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Sciences

Strategies of plasmid DNA production and their influence on therapeutic applications

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"Always remember that striving and struggle precede success, even in the dictionary."

Sarah Ban Breathnach

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Resumo alargado

A terapia génica e as vacinas de DNA são abordagens promissoras para a prevenção e possível cura de doenças como cancro, infeções virais, como HIV e hepatite, doenças cardiovasculares, entre outras. Os constantes progressos no aumento da eficácia na transfecção de moléculas de DNA plasmídico (pDNA), faz com que este vetor não viral seja visto cada vez mais como um vetor viável para uso em terapia génica ou vacinação com DNA. Assim sendo, torna-se relevante a obtenção de elevadas quantidades de DNA plasmídico com elevado grau de pureza para ser posteriormente usado em aplicações terapêuticas. Para atingir este objetivo, é necessária a combinação entre a otimização das condições de crescimento para a produção de DNA plasmídico e a eficiência do processo de purificação. Apesar de, até à data, grande parte da pesquisa se ter focado no desenvolvimento do processo de purificação; o passo limitante do processo global continua a ser a produção de pDNA, que quando otimizada permite melhorar os processos de purificação subsequentes pela redução da fração de contaminantes (DNA genómico, RNA, proteínas e endotoxinas). A *Food and Drug Administration* (FDA) reconhece que as conformações circular aberta e linear são, terapeuticamente, menos eficazes que a conformação superenrolada. Assim, torna-se importante que, nos processos de purificação, se efetue a separação entre as outras isoformas e o plasmídeo superenrolado e, uma vez que esta operação pode ser bastante difícil durante a purificação, o processo de fermentação deve ser otimizado de modo a produzir uma elevada percentagem de plasmídeo na conformação superenrolada.

O design e otimização de um processo de produção engloba múltiplas etapas de seleção tendo em vista a escolha do bioprocesso com o maior rendimento em termos de DNA plasmídico. A primeira etapa neste design é a escolha da estirpe hospedeira e do vetor a utilizar para a produção do plasmídeo contendo a sequência terapêutica de interesse. Apesar de já terem sido propostos outros microrganismos para produção recombinante de biomoléculas, a bactéria *Escherichia coli* continua a ser a mais utilizada para este fim. Atualmente, devido à constante evolução da biologia molecular e engenharia genética, encontra-se disponível uma grande variedade de estirpes recombinantes de *E. coli* e de vetores otimizados para a produção de DNA plasmídico. Para a produção de pDNA, as estirpes de *E. coli* usadas possuem, geralmente, mutações em genes de recombinação e de produção de endonucleases, proporcionando uma maior estabilidade do vetor. Em relação aos vetores, têm sido propostas novas estratégias para uma produção mais eficiente e para um aumento da segurança destes vetores, reduzindo os elementos procariotas e melhorando o controlo da integração da sequência terapêutica no genoma do hospedeiro. De entre estas novas estratégias destacam-se os plasmídeos minicirculares, os plasmídeos de tamanho reduzido (MIDGE) e ainda a introdução do transposão "Sleeping Beauty". Relativamente à produção de pDNA em *Escherichia coli*, existem dois fatores que podem influenciar consideravelmente o processo de

produção: a estabilidade plasmídica e o stress metabólico imposto à estirpe hospedeira pela manutenção e replicação do plasmídeo. Estes dois fatores podem levar a uma diminuição da produtividade do processo devido ao aumento da instabilidade do plasmídeo a vários níveis (segregacional, estrutural e relativamente às isoformas) e a uma redução do crescimento da estirpe hospedeira devido a uma limitação da capacidade biossintética e produção de energia das células.

Após uma seleção preliminar do sistema de expressão, tendo em conta todos os fatores já descritos, o passo seguinte consiste na formulação de um meio de cultura contendo os componentes apropriados, não só para o crescimento, como também para a produção de DNA plasmídico. Estes componentes incluem uma fonte de carbono, uma ou várias fonte(s) de azoto, um sistema tamponante, geralmente contendo fosfatos e vários elementos vestigiais (cálcio, ferro, molibdénio, entre outros). No caso particular da produção de pDNA, o meio de cultura também deve conter sulfato de magnésio, numa concentração de aproximadamente 80 mM, uma vez que este parece causar um aumento da produção da isoforma superenrolada. Após definição do meio de cultura, o próximo passo é a definição das condições de cultura, como pH, teor de oxigénio dissolvido e temperatura. A definição das condições de cultura não deve ser efetuada apenas tendo em conta a obtenção de elevadas quantidades de biomassa, mas também a produção de DNA plasmídico. Por exemplo, está descrito que temperaturas mais elevadas (37-42 ºC) e teores de oxigénio dissolvido mais baixos causam um aumento da produção de pDNA; contudo, estas mesmas condições também são responsáveis por uma diminuição do crescimento de *E. coli*, levando à produção de uma menor quantidade de biomassa.

A otimização das condições de cultura, por si só, leva, geralmente, a um aumento da produção de pDNA. Todavia, têm sido propostas várias estratégias de indução de DNA plasmídico que poderão conduzir a maiores produtividades, uma vez que estas causam a amplificação seletiva de pDNA, aumentando o número de cópias de plasmídeo por célula e, por conseguinte, levam a um aumento da produção de pDNA com uma concomitante redução da quantidade de contaminantes para os processos subsequentes de purificação. Estas estratégias de indução incluem a adição de cloranfenicol e adenosina monofosfato (AMP), limitação de aminoácidos e aumento de temperatura. Após seleção de todos estes parâmetros, a transição para bioreactor torna necessária a escolha da estratégia de fermentação apropriada. De entre as estratégias de fermentação descritas para a produção de pDNA, nomeadamente "batch", "fed-batch" e contínuo; a mais utilizada e descrita como aquela que possibilita uma maior produtividade de pDNA é a fermentação em modo "fed-batch". A fermentação em "fed-batch" é a mais utilizada para a produção de pDNA uma vez que possibilita a obtenção de elevadas densidades óticas e baixa acumulação de produtos tóxicos resultantes do metabolismo como o acetato, por exemplo.

Uma vez que há diversos fatores que podem influenciar um bioprocesso para produção de DNA plasmídico, torna-se necessário desenvolver novas metodologias e técnicas que forneçam uma análise do processo num espaço de tempo que permita ao investigador alterar o seu processo a fim de atingir uma maior produtividade. Tendo em consideração que o stress metabólico imposto pelo plasmídeo bem como a instabilidade plasmídica são potenciais fatores limitantes para um processo de produção de pDNA, o desenvolvimento de novas técnicas de monitorização, bem como a aplicação de técnicas já existentes, deve ter em conta a análise destes dois fatores. Estes métodos de monitorização, para além de serem utilizados para uma melhor compreensão da performance dos bioprocessos, também permitem a identificação de possíveis alvos para engenharia metabólica das estirpes e vetores a fim de reduzir o stress metabólico e a instabilidade plasmídica, conduzindo à obtenção de maiores produtividades, reduzindo assim o custo global do processo.

Assim sendo, o principal objetivo desta tese foi a otimização da produção de DNA plasmídico em *Escherichia coli*, numa primeira fase em erlenmeyer e, posteriormente, em bioreactores de bancada. Primeiramente foi estudada a influência das condições de cultura na produção de pDNA, utilizando um meio de cultura semi-definido previamente desenvolvido pelo nosso grupo. Seguidamente, foram desenvolvidas novas metodologias para a análise da fisiologia celular do hospedeiro e estabilidade segregacional do plasmídeo, uma vez que o primeiro estudo evidenciou que as condições de cultura poderiam estar a influenciar o stress metabólico da célula e a estabilidade do plasmídeo. Após o desenvolvimento destas metodologias de citometria de fluxo e PCR em tempo real, a análise da fisiologia celular e estabilidade plasmídica foi aplicada a um estudo comparativo entre algumas das diferentes estratégias de indução de pDNA atualmente descritas. O objetivo final do trabalho consistiu na otimização da produção em bioreactor, estudando a influência de diferentes estratégias de fermentação na produção de pDNA, monitorizando a estabilidade plasmídica e a fisiologia celular ao longo do processo.

No design deste bioprocesso para produção de pDNA, a primeira etapa do estudo consistiu, então, no desenvolvimento de um meio semi-definido contendo glicerol como fonte de carbono e triptona como fonte de azoto. Após o desenvolvimento do meio semi-definido, o nosso primeiro objetivo baseou-se no estudo da influência das condições de cultura, como a temperatura e a concentração de triptona, sobre o rendimento global de pDNA em erlenmeyer.

Para os estudos de temperatura, as células foram cultivadas em diferentes temperaturas de crescimento: 30, 32, 37, 40 e 42 ºC. Este estudo revelou que as temperaturas mais elevadas causaram filamentação das células de *E. coli*. A análise de outros parâmetros relacionados com o crescimento celular, como a taxa específica de crescimento e a produção de biomassa, juntamente com a análise da produção de pDNA, revelaram baixas taxas de crescimento e uma menor produção de biomassa para as temperaturas mais elevadas. Estes resultados

levantaram a hipótese de que a ocorrência de filamentação celular poderia ser devida a um aumento do stress metabólico imposto pela manutenção e replicação do plasmídeo na célula hospedeira. A obtenção de maiores rendimentos específicos de pDNA e graus de pureza para as temperaturas mais elevadas, facto este que pode ser interpretado como sinónimo da existência de um maior número de cópias do plasmídeo por célula hospedeira, pode ser vista como uma das causas para este aumento do stress metabólico da célula.

Uma vez que tinha sido descrito que condições de limitação de aminoácidos causavam amplificação de pDNA em *E. coli*, o próximo passo consistiu no estudo da influência de concentrações limitantes (0.5, 1 e 3 g/L) e não limitantes (5 e 20 g/L) de triptona na produção de pDNA. Os resultados obtidos demonstraram que o uso de concentrações reduzidas de triptona levava a um aumento da produção específica de pDNA, demonstrando que a limitação de aminoácidos leva a uma amplificação de pDNA, o que está relacionado com o facto de a estirpe utilizada ser uma estirpe *relA*$^-$.

Devido aos resultados obtidos neste primeiro estudo, havia evidências de que a fisiologia celular seria um fator importante a ter em conta no desenvolvimento e otimização deste processo de produção de pDNA. Assim sendo, a próxima etapa deste trabalho consistiu no desenvolvimento e implementação de metodologias que permitissem uma avaliação da fisiologia celular tendo por base a citometria de fluxo. A escolha da citometria de fluxo como técnica de monitorização deveu-se, sobretudo, ao facto de possibilitar a análise de cada célula da população individualmente e fornecer uma elevada quantidade de dados de forma rápida permitindo, assim, uma análise em linha dos bioprocessos. Uma vez que a análise do DNA bacteriano é considerada uma mais-valia para o controlo de bioprocessos e como ainda não se encontram descritos muitos protocolos para esta análise aplicada a bioprocessos; de seguida, procedeu-se ao desenvolvimento e validação de um protocolo de citometria de fluxo para a monitorização do ciclo celular durante os processos de fermentação de *Escherichia coli*. Este protocolo foi desenvolvido utilizando o fluorocromo DRAQ5, uma vez que, devido às suas características espetrais, não necessita de um citómetro com laser ultra-violeta (UV) e permite ser conjugado com outros fluorocromos.

Apesar de os resultados obtidos demonstrarem que o DRAQ5 era capaz de marcar células de *E. coli* vivas, devido aos baixos valores de fluorescência obtidos e ao facto de não ser possível o estudo do ciclo celular nestas condições, investigou-se a capacidade de análise do ciclo celular em células de *E. coli* fixadas com etanol ou glutaraldeído. Depois de determinar as condições de incubação adequadas, concentração de fluorocromo e agente fixante (etanol), as células de *Escherichia coli* não contendo plasmídeo foram cultivadas em meio semi-definido, a fim de avaliar a aplicabilidade do DRAQ5 para monitorizar o ciclo celular em fermentações bacterianas. Os resultados obtidos demonstraram que esta estirpe de *E. coli*, nas condições referidas, tem um ciclo celular em que alterna entre um e dois cromossomas e,

também, o aumento da população de células com apenas um cromossoma na fase final da fermentação está de acordo com os modelos propostos para o crescimento bacteriano.

Depois de desenvolver esta nova técnica para a monitorização de bioprocessos, foram desenvolvidas outras técnicas para o estudo da fisiologia celular e estabilidade do plasmídeo durante a fermentação, uma vez que estes dois fatores estão intimamente relacionados. A análise da fisiologia celular, para além da análise do ciclo celular, englobou ainda a análise da viabilidade celular através de uma dupla marcação com iodeto de propídeo e bis-(1,3-ácido dibutilbarbitúrico)trimetina oxonol (BOX), que permitiu a análise de três populações distintas: células saudáveis (sem marcação), células com membrana despolarizada (marcação com bis-oxonol) e células com membrana permeabilizada (marcação com iodeto de propídeo). Para a análise da estabilidade segregacional do plasmídeo foi desenvolvimento um método de PCR em tempo real que permitiu a avaliação do número de cópias de plasmídeo (PCN) por célula no decorrer da fermentação, usando um quantificação absoluta efetuada em células de *E. coli* previamente permeabilizadas.

Após a implementação destas técnicas, foi estudado o impacto de diferentes estratégias de indução de DNA plasmídico na performance do bioprocesso. Devido às características da estirpe hospedeira e do plasmídeo utilizados para a otimização da produção, foi possível o estudo comparativo entre diversas estratégias de indução de pDNA como a adição de adenosina monofostafato (AMP), limitação de aminoácidos com ou sem adição de AMP, bem como a indução pela temperatura. Devido às alterações morfológicas previamente observadas pelo nosso grupo, um dos parâmetros avaliado foi a morfologia celular através da medição da dispersão de luz por citometria de fluxo. Esta análise permitiu, mais uma vez, verificar a existência de filamentação celular que terá ocorrido devido à amplificação de pDNA causada pelas diferentes estratégias de indução utilizadas. A análise da viabilidade celular por citometria de fluxo permitiu concluir que, no final da fermentação, a grande maioria das células ainda possuía membranas citoplasmáticas intactas e polarizadas, indicando que as condições utilizadas não terão causado nas células um elevado stress metabólico. Foi ainda estudado o ciclo celular durante a fermentação tendo em vista a obtenção de informações mais detalhadas sobre a heterogeneidade da população bacteriana e possíveis alterações fisiológicas causadas pela amplificação de pDNA. Esta análise da distribuição de DNA permitiu observar que, durante a fermentação, ocorreu um alargamento dos picos dos histogramas de DNA devido ao aumento da heterogeneidade da população, possivelmente como resultado da amplificação de pDNA verificada. Os resultados obtidos para a determinação de PCN demonstraram que as estratégias de indução tendo por base a limitação de aminoácidos permitiram a obtenção de valores de PCN mais elevados no final das fermentações, comparativamente à indução pelo aumento da temperatura. Os elevados valores de PCN obtidos no final das fermentações parecem sugerir que é na fase estacionária do crescimento que se verificam os valores máximos de amplificação do plasmídeo.

Quanto à produção de DNA plasmídico, as produções específicas mais elevadas, expressas em mg pDNA por g de peso seco de células, foram obtidas nas fermentações em que foi utilizada a limitação de aminoácidos conjuntamente com a adição de AMP. A análise por eletroforese de agarose dos lisados alcalinos resultantes das fermentações confirmou, ainda, a integridade do pDNA, que se encontrava maioritariamente na conformação superenrolada, com pequenas quantidades da isoforma circular aberta e sem bandas visíveis de RNA.

A última etapa deste trabalho consistiu no estudo da estratégia de fermentação mais adequada em bioreactor, a fim de aumentar a produtividade do processo. Para o efeito foram testadas fermentações em "batch" e "fed-batch", variando as concentrações das fontes de carbono e azoto em "batch" e utilizando diferentes perfis de alimentação em "fed-batch". Nas fermentações em "fed-batch", o meio de alimentação foi adicionado segundo perfis pré-determinados exponenciais e constantes para obtenção de diferentes taxas de crescimento, sempre inferiores à taxa específica de crescimento máxima. Para além do estudo da influência das estratégias de fermentação na produção de pDNA, também foi estudado o impacto destas na viabilidade celular e na estabilidade segregacional do pDNA utilizando os métodos de monitorização já descritos anteriormente.

Os resultados obtidos demonstraram que as produções de pDNA e biomassa mais elevadas foram obtidas nas fermentações em "fed-batch", tendo os melhores resultados, em termos de biomassa e produção de pDNA, sido obtidos com uma alimentação exponencial com uma taxa de crescimento pré-determinada de 0.2 h^{-1}. Em termos de estabilidade plasmídica, as fermentações em "batch" demonstraram menor instabilidade durante as fermentações, com um aumento do número de cópias de plasmídeo no início da fase estacionária das fermentações; enquanto as fermentações em "fed-batch" mostraram uma maior instabilidade segregacional, devido às constantes flutuações nos valores de PCN no decorrer das fermentações. Em relação à análise da fisiologia celular, também se verificou que a percentagem de células viáveis era mais elevada nas fermentações em "batch" e com uma menor heterogeneidade em termos de ciclo celular. Possivelmente, o prolongado tempo de fermentação, mesmo com a adição constante de nutrientes fez com que a percentagem de células viáveis em "fed-batch" fosse menor. Os resultados obtidos nestas experiências demonstraram que, no desenvolvimento de um bioprocesso de produção de pDNA, as decisões para a escolha do melhor processo não devem ser apenas baseadas na produção de pDNA, mas devem ter em conta outros fatores que possam levar a uma diminuição dos valores de produtividade, como o aumento do stress metabólico e da instabilidade plasmídica. A avaliação destes fatores permite aos investigadores uma visão mais detalhada sobre o bioprocesso em si, de forma a compreender e minimizar os efeitos nocivos destes dois fatores na produção e assim alcançar maiores rendimentos e reduzindo o custo do produto.

Em suma, nesta tese pretendeu-se otimizar e avaliar de uma forma sistemática a produção do plasmídeo pVAX1-LacZ em *Escherichia coli* DH5 alfa, considerando que a fisiologia celular e a

estabilidade plasmídica são dois fatores fulcrais a considerar para o desenvolvimento deste tipo de bioprocessos. Para o efeito foram desenvolvidas várias metodologias que permitiram a obtenção de um conhecimento mais alargado sobre o bioprocesso em si e sobre a melhor forma de atuar para aumentar a sua performance.

Palavras-chave

DNA plasmídico, *Escherichia coli*, fisiologia celular, estabilidade plasmídica, indução, estratégias de fermentação

Abstract

Current developments in gene therapy and DNA vaccination, plasmid DNA vectors are becoming increasingly appealing as therapeutics towards a large number of diseases such as cancer, infectious and cardiovascular diseases. This popularity is creating the demand for high quantities of highly purified plasmid DNA which in turn requires the design of high pDNA yield bioprocesses. However, opposed to recombinant protein production, research on pDNA production is still needed, in order to have a clear comprehension of all the challenges and bottlenecks faced during the production of plasmid DNA (pDNA).

The design of a plasmid DNA production process usually begins with the choice of a suitable culture medium to cultivate the expression system containing the therapeutic plasmid. After defining all medium components, the influence of culture conditions, such as pH, temperature and dissolved oxygen, on biomass and plasmid yields is generally studied. Since the appropriate conditions for maximizing biomass production and plasmid replication are not usually the same, a compromise solution is usually considered in these cases. When designing a large scale plasmid DNA production process, the employment of a correct fermentation strategy is also necessary in order to improve yields while reducing production costs.

