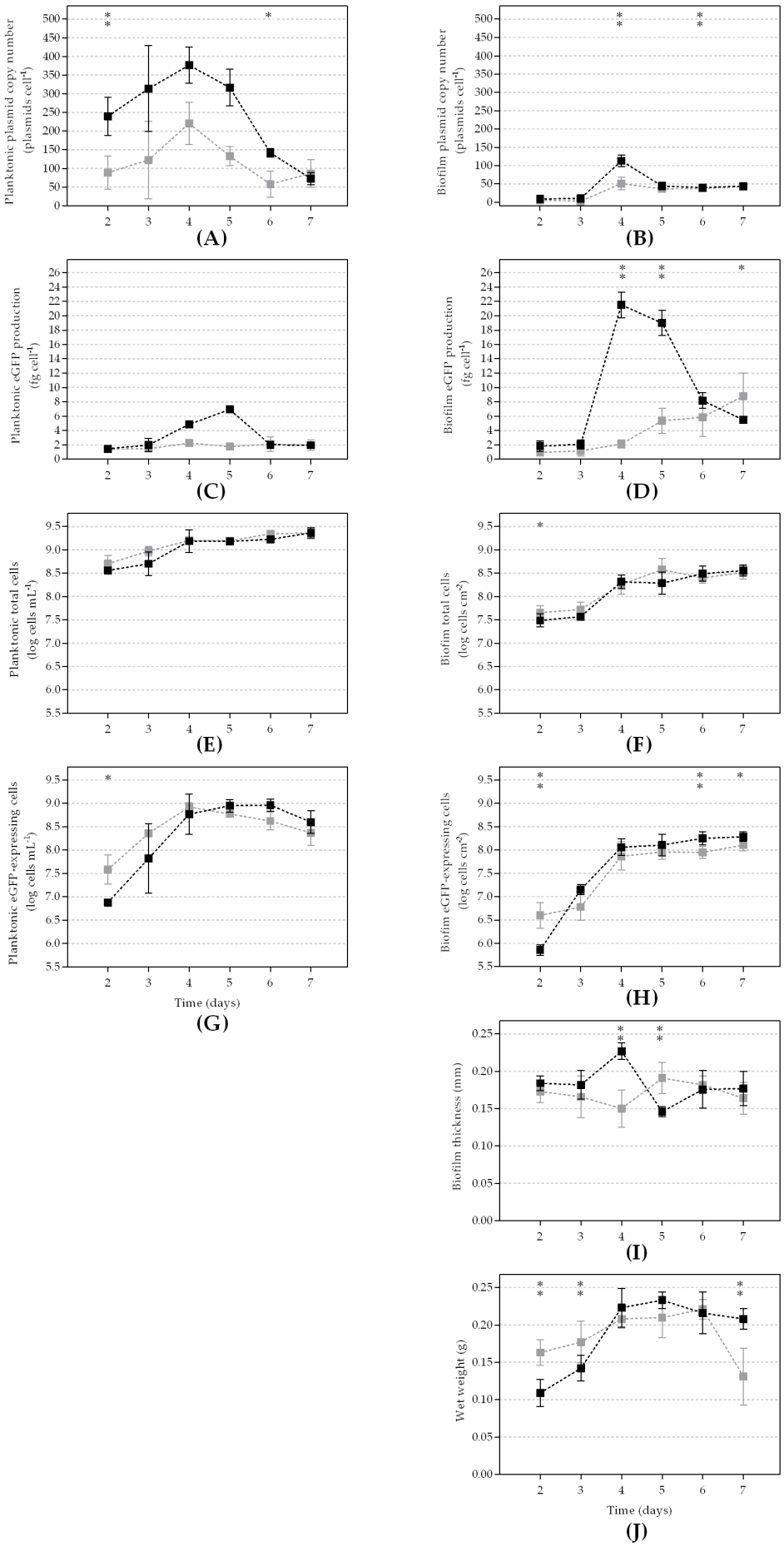

Planktonic plasmid copy number (plasmids cell-1)
(A)
Biofilm plasmid copy number (plasmids cell-1)
(B)
Planktonic eGFP production (fg cell-1)
(C)
Biofilm eGFP production (fg cell-1)
(D)
Planktonic total cells (log cells mL-1)
(E)
Biofim total cells (log cells cm-2)
(F)
Planktonic eGFP-expressing cells (log cells mL-1)
Time (days)
(G)
Biofim eGFP-expressing cells (log cells cm-2)
(H)
Biofilm thickness (mm)
(I)
Wet weight (g)
Time (days)
(J)

Figure 5.1. Planktonic and biofilm parameters at a flow rate of 255 L h^{-1} (*Re* of 4600; dark squares) and 128 L h^{-1} (*Re* of 2300; gray squares): (**A**) planktonic plasmid copy number; (**B**) biofilm plasmid copy number; (**C**) planktonic eGFP production; (**D**) biofilm eGFP production; (**E**) planktonic total cells; (**F**) biofilm total cells; (**G**) planktonic eGFP-expressing cells; (**H**) biofilm eGFP-expressing cells; (**I**) biofilm thickness; and (**J**) biofilm wet weight. The means ± standard deviations (SDs) for at least three independent experiments are illustrated. The statistical analysis corresponding to each time point is also represented with an asterisk for a confidence level of greater than 90% ($p < 0.1$) and with a double asterisk for a confidence level of greater than 95% ($p < 0.05$).

Although higher PCN values were always achieved at the highest flow rate, the plasmid maintenance in both planktonic and biofilm cells seemed to be favoured at a lower *Re*. In fact, for the planktonic cells that were grown at 255 L h^{-1}, a strong reduction was observed between days 4 and 7 (around 81%), while at a flow rate of 128 L h^{-1} plasmid loss was around 61%. Regarding the biofilm cells, the percentage of plasmid lost at 255 L h^{-1} was 63%, whereas at 128 L h^{-1} this reduction was approximately 25%.

The fluorometric analysis of the biofilm cells (Figure 5.1D) showed that the cells grown at the highest *Re* expressed more eGFP and reached the highest production value of 21.5 fg $cell^{-1}$ on day 4. After day 4, the production decreased until the end of the experiment. Additionally, the eGFP production rate that was obtained at the lowest flow rate remained almost constant until day 4 and after that, it started to increase until the end of the experiment and reached a maximum of 8.8 fg $cell^{-1}$. In the planktonic state (Figure 5.1C), the production of eGFP was also higher at the highest flow rate and increased until day 5 (7.0 fg $cell^{-1}$), whereas at the lowest flow rate the eGFP levels remained practically constant over the whole experiment (around 1.8 fg $cell^{-1}$). Furthermore, the protein production rate in the biofilm cells was higher compared to the planktonic cells under both hydrodynamic conditions (3-fold higher for 255 L h^{-1} and 2-fold higher for 128 L h^{-1}).

The planktonic total cells (Figure 5.1E) had similar profiles for both flow rates ($p > 0.1$) and throughout the whole experiment. The quantification of the biofilm total cells (Figure 5.1F) also showed few differences between the hydrodynamic conditions. An initial increase was observed until day 4 and then, the number of cells stabilized from day 4 onwards. Concerning the planktonic eGFP-expressing cells (Figure 5.1G), both flow conditions reached similar cell amounts (8.6×10^8 cells mL^{-1}), although this value was

reached sooner for the flow rate of 128 L h^{-1} (day 4 versus day 5 for 255 L h^{-1}). From that day onwards, the number of cells that expressed eGFP decreased until the end of the experiment. By comparing the number of planktonic cells that were expressing eGFP to the total cell number, the results showed that on average 31% and 26% of the cells expressed eGFP at 255 L h^{-1} and 128 L h^{-1}, respectively (data not shown). The number of biofilm cells that were expressing eGFP (Figure 5.1H) strongly increased between days 2 and 4 and then remained practically constant until the end of the experiment, regardless of the hydrodynamic condition being tested. The number of cells that were expressing eGFP in comparison to the total cell number was on overage 53% higher for the highest flow rate. Under this flow condition, a strong increase was observed until day 5, when 66% of the cells were found to express the protein. Furthermore, for the flow rate of 255 L h^{-1}, the number of biofilm cells that expressed eGFP was on overage 52% higher than the planktonic counterparts.

The biofilm thickness (Figure 5.1I) remained practically constant throughout the experiment for both flow conditions (an average of 176 μm), with a slight increase on days 4 and 5 for 255 L h^{-1} and 128 L h^{-1}, respectively. The biofilm wet weight (Figure 5.1J) was similar between flow rates but had slightly higher values for 255 L h^{-1}. An initial increase was observed until day 4, which then stabilized from that day until the end of the experiment for 255 L h^{-1}, while a decrease was observed on the last day of the experiment for the flow rate of 128 L h^{-1}.

5.4 Discussion

The study of the parameters that influence both biofilm formation and heterologous protein production is a valuable approach for reaching the highest specific recombinant protein expression in *E. coli* biofilms. Previous experiments using the same flow cell system have shown that higher flow velocities increase the external mass transfer [14, 15] but produce thinner and more compact biofilms (compared to laminar conditions) [34] in which the internal mass transfer can be limited [22]. As well as a turbulent regime (flow rate of 255 L h^{-1} and Re of 4600), transient conditions (flow rate of 128 L h^{-1} and Re of 2300) were also tested in the present work.

Doubling the flow rate (from 128 to 255 L h^{-1}) seemed to have no impact on biofilm formation. This result was not expected since it is generally accepted that biofilms that are formed under turbulent conditions are thinner, denser, and stronger than those developed under lower shear stress [15, 29]. This could be related to the presence of an antibiotic resistance gene (kanamycin, KmR) in the plasmid. It is known that these genes are the major genes responsible for increasing the metabolic burden on the host cell [35]. Since stress conditions can favour biofilm formation [36] and, additionally, the presence of a considerable number of eGFP-expressing cells can increase the stress, it is possible that the similar biofilm growth behaviour under the different flow conditions could have been due to these factors [31, 36].

The single-cell analysis showed an increase in the number of eGFP-expressing biofilm cells between days 2 and 5, which represented on average 58% and 37% of the total population for 255 and 128 L h^{-1}, respectively, from this moment until the end of the experiment. It should be noted that, contrary to other studies, the number of eGFP-expressing cells did not correlate with the number of viable cells (Figure S 5.2 of Supplementary Material). In fact, the number of eGFP-expressing cells was found to be higher than viable cells under both flow conditions. This could be explained by the fact that dead cells completely lose eGFP while dying cells gradually leak eGFP from increasingly permeable membranes [37] once a decrease in viability has been observed. The decrease in biofilm viability could be related to the increase in protein production, which imposes a metabolic burden on the host [38, 39]. It is well documented that recombinant protein production affects host metabolism due to the energy and metabolites that are needed for the replication of plasmid DNA and the synthesis of foreign protein [3, 40]. Some of the metabolic changes that can be induced during recombinant protein production are an increase in protease activity and a decrease in growth rate and cell viability [38, 39].

The specific eGFP production was also higher for both planktonic and biofilm cells that were grown at the 255 L h^{-1} flow rate. In suspended cells, several studies have reported that a high agitation rate increases metabolite production in recombinant microorganisms [41-43]. This suggests that a high shear rate is generated under high flow velocities and results in an increase in oxygen and nutrient supply. Some studies of recombinant protein production in immobilised bacterial cells have also demonstrated

the influence of agitation rate on oxygen and nutrient diffusion within the cells and the consequent effects on heterologous protein production [27, 28]. Talabordan and Yang [44] reported that the production of a glucoamylase–green fluorescent fusion protein on a fibrous-bed bioreactor increased when the agitation rate increased to 400 rpm and that a further increase to 600 rpm resulted in the detachment and breakage of fungal mycelia and a consequent increase in protein secretion. Similar to bacterial biofilms, filamentous microorganisms comprise channels that allow fluid circulation when immobilised in reactors [45]. An increase in agitation rate promotes a better mass transfer within the cells [45]. Biofilms are known to be highly heterogeneous and contain cells in different physiological states, which influence gene expression in different zones within the biofilm [19, 46, 47]. In a previous work by our group [22], it was demonstrated that the eGFP-expressing cells in a 3-day *E. coli* biofilm were mostly found at the top of the biofilm. The lower eGFP expression that was obtained at the lowest tested flow rate in this study (128 L h^{-1}) could be related to the absence of oxygen and fresh nutrients in the deeper regions of the biofilm. In agreement with this hypothesis, Araújo et al. [34] showed that mass transfer limitations in *Pseudomonas fluorescens* biofilms occurred under lower velocity conditions (0.1 m s^{-1}, which corresponded to a Reynolds number of 1000). Additionally, the higher eGFP production that was obtained at the higher flow rate was probably related to the high availability of oxygen and nutrients in the bulk fluid, since a direct consequence of increasing flow velocity is that the transport of nutrients and oxygen also increases [48].

Although the specific eGFP production in the biofilms that were grown at the lower flow rate remained almost constant until day 4, an increase was observed from that day onward. This phenomenon could be associated with the slight increase that was observed in PCN and the percentage of eGFP-expressing cells during the same period. It was expected that the higher PCN and the larger number of cells that were expressing the protein would result in higher eGFP levels. Furthermore, the increase in the number of eGFP-expressing cells on day 4 could be related to the simultaneous decrease in biofilm thickness, since the oxygen transfer that is needed for the maturation of eGFP is facilitated in thinner biofilms.

Higher specific eGFP production levels were obtained in biofilm cells, even though they presented a smaller number of plasmid copies compared to the planktonic cells.

These results were in agreement with our previous work [7], in which eGFP production was shown to be higher in biofilm cells, even with 3-fold lower PCN levels. Thus, under the tested hydrodynamic conditions, gene dosage did not affect recombinant protein production. This was probably related to the metabolic burden that was imposed by plasmid replication, which can promote plasmid instability and, consequently, reduce the yield of heterologous protein in planktonic cells [3, 49].

Biofilm environments have been shown to increase plasmid maintenance compared to chemostats [6, 7, 50]. In this work, a higher percentage of plasmid loss was observed in the planktonic state for both flow rates. However, a low flow rate seemed to be advantageous for plasmid retention: in biofilm cells, 25% of plasmid loss was observed at 128 L h^{-1} while at a higher flow rate, this percentage was 2.5-fold higher. The marked decline in PCN on day 5 for the biofilm cells that were grown at 255 L h^{-1} could be related to the higher eGFP production that was observed on days 4 and 5. It is known that higher levels of protein production increase the metabolic burden on the host cell, which can contribute to plasmid loss [3, 51]. Huang et al. [26] reported that biofilm cells that are grown at low agitation rates are associated with high plasmid stability.

5.5 Conclusions

Despite the advantages that biofilm reactors present over suspended cells, limitations in the diffusion of oxygen and nutrients within the biofilm remain a major problem. This study demonstrated that a flow rate had an impact on recombinant protein production in *E. coli* biofilms. Although increasing the flow rate had no major impact on biofilm formation, higher eGFP production and PCN values were obtained at the higher flow rate. The results suggested that the higher hydrodynamics favoured the recombinant protein production in *E. coli* biofilms.

The results that were obtained in this work are essential for the definition of the operating conditions that are needed for heterologous protein production in biofilms systems in order to maximize protein production.

5.6 References

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5.7 Supplementary material

5.7.1 Flow cell system schematization and experimental procedure

The flow cell system is composed of a recirculating tank (planktonic cells), a vertical flow cell reactor (where biofilms are formed), and centrifuge pumps that allow the circulation of the bacterial suspension. The flow cell reactor is a semi-circular Perspex duct (3.0 cm in diameter and 1.09 m in length) with 21 apertures on its flat wall to fit removable Perspex pieces (coupons) to which polyvinyl chloride (PVC) slides (2 x 1 cm) were glued. The biofilms were developed on the upper faces of the PVC slides that were in contact with the bacterial suspension circulating through the system.

E. coli cells were grown by recirculating the bacterial suspension at 30 °C for 7 days at two different flow rates: 255 L h^{-1} (*Re* of 4600) and 128 L h^{-1} (*Re* of 2300). The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with Terrific Broth (supplemented with 20 μg mL^{-1} kanamycin) at a flow rate of 0.025 L h^{-1}.

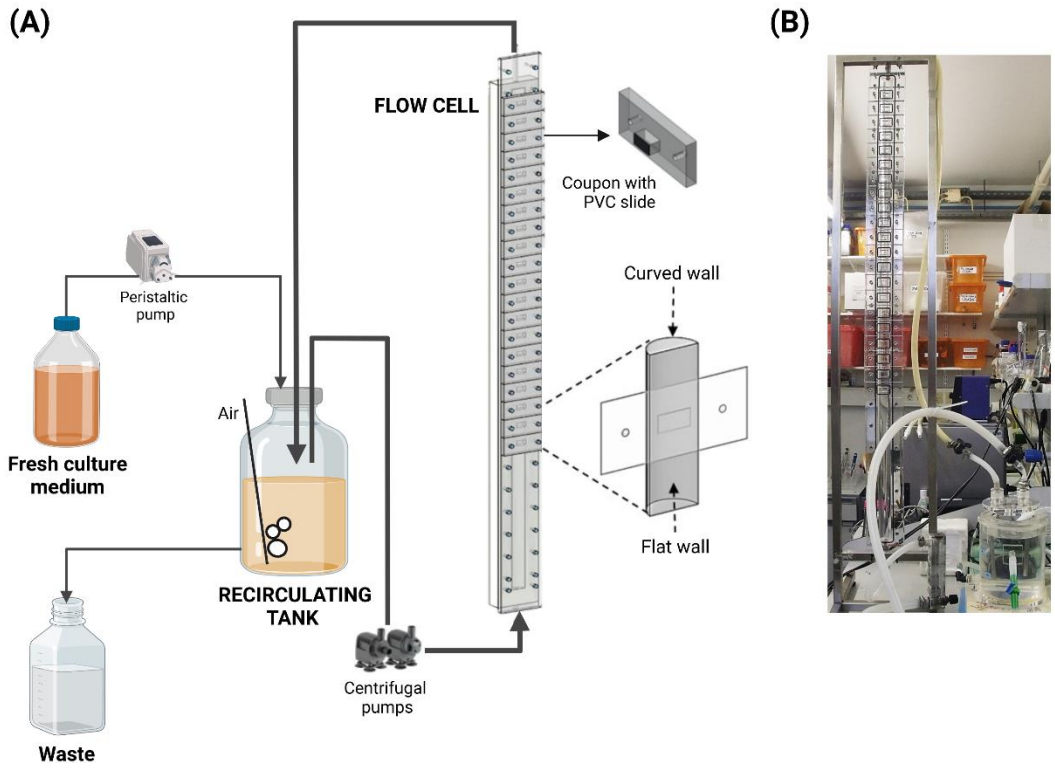


Figure S 5.1. (**A**) Schematic representation and (**B**) photograph of the flow cell system

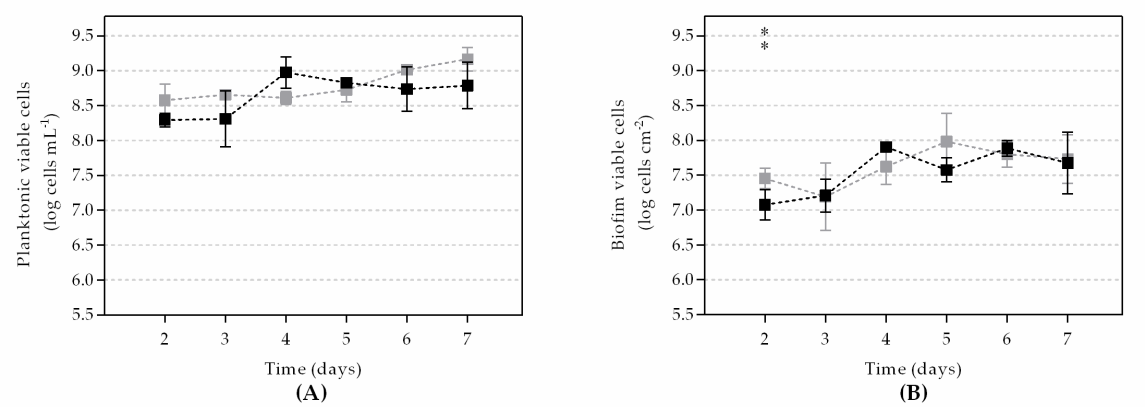


Figure S 5.2. Planktonic (**A**) and biofilm (**B**) viable cells at a flow rate of 255 L h^{-1} (*Re* of 4600, dark squares) and 128 L h^{-1} (*Re* of 2300, grey squares). The means ± standard deviations (SDs) for at least three independent experiments are illustrated. Statistical analysis corresponding to each time point is also represented with an asterisk for a confidence level greater than 90% ($p < 0.1$) and with a double asterisk for a confidence level greater than 95% ($p < 0.05$)

6 Effects of IPTG induction on recombinant protein expression by *Escherichia coli* biofilms

Abstract

The pET system, based on the T7 RNA polymerase (RNAP), is one of the most widely used expression systems in *Escherichia coli*. This work assesses the effect of chemical induction by isopropyl-β-D-1-thiogalactopyranoside (IPTG) on the production of a model recombinant protein encoded in the pET28a plasmid (the enhanced green fluorescent protein, eGFP) in *E. coli* biofilms. Two IPTG concentrations (0.1 and 0.5 mM) and induction times (at days 3 and 5) were tested in biofilms growing on polyvinyl chloride (PVC) coupons inserted into well plates. Results showed that in biofilms induced at day 3, eGFP production was lower than in non-induced biofilms. Higher specific eGFP production was always obtained in biofilm cells induced at day 5, and the inducer avoided the overall decrease of plasmid copy number. Furthermore, increasing IPTG concentration had no beneficial effect on specific eGFP production; on average, slightly higher values were obtained at the lowest IPTG concentration. Therefore, induction at day 5 with 0.1 mM IPTG increased the production of the model recombinant protein and plasmid stability when compared to the other tested conditions and non-induced biofilms.

Keywords: biofilm; *Escherichia coli*; green fluorescent protein; plasmid copy number; IPTG induction.

6.1 Introduction

The Gram-negative bacterium *Escherichia coli* (*E. coli*) remains the most intensively used workhorse for recombinant protein production [1]. Versatile genetic tools are available for high-level protein expression in this host, and expression systems have been developed so that induction is simple and cost-effective [2, 3]. Successful recombinant protein production in *E. coli* involves several decisions regarding the host strain (genetic background and the choice of genetic elements of the expression vector) and the fermentation process (cultivation conditions and purification strategies) [4]. *E. coli* plasmids have been the preferred expression vector since they can attain high gene dosage. Normally, a high plasmid copy number (PCN) leads to high gene dosage, but it does not guarantee a higher protein yield [5]. In suspended cells, it is known that the presence of a high copy number plasmid imposes a metabolic burden that usually results in decreased cellular growth rate, plasmid stability, and consequent protein yield [6, 7]. Interestingly, biofilms have demonstrated potential to increase plasmid stability [8-10].

Several expression systems and promoters have been developed for the expression of heterologous proteins in *E. coli* [11-13]. The pET system has been widely used since the efficient expression of the desired protein can achieve 50% of the total protein [11, 14]. In this system, the gene of interest is cloned behind the T7 promoter, which is only transcribed by the RNA polymerase of the bacteriophage T7. This highly active polymerase is incorporated into the bacterial chromosome at the DE3 locus by a prophage (λDE3) under the transcriptional control of the lacUV5 promoter [15]. The system can be induced by lactose or by a non-hydrolysable analog like isopropyl-β-D-1-thiogalactopyranoside (IPTG) [16].

The use of inducible expression systems is an important variable to consider in increasing overall specific protein production. [17]. IPTG is commonly used for inducing expression from the *lac* promoter and several studies have reported the positive effect of induction on total protein yield [18, 19]. However, optimization of IPTG concentration is not so straightforward since higher inducer concentrations do not necessarily lead to maximum protein expression [20, 21]. Indeed, it is known that the growth rate and metabolic activity of the host cell are often influenced by the inducer [22, 23]. Larentis et al. [24] showed

that IPTG concentration had a negative effect on *Leptospira* Immunoglobulin-like B (LigB) protein expression in *E. coli*. Higher protein yield and productivity were obtained with lower IPTG concentration (0.1 mM). Furthermore, Turner et al. [25] demonstrated that high IPTG concentrations also affect protein solubility. These authors showed that recombinant cyclomaltodextrinase (CDase) was soluble and active with 0.05 mM IPTG, whereas doubling the IPTG concentration (to 0.1 mM) resulted in insoluble and inactive protein. Additionally, high inducer concentrations might also lead to the accumulation of target protein in inclusion bodies [26, 27].

