Process Intensification at the expression system level for the production of 1-phosphate aldolase in antibiotic-free *E. coli* fed-batch cultures

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Keywords: High-cell-density fed-batch cultures, recombinant protein production, antibiotic-free expression system, *Escherichia coli,* bioprocess optimization.

Abstract

To successfully design expression systems for industrial biotechnology and biopharmaceutical applications; plasmid stability, capacity to efficiently synthesize the desired product and the use of selection markers that are acceptable to regulatory bodies are of utmost importance.

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In this work we demonstrate the application of a set of engineered strains – with different features namely, antibiotic vs auxotrophy marker; two-plasmids vs single plasmid expression system; expression levels of the repressor protein (LacI) and the auxotrophic marker (*glyA*) – in high cell density cultures to evaluate their suitability to be used in bioprocess conditions that resemble industrial production. Results revealed that the first generation of engineered strain showed a 50 % reduction in fuculose-1-phosphate aldolase (FucA) production compared to the reference system (165 ± 13 mg FucA·g^{-1} DCW and 806 ± 12 AU·g^{-1} DCW) which is commercially available from QIAGEN and uses an antibiotic selection marker. The over-transcription *glyA* was found to be a major factor responsible for the metabolic burden leading to the decrease in FucA yield.
The second- and third-generation of *E. coli* strains presented an increase in FucA production, being 202 ± 18 mg^{-1} FucA·g^{-1} DCW and 1176 ± 19 AU·g^{-1} DCW, and 1322 ± 19 AU·g^{-1} DCW and 245 ± 13 mg^{-1} FucA·g^{-1} DCW, respectively. Both strains presented a fitness improvement after the tuning of *glyA* expression levels and the deletion of the ampicillin resistance gene (*bla*) from the plasmid were carried out. The third-generation expression system is antibiotic-free, autotrophy-selection based and single-plasmid and is capable to produce FucA at similar levels compared to the original commercial expression system.
Hence, our expression system possesses advantageous features compared to the commercial one and proved to have the potential to become an attractive platform for the production of recombinant proteins in a wide range of industrial biotechnology applications.

1 Introduction

Escherichia coli is the most widely used host for the development of bacterial expression systems for overexpression of recombinant proteins and remains the first choice for laboratory investigations and industrial production of recombinant proteins within the biopharmaceutical sector (Rosano and Ceccarelli, 2014) (Castiñeiras et al., 2018). The main advantages of *E. coli* are: rapid growth rate, capability to reach high cell densities,

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growth on inexpensive substrates, well-characterized genetics and the availability of excellent tools for genetic manipulation (Brown, 1995).

A wide range of expression systems with different features have been developed for recombinant protein production (RPP) in *E. coli* comprising plasmids for constitutive or inducible expression, different mechanisms of induction (e.g. temperature and IPTG-inducible systems), the use of strains with different genotypes (Waegeman and Soetaert, 2011) and additional features to aid production (e.g. use of tuneable strains or co-expression of chaperones) (Marschall et al., 2017). Moreover, expression systems may incorporate fusion tags to increase protein solubility or enable purification using affinity chromatography (Costa et al., 2014). The vast majority of expression systems for lab-based investigations include antibiotic resistance markers that enable screening of positive clones and help in plasmid retention. However, the use of expression systems with antibiotic markers is considered unacceptable by regulatory authorities in relevant areas of industrial biotechnology (e.g. medical, therapeutic and agricultural applications) (Glenting and Wessels, 2005) and follows current trends in improvements in antibiotic use and stewardship to reduce antimicrobial resistance (Mignon et al., 2015).

In order to produce high levels of protein, it is often useful to clone the gene of interest downstream to a well-characterized, strong and tightly regulated promoter. The *E. coli lac* promoter is arguably the most extensively studied (Germán et al., 2019). Within the realm of *E. coli* expression, the T5 promoter system (and its derivatives) available from QIAGEN is one of the most popular choices for protein production (Fernández-Castané at el., 2012a) (Calleja et al., 2014). This IPTG-induced promoter has a high transcription rate that can only be tightly regulated in the presence of high levels of the Lac repressor protein (LacI) which is encoded by the *lacI* gene (Xu and Matthews, 2009). The commercial pQE- vectors commercialised by QIAGEN utilize two *lac* operator (*lacO*) regions in order to guarantee a strong repression under non-induced conditions (Rosano and Ceccarelli, 2014).

It is widely recognized that RPP consumes cellular energy and metabolites (Rahmen et al., 2015), whereby high level expression of heterologous proteins has a direct impact on the central metabolism of cells and causes the activation of stress responses (Weber et al., 2002) (Neubauer et al., 2003). This physiological response is known as the metabolic burden caused by RPP draining the host cell's resources, either in the form of energy

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such as ATP or GTP, or nutrients such as amino acids and nucleotides that are required to express and synthesize the protein of interest and its associated cellular machinery (Glick, 1995). Typically, cells overcome the metabolic burden by triggering stress-response mechanisms that adapt and readjust the metabolism in order to restore functionality and viability. However, such cellular responses often affect negatively growth parameters such as growth rate, biomass yield, and cellular viability, as well as recombinant protein productivity (Carneiro et al., 2012).

Plasmid-based expression systems are not always stable, particularly in cultivation strategies where cells are grown for many generations, i.e. in high-cell-density or continuous cultures (Pierce and Gutteridge, 1985). Hence, vector stability is of utmost importance. Expression systems using two or more plasmids may present high instability, thus enabling their use only as "transient" expression systems (Al-Allaf et al., 2013). During cell growth in high-cell-density cultures, overexpression of recombinant proteins often results in (i) cell aggregation and (ii) bacteria becoming viable but non-culturable (VBNC), a phenotype which means the loss of the ability to form colonies on agar plates (Wyre and Overton, 2014a) (Jeong and Lee, 2003) (Fernández-Castané at el., 2017). Flow cytometry has been extensively used as a rapid and high-throughput analytical technique to determine the viability and physiology of cells and to detect heterogeneities in cultures (Hewitt et al., 1999) (Wyre and Overton, 2014b).

Previous studies carried out in our laboratory developed auxotrophic selection systems based on glycine auxotrophy derived from the commercial two-plasmid pQE-based expression system (QIAGEN), comprising an expression plasmid (pQE) where the T5 promoter regulates expression of the recombinant gene of interest, and pREP4 which expresses *lacI* that allows tight regulation of the T5 promoter on the pQE plasmid (Gronenborn, 1976). The *E. coli glyA* gene encoding serine hydroxymethyl transferase (SHMT), which catalyzes the reversible interconversion of L-threonine and glycine and is essential for cell growth in the absence of glycine (Plamann and Stauffer, 1983), was used as a selective marker. In the 1st generation auxotrophic system (Vidal et al., 2008), *glyA* was cloned into the pQE-FucA vector expressing *fucA*, encoding fuculose-1-phosphate aldolase. The resultant plasmid, pQEαβFucA, was transformed into the glycine auxotroph *E. coli* M15*ΔglyA*[pREP4] yielding M15*ΔglyA*[pREP4] pQEαβFucA. Expression studies in shake flask cultures revealed that this strain presented a slight

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decrease in recombinant protein yield compared to the commercial reference strain (Pasini et al., 2016).

In the 2nd generation of the expression system (named 'Puzzle'), we optimized expression levels of *lacI* and *glyA* using the weak, constitutive J23110 synthetic promoter (Registry for Standard Biological Parts, http://parts.igem.com) and cloned both as a cassette into the pQE-FucA vector, which was transformed into *E. coli* M15Δ*glyA*, removing the need for the pREP4 plasmid thus alleviating metabolic burden. This resulted in improved plasmid maintenance while substantially increasing the production levels of the protein of interest in shake flasks experiments (Pasini et al., 2016). Finally, the 3rd generation strain ("AmpR⁻") was generated by deleting the ampicillin resistance gene (*bla*) from the pQE-FucA plasmid, resulting in solely auxotrophic selection.

In this work, we demonstrate that the 3rd generation of antibiotic-free pQE-derived expression systems developed recently in our laboratory can be successfully used in industrially relevant bioprocessing strategies (i.e. in high-cell-density cultures) for the expression of fuculose-1-phosphate aldolase (FucA), an enzyme of interest for biocatalytic pharmaceutical applications (Clapés et al., 2010) (Koeller and Wong, 2000). Growth and production of FucA was compared in the reference and three generations of auxotrophic systems, and cell physiology was measured using flow cytometry. Our 3rd generation system is advantageous as it does not require antibiotics and comprises a single plasmid.

2 Materials and methods

2.1 Strains and plasmids

The K-12 derived *E. coli* M15 (QIAGEN) and M15Δ*glyA* strains were used for recombinant FucA expression. The strains were stored at -80 °C in cryo-stock aliquots prepared from exponential phase grown cultures in Luria-Bertani (LB) medium. The list of *E. coli* strains, plasmids, and the expression systems used in this study and their abbreviated names are summarized in Table 1.

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Table 1. Bacterial strains and plasmids used in this work and abbreviation for the four *E. coli* expression systems used in this study.

Name	Characteristics	Reference or source
Strains		
M15	K12 derived	QIAGEN (Villarejo and Zabin 1974; Zamenhof and Villarejo, 1972)
M15 *Δ glyA*	Deletion of the *glyA* locus of the chromosome	(Vidal et al. 2008)
Plasmids		
pQE-FucA	pQE-40 derived (QIAGEN) with *fucA* gene cloned	(Pasini et al. 2016)
pREP4	$LacI^q$ kan^R	QIAGEN
pQEαβFucA	pQE-FucA derived with fragment, containing the *glyA* gene, under the P3 promoter transcriptional control	(Pasini et al. 2016)
pQE-FucA_puzzle	pQE-FucA derived with insertion of the cassette (J23110-*lacI*-*glyA*) for *lacI* and *glyA* genes transcription	(Pasini et al. 2016)
pQE-FucA_puzzle_AmpR$^-$	pQE-FucA_puzzle (J23110) derived without the *bla* gene	(Pasini et al. 2016)

Expression system name	Abbreviation	Generation	Number of plasmids
M15[pREP4] pQE-FucA	M15[pREP4]	Reference system	2
M15 *Δ glyA*[pREP4] pQEαβFucA	M15 *Δ glyA*[pREP4]	1st generation system	2
M15Δ*glyA* pQE-FucA_puzzle (J23110)	Puzzle	2nd generation system	1
M15 *Δ glyA* pQE-FucA_puzzle (J23110)_AmpR$^-$	AmpR$^-$	3rd generation system	1

2.2 Culture media

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise indicated. Luria Bertani (LB) medium, containing 10 g·L^{-1} peptone, 5 g·L^{-1} yeast extract and 10 g·L^{-1} NaCl, was used for pre-cultures.