So far, reports on plasmid DNA production in *E. coli* are focused essentially on the influence of different medium composition, fermentation conditions and feeding strategies on overall biomass and pDNA mass and volumetric titres. Nevertheless, due to the complex nature of microbial growth and the application of several modes of operation such as batch, fed-batch and continuous cultivations, the constant monitoring and control of pDNA bioprocesses represents an engineering challenge that should not be disregarded. As a highest yield process may not correspond necessarily to the best fermentation design, the improvement in off-line, at-line and online monitoring techniques should be seen as a crucial task in the design of the fermentation. Two of the most relevant factors for fermentation performance are the existence of host cell metabolic stress and plasmid instability; hence, the characterization of cell physiology and plasmid segregational stability has to be considered and monitored during the process.

With this thesis, we attempted to improve plasmid DNA yields while gaining new insights on plasmid DNA fermentation processes through the use of novel monitoring techniques such as flow cytometry and real-time quantitative PCR.

We started this work studying the influence of growth temperature and tryptone concentration on plasmid DNA production in a previously developed semi-defined medium. The analysis of pDNA yields and *E. coli* morphology revealed that higher pDNA specific yields

were obtained at higher temperatures (37 and 40 ºC). Also, at these temperatures, *E. coli* filamentation was observed, possibly indicating a higher metabolic stress due to higher plasmid replication and higher culture temperatures. When analyzing the influence of tryptone concentration on plasmid yield, the best results were achieved with the lowest tryptone concentration used. The use of limiting tryptone concentrations at a temperature of 37 ºC was shown to be a powerful tool to promote plasmid amplification, keeping the desirable plasmid structure (supercoiled isoform); thus favouring the attainment of product quality. Our results suggest that by using tryptone alone as an amino acid source, pDNA amplification was improved, proving that this strategy is able to increase pDNA yield even at small scale.

Since this first study revealed some evidence of *E. coli* metabolic stress during cultivation, the next task consisted in the development of new flow cytometric methodologies that allowed single cell physiology monitoring during cultivation for a better understanding of cultivation conditions influence in the host metabolic activity. Because one of the parameters that enable a better characterization of cell metabolic cell as well as population heterogeneity is cell cycle progression, in the second part of the work, we developed a flow cytometric method to evaluate cell cycle progression in *E. coli* cultivation using a newly developed far-red dye, DRAQ5. In this study we demonstrated that the use of DRAQ5 as a DNA-specific labelling stain provided an easy assessment of intracellular DNA content and cell-cycle phases in the Gram-negative bacteria *E. coli*.

Besides the previously reported method for cell cycle analysis, another method for assessing cell viability was implemented using flow cytometry. In this method, a propidium iodide/bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) dual staining was used to distinguish between three different populations: healthy cells (no staining), cells with depolarized membranes (stained with BOX) and cells with permeabilized membranes (stained with propidium iodide and BOX). In order to evaluate plasmid copy number (PCN) throughout the fermentation, a real-time quantitative PCR method was developed for absolute plasmid copy number quantification in whole *E. coli* cells. After developing and implementing these methods, we evaluated the impact of several plasmid DNA induction strategies, such as amino acid limitation, AMP addition and temperature up-shift, on cell physiology and plasmid segregational stability. This study showed that all induction strategies caused cell filamentation, due to an increase in forward scatter values, and decreased viability at the end of fermentation, as was seen by an increase in the percentage of depolarized and permeabilized cells. The results also suggest that an amino acid limitation with AMP addition induction strategy resulted in the highest specific yields and, concomitantly, highest PCN values. In conclusion, amino acid limitation-based amplification strategies seemed to be suitable approaches to be implemented at a large scale level since they do not require any

additional energy and also had proved to be efficient in plasmid amplification, without causing any detrimental effects in plasmid stability and cellular viability.

The last step of this work aimed at improving plasmid DNA yield through the study of different batch and fed-batch fermentations in bioreactors. Also, the influence of different glycerol and tryptone concentrations and different non-feedback feeding profiles, namely exponential and constant feed rates, on cell physiology and plasmid stability was evaluated by means of flow cytometry and real-time qPCR, respectively; investigating the potential of these two techniques as valuable tools for bioprocess monitoring and design. The results showed that all fermentation strategies caused a slight decrease of cell viability at the end of fermentation, being this decrease more pronounced in fed-batch fermentations than in batch fermentations. The time-course assessment of plasmid copy number revealed that PCN values suffered an increase at the end of batch fermentations, which is in agreement with our previous results obtained in batch fermentations performed in shake flasks. However, in fed-batch fermentations, there were pronounced fluctuations in PCN values throughout the fermentations, indicating some plasmid segregational instability. As supposed, fed-batch fermentations with exponential or constant feeding profiles yielded higher biomass and plasmid DNA than batch fermentations with the highest biomass and plasmid yields being obtained with a fed-batch strategy with an exponential feed rate of 0.2 h^{-1}. Notwithstanding the high biomass (95.64 OD600) and plasmid yields (344.30 mg pDNA/L) obtained, this fermentation also exhibited higher plasmid instability and lower percentage of viable cells. This work showed that the fermentation strategy used, not only influences product yield, but also cell physiology and pDNA segregational stability. Furthermore, the new findings described herein draw attention towards the relevance of monitoring bioprocess performance and not just overall biomass and product yields.

In conclusion, in this thesis we evaluated and improved pVAX1-LacZ plasmid production in *Escherichia coli* DH5 alpha taking into account not only the overall biomass and plasmid yields, but also considering that cell physiology and plasmid segregational stability, that are two pivotal features to the design and development of these production bioprocesses. In order to study these two factors, several techniques were implemented and were later used to evaluate the influence of several fermentation parameters such as induction strategies and fermentation strategies on overall process performance.

Keywords

Plasmid DNA, *Escherichia coli*, cell physiology, plasmid stability, induction, fermentation strategies

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List of Abbreviations

2D	Two-dimensional
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APEX	Absolute protein expression
ATP	Adenosine-5'-triphosphate
BOX	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol
CFU	Colony forming units
CGA	Capillary gel electrophoresis
CMV	Cytomegalovirus
CpG	Cytosine-Guanine oligodeoxynucleotides
Ct	Cycle threshold
CV	Coefficient of variation
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved oxygen concentration
DRAQ5	Deep red-fluorescing bisalkylaminoanthraquinone number five
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FCM	Flow cytometry
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gly	Glycerol
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
IS	Insertion sequence
LB	Luria Bertani
LPS	Lipopolysaccharides
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
MFA	Metabolic flux analysis

MIDGE	Minimalistic immunogenic defined expression
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
oc	Open circular pDNA
OD	Optical density
OD 600	Cell density
P^- cells	Plasmid-free cells
P^+ cells	Plasmid-bearing cells
PBS	Phosphate buffered saline
PCN	Plasmid copy number
PCR	Polimerase chain reaction
pDNA	Plasmid deoxyribonucleic acid
PI	propidium iodide
ppGpp	Guanosine-5'-diphosphate-3'-diphosphate, guanosine tetraphosphate
pppGpp	Guanosine 5'-triphosphate-3'-diphosphate
qPCR	Quantitative polimerase chain reaction
RNA	Ribonucleic acid
Rnase A	Ribonuclease A
rRNA	Ribosomal ribonucleic acid
SBTS	Sleeping Beauty transposon system
sc	Supercoiled pDNA
SSC	Side scatter
TAAs	Tumour associated antigens
TCA cycle	Tricarboxylic acid cycle
TGGE	Temperature gradient gel electrophoresis
tRNA	Transfer ribonucleic acid
TRS	Trinucleotide repeat sequences
Tryp	Tryptone
UV	Ultra-violet
$Y_{X/glucose}$	Biomass yield from substrate (glucose)
$Y_{X/glycerol}$	Biomass yield from substrate (glycerol)
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

List of Publications

Related to this Thesis

Influence of growth conditions on plasmid DNA production.
Silva F., Passarinha L., Sousa F., Queiroz J.A. and Domingues F.C.
J. Microbiol. Biotechnol. 2009. 19(11): 1408-1414.

The use of DRAQ5 to monitor intracellular DNA in *Escherichia coli* by flow cytometry.
Silva F., Lourenço O., Pina-Vaz C., Rodrigues A.G., Queiroz J.A. and Domingues F.C.
J. Fluoresc. 2010. 20(4): 907-914.

Impact of plasmid induction strategy on overall plasmid DNA yield and *E. coli* physiology using flow cytometry and real-time PCR.
Silva F., Lourenço O., Maia C., Queiroz J.A. and Domingues F.C.
Process Biochem. 2011. 46(1): 174-181.

Plasmid DNA fermentation strategies: influence on plasmid stability and cell physiology.
Silva F., Queiroz J.A. and Domingues F.C.
Appl. Microbiol. Biotechnol. 2011 (accepted for publication)

Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*.
Silva F., Queiroz J.A. and Domingues F.C.
Biotechnol. Adv. 2011 (submitted for publication)

Unrelated to this Thesis

Bacteriostatic versus bactericidal activity of ciprofloxacin in *Escherichia coli* assessed by flow cytometry using a novel far-red dye.
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Phytomed. 2011 (submitted for publication)

Chapter I

1. Introduction

1.1. Gene therapy and DNA vaccination

Gene therapy is defined as the introduction of genetic material to the cells or tissues with the goal of treating a disease, since the introduced genes can provide novel cellular functions, improve or modulate existing ones, or even replace deleterious mutants with functional ones [1]. Gene therapy potential continues to rise and, at present, over 1714 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide, with some of them already in the phase IV of the drug approval process (http://www.wiley.com/ /legacy/wileychi/genmed/clinical, July 2011). In order to achieve successful gene therapy, it is necessary to design an efficient and safe delivery system capable of transferring the therapeutic gene to a specific target tissue or organ [2]. Currently, gene delivery systems can be divided into viral and non-viral vectors [2]. Viral vectors are known to have high gene delivery and expression efficiencies; nonetheless, the limitations associated with these vectors regarding safety, immunogenicity, low transgenic size and high cost [2], have drawn researchers to focus on non-viral vectors, developing new strategies in order to improve their efficiency [3]. Of all non-viral vectors currently used at clinical trials worldwide, naked/plasmid DNA is the most popular non-viral system, accounting for approximately 18.7% of all clinical trials targeting pathologies such as cancers, cardiovascular diseases, ocular diseases, infectious and monogenic diseases as well as autoimmune and neurological disorders (http://www.wiley.com/legacy/wileychi/genmed/clinical, July 2011).

Another DNA-based therapeutics, DNA vaccines, currently represent a new and rapidly progressing area in vaccinology [4]. To date, plasmid DNA (pDNA) vaccines have been reported to induce immunity in several animal models against parasitic, viral, bacterial diseases and cancer. These DNA vaccines can elicit the expression of infectious agent's proteins from the host cell, miming the native infectious agent proteins and are also capable of inducing both humoral and cellular immunity [5]. Nowadays, regarding viral diseases, some of the targets are Human Immunodeficiency Virus (HIV) [6], Hepatitis C virus (HCV) [7], Hepatitis B virus (HBV) [8] and Influenza virus [9]. Regarding to parasitic diseases, some therapies have been proposed for toxoplasmosis [10], leishmaniasis [11] and malaria [12], among others. Also, DNA vaccination has been widely explored to develop new, alternative and efficient vaccines for cancer immunotherapy [13] containing tumor-specific or tumor-associated antigens (TAAs) and additional immune-stimulatory factors such as cytosine-guanine oligodeoxynucleotides (CpG) motifs [14]. The term cancer vaccine might refer either to prophylactic cancer vaccines (to prevent cancer development) or therapeutic cancer

vaccine (to treat existing cancer) [15]. Both these types of vaccines have already been proposed for several cancer types, including prostate [16], colorectal [17] and ovarian cancer [18], as well as melanoma [19] and lymphoma [13].

As gene therapy and DNA vaccines advance towards approval by Food and Drug Administration (FDA), the goal of bioprocesses related with the production of plasmid DNA-based therapeutics is to economically produce adequate quantities of sufficiently pure plasmid DNA suitable for use as vector in clinical applications.

1.2. Plasmid DNA production

The vast majority of plasmid DNA vectors currently used in clinical trials were propagated by growing *Escherichia coli* in a bioreactor [20], although other alternative host systems have been proposed [21]. The primary goal of the fermentation is to maximize the amount of plasmid DNA while minimizing process key contaminants, such as genomic DNA (gDNA), RNA and proteins. In order to improve plasmid DNA yields, there are several factors that should be taken into account. Some of these factors include an appropriate choice of the host strain and vector in order to determining an optimal plasmid/host combination, a formulation of the culture medium that should consider the elemental composition of *E. coli* as well as plasmid DNA replication requirements and an adequate fermentation strategy with the appropriate operating conditions. Concomitantly, an induction strategy can be used in order to maximize final pDNA product by increasing the average plasmid copy number per cell, thus maximizing pDNA purity at harvest. Plasmid DNA can exist in three tertiary structures: supercoiled, open-circular and linear. Open-circular (oc) and linear plasmid isoforms are usually treated as process impurities because these isoforms do not seem to transfect (and hence express) as efficiently as the supercoiled isoform into eukaryotic cells. Therefore, the ultimate goal in a plasmid DNA production bioprocess should be to maximize the average supercoiled-plasmid copy number [22].

1.2.1. Host strain selection

The low pDNA concentration in the *E. coli* cell and the need to remove large quantities of cellular debris, proteins, chromosomal DNA and endotoxins has driven the genetic and process strategies [23-24] to obtain higher plasmid producer *E. coli* strains. Also, plasmid-imposed metabolic burden on the host cell should be considered since metabolic engineering of central pathways can improve process yield. Over the years, the establishment of efficient expression vectors and host-strain systems has had a significant impact on the improvement of plasmid DNA yields, thus improving its downstream purification by reducing contaminants.

In order to address some of the problems related to plasmid instability in *E. coli*, some mutant strains were developed by genetic engineering to improve plasmid segregational and structural stabilities [25]. In pDNA production processes, preferred host strains generally have mutations (Figure 1) in the *relA*, *endA*, and *recA* genes [26-28]. The *endA1* mutation is recommended to prevent plasmid degradation after cell lysis [29] since this enzyme, located in *E. coli* periplasmic space, cleaves double-stranded DNA into oligonucleotides [30]. *E. coli* strains with the *recA* mutation lack the extreme recombination deficiency [31], ensuring insert stability and also decreasing mutation frequency, preventing plasmid structural instability [29]. The *relA* mutant strains have a relaxed response in amino acid starved cells [32] which may lead to plasmid DNA amplification under these conditions [32-33].

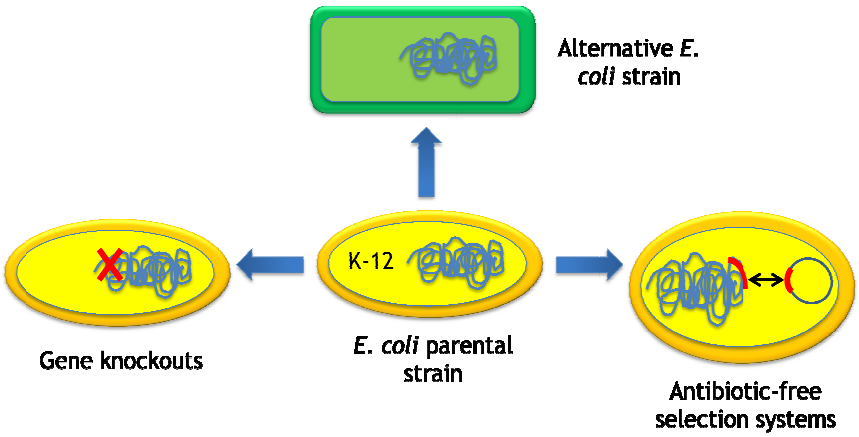

Figure 1: *E. coli* K-12 strain engineering strategies.

Adapted from: [34].

Due to the concerns regarding the use of antibiotics in production processes, effort is being made to create antibiotic-free selection systems and some *E. coli* strains have been manipulated in order to develop an auxotrophy which is complemented with a plasmid encoding the gene responsible for the auxotrophy [35]. For example, *E coli* strains DH1*lacdapD* and DH1*lacp2dapD* allowed an antibiotic-free plasmid selection and maintenance in complex media using repressor titration, since these strains contain an ectopic copy of a *dapD* gene whose expression is controlled by a lac promoter [36].

Another approach to improve plasmid yields in *E. coli* is related with bacterial metabolic engineering (Figure 1) in order to circumvent the detrimental effects on *E. coli* metabolism resultant from high-cell density cultures and plasmid-imposed burden, namely acetate production, low energy production and decreased biosynthesis pathways, that could, eventually, led to cell growth arrest and lower product yields [37-38]. In fact, significant improvement of cell growth and plasmid yields have already been obtained by engineering central carbon metabolism together with the glucose transport system [37] and also by engineering glycolysis flow regulators [39].

It has been described that popular *E. coli* hosts such as DH5 [40] and derivatives [41] (Table 1), and XL1 Blue are suitable for plasmid production [29]. In fact, host strains like DH5 alpha, DH1, or C600, in particular, tend to produce high amounts of plasmid DNA [26]. In recent years, some comparative plasmid DNA production studies in several *E. coli* strains have reported other *E. coli* strains as high pDNA producers, even when compared to DH5α or DH1 strains used in those studies. For instance, in the work described by Yau and collaborators [42], *E. coli* BL21(DE3), a common recombinant protein producer, have shown a five-fold increase in plasmid DNA yield, when compared to strain DH5 alpha. In another study, *E. coli* strains BL21 and SCS1-L (Table 1) have shown an increased plasmid DNA yield when compared to both DH5 alpha and DH1 strains [43]. Although the results obtained by Yau *et al*. and Singer *et al*. comparative studies seem to indicate that other *E. coli* strains could be higher plasmid producers than DH5 alpha strains; so far, *E. coli* DH5 alpha has been described as the more efficient plasmid producer strain in large scale bioprocesses [41] (Table 1).

Table 1: Examples of *E. coli* strains used for large scale plasmid DNA production.

***E. coli* strain**	**Genotype**	**pDNA yield (mg/L)**	**References**
BL21	B F- *dcm ompT hsdS* (r_B^-, m_B^-) *gal*	504	[43]
SCS1-L	*recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1*	599	[43]
JM108	F− *thi Δ(lac-proAB) endA1 gyrA96 relA1 phx hsdR17 supE44 recA*$^-$	938.80	[44]
DH5	*F*-, *end*A1, *hsd*R17(r_K^- m_K^+), *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *rel*A1	1600	[40]
DH5 alpha	*F*$^-$ *end*A1 *gln*V44 *thi*-1 *rec*A1 *rel*A1 *gyr*A96 *deo*R *nup*G Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsd*R17(r_K^- m_K^+), λ^-	2200	[41]

1.2.2. pDNA vector selection

Plasmid DNA constructs used for the purpose of immunization are usually similar to those used for delivery of therapeutic genes. They all have four features in common: firstly, an origin of replication allowing for growth in bacteria (Table 2) [45] and an amplification of large quantities of plasmid DNA for purification [46]; secondly, a prokaryotic selectable marker gene such as an antibiotic resistance gene; thirdly, several eukaryotic transcription regulatory elements that are most often strong viral promoter/enhancer sequences to direct high levels of gene expression in a wide host-cell range; and finally, a polyadenylation sequence [46] to ensure appropriate stabilization of mRNA transcripts [45].

Table 2: Replication origins of plasmid DNA vectors.
Adapted from: [28].