The induction time is also important because the maximum protein yield depends on cell density as well as the physiological state of the cells at the time of induction [28]. Most of the studies have reported that cells induced at the mid-logarithmic growth phase can provide sufficient levels of energy and metabolic precursors for recombinant biosynthesis [29, 30]. On the contrary, Ramirez et al. [18] reported that the production of secreted acylase in *E. coli* under the control of the *lac* promoter was favoured with induction at the early log phase. This strategy can be advantageous in cases where protein production is low and/or does not significantly influence cell growth [31]. Furthermore, it has been reported that for strains whose growth and viability are drastically reduced upon induction, protein production can be favoured by induction at the late-logarithmic or stationary phase [32, 33].

There is a considerable amount of information on the impact of induction conditions in *E. coli* batch cultures, but little is known about their influence on biofilms. Huang et al [34] showed that cell accumulation rates decreased with increasing IPTG concentrations in both planktonic and biofilm cells. Furthermore, the different IPTG concentrations tested (0.17, 0.34 and 0.51 mM) did not affect the maximum β-galactosidase concentration obtained 24 h after induction [34]. More recently, our research group tested the impact of induction with 2 mM IPTG on plasmid stability and eGFP production in a continuous biofilm reactor [35]. The results showed that the inducer avoided the overall decrease in the amount of expressed enhanced green fluorescent protein (eGFP; a decrease of 31% was observed in non-induced cells), although a negative effect on plasmid stability after IPTG induction was observed [35].

Thus, the aim of this work was to determine the optimal IPTG concentration and induction time to achieve the maximum eGFP production in *E. coli* biofilms. For that, two different IPTG concentrations (0.1 and 0.5 mM) and two experimental days (days 3 and 5) were tested considering the preliminary results which used 2 mM IPTG to induce *E. coli* planktonic and biofilm cells containing the pFM23 vector in a flow cell system [36]. The assays were performed in 12-well plates using polyvinyl chloride (PVC) as surface material and Terrific broth (TB) as culture medium since these conditions were the most advantageous to obtain higher specific eGFP production values in *E. coli* biofilms (unpublished). To the best of our knowledge, this is the first study combining the effect of inducer concentration and induction time on heterologous protein expression in sessile cells.

6.2 Material and Methods

6.2.1 Bacterial Strain

E. coli JM109(DE3) (Promega, Madison, WI, USA) carrying the pFM23 (constructed from pET28a; Novagen, Madison, WI, USA), which contains the eGFP gene [36], was used. The transformants were selected on agar medium supplemented with 20 μg mL^{-1} of kanamycin (Eurobio, Orsay, France).

6.2.2 Biofilm Formation Assays

Cells were grown in 250 mL culture in TB medium supplemented with 20 μg mL^{-1} of kanamycin and incubated overnight at 30 ºC and 120 rpm. The bacterial cultures were harvested by centrifugation at 3202 g for 10 min at 25 ºC (Eppendorf Centrifuge 5810R, Hamburg, Germany), and the final concentration was adjusted in fresh TB medium to an optical density at 610 nm of 0.1, corresponding to approximately 10^7 colony-forming units per mL (CFU mL^{-1}).

Biofilm formation assays were performed on PVC coupons with 1 cm diameter (Neves & Neves, Lda, Maia, Portugal) placed inside 12-well plates (VWR International,

Carnaxide, Portugal). The coupons were washed with 70% (v/v) ethanol (VWR, Radnor, PA, USA) solution and then in ultrapure water, and air-dried for 1 h [37]. Additionally, coupons were sterilized through ultraviolet (UV) radiation for 30 min. Double-sided adhesive tape was placed in each plate well and sterilized with UV radiation for 30 min. Finally, the sterile coupons were glued inside the wells [38]. Biofilms were grown at 30 °C and 185 rpm for 9 days in TB supplemented with 20 μg mL^{-1} of kanamycin. The TB medium is composed of 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 2.31 g L^{-1} KH_2PO_4, 12.54 g L^{-1} K_2HPO_4 and 4 mL glycerol [39]. All compounds were purchased from Merck, Algés, Portugal.

Each well was filled with 3 mL of the bacterial suspension and during the experiment, the medium was replaced on days 1, 3, 5 and 7. The influence of IPTG on eGFP production was assessed by inducing the expression system on two different days (days 3 and 5) using two different IPTG concentrations (0.1 and 0.5 mM).

6.2.3 Biofilm Analysis

Sampling was performed on days 3, 5, 7 and 9. On each sampling day, 4 coupons for each tested condition (2 coupons for optical coherence tomography (OCT) analysis, and 2 coupons for total cell, eGFP and plasmid quantification) were analysed.

Biofilm thickness was determined by OCT (Thorlabs Ganymede Spectral Domain Optical Coherence Tomography system with a central wavelength of 930 nm, Thorlabs GmbH, Dachau, Germany) as described by Romeu et. al [40]. Briefly, the culture medium was carefully removed, and the non-adherent cells were washed with 8.5 g L^{-1} NaCl solution. Subsequently, the wells were filled with 3 mL of 8.5 g L^{-1} NaCl solution and imaged. The captured volume was ~ 4 × 4 × 3 mm^3 (509 × 313 × 1024 $pixels^3$) and the refractive index was 1.40. The Image Processing Toolbox from MATLAB 8.0 and Statistics Toolbox 8.1 (The MathWorks, Inc., Natick, Massachusetts, USA) was used to determine the biofilm thickness on each coupon from a minimum of 5 fields of view, and the final values were expressed as μm.

For total cell quantification, biofilms were resuspended in 4 mL of 8.5 g L^{-1} NaCl solution by vortexing (VV3, VWR) for 3 min at maximum rotational speed (2500 rpm) [41].

Biofilm total cells were determined using 4',6-diamidino-2-phenylindole (DAPI) (Merck, Germany) fluorescent stain [42]. Bacterial cell suspensions were firstly filtered through a Nucleopore Track-Etch Membrane of black polycarbonate (pore size of 0.22 μm; Whatman Ltd, UK) and stained with 100 μL of DAPI reagent (0.1 mg mL^{-1}) for 10 min in the dark. Then, stained bacteria were observed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems Ltd, Switzerland). The image processing software ImageJ v1.48 (NHI, USA) was used to estimate the number of total cells on each membrane from counts of a minimum of 15 fields of view, and the final values were expressed as cells cm^{-2} of coupon area.

6.2.4 eGFP quantification

The amount of eGFP-expressing cells was determined by epifluorescence microscopy as described by Gomes et. al [43]. Briefly, biofilm suspensions were filtered and observed using a Leica DM LB2 epifluorescence microscope. The percentage of eGFP-expressing cells was calculated by dividing the number of eGFP-expressing cells by the total cell number.

The specific eGFP production was quantified by the fluorometric method fully described by Mergulhão and Monteiro [36]. Fluorescence was measured in a microplate reader (SpectraMax M2E, Molecular Devices, Inc., UK) with a 488 nm excitation filter and a 507 nm emission filter. The eGFP concentration was calculated through a calibration curve obtained from purified eGFP (range of 0 to 0.31 μg μL^{-1}) and presented as specific eGFP production (fg $cell^{-1}$).

6.2.5 Confocal Laser Scanning Microscopy (CLSM)

The spatial distribution of eGFP-expressing cells in 7-day-old biofilms (non-induced and induced at day 5 with 0.1 mM) was analysed by Confocal Laser Scanning Microscopy (CLSM). All samples were counterstained with 5 μm Syto61 (Invitrogen, France), a cell-permeant red fluorescent nucleic acid marker, for 10 min. Each stained sample was mounted in a microscopic slide and scanned using a 40 × water immersion objective lens

(Leica HCX PL APO CS; Leica Microsystems GmbH, Wetzlar, Germany) in an inverted microscope Leica DMI6000-CS, with excitation wavelengths of 488 nm (argon laser) and 633 nm (helium–neon laser). The emitted fluorescence was recorded within the range of 500–580 nm to collect the eGFP emission fluorescence and 640–730 nm to collect the Syto61 fluorescence. A minimum of 5 stacks of horizontal plane images with a z-step of 1 μm was acquired for each biofilm sample.

Two-dimensional projections of the biofilm structures were reconstructed using the "Section" function of IMARIS 9.9 software (Bitplane AG, Zurich, Switzerland).

6.2.6 Plasmid extraction and quantification

Plasmid DNA was obtained by alkaline lysis and quantified by an analytical high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC) as described by Soares et. al [44]. A 4.6/100 mm HIC source 15 PHE PE column (Amersham Bioscience, Uppsala, Sweden) connected to an HPLC system (Shimadzu, Nexera-i LC-2040C 3D, Duisburg, Germany) was used and the absorbance of the eluate was recovered at 260 nm. Plasmid concentration was calculated by using calibration curves constructed with plasmid DNA standards (ranging from 0.04 to 50 μg mL^{-1}), and the corresponding plasmid copy number (PCN) was then calculated using Equation (1) [45]:

$$\mathrm{PCN} = \frac{6.02 \times 10^{23}\ (\mathrm{copy\ mol^{-1}}) \times \mathrm{DNA\ amount\ (g)}}{\mathrm{DNA\ length\ (bp)} \times 660\ (\mathrm{g\ mol^{-1}\ bp^{-1}})} \quad (1)$$

knowing that the size of plasmid pFM23 is 6053 bp.

6.2.7 Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics version 26 for Windows (IBM SPSS, Inc., Chicago, IL, USA). Descriptive statistics were used to calculate the mean and standard deviation (from at least three biological experiments with two replicates) for biofilm thickness, total cells, percentage of eGFP-expressing cells, specific eGFP concentration, and plasmid copy number. For all response variables, the homogeneity of variances and normality of data was assessed using the Kolmogorov-

Smirnov and Shapiro-Wilk tests. Subsequently, the Kruskal-Wallis test and One-way ANOVA were performed to determine if there were statistically significant differences between the means of all conditions (regarding inducer concentration and the induction time) for each experimental day. The Mann-Whitney test and the t-test were then conducted to compare the control (no induction) with each induction condition for each experimental day. Statistical differences were considered for p-values < 0.05, corresponding to a confidence level of 95% (* and ** indicate $p < 0.05$ and $p < 0.001$, respectively).

6.3 Results

To evaluate the impact of IPTG induction on eGFP production by *E. coli* biofilms, an assay was performed inducing the cells with different IPTG concentrations (0.1 and 0.5 mM) on different days (days 3 and 5).

Biofilm development was monitored by quantifying the total cells and thickness (Figure 6.1A and B, respectively). As can be observed in Figure 6.1A, induction with the lowest IPTG concentration caused a slight increase in the number of total cells upon induction (days 5 and 7 for biofilms induced at days 3 and 5, respectively) compared to non-induced biofilms (with statistical difference on day 5, $p < 0.1$). On the contrary, in biofilms induced with 0.5 mM IPTG, the number of total cells observed upon induction (days 5 and 7, for biofilms induced at days 3 and 5, respectively) were lower than the non-induced biofilms.

Biofilm thickness (Figure 6.1B) significantly increased over time, regardless of the induction day and IPTG concentration. Nevertheless, biofilm thickness upon induction was significantly higher for 0.5 mM IPTG concentration when compared to non-induced biofilms (on average, 38% for biofilms induced on day 3 and 24% for biofilms induced on day 5; $p < 0.001$).

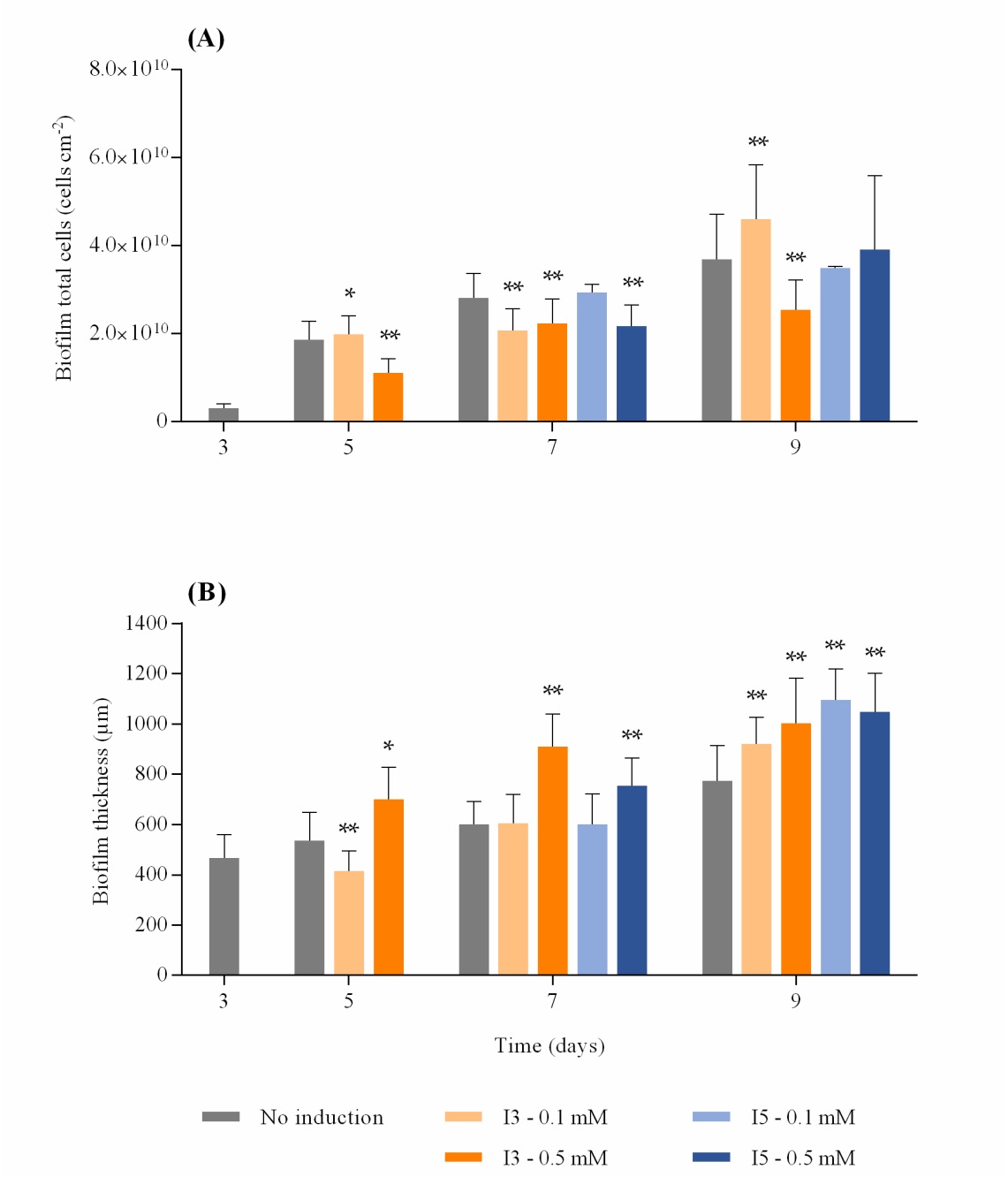


Figure 6.1. Biofilm development parameters: (**A**) total cells and (**B**) thickness. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 95% (* and ** indicate $p < 0.05$ and $p < 0.001$, respectively) compared to the non-induction condition. Different induction conditions were represented as: I3 – 0.1 mM (induction at day 3 with IPTG concentration of 0.1 mM); I3 – 0.5 mM (induction at day 3 with IPTG concentration of 0.5 mM); I5 – 0.1 mM (induction at day 5 with IPTG concentration of 0.1 mM); and I5 – 0.5 mM (induction at day 5 with IPTG concentration of 0.5 mM).

Analyses of eGFP-expressing cells (Figure 6.2A) showed that at the beginning of the experiment, the percentage of cells expressing the protein on non-induced biofilms was 54% and decreased until day 9 (30%). Regarding eGFP expression in cells induced at day 3, a lower percentage of cells expressing the protein were obtained throughout the experiment for both IPTG concentrations compared to non-induced cells. On the contrary, the percentage of cells expressing eGFP on biofilms induced at day 5 slightly increased compared to non-induced cells, achieving 43% and 38% at day 7 for 0.1 and 0.5 mM IPTG, respectively (percentage of eGFP-expressing non-induced cells at day 7 was 33%).

By analysing the specific eGFP production (Figure 6.2B), it can be observed that the lowest IPTG concentration (0.1 mM) seemed to favour protein production in biofilms induced at day 5. For this inducer concentration, the specific eGFP production increased from 78 fg $cell^{-1}$ (without induction, on day 5) to 92 fg $cell^{-1}$ on day 7 (with induction on day 5). Unexpectedly, the amount of eGFP production in biofilms induced on day 3 was lower than the leaky production from the non-induced biofilms in all experiment.

On average, biofilm cultures had higher PCN values upon induction, regardless of the induction day and IPTG concentration. The early induction on day 3 resulted in the highest PCN values on day 5 (466 and 512 PCN $cell^{-1}$ for 0.1 and 0.5 mM IPTG, respectively), followed by a strong plasmid loss (around 61 and 39% for both 0.1 and 0.5 mM IPTG, respectively) on day 7, and from that on PCN levels increased to 423 and 334 PCN $cell^{-1}$ for 0.1 and 0.5 mM IPTG, respectively. In biofilms induced at day 5, an initial PCN increase was observed with a maximum value of 326 PCN/cell for 0.1 mM IPTG and 339 PCN/cell for 0.5 mM IPTG on day 7. Contrary to the non-induced biofilms, which had their PCN reduced by about 60% throughout the experiment, there was no plasmid loss in biofilms induced at day 5.

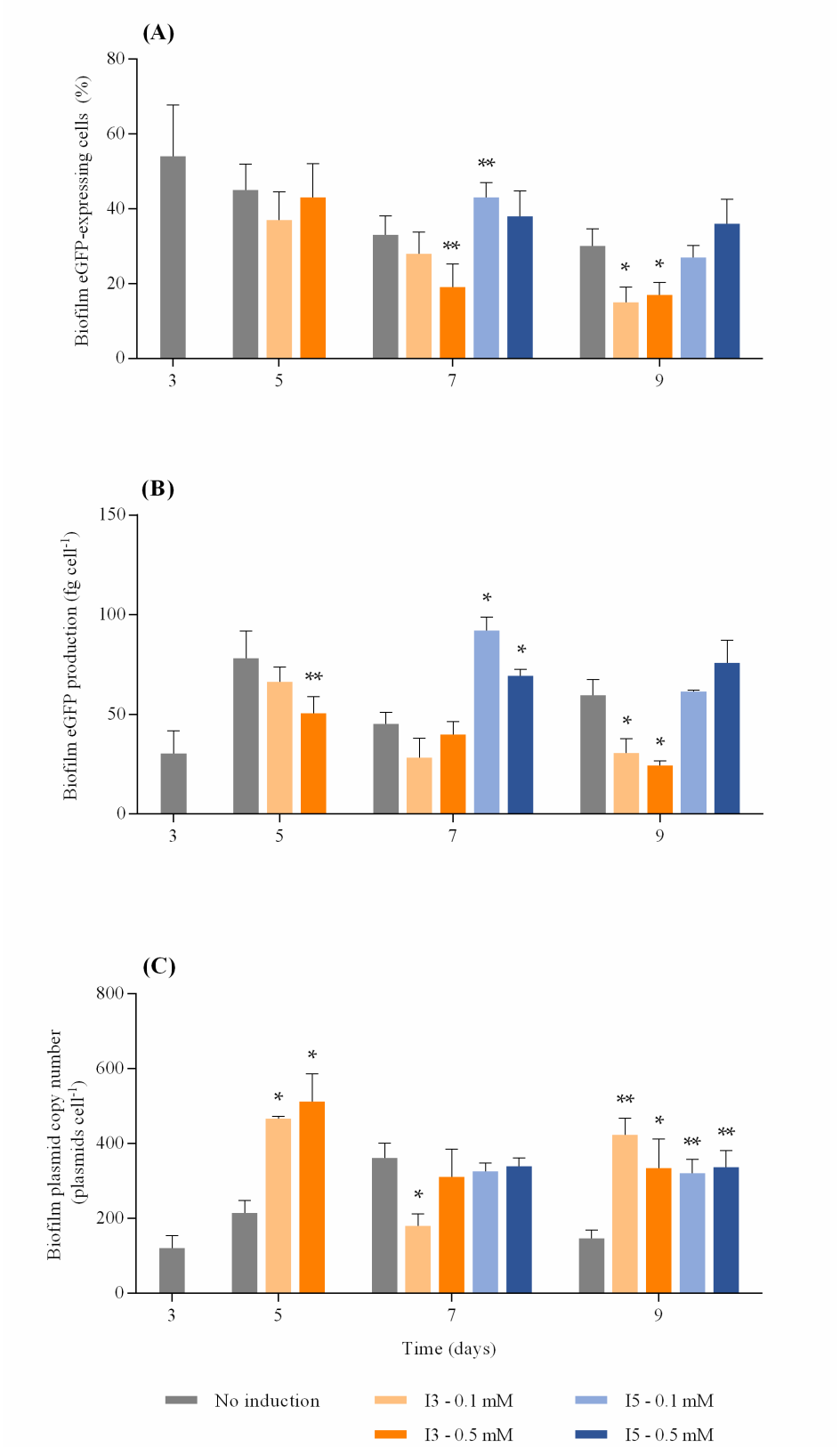


Figure 6.2. (**A**) eGFP-expressing cells, (**B**) specific eGFP production, and (**C**) plasmid copy number in biofilm cells. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 95% (* and ** indicate $p < 0.05$ and $p < 0.001$, respectively) compared

to the non-induction condition. Different induction conditions were represented as: I3 – 0.1 mM (induction at day 3 with IPTG concentration of 0.1 mM); I3 – 0.5 mM (induction at day 3 with IPTG concentration of 0.5 mM); I5 – 0.1 mM (induction at day 5 with IPTG concentration of 0.1 mM); and I5 – 0.5 mM (induction at day 5 with IPTG concentration of 0.5 mM).

To elucidate the population heterogeneity caused by IPTG addition, a confocal microscopy analysis was performed on 7-day-old biofilms for non-induced and induced samples at day 5 with 0.1 mM IPTG (Figure 6.3), since these were the induction conditions where the highest values of specific protein production and eGFP-expressing cells were obtained (Figure 6.2B and A, respectively). Most of the *E. coli* cells in the induced biofilm were expressing eGFP (Figure 6.3B), unlike the non-induced biofilm, where a higher fraction of cells were marked in red (Figure 6.3A). This observation confirmed the quantitative data extracted from the single-cell analysis (Figure 6.2A). Moreover, while in the representative image of the induced biofilm, the few non-expressing cells (that emitted the red signal) are randomly distributed in the verticality of the biofilm (Figure 6.3B), a spatial distribution pattern of the two cell populations was observed in the non-induced biofilm (Figure 6.3A). Here, a zone of bright green fluorescence was observed in the upper part of the biofilm, whereas the interior regions of the biofilm lacked eGFP-expressing cells (Figure 6.3A).

