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Engineering of *Escherichia coli* strains for plasmid biopharmaceutical production: scale-up challenges.

Geisa A. L. Gonçalves[1,2,3], Kristala L. J. Prather [1,4], Gabriel A. Monteiro[1,2,3], Duarte M. F. Prazeres[1,2,3*]

[1]MIT-Portugal Program

[2]Department of Bioengineering, Instituto Superior Técnico (IST), Lisbon, Portugal

[3]IBB-Institute for Biotechnology and Bioengineering, Center for Biological and Chemical Engineering, IST, Lisbon, Portugal

[4]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

*Corresponding Author

Avenida Rovisco Pais
1049-001 Lisboa
Portugal
Phone: +351 - 218 419 133
Fax: +351 - 218 419 062
miguelprazeres@ist.utl.pt

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Abstract

Plasmid-based vaccines and therapeutics have been making their way into the clinic in the last years. The existence of cost-effective manufacturing processes capable of delivering high amounts of high-quality plasmid DNA (pDNA) is essential to generate enough material for trials and support future commercialization. However, the development of pDNA manufacturing processes is often hampered by difficulties in predicting process scale performance of *Escherichia coli* cultivation on the basis of results obtained at lab scale. This paper reports on the differences observed in pDNA production when using shake flask and bench-scale bioreactor cultivation of *E. coli* strains MG1655*ΔendAΔrecA* and DH5α in complex media with 20 g/L of glucose. MG1655*ΔendAΔrecA* produced 5-fold more pDNA (9.8 mg/g DCW) in bioreactor than in shake flask (1.9 mg/g DCW) and DH5α produced 4-fold more pDNA (8 mg/g DCW) in bioreactor than in shake flask (2 mg/g DCW). Accumulation of acetate was also significant in shake flasks but not in bioreactors, a fact that was attributed to a lack of control of pH.

Introduction

Plasmid-based biopharmaceuticals have been studied extensively in the clinic in the last two decades as a new way to treat inherited or acquired diseases, or to confer protection against infectious diseases. Four plasmid DNA (pDNA) biopharmaceuticals have already been licensed for use in the veterinary field which include prophylactic vaccines to prevent West Nile virus infection in horses [1] and hematopoietic necrosis virus infection in farmed salmon [2], and therapeutic vaccines to treat canine malignant melanoma [3] and reduce fetal loss in swine [4]. In anticipation of future successes and of a growth of the plasmid biopharmaceutical market, a number of researchers in academia and industry have focused their attention on the establishment of efficient and cost-effective manufacturing processes capable of delivering high amounts of high-quality pDNA. Key developments include new delivery methods, fermentation strategies, improved media formulations and genetically engineered vectors and *Escherichia coli* strains [5-8].

E. coli host strains of the K-12 and B type such as DH5, DH5α, DH10B, MG1655, JM108, JM101 and BL21 have all been used for pDNA production [9-12]. Strain background and carbon source choice have been identified as two critical elements to consider when engineering new *E. coli* strains for pDNA production [10]. Nevertheless, the translation of the benefits observed in the laboratory with improved strains to process scale remains a challenging, but crucial task. The majority of the process development studies are conducted firstly in shake flasks and only then in bench-scale bioreactors with advanced monitor control [11]. While this approach is popular, previous studies have already demonstrated that productivity data obtained from shake flask experiments often fail to predict the outcome of pDNA production in bench-scale bioreactors [12]. This study addresses the challenge of scaling-up pDNA

production processes on the basis of shake flask experiments. More specifically, we discuss key factors in the control of fermentation that may affect *E. coli* behavior and the main reasons for the divergence between shake flask and bioreactor data. As model pDNA producers we use MG1655*ΔendAΔrecA*, a strain with a wild-type genetic background and deletions in the *endA* and *recA* genes, and DH5α, a commonly used laboratory strain which is characterized by a highly mutagenized genetic background [10].