Parent Origin	Regulation	High copy derivation	Therapeutic plasmids	References
pMB1	Antisense RNAI binds RNAII. Rop accessory protein stabilizes this interaction	pUC origin (Rop deletion and second site mutation that alters RNAI/II interaction and induces plasmid copy number at 37-42 ºC, but not 30-32 ºC	pcDNA3, pDNAVACC, pVAX1-LacZ	[47-49]
		pUC origin with second site enhancer increases copy number 14-50%	pDNAVACCUltra	[49]
		Rop deletion	pKKH	[50]
		G to T mutation (extends RNAI, attenuating repressor, not conditional)	pBR322 derivatives	[51]
ColE1	Antisense RNAI binds RNAII. Rop accessory protein stabilizes this interaction	Rop deletion and second site mutation that alters RNAI/RNAII interaction and induces plasmid copy number at 37-42 ºC, but not 30-32 ºC.	pAClacLink	[52]
R6K (ori α, ori ß, ori γ)	π rep protein binds iteron, copy number dependent activation (low) or repression (high)	Host strain pir-116 mutant (π rep protein copy-up mutation in oligomerization domain removed from plasmid and provided in trans from chromosome)	pBoost, pCOR	[53-54]
R1	RepA initiator protein binds non repeated target. Antisense copA repressor binds RepA leader (CopT). Auxiliary CopB protein represses RepA expression	High temperature inducible copy number using dual origin RepA controlled by temperature inducible lambda P_R promoter and temperature sensitive lambda repressor controlled	pColV2-K94	[55]
pKL1	RepA initiator protein represses repA transcription as hexamer	RepA initiator protein overexpression on separate plasmid or on chromosome	pKL1 RepA	[56]

Nowadays, problems with pDNA integration into the host genome have arisen, and a possible way to circumvent this problem is to eliminate all non-essential regions from the plasmid vector (Figure 2), such as the bacterial elements. The design of these minimized vectors has led to the generation of minimalistic immunogenic defined expression (MIDGE) vectors and minicircle plasmid vectors. The MIDGE vectors lack any non-essential and potentially detrimental backbone sequences, comprising only a eukaryotic expression unit and terminal hairpin cap nucleotides for protection against nucleases [57]. Regarding the generation of minicircle plasmids, there is a parental plasmid, containing both bacterial and eukaryotic expression elements that suffers site-specific recombination inside recombinant *E. coli* cells, leading to the formation of two circular molecules: one that is devoid of all bacterial elements (called minicircle) and the second one containing all of these elements (called miniplasmid) [57]. Both these minimized vectors have shown a better performance when compared to typical plasmid vectors in terms of transfection efficiency, expression levels and bioavailability [58-59].

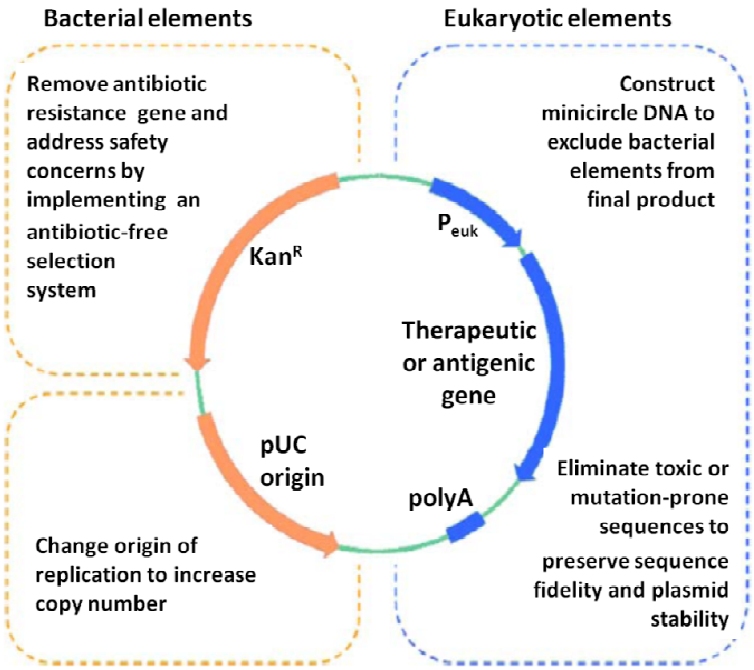

Figure 2: Vector engineering strategies.

Source: [34]

Vectors for DNA vaccination

The vectors intended for DNA vaccination are known to generate both humoral and cell-mediate immune responses in a wide variety of species for numerous diseases [60]. With the purpose of developing a DNA vaccine, the pDNA vector should also contains specific nucleotide sequences such as unmethylated cytosine-phosphate-guanine (CpG) dinucleotides (termed CpG motifs) which play an important role in the immunogenicity of these vaccines, causing an augmentation of the immune responses against the expressed antigen [46]. These CpG motifs can activate, directly or indirectly, several types of cells, due to the fact that they are recognized by effector cells of the immune system containing the toll-like receptor (TLR) 9, resulting in an immunostimulatory cascade, activating B-cells to proliferate or secrete antibodies [61-62], which leads to augmented immune responses to DNA vaccine vectors. Other known adjuvants of DNA vaccine vectors immunogenicity are the Kozak sequences. Although not being responsible for the development of an immunomodulator effect, these sequences might led to an increased gene expression level, improving antigen production in eukaryotic cells [63]. Other approach to modulate and/or enhance an immune response is related with the co-expression of cytokines, chemokines and co-stimulatory molecules or other strategies in order to direct antigens to the appropriated sites for immune modulation [63].

One of the major problems when designing a DNA vaccine is the assurance of its structural stability, since repeated DNA motifs, such as CpG or even polyA motifs, presented in the vector backbone, are more prone to suffer genetic rearrangements [57]. In order to tackle some of these limitations, a family of DNA vaccine vectors, optimized and minimized to comply with FDA guidelines regarding content and elimination of extraneous materials, was constructed [49]. These new DNA vaccine vectors, the pDNAVACCultra vector family, have the advantage of facilitating the cloning of genes or epitopes of interest and improving stability and yield. Moreover, a broad range of target genes can be introduced in these vectors since the origin of replication and the antibiotic-resistance gene are flanked by prokaryotic transcriptional terminators [49]. In the case of these vectors, it was demonstrated an higher expression levels when the antibiotic-resistance gene is distal to the eukaryotic promoter [49].

Vectors for gene therapy

In gene therapy, vector design is a major contributor to the safety of both production and therapy. Efforts to improve the plasmid backbone have focused on the enhancement of transgene expression or plasmid copy number [64] and recently, in the development of plasmid lacking the bacterial elements, such as MIDGE and minicircle vectors [57]. The ColE1-derived bacterial plasmids are currently used in gene therapy experiments and clinical trials. However, due to concerns on the safety and containment of such genetic material, mainly related to the deleterious effects of the ColE1 origin of replication and the antibiotic

resistance marker, the clinical use of such plasmids could potentially lead to their dissemination in the environment or even integration in the patient genome [64].

In order to overcome the detrimental effects posed by ColE1 plasmids, research has focused on the design of more efficient and safer plasmid vectors. Soubrier and collaborators have developed a new vector, the pCOR plasmid, by completely redesigning the plasmid backbone, resulting in improved properties in terms of biosafety while achieving efficient gene expression in vivo [65]. This new plasmid backbone contains a conditional origin of replication (pCOR) derived from R6K type of plasmid, which restricts pCOR amplification to a specific bacterial strain, greatly reducing these plasmid propagation in the environment. In addition, pCOR plasmid selection is antibiotic-free [65]. This pCOR/bacterial host co-dependent system establishes new features and significant safety improvements that are highly suitable for the production and use of plasmid DNA for human gene therapy [64].

In recent years, other plasmid vectors, based on ColE1 vectors have been developed containing the Sleeping Beauty transposon system (SBTS) [66-67]. Many researchers consider these vectors as the leading non-viral vector for gene therapy, since they are able to combine the advantages of viruses and naked DNA [68]. The SBTS consists of two components: a transposon containing a gene-expression cassette and a source of transposase enzyme [69]. When delivered to effector cells, this transposase is capable of transferring the transposon containing the gene of interest into the effector cell chromosomal DNA, thus attaining a long-term expression of the therapeutic gene [69]. SBTS vectors can be maintained and propagated as plasmid DNA vectors, rendering them simple and inexpensive vectors to manufacture [68]. These vectors are not prone to incorporate mutations and can tolerate larger (up to 8 kb) and more complex transgenes, including those containing repeated DNA motifs and they even eliminate the risk of rearrangements of the expression cassette, integrating the gene of interest into chromosomal DNA in an intact form [68].

Vector size

Another important characteristic of plasmid DNA vectors is their size since it can impact both upstream and downstream processing, as well as gene transfer efficiency. There are several studies focusing on the influence of plasmid size in recombinant microbial fermentations and, in fact, there is evidence that plasmid size can affect *E. coli* growth and product yield [70-71]. The study performed by Cheah and co-workers revealed that, as plasmid size increased, maximum cell density decreased and, with the largest plasmid used, cell death was accelerated after the stationary phase was reached [70]. Moreover, the largest plasmid was harboured at significantly fewer copies per cell than smaller plasmid at all phases of growth, thus decreasing product yield [70]. Another study by Khosravi and co-workers also demonstrated that, during logarithmic growth in shake flasks, oxygen uptake by *E. coli* containing the largest plasmid (8.7 kb) was 2.5 times higher that of *E. coli* strain containing

the smaller plasmid (2.7 kb) [71]. On opposite to the previous study conducted by Khosravi *et al.*, a more recent study performed by Kay and collaborators have shown that plasmid size does not significantly affect oxygen demand, and that this increased oxygen demand is caused by increased product formation [72]. Although the results regarding the influence of plasmid size on fermentation performance are not consensual, many efforts have been made to reduced plasmid size and, at the same time, increase their expression levels and copy number [49, 65].

pDNA vector stability

The transformation of a plasmid vector containing exogenous genes into *Escherichia coli* always brings a series of physiological burdens that eventually affect plasmid stability [73]. Plasmid instability usually denotes a considerable decrease in plasmid DNA productivity, finally leading to poor economics of the whole bioprocess [73]. Plasmid instability usually originates from either structural instability caused by changes in the plasmid itself, such as point mutation, deletion, insertion or rearrangement in the plasmid DNA; or segregational instability caused by defective partioning of plasmids between the daughter cells during cell division [74]. Studies have shown that plasmid instability is determined by many factors such as plasmid load, plasmid copy number, replication patterns, substrate type, medium composition, host background [75], culture conditions and culture temperature [76] (Table 3).

Table 3: Factors affecting plasmid stability.

Type of plasmid instability	Factor	Reference
Structural	Plasmid size	[77]
	PolyA sequences	[78]
	Direct repeats	[79]
	Inverted repeats	[80]
	Insertion sequences	[81]
	Culture conditions	[82]
	Increased expression of transposons and IS-elements	[83]
Segregational	Host strain metabolic burden	[84]
	Plasmid multimers	[85]
	Lack of selective pressure	[86]
	Dissolved oxygen concentration	[86-87]
	Low pH	[88]
	Culture medium composition	[89]
	Fermentation strategy	[90]
Isoform	Culture conditions	[91-92]
	Culture media	[23]
	Host strain growth phase	[93]

Plasmid structural stability

Although structural instability is known to occur in recombinant *E. coli* fermentations, since mutation frequency is kept at a low frequency and standard methods have low mutant detection, this type of plasmid instability is commonly disregarded. However, plasmid DNA molecules present repeated DNA motifs that are prone to suffer structural instability. In addition, there are other factors affecting plasmid structural stability such as plasmid size [77], polyA sequences [78], direct repeats [79], inverted repeats [80] and insertion sequences [81]. Also, environmental stresses such as antibiotic concentration, medium composition, temperature shifts and oxygen fluctuations can elevate the rate of spontaneous point mutations or promote recombinations [82]. Structural instability could additionally arise due to the fact that plasmid DNA maintenance and replication is known to increase the expression of *E. coli* transposons, insertion sequence (IS) elements and phage-related genes which result in increased genetic variation [83]. One of the recent approaches to circumvent plasmid structural instability in recombinant hosts was based on the removal of recombinogenic or mobile DNA and cryptic virulence genes from the host genome [94].

Plasmid segregational stability

In the fermentation process, recombinant plasmid segregational instability is related to the metabolic burden suffered by the engineered host strain. Firstly, the transcription and replication are competitive processes in prokaryotic cells so, increased transcription blocks the segregation of plasmid molecules and, also, overloads the bacterial ability to repair plasmid DNA [84]. Secondly, the instability of the recombinant plasmid may be a result of the slower growth rates exhibited by plasmid-carrying cells when compared to plasmid-free cells, mostly due to the additional burden caused by plasmid maintenance and replication [84].

Results have shown that plasmid DNA stability is affected by dissolved oxygen concentration (DOC), showing that a decrease in DOC level in growth medium (below a threshold value) could adversely affect the maintenance of recombinant plasmids [86-87]. A low pH medium may cause stress to the bacterial cells, also resulting in plasmid loss from the high-density culture [88]. Another factor that may influence plasmid segregational stability is culture media composition [86, 89] since it has been described that the use of complex nitrogen sources increased plasmid stability [95]. The fermentation strategy chosen for the bioprocess also seems to play a role in affecting plasmid stability [90]. Another well-known cause of plasmid instability is the accumulation of plasmid multimers which leads to a decrease in the number of segregating units, increasing the probability of plasmid loss [85]. Although multimers do not arise frequently by homologous recombination, they accumulate rapidly by over-replication and plasmid-free cells arise at high frequency from these multimer-only cells [96].

In order to improve segregational plasmid stability, a number of strategies have been described. One of these approaches, the increase of antibiotic concentration in growth medium has failed as a strategy to improved segregational stability, due to stress induced by recombinant protein production and antibiotic degradation in growth medium [86]. Others studies also corroborate this fact by demonstrating that growth in a selective environment did not increase the stability as was expected [97]. However, other studies have shown that under the resistance-selective pressure, plasmid-carrying cells remained at higher level that under nonselective pressure after induction [84]. Due to this and other limitations, antibiotic-free systems with similar or even higher plasmid stability have been developed using auxotrophic bacterial strains [98], repressor-titration systems [99], alteration of the expression of essential growth genes [100-101] and selection with other biocide agents [102].

Plasmid isoforms stability

Another important type of plasmid stability is isoform stability, mainly in plasmid DNA production processes since the supercoiled (sc) plasmid isoform, also designated covalently closed circular (ccc) isoform [103], is known to produce higher levels of in vitro and in vivo transgene expression when compared with open circular (oc) or linear isoforms [104-105] (Figure 3).

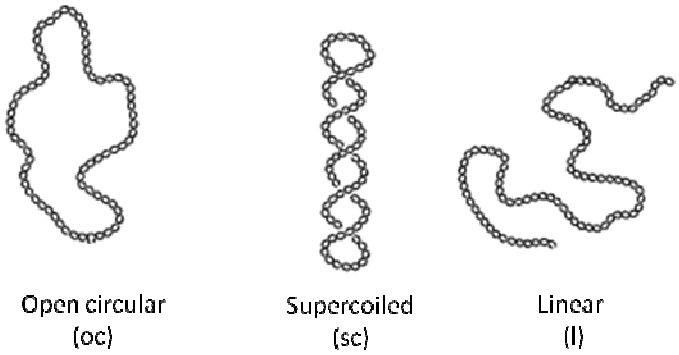

Figure 3: Plasmid DNA isoforms.

Adapted from: [106].

Furthermore, there is a greater risk of the linear isoform to integrate into the host genome, leading to undesirable deleterious effects [107]. Thus, the use of pDNA preparations containing predominantly the sc isoform on cell transfection is highly desirable. DNA super coiling is known to vary in response to environmental conditions such as pH, osmolarity [91], nutrient supply and starvation and, even, lack of oxygen [92]. It has been described that plasmid DNA topology varies throughout *E. coli* fermentations, showing that the percentage of the sc isoform remains constant during most of the exponential phase (up to 10 h) [93]. However, upon entry into the stationary phase of growth, the percentage of the sc isoform decreases, as a response to bacterial DNA supercoiling control [108]. In addition, different

growth media have been shown to produce major differences in the percentage of supercoiled pDNA obtained at the end of fermentation [23].

1.2.3. Influence of pDNA on host strain physiology

Plasmid DNA maintenance and replication can induce a "metabolic burden" in *E. coli* host cell physiology and metabolism and can also elicit several stress responses (Table 4). The relevance of *E. coli* growth parameters can be deduced from the fact that, although parameters like stable maintenance of recombinant plasmid and plasmid copy number are primarily a function of genetic makeup of the host and vector system, these are also known to be greatly affected by cultivation techniques and media composition [86]. Several research studies have shown that plasmid-bearing cells (P^+ cells) exhibited lower specific growth rates than plasmid-free cells (P^- cells), resulting in lower biomass yields at the end of fermentation [38, 109]. It has been described that the degree of specific growth rate decrease is dependent on plasmid type and copy number [72, 110]. Also, it has been reported that this growth-arrested cells can experience difficult carbon and nitrogen assimilation due to the activation of stress responses [111].

Table 4: Alterations in *E. coli* biological levels due to plasmid-imposed metabolic burden.

Physiological alterations	**References**
At culture level	
Reduced specific growth rates	[38, 109]
Reduced biomass yield	[38]
Reduced plasmid stability	[73]
At cellular level	
Cell filamentation	[76, 112]
Cell viability	[48]
Cell cycle alterations	[48, 113]
Activation of stress responses	[111]
Metabolic alterations (Molecular level)	
Increased TCA cycle	[38, 83, 110, 114]
Increased/Decreased glycolysis	[38, 110, 114]
Decreased ribosome synthesis	[114]
Increased heat-shock response	[38, 114]
Increased stringent response	[32]
Increased carbon transport	[38, 110]
Decreased pentose-phosphate pathway	[110]
Decreased gluconeogenisis	[110]
Increased ribosome related genes	[83]
Increased/Decreased amino acid synthesis	[38, 83]
Decreased motility elements (e.g. fimbriae)	[83]
Increased/Decreased ATP production	[38, 83]
Nucleotide synthesis	[38]

One of the frequent observations during plasmid DNA production in *E. coli* is cell filamentation [48, 76, 112]. Cell filamentation is seen when cells elongate and replicate their DNA, but are not able to septate and divide [115]. This filamentation causes a decrease in growth rate or absence of further cell division, eventually leading to low biomass and productivity. Since filamentation is caused by the blockage of cell division, it was reasoned that essential cell division proteins might be inactivated due to plasmid production. In fact, Ow *et al*. (2006) had described that some genes coding for proteins involved in cell wall synthesis are downregulated in plasmid-bearing cells and there is evidence that the amplification of an essential cell division protein, FtsZ, could suppress cell filamentation in recombinant *E. coli* [112]. Also, cell filamentation can be a result of the SOS stress response [116] and this phenomenon seems to be related with increased plasmid segregational instability [117]. Another detrimental effect of the metabolic burden imposed by plasmid DNA

maintenance and replication is a reduced cellular viability in plasmid-bearing cells [118] probably as a result of the increased stress suffered by these cells [119].

Metabolic consequences of pDNA maintenance and replication

The term "metabolic burden" is defined as the amount of resources (raw material and energy) that are taken from the host cell metabolism for maintenance and replication of the foreign DNA [120]. The response of cells under energy limiting conditions is very complex and includes the activation of alternative pathways for energy generation [121]. It has already been reported that the presence of plasmids can induce host metabolic burden by altering cell regulatory status through a complex interaction between host and vector [118, 122], but a more complete picture of the changes that occur in plasmid-bearing cells is needed. With the recent arrival of high-throughput analytical tools, the accumulation of vast amounts of genome, transcriptome, proteome, metabolome and fluxome data has enabled researchers to gain a deeper knowledge on plasmid-induced "metabolic burden", allowing them to simulate *E. coli* metabolic behaviour under specific conditions as well as to identify possible targets for a more systematic design and implementation of metabolic engineering strategies towards the improvement of bioprocess yields [39, 109]. To date, there are only a few published reports addressing the metabolic burden caused by the plasmid itself on *Escherichia coli* metabolism (Table 4).