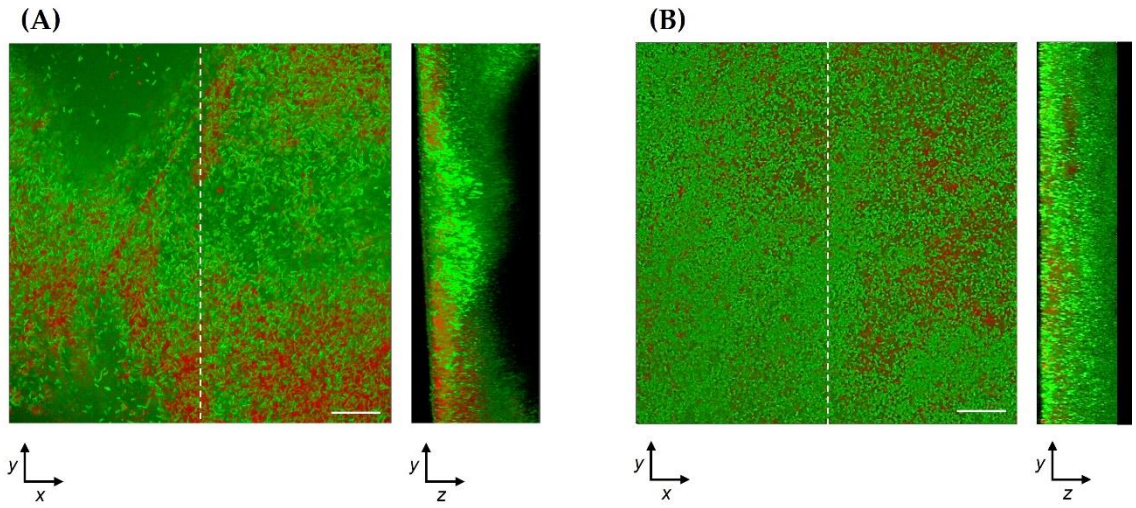


Figure 6.3. Spatial organization of 7-day-old biofilms: (**A**) non-induced and (**B**) induced samples at day 5 with 0.1 mM IPTG. These are section views of the CLSM images where the eGFP-expressing cells are labelled in green and the non-expressing cells are countermarked in red with Syto61. The dotted white lines indicate the vertical section (scale bar = 50 μm).

6.4 Discussion

To increase the yield and productivity of recombinant protein expression in *E. coli* biofilms, different IPTG conditions (concentration and induction time) were tested.

As far as biofilm formation is concerned, the addition of the lowest IPTG concentration tested (0.1 mM) seemed to have no impact, regardless of the induction day. On the contrary, biofilm development was affected by the highest IPTG concentration (0.5 mM) on both induction days. In general, although there was a decrease in the number of total cells of the induced biofilm compared to the non-induced samples, the biofilm thickness increased. The increase of thickness may be related to the higher extracellular polymeric substances (EPS) production, probably resulting from the higher availability of cellular resources such as amino acids, nucleotides, and metabolic energy. It has been suggested that recombinant protein production competes with the production of EPS content for metabolic intermediates and energy sources [46].

Gomes et al. [35] reported that the IPTG exposure did not significantly change the total and viable cells in *E. coli* JM109(DE3) biofilms. This non-accumulation effect was also observed by Huang et al [34] in *E. coli* DH5α(pTKW106) induced with 0.51 mM IPTG. Studies on suspended cells have reported a reduction in cell growth upon induction in some cultures of recombinant *E. coli* [47, 48]. It is known that the induction causes a metabolic burden on the host, which results in the inhibition of cell growth due to the cellular resources needed for the production of the target protein [4, 6]. Furthermore, Babaeipour and colleagues [29] showed that the growth rate of recombinant *E. coli* BL21 (DE3) (pET3a-hifnγ) decreased with the increase of IPTG concentration. Also, Bentley et al. [30] demonstrated that the growth rate of *E. coli* DH5aF'IQ [pKK262-11] was slightly affected when cells were induced with less than 0.1 mM of IPTG while using a concentration of 1.2 mM resulted in a growth rate decrease of 60%.

Results of eGFP concentration showed that the expression levels from biofilms induced at day 3 were lower than the leaky expression from the non-induced cells and decreased over time, indicating that heterologous protein production from these biofilms did not benefit from IPTG induction. IPTG was added in a single pulse and was present in the culture medium for a maximum period of 2 days. Thus, an increase in the specific

eGFP concentration was not expected after this time due to the depletion of IPTG. Despite this observation, it has been suggested that the addition of IPTG may be detrimental to the expression of recombinant proteins when using high-copy plasmids [49]. Although the pET plasmid is considered a medium-copy plasmid, it was shown that upon induction PCN reached around 500 copies. Additionally, protein expression may also be limited by the translational machinery of the cell [19, 50]. Gomes et al. [35] observed that total RNA levels decreased upon induction, which could be an indicator that the overall protein translation activity is decreased in induced cells.

The addition of IPTG at day 5 avoided the overall decrease of eGFP expression over the experiment since only biofilms induced at day 5 exhibited higher eGFP production than non-induced cells. In general, suspended cells are induced in the exponential growth phase since, at this point, cells are usually metabolically more active, providing sufficient levels of energy and metabolic precursors for recombinant biosynthesis [29, 30]. In biofilm cultures, cells usually remain in their metabolically active state for a longer period. Rahman et al. [51] reported that the higher protein concentration obtained in biofilms was related to the fact that after 6 days, biofilm cells remained metabolically active. Despite this fact, it is known that recombinant protein production depends on both biomass concentration and specific cellular product yield [52]. Thus, the high eGFP production level of biofilms induced at day 5 may be related to the higher metabolically active cell number present at the induction point.

This work suggests that the lowest IPTG concentration tested favoured eGFP production in biofilm cells (a maximum of 92 fg cell^{-1} was obtained at day 7 for biofilms induced with 0.1 mM IPTG). Also, several studies using suspended cells reported that low IPTG concentrations enhance recombinant protein production since induction causes a metabolic burden on the host cell, which can affect its growth rate and metabolic activity [22-24]. The present results are different from those obtained by Huang et al. [34] who showed that different IPTG concentrations (0.17, 0.34, and 0.51 mM) did not affect the maximum β-galactosidase concentration obtained in *E. coli* biofilms after induction (about 0.1 pg cell^{-1}) [34].

Plasmid stability is a key issue in recombinant protein production, and it is known that IPTG induction can affect plasmid stability in *E. coli* batch cultures [22, 53]. Although

some studies have reported on the impact of IPTG induction on plasmid stability in *E. coli* biofilms [34, 35], little is known about the influence of different IPTG concentrations and induction time points. In this work, higher specific eGFP production was obtained in biofilms induced at day 5, although more PCN were found in biofilms induced at day 3. This revealed that under tested conditions, gene dosage did not affect protein production. This was probably related to the high metabolic burden imposed by plasmid replication, resulting in segregational and structural plasmid instability, and reduction of yield and activity of the recombinant protein [6, 54]. On the contrary, biofilms induced at day 5 presented lower PCN, resulting in lower stress for the host and, consequently higher protein production. Additionally, similar PCN was obtained for both IPTG concentrations in biofilms induced at day 5. Thakker et al. [55] showed that the higher PYC activity obtained with pHL413-Km plasmid was not related to PCN, once they tested two different plasmids which presented the same copy number and stability. Thus, higher protein production might be caused by mRNA stability or translation efficiency [55].

6.5 Conclusions

Inducible expression systems have been widely used to increase recombinant protein production in suspended cells. However, little is known about the influence of chemical induction in biofilm cells. Biofilm formation using lower concentrations of IPTG seems to be the most advantageous strategy to obtain the highest specific eGFP production in *E. coli* biofilms. Furthermore, a later induction showed to be more suitable for long-term protein production.

Having determined the concentration of IPTG and the induction time that achieved the highest specific eGFP production in *E. coli* biofilms formed in a high throughput system, further assays consist in using these conditions to produce recombinant proteins in *E. coli* biofilms developed in a flow cell system.

6.6 References

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7 Concluding remarks and future perspectives

7.1 Concluding remarks

Efficient strategies to produce recombinant proteins are gaining increasing importance since these proteins are required for several applications ranging from industrial settings to laboratory research. Biofilm research is usually focused on their deleterious effects in health and industrial sectors. Nevertheless, biofilms have been advantageously used in wastewater treatment and bioremediation and, more recently, in the production of valuable compounds such as solvents, organic acids, and enzymes. Biofilm reactors have many advantages over planktonic cells, as they (i) are able to retain 5 to 10 times more biomass per unit volume, thus increasing the production rates, (ii) reduce the risk of cell washout when operating at high dilution rates during continuous fermentation, (iii) provide a protective environment against extreme conditions of pH and temperature, hydraulic shock and toxic substances, and (iv) enhance plasmid stability [1]. In previous work from our group, it was shown that *E. coli* biofilms can be used for the production of recombinant proteins using the enhanced Green Fluorescent Protein (eGFP) as a model system [2, 3]. Biofilm formation and recombinant protein production can be affected by several environmental factors, such as nutrient medium composition, and hydrodynamic conditions. Additionally, inducible expression systems can be used to increase the expression level of the target protein. Therefore, the main goal of this thesis was to assess the effect of medium composition, hydrodynamic conditions, and chemical induction on recombinant protein expression in biofilm systems, using eGFP as a model protein and biofilms formed by transformed *E. coli* JM109(DE3) strain.

This work started with the comparison of eGFP production in *E. coli* planktonic and biofilm cells by fluorometry (a bulk method) and epifluorescence microscopy (a single-cell method). This combined technology allows the determination of important production parameters, such as the number of cells expressing the recombinant protein within a bacterial population. The results showed that *E. coli* biofilms cells can produce

eGFP at a much higher level than planktonic cells and that was associated with the highest potential of biofilms in retaining the plasmid.

Since the influence of environmental factors can affect both biofilm formation and recombinant protein production, the influence of nutrient medium composition and hydrodynamic conditions were assessed. Biofilm formation was favoured in M9ZB, although the number of eGFP-expressing cells and the eGFP yield were higher in LB in both planktonic and sessile cells. Moreover, the plasmid copy number was assessed by a chromatographic method, and results indicated that the biofilm environment seems to be advantageous for plasmid retention. This study confirmed that heterologous protein production in *E. coli* biofilms is affected by nutrient conditions, indicating that higher nitrogen content increases eGFP production while the presence of glucose (carbon source) in the medium had an inhibitory effect on protein expression in this particular system.

Following the study of nutrient medium composition, different flow rates were tested to evaluate the impact of hydrodynamic conditions on eGFP. Increasing the shear stress, from 0.1 to 0.3 Pa, had no major effects on the biofilm cell number and viability. However, with regard to eGFP production, it was possible to conclude that higher shear stress increased specific production levels and plasmid copy numbers in biofilm cells. This study demonstrated that, in this flow system, using turbulent flow conditions (Re of 4600) instead of transient flow conditions (Re of 2300) favoured recombinant protein production.

After defining the best culture medium and hydrodynamics, experiments were performed in order to assess the best IPTG induction regime (inducer concentration and induction time) to produce heterologous protein in *E. coli* biofilms. Induction at day 5 with low concentrations of IPTG seems to be the most advantageous strategy to obtain the highest specific eGFP production in long-term assays.

This work presents a contribution to the study of recombinant protein production in *E. coli* biofilms, which have already shown potential to be used as protein factories. The results obtained in this optimization work are important to define the operating conditions to be used in further studies focused on the synthesis of other heterologous proteins in this *E. coli* biofilm system.

7.2 Future perspectives

To establish guidelines for recombinant protein production using this biofilm system, several aspects require further research. This section explores the limitations and opportunities for extending the knowledge on the effect of the tested conditions on protein production.

It is known that a major problem of biofilm reactors is the limitation in the diffusion of oxygen and nutrients within the biofilm [4]. Mass transfer inside biofilms occurs by diffusion, which is dependent on biofilm architecture. In this thesis, it was shown that hydrodynamic conditions had no major effect on biofilm formation, and higher eGFP production was obtained in biofilms formed under turbulent flow conditions. Single-cell analysis revealed that 31% of the whole cells are producing eGFP. Thus, it would be interesting to apply techniques like confocal microscopy to obtain information about the spatial distribution of eGFP-expressing cells inside the biofilm. Furthermore, since hydrodynamics did not significantly affect biofilm density and thickness, the higher eGFP expression obtained at the higher flow rate could be related to the increased mass transfer from the bulk solution to the biofilm surface (higher external mass transfer coefficient, Km) as a direct consequence of increasing flow velocity [5, 6]. It was also been described that turbulence facilitates high substrate diffusion in biofilms [7], including the molecular oxygen needed for the eGFP to become fluorescent. Therefore, it would be also interesting to assess higher turbulent conditions.

It would also be interesting to apply techniques like flow cytometry to obtain information on the metabolic state of eGFP-expressing cells inside the biofilm. It is expected that more metabolically active cells are more likely to be eGFP-producing cells as they have more biosynthetic precursors, energy, and other cellular components available for the synthesis of the foreign protein.

To investigate the impact of IPTG induction on eGFP production in *E. coli* biofilms, screening assays on 12-well plates were performed in order to achieve the optimal induction concentration and induction time. In this work, it was found that low IPTG concentration and induction at day 5 seemed to be the best conditions for long-term assays. Microtiter plates have been used as a high throughput screening platform, since

this platform requires low volumes, is easy to handle, and enables the study of different conditions [8]. However, they present some limitations, such as the addition of IPTG in a single pulse being present in the culture medium only for two days. Thus, the effect of these parameters should also be evaluated on the flow cell system.

It is well known that plasmid stability is an important issue in recombinant protein production [9], but little is known about the influence of biofilm development on plasmid copy number and recombinant protein expression. During the optimization of the different culture conditions, it was possible to observe that the biofilm environment increased plasmid retention, and eGFP production was not related to gene dosage. Thus, it will be interesting to perform a full molecular analysis, including experimental data on recombinant protein transcription, translation, and cellular metabolic activity to elucidate the mechanisms of heterologous protein production in biofilm cells.

Additionally, recombinant protein production imposes a metabolic burden [10] on the host cell, which probably disturbs the global gene expression. However, little is known about the gene expression profile in *E. coli* biofilms producing a recombinant protein. Thus, it would be interesting to use transcriptome and proteomic analysis to study the response of sessile cells to foreign protein expression with changes in gene expression profile and synthesis of host cell proteins. This established relation can contribute to the development of a better *E. coli* strain for protein production in biofilms.

The increasing requirement for recombinant proteins led to the development of new efficient strategies for their production. In this work, promising results were obtained for eGFP production in this biofilm system. Therefore, it will be interesting to assess the expression of a different model protein (β-galactosidase) using the best conditions previously achieved, with the objective of establishing more guidelines for recombinant protein production using this biofilm system. The enzyme β-galactosidase, most commonly known as lactase, catalyzes the hydrolysis of lactose into glucose and galactose, being fundamental in the pharmaceutical and food industries [11, 12].

7.3 References

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Annex A - Publications from this PhD.

A.1 - Recombinant protein expression in biofilms (2019) 10.3934/microbiol.2019.3.232 (chapter 2)

A.2 - Comparing the recombinant protein production potential of planktonic and biofilm cells (2018) https://doi.org/10.3390/microorganisms6020048 (chapter 3)

A.3 - The Influence of Nutrient Medium Composition on *Escherichia coli* Biofilm Development and Heterologous Protein Expression (2021) https://doi.org/10.3390/app11188667 (chapter 4)

A.4 - Hydrodynamic Effects on Biofilm Development and Recombinant Protein Expression (2022) https://doi.org/10.3390/microorganisms10050931 (chapter 5)

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Review

Recombinant protein expression in biofilms

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Abstract: Biofilm research is usually focused on the prevention or control of biofilm formation. Recently, the significance of the biofilm mode of growth in biotechnological applications received increased attention. Since biofilm reactors show many advantages over suspended cell reactors, especially in their higher biomass density and operational stability, bacterial biofilms have emerged as an interesting approach for the expression of specific proteins. Despite the potential of biofilm systems, recombinant protein production using biofilms has been scarcely investigated for the past 25 years. Our group has demonstrated that *E. coli* biofilms were able to produce a model recombinant protein, the enhanced green fluorescent protein (eGFP), at much higher levels than their planktonic counterparts. Even without optimization of cultivation conditions, an attractive productivity was obtained, indicating that biofilm cultures can be used as an alternative form of high cell density cultivation (HCDC). *E. coli* remains one of the favorite hosts for recombinant protein production and it has been successfully used in metabolic engineering for the synthesis of high value products. This review presents the advantages and concerns of using biofilms for the production of recombinant proteins and summarizes the different biofilm systems which have been described for this purpose. The relative advantages and disadvantages of the four microbial hosts tested for recombinant protein production in biofilms (two bacteria and two filamentous fungi) are also discussed.

Keywords: recombinant protein; green fluorescent protein; biofilm; bacteria; *Escherichia coli*; filamentous fungi

1. Introduction

Recombinant proteins are synthesized in a host cell which is usually of a different species from the source of the DNA encoding them [1] and in that case they can be called heterologous proteins. Recombinant proteins have wide applications in medicine, research and biotechnology. With the development of recombinant protein methodology, it is possible to clone genes encoding proteins from different organisms and express them in other organisms at much higher levels than those naturally achieved [1,2], leaving behind the necessity of huge amounts of animal and plant tissues or volumes of biological fluids [3].

To obtain recombinant proteins, the gene encoding the protein is isolated and introduced into an expression vector, afterwards it is transformed into the chosen host system [1,3]. An important step in recombinant protein production is the choice of the ideal expression system. A large number of protein expression hosts are available, such as bacteria, yeast, filamentous fungi, and mammalian, plant and insect cells [4,5]. It is also important to select the most suitable expression vector, which is composed by a set of genetic elements that affect both transcriptional and translational steps of protein production, namely the origin of replication, promoter, ribosome binding site, start codon, transcriptional terminator, and selective marker [3].

Recombinant proteins have been mostly produced in suspended cultures. The insertion of the gene of interest into a multicopy plasmid may result in high recombinant protein expression [1]. Nevertheless, this may impose a metabolic burden on the host cell due to the energy and metabolites needed for the replication of plasmid DNA and synthesis of recombinant proteins [1,6]. In planktonic cells, this added metabolic burden may decrease the cellular growth rates and biomass yield, besides affecting the yield and activity of the desired protein [6]. In order to overcome these problems, a strategy based on the use of biofilm systems has been studied. Microbial biofilms are communities of microorganisms, single or multispecies, attached to surfaces which are embedded in a self-produced matrix of extracellular polymers [7–9]. Biofilms are usually known for their negative effects on health and industrial sectors as they can cause diseases, equipment corrosion, local clogging, heat transfer resistance and product contamination in food processing environments [7,10]. However, biofilms have beneficial use in wastewater treatment [11] and are being tested for the production of solvents, organic acids and enzymes [12–14]. The biological organization of biofilms provides them with many advantages over the suspended cells, including high cell density and protection against hostile conditions [15]. Furthermore, the presence of expression vectors in the sessile cells has shown to increase biofilm formation and lead to higher production of recombinant proteins compared to planktonic cells [16–19].

This review will focus on the production of recombinant proteins by biofilms. The main studies using biofilm systems for the production of recombinant proteins will be explored, as well as the differences between producing recombinant proteins in suspended and biofilm cultures. The advantages and disadvantages of using different microbial hosts in the production of recombinant proteins, namely *Escherichia coli*, *Bacillus subtilis*, *Aspergillus niger* and *Aspergillus oryzae*, will also be addressed.

2. Recombinant protein expression in suspended and biofilm cultures

Biofilm systems have many advantages when compared with suspended growth systems, especially in their higher biomass density and operation stability. Biofilm reactors are able to retain 5 to 10 times more biomass per unit volume of reactor [15], thus increasing the production rates and reducing the risk of cell washout when operating at high dilution rates during continuous fermentation. Furthermore, biofilms provide a protective environment to the cells as the extracellular matrix protects them against extreme conditions of pH and temperature, contaminations, hydraulic shock, antibiotics and toxic substances [15]. Another advantage of biofilm cultures is that cells can be easily separated from the liquid by sedimentation or filtration, resulting in more efficient downstream processes [13].

Recombinant protein production in biofilms was first described 25 years ago by Huang et al. [20–22], who showed that the maximum β-galactosidase concentration obtained in *E. coli* DH5α suspended cells was higher than that obtained in biofilm cells (0.47 vs. 0.12 pg/cell) [21]. On the other hand, the

probability of plasmid loss in a biofilm culture was greater than that observed in suspended batch cultures [21]. These results suggested that the production of extracellular polymeric substances (EPS) by biofilm cells may compete with plasmid maintenance/replication and expression of an heterologous plasmid-encoded protein for metabolic intermediates and energy sources, a competition that suspended cells do not experience [21]. About 15 years later, O'Connell and colleagues [17] contradicted this previous work by demonstrating that the *E. coli* biofilm environment enhanced the heterologous protein production when compared to planktonic cells. This study was carried out with *E. coli* ATCC 33456 pEGFP, a strain that formed a robust biofilm and harbored a high copy number modified pUC vector encoding enhanced green fluorescent protein (eGFP). Additionally, cultivation of *E. coli* as a biofilm was found to have a beneficial effect on high copy number plasmid maintenance compared to chemostats [17], which increased the gene dosage and may have contributed to the fact that 90% or more of biofilm cells produced significant levels of eGFP after 6 days, even in the absence of selective pressure. The reason for the enhanced plasmid maintenance in biofilms is that sessile cells tend to grow more slowly than their planktonic counterparts [23], leading to fewer cell divisions and consequently less plasmid partitioning. With infrequent cell division, less energy is directed towards replication, reducing the metabolic burden of plasmid maintenance on the cell. More recently, our research group assessed the potential of *E. coli* JM109(DE3) biofilms in the expression of the model protein eGFP from a pET-based vector [16,18,24,25]. It was found that the specific protein production from *E. coli* biofilm cells was about 30 fold higher than in planktonic state (5.8 vs. 0.18 fg/cell) [16]. When lysogeny broth (LB) was tested, which is a common culture medium used for recombinant protein expression with the pET system, the difference between the specific eGFP production of biofilms and suspended cells decreased to 10 times (12 and 1.2 fg/cell in biofilm and cell suspension, respectively) [18,25]. Furthermore, the percentage of planktonic eGFP-expressing cells oscillated around 5%, whereas for biofilms eGFP-expressing cells represented 21% of the total cell population [18]. The higher productivity of *E. coli* biofilms is probably related to their higher potential in maintaining the plasmid within the cells. In fact, in planktonic cells, the frequency of plasmid-containing bacteria was on average 0.33, while in the biofilm this parameter rose to approximately 0.90 [18].

In addition to *E. coli*, another bacteria - *Bacillus subtilis* - was studied for recombinant protein production in biofilms. The biofilm was reported as a new fermentation technique wherein the iturin A concentration almost doubled that obtained in the submerged culture [26]. This result was explained by the fact that biofilm cells remain in their metabolically active state for a longer period of time and maximize nutrient utilization [26].

The comparative analysis of classical fermentation and biofilm reactors for the production of recombinant proteins was also performed for the filamentous microorganisms *A. niger* and *A. oryzae* [27,28]. Talabardon and Yang [28] cultured a recombinant strain of *A. niger* containing the gene coding for GLA-GFP (glucoamylase-green fluorescent protein) fusion protein on cotton cloth in two different biofilm reactor configurations. They revealed that the biofilms formed in these conditions produced 10 fold more fusion protein than free-living pellets [28]. Zune et al. [27] also showed that the fluorescence signal measured from the same fusion protein produced in *A. oryzae* biofilm reactors was two times higher than that obtained in a tank operating at a low stirrer rate. In the case of filamentous microorganisms, the immobilization in biofilm reactors leads to decreased medium viscosity and consequently to an enhanced nutrient and oxygen transfer [29,30], which in turn may have increased the product yield. At the same time, the internal structure of fungal biofilms comprises channels in the hyphal layers that allow fluid circulation and promote a better mass transfer in comparison with the more compact

structure of the pellets found in submerged culture [31]. Another reason for the higher recombinant protein levels found in *Aspergillus* biofilm cultures is the low protease secretion [32,33].

Although biofilm reactors show several advantages for recombinant protein production over planktonic cultures, there are some limitations, particularly in the diffusion of substrates to the bacterial cells. Usually bacterial biofilms contain multiple layers of cells and their thickness may vary from a few to many μm. In order for the cells to be active in the production of value-added compounds, nutrients and substrate must diffuse/penetrate to the inner layers of the biofilm. However, it is known that sometimes the nutrients and substrate are used up by the outer cell layers before they reach the innermost layers [14,24]. According to a previous work from our research group [24], eGFP-producing biofilms are highly heterogeneous, with the cells actively producing the recombinant protein restricted to the top layer of the biofilm. This is probably due to the lack of oxygen penetration inside the biofilm, which is necessary for eGFP maturation, and/or mass transfer limitation of nutrients. Hence, the operating conditions should be optimized in order to obtain a porous biofilm, thus facilitating the access of the bottom layer to oxygen and fresh nutrients, which could then be shifted to a productive state [14,24,34]. Nevertheless, some authors have shown that diffusion limitations are not always harmful to recombinant protein expression in biofilms [21,35]. Mass transfer limitations of nutrients may cause lower cell growth rates inside the biofilm, thereby contributing to increased plasmid stability [21,35].