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Defined Medium (DM) used for shake flasks cultures and bioreactors contained per liter: 5 g glucose; 2.97 g K_2HPO_4; 0.60 g KH_2PO_4; 0.46 g NaCl; 0.75 g $(NH_4)_2SO_4$; 0.11 g $MgSO_4{\cdot}7H_2O$; 0.006 g $FeCl_3$; 0.025 g thiamine; 1.44 g $CaCl_2{\cdot}2H_2O$; and 0.7 mL of trace elements solution (TES). TES contained per liter: 0.04 g $AlCl_3{\cdot}6H_2O$; 1.74 g $ZnSO_4{\cdot}7H_2O$; 0.16 g $CoCl_2{\cdot}6H_2O$; 2.18 g $CuSO_4{\cdot}5H_2O$; 0.01 g H_3BO_3; 1.42 g $MnCl_2{\cdot}6H_2O$; 0.01 g $NiCl_2{\cdot}6H_2O$; and 0.23 g $Na_2MoO4{\cdot}5H_2O$.

Stock solutions of kanamycin and chloramphenicol were dissolved in dH_2O at a concentration of 100 $mg{\cdot}mL^{-1}$ and 30 $mg{\cdot}mL^{-1}$, respectively and stored at -20 °C. Ampicillin was dissolved in absolute EtOH and prepared at a concentration of 100 $mg{\cdot}L^{-1}$and stored at -20 °C. IPTG stock was prepared at 100 mM, and stored at -20 °C.

Vitamins, antibiotics, TES, $FeCl_3$, $MgSO_4{\cdot}4H_2O$, $CaCl_2{\cdot}2H_2O$ and inducer were sterilized by filtration (0.2 μm syringe filter made from a blend of cellulose esters, Sartorius). Glucose and saline solutions were separately sterilized by autoclaving at 121°C for 30 min.

Feeding medium for fed-batch phase contained per litre: 490 g glucose; 9.56 g $MgSO_4{\cdot}7H_2O$; 0.49 g $FeCl_3$; 0.33 g thiamine; 0.10 g $CaCl_2{\cdot}2H_2O$; and 63 mL of TES. Phosphates (P) were not included in the feeding solution in order to avoid co-precipitation with magnesium salts. Instead, a 5-mL pulse of a concentrated phosphates solution containing 500 $g{\cdot}L^{-1}$ K_2HPO_4 and 100 $g{\cdot}L^{-1}$ KH_2PO_4 was added every 30-OD_{600} step increase based on a calculated biomass from phosphates yield ($Y_{X/P}$) of 18 g DCW g P^{-1} (Vidal et al., 2003).

2.3 Cultivation conditions

Cryo-stocks stored at -80 °C, were used to inoculate 50 mL Falcon tubes with 15 mL of LB medium supplemented with the corresponding antibiotic when necessary. Growth was performed overnight at 37 °C at 200 rpm.

Three mL of overnight pre-inoculum were transferred into a 500 mL conical flask containing 100 mL of DM, following the same growing conditions as pre-inoculum cultures for approximately 4.5 hours until an OD_{600} of 1.1 – 1.2 was achieved.

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Bioreactor experiments were carried out using two types of stirred tank bioreactors: Braun Biotech Int. Biostat® B (Germany) and Electrolab Fermac 310/60 (Tewkesbury, UK). For both bioreactors, the temperature was maintained at 37 °C and the pH was kept at 7.00 ± 0.05 by adding 15 % (v/v) NH_4OH solution (NH_4^+ from base addition served also as a nitrogen source). Dissolved oxygen level (pO_2) was kept above 60 % saturation. After glucose was depleted during the batch phase, feeding was started with the addition of the feed solution.

Induction of cultures was carried out by a single-pulse of 70 µM IPTG as previously described by (Fernández-Castané et al., 2012b). 1.5 mL samples were removed from the bioreactor every 10-30 min intervals during the induction period. One sample was removed prior to induction for the measurement of basal FucA expression.

For experiments performed in the Biostat® B bioreactor, a volume of 80 mL of the inoculum culture were added to 720 mL of DM in a 2 L-vessel (Calleja et al., 2014)(Vidal et al., 2005). pO_2 set point was fixed at 60 % saturation and pO_2 was controlled by adapting the stirring speed between 350 and 1120 rpm and by supplying air (enriched with pure oxygen when necessary) at a flow rate of 1.5 vvm. A microburette was used for the discrete addition of feed. The pH was set at 7 and maintained by adding 15 % (v/v) NH4OH solution.

For experiments performed in the Fermac 310/60 bioreactor, the volume of inoculum was 200 mL and the DM volume was 1300 mL, making up to a total of 1500 mL batch volume in a 5 L-jar equipped with 4 baffles and an agitator with 2 six-bladed Rushton turbines. Aeration was achieved by sparging air from below the lower impeller at a rate of 3 $L \cdot h^{-1}$ (2 vvm). pO_2 was measured online using a D150 Oxyprobe (Broadley James) and was maintained above a set point of 50 % by increasing agitation to a maximum of 1000 rpm from a minimum of 200 rpm. Off-gas passed through a condenser, autoclavable 0.22-µm filter (Sartorius, Goettingen, Germany) and HEPA filter (Millipore, Darmstadt, Germany). pH was measured using an F-695 FermProbe (Broadley James). The temperature was maintained at 37 °C by a heating jacket and coldfinger. The pH was set at 7 and maintained by adding 15 % (v/v) NH4OH solution.

Bioreactor off gasses were automatically collected for subsequent compositional analysis using a PrimaDB gas-chromatograph mass spectrometer (GC-MS) (Thermo) and

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compared to atmospheric air in order to calculate the O_2 uptake rate (OUR), CO_2 evolution rate (CER), and subsequent estimation of the respiratory quotient (RQ). This data was logged automatically by GasWorks v1.0 (Thermo). The increase of pO_2 ,O_2 and pH values, together with a decrease in CO_2 values, indicate the complete consumption of the 10 $g{\cdot}L^{-1}$ of initial glucose and the end of the batch phase (Figure S2-1), (Sooan at al., 2009).

The feeding strategy was performed to maintain the specific growth rate (μ_{fix}) constant through the fed-batch phase, using a pre-defined exponential feeding profile (Pinsach et al., 2008). Feeding was manually and periodically interrupted and restarted in order to avoid glucose accumulation during the induction phase.

2.4 Analytical methods

Cell concentration was determined by optical density measurements at 600 nm (OD_{600}) using a spectrophotometer (Uvicon 941 Plus, Kontrol and Evolution 300 UV–vis, Thermo Scientific, for the Biostat® B and the Fermac 310/60 bioreactors, respectively). OD_{600} values were correlated to biomass concentration expressed as Dry Cell Weight (DCW), being 1 OD_{600} equivalent to 0.3 $gDCW{\cdot}L^{-1}$ (Pinsach et al., 2008).

Glucose and acetate concentration in the fermentation broth were analysed. One milliliter of culture medium was separated from biomass by centrifugation at 14 000 rpm for 6 min and filtered (0.45 μm membrane filter of cellulose esters, Millipore) prior to analysis. Glucose concentration was determined enzymatically using an YSI 2070 biochemical analyser (Yellow Spring Systems). Acetic acid was analyzed by HPLC (Hewlett Packard 1050) equipped with an ICSep COREGEL 87H3 ICE-99-9861 (Transgenomic) column and IR detector (HP 1047), using 6 M H_2SO_4 (pH 2.0) as mobile phase at a flow rate of 0.3 $ml{\cdot}min^{-1}$. The column was kept at 40 °C.

The maximum specific growth rate (μ_{max}) of the strains was estimated from microbial growth curves. Equation [1] shows the relationship between the cell concentration (X), maximum specific growth rate (μ_{max}) and time (t). Log-linearized Eq. [2] yields a linear relationship where the μ_{max} is represented by the slope of the linear portion in the plot of the natural log of cell concentration versus time.

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$$X_t = X_0 \cdot e^{\mu_{max} \cdot t} \qquad [1]$$

$$lnX_t = lnX_0 + \mu_{max} \cdot t \qquad [2]$$

where X_0 and X_t are the OD_{600} or cell concentration at the start and during the exponential phase of growth.

The biomass yield, $Y_{X/S}$ was calculated using the following equation:

$$Y_{X/S} = \frac{(DCW_{max} - DCW_0)}{(Glc_0 - Glc_f)} \qquad [3]$$

DCW_{max} and DCW_0 ($g \cdot L^{-1}$) are the maximum and the initial biomass values, respectively. Glc_0 and Glc_f ($g \cdot L^{-1}$) are initial and final value of glucose concentration, respectively.

2.5 FucA quantification

Samples from fermentation broth were withdrawn, adjusted to a final OD_{600} of 4, centrifuged and subsequently processed as described elsewhere (Durany et al., 2005) (Vidal et al., 2008). Briefly, pellets were resuspended in 100 mM Tris HCl (pH 7.5). Cell suspensions were placed in ice and sonicated (Vibracell™ model VC50 (Sonics & Materials)), over four pulses of 15 seconds each at 50 W with 2 minutes intervals in ice between each pulse. Cellular debris was then removed by centrifugation and the cleared supernatant was collected for FucA analysis. One unit of FucA activity is defined as the amount of enzyme required to convert 1 μmol of fuculose-1-phosphate in DHAP and L-lactaldehyde for minute at 25 °C and pH 7.5 (Vidal et al., 2008).