Material and Methods

Strains and plasmids

The bacterial strains MG1655*ΔendAΔrecA* (*F⁻ λ⁻ ilvG rfb-50 rph1ΔendAΔrecA*) and DH5α (F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1) were obtained from the Prather Lab, MIT [10] and from Invitrogen, respectively. Strains were transformed with pVAX1GFP, a 3697 bp plasmid derived from pVAX1LacZ (Invitrogen, Carlsbad, CA) as described previously [13].

Shake flask cultures

Transformed strains were maintained on agar plates containing 30 µg/mL of kanamycin. Seed banks and shake flask cultures were prepared as previously described [10] and glucose was used as the primary carbon sources. Samples were withdrawn periodically to quantify biomass, glucose, acetate, and pDNA.

Bioreactor cultures

Batch fermentations were performed in a Fermac 360 Bioreactor (Electrolab), with a working volume of 1.1 L. Pre-inoculum was prepared by transferring cells from seed bank (1% v/v) to 5mL of complex medium [Bacto peptone, 10 g/L; yeast extract, 10 g/L; $(NH_4)_2SO_4$, 3 g/L; K_2HPO_4, 3.5 g/L; KH_2PO_4, 3.5 g/L; thiamine, 199 mg/L; $MgSO_4$, 1.99 g/L; trace element solution, 1 mL/L supplemented with 30 µg/mL of kanamycin. Cells were grown overnight at 37ºC and 250rpm. Next, an inoculum was prepared in 100mL of complex medium with 1% of pre-inoculum culture and grown to early exponential phase (OD_{600} ~1.5) at 37ºC and 250 rpm. 1L of complex medium [10] was autoclaved in the bioreactor and medium supplements and kanamycin 30 (mg/L) were added in the inoculation day. The reactor was inoculated to an initial OD_{600} of 0.1 using the prepared inoculum. The dissolved oxygen set-point was controlled at 30% using a cascade to agitation (250 rpm to 800 rpm) and air was provided at a flow rate of 1 vvm. The pH was controlled at 7.10 using 1 M NaOH and 1 M H_2SO_4. Antifoam was manually added as required. Samples were taken periodically from the bioreactor to quantify biomass, glucose, acetate, and pDNA.

Plasmid DNA quantification

Plasmid DNA was quantified from crude alkaline lysates prepared from cell pellets (OD_{600nm} = 10) using the hydrophobic interaction HPLC method described before by Diogo *et al.* (2003) [14]. A 15 PHE PE HIC column (4.6mm×10 cm) from GE Healthcare was firstly equilibrated with 1.5 M ammonium sulfate in 10 mM Tris, pH 8.0 (1 ml/min). Thirty µL of lysate samples were injected and isocratic elution was performed with the equilibration buffer for 1.4 min and then with 10 mM Tris, pH 8.0 buffer for 0.9 min. At 2.3 min, the column was re-equilibrated with 1.5 M ammonium sulfate. Plasmid concentration was determined from a calibration curve (5–100 µg/ml).

Metabolite quantification

The concentration of glucose and acetic acid were determined by HPLC analysis on a Hitachi LaChrom Elite HPLC system equipped with a Rezex ROA Organic Acid Column and refractive index detector (RID). Culture samples were centrifuged at 10000 rpm for 15 minutes, and supernatant samples were injected in the column. The HPLC method was run at a constant flow rate of 0.5 mL/min with 5 mM H_2SO_4 as the mobile phase, at 65ºC for 20 min.