An additional drawback of recombinant protein production in biofilm cultures may come from the toxicity of the protein, which arises when it performs an unnecessary and detrimental function in the host cell [3]. Another aspect to consider is the location of the recombinant protein since the biofilm approach is more appropriate if the protein is released from the biofilm and can be captured afterwards from the culture medium [36]. If the recombinant protein is located intracellularly, a "milking" process must be established to operate the reactor in a continuous mode. This is a challenging task, in particular with regard to process engineering and economics of the process.

The Strengths, Weaknesses, Opportunities and Threats (SWOT) analysis of the application of biofilm reactors in recombinant protein production is presented in Figure 1.

3. Application of biofilms in recombinant protein production

The biofilm may provide distinct characteristics for the production of valuable compounds such as recombinant proteins [16,17]. Despite the potential of this expressing system, recombinant protein production using biofilms has been scarcely investigated during the last decades. To the best of our knowledge, there are only seven studies from independent groups on this topic. The published work is summarized in Table 1.

The expression of recombinant proteins in biofilms was first reported by Huang et al. [20,21,37], who have studied the production of β-galactosidase in *E. coli* DH5α cells carrying a plasmid containing the *tac* promoter. These authors have studied the plasmid retention and expression in both suspended and biofilm cells growing in a parallel-plate flow cell (PPFC). During the course of the experiments, they found that the cell accumulation rates decreased with increasing induction levels for both suspended and biofilm cells [21]. Furthermore, the production of β-galactosidase in both types of *E. coli* cells increased with induction (no basal expression was detected). Three different IPTG concentrations (0.17, 0.34 and 0.51 mM) were tested to determine the optimal inducer concentration. In biofilm cells, different IPTG concentrations did not affect the maximum β-galactosidase concentration obtained 24 h after induction (about 0.1 pg/cell) [21]. Huang et al. [37] also shown that the concentration of nitrogen and carbon in the growth medium affected biofilm

formation and plasmid stability. When the carbon/nitrogen ratio on the nutrient supply increased, the biofilm cell density decreased, while the probability of plasmid loss increased [37]. Additionally, it was shown that the synthesis rates of β-galactosidase mRNA were higher at higher expression levels, increasing 4 fold under 0.17 mM IPTG, and almost 12 fold under 0.34 and 0.51 mM IPTG. However, the β-galactosidase production did not increase at the same proportion of mRNA synthesis rate. It is possible that the synthesized mRNA was much less stable at high expression levels [20].

STRENGTHS	**WEAKNESSES**
High biomass density Operational stability High production rates Protective environment against extreme conditions Can be operated for longer periods Ease of biomass-liquid separation Plasmid stability Plasmid copy number maintained or increased	Limited transfer of oxygen and nutrients Control of biofilm thickness is hard Process scalability Structural or segregational instability of the plasmid under different culture conditions
OPPORTUNITIES	**THREATS**
Biofilm cultures offer high reaction rates and are economical, so they can be employed to decrease production costs of various recombinant products on a large scale Considerable knowledge on the design and operation of biofilm reactors is available since they are being used in a number of full-scale applications for industrial and wastewater treatment Most of the microorganisms applied in biotechnology can be grown attached to a surface The stable maintenance of high copy number vectors by biofilms can benefit metabolic engineering processes (e.g. rational design of multispecies biofilms for recombinant protein production)	Competition with established production processes using suspended cultures Additional downstream challenges for separating the cells from the surface (intracellular proteins)

Figure 1. SWOT analysis of biofilm cultures for recombinant protein production.

Table 1. Overview of the published work on the production of recombinant proteins in biofilms.

Host	Recombinant protein	Cultivation conditions	Recombinant protein production	Reference
E. coli DH5α	β-galactosidase	PPFC Glass slides 37 ℃ Supplemented M9 minimal medium	0.08–0.12 pg/cell	[20,21,37]
E. coli ATCC 33456	eGFP	PPFC Cover glass 37 ℃ LB medium Laminar flow	0.01–0.16 g/L	[17]
E. coli JM109(DE3)	eGFP	Flow cell system PVC surfaces 30 ℃ DM and LB media Turbulent flow	5.8 fg/cell and 0.22 g/L for DM supplemented with 20 μg/mL kanamycin 12 fg/cell for LB supplemented with 20 μg/mL kanamycin	[16,18,24, 25]
Bacillus subtilis	iturin A	24-well plates 28 ℃ LB medium	0.6 g/L	[26]
Bacillus subtilis	mCherry, EgTrp and EgA31 (part of the TasA-mCherry, TasA-EgTrp and TasA-EgA31 fusion proteins, respectively)	Well plates with 22 mm^2 surface area 30 ℃ MSgg medium	n.a.	[38]

Continued on next page

Host	Recombinant protein	Cultivation conditions	Recombinant protein production	Reference
Aspergillus niger	GFP (part of the GLA-GFP fusion protein)	Static and RFB reactor Cotton cloth attached to a stainless steel cylinder Modified Vogel's medium 25 ℃	0.78 g/L	[28]
Aspergillus oryzae	GFP (part of the GLA-GFP fusion protein)	Reactor based on a stainless steel structured packing 30 ℃	n.a.	[27]

Notes: n.a: not available. *Abbreviations:* PPFC: parallel-plate flow cell; RFB: rotating fibrous bed; LB: lysogeny broth; DM: diluted medium; eGFP: enhanced green fluorescent protein; TasA-EgTrp: TasA-tropomyosin peptide; TasA-EgA31: TasA-paramyosin peptide; GLA-GFP: glucoamylase-green fluorescent protein; PVC: polyvinyl chloride.

In 2007, O'Connell et al. [17] described the first system for high level heterologous protein production in *E. coli* biofilm cells. These authors used an *E. coli* strain containing the plasmid pEGFP to investigate the production of eGFP in a chemostat (planktonic cells) and in a PPFC reactor (biofilm cells). They detected different populations of sessile cells: strongly producing cells (capable of producing 0.16 g/L of eGFP), moderately producing cells (capable of producing 0.01 g/L of eGFP) and non-producing cells. In contrast with Huang et al. [20,21], the results of this work indicated that the biofilm environment enhanced both plasmid maintenance and heterologous protein concentration when compared to planktonic cells [17]. This study also found that the addition of low antibiotic concentrations to biofilm populations increased these beneficial effects, enabling them to obtain 60% of strongly producing cells [17].

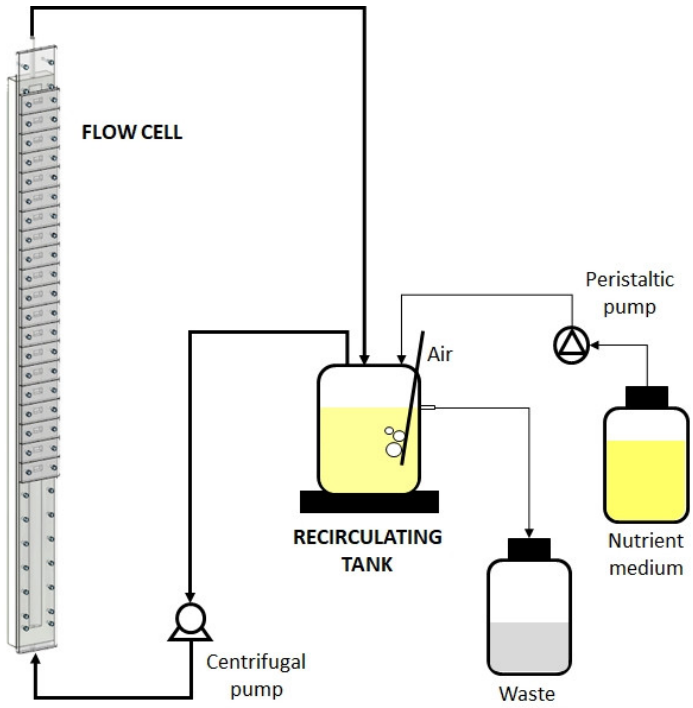


Figure 2. Schematic representation of the biofilm producing system.

The diluted medium (mainly composed by 0.55 g/L glucose, 0.25 g/L peptone and 0.125 g/L yeast extract) was described as a good medium for biofilm formation [43], whereas LB is frequently used for the expression of recombinant proteins and provides abundant carbon and nitrogen (10 g/L tryptone and 5 g/L yeast extract) [46]. This study reported a 2 fold increase in the eGFP production of biofilms developed in LB when compared to what was obtained in DM [25]. Regarding the effect of antibiotic concentration, the increase from 20 to 30 μg/mL had no effect on the amount of recombinant protein produced by the biofilm [25]. Thus, among the tested conditions, LB supplemented with 20 μg/mL of kanamycin seemed to be the most advantageous medium to obtain the highest specific eGFP production in *E. coli* biofilms [25]. Our research group also examined the eGFP expression during biofilm development at the single-cell scale, showing that the biofilm population became increasingly heterogeneous over the course of the experiment [24]. Three different types of biofilms were observed: one with a homogeneous population (between days 3 and 5), a second with a moderately homogeneous population (between days 6 and 8), and the third with a strongly heterogeneous population (between days 9 and 11). These results are consistent with those obtained by O'Connell et al. [17], who studied the dynamics of protein fluorescence during biofilm development. Moreover, by confocal microscopic analysis (Figure 3), we also showed that the majority of the eGFP-expressing cells were located in the liquid interface of the biofilm. It is known that the spatial organization of biofilms can affect the diffusion of oxygen and nutrients, resulting in heterogeneous populations in terms of metabolic and phenotypic behaviors [47,48]. Lenz et al. [49] observed a spatially non-uniform pattern of GFP expression in *Pseudomonas aeruginosa* biofilms. These authors demonstrated that the amount of GFP mRNA was higher in the external layers of the biofilm, which is associated with the zone of active GFP fluorescence visualized at the top of the biofilm [49]. This is probably related with the higher access of sessile cells to the oxygen required to the final stage of protein folding [50].

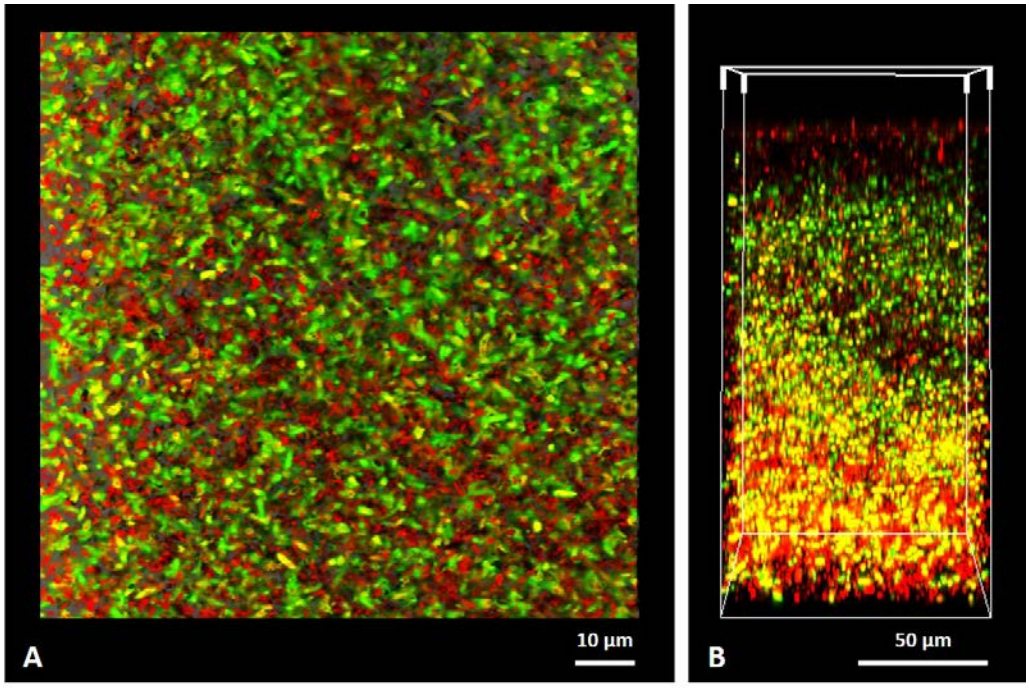


Figure 3. Spatial heterogeneity of a 11-day-old biofilm formed by *E. coli* JM109(DE3) expressing the recombinant protein eGFP: (A) top view and (B) three-dimensional view of the confocal laser scanning microscopy (CLSM) images. The eGFP-expressing cells are labelled in green and the non-expressing cells are countermarked in red with Syto61.

The production of recombinant proteins in biofilms of the Gram-positive bacterium *Bacillus subtilis* has also been studied [26,38]. Rahman et al. [26] used a transformant strain of *B. subtilis* 168, containing wild *sfp*, the *itu* operon and *degQ* for the production of the lipopeptide iturin A. These authors reported an iturin A concentration in the biofilm of approximately 0.6 g/L in steady state conditions, which is higher than the concentration of 0.4 g/L obtained in the cell suspension [26]. Later on, Vogt et al. [38] developed a strategy based on the TasA protein for the display of a heterologous protein within the *B. subtilis* biofilm. They engineered a fusion protein with TasA and the red fluorescent protein mCherry, and showed that this fusion protein was abundant and homogeneously distributed within the biofilm. TasA is one of the crucial matrix proteins responsible for biofilm formation and is dependent on the tapA-sipW-tasA operon [51]. The same authors also produced fusion proteins of TasA with *Echinococus granulosus* antigenic peptides, paramyosin and tropomyosin [38]. The results showed that the antigens were expressed and could be efficiently located in the biofilm matrix. Additionally, it was demonstrated that the spores produced by the recombinant strain were physically and morphologically identical to the wild-type strain, which could be an excellent strategy to preserve the integrity of heterologous proteins and facilitate their transport to the desired location [38].

In 2005, Talabardon and Yang [28] used a recombinant *A. niger* strain containing a gene encoding the glucoamylase-GFP (GLA-GFP) fusion protein to study the GFP and glucoamylase protein production in suspension and immobilized biofilm cells. They used different culture systems: free-cell cultures in conventional stirred-tank bioreactors grown in pellet form and cells immobilized in cotton cloth grown in a mycelial form in a rotating fibrous bed (RFB), to study the influence of the fungal morphology in the expression of the fusion protein. These authors found that the amount of glucoamylase and GFP produced by the immobilized cells of *A. niger* was 0.8 g/L, about 10 times

more than in suspended cells. Later, Zune et al. [27] used a tank with a metal structured packing as a fungal biofilm reactor for the production of the GLA-GFP fusion protein by *A. oryzae*. The fluorescence measurements indicated that protein production in the biofilm reactor was similar to that of suspended cell cultures under higher shear stress conditions. However, the western blot analysis revealed that the band corresponding to the fusion protein in submerged conditions was thicker than that under biofilm conditions. This result could be explained by the operating conditions in the submerged reactor, namely the acid pH which could promote biomass leakage and affect the quality of the protein. Based on the results obtained in the shake flasks, the authors investigated the production of the fusion protein in two different configurations of the biofilm bioreactor, one with full immersion of the fungal biofilm in the liquid medium and other with periodic immersion of the biofilm [27]. Although both biofilm reactors reached satisfactory protein productivities, a 2-D electrophoresis analysis indicated that the quality of the fusion protein was higher in the fully immersed bioreactor [27].

4. Hosts for recombinant protein expression in biofilms

A myriad of organisms (or their derivatives) can be chosen as hosts for recombinant protein production. The list includes bacteria, yeast, filamentous fungi, mammalian, plant and insect cells or transgenic organisms (plants and animals) [52]. Although there is a lot to choose from, the selection of the most appropriate expression host is often limited to a few options given the intrinsic characteristics of the protein and the process to be developed. For low scale protein production (mainly for research purposes), intrinsic attributes of the protein are usually the most important parameter, but for large scale production (industrial scale) other criteria are also relevant. A summary of the factors that influence the host selection for low and industrial scale can be found in Table 2.

In either production scale, the intrinsic characteristics of the protein are of the utmost importance. These include the requirement for post-translational modifications such as glycosylation, disulfide bond formation, phosphorylation, acetylation, acylation, carboxylation and other modifications that can affect folding and activity [53]. Some of these modifications can be performed *in vitro,* namely in refolding strategies, but this is often not ideal. The production level is also very important in both cases because if a protein is inherently unstable in a given host, or if the host is for any reason incapable of expressing it in reasonable amounts, another system must be used. This may be related to the codon bias of the gene (which can be easily solved), but also to the instability of the produced mRNA, the susceptibility to attack by host proteases (protease deficient hosts are sometimes available), the incompatibility with secretion, or the deficient folding and aggregation in a particular host or cellular compartment. The technical expertise of the staff/researcher may also be an important factor. In the lab, prior experience with a certain system may introduce a certain bias to a particular host and in industrial settings, a small diversity of "cell factories" is often used in a given facility so that the staff becomes highly experienced in dealing with the peculiarities of each system.

The cell growth rate is important in both production scales, but it has a much larger impact in industrial production. If the cell growth is slow, the bioreactor must be operated during a longer period and this may affect the scheduling of the unit operations in a production plant. Since in some cases downstream processing equipment is shared by different production lines, an increased bioreactor operation time may impact on the possibility to integrate the production of that particular protein in the industrial unit which may be producing several products. The time for setup and development is also important in both production scales and it concerns the time required to go from gene to product. Since in industrial production, protein products often require approval by regulatory

bodies (e.g., the Food and Drug Administration, FDA) and this approval process must follow the process development, this issue becomes critical in the industrial case and is somewhat less important at the lab scale. The scalability of the process is also important at both production levels, but has a more profound effect on industrial production. At the host level, scalability means that the cells will be able to withstand very demanding cultivation conditions, particularly concerning the sensitivity to shear forces, tolerance to deficient oxygen mixing and heat and mass transport limitations that are common in large scale reactors. Additionally, the possibility of performing fed-batch fermentation in HCDC mode is also an advantage of some of the hosts.

For large scale production and commercialization, issues like the safety component are also important. If possible, the organism should have a GRAS (Generally Regarded As Safe) status so that additional precautions are not necessary and even cheaper (e.g., non-explosion proof) equipment or handling procedures can be adopted [54]. The issues surrounding regulation are also critical as many protein products must be approved by regulatory bodies as mentioned before. The regulatory bodies will make sure that the protein product is safe and it can provide the claimed effect before commercialization. Hosts with a very solid track record of approval are most likely to get faster and favorable decisions from the regulatory bodies. If a protein product is under patent protection or even if the process of producing the desired protein is protected, the inventors may be entitled to royalties until the patent expires. Thus, it is important to guarantee that the product or the production process does not infringe pre-existing patents [55]. The cost of goods is particularly important in large scale production because of the amount of culture medium components and also other components required during downstream or formulation. Particular attention is given to the culture medium, which sometimes is very expensive due to the metabolic requirements of the host organism. However, in some cases, it can include wastes and by-products of other industries as a cost reduction strategy.

Table 2. Factors that influence the selection of the most appropriate expression host for low and industrial scale production.

Low level production	Industrial production
Intrinsic protein properties	Intrinsic protein properties
Level of production	Level of production
Technical expertise	Technical expertise
Cell growth	Cell growth
Time for setup and development	Time for setup and development
Scalability potential	Scalability potential
	Safety component
	Regulatory issues
	Patenting issues
	Cost of goods

For the purpose of this review, we are going to focus our attention on microbial systems since recombinant protein production in biofilms is being explored in this context. A closer look at the available literature shows that from the many microbial hosts that are currently used for recombinant protein production in the planktonic state, only a few have ever been tested for recombinant production in biofilms (Table 1). Table 1 shows only four microbial hosts, two bacteria and two filamentous fungi. The relative advantages and disadvantages of each system will be discussed below and summarized in Table 3.

4.1. Escherichia coli

The Gram-negative bacterium *E. coli* has been the most intensively used workhorse for recombinant protein production since the 1980s. The profound knowledge that has accumulated about this organism and the associated genetic methodologies still make it a highly versatile host capable of adaption to different production requirements [56]. *E. coli* grows fast (doubling times of 20 min can be achieved) in non-expensive culture media [3,57,58], and the fermentation is easy to scale-up. In this host, there is no unintended glycosylation in non-engineered strains, no viral contamination risk (with viruses that can affect humans) and it has a very solid track-record in product approvals by the FDA. Additionally, some strains have a GRAS status, it can accumulate recombinant proteins at more than 20% of the total cellular protein content, a very well-established fermentation know-how is available and it is amenable to HCDC [58]. Although there are many available expression strains, some of them have been specifically manipulated to be used with particular expression systems [3].

Protein production can be targeted to different locations (cytoplasm, periplasm, inner/outer membrane, or culture medium) with significant impact in downstream processing [59]. Although extracellular production can greatly reduce the complexity of the production process, the low secretion capacity of *E. coli* has always been considered as a major drawback. Different techniques can be used to circumvent this limitation such as the engineering of dedicated secretion systems existing in pathogenic strains, the use of native translocation mechanisms eventually using carrier-proteins, or cell envelope mutants or the co-expression of lysis-promoting proteins [4,59].

The inability to perform glycosylation and several other post-translational modifications have also been a hallmark of *E. coli*. Although it was initially thought that bacteria were not capable of producing glycosylated proteins, the discovery of an N-linked glycosylation system in *Campylobacter jejuni* changed this perception. This system was transferred to *E. coli* [60] and the production of glycosylated proteins at a scale of 5 L was made possible [61]. There are still several significant challenges to be overcome in the production of glycosylated proteins in *E. coli*. Not only sometimes glycosylation is incomplete, but also its efficiency can be very low and heterogeneity can be found if more than one glycosylation site is present. Additionally, the production of glycosylated proteins was shown to negatively affect cell growth [4]. Since most of the eukaryotic proteins are N-terminally acetylated, this modification is very important in recombinant protein production in prokaryotic systems as acetylation has shown to affect several cellular processes. Also, phosphorylation is very important for the activity of many recombinant proteins and it was shown that either by co-expressing chaperone proteins or by performing gene fusions, it was possible to obtain some acetylated and phosphorylated proteins in *E. coli* [4].

Other drawbacks are the possibility of acetate accumulation, which has a toxic effect, although this can be controlled by the oxygen level and careful choice of the expression strain. Additionally, many proteins are produced as insoluble inclusion bodies, which are inactive and require refolding. Although it has been argued that recombinant protein production in inclusion bodies decreases the deleterious effects associated with protein toxicity (as the protein is not active), it must be remembered that high-level recombinant protein production is always toxic to the cells due to the metabolic burden associated with the expenditure of energy, amino acids and other precursor molecules. In order to obtain an active protein from these inclusion bodies, it is often necessary to remove them from the cells, the proteins must be solubilized by denaturants that cause protein unfolding, and disulfide bonds must be broken using reducing agents. Refolding than proceeds by removing the denaturant and reducing agent followed by renaturation with oxidation agents [5]. A

further disadvantage of *E. coli* is the accumulation of lipopolysaccharides (LPS), requiring additional purification steps as some of these substances are pyrogenic in humans [62].

Another possible application of biofilms as a system to produce valuable proteins is the possibility of expressing membrane proteins. These play key roles in many diseases and about 70% of all drugs act on membrane proteins [63]. A particular type of membrane proteins (helical bundle type) is difficult to produce in inclusion bodies and must be produced in a way that the protein is inserted into the membrane from where it can be purified. This may be problematic because the capacity of the translocation machinery of an *E. coli* cell may be saturated, thus preventing efficient transport to the cell envelope [64,65]. In order to circumvent this problem, different strains were developed for membrane protein expression in which expression rates can be tuned to match the capacity of secretion systems [66], and also omitting the inducer in chemically inducible promoter systems has been tested with success [67]. These systems are based on expressing the recombinant protein at a lower rate and since biofilm cells are usually at a lower metabolic state then their planktonic counterparts, it is tempting to use them for this purpose. On the other hand, biofilm formation depends on the production of EPS and the transport of these molecules to the cell exterior will take its share of the translocation capacity of the cell.