Total protein content was determined by means of the Bradford method using a Coomassie® Protein Assay Reagent Kit (Thermo Scientific). To quantify the amount of FucA relative to total intracellular soluble proteins, NuPAGE® 12 % Bis–Tris gels were used according to the manufacturer's instructions (Invitrogen). Protein concentration was quantified using Kodak Digital Science® 1D 3.0.2 densitometry software.

Average values of triplicates experiments were plotted with error bars. The error indicates the confidence interval of 90 %.

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2.6 Flow cytometry (FCM)

Bacterial samples taken directly from the bioreactor were resuspended in phosphate-buffered saline (PBS) to a final concentration of $10^5 – 10^6$ cells mL^{-1} and then analysed directly or after staining with various fluorescent dyes using a BD Accuri™ C6 flow cytometer (Becton, Dickinson and Company, Oxford, UK). Samples were stained with: bis (1,3-dibutybarbituric acid) trimethine oxonol (0.05 µg BOX mL^{-1}) to determine membrane potential and propidium iodide (4 µg PI mL^{-1}) to determine membrane integrity; or Congo red (40 µg CR mL^{-1}) to determine amyloid content and thus presence of inclusion bodies. During FCM on fluorescently labelled cells, samples were excited using a 488 nm solid-state laser and fluorescence was detected using two different filters: a 533/30 BP (filter FL1-A) for BOX; and a 670 LP filter (FL3-A) for PI and CR. Particulate noise was eliminated using a FSC-H threshold. 20,000 data points were collected at a maximum rate of 2,500 events·s^{-1}. Data were analyzed using BD CFlow software.

3 Results

3.1 Comparison of cell growth, substrate consumption and acetate formation

The performance of the three generations of auxotrophic expression systems (Table 1) were compared to that of the initial QIAGEN pQE/pREP4 system in fed-batch fermentations for the production of FucA. These strains had previously only been compared in shake flask cultures (Pasini et al., 2016). In this study, triplicate fed-batch fermentations were performed using defined medium and glucose as a carbon source in BioStat bioreactors to determine the robustness of each expression system in terms of growth yield, plasmid stability and recombinant protein production.

The time-profiles of the biomass, glucose consumption, FucA mass and specific activity and acetate production of the four expression systems evaluated in this work are represented in Figure 1. Key fermentation parameters are listed in Table 2.

The comparison of the maximum specific growth rate (μ_{max}) in the batch phase between the reference commercial M15[pREP4] *E. coli* strain with the three M15*ΔglyA*-derived

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generations, shows that the latter present slightly lower μ_{max}, decreasing from 0.49 ± 0.01 h^{-1}, of the reference strain, to 0.44 ± 0.01 h^{-1}, 0.45 ± 0.01 h^{-1} and 0.41 ± 0.01 h^{-1} of the 1st, 2nd and 3rd generations, respectively (Table 2). Furthermore, in both the reference and the 1st generation strains, the concentration of the biomass progressively increased until 2.5 h after induction and then stopped around 4 h after induction, reaching a final value of 40 g DCW·L^{-1}. While for the 3rd generation strain the fermentation ended with a final biomass concentration of 53.5 ± 0.7 g DCW·L^{-1}, with a concentration of dissolved oxygen dropped below 10 %, after 26.3 h (Figure 1).

Substrate uptake rates (q_S) along the induction phase for both reference and 1st generation strains were calculated, being 0.37 ± 0.04 and 0.50 ± 0.13 g Glc·g^{-1} DCW·h^{-1}, respectively. As a consequence, the 1st generation strain accumulated higher amounts of acetate throughout, reaching a final concentration of 0.54 ± 0.03 g·L^{-1}. Both strains started to accumulate glucose and acetate in the medium after 2 h of induction, remaining below inhibitory levels (32 g·L^{-1} and 2.4 g·L^{-1}, respectively) (Figure 1A-B) (Kazan et al., 1995) (Phue et al., 2005). It is widely known that glucose acts as catabolite repressor, thus repressing the genes involved in metabolizing other sugars, and in IPTG-inducible systems, to repress the expression of recombinant proteins (Amma et al., 2018). The accumulation of glucose and acetate during the induction phase occurred at the same time as a decrease in the FucA activity and production in the first-generation system (Figure 1B).

This accumulation is probably due to the high-energy demand imposed to the cell for the synthesis of the recombinant protein. Recently, it has been demonstrated that this “metabolic burden” is not caused by energy limitation, on the contrary this is due to restrictions in anabolic functions (Weber et al., 2021). This energy excess due to RPP causes a reduction of catabolic carbon processing hence, i) affecting negatively the growth rate; ii) enhancing acetate formation and iii) reducing the carbon substrate uptake; evidenced by the accumulation of glucose (Weber et al., 2002) (Weber et al., 2021).

The feeding strategy of our fed-batch cultivations was pre-set to maintain constant values of μ and $Y_{X/S}$, and these were not modified during the induction phase, that is, the substrate feeding program assumed these parameters constant even though they changed as a result of the metabolic imbalance generated by RPP, leading to an

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accumulation of glucose into the medium. When this state was reached, glucose feeding was manually stopped to avoid its further accumulation and reestablished once the residual glucose was depleted.

Remarkably, the 2nd and 3rd generation strains (each with a single plasmid), unlike the previous two-plasmid systems, did not generate acetate or accumulate glucose during the induction phase (Figure 1C-D). Thus, the tuning of *lacI -glyA* expression by the weaker constitutive J23110 promoter, leads to a reduction in the transcription levels of these genes, resulting into a reduction of the metabolic load imposed to the cells.

Table 2 Calculated maximum specific growth rate and biomass from substrate yield of the *E. coli* strains used in this work along the batch phase.

E. coli strains	μ_{max} (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)
M15[pREP4] (Reference strain)	0.49 ± 0.02	0.43 ± 0.04
M15Δ*glyA*[pREP4] (1st generation strain)	0.44 ± 0.01	0.36 ± 0.06
Puzzle (2nd generation strain)	0.45 ± 0.01	0.37 ± 0.09
AmpR- (3rd generation strain)	0.41 ± 0.01	0.35 ± 0.02

3.2 Determination of FucA expression levels

In order to evaluate the levels of FucA production in fed-batch cultures of the different strains, specific protein production ($mg \cdot g^{-1}$DCW) and activity ($AU \cdot g^{-1}$ DCW) were measured as shown in Figure 1 and 2. Overall, FucA activity and FucA production decreased more than 35 % when comparing the 1st generation to the reference strain, which reached a final production of 240 ± 16 mg FucA·g^{-1} DCW, with an activity of 1214 ± 47 AU·g^{-1} DCW. These values were reduced to 165 ± 13 mg FucA·g^{-1} DCW and 806 ± 12 AU·g^{-1} DCW respectively, in the 1st generation strain (Figure 2). These effects may be caused by the increase in the metabolic burden due to the maintenance of the pQEαβFucA expression vector in 1st generation auxotrophic, strain. The *glyA* gene, even though encoded by the low-copy number plasmid (ColE1 replication origin) under the

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control of the P3 from the gen bla_{TEM-1A} (Lartigue et al., 2002), leads to substantially higher amounts of SHMT protein product which accumulates in the cytoplasm compared to the reference strain which contains a single copy genomic copy of *glyA* (Pasini et al., 2016).

The maximal FucA specific productivity, both in terms of activity and mass, was highest for the reference strain, being 62.04 ± 0.26 $AU \cdot g^{-1}$ $DCW \cdot h^{-1}$ and 14.95 ± 0.39 $mg \cdot g^{-1}$ $DCW \cdot h^{-1}$, respectively (Table 3). Clearly, the 1st generation strain showed reduced specific productivity of 44.02 ± 0.68 $AU \cdot g^{-1}$ $DCW \cdot h^{-1}$ and 7.61 ± 0.17 $mg \cdot g^{-1}$ $DCW \cdot h^{-1}$. A slight improvement was obtained in the 3rd generation strain, obtaining a final value of 48.51 ± 0.31 $AU \cdot g^{-1}$ $DCW \cdot h^{-1}$ and 8.95 ± 0.39 $mg \cdot g^{-1}$ $DCW \cdot h^{-1}$. Nevertheless, this strain showed volumetric productivities in terms of enzymatic activity comparable to the reference strain, despite still being 22 % lower in terms of enzyme mass.

Unlike the 1st generation, previous results in shake flasks showed that FucA production improved in the 2nd and 3rd generation expression strains, both of which have a single plasmid, compared to the reference (Pasini et al., 2016). The results obtained in shake flask were consistent with the results presented in this study in bioreactor cultures where the maximum FucA specific production and specific activity increased up to 202 ± 18 mg^{-1} $FucA \cdot g^{-1}$ DCW and 1176 ± 19 $AU \cdot g^{-1}$ DCW for the 2nd generation, and 1322 ± 19 $AU \cdot g^{-1}$ DCW with a specific mass of 245 ± 13 mg $FucA \cdot g^{-1}$ DCW for the 3rd generation strain (Figure 2). Comparing these values with those obtained with the reference strain, both the specific activity and the amount of the recombinant protein are comparable.

Remarkably, the *ΔglyA* strain generations based on a single-plasmid expression system allow for an improvement of FucA production levels compared to the two-plasmid system. In particular, the down-regulation of the *glyA* gene is a key factor that is likely to contribute to improve the regulation of *fucA* expression thus, leading to a higher FucA specific activity. These conclusions are in accordance with the results obtained with the 3rd generation strain. When comparing the 1st and the 3rd generations, FucA specific activity increased more than 60 % (from 806 ± 12 to 1322 ± 19 $AU \cdot g^{-1}$ DCW) and FucA specific mass increased c.a. 50 % (from 165 ± 13 to 245 ± 13 $mgFucA \cdot g^{-1}$ DCW) (Figure 2).