Results

We previously demonstrated the importance of strain background and carbon source selection in the creation of new *E. coli* strains for pDNA production [10]. On the basis of shake flask experiments, the MG1655*ΔendAΔrecA* strain was identified as a potential high-producer of pDNA when glycerol was used as the primary carbon source [10]. Nevertheless, the same strain did not produce large amounts of pDNA in glucose, especially when the initial concentration was 20 g/L. The MG1655*ΔendAΔrecA* and DH5α strains were also found to produce more pDNA when the initial concentration of glucose was 5 g/L when compared with 10 g/L or 20 g/L [10]. This inhibitory effect of glucose in pDNA production during shake flask cultivation could result from a lack of control of important parameters such as the pH and dissolved oxygen during batch cultivation. We also observed that both strains produced higher amounts of acetate when the initial concentration of glucose was increased from 5 g/L to 20 g/L in shake flask.

In this study we confirmed that MG1655*ΔendAΔrecA* and DH5α grown in shake flasks at 20 g/L of glucose produced small amounts of pDNA (~2 mg/g DCW) and high

amounts of acetate (~1.3-3.0 g/L). However, when both strains were grown in a controlled bioreactor with 20 g/L of initial glucose, the specific pDNA productivity of MG1655*ΔendAΔrecA* and DH5α pDNA increased 5 and 4 fold, respectively (Table 1). The results also show that when the two strains were grown in shake flasks, acetic acid accumulated from hour 12 to hour 24, a situation which could cause the inhibition of biomass and pDNA formation as well as the degradation of pDNA. In bioreactors, by contrast, both strains were able to consume all the acetic acid formed once glucose was depleted (Figure 1). The strain MG1655*ΔendAΔrecA* produced higher amounts of pDNA (~90 mg/L) when compared to DH5α (~75 mg/L). Although the consumption rate of acetic acid is lower and less pDNA is produced by DH5α when compared to MG1655*ΔendAΔrecA* (Figure 1), the specific pDNA productivity obtained with DH5α at bioreactor scale (8.02 mg pDNA /g DCW) is significantly higher when compared with shake flask cultivation (2.08 mg pDNA /g DCW). This demonstrates the importance of control fermentation parameters in pDNA production.

Discussion

Development of pDNA manufacturing processes is often hampered by difficulties in predicting process scale performance of *E. coli* cultivation on the basis of results obtained with shake-flasks at lab scale [15-18]. Experience shows that a particular strain which produces low amounts of pDNA in flask experiments can turn out to be a high-yield pDNA producer at full scale production [12]. According to literature data, volumetric pDNA titers obtained in shake-flask and small-scale fermentations seldom exceed the 150-250 mg/L [10,11,23], whereas titers of the order of 1000-2600 mg/L have been reported in high-yield, preindustrial fermentations [6, 12,16-18]. For example, Carnes *et al.* have pushed pDNA productivity limits of a DH5α

strain up to 2600 mg/L by using a combination of optimized media, reduced temperature, nutrient limited growth during biomass accumulation and a slow linear temperature ramp induction [6]. Attention is thus necessary so that potential high-producer pDNA strains are not excluded on the basis of data obtained from experiments conducted in shake flasks or smaller tubes without control of specific process variables. While O'Kennedy *et al.* [15] have previously demonstrated that similar pDNA yields are obtained at mid exponential phase when using the same medium in shake flasks and bioreactor, plasmid degradation occurred at the end of the fermentation in shake flasks. Once again this could be associated with a lack of control of important parameters in shake flasks, such as pH.

The production experiments reported here highlight the scalability issues referred above, indicating clearly that pDNA productivity obtained when growing *E. coli* strains on glucose in a controlled bioreactor is several fold higher when compared with shake flask productivity (Table 1). The differences can be attributed to a lack of control of specific variables like pH and dissolved oxygen. According to the data obtained in this study (Table 1), acetate formation is likely to play an important role in the inhibition of pDNA production at small scale (shake flask), when pH control is absent. A previous study has demonstrated that the protonated form of acetate is able to cross the cell membrane and to uncouple the proton motive force at pH values below the pKa of the acid. Moreover, acetate can still be toxic and inhibit biomass formation at neutral pH when high amounts of the acid are accumulated [19]. This phenomenon is also related to the capacity of each cell to metabolize the acetate, since most of the *E. coli* cultures reutilize acetate when glucose has been consumed. Thus, although the effect of acetate in large scale pDNA production has been reported as minimal [6], choosing a cultivation method and *E. coli* strain that minimize acetate production could