4.2. Bacillus subtilis

This organism is arguably the best studied Gram-positive bacterium [68] and has been intensively used for the production of different enzymes like proteases and amylases, mainly for detergents and the food industry. The production of these enzymes is also regulated, but criteria are less stringent than when producing recombinant proteins for therapeutic application. A major advantage of *B. subtilis* when compared to *E. coli* is that it does not have an LPS-containing outer membrane [5], which is important as LPS are pyrogenic in humans [62]. Several homologous proteins have been produced in this host [5], but sometimes the expression of heterologous proteins is more challenging [69,70]. Several strains of *B. subtilis* have been sequenced and they not produce toxins [5]. *Bacillus* species are capable of fast growth on cheap carbon sources and are robust for use in industrial fermentations [71]. The secretion capacity of the system is high [72], which facilitates downstream processing. Several *Bacillus* strains produce different proteases [69], but some protease-deficient strains are available. This host is generally regarded as a potentially safe bacterium for industrial and pharmaceutical applications [68]. Plasmid instability can also be an issue when working with this organism, but it has been shown that this disadvantage can be overcome by using plasmids with theta replication [69,72]. For recombinant protein production, *B. subtilis* is being intensively used and current development strategies include promoter engineering, signal peptide engineering to increase secretion efficiency and development of protease-defective strains [68]. Further knowledge must also be gathered about the physiological responses of producing strains during large-scale fermentation, and there is a lack of detailed knowledge on protein-protein interactions and the post-translational regulation network [71]. Operational challenges like the production of large amounts of foam, sporulation under nutrient starvation [70] and high maintenance metabolism are being addressed through genetic manipulation [71].

4.3. Aspergillus niger and *A. oryzae*

A. niger and *A. oryzae* are considered non-mycotoxin producers, although low levels of mycotoxins have been found in particular fermentation conditions [73]. Both species have a GRAS status due to long-term use without pathogenicity.

These are very attractive hosts for recombinant protein production since they can secrete high levels of active proteins with post-translational modifications such as glycosylation [5]. Additionally, it has been shown that some strains have a similar glycosylation pattern to that of human cells [54,73] and that these species can grow at high rates and to high densities in commercial bioreactors [74].

Although the production of homologous proteins is sometimes successful, the production of heterologous proteins is often difficult [75,76]. Secretion of some recombinant proteins is low in some cases, and production seems to be limited by transcriptional and post-translational metabolic steps [5,54]. There is still limited knowledge about the details of protein secretion and modification in these organisms. Also, foreign DNA is sometimes quickly degraded and expression rates are often reduced due to rapid RNA degradation [76], or processing. Additionally, the glycosylation pattern affects folding and secretion, and therefore incorrectly glycosylated proteins are degraded [54]. Being saprophytic organisms, these organisms naturally secrete large amounts of proteases that degrade foreign proteins [73,75] and cell productivity is highly affected by the growth morphology.

Despite the challenges posed by broth rheology when it comes to mass transfer, non-filamentous, less viscous, low-protease producing strains have been developed [5,74]. *Aspergillus* species are good candidates for heterologous protein production and other foreign metabolites, thus acting as cell factories [77].

Table 3. Advantages and disadvantages of the different hosts used for recombinant protein production in biofilms.

Host	Advantages	Disadvantages
Escherichia coli	Proven track record in FDA approvals Many available expression systems Simple, well-understood genetics Inexpensive culture media Fermentation easy to scale up No unintended glycosylation No viral or prion contamination risk Ease of handling, GRAS status Fast proliferation High expression levels Very well-established fermentation know-how Amenable to HCDC Protein can be directed to different cellular locations	Proteins with disulfide bonds are difficult to express Production of glycosylated proteins is challenging Phosphorylated or acetylated proteins are difficult to produce Proteins produced with endotoxins Acetate formation resulting in cell toxicity Proteins produced as inclusion bodies Inactive proteins require refolding Protein stability can be low due to proteolytic degradation Protein secretion is challenging
Bacillus subtilis	Strong secretion capacity Ease of genetic manipulation Genetically well-characterized systems Superior growth characteristics Metabolically robust GRAS status No production of toxins Outer membrane devoid of LPS Fast growth on cheap medium	Protein instability due to host proteases Sporulation can be an issue Excessive foaming High maintenance metabolism

Continued on next page

Host	Advantages	Disadvantages
Aspergillus niger and *A. oryzae*	Enormous nutritional flexibility Able to perform complex post-translational modifications Glycosylation pattern may be similar to mammalian cells Some strains have a GRAS status Very high secretion potential HCDC is possible	Secretion levels are sometimes low Production can be limited by transcription Some strains encode many proteases Possibility of obtaining a highly viscous culture broth mRNA stability can be an issue Plasmid stability may be an issue Lack of knowledge about physiology

Abbreviations: FDA – Food and Drug Administration; GRAS – Generally Regarded As Safe; HCDC – high cell density cultivation; LPS – lipopolysaccharides.

5. Conclusions

Biofilm reactors present many advantages over suspended cell reactors, including higher cell densities and long-term stability as required for continuous processing. Furthermore, according to the work published on recombinant protein production, microbial biofilm systems are sometimes able to produce recombinant proteins at attractive levels. Nevertheless, several elements that affect the overall efficiency of biofilm reactors, especially limitations in the diffusion of nutrients and oxygen, call for careful implementation of strategies specifically targeted for increasing recombinant protein production in these systems.

Additionally, there are several hosts that can be chosen for recombinant protein production in biofilms. However, the selection of the most appropriate expression host is limited to a few options given the characteristics of the protein and the process to be developed. In this review, we focused on two bacteria (*E. coli* and *B. subtilis*) and two filamentous fungi (*A. niger* and *A. oryzae*) since they have already been tested for recombinant production in biofilms. *E. coli* remains one the favorite hosts for recombinant protein production at lab and industrial scale since it offers many advantages over the other host systems, namely fast growth at high cell densities, well-characterized genetics, and availability of a large number of cloning vectors.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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Comparing the Recombinant Protein Production Potential of Planktonic and Biofilm Cells

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Abstract: Recombinant protein production in bacterial cells is commonly performed using planktonic cultures. However, the natural state for many bacteria is living in communities attached to surfaces forming biofilms. In this work, a flow cell system was used to compare the production of a model recombinant protein (enhanced green fluorescent protein, eGFP) between planktonic and biofilm cells. The fluorometric analysis revealed that when the system was in steady state, the average specific eGFP production from *Escherichia coli* biofilm cells was 10-fold higher than in planktonic cells. Additionally, epifluorescence microscopy was used to determine the percentage of eGFP-expressing cells in both planktonic and biofilm populations. In steady state, the percentage of planktonic-expressing cells oscillated around 5%, whereas for biofilms eGFP-expressing cells represented on average 21% of the total cell population. Therefore, the combination of fluorometric and microscopy data allowed us to conclude that *E. coli* biofilm cells can have a higher recombinant protein production capacity when compared to their planktonic counterparts.

Keywords: green fluorescent protein; *Escherichia coli*; biofilm; planktonic; flow cell reactor

1. Introduction

Recombinant proteins are currently used in different biotechnological industries and are produced in large amounts in bioreactors [1]. An important step in recombinant protein production is to choose the ideal expression system and factors like protein quality, functionality, production speed, and yield should be considered [2]. *Escherichia coli* has been widely used for the production of recombinant proteins due to its fast growth at high cell densities, minimal nutrient requirements, well-characterized genetics, and the availability of a large number of cloning vectors [2,3]. In the early 1980s, the Food and Drug Administration (FDA) approved the first recombinant insulin produced in *E. coli* [4] and since then this bacterium is one of the organisms of choice for the production of several commercial recombinant proteins [5].

Most of the research on the expression of recombinant proteins has been based on planktonic bacteria grown in liquid cultures. However, the natural state for many bacteria is to live in communities of sessile cells forming biofilms [6]. Biofilms are communities of surface-attached microorganisms encased in a self-produced extracellular matrix [7]. Such biological organization provides distinct characteristics to bacteria compared to their planktonic counterparts [8]. For instance, local environmental conditions arising as a result of mass transport limitations, intercellular signaling, and other phenomena, may induce biofilm cells to modulate expression of genes differently than in suspended populations [9]. The expression of recombinant proteins in *E. coli* is commonly accomplished by inserting the gene of interest into a multicopy plasmid [10] that imposes a metabolic burden on the host cell [11]. In planktonic cells, this added metabolic burden may decrease cellular growth rates and biomass yields [11], particularly when the plasmid vector is used for the direct

production of a recombinant protein [12]. Conjugative [13–17] and non-conjugative plasmids [18–21] were shown to increase biofilm formation. For example, our research group has previously shown that the presence of the non-conjugative plasmid pET28A in *E. coli* JM109(DE3) cells increased biofilm formation when compared to a non-transformed strain [18].

In 2007, O'Connell et al. [22] examined the production of eGFP in a chemostat with planktonic cells and in a parallel plate flow cell (PPFC) reactor with biofilm cells. This was the first experiment showing a high-level production of a heterologous protein in *E. coli* biofilms. Although the number of studies on the effect of recombinant protein expression on sessile cells is still scarce, it has been suggested that the biofilm environment benefits recombinant protein production [18,23].

The aim of this work was to compare the production of a model recombinant protein (enhanced green fluorescent protein, eGFP) between *E. coli* planktonic and biofilm cells. In the present study, the comparison between planktonic and biofilm cells was explored using a novel single-cell methodology which combines the common epifluorescence microscopy with an image analysis tool. Additionally, the plasmid maintenance in both planktonic and sessile cells was also assessed for the first time.

2. Materials and Methods

2.1. Bacterial Strain

The *E. coli* strain JM109(DE3) from Promega (Madison, WI, USA) was transformed with the plasmid pFM23 (constructed from pET28A vector; Novagen, Madison, WI, USA) for the cytoplasmic production of eGFP [24]. This plasmid contains a kanamycin resistance gene and a pMB1 origin of replication (medium-copy number replicon), and uses the T7 promoter for transcription of the foreign gene, which can be induced by lactose or its non-hydrolyzable analogue isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.2. Flow Cell System and Experimental Conditions

A reactor system comprising a recirculating tank (for planktonic cells), a vertical flow cell reactor (where biofilms are formed), and peristaltic and centrifuge pumps that allow the circulation of the bacterial suspension was operated as described by Gomes et al. [25]. The flow cell reactor is a semi-circular Perspex duct with apertures on its flat wall to fit removable Perspex pieces (coupons) to which polyvinyl chloride (PVC) slides were glued. The biofilms were developed on the upper faces of the PVC slides that were in contact with the bacterial suspension circulating through the system.

E. coli cells were grown by recirculating the bacterial suspension at 30 °C for 11 days under turbulent flow (Reynolds number of 4600) [25]. The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with lysogeny medium (LB-Miller; Sigma, St. Louis, USA) supplemented with 20 μg mL^{-1} kanamycin at a flow rate of 0.025 L h^{-1} [25].

2.3. Biofilm and Planktonic Monitoring

The system was stopped daily and a coupon was carefully removed from the flow cell for biofilm sampling. The biofilm wet weight and thickness were firstly determined by comparing the coupon weight prior to the start of the experiment and on the sampling day, and by using a digital micrometer (VS-30H; Mitsubishi Kasei Corporation, Tokyo, Japan) [26], respectively. Then, the biofilm was scrapped off the coupon using a pipette tip and resuspended in 25 mL of sterile saline solution (0.85% NaCl) for total and viable cell quantification and eGFP analysis.

Biofilm total (viable plus non-viable) and viable cell counts were determined using the Live/Dead® BacLight™ bacterial viability kit (Syto9/propidium iodide; Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal) as fully described in Gomes et al. [25]. Briefly, bacterial cells from the biofilm suspension were filtered through a Nucleopore Track-Etch Membrane of black polycarbonate (pore size of 0.22 μm; Whatman Ltd., Buckinghamshire, UK), stained for 10 min in the dark and observed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems

Ltd, Heerbrugg, Switzerland). The image processing software ImageJ v1.48 (National Institutes of Health, Bethesda, MD, USA) was used to estimate the number of viable and non-viable cells on each membrane from counts of a minimum of 15 fields of view. The final values of biofilm total cells were expressed as log cells cm^{-2} of coupon area and the percentage of biofilm viability on each day was calculated by dividing the viable cell counts by the total cell number.

The eGFP expression in biofilm cells was assessed by both epifluorescence microscopy [25] and fluorometry [24]. For the microscopic method, the biofilm suspension was filtered and observed using a Leica DM LB2 epifluorescence microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland). Fifteen fields of view were photographed for each sample in order to estimate the number of eGFP-expressing cells [25]. The percentage of eGFP-expressing cells in the biofilm was then calculated by dividing the number of eGFP-expressing cells by the total number of cells. The specific eGFP production in biofilms was quantified by the fluorometric method fully described by Mergulhão and Monteiro [24]. Briefly, a volume of biofilm detached cells corresponding to an equivalent optical density (OD) of 1 at 610 nm was centrifuged. The pellet was resuspended in 100 µL of Buffer I (50 mM Na_2HPO_4, 300 mM NaCl, pH 8) and added to 100 µL of Buffer I in a 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium). Fluorescence was measured in a microplate reader (SpectraMax M2E; Molecular Devices, Inc., Berkshire, UK) with a 488 nm-excitation filter and a 507 nm-emission filter. Calibration curves were constructed with purified eGFP and the final values were presented as specific eGFP production (fg $cell^{-1}$). An OD_{610nm} of 1 corresponds to a cellular concentration of 7.6×10^8 cells mL^{-1}.

For monitoring the planktonic cells, a sample was taken from the recirculating tank to first assess the OD_{610nm} and the dissolved oxygen (OX 4100 H; VWR, Alfragide, Portugal). Total and viable cell counts, eGFP-expressing cell counts and specific eGFP production were also assessed as previously described for the biofilm cells (Figure 1).

2.4. Measurement of Plasmid Maintenance

Planktonic and biofilm sample suspensions were suitably diluted with saline solution and spread on both selective (supplemented with 20 µg mL^{-1} kanamycin) and non-selective agar plates (Plate Count Agar, Merck, Lisboa, Portugal) to form between 10 and 300 colony forming units (CFU) per plate. Colony enumeration was carried out after 24-h incubation at 30 °C. The number of CFU on selective plates corresponds to the number of culturable cells carrying the plasmid pFM23, whereas the CFU detected on non-selective plates are equivalent to the total number of culturable cells, i.e., plasmid-bearing cells plus plasmid-free cells (see Supplementary Material, Figure S1). The maintenance of plasmid pFM23 in both planktonic and biofilm cells was determined as the ratio between the number of plasmid-bearing cells and the total number of culturable cells.

2.5. Statistical Analysis

The results presented in Figure 1 originated from averages of triplicate sets obtained in independent experiments. Standard deviations (SDs) on the triplicate sets were calculated for each day and parameter. For planktonic growth (Figure 1a,b), the following SD averages were obtained: SD < 16% for OD_{610nm} and dissolved oxygen, SD < 4% for total cell counts, and SD < 5% for planktonic viability. Concerning biofilm formation (Figure 1d,e), the following SD averages were obtained: SD < 23% for biofilm wet weight, SD < 20% for biofilm thickness, SD < 3% for biofilm total cells, and SD < 4% for biofilm viability. Regarding the eGFP quantification (Figure 1c,f), SD < 19% for planktonic eGFP production, SD < 4% for planktonic eGFP-expressing cells, SD < 8% for biofilm eGFP production, and SD < 4% for biofilm eGFP-expressing cells were obtained.

Paired *t*-test analysis was performed based on a confidence level of 95% (differences reported as significant for p values < 0.05).

3. Results

In this work, two fluorescence-based techniques (fluorometry and epifluorescence microscopy) were used with the aim of comparing the eGFP production in planktonic and biofilm populations grown in a flow cell system.

Planktonic growth was monitored over 11 days by measuring the OD_{610nm} and dissolved oxygen in the recirculating tank (Figure 1a) and by quantifying the total cell number and cell viability (Figure 1b). The OD_{610nm} increased until day 4, which is in agreement with the corresponding decrease in the oxygen concentration in the recirculating culture over the same experimental period. Between days 4 and 6, the OD_{610nm} decreased and then stabilized from day 6 onwards. The same stabilization was observed in the oxygen consumption profile with dissolved oxygen levels around 2 mg L^{-1}, thus it can be concluded that the flow cell system was in steady state between days 6 and 11. Looking at Figure 1b, the total number of planktonic cells remained practically constant over the experiment. However, the percentage of viability varied significantly (Figure 1b) due to oscillations on the number of viable cells during the experimental time (data not shown). Between days 4 and 6, a strong decrease in planktonic viability (from 92% to 42%) was observed. But from day 8 onwards, the planktonic viability increased significantly, reaching almost 100% at the end of the experiment.

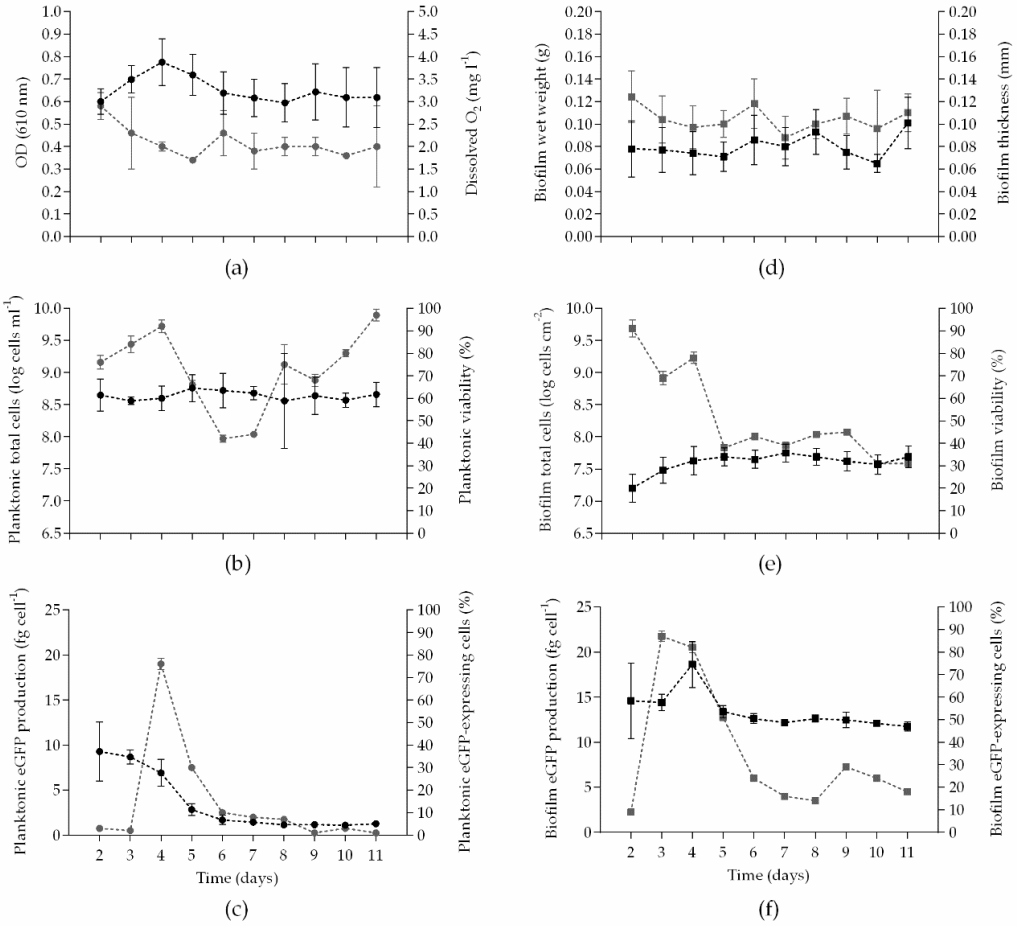


Figure 1. Time-course evolution of planktonic and biofilm parameters: (**a**) OD_{610nm} and dissolved oxygen in the recirculating tank; (**b**) planktonic total cells and planktonic viability; (**c**) planktonic eGFP production and percentage of planktonic eGFP-expressing cells; (**d**) biofilm wet weight and thickness; (**e**) biofilm total cells and biofilm viability; (**f**) biofilm eGFP production and percentage of biofilm eGFP-expressing cells. Black circles (●) and squares (■) on the left y-axis, and grey circles (●) and squares (■) on the right y-axis. The means ± SDs for three independent experiments are illustrated.

Regarding the biofilm growth, it was monitored by determining the wet weight and thickness (Figure 1d), and by quantifying the total cell number and cell viability (Figure 1e), as performed for planktonic cells. Biofilm wet weight remained practically constant during the experimental time, but a slight increase was observed in the last day of the experiment. The evolution of biofilm thickness was similar to the wet weight with average values around 0.104 mm. Figure 1e shows that the total number of biofilm cells increased slightly between days 2 and 5, remaining constant until the end of the experiment. Concerning biofilm viability (Figure 1e), it is possible to observe a marked reduction in cell viability until day 5 (38%) that resulted in viability percentages of on average 39% until day 11. Note that lower viability percentages were found for biofilms (Figure 1e) when compared to planktonic population (Figure 1b).

By analysing the specific eGFP production (Figure 1c,f), it can be seen that sessile cells produced more recombinant protein than those grown in planktonic state. The biofilm environment enhanced the specific eGFP expression about 10-fold when compared to planktonic cells in steady state (statistically significant differences were confirmed for all experimental days, $p < 0.05$). Additionally, epifluorescence microscopy was used to determine the percentage of eGFP-expressing cells in both planktonic (Figure 1c) and biofilm populations (Figure 1f). The percentage of eGFP-expressing cells in planktonic state started to increase at day 3, reaching a maximum value of 76% at day 4 and then decreasing abruptly to values around 5% (Figure 1c). The equivalent biofilm curve (Figure 1f) also shows an initial increase in the percentage of expressing cells (with a maximum value of 87% between days 3 and 4), followed by a strong reduction (of approximately 58%) until day 6. In steady state, the percentage of planktonic expressing cells oscillated around 5%, whereas for biofilms, eGFP-expressing cells represented on average 21% of total cell population. Combining this information with the fluorimetric data, it is possible to conclude that *E. coli* biofilm cells can have a higher recombinant production capacity when compared to their planktonic counterparts under the tested conditions. This may be related with the highest potential of *E. coli* biofilms in maintaining the plasmid pFM23 within cells. In fact, in planktonic cells, for which the total cell number was constant over the course of the experiment (Figure 1b), the frequency of plasmid-containing bacteria was on average 0.33, while in the biofilm this parameter rose to approximately 0.90 in steady state (from day 5 onwards, Figure S1).

4. Discussion

In this study, the production of a model recombinant protein (eGFP) in *E. coli* planktonic and biofilm cells was evaluated by fluorometry (a bulk method) and epifluorescence microscopy (a single-cell method).

We found that the specific eGFP production was lower in planktonic cells and this may have contributed to the higher viability of these cells when compared to biofilm cells. It is well documented that cells bearing a plasmid may suffer from a metabolic burden as cellular resources are being used for its replication and for the expression of plasmid-encoded genes [11,27,28]. In particular, a decreased cellular viability of plasmid-bearing cells and an increased protease activity are some stress signals suffered by these cells [29,30].