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Table 3 Specific productivity and volumetric productivity for both FucA activity and mg for the 4 strains presented along this work

E. coli strains	Specific productivity		Volumetric productivity	
	AU FucA $\cdot g^{-1}DCW \cdot h^{-1}$	mgFucA $\cdot g^{-1}$ DCW$\cdot h^{-1}$	AU FucA $\cdot L^{-1} \cdot h^{-1}$	mgFucA $\cdot L^{-1} \cdot h^{-1}$
M15[pREP4] (Reference strain)	62.04 ± 0.26	14.95 ± 0.39	2536 ± 44	611 ± 24
M15Δ*glyA*[pREP4] (1st generation strain)	44.02 ± 0.68	7.61 ± 0.17	1652 ± 13	241 ± 80
Puzzle (2nd generation strain)	45.46 ± 0.26	7.86 ± 0.18	2328 ± 67	407 ± 19
AmpR^{-} (3rd generation strain)	48.51 ± 0.31	8.95 ± 0.39	2597 ± 56	479 ± 26

3.3 SHMT production levels

A potential drawback of using SHMT as an auxotrophic marker is accumulation to high concentrations, leading to metabolic burden, and resultant decreases in biomass and recombinant protein production (Vidal et al., 2008) (Pasini et al., 2016). The 2nd and 3rd generation strains aimed to reduce SHMT levels by regulating *glyA* expression from the weak, constitutive, J23110 promoter (Pasini et al., 2016).

Figure 3 depicts the concentration of the SHMT protein both pre-induction and during the induction phases in high-cell-density fed-batch cultures carried out in Figure 1. It can be seen how the SHMT levels are around 130 mg SHMT$\cdot g^{-1}$DCW in the 1st generation strain which corresponds to a *ca*. 4.5-fold increase compared to the reference system (20 mg SHMT$\cdot g^{-1}$DCW). The constitutive high SHMT production can be clearly observed in Figure S1A, which as an example presents the SDS-PAGE analysis of the samples taken during the induction phase of the 1st generation fed-batch culture. Hence, it is clear that the *glyA* gene encoded on a high-copy number plasmid leads to substantially higher amounts of SHMT accumulated in the cytoplasm compared to the reference strain containing a single genomic copy of *glyA*.

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However, by tuning *glyA* expression levels, SHMT production decreased in the single plasmid constructs (2nd and 3rd generation), resulting in an overall reduced metabolic burden (Pasini et al., 2016). As it can be seen in Figure 3 and Figure S1B, even though there is constitutive overexpression of the SHMT protein in both pre-induction and induction phases, for the 2nd and 3rd generation strains, these values were almost 50 % reduced compared to the 1st generation strains. Thus, these data confirm that the reduction of SHMT production is observed in both bioreactors and shake flasks. As an explanatory example, 85 ± 0.5 and 72 ± 13 mg SHMT ·g DCW^{-1} were produced after 4 h of induction for the 2nd and 3rd generation strains, respectively. In contrast 132 ± 4 mg SHMT ·g DCW^{-1} was produced for the reference strain (Figure 3). These findings are an equivalent to a 1.6-fold reduction in the accumulation of the SHMT protein in the cytoplasm as a result of replacing the strong P3 promoter with the weaker J23110 constitutive promoter (Pasini et al., 2016). Therefore, this enabled a reduction of the metabolic burden imposed to the cells. These results are in accordance with the observations by (Mairhofer et al., 2013), who demonstrated that the folding machinery is severely overstrained in plasmid-based expression systems compared with plasmid-free cells (i.e. with a single copy of the gene of interest integrated into the genome) and this is due to different expression vector dosage.

As stated above, similarities can be observed among all the strains when comparing the fed-batch culture results presented here with the previously experiments performed in shake flask cultures (Pasini et al., 2016): i) the reference strain presents a slightly higher maximum specific growth rate compared to the new set of engineered strains; ii) the SHMT production increased significantly when moving from the reference to the to the 1st generation strain; iii) tuning *glyA* levels is a key factor to allow for improved fucA expression regulation, leading to a higher FucA specific activity in the 2nd and 3rd generations strains; iv) the 2nd and 3rd generation strains presented a reduction in the amount of acetate production and glucose accumulation.

3.4 Flow cytometry study of physiological responses caused by FucA expression

To gain new insights into the physiology of the strains upon FucA overexpression, flow cytometry analysis was performed on the high-cell-density fed-batch culture

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experiments performed in Fermac 310/60 bioreactors. The time profiles of the biomass, pH, pO_2 and CO_2, O_2 and RQ of the four expression systems evaluated in these bioreactors are represented as an example in Figure S2. Importantly, pO_2 time profile between the set of experiments carried out in Biostat® B and the Fermac 310/60 bioreactors were similar.

Propidium iodide (PI), which only stains cells with compromised membranes, and bis-oxanol (BOX), which only stains cells with a collapsed membrane potential, were used to determine viability (Figure 4). Cells were classified as 'healthy' with a membrane potential (PI^-/BOX^-), 'damaged', being intact but with no membrane potential (PI^-/BOX^+), or 'dead', having a non-intact membrane and no membrane potential (PI^+/BOX^+).

As can be seen in Figure 4A, from the pre-induction sample to the end of the fermentation, the reference strain presented a significant percentage of unhealthy and dead cells even under uninduced condition. The high proportion of damaged or dead cells present before the induction stage is probably due to the presence of the antibiotic in the medium which is used to maintain plasmid stability (Feizollahzadeh et al., 2017). The percentage of damaged cells remained relatively constant through growth (3-5 %). The percentage of dead cells reached the maximum value (18 %) after 15 minutes of induction which indicates the stress caused to the cells by the induction of FucA expression.

For the two-plasmid based expression systems (the reference and the 1St generation strains) it can be seen that at the end of the fed-batch cultures the proportion of damaged and dead cells increased (Figure 4A-B). It is well known that the production of a foreign protein causes an additional stress for the host strain and leads to a decrease in the overall cell fitness (Hoffmann and Rinas, 2004). The dead population at the end of the fermentation corresponded to 16 % and 8 % for the reference and 1st generation strains, respectively. This reflects the stress caused to the cells due to the induction. The state of the cells of the reference strain results more unhealthy than the 1st generation strain probably due to a higher FucA overexpression and also due to the presence of the antibiotic. In addition to PI&BOX staining, we also stained cells with Congo Red (CR), which stains amyloids such as inclusion bodies (IBs; Carrió et al., 2005). Figure 5A-B shows that, for the reference and 1st generation strains, there were around 1 % of cells containing IBs; for the 1st generation strain, this increased after 3 hours of induction.

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FCM data reveal that for the 2nd generation strain, Puzzle, more than 95 % of cells were healthy throughout the fermentation (Figure 4C). Similar observations were obtained for the 3rd generation strain (AmpR$^-$) (Figure 4D), with even fewer unhealthy cells (96 % healthy throughout). Almost no change in PI or BOX staining was observed through the induction phase for the 2nd and 3rd generation strains, indicating that physiology was not negatively impacted by RPP.

Only at the very end of the fermentation a slight increase of both damaged and dead cells appears, for the Puzzle and the AmpR$^-$ strains, indicating an initial loss of viability. This is in accordance with previous works, where the number of cells identified as either being stressed or dead increased at the end of the fermentation, due to stress condition (Lewis et al., 2004). This increase is probably due to the non-optimal conditions presented at the end of the fermentation, in particular the low pO_2 levels due to the high oxygen demands of the culture (data not shown). This stress condition can also be observed in the FCM analysis of cells stained with Congo red (CR) (Figure 5C-D), showing an increase in the proportion of cells containing IBs (up to 2 % and 3 % of cells, respectively). This result can be explained by an increase of the stress condition of the protein folding machinery resulting in an increase of aggregated protein. This can be reflected also in the specific enzyme activity values of the strains presented in this study, being: 5.06 ± 0.01 UA·mg^{-1} for the reference strain; 4.88 ± 0.01 UA·mg^{-1} for the 1st generation; 5.82 ± 0.01 UA·mg^{-1} for the 2nd generation; and 5.40 ± 0.01 UA·mg^{-1} for the 3rd generation. These results indicate that the 1st generation strain presents the lower FucA enzyme activity/protein concentration ratio indicating a lower folded enzyme activity, in accordance with the CR results that indicate the higher amount of IBs. It is interesting to note that the 2nd generation strain showed the higher specific enzyme activity.

Bacterial exposure to environmental stress (including glucose/oxygen availability) often triggers protein misfolding and formation of aggregates as IBs (Kwiatkowska et al, 2008) (Schramm et al., 2020). These results are consistent with the those presented by Castellanos-Mendoza et al., where IB formation (as detected using CR) increased at uncontrolled pH conditions (Castellanos-Mendoza et al., 2014).

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The presence of IBs is generally not desirable in recombinant production of enzymes because a further refolding step during purification is often required (Vallejo and Rinas, 2004). In future studies, a trade-off between maximising protein production and correct folding of the enzyme should be taken into consideration if further downstream processing – harvesting, purification and application of FucA – is intended (Mukhopadhyay, 1997) (Kim at el., 2020).

4 Conclusion

We have demonstrated that by tuning the expression levels of the repressor (*lacI)* and the auxotrophic gene (*glyA)* using synthetic biology tools and methods, the strains developed in our lab can be used for efficient and stable expression of an industrially relevant enzyme namely, FucA; in cultivation conditions that resemble industrial production settings. FucA production (mg FucA ·g DCW^{-1}) in the optimized 3rd generation strain was c.a. 5 %, 50 % and 10 % higher comparing with the reference, 1st generation and 2nd generation strains, respectively. FucA activity (AU·g^{-1}DCW) was increased through the different stepwise improvements performed along this work. The best performing engineered strain reached more than 60 % higher values in terms of activity compared to the 1st generation of M15Δ*glyA* strain.

Tuning of *glyA* transcriptional levels using the weak constitutive promoter J23110 resulted in the reduction of more than 50 % of SHMT expression levels in the single plasmid expression system strains, led to a reduction of the energy demand towards SHMT production thus, redirecting nutrients/energy for recombinant FucA production.