Metabolic burden could arise due to the extra biosynthetic demands for plasmid synthesis, or the perturbation of *E. coli* regulatory systems affecting central metabolic pathways. Regarding carbohydrate metabolism, P^+ cells increase the number of several enzymes involved in the tricarboxylic acid cycle (TCA cycle) [38, 83, 110, 114]. The results obtained for the glycolytic pathway are not consensual: while some authors reported a decrease of enzymes and gene expression of enzymes involved in this pathway [114 2006 #26], others reported an increased expression of the glycolytic genes [110]. The genes involved in carbon transport also have an increased expression in P^+ cells, namely the ones responsible for glucose uptake [38, 110]. The study of the genes involved in energy metabolism are not concordant, since one study reported an increased expression of ATP synthesis genes [83] while another one revealed that a number of aerobic proteins involved in ATP production have lower expression in P^+ cells [38].

When analysing genes and proteins involved in amino acid and protein synthesis/metabolism, the results are not consensual again, since some authors described a decrease of proteins involved in protein translation, including 30S and 50S ribosome subunits, other ribosome-related proteins and even in amino acid synthesis [38, 114]; whereas another study have shown an increased expression in ribosome-related genes and amino acid synthesis genes [83]. Interestingly, although the host cell is continuously producing plasmid DNA, the expression of purine and pirimidine synthesis genes is lower in P^+ cells [38].

Other metabolic pathways shown to be altered due to plasmid presence comprise the pentose-phosphate pathway, the glyoxylate shunt, gluconeogenisis, all of them being downregulated in P^+ cells [110]. These studies also provided further evidences to support P^+ cells filamentation, as cell wall synthesis genes, including the ones involved in cell division, have lower expression in P^+ cells [38]. The levels of heat-shock proteins and heat-shock genes are also higher in P^+ cells [38, 114], corroborating the fact that plasmid-induced stress response in the host cell is similar to the response induced by heat-shock [121]. The study performed by Wang and co-workers also revealed that P^+ cells had higher expression of acetate synthesis genes, leading to an increased acetate production [110], a metabolic by-product of *E. coli* fermentations which is known to influence *E. coli* physiology [123]. Another parameter that has been described to contribute to plasmid-induced metabolic burden is the increased synthesis of plasmid-encoded proteins such as antibiotic resistance proteins used for selection of plasmid-bearing cells [124-126].

Although not consensual, probably due to the different host strains and plasmid vectors used throughout these studies; taken together, these results showed that host cells might adopt different respiratory pathways for energy production when carrying plasmids and that most of the biosynthetic pathways are diminished in these cells.

Induction of stress responses

The maintenance of a plasmid often induces a stress response similar to the response induced by heat-shock and amino acid starvation, the later designated as the stringent response [127] (Table 4). Stress induced by plasmid maintenance and replication is often related to plasmid copy number [128] while the main perturbation can be attributed to genes encoded by the plasmid and even constitutively expressed genes such as antibiotic resistance genes [125-126] as previously discussed.

Heat-shock response

One of the most widely studied stressful environmental condition in *Escherichia coli* is elevated temperature, which elicits a cellular response termed the heat-shock response [111, 129]. The heat-shock response is controlled by the alternative factor σ^{32} [130] that is responsible for altering the expression of transcription factors and executing several roles in cell homeostasis. This response is characterized by the increased synthesis of several proteins designated as heat-shock proteins that are coded by the σ^{32} regulon [131]. Many heat-shock proteins are chaperones (ClpB, DnaK/J and GroEL/S) that promote protein folding, while others are proteases (Lon, ClpP, ClpC, HslV (ClpY), HslU, ClpQ and FtsH), which degrade unfolded or damaged proteins [111].

The activation of the heat-shock response causes the triggering of physiological events such as a decrease in the growth rate, due to the down-regulation of energy and protein

translation genes [111], changes in cell membrane due to modifications in lipid/integral membrane proteins ratio [132] and can, even, cause ribosome destruction. It has been described that, besides increased temperature, many other stresses can also cause the heat-shock response in *E. coli* such as viral infections, recombinant protein production [124] and plasmid maintenance and replication [38].

Stringent response

Another commonly described stress response is the stringent response that is elicited by the expression of the *relA* gene product [133]. The stringent response results in the formation of the signal and effector molecule guanosine tetraphosphate (ppGpp), which then triggers numerous reactions [134]. For instance, ppGpp may either stop energy consuming synthesis processes, such as the major part of protein, rRNA, tRNA, pDNA synthesis or activate the synthesis of specific proteases and other proteins to overcome stress situations [134].

One of the conditions that induce the stringent response is amino acid starvation. Under these conditions, the product of the relA gene is activated to produce specific signal nucleotides, guanosine 5'-triphosphate-3'-diphosphate (pppGpp) and guanosine 5'-diphosphate-3'-diphosphate (ppGpp). These nucleotides interact with RNA polymerase causing dramatic changes in the efficiency of transcription form various promoters [32]. *E. coli* mutants in the *relA* gene do not produce ppGpp during amino acid starvation, leading to a decrease in the ppGpp level in amino acid-starved *relA*$^-$ cells which is designated as the relaxed response [32]. In *E. coli* wild-type cells, the stringent response often leads to inhibition of plasmid DNA replication. However, in relA$^-$ mutant *E. coli* cells, plasmid DNA replication can proceed efficiently under amino acid starvation conditions [135-137].

SOS response

DNA repair systems, such as nucleotide excision repair (NER) play an important role in the detection and removal of unusual DNA structures. In bacteria, the NER system is related to the overall response to DNA damage which is coordinated by a network of reactions named the SOS response. This response is described to occur in cells exposed to massive DNA damage or under specific physiological stress such as pH alterations, nutrient starvation and transition from exponential to stationary growth [138]. When this stress response is activated, cell division is halted to give cells time to repair DNA damages and complete ongoing replication [138]. The regulation of the SOS regulon is based on the interaction between two crucial gene products: LexA and RecA [139]. All the key proteins involved in *E. coli* NER are induced during the SOS response.

Previous studies have identified that long DNA trinucleotide repeat sequences (TRS) contained in plasmids influence the growth of the host strain harbouring them, as well as DNA metabolism and cell division [140-141]. Besides the NER genes, one of the first genes to be

induced as a consequence of SOS response activation is sfiA (sulA), which encodes an inhibitor of septum formation [142]. This inhibition of cell wall synthesis causes high cell filamentation and is indicative of the induction of the SOS response [138]. Plasmids containing long TRS are known to induce the SOS response in *E. coli* [138]. Also, the SOS response can be induced by a single plasmid-encoded protein such as the replication initiator protein of plasmid pSC101, RepA [143].

1.2.4. Culture media

The three general types of media that are used to perform fermentation studies can be grouped as minimal (defined) media, composed only of salts, a carbon source, a nitrogen source and trace elements; rich (complex) media, containing in addition to salts and carbon source one or several complex extracts [26] and semi-defined media which is a defined media containing one or more complex extracts [29]. These extracts are most commonly from protein (e.g. casein peptone, tryptone and casamino acids), meat (meat peptone) and yeast (yeast extract), or plant material (e.g. soybean peptone) and their exact composition is not known [26]. There is some evidence that defined media tend to reduce segregation rate-associated plasmid loss, whereas complex media seem to reduce growth rate-associated plasmid instability at least in *Bacillus subtilis* and *Saccharomyces cerevisiae* [89, 144-146].

In *E. coli* fermentation processes, complex media caused an elevated loss of plasmid DNA, mainly in the supercoiled form [23], resulting in low specific pDNA yields [147] whereas defined media yield low specific growth rates, resulting in low productivity levels due to prolonged cultivation time [40]. Regarding semi-defined media, it has been described that these types of culture media are able to support the higher plasmid stability [147], allowing the attainment of high levels of specific and volumetric productivities [29].

Culture media formulation

Escherichia coli is a microorganism that grows in both rich complex organic media and salt-based chemically defined media as long as carbon source is present [147]. Besides an energy source, *E. coli* requires nutrients for the biosynthesis of cellular matter, formation of products and maintenance; so, medium components must supply the nutrients needed to accomplish all these functions. The vital chemical elements needed for the cultivation of *E. coli* are hydrogen, carbon, nitrogen, oxygen, sodium, magnesium, phosphorus, potassium and calcium [148] considering that these elements have specific functions during the bioactivity of cell growth and plasmid yield [148]. Sodium, potassium, magnesium and calcium are cellular cations and cofactors for some enzymes. Phosphorous constitutes phospholipids, coenzymes and nucleotides in nucleic acids [148]. As a source of phosphorous, potassium or sodium phosphates are generally used and, in certain proportions, they can also function as

buffering agents [23, 29, 40, 149]. Magnesium sulphate heptahydrate is often the source of both magnesium and sulphur in plasmid DNA production processes [40, 149-150] and it has been found that high concentrations (approximately 80 mM) are beneficial to production of supercoiled plasmid monomers [151]. In general, culture media should also contain small amounts of calcium, copper, cobalt, iron, manganese, molybdenum, and zinc that are supplied by adding a trace minerals solution (Table 5) or just by adding a complex nutrient, such as yeast extract, since these minerals are usually present as impurities in these nutrients. If needed, the adjustment in medium osmolarity can be performed with sodium chloride [29].

Table 5: Examples of trace minerals solutions used in pDNA production processes.

$FeCl_3.6H_2O$		
$MnCl_2.4H_2O$		
$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$FeCl_3.6H_2O$
$ZnCl_2$	$MnSO_4.4H_2O$	$ZnCl_2$
$CoCl_2.6H_2O$	$ZnSO_4.7H_2O$	$CoCl_2.6H_2O$
$Na_2MoO_4.2H_2O$	$CoCl_2.6H_2O$	$Na_2MoO_4.2H_2O$
$CaCl_2.2H_2O$	$CaCl_2.2H_2O$	$CaCl_2.2H_2O$
$CuSO_4.5H_2O$	$CuSO_4.2H_2O$	$CuCl_2.2H_2O$
H_3PO_4		H_3BO_3
$K_3C_6H_5O_7.H_2O$		
$Na_3C_6H_5O_7.2H_2O$		
[152]	[27]	[149]

The type and concentration of all the above mentioned ingredients in cultivation medium directly affects the amount of biomass produced, as well as plasmid volumetric and specific yields. Also, medium composition can have influence on host cell physiology by influencing their intricate regulatory systems, and could eventually contribute to control plasmid copy number [148].

When formulating a culture media, there are two main ingredients that should be taken into account: the carbon and nitrogen sources. Another important parameter that researchers should taken into account is the ratio between carbon and nitrogen sources, since a study carried out by O'Kennedy and co-workers using a semi-defined medium showed that C/N ratio significantly affected plasmid specific yields, with higher specific yields being obtained at intermediate C/N ratios (2.78/1.0) [23].

Carbon source

A carbon source provides energy and biomass and is usually the limiting nutrient in fed-batch cultures [29]. Besides the two carbon sources normally used for plasmid DNA production,

glucose and glycerol, there has been some research with alternative carbon sources such as maltose, mannitol, lactose and sucrose [153-154]. In fact, in a study performed by Zheng and collaborators, it was found that the addition of lactose, sucrose or mannitol to the culture medium increased plasmid DNA yield up to 4 times compared to the use of glycerol or glucose [153]. Despite the interesting results obtained by this study, glucose and especially glycerol, continue to be used in the large scale production of plasmid DNA [40-41]. Although glucose is very efficiently metabolized and biomass yield coefficients (carbon) are slightly higher ($Y_{X/glucose}$=0,506) than those obtained when glycerol is used ($Y_{X/glycerol}$=0,430) [155], high levels of glucose are known to cause undesirable acetate production, which can prevent high cell density growth of *E. coli* [156], due to metabolic overflow [157]. In plasmid DNA production, glycerol is often used as preferred carbon source [40, 152], since it does not cause as much acetate accumulation as glucose [157], so it can be used at higher concentration without becoming inhibitory and it also provides reduced growth rates which not only eases the cellular competition for carbon and energy sources but also provides time for plasmid DNA replication to synchronize with cell division [158-159]. Xu and collaborators [154] also reported that when glycerol was supplemented into the culture medium, the specific plasmid DNA productivity was improved.

Nitrogen source

A nitrogen source is used for biosynthesis of nitrogenous compounds such as amino acids, purines and pyrimidines. Under carbon limitation conditions, amino acids can also be used as an alternative carbon source, resulting in ammonia accumulation. The bacterial requirements for nitrogen can be satisfied by inorganic or organic sources [29] (Table 6). Inorganic ammonia and ammonium salts such as ammonium chloride or sulphate are used in minimal or defined media, with some research stating that the addition of ammonium chloride to the culture medium even improves plasmid DNA yield [160]. Semi-defined media or complex media supply nitrogen either partly or entirely from complex components such as yeast extract, peptone, tryptone and/or casamino acids [23, 41, 76]. In order to choose an appropriate complex nitrogen source, the amino acid content should be taken into account. For instance, aspartic acid, along with glutamine and glycine serve as nitrogen donors for the synthesis of nucleotides, leading to the formation of nucleic acids (RNA, DNA and plasmid DNA) [150] and tyrosine, together with phenylalanine, can even decrease plasmid stability [95]. A study compared the use of casamino acids and Luria Bertani (LB) medium (containing yeast extract and tryptone) in *E. coli* plasmid DNA production and it was found that in comparison to LB medium, the medium containing casamino acids was able to support lower growth rates, higher cell densities, improved plasmid stability and a higher proportion of supercoiled pDNA [23]. The use of other elemental organic nitrogen sources such as isoleucine, combination of isoleucine with proline [27] or monosodium glutamate in a defined medium also allowed the attainment of high plasmid DNA yields (up to 1.6 g/L) [40, 149].

Yeast extract is generally used in media formulations for plasmid production in *E. coli*. It has been shown that high amounts of yeast extract could promote plasmid DNA production [26], leading to the production of high plasmid DNA titres. It was also found that, for the case of plasmid DNA production in *Saccharomyces cerevisiae*, high yeast extract concentrations promoted plasmid instability [161]. Despite containing a mixture of amino acids, peptides, water soluble vitamins, yeast extract also contains carbohydrates, which is a limiting factor when defining a carbon source consumption rate, since one could not determine the exact concentration of the carbohydrates in yeast extract; for this reason, other alternative nitrogen sources, devoid of carbohydrates, such as casein hydrolysates should be preferred [76].

Being an amino acid source, tryptone is not commonly used as a nitrogen source in culture media for plasmid DNA production, although it could provide several advantages: tryptone is carbohydrate deficient unlike yeast extract, which is very useful when trying to establish the consumption rate of a previously established carbon source. Tryptone shows an elevated percentage of aspartic acid [150]; and also shows low percentage of tyrosine, when compared with casamino acids [95].

Another important feature regarding nitrogen source is the fact that amino acid limitation or starvation can cause plasmid amplification using either amino acids or complex nitrogen sources in the culture medium [48, 137, 162].

Table 6: The influence of nitrogen source in plasmid DNA production processes.

Nitrogen Sources	Volumetric yield (mg/L)	Specific yield (mg/g)	References
Yeast extract	1497	17.5	[152]
	250	ND	[26]
	98	1.7	[163]
Casamino acids	5.2[a]	9.12	[90]
L-isoleucine + L-proline	590	15	[27]
Monosodium glutamate	1600	39	[40]
Tryptone	ND	20.94[a]	[48]
	62.6	17.1	[148]
Yeast extract + Tryptone	66.9	12.4	[164]
	13.65[a]	9.10	[165]
	120	19.2	[166]
Monosodium Glutamate + Ammonium Sulphate	45	ND	[160]

[a]Production in shake flasks

ND Not determined

1.2.5. Culture conditions

pH

Generally, classical *E. coli* cultivation uses a pH of 7 as such pH promotes fast bacteria growth [149, 152]. A study performed by O'Mahony *et al.*, 2007 [26] investigated the effect of the culture pH, ranging from 6.5 to 7.5 on biomass production and plasmid titres and they concluded that an initial pH between 7.0 and 7.2 is optimal for cell growth but resulted in low plasmid titres. Also, when the culture pH is acidic in the beginning, plasmid production is also low, due to the fact that the bacteria are not able to multiply and there is an increased ATP consumption in order to balance intracellular and extracellular pH [167]. In this study performed by O'Mahony and collaborators, the best results both in terms of absolute titres and specific plasmid yield were observed for initial pH values of 6.7-6.9 and 7.3-7.5. These results are in agreement with a more recent study by Ongkudon and co-workers that studied the pH influence (6.0 to 8.5) on plasmid DNA yield and also found that a pH of 7.5 yielded higher plasmid volumetric and specific yields, while causing a slight decrease in the specific growth rate [165].

Dissolved oxygen

Operational parameters that regulate *E. coli* growth in batch cultures, such as the dissolved oxygen concentration (DOC), can be used to control and optimize plasmid production [163] and even alleviate *E. coli* metabolic burden. Results have shown that plasmid DNA stability is affected by dissolved oxygen concentration, since a specific level of dissolved oxygen is required for maintaining plasmid stability [89]. Most fermentation processes for pDNA production in *E. coli* uses a DOC set point of 30% [149, 152], but the choice of the best DOC level for a given system should be made as a compromise solution among several factors, such as plasmid copy number, productivity, impact in the downstream processing and costs. It was demonstrated that the use of low dissolved oxygen levels clearly increased the number of plasmid copies per cell, consequently leading to higher specific plasmid concentrations and to a further higher purity of cell lysates [164]. Nevertheless, plasmid productivity was higher at higher dissolved oxygen levels, owing to a faster cell growth which caused reduced fermentation time [164]. Despite Passarinha and co-workers have shown that lower dissolved oxygen concentrations yielded higher plasmid copy number per cell, another study has also shown that dissolved oxygen concentration should not be too low, since below a specific dissolved oxygen threshold, plasmid segregational instability increases [86-87]. In conclusion, an optimal DOC should be set for each plasmid DNA fermentation process.

Temperature

The temperature effect on plasmid production, which has been observed repeatedly in the past, is most likely linked to a nutrient effect. Below the optimal temperature of growth (37 ºC in case of most *E. coli* strains), the cell membrane has a reduced ability to actively

transport nutrients into the cell, producing an effect of near starvation [168]. Moreover, the effect of temperature on pDNA production seems to depend on the type of vector used [144, 169] since, with some vectors, a temperature up-shift can selectively amplify plasmid DNA [152]. These temperature-inducible vectors have also been successfully used for recombinant protein production thus avoiding the use of expensive and toxic chemical inducers such as isopropyl ß-D-1-thiogalactopyranoside (IPTG) [170]. Although increased culture temperature has proven to be beneficial for increasing plasmid DNA yield, one should also consider the detrimental effects of this elevated temperature on *E. coli* physiology, due to the activation of the heat-stress response [111], resulting in lower *E. coli* growth rates and biomass yields [76]. Another deleterious effect of increasing culture temperature is related to increased plasmid isoform instability, leading to a decrease in the supercoiled isoform even at 42 ºC [92]. Taken all these effects into account, culture temperature should be chosen as a conciliated solution between high biomass yield and high plasmid yield and quality.