The expression of recombinant proteins in *E. coli* biofilms was first reported by Huang et al. [20,21,31], who studied the production of β-galactosidase in a chemostat and in a parallel plate flow. These authors found that the recombinant protein was successfully produced in biofilm cells, although at a lower level than in planktonic cells [20,31]. In contrast, our results show that the *E. coli* biofilm environment enhanced eGFP production when compared to planktonic cells. Furthermore, biofilms presented a higher frequency of plasmid-bearing cells than the planktonic population. These results corroborate those obtained in a previous study of O'Connell et al. [22] who produced eGFP in a chemostat (planktonic cells) and in a PPFC reactor (biofilms). The results of their work indicated that the biofilm environment enhanced both plasmid maintenance and cellular GFP concentrations when compared to planktonic cells. Continuous biofilm cultures for recombinant protein production can be more beneficial for retention of plasmid-bearing cells than chemostats [22] since cells in biofilms

tend to grow more slowly than their planktonic counterparts [32], leading to fewer divisions and correspondingly less plasmid segregation. Other authors have also shown that the plasmid loss is more significant in planktonic populations [33–35]. More recently, our research group found that the volumetric productivity of the biofilms developed in the flow cell system is already within the range that can be obtained by conventional high cell density cultures, even before optimization of cultivation conditions [23].

During recombinant protein production, it is essential to monitor the amount of target protein produced in order to find the best processing conditions. The use of a fluorescent protein as a model recombinant protein enables bulk and single-cell quantification. Bulk production can be assessed by fluorometry, which is a highly sensitive, specific, and simple method with low instrumental costs when compared to other techniques, like flow cytometry, that is also used to follow recombinant protein expression [36,37]. On the other hand, single-cell analysis by epifluorescence microscopy can provide information about the heterogeneity level of protein expression within a cell population [38]. In this work, the combination of these two techniques was found to be very useful in monitoring recombinant protein production in both planktonic and sessile cells.

5. Conclusions

This study revealed that the specific recombinant protein production and the percentage of eGFP-expressing cells were higher in *E. coli* biofilms than in cell suspension, which was associated with the highest potential of biofilms in retaining the plasmid.

When combined with a bulk method such as fluorimetry, epifluorescence microscopy and the corresponding image analysis is an innovative tool for determining important production parameters in both planktonic and sessile cells, namely the number of cells expressing the recombinant protein within a bacterial population. The methodology presented here is a simple, fast and low-cost way of obtaining information about the heterogeneity level of protein expression within a cell population and it can be further used to find the best processing conditions for recombinant protein production in biofilm reactors.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2607/6/2/48/s1, Figure S1: Planktonic (a) and biofilm (b) population dynamics of plasmid-bearing cells (●) and total culturable cells (plasmid-bearing cells plus plasmid-free cells) (○). The means ± SDs for three independent experiments are illustrated.

Author Contributions: L.C.G. and F.J.M. conceived and designed the experiments; L.C.G. performed the experiments; L.C.G. and A.S. analyzed the data; A.S. wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

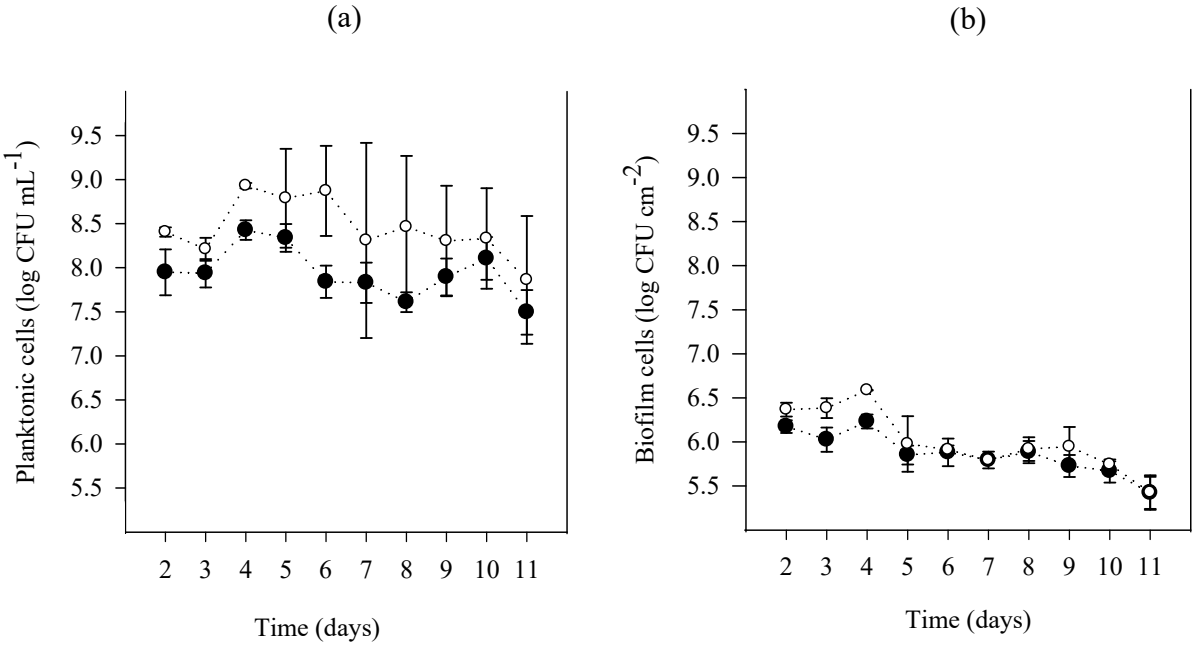


Figure S1. Planktonic (a) and biofilm (b) population dynamics of plasmid-bearing cells (●) and total culturable cells (plasmid-bearing cells plus plasmid-free cells) (○). The means ± SDs for three independent experiments are illustrated.

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The Influence of Nutrient Medium Composition on *Escherichia coli* Biofilm Development and Heterologous Protein Expression

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Abstract: In the present study, the effects of different nutrient media on the development of *Escherichia coli* biofilms and the production of a heterologous protein were examined. *E. coli* JM109(DE3) cells transformed with pFM23 plasmid carrying the gene for enhanced green fluorescent protein (eGFP) expression were used. Cells were grown in two different culture media, Lysogenic Broth (LB) and M9ZB, in a flow cell system for 10 days. Epifluorescence microscopy, fluorimetry, and a high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC) were used to assess bacterial growth, plasmid copy number (PCN), and eGFP production in both planktonic and biofilm cells. The results showed that biofilm development was favored in M9ZB medium when compared with LB. However, the number of eGFP-expressing cells was higher in LB for both planktonic and sessile states (two-fold and seven-fold, respectively). In addition, the PCN in biofilm cells was slightly higher when using LB medium (on average, 29 plasmids per cell versus 20 plasmids per cell in M9ZB), and higher plasmid stability was observed in biofilms formed in LB compared to their planktonic counterparts. Hence, *E. coli* biofilms grown in LB enhanced both plasmid stability and capacity to produce the model heterologous protein when compared to M9ZB.

Keywords: biofilm; flow cell system; *Escherichia coli*; green fluorescent protein; culture medium; plasmid copy number

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1. Introduction

Escherichia coli has been one of the most used organisms for heterologous protein production due to its inexpensive and fast high-cell density cultivation, well-known genetics, and large number of cloning vectors [1–3]. In addition to the host strain, factors such as the expression vector, cultivation conditions (medium composition, temperature, pH, and induction conditions), and purification strategies are essential for the successful production of heterologous proteins [4].

E. coli plasmids have been the preferred expression vectors because of the high gene dosage that can be attained and the capability to control the levels of gene expression through inducible promoters [5]. Although a high plasmid copy number (PCN) may ensure a high gene dosage, this does not always mean a high level of target heterologous protein [5,6]. Studies in suspension cell cultures have demonstrated that high plasmid copy number imposes a metabolic burden on the host cell, which reduces the bacterial growth rate and favors plasmid instability, thereby decreasing the overall protein yield [7,8].

The use of biofilm cells in recombinant protein production has been scarcely studied, but some promising results have been obtained by our research group in the last few

years [6,9–11]. The biological organization of biofilms provides them with many advantages over cells grown in suspension cell cultures, including high cell density, protection against hostile conditions, and operation stability, particularly when biofilms are used in biotechnological processes for the production of value-added compounds [12]. In addition, the presence of plasmids has been shown to stimulate biofilm formation and lead to a higher production of recombinant proteins compared to planktonic cells [10–15]. Our group has demonstrated that cells expressing eGFP (enhanced green fluorescent protein) formed more biofilm than non-expressing cells [10]. Moreover, *E. coli* biofilms were able to produce eGFP at a much higher level (30-fold) than their planktonic counterparts [10]. Furthermore, it has been shown that biofilm cultures have a beneficial effect on the maintenance of high and medium copy number plasmids [6,16]. O'Connell et al. [16] reported that continuous biofilm cultures of *E. coli* ATCC 33456 containing the pEGFP plasmid (a pUC family vector) maintained a high PCN over extended periods, while in chemostat a significant plasmid loss was observed. More recently, we showed that although the PCN was higher in planktonic cells, a significant PCN reduction (around 89%) was observed in planktonic cells after 5 days of flow cell operation, while no plasmid loss was observed in *E. coli* JM109(DE3) biofilms [6].

Different strategies have been explored to achieve maximum yields of heterologous proteins in suspended cell reactors, including by (1) changing the expression vector and/or the host, (2) optimizing the culture parameters (medium, pH, and temperature) and induction conditions (inducer concentration and temperature) of the recombinant strain, (3) co-expressing other genes such as chaperones, and (4) adjusting the synthesis of stress-related sigma factors and/or deleting specific gene sequences such as proteases, which may promote both the quantity and quality of the desired protein [2,3,5,17–19]. Nonetheless, the investigation of productive biofilms remains scarce. One of the parameters that can affect both biofilm formation and the level of heterologous protein expression is the culture medium composition. Several reports have demonstrated that the nutritional medium content affects biofilm formation [11,20–22]. Some authors have shown that an increase in nutrient levels promotes biofilm formation [12,13]. However, contradictory results showing that high nutrient concentration inhibits biofilm formation were also reported [14,15]. Regarding the effect of nutrient medium composition on heterologous protein production, most of the studies were performed in planktonic conditions [23–25]. In these conditions, it has been demonstrated that the increased concentration of some salts, peptone, and yeast extract can increase the production of the desired protein [26,27]. Furthermore, Matsui et al. [28] showed that the use of tryptone and yeast extract can increase plasmid stability. However, it is known that the high-level expression of heterologous protein in suspended cells often promotes a metabolic burden in the host cell that can affect plasmid stability and consequently the protein yield [29].

The main aim of this study was to determine the impact of different nutrient media compositions on biofilm development, plasmid stability, and eGFP expression in *E. coli* biofilm and planktonic cells. For that, *E. coli* JM109(DE3) cells transformed with the pFM23 plasmid for eGFP expression were used. Biofilm formation in two growth media, Lysogenic Broth (LB) and M9ZB, was carried out in a flow cell system for 10 days. LB medium is a rich medium that is frequently used for *E. coli* growth and expression of heterologous proteins [30]. M9ZB medium is the combination of M9 and ZB media, which are a minimal growth medium used for bacterial cultures and a medium recommended for the preparation of competent *E. coli* cells, respectively. M9ZB is also used for the cultivation of *E. coli* recombinant strains [31]. As far as we know, this is the first time that the M9ZB medium has been tested for biofilm formation and heterologous protein expression in sessile cells. On the other hand, this work enriches the group's previous studies [6,11] with the assessment of the population of planktonic and biofilm cells that produce the protein of interest by a single-cell analysis, and the evaluation of plasmid stability by determining the PCN in both types of cells by a chromatographic method.

2. Materials and Methods

2.1. Bacterial Strain

The pFM23 plasmid (created from pET28A, Novagen, Madison, WI, USA) containing the eGFP gene was transformed by heat shock in *E. coli* JM109(DE3) (Promega, Madison, WI, USA) for the intracellular production of eGFP [32]. Transformants were selected on agar medium supplemented with 20 μg mL^{-1} kanamycin (Eurobio, Orsay, France).

2.2. Flow System

A flow cell system (Figure 1) containing a recirculating tank (planktonic cells) and a flow cell composed of removable coupons with polyvinyl chloride (PVC) slides for biofilm formation was operated as described by Gomes et al. [9]. Briefly, *E. coli* cells were recirculated at 30 °C for 10 days under turbulent flow conditions (Reynolds number = 4600) [9], which are the hydrodynamic conditions previously used by the group [9,10,22,33] and completely characterized by computational fluid dynamics [34]. The tank was aerated and continuously fed with fresh medium at a flow rate of 0.025 L h^{-1} [9].

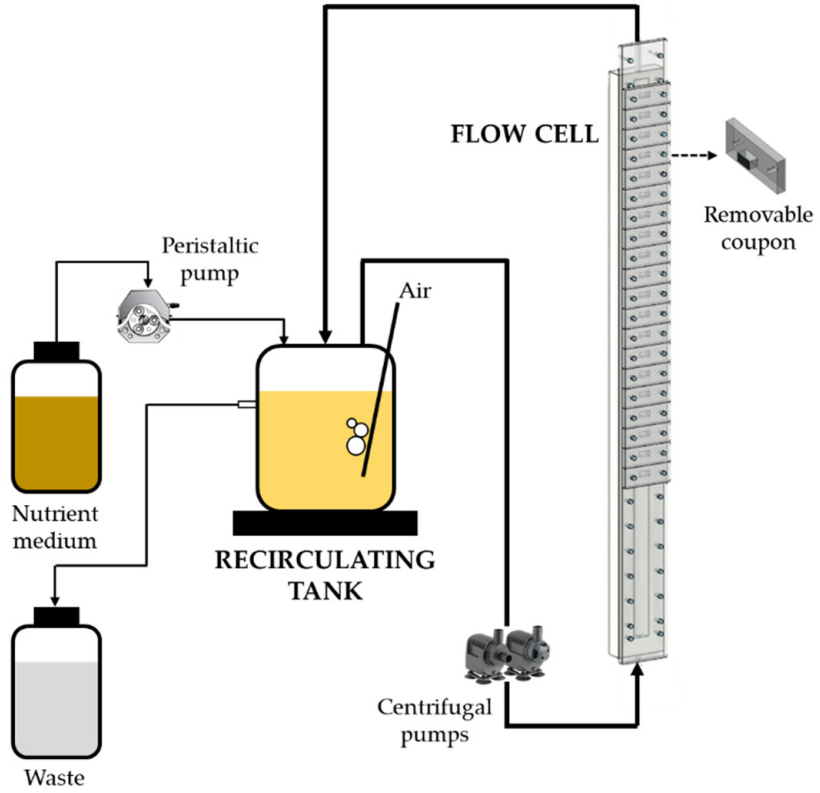


Figure 1. Schematic representation of the flow cell system (adapted from [3]).

To determine the effect of culture medium on biofilm formation and eGFP expression, two different media were tested: LB-Miller (Sigma, St. Louis, MO, USA) and M9ZB medium. According to the supplier, LB-Miller is made of 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl. The M9ZB medium was prepared as indicated by the Novagen pET System manual and Studier et al. [30,35]. The mixture of 10 g L^{-1} N-Z-amine A and 5 g L^{-1} NaCl was first prepared. Then, 100 mL of 10× M9 salts solution (10 g L^{-1} NH_4Cl, 30 g L^{-1} KH_2PO_4, 60 g L^{-1} $Na_2HPO_4 \cdot 7H_2O$), 1 mL of 1 M $MgSO_4$ solution, and 10 mL of 400 g L^{-1} glucose solution were added. All solutions were prepared and autoclaved separately. All compounds were purchased from Merck, Algés, Portugal. The comparison of carbon and nitrogen sources and amounts between media are presented in Table S1 in Supplementary Material. Both liquid media were supplemented with 20 μg mL^{-1} kanamycin.

2.3. Biofilm and Planktonic Analysis

The system was stopped every day to allow biofilm sampling and restarted using the same flow rate. The biofilm thickness was first determined by using a digital micrometer [22]. Then, it was resuspended in 25 mL of 8.5 g L^{-1} NaCl solution for total and culturable cell quantification, and eGFP and plasmid analysis. For biofilm culturability, samples were spread on solid growth medium (PCA; Merck, Algés, Portugal) supplemented with 20 μg mL^{-1} kanamycin, and colony enumeration was performed after incubation at 30 °C. Biofilm total cells were determined with the Live/Dead® BacLight™ bacterial viability kit (Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal) as described in Gomes et al. [9]. The final values of biofilm total and culturable cells were expressed as log cells cm^{-2} and log CFU cm^{-2}, respectively.

For planktonic fraction, a 10 mL sample was taken from the recirculating tank daily, and total and culturable cell numbers were assessed by the same methodology as biofilm cells. The final values of planktonic total and culturable cells were presented as log cells mL^{-1} and log CFU mL^{-1}, respectively.

2.3.1. Quantification of eGFP-Expressing Cells and Specific Protein Concentration

The number of eGFP-expressing cells in planktonic and biofilm cells was assessed by epifluorescence microscopy as indicated by Gomes et al. [9]. The percentage of eGFP-expressing cells was calculated by dividing the number of expressing cells by the total cell number.

The specific eGFP production was analyzed by fluorimetry as fully described by Mergulhão and Monteiro [32]. The eGFP fluorescence was measured using a microplate reader (SpectraMax M2E, Molecular Devices, Inc., Wokingham, UK) with an excitation filter of 488 nm and an emission filter of 507 nm. Then, the eGFP concentration was calculated through a standard curve obtained from purified eGFP serially diluted (range of 0 to 0.31 μg μL^{-1}) and the final values were presented as specific eGFP production (fg $cell^{-1}$).

2.3.2. Plasmid Extraction and Quantification

Plasmid DNA was extracted by alkaline lysis as described by Alves et al. [36]. Briefly, bacterial pellets were resuspended in 100 μL of Solution I (50 mM glucose, 25 mM Tris–HCl, 10 mM EDTA, pH 8). Alkaline lysis was performed by adding 100 μL of Solution II (0.2 M NaOH, 1% (*w*/*v*) SDS) and incubating the mixture for 10 min at room temperature. Cellular debris, genomic DNA and proteins were precipitated by adding 100 μL of Solution III (5 M acetate/3 M potassium, pH 5) and incubating for 10 min in ice. The precipitate was removed by centrifuging twice at 20,000× *g* for 30 min at 4 °C (Centrifuge 5424, Eppendorf, Hamburg, Germany).

Plasmid quantification in biofilm and planktonic samples was performed according to the analytical high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC) described by Diogo et al. [37]. A 4.6/100 mm HIC source 15 PHE PE column (Amersham Bioscience, Uppsala, Sweden) was connected to an HPLC system (Shimadzu, Nexera-i LC-2040C 3D, Duisburg, Germany) and equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl buffer, pH 8. Then, 50 μL of each sample was injected and eluted at 1 mL min^{-1}. After 1 min, the elution buffer was changed to 10 mM Tris–HCl without ammonium sulfate and maintained for 0.8 min in order to elute all bounded species. Finally, the elution buffer was changed to the equilibration buffer and maintained for 8.2 min to re-equilibrate the column for the next run. The absorbance of the eluate was recorded at 260 nm. The plasmid concentration of the samples was calculated using calibration curves constructed with serial dilutions of plasmid DNA standards (ranging from 0.04 to 20 μg mL^{-1}). The calibration curves were established among the peak area

appearing between 0.67 and 0.7 mL of elution volume versus the concentration of plasmid DNA standard [37]. Then, the plasmid copy number (PCN) was calculated using Equation (1) [38]:

$$\mathrm{PCN} = \frac{6.02 \times 10^{23} \left(\text{copy mol}^{-1}\right) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \left(\text{g mol}^{-1}\,\text{bp}^{-1}\right)} \quad (1)$$

where the size of pFM23 plasmid is 6053 bp.

2.4. Statistical Analysis

The results originated from averages of independent experiments for each culture condition (LB and M9ZB). All reported data are presented as mean ± standard deviation (SD) from at least three experiments with triplicates.

Paired *t*-test analysis was conducted to determine the differences between culture media on each experimental day. Statistically significant differences were reported for p values < 0.1 (corresponding to a confidence level of 90%) and <0.05 (corresponding to a confidence level of 95%).

3. Results

In this study, two different nutrient media were used with the aim of comparing eGFP production and plasmid stability in biofilm and planktonic populations growing in a flow cell system.

The planktonic fraction was monitored by quantifying the total and culturable cells (Figure 2A,C, respectively). Analysis of the planktonic total cells (Figure 2A) indicated that there were very few statistical differences in *E. coli* growth in both media (only one of the eight experimental points has $p < 0.05$). Despite the few statistical differences, from day 5 onwards, it seems to be a higher cell number of bacteria when growing in LB medium, with on average 29% more cells being present in LB than in M9ZB until the end of the experiment. Regarding the number of planktonic culturable cells (Figure 2C), similar behavior was observed for both media, but slightly higher CFU mL^{-1} values were obtained in M9ZB in all experimental points (on average 62%) when compared to LB.

The impact of nutrient medium composition on biofilm development was assessed by quantifying total and culturable cells and determining the biofilm thickness (Figure 2B,D,E, respectively). Globally, the amount of biofilm total cells (Figure 2B) was higher for M9ZB (on average 8.25 log cells cm^{-2}) than for LB (on average 8.0 log cells cm^{-2}) with statistically significant differences for five of the eight experimental points ($p < 0.1$). When comparing biofilm culturable cells (Figure 2D), higher values were also obtained for M9ZB (on average 65%) with statistical differences in most time points ($p < 0.1$ for days 6 and 10, and $p < 0.05$ for days 3, 5, 7 and 9). Biofilm thickness (Figure 2E) was similar between growth media until day 5, but thereafter, the biofilms developed in M9ZB were thicker than biofilms formed in LB (176 μm versus 161 μm, respectively), which is most likely a result of their higher cell density (Figure 2B,D).

Looking at the results of heterologous protein expression (Figure 3), the number of planktonic cells expressing eGFP relative to the number of total cells (Figure 3A) was slightly higher for LB medium in most experimental points. The number of eGFP-expressing cells in both media increased until day 6 when on average 27% of the total cells produced eGFP. Regarding the percentage of eGFP-expressing cells in biofilm (Figure 3B), it can be seen that in M9ZB, this parameter remained almost constant throughout the experiment (around 7%, with the maximum value of 13% on day 6), while in LB, a strong increase was observed until day 6 when 66% of the total cells expressed eGFP ($p < 0.05$). From day 6 onwards, the amount of expressing cells decreased about 20% and was nearly similar until the end of the experiment. Furthermore, in the LB medium, the number of sessile cells expressing eGFP was about 54% higher than in *E. coli* suspensions, while in M9ZB, the number of expressing cells in the biofilm was similar to its planktonic counterparts (Figure 3A,B).