be advantageous considering the total mass balance of carbon. The lack of control of dissolved oxygen in shake flasks can also explain the poor performance observed in shake flasks. Obviously, oxygen transfer rate should be controlled during pDNA production in order to maximize biomass formation [20] and volumetric productivity of pDNA. The use of baffled shake flasks could improve oxygen transfer rate of the cell culture and hopefully contribute to a production performance that is closer to the one observed in bioreactors. In addition, recent studies have shown the importance of an optimal growth rate to increase pDNA yields [21].

While data obtained from laboratory scale bioreactor experiments is clearly more reliable from a process point of view, screening process conditions, strains and media composition on the basis of bioreactor operation is time-consuming, labor intensive and expensive. In the future, development of pDNA production processes could be streamlined by using micro-devices with tight control of important parameters such as pH, oxygen and feeding strategy. Recent results demonstrate that data obtained with such devices can predict bench-scale bioreactor pDNA production successfully [22]. Nevertheless, improving methodologies and platforms more commonly used for pDNA production at small scale (e.g. shake flask cultivation, micro-well plates) would be of high importance to speed up process development in a short term [10,23].

Conclusion

The development of plasmid production processes relies heavily on shake flask experiments as a means to evaluate the performance of new strains, vectors and cultivation media. However, productivity data obtained from shake flask experiments often fails to predict the outcome of pDNA production in bench-scale bioreactors, as demonstrated in this work. Significant differences were observed between shake flask

and bench-scale bioreactor cultivation of *E. coli* strains MG1655*ΔendAΔrecA* and DH5α during plasmid DNA production. The accumulation of acetate and reduced pDNA and biomass production seen during shake flask cultivation are attributable to the lack of control of pH and dissolved oxygen. The results suggest that manual control of pH could be adopted as a fast and easy solution to improve pDNA production in shake flasks, especially when the system produces high amounts of acetate. Other alternatives include the choice of a carbon source that minimizes acetate production, such as glycerol or the adoption of a strain that reduces acetate production, such as GALG20 (a *pgi*- strain). In this situation the results obtained in shake-flasks are more likely to predict the performance of cell production at process scale. While data generated with lab-scale bioreactors is more reliable, this approach is time-consuming, labor intensive and expensive. Clearly, new platforms (e.g. controlled micro-devices) and approaches are needed to speed up and improve process development of plasmids and other biopharmaceuticals.

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Figure legends

Figure 1. pDNA production, biomass formation, glucose consumption and acetate accumulation, during batch fermentation of MG1655*ΔendAΔrecA* (A) and DH5α (B).

Table1. Comparison of batch production of plasmid DNA in shake flask and bench-scale bioreactor. Experiments were conducted in rich media with 20 g/L of glucose. Average value ± standard error of mean (SEM) is shown.

Strain	Scale	pDNA (mg/g DCW)		Biomass (g/L)		Acetate (g/L)	
		12h	24h	12h	24h	12h	24h
MG1655*ΔendAΔrecA*	Shake flask	2.85 ± 0.87	1.93 ± 0.34	3.56 ± 0.40	4.12 ± 0.17	1.50 ± 1.51	3.04 ± 1.66
	Bioreactor	6.47 ± 1.29	9.74 ± 1.76	6.76 ± 1.24	11.75 ± 2.47	6.12 ± 0.24	0
DH5α	Shake flask	2.41 ± 0.56	2.08 ± 0.27	3.48 ± 0.21	3.81 ± 0.15	1.31 ± 0.57	2.13 ± 1.42
	Bioreactor	4.20 ± 1.36	8.02 ± 0.86	6.49 ± 0.98	10.25 ± 1.76	6.79 ± 1.03	3.91 ± 0.64