1.2.6. Induction strategies

To date, several strategies aimed to increase plasmid copy number in the host cell, also called pDNA induction strategies, have been described. These inductions strategies include the use of temperature up-shift, addition of chloramphenicol, amino acid starvation or limitation, addition of adenosine monophosphate (AMP) and a combination between amino acid limitation and AMP addition [48].

Temperature up-shift

The vast majority of therapeutic plasmids used for gene therapy and DNA vaccination contain a ColE1 or pBR322-derived replication origin. These origins of replication can generate high-copy number derivatives with mutations affecting copy-number regulation, such as *rop* (repressor of primer gene) deletion [171] and G→A point mutation [172]. This point mutation can be employed to induce selective plasmid amplification by growth at higher temperatures (37 to 42 ºC) with pUC and pMM1 [173] replication origins.

When using this induction strategy, researchers should take into account that prolonged cultivation of *E. coli* at high temperatures, such as 42 ºC, can lead to the activation of the heat-shock response [111], resulting in lower biomass and product yields [76]. In order to prevent these detrimental temperature effects, Carnes and collaborators developed a fermentation process consisting of two phases: in the first phase *E. coli* was grown at lower temperatures (30 ºC), in order to produce biomass, and in the second phase, the temperature was increased to 42 ºC for plasmid DNA induction [152]. Later research on this process also showed that the addition of a cooling step after the induction at 42 ºC caused an increase in plasmid DNA yields [41]. Other studies also using temperature up-shift concluded that *E. coli*

growth at low temperatures (30 ºC) could led to increased plasmid segregational instability [48] and, therefore, moderate growth temperatures should be preferred [26]. Recently, another study describing the use of temperature up-shift induction strategy (the first phase of growth at 35 ºC and the second one at 45 ºC) with a higher culture medium pH (approximately 7.5) reported significantly increased pDNA yields [165].

Despite the evidences for a temperature increase in the first phase of the process, this induction strategy with a temperature up-shift from 30 ºC to 42 ºC continues to be the most successful, since a large scale pDNA fermentation process is capable of producing up to 2.2 g pDNA/L [41].

Amino acid limitation and starvation

When subjected to nutrient limitation, such as amino acids, *E. coli* activates the stringent response (Figure 4). The first event of this response is production of the specific signal nucleotide, ppGpp, a product of the *relA* gene, which interacts with RNA polymerase, leading to global changes in bacterial physiology [174]. Since one of the key genes responsible for the activation of the stringent response is the *relA* gene, *E. coli* mutants lacking this gene were readily created in order to prevent the harmful effects of the stringent response. Due to this mutation, *E. coli relA* mutants do not accumulate ppGpp upon amino acid starvation/limitation and the level of this nucleotide can even decrease in these conditions, resulting in the so-called relaxed response [174]. This relaxed response that is known to occur in situations of amino acid starvation/limitation leads to continued plasmid DNA replication by promoting the interaction of uncharged tRNAs, a consequence of the lack of amino acids, with RNA I or RNA II. This interaction prevents the association between RNA I and RNA II, which is known to inhibit plasmid replication, which ultimately results in increased plasmid replication [32]. Since plasmid DNA replication continues in the relaxed response, elicited by amino acid starvation or limitation, and that there is a simultaneous growth decrease or even arrest due to the lack of these same nutrients, the amount of plasmid DNA in the cell will increase, thus causing plasmid DNA amplification [135].

In *E. coli* strains containing the *relA* gene, when the stringent response is activated the deleterious effects of this stress response in plasmid DNA replication can be avoided if these strains are *rop*/*rom* mutants. This advantageous effect of the *rop*/*rom* gene mutation is due to the fact that this gene product, the Rop/Rom protein, is no longer capable of preventing plasmid replication and, therefore, plasmid replication can continue even when the stringent response in activated in conditions of amino acid limitation [175].

Figure 4: A model for the regulation of replication of ColE1-like plasmids in amino acid-starved stringent and relaxed strains of *Escherichia coli* mediated by uncharged tRNA molecules and the Rom protein.

Adapted from [32]

Previous works, using this induction strategy, have reported the influence of amino acid limitation in pDNA amplification [135, 174]. These studies proposed that, by using an appropriate amino acid limitation or starvation, it is possible to amplify plasmids containing ColE1-type [137] and even bacteriophage γ-derived [174] origins of replications. For the amino acid starvation experiences, *E. coli* was grown in minimal medium supplemented with several amino acids and, afterwards, the amino acids were removed from the culture medium by centrifugation of that culture and subsequent resuspension of the cells in a medium devoid of the appropriate amino acid [136]. For the achievement of amino acid limitation, bacterial cells were cultured in medium containing casamino acids [135] or LB medium (with yeast extract and tryptone) [162] until the stationary phase of growth was reached and, afterwards, the cultivation was prolonged for several hours until the obtention of constant pDNA amplification values. Other recent study using a semi-defined medium containing tryptone as nitrogen source also showed that this strategy is capable of yielding higher plasmid

amplification when compared to the temperature up-shift induction strategy previously described [48]. This could represent a significant advantage for large-scale production of plasmid DNA, since this strategy only requires a prolonged cultivation time, without the need for extra energy requirements, such as an increase in bioreactor temperature.

Addition of chloramphenicol

In the presence of a high level of chloramphenicol, ColE1 DNA continues to replicate by a semi-conservative mechanism in *Escherichia coli* cells for 10 to 15 hr, after chromosomal DNA synthesis has terminated [176]. A study has shown that the number of copies of ColE1 DNA molecules that accumulate under these conditions approaches about 3,000 copies per cell, representing a 125-fold increase over the normal level of 24 copies per cell [176]. This strategy is particularly convenient for studying the mechanism of DNA replication [176]. However, nowadays, this system is not applied due to the toxic effects of chloramphenicol and the difficult implementation of this strategy in a large-scale bioprocess.

Addition of AMP

AMP, like other nucleotides, cannot directly cross the bacterial cell envelope. It is first converted to adenosine, which enters the periplasm, and then to adenine, which crosses the inner membrane and is a prerequisite for the synthesis of intracellular AMP in the reaction with phosphoribosyl pyrophosphate [162]. Although AMP is not capable of entering the bacterial cell, this molecule plays an important role in various metabolic pathways, acting as a signal molecule [177].

Wang and collaborators studied the effect of AMP, adenine, adenosine, ADP, ATP, ribose, potassium pyrophosphate and sodium phosphate on plasmid DNA amplification in both *E. coli* *relA*$^+$ and *relA*$^-$ mutants [162]. These molecules were chosen since it appears that intracellular re-synthesis of AMP (which employs products of AMP conversion, performed during the uptake into *E. coli* cells) is crucial for effective ColE1 amplification in the stationary phase of growth of *relA*$^-$ mutant *E. coli* strains [162]. This study also tested the possible combination between amino acid limitation or starvation and all the chemicals mentioned earlier, envisioning a possible increase on plasmid DNA amplification, due to the mechanism of ColE1 replication and the attachment of an amino acid to an uncharged tRNA molecule [162]:

$$\text{Amino acid} + \text{ATP} \leftrightarrow \text{aminoacyl-AMP} + \text{PPi} \quad (1)$$

$$\text{Aminoacyl-AMP} + \text{tRNA} \leftrightarrow \text{aminoacyl-tRNA} + \text{AMP} \quad (2)$$

Therefore, the authors hypothesized that the addition of AMP or its precursor molecules (adenine, adenosine, ADP, ATP, ribose, potassium pyrophosphate and sodium phosphate) to the culture medium will contribute to the generation of more uncharged tRNAs, that are responsible for promoting plasmid DNA replication in *relA*$^-$ mutants, by preventing the

interaction between RNAI and RNAII [162]. The results showed that AMP addition caused the most effective plasmid DNA amplification in comparison to the addition of AMP precursors. However, no AMP-induced amplification of ColE1 plasmid DNA was observed in the *relA*$^+$ mutant, even in conditions of amino acid limitation or starvation [162]. On the contrary, AMP-induced plasmid amplification occurred in *relA*$^-$ mutant cultures and this amplification was even higher (more than 6-fold) when AMP addition was combined with amino acid starvation or limitation [162].

Our study comparing amino acid limitation, AMP addition, amino acid limitation with AMP addition and temperature up-shift plasmid DNA induction strategies using a pMB1-derived plasmid and a *relA*$^-$ *E. coli* strain proved that amino acid limitation with AMP addition was the most effective pDNA amplification strategy for the expression system in question [48].

1.2.7. Culture strategies

For plasmid DNA production, three culture strategies have been proposed: batch, fed-batch with different feed profiles and continuous cultures. There are several studies discussing the influence of culture strategy on final plasmid DNA yield [172, 178-180]; however, only a few reports address the influence of culture strategy on other pDNA bioprocess features. For instance, two recent studies have addressed the influence of culture strategy on final plasmid DNA quality [90] and *E. coli* physiology and plasmid stability [181]. This first study demonstrated that higher growth rates in fed-batch culture were associated with lower percentages of supercoiled pDNA and also that sc pDNA yields and the percentages of sc pDNA were higher in fed-batch cultures when compared to the ones obtained in batch cultures [90]. The other study reported that fed-batch cultures, although yielding higher plasmid DNA amounts, exhibited lower plasmid DNA segregational stability and cell viability, with higher fluctuations in those parameters throughout the fermentation in comparison to the ones obtained in batch cultures [181].

Batch culture

The main advantage of batch culture is its simplicity. All nutrients for cell growth and plasmid production are present at the time of inoculation. The use of a suitable inoculum (1–5% of the culture volume), in the exponential phase of growth, is recommended in order to minimize the lag phase of growth. During the exponential phase of growth, all nutrients are in excess; thus the specific growth rate will be essentially the maximum specific growth rate (μ_{max}). Since slower growth rates are desirable for plasmid production, some strategies must be adopted in order to reduce the growth rate in batch cultures such as the use of lower culture temperatures and the addition of glycerol instead of glucose [179].

When compared to fed-batch strategies, current batch methods are somehow inefficient in both overall plasmid yield and in the conversion of raw materials to product. Also, the concentration of substrates, such as glycerol, yeast extract and peptones is usually very high, increasing process costs, since these materials may be expensive [180]. When *E. coli* is grown under batch conditions, due to the rapid utilization of carbon sources from the culture medium, metabolic overflow can occur. This overflow might result in an increased production of metabolic by-products such as acetate and carbon dioxide, which are known to have repressive effects on culture growth [123, 182]. Due to the lower biomass yields, plasmid DNA volumetric yields will also be lower, causing a decrease in process overall productivity [180, 183]. Some recent works using batch fermentation strategies have reported plasmid DNA yields ranging from 62.6 mg pDNA/L to 230 mg pDNA/L, which are fairly lower than the ones obtained in fed-batch fermentations [152, 181, 184].

Continuous cultures

Continuous culture may be desirable for manufacturing very large quantities of several recombinant products, such as proteins or even plasmid DNA, since an increased productivity can be obtained without the need for increasing culture volume or the number of fermentations [29]. The continuous culture also provides a constant environment that may be optimized for plasmid replication and can be more productive compared to batch operation [185].

To date, continuous culture of *E. coli* for plasmid DNA production has been frequently used with the purpose of studying plasmid stability [186-188] with only a few reports about large-scale continuous plasmid DNA production. For instance, Carnes (2007) described a new fermentation process for continuous production of plasmid DNA using recombinant *E. coli* DH5 alpha grown in a semi-defined media. This process has two stages, each of them occurring in a different culture vessel [189]. In summary, in the first stage, growth temperature is in the range of 30-32 ºC, in order to produce biomass and maintain plasmid stability and, in the second stage, growth temperature is in the range of 36-45 ºC in order to induce plasmid amplification to high plasmid yields [189]. By using this strategy, the overall plasmid productivity was 1.2 g of pDNA per hour, after the achievement of a steady state in the first vessel [189], being this the higher productivity value attained in continuous fermentation to date.

However, in the case of plasmid DNA production, it could be disadvantageous to operate in continuous mode for long periods of time due to increased plasmid DNA instability [186] that may result in a productivity decrease as run time increases [185]. This productivity decrease is due to the takeover of plasmid-free cells over plasmid-bearing cells, since they have lower metabolic burden and are able to replicate at higher rates. One could think that the existence of selective pressure in the culture medium could alleviate this plasmid instability.

However, several studies have shown that, even with antibiotics in the culture medium, there is a loss of selective pressure under intensive culture conditions such as continuous culture due to antibiotic degradation or inactivation. This could lead to product yield reduction due to segregational plasmid instability [35] and even contamination due to extended cultivation time [29].

Fed-batch culture

Although batch and continuous cultures have been proposed for plasmid DNA production, fed-batch cultures continues to be the preferred cultivation method for this production bioprocess [40, 90, 152, 163]. Fed-batch fermentations begin with a batch phase where cells are grown until nutrient exhaustion, typically the carbon source. After this nutrient exhaustion, the fed-batch phase begins with the controlled addition of a limiting nutrient allowing a greater control of culture growth rates. This growth rate control is especially important in plasmid DNA production processes since high growth rates [158-159] as well as very low growth rates are generally associated with higher plasmid instability [190]. Thus growth rate should be kept to a value close to the optimal value for the culture conditions used to prevent plasmid segregational instability. Fed-batch operations also provide higher biomass yields and, concomitantly higher product yields than batch cultures, because substrate is supplied at a rate such that leads to the nearly total consumption of the substrate. As a result, conversion of substrate to biomass is very efficient while residual substrate concentration never reaches inhibitory concentrations [29] which prevents metabolic overflow from excess substrate, reducing the formation of inhibitory acetate [157]. A very important aspect in setting up the fed-batch process is the inoculum, considering that if the inoculum is not produced correctly it will not be possible to stabilise the culture in the control range of pH and dissolved oxygen, for instance [26].

Nutrient feeding strategies

For the controlled addition of limiting nutrient in the fed-batch phase, a number of nutrient feeding strategies have been explored as a means to control cell growth in these cultures. Feeding strategies can be divided into two groups: non-feed-back control and feed-back control [178]. The non-feed-back feeding strategies includes controlling the nutrient feeding rate according to a pre-determined constant, linear, stepwise, or exponential profile [26, 40, 152] or even combinations among these profiles. The design of these feeding profiles should take into account the maximum specific growth rate of the culture (μ_{max}), since if the addition rate is higher than $\mu_{máx}$, substrate consumption will not be so efficiently, leading to substrate accumulation and possibly decreasing product yield. This type of feeding scheme also requires that the cell performs reproducibly each time in both the inoculum and early batch culture stages to warrant the match of the feeding rate to the actual cell growth rate. This is a kind of very stringent control which may work very consistently and yield very

reproducible results [152], but could easily get out of track if cell activity changes due to any environmental fluctuation [178].

Feed-back control, on the other hand, can react based on demand and is directly correlated to the cell activities actually existing throughout the fermentation. Control parameters which have been used for feed-back feeding strategies include pH, pCO_2, cell density measurement or dissolved oxygen (DO) [191-196] and a combination of pH and dissolved oxygen [163]. The DO-stat and pH-stat methods are fairly easy to implement because most standard bioreactors include DO and pH monitoring. Trends in DO and pH can indicate whether substrate is available to the cells: exhaustion of substrate decreases oxygen uptake, so DO concentration in the medium rises and pH will also rise because of an increase in cellular excretion of ammonium ions [163]. In these feeding strategies, feeding would be triggered when either DO or pH rises above a set threshold [163] and, in these cases, growth rate can then be adjusted by changing the threshold values [29].

Some examples of adopted fed-batch strategies (Figure 5)

O'Mahony *et al*. used a fed-batch process in semi-defined medium containing glucose and yeast extract [26]. They used a batch phase that was aimed at plasmid DNA production and not to biomass production, followed by an adaptative feeding strategy based on pH-DO feed-back controls [26] which led to plasmid yields of 250 mg/L [26].

Listner *et al*. reported the development of a pDNA production process, based on a defined cultivation medium using a constant feeding strategy, resulting in cultivation conditions that supported volumetric plasmid titres of more than 1600 mg/L, while achieving specific yields ranging from 25 to 32 mg/g dcw [40]. When used for the production of clinical supplies, this novel process demonstrated applicability upon scale-up in 2,000 L bioreactors [40].

Carnes *et al*. used an inducible fed-batch process in semi-defined medium containing glycerol and yeast extract with exponential and constant feeding strategies [152]. Plasmid induction was performed with a temperature up-shift from 30 to 42 ºC [152]. The constant feeding strategy led to plasmid yields of 620 mg/L [152] and the exponential feeding strategy, set at a desired growth rate of 0.12 h^{-1}, led to plasmid yields of up to 2200 mg/L [41].

Singer *et al*. also evaluated plasmid DNA production in several *E. coli* strains cultivated in a defined medium using a fed-batch strategy with an exponential glucose feeding rate for attaining pre-determined specific growth rates of 0.14 and 0.28 h^{-1}. The results showed that maximum plasmid yields, of approximately 600 mg pDNA/L were obtained with *E. coli* SCS1-L with an exponential feeding of 0.28 h^{-1} [43].

Mairhofer *et al*. attained a plasmid DNA volumetric yield of 938.80 mg/L and a maximum specific yield of 25.85 mg/d dcw by cultivating an *E. coli* strain JM108 harbouring a selective

marker-free plasmid, in a defined medium using a fed-batch strategy with an exponential feeding profile of 0.1 h^{-1} [44].

Phue *et al*. studied fed-batch production of plasmid DNA in several *E. coli* strains cultivated in a semi-defined medium containing glycerol or glucose as carbon sources. They found that maximum plasmid yields (1923 mg/L) were obtained by growing an engineered *E. coli* BL21 in semi-defined medium containing glycerol as carbon source using a fed-batch strategy with a DO-feed-back feeding strategy and plasmid DNA induction by temperature up-shift from 30 to 42 ºC [197].

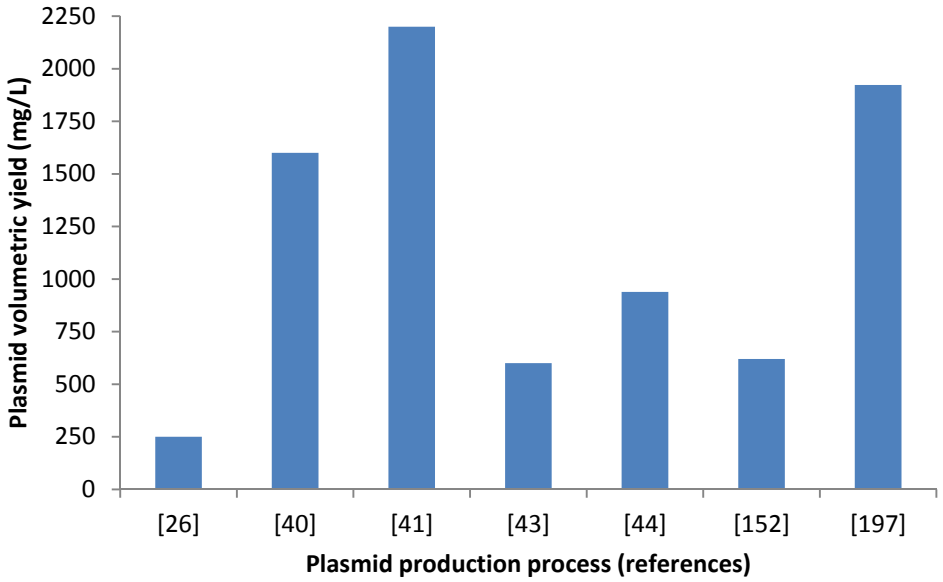

Figure 5: Plasmid yields from several fermentation processes.