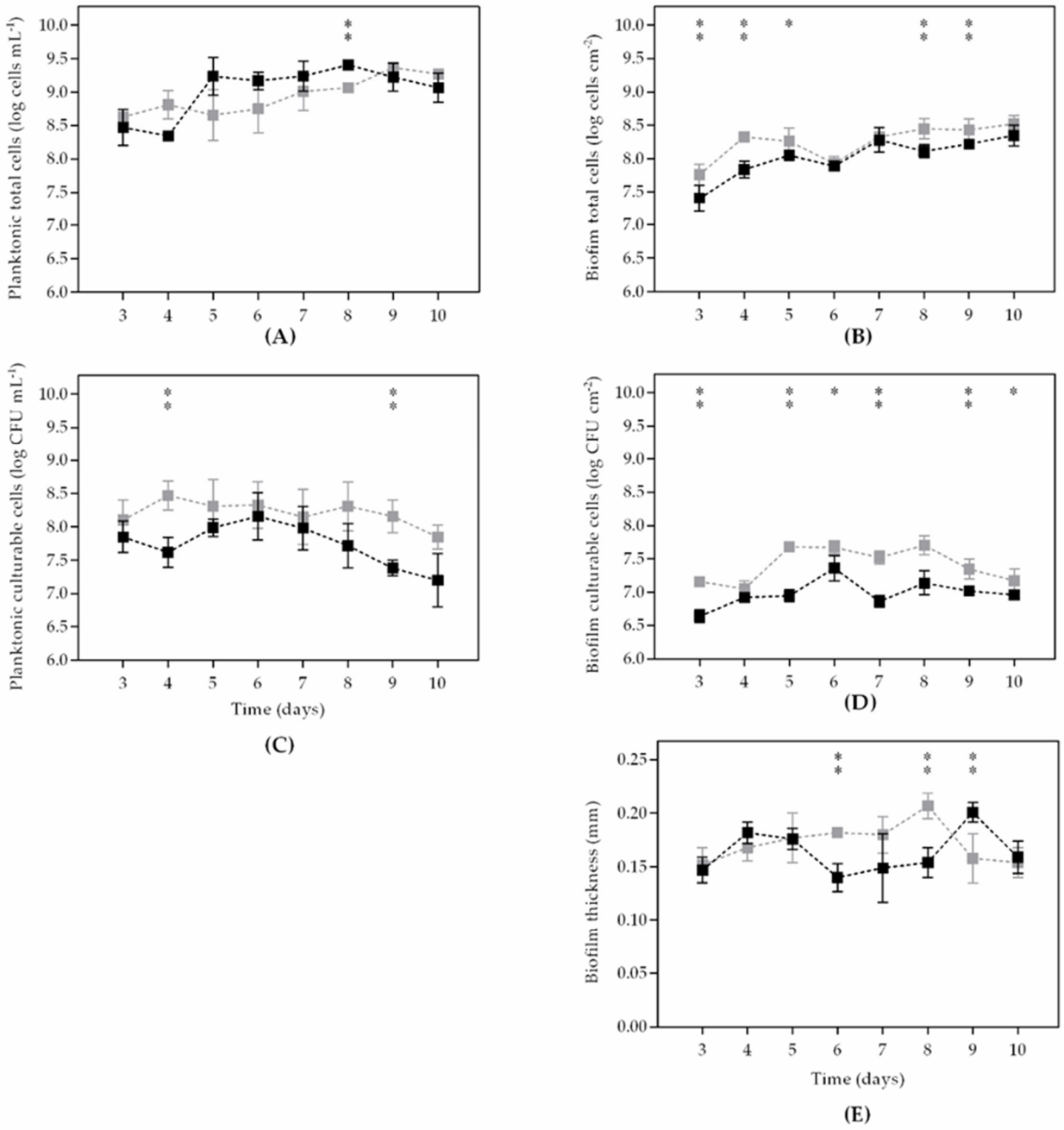


Figure 2. Planktonic and biofilm monitoring in LB (black squares) and M9ZB (gray squares): (**A**) planktonic total cells, (**B**) biofilm total cells, (**C**) planktonic culturable cells, (**D**) biofilm culturable cells, and (**E**) biofilm thickness. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 90% (an asterisk indicates $p < 0.1$) and 95% (a double asterisk indicates $p < 0.05$).

The specific eGFP values (Figure 3C,D) showed that for both planktonic and biofilm states, the cells grown in LB synthesized more protein than cells grown in M9ZB. On average, the eGFP production in LB medium was eight-fold and three-fold higher for planktonic and biofilm cells, respectively, than in M9ZB. Additionally, in M9ZB, the eGFP levels remained practically constant over time for both planktonic and biofilm states (around 0.31 fg cell^{-1} and 1.51 fg cell^{-1}, respectively), whereas in LB, the specific eGFP concentration increased until days 5 and 7 for planktonic and biofilm environments, respectively, reaching a maximum of 4.31 fg cell^{-1} and 15.96 fg cell^{-1}, and then it started to decrease, reaching the steady state.

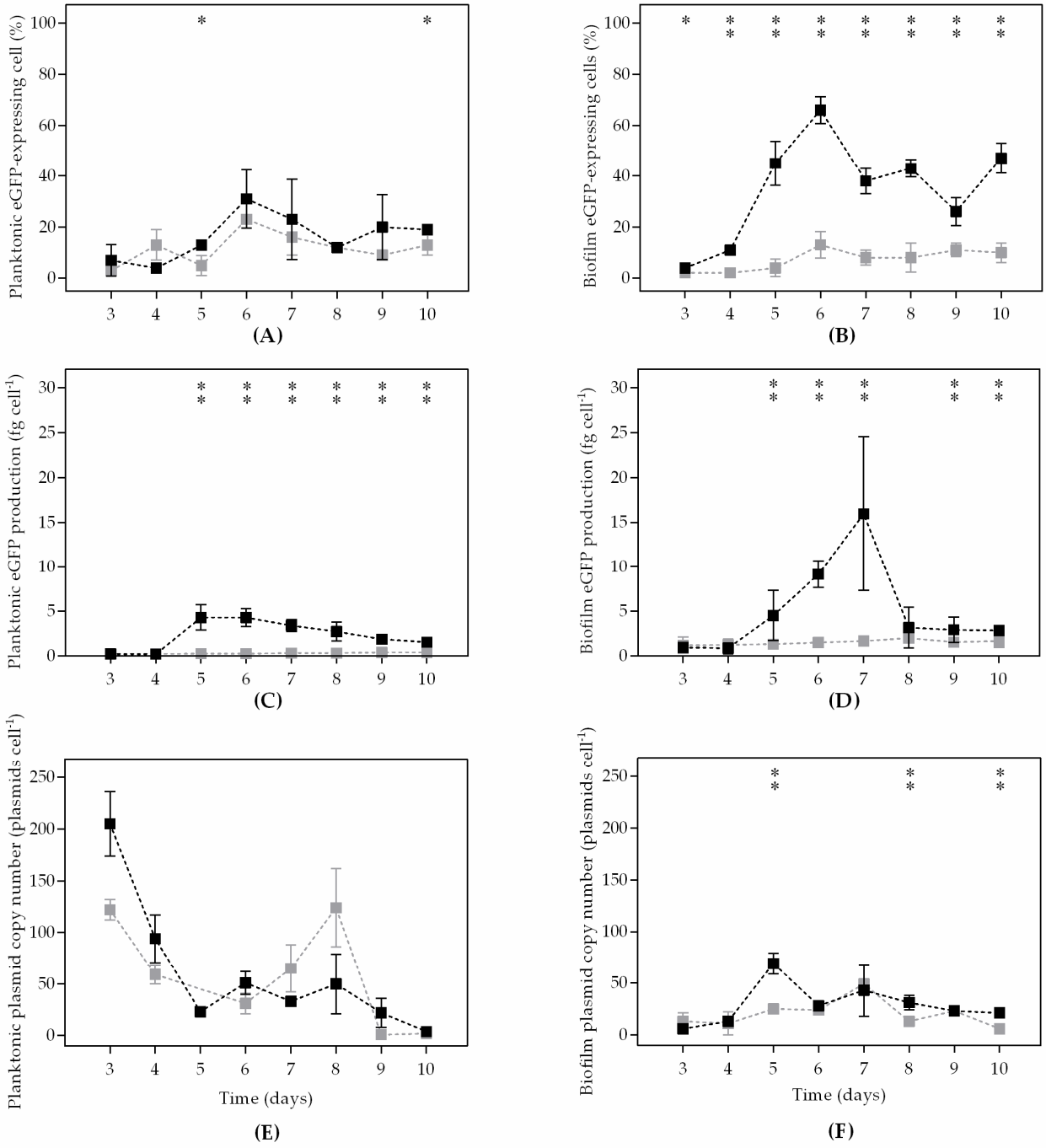


Figure 3. Planktonic and biofilm monitoring in LB (black squares) and M9ZB (gray squares): (**A**) planktonic eGFP-expression cells, (**B**) biofilm eGFP-expression cells, (**C**) planktonic eGFP production, (**D**) biofilm eGFP production, (**E**) planktonic plasmid copy number, (**F**) biofilm plasmid copy number. The means ± standard deviations (SDs) for at least three independent experiments are presented. Statistically significant differences to each time point were considered for a confidence level greater than 90% (an asterisk indicates $p < 0.1$) and 95% (a double asterisk indicates $p < 0.05$).

In general, the PCN (Figure 3E,F) was higher in planktonic cells for both culture media (on average three-fold for M9ZB and two-fold for LB). In planktonic cells grown in LB (Figure 3E), a strong reduction in PCN (of around 89%) was observed until day 5, and from then on, the PCN levels were kept between 4 and 51 plasmids per cell. On the other hand, in planktonic cells grown in M9ZB medium, following an initial decrease by 75% until day 6, the PCN increased until day 8 to the values registered at the start of the experiment,

decreasing to about 2 plasmids per cell in the next two days. In biofilm cells (Figure 3F), the PCN reached its maximum at days 5 and 7 in LB and M9ZB, respectively. Then, sessile cells in LB lost about 59% of plasmid content, maintaining values around 29 plasmids per cell until the end of the assay, while in M9ZB, this reduction was about 75% with cells keeping on average 14 plasmids inside them.

4. Discussion

Heterologous protein production using *E. coli* biofilms has shown to be advantageous for many reasons, including higher plasmid copy number [16] and a higher level of protein production compared to their planktonic counterparts [10,39]. However, the most important goal of heterologous protein production is to achieve high protein production at low cost. Several parameters, such as nutrient medium composition, temperature, hydrodynamic conditions, and surface properties can affect biofilm formation [11,22,40] and consequently influence the final protein yield.

In a previous study [11], our research group compared the effect of two culture media—a diluted medium (DM) and LB—and concluded that biofilms grown in LB produced more eGFP than those grown in DM. In this preliminary work from 2017, primacy was given to culture media typically used to promote biofilm growth such as DM [22,33], while here, there was a concern in testing media recommended for the high expression of recombinant proteins in *E. coli* such as the M9ZB.

Planktonic results showed a higher number of culturable cells in M9ZB compared to LB medium. This could be related to the presence of glucose in M9ZB medium, which is a compound that is absent in the LB medium used. Previously, Tripathi et al. [41] also found that a culture of *E. coli* DH5α cells harboring the recombinant plasmid pMAL-c2X achieved higher cell densities in media with a high concentration of glucose, such as Tryptone Yeast (TY) medium, Terrific Broth (TB), and Super Broth (SB).

Biofilm formation was also favored in the M9ZB medium, as indicated by the higher values of both culturable and total cells when compared to LB. Several studies have shown that the nutritional content of the medium, namely carbon and energy substrates, affects biofilm formation [42,43]. Dewanti and Wong [44] observed that using a minimal salts medium (MSM) supplemented with glucose resulted in a stable biofilm with shorter bacterial cells and a thicker extracellular matrix. Buhler et al. [13] have also reported that increasing the glucose concentration of culture medium can promote the growth of *E. coli* nutrient-depleted biofilms.

Concerning heterologous protein production, a higher number of cells expressing eGFP and higher specific eGFP concentrations were found in LB for both planktonic and sessile cells. Complex media, such as LB, contain yeast extract and tryptone that are known to provide biosynthetic precursors, such as vitamins and amino acids, that support protein expression in *E. coli* [26,27]. Furthermore, yeast extract can also be responsible for reducing the cellular stress (protease production) during the synthesis of recombinant proteins [45]. Similar to what was observed in the present work with LB, Goyal et al. [26] found that a synthetic medium containing yeast extract and tryptone resulted in higher Streptokinase protein concentration in *E. coli*, without increasing cell density [26].

The lower eGFP expression in M9ZB medium could be related to the presence of glucose in the medium by repressing the lac promoter [30,46]. In the present work, a pET system was used, where the eGFP gene is transcribed by T7 RNA polymerase, which is regulated by the lacUV5 promoter [4]. It has been shown that glucose reduced the basal expression by reducing cyclic adenosine monophosphate (cAMP) levels, resulting in a low transcription from lacUV5 promoter [46]. Jevševar et al. [47] confirmed that glucose supplementation repressed the expression of recombinant human granulocyte colony-stimulating factor (hG-CSF). On the other hand, it was revealed that the addition of yeast extract to the culture media increased the background expression from the lac promoter [48,49].

Regardless of the culture medium used, there was an increase in the percentage of eGFP-expressing cells in both planktonic and biofilm between days 5 and 7. This phenomenon may be associated with the higher number of culturable cells also detected in the same period. It is expected that metabolically active cells such as the culturable cells are more likely to be eGFP-producing cells, as they have more biosynthetic precursors, energy, and other cellular components available for the synthesis of the foreign protein. In the particular case of biofilms grown in LB medium, the higher levels of expressing cells on days 5–7 may also be related to the simultaneous decrease in biofilm thickness, since the transfer of oxygen for the maturation of the protein under study is facilitated in thinner biofilms [9].

Plasmid stability is an important factor in heterologous protein production [5]. In this work, high specific eGFP concentration was obtained in biofilm cells, although on average higher copies of plasmid were found in planktonic cells. These results suggest that the gene dosage was not responsible for the expression of eGFP. The same phenomenon was observed by Gomes et al. [6], where biofilm cells had more eGFP concentration even though they presented seven-fold lower PCN than planktonic cells. In the present work, the low eGFP expression levels in planktonic cells for both media may be caused by the high metabolic burden linked to plasmid replication. This added metabolic burden usually causes structural and segregational plasmid instability as well as several metabolic changes in the host cell, which may reduce the yield and activity of the heterologous protein [29,50].

Although a small number of plasmids were found in biofilm cells in LB, the biofilm environment seems to be advantageous for plasmid retention, since the PCN decrease was more accentuated in suspended cells than in the biofilms formed in the flow cell system (89% versus 59% reduction). Similar to our results, O'Connell et al. [16] demonstrated that *E. coli* cultivation as a biofilm has a positive effect on high copy number plasmid maintenance compared to chemostats. The reason for the higher plasmid maintenance in biofilms is that sessile cells tend to grow more slowly than planktonic cells [51], leading to fewer cell divisions and consequently less plasmid partitioning. Furthermore, it was reported that the use of yeast extract and tryptone as nitrogen sources may result in higher plasmid stability [28].

The results obtained in this optimization work are essential to define the operating conditions to be used in further studies focused on the synthesis of other heterologous proteins of different sizes and involved in synthetic pathways for the production of metabolites of high market value.

5. Conclusions

Biofilm cells have shown potential to be used as microbial cell factories, although the influence of environmental factors in the production of high added-value compounds has barely been explored. This study confirmed that heterologous protein production in *E. coli* biofilms is affected by nutrient conditions. Even though the biofilm development was favored in M9ZB medium when compared with LB, the number of eGFP-expressing cells and the eGFP yield were higher in LB, which is the medium with higher nitrogen content (yeast extract and tryptone). In addition, the PCN in biofilm cells was slightly higher when using LB. On the other hand, the use of M9ZB medium, which has glucose as carbon source, had an inhibitory effect on eGFP expression.

This work is a contribution to the study of operational conditions affecting heterologous protein production in biofilms systems in order to maximize its production.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/app11188667/s1, Table S1: Comparison of carbon and nitrogen sources and corresponding concentrations between M9ZB and LB media.

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Supplementary Material

Table S1. Comparison of carbon and nitrogen sources and corresponding concentrations between M9ZB and LB media.

		M9ZB	**LB**
Carbon	Source	Glucose	Tryptone and yeast extract
	Concentration	1.6 g L^{-1} *	Residual **
		About 100% higher in M9ZB	
Nitrogen	Source	Amine and ammonium chloride	Tryptone and yeast extract
	Concentration	1.37 g L^{-1} *	1.59-1.99 g L^{-1} *
		16-45% higher in LB	

*based on suppliers; **based on Podpora et al. [1]

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Hydrodynamic Effects on Biofilm Development and Recombinant Protein Expression

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Abstract: Hydrodynamics play an important role in the rate of cell attachment and nutrient and oxygen transfer, which can affect biofilm development and the level of recombinant protein production. In the present study, the effects of different flow conditions on the development of *Escherichia coli* biofilms and the expression of a model recombinant protein (enhanced green fluorescent protein, eGFP) were examined. Planktonic and biofilm cells were grown at two different flow rates in a recirculating flow cell system for 7 days: 255 and 128 L h^{-1} (corresponding to a Reynolds number of 4600 and 2300, respectively). The fluorometric analysis showed that the specific eGFP production was higher in biofilms than in planktonic cells under both hydrodynamic conditions (3-fold higher for 255 L h^{-1} and 2-fold higher for 128 L h^{-1}). In the biofilm cells, the percentage of eGFP-expressing cells was on average 52% higher at a flow rate of 255 L h^{-1}. Furthermore, a higher plasmid copy number (PCN) was obtained for the highest flow rate for both planktonic (244 PCN/cell versus 118 PCN/cell) and biofilm cells (43 PCN/cell versus 29 PCN/cell). The results suggested that higher flow velocities promoted eGFP expression in *E. coli* biofilms.

Keywords: biofilm; flow cell system; *Escherichia coli*; green fluorescent protein; hydrodynamic conditions; plasmid copy number

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1. Introduction

Recombinant DNA methodology is very important for biotechnological industries because by using this technology, it is possible to produce proteins at much higher levels than are found naturally [1,2]. Although most heterologous proteins are produced on an industrial scale in suspended cells, it is known that the high-level production of heterologous proteins using planktonic cells has an associated metabolic burden on the host cell that can affect plasmid stability and protein yield [3]. Bacterial biofilms have recently received increased attention in terms of the production of added value compounds since they present many advantages over their free and planktonic counterparts, such as a higher cell density, protection against hostile conditions, higher operation stability [4,5], and greater plasmid stability [6,7]. Biofilms are communities of surface-attached microorganisms that are encased in a self-produced matrix of extracellular polymeric substances (EPS) [8]. Bacterial biofilms can contain multiple cell layers and their thickness can vary from a few to many μm [8]. The biological organization of biofilms can promote different rates of growth and metabolic activity within the biofilm cells, which can be a consequence of different diffusion rates of oxygen and nutrients inside the biofilms [9]. Microbial adhesion and the subsequent biofilm development can be affected by several factors, such as nutrient levels, surface properties, and hydrodynamic conditions [10–13].

Hydrodynamics is one of the most important factors that affects biofilm development since it determines the shear stress on the surfaces on which the biofilms form and the mass transfer of nutrients, oxygen, and bacteria from the bulk medium to the biofilm [14–16]. A higher flow velocity enables a higher external mass transfer, which means that more nutrients and oxygen are brought to the surface of the biofilm [15]. Hydrodynamic conditions also influence biofilm formation and structure due to the shear forces, which can modulate microbial cell adhesion to and detachment from a given surface [17]. A higher shear stress usually results in thinner, denser, and stronger biofilm, while lower shear stress conditions result in biofilms with thick multilayer structures [8,18]. Although a higher flow velocity increases the supply of nutrients and oxygen, the biofilm density can reduce the diffusion or penetration of the substrate into the inner layers of the biofilm [19].

The successful production of recombinant proteins in bacterial cells depends on sufficient nutrients and oxygen supply. Although biofilm reactors have been shown to have several advantages over planktonic cultures for recombinant protein production, the diffusion of substrates into the sessile cells is still a significant limitation [5,20]. It has also been shown that nutrients are mostly consumed by the outer cell layers before reaching the inner layers of the biofilm [19,21]. Recently, our research group demonstrated that the eGFP expression in *E. coli* biofilms is highly heterogeneous, with the cells that express the protein being located mostly on the top of the biofilm [22]. This is probably due to limitations in the mass transfer of oxygen and nutrients from the bulk liquid to the biofilm. Nevertheless, Huang et al. [23] showed that diffusion limitations can have a beneficial effect on plasmid stability since they can lower the cell growth rates inside the biofilm. Recombinant protein production in biofilm cells has been mostly performed in static conditions or using low laminar flow rates (Reynolds numbers (*Re*) of 20 and 32) [6,23–25]. Despite the lack of information on recombinant protein production in biofilms, it has been reported that agitation rate plays an important role in nutrient and oxygen diffusion within immobilized cells systems [26]. By using immobilized cells in sodium alginate for the production of recombinant aspartase, Singh and Yadav [27] demonstrated that a higher production rate (1234 U/g wet cells) was obtained with an agitation rate of 250 rpm compared to the lower agitation of 130 rpm (534 U/g wet cells). However, a high agitation rate (270 rpm) decreased the production to 931 U/g wet cells. Nevertheless, Man et al. [28] observed that an increased agitation rate negatively affected plasmid stability in the immobilized cells. However, the higher plasmid stability that was obtained at lower agitation rates was not associated with the increased excretion of the cyclodextrin glucanotransferase enzyme [28].

The aim of this work was to investigate the effects of flow rate variation on the development of *E. coli* biofilms and eGFP expression. For that, two flow conditions were tested: 255 and 128 L h^{-1} (corresponding to *Re* of 4600 and 2300, respectively). The flow cell system that was used in this study has been extensively characterized in terms of its hydrodynamics [15,16,29,30]. Turbulent conditions (*Re* of 4600) have already been tested in previous works using the same flow cell system for eGFP production [22,31], but laminar ($Re < 2300$) and transient conditions ($2300 < Re < 4000$) could also be achieved with this system. The variations in this operational parameter are discussed here for the first time.

2. Materials and Methods

2.1. Bacterial Strain

The *E. coli* JM109(DE3) (Promega, Madison, WI, USA) was transformed by heat shock with the plasmid pFM23 (constructed from pET28A; Novagen, Madison, WI, USA), which contained the eGFP gene [32]. The transformants were selected on an agar medium that was supplemented with 20 μg mL^{-1} of kanamycin (Eurobio, Orsay, France).

2.2. Flow Cell System and Experimental Conditions

A flow cell system (Figure S1 of Supplementary Materials) composed of a vertical semicircular flow cell with removable coupons on which biofilms were formed and a recirculating tank (planktonic cells) was used, as described by Gomes et al. [22]. *E. coli* cells

were grown by recirculating the bacterial suspension at 30 °C for 7 days at two different flow rates: 255 L h^{-1} (*Re* of 4600) and 128 L h^{-1} (*Re* of 2300). The Reynolds number was defined as (Equation (1)):

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

where ρ is the density of water at 30 °C (kg m^{-3}), U is the linear flow velocity (m s^{-1}), D_h is the hydraulic equivalent diameter of the semicircular flow cell ($D_h = \pi D/(2+\pi) = 1.83$ cm) of diameter D (m), and μ is the dynamic viscosity of water at 30 °C (kg (m^{-3} s^{-1})).

The average wall shear stress (τ_w) values for the two tested flow conditions (*Re* of 4600 and 2300) were estimated using the dimensionless Darcy friction factor (f), which was obtained from the work of Teodósio et al. [16], and applying Equation (2):

$$f = \frac{4\tau_w}{\rho U^2/2} \quad (2)$$

where ρ is the density of water at 30 °C (kg m^{-3}) and U is the linear flow velocity (m s^{-1}). A *Re* of 4600 and 2300 in the flow cell system corresponded to τ_w values of approximately 0.3 and 0.1 Pa, respectively.

The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with Terrific Broth (TB) medium, which was supplemented with 20 µg mL^{-1} of kanamycin, at a flow rate of 0.025 L h^{-1} [22]. In a recent screening test, our research group found that the eGFP production was higher using the TB medium compared to Lysogenic Broth (LB) (data not shown). The TB medium is composed of 12 g L^{-1} tryptone, 24 g L^{-1} yeast extract, 2.31 g L^{-1} KH_2PO_4, and 12.54 g L^{-1} K_2HPO_4. All compounds were purchased from Merck, Algés, Portugal.

2.3. Biofilm and Planktonic Monitoring

For the biofilm sampling, the system was stopped every day for coupon removal and was then carefully restarted at the same flow rate [22]. The biofilm wet weight and thickness were firstly determined by subtracting the coupon weight on the sampling day from the weight prior to the start of the experiment and by using a digital micrometer, respectively [29]. Then, the biofilm was resuspended in 25 mL of 8.5 g L^{-1} NaCl solution for total, viable, and eGFP-expressing cells quantification and also for eGFP production and plasmid analysis. The biofilm total and viable cells were determined using the Live/Dead® BacLight™ bacterial viability kit (Invitrogen Life Technologies, Alfagene, Carcavelos, Portugal), as fully described by Gomes et al. [22]. Briefly, each biofilm cell suspension was filtered through a Nucleopore Track-Etch Membrane of black polycarbonate (pore size 0.22 µm; Whatman Ltd., Banbury, UK), stained for 7 min in the dark, and then observed using an epifluorescence microscope (Leica DM LB2; Leica Microsystems Ltd., Heerbrugg, Switzerland) that was connected to a Leica DFC300 FX camera. ImageJ v1.48 software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the number of total and viable cells from the counts of a minimum of 15 fields of view per sample and the final values were expressed as log cells cm^{-2}.