To conclude, the optimized 3rd generation strain presented a reduction of the metabolic burden and an improvement of the strain fitness and overall performance of the expression. The expression levels were similar to the commercial strain, but our novel expression system present advantageous features: i) it is a single-plasmid tightly regulated expression system; ii) presented significant reduced metabolic burden to the cells, resulted in no acetate production nor glucose accumulation and in healthy cells

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with intact and polarized cytoplasmatic membrane all along the fermentation; iii) no antibiotic is needed.

Remarkably all this is achieved by means of i) a system with reduced number of genetic components and, importantly, ii) antibiotic free, that makes it suitable for biopharmaceutical and agritech applications.

We envisage that our antibiotic-free expression system has the potential to become an attractive platform for the production of a wide range of proteins as well as for other application where the use of antibiotics is restricted, such as vaccine delivery (Saubi et al., 2014).

Acknowledgements

This work was supported by the Spanish MICINN, project number CTQ2011-28398-CO2-01 and the research group 2009SGR281 and by the Bioprocess Engineering and Applied Biocatalisys Group, Department of Chemical, Biological and Environmental Engineering of the Universitat Autònoma de Barcelona, Cerdanyola del Valles (Spain).

M. P. acknowledges the Universitat Autònoma de Barcelona for the pre-doctoral fellowship and EMBO for a Short Term Fellowship.

Ethical statement/conflict of interest

All authors concur with the submission and agree with its publication. The authors declare that they have no conflict of interest.

The authors confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere.

The manuscript does not contain experiments using animals or human studies.

Authors' contributions

MP: Performed all experiments, acquisition and analysis of all the data, as well as drafting the manuscript. AFC, GC and PF: Contributed to the overall conceptual design of the study and data interpretation, as well as in drafting and revision of the manuscript.

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TWO: Contributed to the conceptual design of the study and manuscript editing. All Authors read and approved the manuscript.

References

Al-Allaf, Faisal A., O. E. Tolmachov, L. P. Zambetti, V. Tchetchelnitski, and H. Mehmet, "Remarkable stabilityu of an instability-prone lentiviral vector plasmid in *Escherichia coli* Stbl3" *Biotech* 3(1): 61-70, 2013. doi: 10.1007/s13205-012-0070-8

Ammar, Ehab M., X. Wang, and C. V. Rao, "Regulation of metabolism in Escherichia coli during growth on mixtures of the non-glucose sugars: arabinose, lactose, and xylose", *Sci Rep* 8:609, 2018. doi: 10.1038/s41598-017-18704-0

Baneyx, François, "Recombinant protein expression in *Escherichia coli*," *Curr Opin Biotechnol* 10(5): 411–421, 1999. doi: 10.1016/s0958-1669(99)00003-8.

Brown, Terrence A., "Gene Cloning: an Introduction", Wiley-Blackwell, 4th edition, 1995.

Calleja, Daniel, A. Fernández-Castañé, M. Pasini, C. de Mas, and J. López-Santín, "Quantitative modeling of inducer transport in fed-batch cultures of Escherichia coli," *Biochem Eng J* 91: 210–219, 2014. doi: 10.1016/j.bej.2014.08.017

Carneiro, Sónia, E. C. Ferreira, and I. Rocha "Metabolic Responses to recombinant Bioprocesses in *Escherichia coli*" *J Biotech* 164(3): 96-408, 2012. doi: 10.1016/j.jbiotec.2012.08.026

Carrió, Mar, N. Gonzalez-Montalbán, A. Vera, A. Villaverde, and S. Ventura, "Amyloid-like properties of bacterial inclusion bodies", *J Mol Biol* 3447(5): 1025–37, 2005. doi: 10.1016/j.jmb.2005.02.030

Castellanos-Mendoza, Andrea, R. M. Castro-Acosta, A. Olvera, G. Zavala, M. Menodza-Vera, E. Garcia-Hernandez, A. Alagon, M.A. Trujillo-Roldan, and N. A. Valdez-Cruz, " Influence of pH control in the formaiton of inclusion bodies during production of recombinant sphyingomyelinase-D in *Escherichia coli*", *Microb Cell Fact* 13: 137, 2014. doi: 10.1186/s12934-014-0137-9

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ORIGINAL UNEDITED MANUSCRIPT

Castiñeiras, Tania S., S. G. Williams, A. G. Hitchcock, and D. C. Smith "*E. coli* strain engineering for the production of advanced biopharmaceutical products", *FEMS Microbiology Letters* 365(15): 162, 2018. doi: 10.1093/femsle/fny162

Clapés, Pere, W. D. Fessner, G. A. Sprenger, and A. K. Samland "Recent progress in stereoselective synthesis with alsolases", *Curr Opin Chem Biol* 14(2):154-157, 2010. doi: 10.1016/j.cbpa.2009.11.029

Costa, Sofia, A. Almeida, A. Castro, and L. Domingues, "Fusion tags for protein solubility, purification, and immunogenicity in Escherichia coli: the novel Fh8 system" *Front Microbiol* 19(5):63, 2014. doi:10.3389/fmicb.2014.00063

Durany, Olga, C. de Mas, and J. López-Santín, "Fed-batch production of recombinant fuculose-1-phosphate aldolase in E. coli," *Process Biochem* 40(2):707–716, 2005. doi: 10.1016/j.procbio.2004.01.058

Feizollahzadeh, Sadegh, S. Kouhpayeh, I. Rahimmansh, H. Khanahmad, F. Sabzehei, M. Ganjalikhani-hakemi, A. Andalib, Z. Hejazi, and A. Razaei, "The increase in preotein and palmsid yields of *E. coli* with optmized concentration of Ampicillin as selection marker" *Iran J Biotechnol* 15(2):128-134, 2017. doi: 10.15171/ijb.1467.

Fernández-Castané, Alfred, C.E. Vine, G. Caminal, and J. López-Santín, "Evidencing the role of lactose permease in IPTG uptake by *Escherichia coli* in fed-batch high cell density cultures", *J Biotechnol* 157(3):391-398, 2012a. doi: 10.1016/j.jbiotec.2011.12.007.

Fernández-Castané, Alfred, G. Caminal, and J. López-Santín, "Direct measurement of IPTG enable analysis of the induction of *E. coli* in high cell density cultures", *Microb Cell Fact* 11:58, 2012b. doi: 10.1186/1475-2859-11-58.

Fernández-Castané, Alfred, H. Li, O. R. T. Thomas, and T. W. Overton, "Flow cytometry as a rapid analytical tool to determine physiological responses to changin O_2 and iron concentration by *Magnetospirillum gryphiswaldense* strain MSR-1", *Sci Rep* 7:13118, 2017. doi: 10.1038/s41598-017-13414-z.

Garcia-Junceda, Eduardo, S. Gwo-Jenn, S. Takeshi, and C. H. Wong, "A New Strategy for the Cloning , Overexpression and One Step Purification of Three DHAP-Dependent Aldolases: Rhamnulose-1-Phosphate Aldolase, Fuculose-1-Phosphate Aldolase and Tagatose-1,6-Diphosphate aldolase" *Biorg Med Chem* 3(7):945–953, 1995. doi: 10.1016/0968-0896(95)00077-t.

Germán, L. Rosano, E. S. Morales, and E. A. Ceccarelli, "New tools for recombinant protein production in *Escherichia coli*: a 5-year update" *Protein Sci* 28(8):1412-1422, 2019. Doi: 10.1002/pro.3668.

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ORIGINAL UNEDITED MANUSCRIPT

Glick, Bernard R., “Metabolic Load and heterologous Gene Expression” *Biotechnol Adv* 13(2):247–261, 1995. doi: 10.1016/0734-9750(95)00004-A.

Glenting, Jacob and S. Wessels, “Ensuring safety of DNA vaccines” *Microb Cell Fact* 4:26, 2005. doi: 10.1186/1475-2859-4-26.

Gronenborn, Bruno, “Overproduction of phage lambda repressor under control of the lac promotor of *Escherichia coli.*” *Mol Gen Genet* 148(3):243–50, 1976. doi: 10.1007/BF00332898.

Hewitt, Christopher J., G. Nebe-von Caron, A. W. Nienow, and C. M. Mcfarlane, “The use of multi-parameter flow cytometry to compare the physiological response of Escherichia coli w3110 to glucose limitation during batch, fed-batch and continuous culture cultivations”. *Journal of Biotechnology* 75:251–64, 1999. doi: 10.1016/s0168-1656(99)00168-6

Hoffmann, Frank, and U. Rinas, “Stress induced by recombinant protein production in *Escherichia coli*” *Adv Biochem Eng Biotecnol* 89:73-92, 2004. doi: 10.1007/b93994.

Jeong, Ki Jun, and S. Y. Lee, “Enhanced production of recombinant proteins in Escherichia coli by filamentation suppression.” *Appl Environ Microb* 69(2):pp. 1295– 98, 2003. doi: 10.1128/AEM.69.2.1295-1298.2003.

Kim, Juhyun, A. Darlington, M. Salvador, J. Utrilla, and J. I. Jimenez, “Trade-offs between gene exession, growth and phenotypic diversity in microbial populations”, *Curr Opin Biotechnol* 62:29-37, 2020. doi: 10.1016/j.copbio.2019.08.004.

Kazan, Dilek, A. Çamurdan, and A. Hortaçsu, “The effect of glucose concentration on the growth rate and some intracellular components of a recombinant *E. coli* culture”, *Proc Biochem* 30(3):269-273, 1995. doi: 10.1016/0032-9592(95)85008-2.

Koeller, Kathryn M. and C. H. Wong, “Caomplex carbohydrate synthesis tools for glycobiologists: enzyme-based approach and programmable one-pot strategies”, *Glycobiology* 10(11):1157-69, 2000. doi: 10.1093/glycob/10.11.1157.

Kwiatkowska, Joanna, E. Matuszewska, D. Kuczyńska-Wiśnik, and E. Laskowska, "Aggregation of *Escherichia coli* proteins during stationary phase depends on glucose and oxygen availability". *Res Microbiol* 159(9-10):651-7, 2008. doi:10.1016/j.resmic.2008.09.008.