1.3. Monitoring techniques for recombinant process control

In order to fulfil the growing demand for bio-based products, such as recombinant proteins or plasmid DNA, and due to the complex nature of microbial growth and product formation in recombinant fermentations, bioprocess monitoring represents an ever-increasing engineering challenge. In order to achieve maximal exploitation of the production organism, the advancement of bioprocesses towards the improvement of monitoring and control tools is crucial to increase product yield and quality, so that production costs can be reduced.

Despite this ever-growing demand for better, more sensitive bioprocess monitoring tools, the currently available techniques and tools can provide researchers with a substantial amount of information towards bioprocess optimization. Most of these techniques are based in bioprocess at-line or off-line monitoring through sampling of the culture media; however, to achieve further optimization, it is necessary that these techniques evolve to ensure real-time

monitoring of all critical parameters. When focusing on plasmid DNA production processes, there are two fundamental key parameters that must be constantly monitored in order to achieve higher plasmid DNA yields: host cell physiology and plasmid stability.

1.3.1. Monitoring *E. coli* cell physiology

In order to monitor cell physiology several techniques have been proposed such as flow cytometry and fluorescence-based techniques, proteomic and genomic analysis and metabolic flux analysis with labelled glucose (Table 7).

Regarding the study of bacterial physiology, fluorescence-based techniques such as flow cytometry or two-dimensional (2D) fluorometry have gained relevance in recent years. Two-dimensional (2D) fluorometry and other fluorescence spectroscopy methods potentially represents a useful option for online analysis of bioprocesses due to the fact that this is a non-destructive, non-invasive and relatively inexpensive analytical method with relative high sensitivity and selectivity to detect changes in the various fluorophores typically found in culture media such as amino acids (tryptophan, tyrosine, and phenylalanine), vitamins (pyridoxine, riboflavin) and cellular cofactors [flavin adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide (NADH) and its phosphate derivative (NADPH)] [198]. In fact, these characteristics have justified its use in real-time monitoring of numerous bacterial and yeast fermentations [97, 199-200].

Although the goal of flow cytometry and other fluorescence techniques is to be able to monitor both the isolated cell and the overall population, a deeper knowledge of what is happening inside the bacterial cell is needed. Expression profiling by DNA microarrays is a powerful tool for analysing gene expression at a genomic scale and can be used to compare global changes in gene expression that occur in response to environmental conditions or to compare the effects of genetic changes on gene expression [201]. DNA microarrays can simultaneously measure the expression level of thousands of genes within a particular mRNA sample [202], namely those involved in *E. coli* biosynthetic pathways, transport, energy metabolism, stress response, carbohydrate metabolism, transcriptional regulation and cell division [38]. So far, the high-throughput expression profiling enabled DNA microarrays to be successfully used to quantify gene expression alterations in *E. coli* as a result of heat-shock, cold-shock, high cell density cultures and plasmid-induced metabolic stresses [38, 83, 111, 170]. Despite being an efficient tool to provide a complete picture of the physiological state of stressed cells, DNA microarrays are time-consuming and their complex manufacture hamper these methods to be useful for online monitoring [203].

Table 7: Techniques for monitoring *E. coli* metabolic burden.

Technique	Application	Advantages	Limitations	References
Flow cytometry	Cell size Cell viability Cell cycle	Fast and sensitive Single cell analysis Multi-parametric analysis	At-line or off-line monitoring Invasive technique Relatively expensive	[48, 113, 204-210]
Fluorescence spectroscopy	Metabolic changes by measuring intracellular NADH, FAD, tryptophan and pyridoxine	Non-destructive Non-invasive Relatively inexpensive Enables online monitoring	Whole population analysis	[97, 198-200, 211-212]
DNA microarrays	Transcriptional analysis of *E. coli* gene alteration	High-throughput technique Fast and sensitive	Expensive Laborious sample treatment Off-line analysis	[83, 201-203]
2D polyacrylamide electrophoresis	Proteomic analysis of *E. coli*	Analysis of large datasets Relatively inexpensive Visual proteome representation (isoform identification)	Off-line analysis Laborious sample treatment and protocol Low detection sensitivity Requires confirmation by mass spectrometry	[114, 213-215]
^{13}C flux analysis	Recombinant *E. coli* metabolic flux analysis	Relatively inexpensive More accurate results for metabolic engineering Efficient flux computation Enables gross error detection	Requires MS or NMR analysis Relatively laborious Off-line analysis	[110, 216-217]

Although DNA microarrays provide information about gene expression, protein expression is not monitored, which can lead to misleading results due to a possible impairment of translation mechanisms. Most of quantitative proteomics studies have relied on two-dimensional (2D) gel electrophoresis combined with protein identification by mass spectrometry (MS) to analyze large datasets of proteins from complex protein mixtures [213-214]. This technique was already successfully applied to bioprocess monitoring in order to evaluate stress response on protein level due to heat-shock, cold-shock, oxidative stress, nutrient deprivation and plasmid-induced stress in recombinant *E. coli* fermentations [114, 134, 218-219]. Although 2D electrophoresis has promoted several metabolic studies in recombinant *E. coli*, the limited dynamic range caused by low detection sensitivity, the saturation of protein staining, and insufficient spot resolution from overlapping and co-migrating protein spots have impaired the accuracy and depth of protein quantitation in 2D gels [215]. In order to circumvent such limitations, alternative protein quantitation strategies have been proposed such as shotgun proteomics [220], peptide or protein labelling, and label-free strategies [215] such as absolute protein expression (APEX) technique [221]. Although being inexpensive and versatile, 2D electrophoresis and other MS based proteomic analysis do not provide a high-throughput platform for protein analysis. Therefore, in order to bypass this limitation, protein microarray technologies were developed to expand our understanding of protein function, quantitative proteomics, molecular interactions and protein profiling [222-223].

Whereas transcriptional and translational analysis provided clues about the redirection of metabolic pathways, there is the need to specifically detail all metabolic fluxes ongoing inside the cell. In recent years, metabolic flux analysis (MFA) has become one of the major tools in metabolic engineering [224]. The goal of MFA is the detailed quantification of all metabolic fluxes in the central metabolism of a microorganism [224]. The result is a flux map that shows the distribution of anabolic and catabolic fluxes that comprise the metabolic network, forming a major basis for analyzing and redesigning metabolic networks [217]. One of such flux analysis is based on ^{13}C MFA which has been used in analyzing common encountered underdetermined metabolic networks [217]. This analysis was already successfully applied to glucose-limited *Escherichia coli* chemostat culture for the analysis of intracellular fluxes in glycolysis, pentose-phosphate pathway and TCA cycle [217]. This methodology was also applied to the study of *E. coli* plasmid-induce metabolic burden using ^{13}C-labelled glucose in combination with microarray analysis to study the influence of plasmid DNA on central metabolism by comparison to plasmid-free cells [110]. Although being able to generate relevant data for bioprocess design, metabolic flux analysis requires MS or NMR analysis, is relatively laborious and only enables an off-line analysis of bioprocesses.

Flow cytometric monitoring of cell physiology

The evaluation of cell physiology is extremely relevant in the investigation of the impact of plasmids in host cell metabolism. Moreover, the presence of a high number of dead or dormant cells during cultivation will have a detrimental effect on the synthesis of any desired product [225]. Flow cytometry (FCM) is emerging as an important tool in microbiology [226-227]. Bacteria are relatively difficult to analyze by flow cytometry, mostly due to their reduced size and low concentrations of constituents [228]. However, technical advances in instrumentation and methodology are leading to an increased popularity of flow cytometry and to an extended range of its applications to biotechnology [48, 113, 204-208] enabling the characterization of individual cells in populations, according to properties such as DNA content, protein content, viability and enzyme activities [209], among others (Table 8).

Due to the single cell analysis enabled by flow cytometry, this technique provides information about population heterogeneity, which is of great relevance in bioprocess monitoring due to the fact that bacterial populations are often heterogeneous and contain several subsets of cells with different properties [205, 229]. Without the use of a fluorescent stain, flow cytometry allows the evaluation of cell size and complexity through forward (FSC) and side scatter (SSC) light measurements, respectively. Since FSC is an indicator of cell size, this parameter is useful to evaluate cell filamentation, a common phenomenon in recombinant fermentations [48, 76]. In bacteria, SSC can be used to assess the properties of the cell wall, ribosome content or amount of macromolecules per cell [230]. SSC measurements were also used to distinguish between dividing and non-dividing bacteria, since this value was found to be considerably higher in dividing than in non-dividing cells [230].

For viability and physiological state assessment, a number of fluorescent stains were made available in recent years (Table 8). Propidium iodide incorporation is commonly used as an indicator of bacterial viability, since propidium iodide can only enter in bacterial cells with permeabilized membranes where it binds to nucleic acids. Other cell functions commonly assessed by flow cytometry comprise membrane polarization, enzyme activity and pump activity [231]. Another important cell feature that can be analysed by flow cytometry is cell cycle [232]. Flow cytometric cell cycle evaluation is performed through the analysis of bacterial DNA content stained with a DNA-specific fluorescent stain, such as DAPI, Hoechst dyes or DRAQ5, or using a combined RNase treatment with a nucleic acid fluorescent stain such as propidium iodide [48, 233-235]. The resulting DNA fluorescence histograms provide information about the duration of cell cycle phases as well as the proportion of cells within each phase, thus giving pivotal information about the specific proliferation behaviour of the host strain [48, 234]. Another recent method for evaluating cell proliferation was described by Roostalu and co-workers [230] using GFP. In this method, GFP expression is induced in the host cell containing the GFP-expressing plasmid and, after the removal of the inducer, these cells are placed in fresh medium and the decrease in GFP fluorescence is monitored by flow

cytometry. This decrease in GFP fluorescence is a good indicator of cell division since it could only be the result of the reduction in the number of GFP molecules per cell that can only happen when cells divide.

Overall, the vast majority of flow cytometric measurements described for recombinant bioprocesses provide the means to perform at-line or off-line monitoring. Notwithstanding, given the diversity of important cellular functions that can be assessed by flow cytometry and the speed with which these analysis can be performed, this technique can be a valuable tool for online process control, thus avoiding sampling procedures and obtaining information during the production process in order to perform the necessary alterations [124]. To achieve this online monitoring ability, flow cytometers have been coupled to a flow-injection analysis and flow cytometric methods have been automated [236-239].

Table 8: Flow cytometry analysis of cellular functions and intracellular content in microbial cells.

Parameter/Application	Fluorescent stain	References
Membrane permeability	Propidium iodide (PI)	[48, 240-241]
	SYTOX Orange	[242]
	TO-PRO 3	[243]
	Ethidium monoazide	[244]
Cell size	----	[48]
Total cell counts	SYTO 9	[241]
	SYTO 17	[245]
	SYTO 13	[245]
Total RNA content	PI with DNase treatment	[246]
	SYTO RNAselect	[247]
Glucose uptake activity	2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG)	[248]
Total protein content	Fluorescein isothiocyanate (FITC)	[249]
Specific protein content	GFP-reporter gene	[250]
	Quantum Red-labelled specific antibody	[249]
DNA content (cell cycle)	Ethidium bromide with mithramycin A	[234]
	Hoechst 33258	[249]
	Vybrant DyeCycle Ruby	[210]
	DRAQ5	[204]
Membrane potential	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol	[48, 240]
	Rhodamine 123 (Rh 123)	[245]
	3,3'-diethyloxacarbocyanine iodide (DiOC2(3))	[243]
Efflux activity	Ethidium bromide	[225, 231]
Total lipid content	Nile Red	[251]
Metabolic activity	ChemChrome V6 (CV6)	[252]
	5-cyano-2,3-ditolyl tetrazolium chloride (CTC)	[253]
	Fluorescein diacetate (FDA)	[254]

1.3.2. Monitoring plasmid stability

Plasmid stability is a key issue in *E. coli* recombinant fermentations, both for protein production and for DNA therapeutics since it affects product yield and quality. The main two types of plasmid stability commonly considered in recombinant fermentations are structural and segregational stability; however, when dealing with plasmid DNA-based therapeutics, isoform stability is also of great relevance [93]. Concerning plasmid stability, monitoring techniques include agarose gel visualization, standard and real-time PCR, capillary gel electrophoresis, fluorescence-based techniques and hybridization/chip-based methods (Table 9).

When assessing plasmid structural stability, the method for analysing the structural change depends on the kind of alteration. The methods described herein for mutational analysis of plasmid DNA are commonly used molecular biology methods that have been extensively used to determine mutations in eukaryotic cells. Insertions and deletions can be easily detected by restriction analysis and subsequent comparison of the fragments length by agarose or polyacrylamide gel electrophoresis [255]. This mutational fragment analysis can also be performed using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) after enzyme restriction [256].

According to the European Pharmacopoeia [257], when working with plasmid based expression systems, the structural integrity of the plasmid should be verified from time to time by sequencing the whole nucleotide sequence of the plasmid and also, restriction endonuclease digestion should be performed with sufficient resolution to verify that the structure of the plasmid is unaltered after cultivation in bacterial cells [257]. The determination of point mutations is much more complicated than deletion or insertion detection. Besides DNA sequencing methods, point mutations can be determined using DNA sensors [258] where DNA mutations are visualized due to the selectivity of the hybridization reactions between the immobilized template DNA and sample DNA [259]. When working with known DNA segments, point mutations can be detected by PCR followed by gel electrophoresis and also by temperature gradient gel electrophoresis (TGGE). Capillary electrophoresis is also able to separate and verify point mutations. Although capillary electrophoresis is based on ultra-violet (UV) absorption measurements of samples, in order to improve the sensitivity of this method, it is needed to use fluorescent additives [260]. The use of several defined sequences with specific markers allows the simultaneous determination of point mutations in different regions of long DNA fragments [261].

Traditionally, identification of plasmid isoforms has been routinely assessed by agarose gel electrophoresis with ethidium bromide staining due to the distinct migration pattern of each isoform in the gel [76]; however, additional analytical techniques have been described for this purpose, such as chromatographic techniques and capillary gel electrophoresis (CGE)

[262-263]. Both these methods are preferred over agarose gel electrophoresis quantification owing to the fact that this technique is time-consuming and involves a manual procedure while chromatography and capillary gel electrophoresis can be subjected to automation and provide a standard processing of the data obtained [264]. Concerning the chromatographic methods, isoform quantification is mainly performed using anion-exchange chromatography [265-266] although recently an affinity-based chromatographic approach has been described for the quantification of the supercoiled isoform in *E. coli* cell lysate samples [267]. However, so far, the vast majority of these chromatographic methods have focused on the analysis of the supercoiled and open circular plasmid DNA isoforms. Despite being a more time-consuming technique, a method based on capillary gel electrophoresis was described for the successful analysis of all three plasmid DNA isoforms [264].

Plasmid segregational stability monitoring

Regarding plasmid segregational stability, the most frequent type of stability studied in recombinant fermentations, monitoring methods have evolved greatly. One of the first methods for plasmid stability monitoring was based on classical microbiology techniques and took advantage of the antibiotic resistance marker contained in the plasmid for selection purposes [101, 268-270]. This method, although cheap, simple and reproducible, was very time consuming and did not allow researchers to have a fast knowledge whether the process conditions had impact on plasmid segregational stability.

With the development of molecular biology techniques, several methods for plasmid copy number (PCN) assessment, another way for segregational stability monitoring, were developed. These methods can be divided in two major groups: indirect or direct determinations [271]. One example of an indirect method is based on the determination of plasmid copy number by measuring the activity of a reporter protein coded by the plasmid, that is later normalized to the number of bacterial cells contained in the fermentation sample [272]. With respect to direct methods, they are usually based in the quantification of plasmid and chromosomal DNA quantities followed by the calculation of the ratio between them. These methods include commonly used methods for nucleic acid analysis and quantification such as caesium chloride centrifugation, Southern blot hybridization, high performance liquid chromatography (HPLC), capillary electrophoresis [273] and agarose gel electrophoresis.

Regarding direct methods, agarose gel electrophoresis continues to be the most frequently used method for PCN determination. In these methods, the fermentation sample is subjected to cell lysis with subsequent gDNA and pDNA extraction [274]. After the extraction procedure, samples are placed on an agarose gel for nucleic acid analysis and quantification by band density [274]. Notwithstanding, this method requires DNA extraction, which can led to an inaccurate PCN determination due to incomplete recovery and the variability in the

extraction and precipitation procedures. Also, agarose gel electrophoresis is a simple and low cost technique; however, due to the lysis and extraction procedures, this process is somehow laborious. With the recent development of PCR methodologies, real-time quantitative PCR (qPCR) technology offers a fast and reliable quantification of any target sequence in a sample [275] which continues, to date, to be the most sensitive and precise method for nucleic acid quantitation.

When assessing PCN by real-time quantitative PCR, there are two types of quantification that can be used: absolute and relative quantification [276]. Absolute quantification determines the exact copy concentration of target gene by relating the cycle threshold (Ct) value to a standard curve [276]. Relative quantification presents the amount of target gene in a sample relative to another sample (a calibrator), which contains both target and reference genes within a constant ratio [276]. These two types of quantification can be successfully applied to assess plasmid copy number in bioprocesses, yielding similar PCN results [276]. However, the method described earlier still required DNA extraction procedures that could have a detrimental effect on plasmid copy number. In order to avoid this extraction step, in recent years, some protocols for PCN assessment by real-time qPCR using whole cells, either bacterial [48, 271] or eukaryotic [277] were developed.

The methods described by Carapuça *et al*. [277] and Silva *et al*. [48] use a combined relative and absolute quantification. In these cases, PCN standards are prepared by spiking purified plasmid DNA with non-transformed cells. The standard curve obtained with these standards is, afterwards, used to quantify PCN in fermentation samples, keeping cell concentration constant and equal to the one used to prepare the standards. Skulj and collaborators [271] described another relative quantification method where PCN is determined taking into account different amplification efficiencies for both genomic and plasmid DNA.

Overall, real-time quantitative PCR on whole cells provide a feasible approach for time-course monitoring of plasmid copy number since it only requires sampling and, possibly, a dilution in order to maintain cell density at a constant value. Since the real-time qPCR assay itself can take approximately one hour, these approaches using whole cells can be used for at-line monitoring of recombinant bioprocesses.

Table 9: Techniques for monitoring plasmid DNA stability.

Technique	Application	Advantages	Limitations	References
Plasmid DNA stability				
Classical microbiology techniques	Determination of *E. coli* percentage of P^+ cells	Simple Low cost	Time-consuming Off-line analysis	[101, 268-270]
Agarose gel electrophoresis	Determination of plasmid segregational stability, isoform stability	Simple Relatively inexpensive Fast Enables visual observation	Off-line analysis Low sensitivity Lower reproducibility Requires sample treatment	[48, 76, 255, 274]
Real-time quantitative PCR	Determination of relative and absolute PCN	Whole cell analysis (no cell lysis) Relatively fast Simple Time-course monitoring	At-line analysis Relatively expensive	[48, 271, 276-277]
DNA sensors	Determination of point mutations	Fast Sensitive Selective	Off-line monitoring Requires sample treatment Expensive	[258-259].
Capillary electrophoresis	Determination of mutations and plasmid isoforms	Sensitive (using fluorescent stains) Possible automation High resolution	Requires sample treatment At-line monitoring	[260-264]
Liquid chromatography	Quantification of plasmid DNA isoforms	Isoform quantification Possible automation Low cost High reproducibility	No visual observation Lack of resolution among the three plasmid isoforms	[265-267]

1.4. Aims of the Thesis

Despite the growing interest on plasmid DNA as a therapeutic, much of the bioprocess designs proposed focus in the downstream processing of plasmid DNA. Despite all the effort done in the last decade in order to improve plasmid DNA yield in recombinant microorganisms such as *Escherichia coli*, the best yield does not correspond necessarily to the best process performance. Therefore, the first goal of this thesis was to develop and apply new tools to study cell physiology and plasmid stability, together with overall plasmid yield, in order to improve process performance. The specific goals were:

- To assess the influence of culture conditions on plasmid DNA yield;
- To develop new methods to study cell growth during fermentation using flow cytometry;
- To evaluate the impact of induction strategies on cell physiology, plasmid stability and plasmid DNA yield;
- To optimize plasmid DNA yields using a semi-defined medium and several fermentation strategies, evaluating their influence on cell physiology and plasmid stability.