For planktonic cells, a daily 10 mL sample was taken from the recirculating tank to assess the total and viable cell numbers and to perform eGFP and plasmid analysis. The total and viable cell numbers were assessed using the same methodology as for the biofilm cells and the final values were presented as log cells mL^{-1}.

2.3.1. Assessment of eGFP-Expressing Cells and Specific Protein Concentrations

The eGFP-expressing cells were observed and counted using epifluorescence microscopy with a Leica DM LB2 epifluorescence microscope, as described by Gomes et al. [22]. The final values were presented as log cells mL^{-1} and log cells cm^{-2} for planktonic and biofilm samples, respectively.

The specific eGFP production was analyzed using the fluorometric method that was described by Mergulhão and Monteiro [32]. A microplate reader (SpectraMax M2E, Molecular Devices, Inc., Wokingham, UK) was used to measure the eGFP fluorescence using an excitation filter of 488 nm and an emission filter of 507 nm. The eGFP concentration was then calculated using a standard curve that was obtained from purified eGFP (ranging from 0 to 0.31 μg μL^{-1}), and presented as specific eGFP production (fg $cell^{-1}$).

2.3.2. Plasmid Extraction and Quantification

The plasmid DNA was extracted using alkaline lysis and quantified using the analytical high-performance liquid chromatography (HPLC) method based on hydrophobic interaction chromatography (HIC), as fully described by Soares et al. [7]. A 4.6/100 mm HIC source of 15 PHE PE columns (Amersham Bioscience, Uppsala, Sweden) that was connected to an HPLC system (Shimadzu, Nexera-i LC-2040C 3D, Duisburg, Germany) was used. The plasmid concentration was calculated using calibration curves that were constructed from the serial dilutions of the plasmid DNA standards (ranging from 0.04 to 20 μg mL^{-1}). Then, the corresponding plasmid copy number (PCN) was calculated using Equation (3) [33]:

$$PCN = \frac{6.02 \times 10^{23} \left(\text{copy mol}^{-1}\right) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \left(\text{g mol}^{-1}\,\text{bp}^{-1}\right)} \quad (3)$$

where the size of plasmid pFM23 was 6053 bp.

2.4. Statistical Analysis

The results that are presented in Figure 1 and Figure S1 (Supplementary Materials) were the averages of at least three independent experiments for each flow rate condition (255 and 128 L h^{-1}). All reported data are presented as the mean ± standard deviation (SD) from the three replicates of each independent experiment.

A paired *t*-test analysis using GraphPad Prism 6.01 software (San Diego, CA, USA) was performed based on a confidence level of 90% (differences are reported as significant for p values < 0.1) and a confidence level of 95% (differences are reported as significant for p values < 0.05).

3. Results

In this study, the effects of hydrodynamics on eGFP production and plasmid stability in *E. coli* planktonic and biofilm cells were assessed by testing two different flow rates: 255 L h^{-1} (*Re* of 4600) and 128 L h^{-1} (*Re* of 2300).

The number of plasmids per cell (PCN/cell; Figure 1A,B) was higher at the highest flow rate for both planktonic and biofilm cells (on average 2- and 1.5-fold higher, respectively). In planktonic cells (Figure 1A), PCN increased until day 4, on which it reached a maximum value of 377 PCN/cell for the highest flow rate and 221 PCN/cell for the lowest flow rate that was tested. After that PCN peak, a continuous decrease was observed until the end of the experiment. The same initial behavior was observed in the biofilm cells (Figure 1B), with a maximum value of 113 PCN/cell for the highest flow rate and 51 PCN/cell for the lowest flow rate on day 4. From day 4 onwards, PCN decreased and reached a steady-state of 42 PCN/cell for the highest *Re* and 38 PCN/cell for the lowest *Re*.

Although higher PCN values were always achieved at the highest flow rate, the plasmid maintenance in both planktonic and biofilm cells seemed to be favored at a lower *Re*. In fact, for the planktonic cells that were grown at 255 L h^{-1}, a strong reduction was observed between days 4 and 7 (around 81%), while at a flow rate of 128 L h^{-1} plasmid loss was around 61%. Regarding the biofilm cells, the percentage of plasmid lost at 255 L h^{-1} was 63%, whereas at 128 L h^{-1} this reduction was approximately 25%.

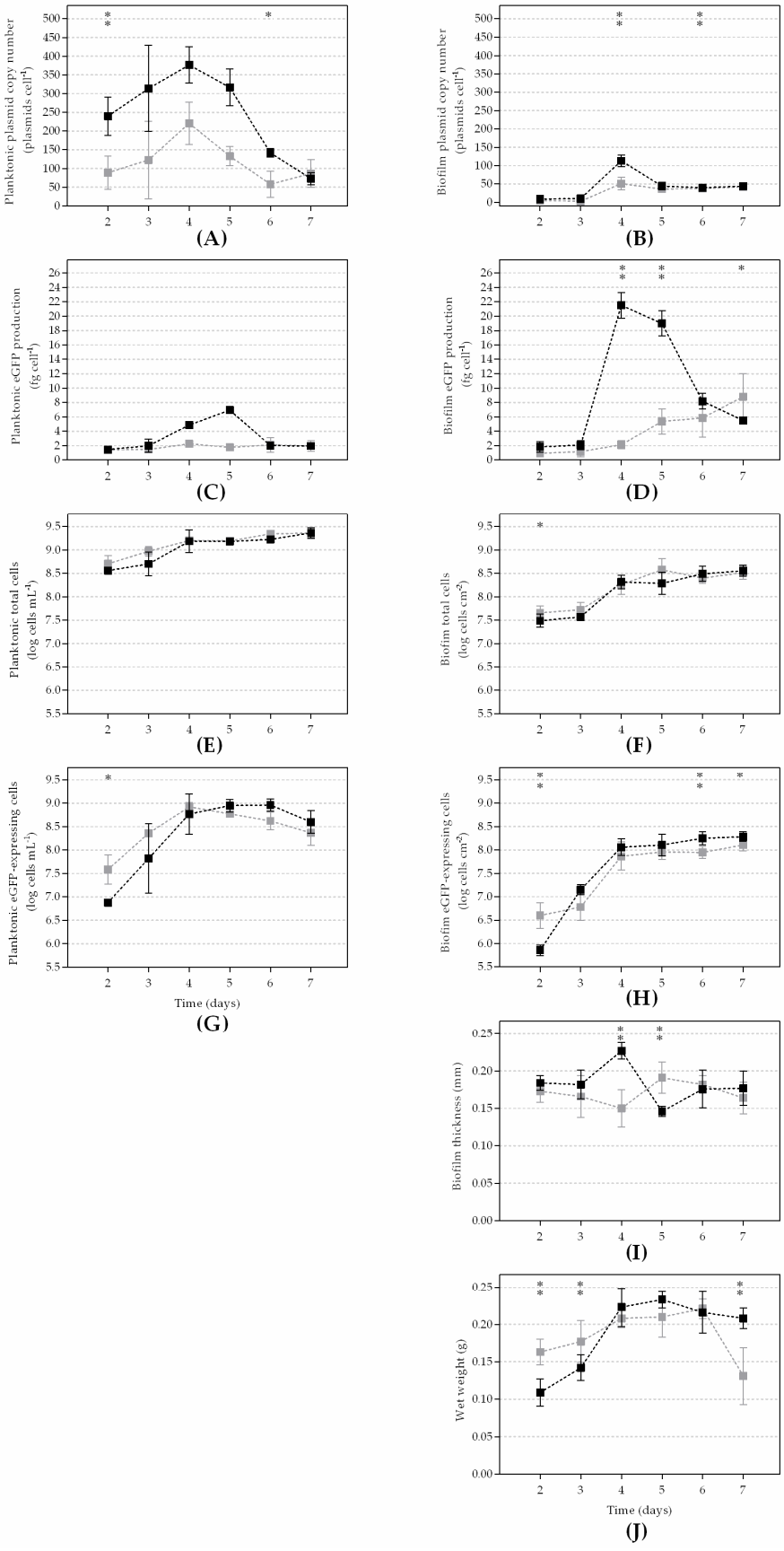


Figure 1. Planktonic and biofilm parameters at a flow rate of 255 L h^{-1} (*Re* of 4600; dark squares) and 128 L h^{-1} (*Re* of 2300; gray squares): (**A**) planktonic plasmid copy number; (**B**) biofilm plasmid

copy number; (**C**) planktonic eGFP production; (**D**) biofilm eGFP production; (**E**) planktonic total cells; (**F**) biofilm total cells; (**G**) planktonic eGFP-expressing cells; (**H**) biofilm eGFP-expressing cells; (**I**) biofilm thickness; and (**J**) biofilm wet weight. The means ± standard deviations (SDs) for at least three independent experiments are illustrated. The statistical analysis corresponding to each time point is also represented with an asterisk for a confidence level of greater than 90% ($p < 0.1$) and with a double asterisk for a confidence level of greater than 95% ($p < 0.05$).

The fluorometric analysis of the biofilm cells (Figure 1D) showed that the cells grown at the highest *Re* expressed more eGFP and reached the highest production value of 21.5 fg cell^{-1} on day 4. After day 4, the production decreased until the end of the experiment. Additionally, the eGFP production rate that was obtained at the lowest flow rate remained almost constant until day 4 and after that, it started to increase until the end of the experiment and reached a maximum of 8.8 fg cell^{-1}. In the planktonic state (Figure 1C), the production of eGFP was also higher at the highest flow rate and increased until day 5 (7.0 fg cell^{-1}), whereas at the lowest flow rate the eGFP levels remained practically constant over the whole experiment (around 1.8 fg cell^{-1}). Furthermore, the protein production rate in the biofilm cells was higher compared to the planktonic cells under both hydrodynamic conditions (3-fold higher for 255 L h^{-1} and 2-fold higher for 128 L h^{-1}).

The planktonic total cells (Figure 1E) had similar profiles for both flow rates ($p > 0.1$) and throughout the whole experiment. The quantification of the biofilm total cells (Figure 1F) also showed few differences between the hydrodynamic conditions. An initial increase was observed until day 4 and then, the number of cells stabilized from day 4 onwards. Concerning the planktonic eGFP-expressing cells (Figure 1G), both flow conditions reached similar cell amounts (8.6×10^8 cells mL^{-1}), although this value was reached sooner for the flow rate of 128 L h^{-1} (day 4 versus day 5 for 255 L h^{-1}). From that day onwards, the number of cells that expressed eGFP decreased until the end of the experiment. By comparing the number of planktonic cells that were expressing eGFP to the total cell number, the results showed that on average 31% and 26% of the cells expressed eGFP at 255 L h^{-1} and 128 L h^{-1}, respectively (data not shown). The number of biofilm cells that were expressing eGFP (Figure 1H) strongly increased between days 2 and 4 and then remained practically constant until the end of the experiment, regardless of the hydrodynamic condition being tested. The number of cells that were expressing eGFP in comparison to the total cell number was on overage 53% higher for the highest flow rate. Under this flow condition, a strong increase was observed until day 5, when 66% of the cells were found to express the protein. Furthermore, for the flow rate of 255 L h^{-1}, the number of biofilm cells that expressed eGFP was on overage 52% higher than the planktonic counterparts.

The biofilm thickness (Figure 1I) remained practically constant throughout the experiment for both flow conditions (an average of 176 µm), with a slight increase on days 4 and 5 for 255 L h^{-1} and 128 L h^{-1}, respectively. The biofilm wet weight (Figure 1J) was similar between flow rates but had slightly higher values for 255 L h^{-1}. An initial increase was observed until day 4, which then stabilized from that day until the end of the experiment for 255 L h^{-1}, while a decrease was observed on the last day of the experiment for the flow rate of 128 L h^{-1}.

4. Discussion

The study of the parameters that influence both biofilm formation and heterologous protein production is a valuable approach for reaching the highest specific recombinant protein expression in *E. coli* biofilms. Previous experiments using the same flow cell system have shown that higher flow velocities increase the external mass transfer [14,15] but produce thinner and more compact biofilms (compared to laminar conditions) [34] in which the internal mass transfer can be limited [22]. As well as a turbulent regime (flow rate of 255 L h^{-1} and *Re* of 4600), transient conditions (flow rate of 128 L h^{-1} and *Re* of 2300) were also tested in the present work.

Doubling the flow rate (from 128 to 255 L h^{-1}) seemed to have no impact on biofilm formation. This result was not expected since it is generally accepted that biofilms that are

formed under turbulent conditions are thinner, denser, and stronger than those developed under lower shear stress [15,29]. This could be related to the presence of an antibiotic resistance gene (kanamycin, KmR) in the plasmid. It is known that these genes are the major genes responsible for increasing the metabolic burden on the host cell [35]. Since stress conditions can favor biofilm formation [36] and, additionally, the presence of a considerable number of eGFP-expressing cells can increase the stress, it is possible that the similar biofilm growth behavior under the different flow conditions could have been due to these factors [31,36].

The single-cell analysis showed an increase in the number of eGFP-expressing biofilm cells between days 2 and 5, which represented on average 58% and 37% of the total population for 255 and 128 L h^{-1}, respectively, from this moment until the end of the experiment. It should be noted that, contrary to other studies, the number of eGFP-expressing cells did not correlate with the number of viable cells (Figure S2 of Supplementary Materials). In fact, the number of eGFP-expressing cells was found to be higher than viable cells under both flow conditions. This could be explained by the fact that dead cells completely lose eGFP while dying cells gradually leak eGFP from increasingly permeable membranes [37] once a decrease in viability has been observed. The decrease in biofilm viability could be related to the increase in protein production, which imposes a metabolic burden on the host [38,39]. It is well documented that recombinant protein production affects host metabolism due to the energy and metabolites that are needed for the replication of plasmid DNA and the synthesis of foreign protein [3,40]. Some of the metabolic changes that can be induced during recombinant protein production are an increase in protease activity and a decrease in growth rate and cell viability [38,39].

The specific eGFP production was also higher for both planktonic and biofilm cells that were grown at the 255 L h^{-1} flow rate. In suspended cells, several studies have reported that a high agitation rate increases metabolite production in recombinant microorganisms [41–43]. This suggests that a high shear rate is generated under high flow velocities and results in an increase in oxygen and nutrient supply. Some studies of recombinant protein production in immobilized bacterial cells have also demonstrated the influence of agitation rate on oxygen and nutrient diffusion within the cells and the consequent effects on heterologous protein production [27,28]. Talabordan and Yang [44] reported that the production of a glucoamylase–green fluorescent fusion protein on a fibrous-bed bioreactor increased when the agitation rate increased to 400 rpm and that a further increase to 600 rpm resulted in the detachment and breakage of fungal mycelia and a consequent increase in protein secretion. Similar to bacterial biofilms, filamentous microorganisms comprise channels that allow fluid circulation when immobilized in reactors [45]. An increase in agitation rate promotes a better mass transfer within the cells [45]. Biofilms are known to be highly heterogeneous and contain cells in different physiological states, which influence gene expression in different zones within the biofilm [19,46,47]. In a previous work by our group [22], it was demonstrated that the eGFP-expressing cells in a 3-day *E. coli* biofilm were mostly found at the top of the biofilm. The lower eGFP expression that was obtained at the lowest tested flow rate in this study (128 L h^{-1}) could be related to the absence of oxygen and fresh nutrients in the deeper regions of the biofilm. In agreement with this hypothesis, Araújo et al. [34] showed that mass transfer limitations in *Pseudomonas fluorescens* biofilms occured under lower velocity conditions (0.1 m s^{-1}, which corresponded to a Reynolds number of 1000). Additionally, the higher eGFP production that was obtained at the higher flow rate was probably related to the high availability of oxygen and nutrients in the bulk fluid, since a direct consequence of increasing flow velocity is that the transport of nutrients and oxygen also increases [48].

Although the specific eGFP production in the biofilms that were grown at the lower flow rate remained almost constant until day 4, an increase was observed from that day onward. This phenomenon could be associated with the slight increase that was observed in PCN and the percentage of eGFP-expressing cells during the same period. It was expected that the higher PCN and the larger number of cells that were expressing the protein would

result in higher eGFP levels. Furthermore, the increase in the number of eGFP-expressing cells on day 4 could be related to the simultaneous decrease in biofilm thickness, since the oxygen transfer that is needed for the maturation of eGFP is facilitated in thinner biofilms.

Higher specific eGFP production levels were obtained in biofilm cells, even though they presented a smaller number of plasmid copies compared to the planktonic cells. These results were in agreement with our previous work [7], in which eGFP production was shown to be higher in biofilm cells, even with 3-fold lower PCN levels. Thus, under the tested hydrodynamic conditions, gene dosage did not affect recombinant protein production. This was probably related to the metabolic burden that was imposed by plasmid replication, which can promote plasmid instability and, consequently, reduce the yield of heterologous protein in planktonic cells [3,49].

Biofilm environments have been shown to increase plasmid maintenance compared to chemostats [6,7,50]. In this work, a higher percentage of plasmid loss was observed in the planktonic state for both flow rates. However, a low flow rate seemed to be advantageous for plasmid retention: in biofilm cells, 25% of plasmid loss was observed at 128 L h^{-1} while at a higher flow rate, this percentage was 2.5-fold higher. The marked decline in PCN on day 5 for the biofilm cells that were grown at 255 L h^{-1} could be related to the higher eGFP production that was observed on days 4 and 5. It is known that higher levels of protein production increase the metabolic burden on the host cell, which can contribute to plasmid loss [3,51]. Huang et al. [26] reported that biofilm cells that are grown at low agitation rates are associated with high plasmid stability.

5. Conclusions

Despite the advantages that biofilm reactors present over suspended cells, limitations in the diffusion of oxygen and nutrients within the biofilm remain a major problem. This study demonstrated that a flow rate had an impact on recombinant protein production in *E. coli* biofilms. Although increasing the flow rate had no major impact on biofilm formation, higher eGFP production and PCN values were obtained at the higher flow rate. The results suggested that the higher hydrodynamics favored the recombinant protein production in *E. coli* biofilms.

The results that were obtained in this work are essential for the definition of the operating conditions that are needed for heterologous protein production in biofilms systems in order to maximize protein production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10050931/s1, Figure S1: (A) Schematic representation and (B) photograph of the flow cell system, Figure S2: Planktonic (A) and biofilm (B) viable cells at the 255 L h^{-1} flow rate (*Re* of 4600; dark square) and 128 L h^{-1} flow rate (*Re* of 2300; gray square). The means ± standard deviations (SDs) for at least three independent experiments are illustrated. The statistical analysis corresponding to each time point is also represented with a double asterisk for a confidence level of greater than 95% ($p < 0.05$).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available yet as some datasets are being used for additional publications.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Material

Flow cell system schematization and experimental procedure

The flow cell system is composed of a recirculating tank (planktonic cells), a vertical flow cell reactor (where biofilms are formed), and centrifuge pumps that allow the circulation of the bacterial suspension. The flow cell reactor is a semi-circular Perspex duct (3.0 cm in diameter and 1.09 m in length) with 21 apertures on its flat wall to fit removable Perspex pieces (coupons) to which polyvinyl chloride (PVC) slides (2 x 1 cm) were glued. The biofilms were developed on the upper faces of the PVC slides that were in contact with the bacterial suspension circulating through the system.

E. coli cells were grown by recirculating the bacterial suspension at 30 °C for 7 days at two different flow rates: 255 L h^{-1} (Re of 4600) and 128 L h^{-1} (Re of 2300). The recirculating tank was aerated using an air pump (flow rate of 108 L h^{-1}) and continuously fed with Terrific Broth (supplemented with 20 μg mL^{-1} kanamycin) at a flow rate of 0.025 L h^{-1}.

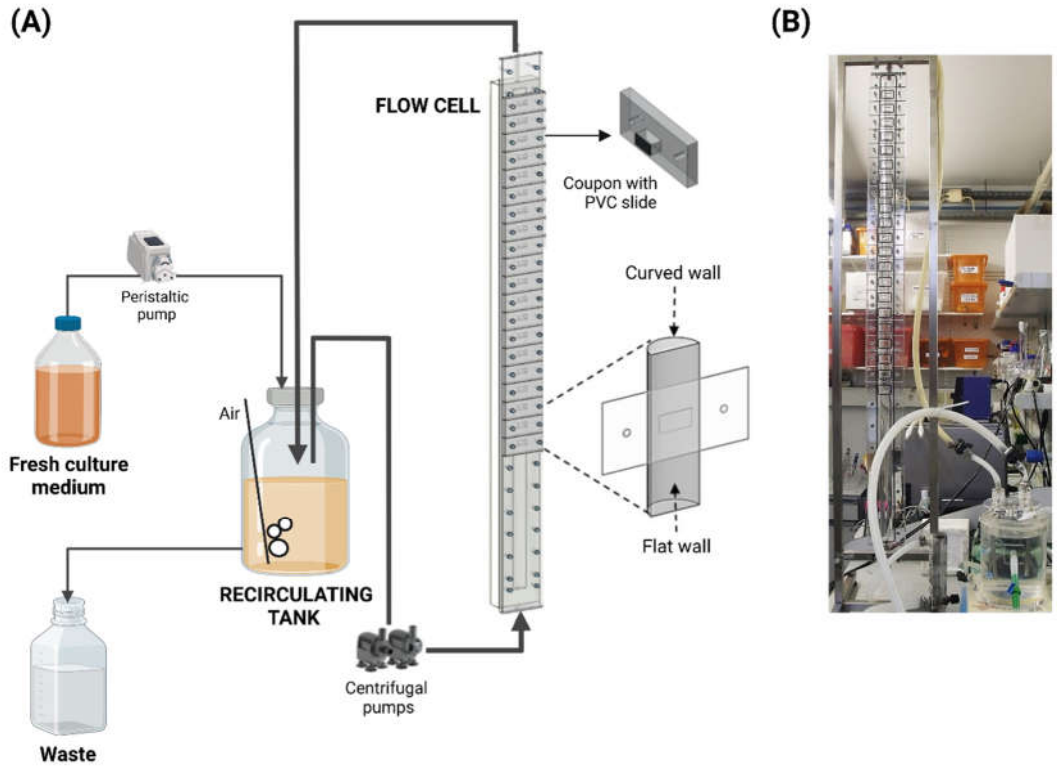


Figure S1. (A) Schematic representation and **(B)** photograph of the flow cell system.

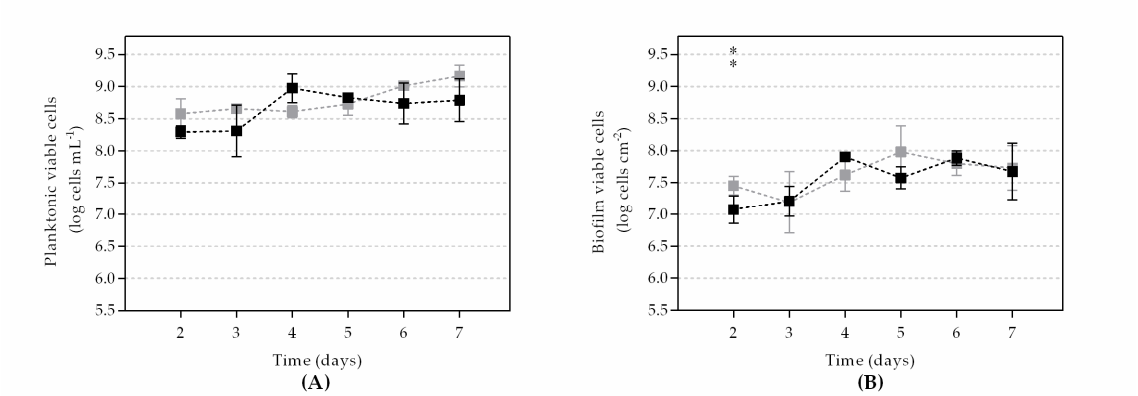


Figure S2. Planktonic **(A)** and biofilm **(B)** viable cells at a flow rate of 255 L h^{-1} (*Re* of 4600, dark squares) and 128 L h^{-1} (*Re* of 2300, grey squares). The means ± standard deviations (SDs) for at least three independent experiments are illustrated. Statistical analysis corresponding to each time point is also represented with an asterisk for a confidence level greater than 90% ($p < 0.1$) and with a double asterisk for a confidence level greater than 95% ($p < 0.05$).