Lartigue, Marie Frederique, W. Leflon-Guibout , L. Poirel L, P. Nordmann, and M. H. Nicolas-Chanoine, “HPromoters P3, Pa/Pb, P4, and P5 upstream from bla(TEM) genes and their relationship to beta-lactam resistance”

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Downloaded from https://academic.oup.com/jimb/advance-article/doi/10.1093/jimb/kuac018/6601392 by guest on 06 June 2022

Antimicrob Agents Chemother 46(12):4035-7, 2002. doi: 10.1128/AAC.46.12.4035-4037.

Lewis, Gareth, I. W Taylor, A.W. Nienow, and C. J. Hewitt, “The application of multi-parameter flow cytometry to the study of recombinant *Escherichia coli* batch fermentation processes”, *J Ind Microbiol Biotechnol* 31(7):311–322, 2004. doi:10.1186/1475-2859-4-1.

Liu, Min, X. Feng, Y. Ding, G. Zhao, H. Liu, and M. Xian, “Metabolic engineering of *Escherichia coli* to improve recombinant protein production,” *App Microbiol Biotechnol* 99:10367-10377, 2015. doi: 10.1007/s00253-015-6955-9.

Mairhofer, Juergen, T. Scharl, K. Marisch, M. Cserjan-Puschmann, and G. Striedner, “Comparative Transcription Profiling and In-Depth Characterization of Plasmid-Based and Plasmid-Free *Escherichia coli* Expression Systems under Production Conditions,” *Appl Environ Microbiol* 79(12):3802–12, 2013. doi: 10.1128/AEM.00365-13.

Marschall, Lukas, P. Segmeister, and C. Herwig, “Tunable recombiant protein expression in E. coli promoter systems and genetic constraints” *Appl Mircobiol Biotechnol* 101(2):501-512, 2017. doi: 10.1007/s00253-016-8045-z.

Mignon Charlotte, R. Sodoyer, and B. Werle, "Antibiotic-free selection in biotherapeutics: now and forever" *Pathogens* 4(2):157-181, 2015. doi:10.3390/pathogens4020157.

Mukhopadhyay, Asok, “Inclusion bodies and purification of proteins in biologically active forms”, *Adv Biochem Eng Biotechnol* 56:61-109, 1997. doi: 10.1007/BFb0103030.

Neubauer, Peter, H. Y. Lin, and B. Mathiszik, “Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in *Escherichia coli*.” *Biotechnol Bioeng* 83(1):53–64, 2003. doi: 10.1002/bit.10645.

Pasini, Martina, A. Fernández-Castané, A. Jaramillo, C. De Mas, G. Caminal, and P. Ferrer, “Using promoter libraries to reduce metabolic burden due to plasmid-encoded proteins in recombinant *Escherichia coli*” *N Biotechnol* 33(1):78-90, 2016. DOI: 10.1016/j.nbt.2015.08.003.

Phue, Je-Nie and J. Shiloach, “Impact of dissolved oxygen concentration on aceate accumulation and physiology of *E. coli* BL21, evaluating transciption levels of key genes at different dissolved oxygen conditions” *Metab Eng* 7(5-6):353-63, 2005. doi: 10.1016/j.ymben.2005.06.003.

Downloaded from https://academic.oup.com/jimb/advance-article/doi/10.1093/jimb/kuac018/6601392 by guest on 06 June 2022

ORIGINAL UNEDITED MANUSCRIPT

Pierce, John and S. Gutteridge, “Large-scale preparation of ribulosebisphosphate carboxylase from a recombinant system in *Escherichia coli* characterized by extreme plasmid instability” *Appl Env Microbiol* 49(5):1094-1100, 1985. doi:10.1128/aem.49.5.1094-1100.

Pinsach, Jaume, C. de Mas, and J. López-Santín, “Induction strategies in fed-batch cultures for recombinant protein production in *Escherichia coli*: Application to rhamnulose 1-phosphate aldolase,” *Biochem Eng J* 41(2): 181–87, 2008. doi: 10.1016/j.bej.2008.04.013

Plamann, Michael D., and G. V. Stauffer, “Characterisation of the *Escherichia coli* gene for serine hydroxymethyltransferase,” *Gene* 22(1)9–18, 1983. doi: 10.1016/0378-1119(83)90059-8.

Rahmen, Natalie, A. Fulton, N. Ihling, M. Magni, K. Jaeger, and J. Büchs et al. “Exchange of single amino acids at different positions of a recombinant protein affects metabolic burden in *Escherichia coli.*” *Microb Cell Fact* 14:10, 2015. doi: 10.1186/s12934-015-0191-y.

Rosano, German L. and E.A. Ceccarelli, “Recombinant protein expression in *Escherichia coli*: advances and challenges.” *Front Microbiol* 5: 172, 2014. doi: 10.3389/fmicb.2014.00172.

Saubi, Narcís, E. Gea-Mallorquí, P. Ferrer, C. Hurtado, S. Sánchez-Úbeda, Y Eto, J.M. Gatell, T. Hanke, and J. Joseph, "Engineering new mycobacterial vaccine design for HIV-TB pediatric vaccine vectored by lysine auxotroph of BCG". *Mol Ther Methods Clin Dev*, 1:14017, 2014. doi: 10.1038/mtm.2014.17. eCollection 2014.

Schramm, Frederic D, K. Schroeder and K. Jonas, “Protein aggregation in bacteria” *FEMS Microbiol Rev* 44(1):54-72, 2020. doi:10.1093/femsre/fuz026

Sooan, Shin, D. Chang and J. G. Pan, “Acetate consuption activity directly determines the level of acetate accumulation during Escherichia coli W3110 growth” *J Microbiol Biotechnol* 19(10):1127-34, 2029. doi: 10.4014/jmb.0902.0097

Vallejo, Luis Felipe and U. Rinas, “Strategies for the recovery of active proteins through refolding of bacterial inclusio body proteins”, *Microb Cell Fact* 2;3(1):11, 2004. doi: 10.1186/1475-2859-3-11.

Vidal, Luis, O Durany, T. Suau, P. Ferrer, and G. Caminal, “High-level production of recombinant His-tagged rhamnulose 1-phosphate aldolase in *Escherichia coli*” *J Chem Technol Biotechnol* 78(11): 1171–179, 2003.

Vidal, Luis, P. Ferrer, G. Alvaro, M. D. Benaiges and G. Caminal, “Influence of induction and operation mode on recombinant rhamnulose 1-phosphate

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aldolase production by *Escherichia coli* using the T5 promoter" *J. Biotechnol* 118:75-87, 2005.

Vidal, Luis, J. Pinsach, G. Striedner, and P. Ferrer, "Development of an antibiotic-free plasmid selection system based on glycine auxotrophy for recombinant protein overproduction in *Escherichia coli*" *J Biotechnol* 134(1-2):127–36, 2008. doi:10.1016/j.jbiotec.2008.01.011.

Villarejo, M.R. and I. Zabin, "β -galactosidase from termination and deletion mutant strains". *J. Bacteriol*. 120(1): 466-474, 1974. doi:10.1128/jb.120.1.466-474.1974

Waegeman, Hendrik and W. Soetaert, "Increasing recombinant protein production in *Escherichia coli* through metabolic and genetic engineering", *J In. Microbio. Biotechnol* 38(12): 1891-910, 2011. doi: 10.1007/s10295-011-1034-4.

Weber, Jan, F. Hoffmann, and U. Rinas, "Metabolic adaptation of *Escherichia coli* during temperature-induced recombinant protein production: 2. Redirection of metabolic fluxes," *Biotechnol Bioeng* 80(3):320–30, 2002. doi: 10.1002/bit.10380.

Weber, Jan, Z. Li. and U. Rinas, "Recombinant protein production provoked accumulation of ATP, fructose-1,6-bisphosphate and pyruvate in *E. coli* K12 strain TG1", *Microb Cell Fact* 20, 169, 2021. doi:10.1186/s12934-021-01661-9

Wyre, Chris, and T. W. Overton, "Flow cytometric analysis of *E. coli* on agar plates: implications for recombinant protein production." *Biotechnol Lett* 36(7):1485–94, 2014a. doi: 10.1007/s10529-014-1511-8.

Wyre, Chris, and T. W. Overton,"Use of a stress-minimisation paradigm in high cell density fed-batch Escherichia coli fermentations to optimise recombinant protein production". *J Ind Microbiol Biotechnol* 41(9):1391-404, 2014b. doi: 10.1007/s10295-014-1489-1.

Xu, Jia and K. S. Matthews, "Flexibility in the inducer binding region is crucial for allostery in the *Escherichia coli* lactose repressor", *Biochemistry* 48(22):4988–98, 2009. doi: 10.1021/bi9002343.