Chapter II

2. Results

2.1. Influence of growth conditions on plasmid DNA production

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2.1.1. Abstract

The obtention of high yields of purified plasmid DNA is viewed as essential issue to be considered towards efficient production of DNA vaccines and therapeutic plasmids. In this work, *Escherichia coli* DH5α bearing pVAX1-LacZ plasmid was grown in a developed semi-defined medium at different temperatures and tryptone concentrations. Analysis of pDNA yields and *E. coli* morphology revealed that at higher temperatures (37 and 40 ºC) higher specific yields and *E. coli* filamentation were obtained. However, the best results were achieved when a lower tryptone concentration was used. This approach was shown to be a powerful tool to promote plasmid amplification, keeping the desirable plasmid structure and favor the attainment of quality. Our results suggest that by using tryptone alone as an amino acid source, pDNA amplification was improved and a specific yield of 20.43 mg pDNA/g dcw was achieved, proving that this strategy can improve pDNA yield even at small scale.

Keywords: plasmid DNA; Fermentation; Filamentous Bacteria; Microbial Growth; Temperature; Tryptone

2.1.2. Introduction

The expansion and significant understanding of DNA technology is conducting to an increased relevance of nucleic acids in biological sciences. Indeed, the development of DNA-based biopharmaceuticals to be applied in several clinical therapies, like gene therapy or DNA vaccination is attaining impact within original research and industry projects [1-2]. For gene therapy applications, non-viral vectors based on bacterial plasmid DNA, normally produced in *Escherichia coli* (*E. coli*), would be preferred since they offer a safer method for delivering large quantities of genetic material to cells [3-4]. Despite these vectors were firstly characterized as inefficient in transfection studies [5], the increased effectiveness recently achieved have made naked DNA gene transfer a suitable method for gene therapy [6]. Thus, for a best design of all the process a deep knowledge of pDNA technology in the host cell must be achieved. In fact, high yields of highly purified plasmid DNA product is viewed as

essential issue to be considered towards efficient production of DNA vaccines and therapeutic plasmids [7].

Since pDNA accounts for less than 3 % of the total contents of an *E. coli* cell (depending on plasmid size and copy number) [3], the main goal in the upstream stage relies in maximizing the amount of pDNA produced, which could be accomplished by maximizing the final cell concentration and the average plasmid copy number [8]. In general, the improvement of plasmid copy number production and more specifically the reduction in contaminants can have an accentuated impact in all downstream processing, leading to easier and reliable economic purification processes. In order to improve plasmid copy number on *E. coli* cell, several approaches have been described. These strategies include the use of a temperature up-shift [9-10], addition of chloramphenicol [11], amino acid starvation or limitation [12-13] and reduced growth rates [14-15].

It is well known that optimal purity can be achieved by maximizing the average supercoiled-plasmid copy number [16]. So, plasmid amplification can lead to higher pDNA specific yields, which is translated in low percentage of key contaminants present in *E. coli* lysate such as RNA, denatured genomic DNA, proteins and lipopolysaccharides (LPS) [17] as well as other forms of pDNA, therapeutically less effective. In this way, the pDNA should be mainly produced in the supercoiled isoform, since regulatory agencies recognize that other forms of DNA, including the open circular and linear forms, may be less effective than the supercoiled form [3]. Although all these forms are produced during host cell growth, the open circular and linear forms may also result from damage in the supercoiled structure at any stage in the process [18-19]. Some new purification strategies of the supercoiled plasmid DNA isoform have been proposed [20-21].

All these concepts are only possible by manipulation of well known microorganisms. In fact, *E. coli* recombinant strains are industrially important microorganisms and largely used in bioprocesses, for being easy to produce and because of their well characterized genetics and cellular metabolism [22]. In this work, the influence of growth temperature and tryptone concentration on plasmid DNA amplification, using a previously developed semi-defined fermentation medium, was studied.

2.1.3. Materials and Methods

Plasmid and bacterial strain

The bacterial host for plasmid pVAX1-*LacZ* was *E. coli* DH5 alpha [F^- ϕ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, thi-1, gyrA96, relA1, tonA]. The plasmid pVAX1-*LacZ* (Invitrogen, Carlsbad, U.S.A) is 6.05 kbp long and has a pUC origin of replication. It also contains a cytomegalovirus (CMV) promoter and a kanamycin resistance gene for selection purposes. The host strain was maintained in 30% (v/v) glycerol at -80 ºC.

The bacterial strain is a relA mutant ($relA^-$) which, in response to amino acid starvation or limitation, causes pDNA amplification [12]. The plasmid contains an origin of replication, which allows an increased plasmid copy number at 37-42 ºC, but not at 30-32 ºC [9].

Fermentation media

The expression system was cultivated in a semi-defined medium developed in this work containing (per L of water): KH_2PO_4, 2.75 g; Na_2HPO_4, 5.5 g; NaCl, 0.5 g; citric acid monohydrate, 1.63 g; potassium citrate tribasic monohydrate, 2.0 g; sodium citrate dehydrate, 0.67 g; glycerol, 30 g and tryptone, 20 g. Glycerol is used as the carbon source and tryptone as a nitrogen source. A 5 M NaOH solution was used to adjust the pH to 7.2, prior to autoclaving. After sterilization, 1 mL of a kanamycin solution 30 mg/mL, 4.15 mL of a supplement solution, and 1 mL of a trace minerals solution, were added separately after filter sterilization. The supplement solution contained (per L of water): thiamine-HCl, 24 g; $MgSO_4.7H_2O$, 240 g and the trace mineral solution contained (per L of 1.2 M hydrochloric acid): $FeCl_3.6H_2O$, 27 g; $ZnCl_2$, 2 g; $CoCl_2.6H_2O$, 2 g; $Na_2MoO_4.2H_2O$, 2 g; $CaCl_2.2H_2O$, 1 g; $CuCl_2.2H_2O$, 1.3 g; and H_3BO_3, 0.5 g. In all studies performed, cultures were started with an OD_{600} of approximately 0.2, grown in 250 mL shake flasks containing 62.5 mL of medium, at 250 rpm, and using the appropriate temperature in each experiment. Growth was suspended at late log phase.

Tryptone limitation

Growth was performed in the semi-defined medium developed in this work, but supplemented with different tryptone concentrations. Tryptone is composed by a mixture of amino acids and is used as nitrogen source. In order to alleviate the interference of tryptone concentration present in the pre-fermentation medium, the bacterial cells from the medium with an approximate OD of 2.6 were harvested by centrifugation of the culture at 5445 x *g* during 30 min at 4 ºC and the pellet was washed twice with an equal volume of 0.9 % NaCl and resuspended in the semi-defined medium [23]. Amino acid limitation was achieved by the cultivation of bacteria in the semi-defined medium containing different tryptone concentrations (g/L): 0.5, 1.0, 3.0 and 5.0. Cells were cultivated until the stationary phase of growth was reached. In order to calculate the amplification factor all samples were collected

in the stationary phase of growth to prevent calculation errors, since plasmid DNA content in bacterial cell during fermentation is highly dynamic, leading to a strong increase of the specific pDNA yield in the middle of fermentation time [24].

To ensure that tryptone was the limiting nutrient, glycerol concentration was kept to a non limiting level.

Determination of cell density and dry cell weight

Cell density (OD_{600}) was measured spectrophotometrically. For dry cell weight assay, aliquots (1 ml) of fermentation culture were centrifuged at 10 000 x *g* for 10 min in pre-weighed tubes and the pellet washed twice with an equal volume of 0.9 % (w/v) NaCl solution. Pellets were dried to a constant weight at 85 °C for, at least, 24 h [25]. The dry cell weight was calculated from the average of three independent samples. One unit of OD_{600} was found to correspond to a dry cell weight of 0.25 g/L.

Lysis and primary isolation

Cells were recovered by centrifugation and stored at -20 ºC. Cell pellets were lysed using a modified alkaline lysis protocol as described previously [20]. Briefly, the bacterial pellets were resuspended in 10 mL of 50 mM glucose, 25 mM Tris-HCl and 10 mM EDTA (pH 8.0). Lysis was performed by adding 10 mL of a 200 mM NaOH and 1% (w/v) SDS solution. After 5 min of incubation at room temperature (20 ºC), cellular debris, genomic DNA and proteins were precipitated by gently adding and mixing 8 mL of prechilled 3 M potassium acetate (pH 5.0). The precipitate was removed by centrifuging twice at 20 000 x *g* for 30 min in the first centrifugation and 15 min in the second one at 4 ºC with a Sigma 3-18K centrifuge. The pDNA in the supernatant was precipitated by adding 0.7 vol. of propan-2-ol. The pDNA was recovered by centrifugation at 15 000 x *g* for 30 min at 4 ºC. The pellets were then redissolved in 0.5 mL of 10 mM Tris/HCl buffer (pH 8.0). Next, $(NH_4)_2SO_4$ was dissolved in the pDNA solution up to a final concentration of 2.5 M, followed by 15 min incubation on ice. Precipitated proteins and RNA were removed by centrifugation at 10 000 x *g* for 20 min at 4 ºC. After a suitable dilution, the supernatant was loaded onto the HPLC system and the agarose gel.

Analytical chromatography

HPLC was used to measure pDNA concentration and purity in *E. coli* lysates according to the method described by Diogo *et al*. [26]. A 4.6/100 mm HIC Source 15 PHE PE column from Amersham Biosciences was connected to a Waters HPLC system (Waters, Milford, MA, U.S.A.). The column was initially equilibrated with 1.5 M $(NH_4)_2SO_4$ in 10 mM Tris/HCl (pH 8.0). Samples (20 μL) were injected, and the column was eluted at a flow rate of 1 mL/min. After injection, elution with 1.5 M $(NH_4)_2SO_4$ in buffer occurred for 2 min, then the elution buffer was instantaneously changed to 0 M $(NH_4)_2SO_4$. This last elution condition was maintained for

5 min to elute bound species. The absorbance of the eluate at 254 nm was continuously recorded.

The concentration of pDNA in each sample was calculated using a calibration graph constructed with pDNA standards (1-400 mg/L) purified with a commercial Qiagen kit (Hilden, Germany) according to the manufacturer instructions. The degree of purity was defined as the percentage of the pDNA peak related to the total area of all chromatographic peaks. pDNA concentration and purity degree were calculated as the mean of three independent samples.

Agarose-gel electrophoresis

An agarose gel electrophoresis analysis (110V, 40 min) was performed using 0.8 % agarose gel in TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) buffer in the presence of 0.5 µg/mL ethidium bromide.

2.1.4. Results and Discussion

To improve specific pDNA yields and, consequently, reduce *E. coli* host contaminants, the most suitable approach is a strategy that leads to plasmid amplification in order to maximize plasmid copy number by *E. coli* cell and to minimize the metabolic burden imposed by plasmid replication. Two strategies were evaluated: growth temperature and amino acid limitation.

In order to establish the best growth temperature, cells were grown at 30, 32, 37 and 40 ºC. Typically, individual cells of *E. coli* are rod-shaped and are approximately 800 nm wide and 2.5 µm long [27]. In fact, the cells grown at 30 and 32 ºC (Figure 6A and 6B, respectively) exhibit this typical morphology. In contrast, cells grown at 37 and 40 ºC (Figure 6C and 6D, respectively) show a filamentous morphology essentially found when cells elongate and replicate their DNA, but do not septate and divide [27].

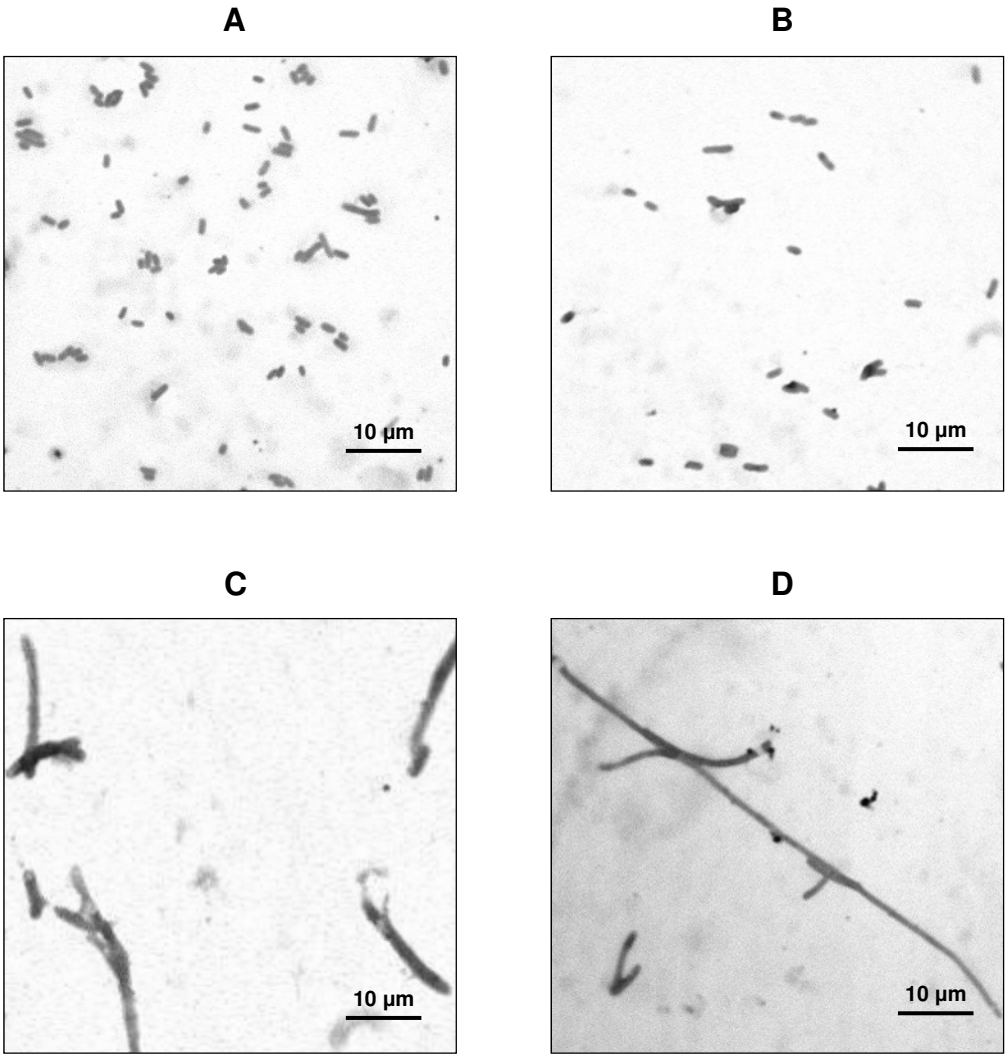

Figure 6: Safranin staining of cellular suspension collected at late log phase of *E. coli* DH5 alpha harbouring plasmid pVAX1-LacZ.

Growth was performed in a semi-defined media containing glycerol 30 g/L and tryptone 20 g/L at different temperatures:(A) 30 ºC; (B) 32 ºC; (C) 37 ºC; (D) 40 ºC.

With relation to *E. coli* morphology, it has been described that nutrient starved cells [28] do not apparently exhibit filamentous structure, but this phenomenon is activated as a stress response to oxidative stress [29] or to an imposed metabolic burden. It is known that a high-copy number plasmid maintenance and replication imposes a metabolic burden in *E. coli* DH5 alpha, resulting in down regulation of cell wall biosynthetic genes [30]. In practice, this effect is often associated with reduced growth rates and final biomass concentration [30], supporting the results obtained for the temperatures of 37 and 40 ºC that showed lower specific growth rate and a decrease in the final biomass concentration (Table 10), when compared with the lower temperatures under study.

Table 10: Effect of growth temperature in specific growth rate, final biomass concentration, plasmid yield and purity degree.

Growth temperature (ºC)	Specific growth rate (h^{-1})	Final biomass concentration (g dcw/L)	Specific yield[a] (mg pDNA/g dcw)	Purity degree[a] (%)
30	0.24	2.20	1.22 ± 0.03	12.06 ± 0.17
32	0.25	2.44	1.60 ± 0.04	13.91 ± 0.17
37	0.17	1.93	4.60 ± 0.09	35.68 ± 0.74
40	0.15	1.31	5.56 ± 0.13	45.72 ± 0.71

[a] Mean results of three experiments are presented ±standard deviation (SD).

Furthermore, due to the presence of a replication origin pUC, higher specific pDNA yields (4.60 and 5.56 mg pDNA/g dcw) and purity degrees (35.68% and 45.72%) were obtained at 37 and 40ºC, respectively (Table 10). This result can be probably interpreted as synonyms of higher plasmid copy number by host cells at these temperatures. In fact, the enhanced plasmid amplification induced by temperature can impose an elevated metabolic burden, probably caused by an inhibition of cell wall synthesis [30] leading to an accentuated filamentation of cells (Figure 6C and 6D). As this filamentation is not observed when plasmid-free *E. coli* DH5 alpha is grown in the same temperature set conditions (data not shown), we can conclude that this morphology variation is not temperature-induced, corroborating the idea of increased pDNA amplification at higher growth temperature. Other studies have already documented an existing filamentation in *E. coli* cells grown at 37 ºC [9], but they did not evaluate if this filamentation was a stress response of the host strain itself to growth conditions or an imposed metabolic burden by plasmid DNA replication, as our results suggests.

In contrast, the experiments performed at 30 and 32 ºC showed lower specific pDNA yields (1.22 and 1.60 mg pDNA/g dcw) and purity degrees (12.06% and 13.91%), respectively (Table 10). This drop off in these parameters values and the higher percentage of RNA as a contaminant (Figure 7, lanes 1 and 2), suggest that plasmid amplification occurred with lower extent at these temperature conditions, what is in accordance with the behaviour previously described [9], and could also explain the typical *E. coli* morphology observed (Figure 6A and 6B). Despite the percentage of RNA seems higher at 37 ºC (Figure 7, lane 3), the high values obtained for purity degree and specific yield proves that this fact was caused by the increased cell growth and plasmid production, in the supercoiled pDNA form in detriment of open circular pDNA structure.

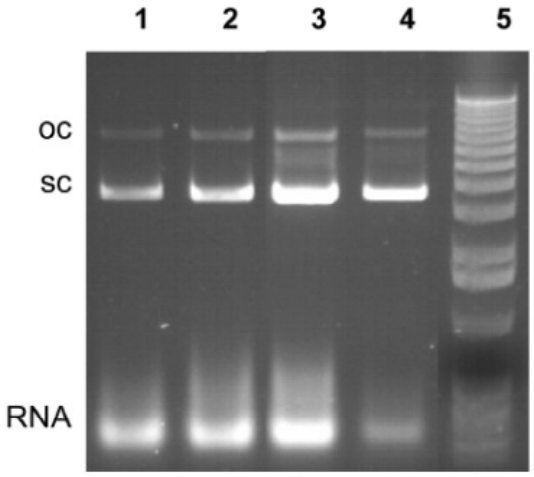

Figure 7: Agarose gel electrophoresis of cell lysate resultant from fermentations at different temperatures.