Zamenhof, Patrice J. and M. Villarejo, "Construction and properties of *Escherichia coli* strains exhibiting α-complementation of β-galactosidase fragments in vivo", *J. Bacteriol* 110: 171, 1972. doi: 10.1128/jb.110.1.171-178.1972

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Figure Caption

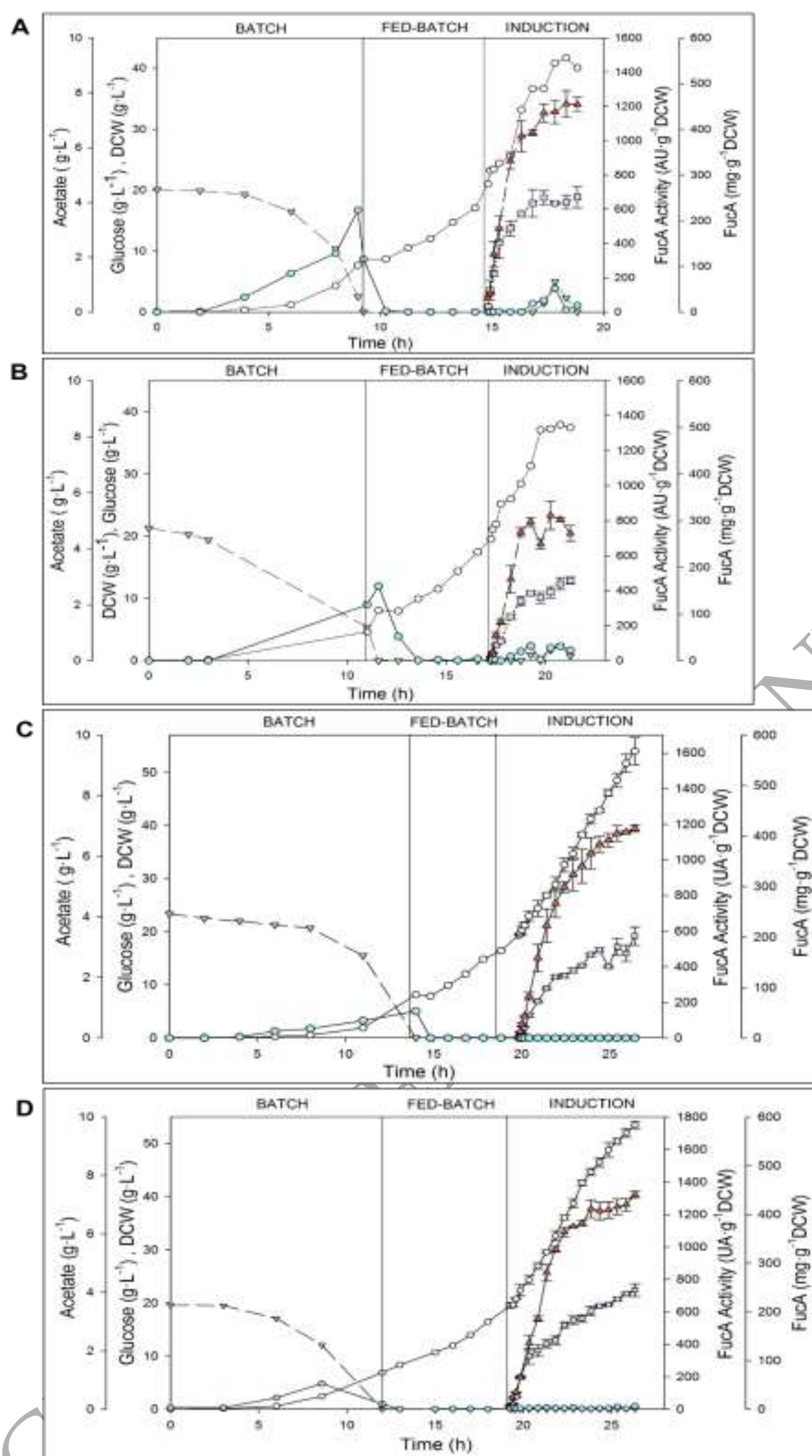

Figure 1 Time profiles of fed-batch cultures of FucA production strains performed under the following conditions: [IPTG], 70 µM; X_{ind}, 20 g·L^{-1}; μ_{fix}, 0.22 h^{-1}. **(A)** Reference strain, M15[pREP4] pQE-FucA; **(B)** 1st generation, M15Δ*glyA*[pREP4]; **(C)** 2nd generation, Puzzle;

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(D) 3rd generation, AmpR-. Key to symbols: (○) Biomass DCW ($g \cdot L^{-1}$); (▽) Glucose concentration ($g \cdot L^{-1}$); (▲) FucA activity ($AU \cdot g^{-1}DCW$); (□) FucA specific mass ($mgFucA \cdot g^{-1}DCW$); and (○) Acetate concentration ($g \cdot L^{-1}$). Batch, fed-batch and induction phases are indicated. Triplicate fermentations were performed and mean ± SD are presented. The arrows indicate the stop of the feeding.

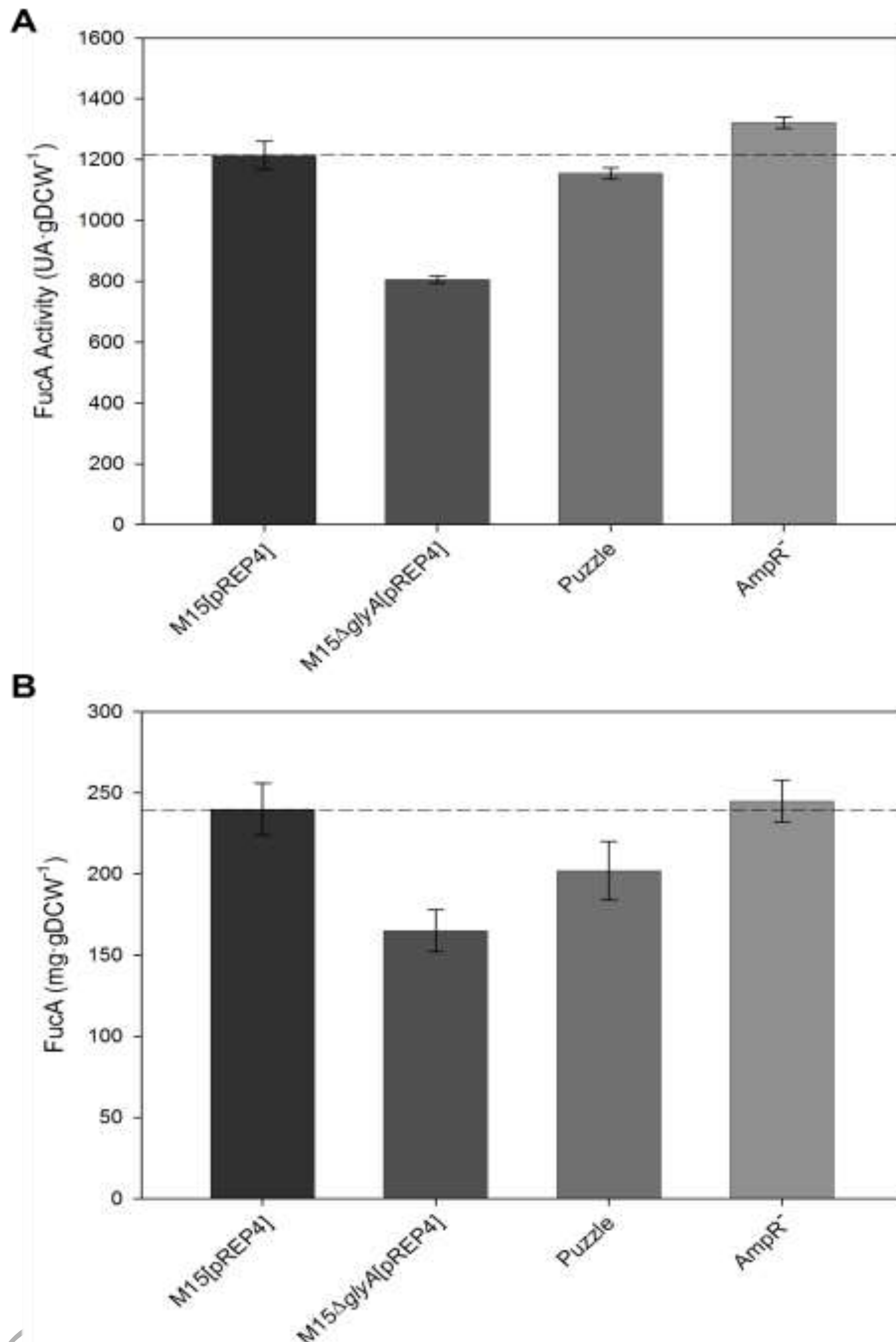

Figure 2 (**A**) Maximum enzyme activity ($AU \cdot g^{-1}DCW$) and (**B**) Maximum specific mass ($mgFucA \cdot g^{-1}DCW$) for the strains presented in this study. The dashed line indicates the reference value corresponding to the reference (M15[pREP4]) strain.

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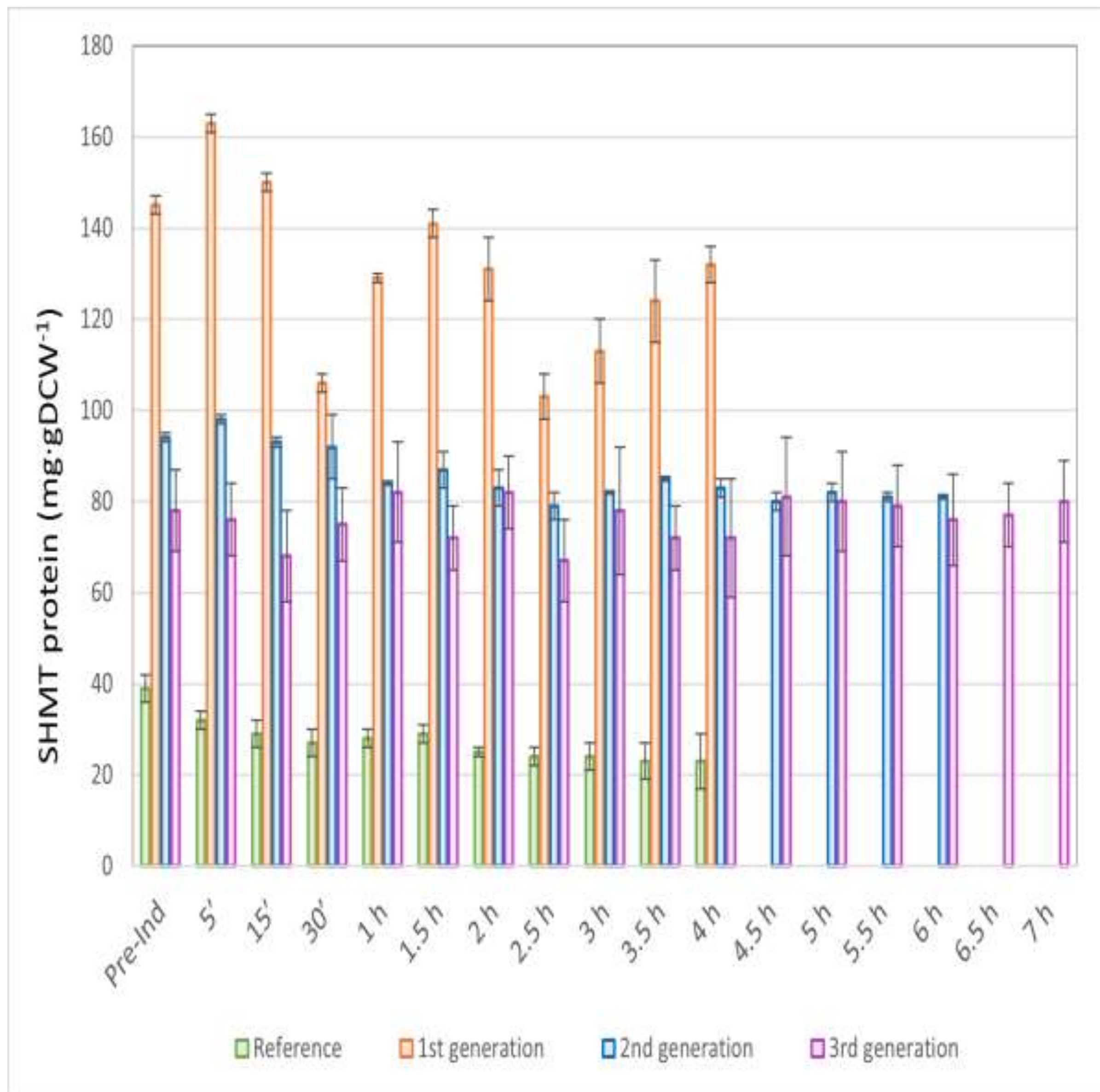

Figure 3 SHMT production (mg·g DCW^{-1}) for the four strains presented along this study during pre-induction (pre-Ind) and induction phase of high-cell-density fed-batch cultures run in triplicate.

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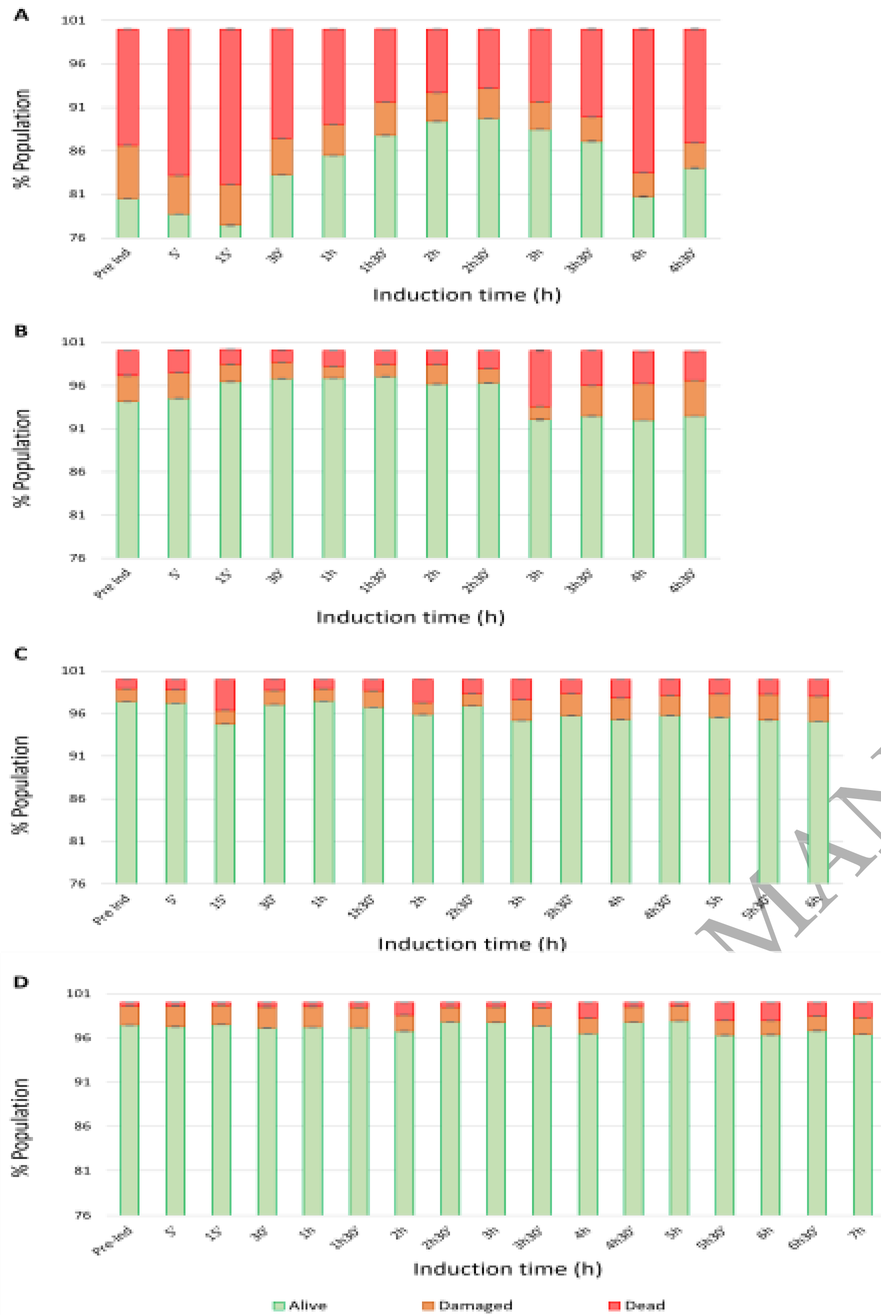

Figure 4 BOX/PI co-staining, indicating the percentage of ( ) Live (PI^-/BOX^-), ( ) Damaged (PI^-/BOX^+) and ( ) Dead (PI^+/BOX^+) cells for the four expression systems presented along this study during pre-induction (pre-Ind) and induction phase (from 5' to 7 h) of duplicate high-cell-density fed-batch cultures using the Fermac 310/60 bioreactor. (**A**) Reference strain; (**B**) 1st generation; (**C**) 2nd generation; (**D**) 3rd generation.

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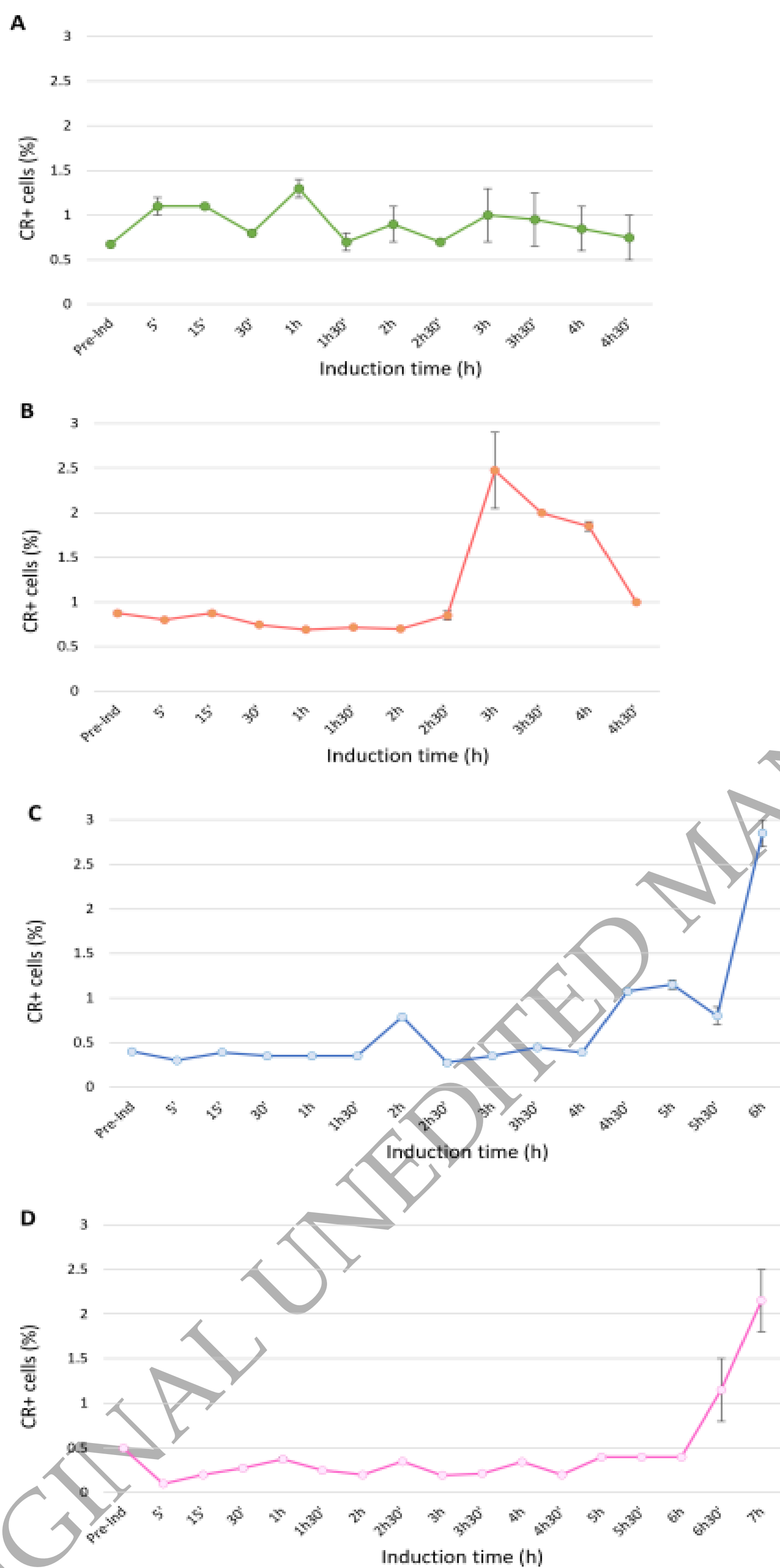

Figure 5 Time profiles of the percentage of Congo red-stained cells as determined by FCM. (**A**) Reference strain; (**B**) 1st generation; (**C**) 2nd generation; (**D**) 3rd generation. Average values and error bars are presented from two independent experiments.

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