The temperatures used were: 30 ºC (lane 1), 32 ºC (lane 2), 37 ºC (lane 3) and 40 ºC (lane 4) and molecular weight marker (lane 5). Abbreviations: oc (open circular pDNA), sc (supercoiled pDNA).

The fermentation performed at 40 ºC exhibited the highest specific yield and purity degree (Table 10), with a lower percentage of RNA (Figure 7, lane 4) and the majority of pDNA in the supercoiled form. Nevertheless, this is not the best process for plasmid DNA production, since the maintenance and its synthesis impose several stress response on the bacterial host, leading to a reduced host growth and viability [31]. In addition, the detrimental effect of this plasmid-imposed metabolic burden can lead to an increase of cells-free plasmid with faster grow kinetics during fermentation. Over time, these non-productive cells become a significant fraction of the population [32] contributing to several disadvantages of industrial relevance such as plasmid loss and reduced productivity [33].

Previous works reported the influence of amino acid limitation in pDNA amplification [23, 34]. They proposed that by using an appropriate amino acid limitation or starvation it is possible to amplify plasmids bearing origin of replication derived from ColE1-type plasmids [35] and bacteriophage γ [23]. Since amino acid starvation leads to the inhibition of bacterial growth, continued plasmid DNA replication should result in its amplification in *relA* mutants. These assays were performed with minimal medium and starved for different amino acids or in medium containing casamino acids [34] or LB medium (with yeast extract and tryptone) [36], where limitation was achieved by cultivation of bacteria to stationary phase of growth.

Being an amino acid source tryptone is not commonly used as a nitrogen source in fermentation media for plasmid DNA production, although it could provide several advantages: tryptone is carbohydrate deficient unlike yeast extract, which is very useful when trying to establish the consumption rate of a previously established carbon source such

as glycerol. Tryptone shows an elevated percentage of aspartic acid, which along with glutamine and glycine serve as nitrogen donors for the synthesis of nucleotides, leading to the formation of RNA, DNA and plasmid DNA [37]; and also shows low percentage of tyrosine, when compared with casamino acids, which could be advantageous, since it was considered that tyrosine, along with phenylalanine, can decrease the stability of the plasmid [38]. In this work, the possibility of studying amino acid limitation by using different tryptone concentration for the most efficient amplification of the plasmid pVAX1-*LacZ* was evaluated.

Some authors described the calculation of an amplification factor as the ratio of plasmid content per bacterial mass after the induction of starvation, to the plasmid content per bacterial mass in the non starved culture [39]. In a similar way, we determined the amplification factor as the ratio of plasmid content per bacterial mass in limitation conditions, to the plasmid content per bacterial mass in non limited culture. Therefore, an amplification ratio above 1 indicates plasmid DNA amplification in the conditions described [34]. The amplification factor was calculated in the cell mass collected in the stationary phase of growth.

In order to compare the influence of tryptone concentration on bacterial growth, different cultivations were done at 37 ºC. The time profiles for cell density, obtained by measuring OD_{600} of samples periodically taken, are shown in Figure 8.

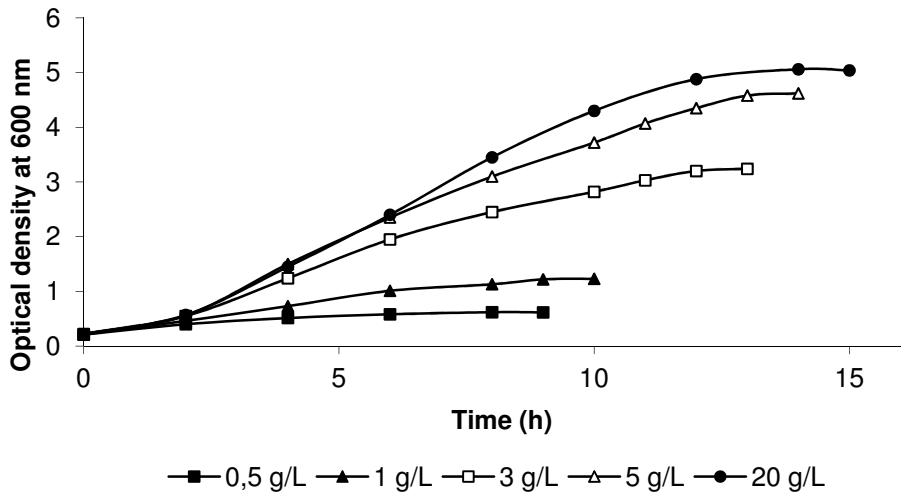

Figure 8: Growth profiles of *E. coli* DH5 alpha harbouring plasmid pVAX1-LacZ performed in shake flasks at 37 ºC and 250 rpm in semi-defined medium containing different tryptone concentrations.

By comparing the growth curves, it is apparent that higher tryptone concentration in the culture medium enhanced bacterial growth. However, as tryptone concentration increases, the pDNA content by bacterial mass, expressed as specific pDNA yield, decreases (Figure 9). Tryptone concentration of 5 g/L was selected as the higher tryptone concentration used since

our preliminary studies proved that higher tryptone concentrations did not promote pDNA amplification, since specific pDNA yield at tryptone concentration of 5 g/L was very similar, for instance, to that obtained for a tryptone concentration of 20 g/L (Figure 9).

When the same experiments were performed at 32ºC, lower specific pDNA yields were obtained, when compared with experiments performed at 37ºC, and tryptone concentration seems not to influence greatly specific yields (Figure 9). These results are in accordance with Wrobel and Wegrzyn's report [34], who reported a decrease in amplification efficiency at low temperatures, when compared with 37 ºC and found that the optimal temperature for amplification was 37 ºC, when pSC101 derived-plasmid was used.

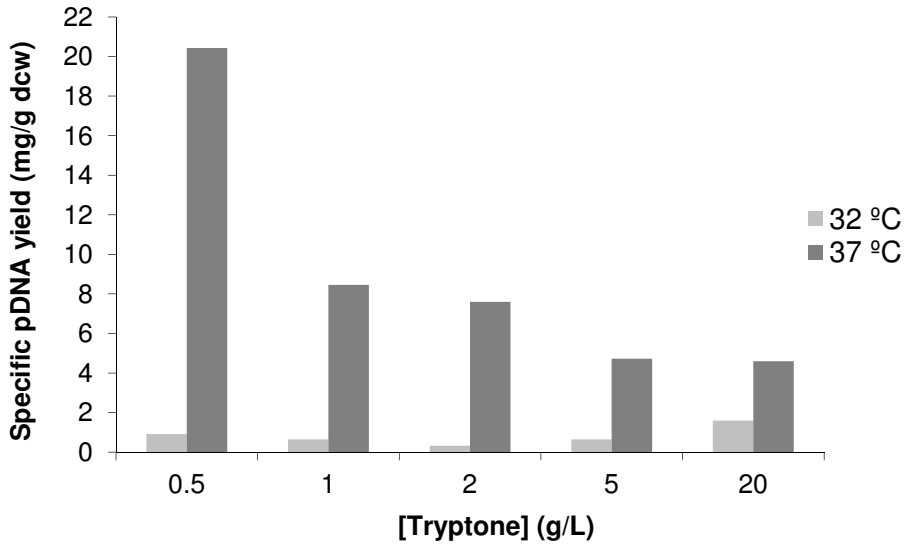

Figure 9: pDNA specific yield variation with different tryptone concentration at 32 and 37 ºC.

The highest specific pDNA yield (20.43 mg pDNA/g dcw) was obtained for a tryptone concentration of 0.5 g/L at 37 ºC, giving an amplification ratio of 4.3. This amplification is mostly due to the fact that *E. coli* DH5 alpha is a relA mutant, in which pDNA replication is promoted under amino acid limitation or starvation [12], despite the fact this strain is not auxotrophic for any amino acid. These results are considerably higher, when compared with studies without any amino acid limitation strategy, which reported values between 6.09 mg pDNA/g dcw [40] and 16.0 mg pDNA/g dcw [41] and even when compared with similar studies using amino acid limitation in LB medium at 37 ºC [36], that obtain an specific plasmid yield of approximately 10.0 mg pDNA/g dcw.

2.1.5. Conclusions

The present work shows that, depending on the growth temperature, different cell morphology specific pDNA yields and plasmid purity levels on *E. coli* lysates were obtained. Higher pDNA/g dcw (4.60 and 5.56 mg) and purity degrees (35.68% and 45.72%) were obtained at 37 and 40ºC, respectively. This result can be probably interpreted as synonyms of higher plasmid copy number by host cells at these temperatures, as the accentuated filamentation observed in cells seems to suggest.

It was also observed in this work that lower tryptone concentrations could improve pDNA specific yields. The highest specific pDNA yield (20.43 mg pDNA/g dcw) was obtained for a tryptone concentration of 0.5 g/L at 37 ºC, giving an amplification ratio of 4.3. However, when the same experiments were performed at 32ºC, lower specific pDNA yields was obtained and tryptone concentration seems not to influence greatly specific yields. So, by using tryptone as source for amino acid limitation experiments at 37ºC, specific pDNA yields suffered a fourfold increase, proving that the pDNA amplification strategy here analysed can improve pDNA yield even at small scale.

By using the conditions established in this work, it was found that the plasmid DNA yields will be improved and with even reduced host cell impurities.

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2.2. The use of DRAQ5 to monitor to monitor intracellular DNA in *Escherichia coli* by flow cytometry

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2.2.1. Abstract

Flow cytometry provides a rapid and high-content multiparameter analysis of individual microorganisms within a population. In the past years, several fluorescent stains were developed in order to monitor DNA content distribution and cell-cycle phases, mainly in eukaryotic cells. Recently, due to its low detection limits, several of these fluorescent stains were also applied to prokaryotic cells. In this study, the ability of a novel far-red fluorescent stain DRAQ5 in assessing intracellular DNA content distribution in *Escherichia coli* DH5α was evaluated. The results showed that a DRAQ5-labelled live *E. coli* suspension can be obtained by incubation of $1x10^6$ cells/mL with 5 μM DRAQ5 in PBS buffer supplemented with EDTA (pH=7.4) during 30 min at 37 ºC. Flow cytometric analysis of fixed *E. coli* cells revealed that ethanol should be used in detriment of glutaraldehyde for DRAQ5 labelling. After the analysis of RNase and DNase digested samples, DRAQ5 was proven to be a specific DNA labelling stain. The present study demonstrates that the use of DRAQ5 as a DNA-labelling stain provides an easy assessment of intracellular DNA content and cell-cycle phases in Gram-negative bacteria such as *E. coli*.

Keywords: Flow cytometry, *Escherichia coli*, DNA content, DRAQ5

2.2.2. Introduction

In recent years, the application of flow cytometry to prokaryotic cells has gained more relevance [1-2]. Although bacteria are relatively difficult to analyze and differentiate by flow cytometry (FCM) owing mostly to their small cell size and similar morphology, this technique has evolved towards a high-throughput capacity of single cell analysis of bacterial populations [3]. This analysis enables the measurement of the distribution of a property or properties within the bacterial population such as DNA and RNA content [4] and bacterial viability [5], which is very helpful in assessing the heterogeneity of the bacterial population.

One of the reasons for heterogeneous bacterial populations results from cell-cycle differences [6]. In bacterial cells, new rounds of chromosomal DNA replication may be initiated before a previous round has been completed. Thus, heterogeneity of DNA content will be larger in prokaryotes when compared with eukaryotes [7]. So, the ability to identify cell cycle position through DNA content analysis is fundamental in applications such as bioprocess monitoring [8] and antimicrobial susceptibility testing [9].

Since the first assays of DNA content in intact cells in order to assess cell cycle phases, a growing number of DNA-binding fluorochromes was made available. Conventionally, propidium iodide or ethidium bromide are used to identify intracellular DNA content. However, cells need to be fixed and RNase digested [8, 10] which results in more laborious protocols. Furthermore, RNase digestion can lead to leakage of intracellular material. Another usual method of DNA-labelling involves the use of bisbenzimide dyes such as Hoechst 33258 and Hoechst 33342 [11-12] that fluoresce bright blue upon binding to DNA, but both require UV excitation. A newly developed far-red cell-permeant high affinity DNA-labelling dye, DRAQ5 (deep red-fluorescing bisalkylaminoanthraquinone number five), has gained popularity in multiparametric flow cytometry, due to the absence of spectral overlap with commonly used fluorescent stains such as FITC or GFP [13].

DRAQ5 has been mostly used in eukaryotes [14-16] with very few reports in prokaryotes. Herrero et al. (2006) and Quirós et al. (2007) reported the use of DRAQ5 for total cell count of yeast and Gram-positive bacteria involved in cider fermentation processes. However, the use and properties of this novel fluorescent stain have not yet been exploited in Gram-negative bacteria.

The aim of this study is to describe the ability of DRAQ5 to stain the DNA of Gram-negative bacteria, using *Escherichia coli* as a model. The possibility of using such stain for the analysis of bacterial cell-cycle is also discussed.

2.2.3. Experimental

Host strain and culture conditions

Escherichia coli DH5 alpha cells were grown in 250 mL shake flasks containing 62.5 mL of semi-defined media [17] in a orbital shaker at 37 ºC at 250 rpm.

Cell fixation

Cells were harvested by centrifugation at 10000 x g for 10 min at 4 ºC. Cells were washed once in 0.1 M Tris, 2 mM $MgCl_2$ buffer (pH 7.4). Ethanol fixation: The cell pellet was resuspended in 300 µL of ice-cold sterile distilled water. A 700 µL volume of ice-cold absolute ethanol was rapidly added, and the suspension was mixed by vigorous pipetting to prevent cell clumping. The sample was then incubated at 4 ºC for 30 min for fixation. Fixed samples were stored at −20°C until they were stained and analyzed on the flow cytometer. Glutaraldehyde fixation: The cell pellet was resuspended in 400 µL of ice-cold sterile distilled water. A 600 µL volume of ice-cold 5 % glutaraldehyde was rapidly added, and the suspension was mixed by vigorous pipetting to prevent cell clumping. The sample was then incubated at

4 ºC for 30 min for fixation. Fixed samples were washed once again in 0.1 M Tris, 2 mM MgCl2 buffer (pH 7.4), immediately stained with DRAQ5 and analyzed on the flow cytometer.

DNase and RNase treatments

Ethanol-fixed cells at a concentration of approximately 10^6 cells/mL (1 mL) were pelleted by centrifugation at 10000 x g for 10 min at 4 ºC and resuspended in one volume (1 mL) of DNase I 1 mg/mL final concentration in 0.1 M Tris, 5 mM $MgCl_2$ and 0.1 M NaCl buffer (pH 7.4) [8]. For RNase treatment, cells were firstly incubated with lysozyme 1.67 mg/mL final concentration, for 2 h at room temperature with shaking. After lysozyme treatment, cells were washed twice in 0.1 M Tris, 2 mM MgCl2 buffer and incubated with 1.25 mg/mL RNase A in 0.1 M Tris, 5 mM $MgCl_2$ and 0.1 M NaCl buffer (pH 7.4) [18]. Cell suspensions were digested with DNase for 1 h at 37 ºC [8] and afterwards with RNase for 3 h at room temperature [18], pelleted by centrifugation at 18000 x g for 10 min at 4 ºC and resuspended in 0.1 M Tris, 2 mM $MgCl_2$ buffer (pH 7.4).

Flow cytometry

DRAQ5 (Biostatus Limited, Leicestershire, U.K.) stock solution (5 mM) was diluted 1:50 with sterile distilled water and stored at 4 ºC. Bacterial samples were analyzed on a BD Biosciences FACSCalibur. Acquisition was performed with CellQuest™ Pro Software and based on light-scatter and fluorescence signals resulting from 15 mW laser illumination at 488 nm and 635 nm. Light scatter measurements were acquired logarithmically, while fluorescence signals were acquired either logarithmically or linearly, depending on the sample. In the case of samples containing live cells, fluorescence data were acquired logarithmically, in order to obtain a higher resolution for low signal intensities; for fixed cells analysis, all fluorescence data were acquired linearly. Signals corresponding to forward and side scatter (FSC and SSC) and fluorescence were accumulated, the fluorescence signal (pulse area measurements) was screened by a FL-4 (661 nm) bandpass filter. Threshold levels were empirically set on SSC to further reduce electronic and small particle noise. The flow cytometer was routinely operated at low flow rate setting (12 µL sample/minute), and data acquisition for a single sample typically took 20 to 30 min. *E. coli* cells were gated according to FSC/SSC parameters. Data analysis was performed using FCS Express version 3 Research Edition (De Novo Software™, Los Angeles, USA).

Sample preparation for flow cytometry

Live cells: In order to obtain the most suitable DRAQ5 incubation time, $1x10^6$ *E. coli* cells were incubated with 5 µM DRAQ5 at 37 ºC for several time periods ranging from 15 to 120 min, in the dark, and washed once in PBS buffer (pH 7.4) before flow cytometric analysis. In order to obtain a maximum of stained cells, $1x10^6$ cells/mL were incubated with serial concentrations of DRAQ5, ranging from 1 to 10 µM in PBS supplemented with 4 mM EDTA (pH

7.4). After the washing step, a sample of each suspension was collected for viability assessment by conventional dilution plating.

Fixed cells: In order to obtain an optimal concentration of dye, $1x10^6$ cells/mL were incubated with serial concentrations of DRAQ5, ranging from 1 to 10 µM in 0.1 M Tris, 2 mM $MgCl_2$ buffer (pH 7.4). Cells were incubated at 37 ºC for 30 min in the dark and washed once in the same buffer before flow cytometric analysis.

Number of replication origins

To determine the number of replication origins in each cell at a given fermentation time, rifampicin 300 mg/mL plus cephalexin 10 mg/mL were added to cells collected from fermentation. Samples were incubated at 37 ºC for 2 h to allow all replication forks to run to completion. After the incubation period, cells were washed twice and ethanol-fixed, as previously described. DNA content of the cells was determined by flow cytometry as described above using the optimal DRAQ5 concentration. The number of chromosome equivalents per cell showed the number of replication origins that were present at the time of drug addition.

Confocal microscopy

E. coli suspensions were deposited in poly(D-lysine)-coated coverslips previously placed in the wells of a 12-well polystyrene plate. The plates were incubated at 37 ºC for 45 min to allow the adhesion of *E. coli* cells to the coverslips. The plates were then washed with PBS buffer and fixed for 10 min at room temperature using 4% paraformaldehyde solution. The plates were washed with PBS buffer and permeabilized for 5 min at room temperature using 1% Triton X-100 solution. The plates were washed with PBS buffer and a 7.5 µM DRAQ5 solution was added to the wells. The plates were then incubated at 37 ºC for 30 min. The plates were washed with PBS buffer and a 6 µg/mL Fluorescein 5(6)-isothiocyanate (FITC, Sigma-Aldrich) solution was added to the wells in order to stain whole cells. The plates were then incubated at 37 ºC for 30 min; afterwards, cells were washed twice and the coverslips were mounted onto glass slides. These slides were then examined under a confocal microscope (x60) in an Olympus FluoView FV10i confocal microscope using the FluoView Ver.2.0b Software.

2.2.4. Results

DRAQ5 staining of live *E. coli* cells

When *E. coli* cells were incubated with 1 µM DRAQ5 in PBS buffer supplemented with 4 mM EDTA (pH=7.4), the percentage of DRAQ5 stained cells reached a plateau after an incubation time of 30 min (Figure 10a). Incubation of *E. coli* cells with DRAQ5 caused, however, a reduction in bacterial viability. When *E. coli* cells were incubated in PBS buffer supplemented with 4 mM EDTA (pH=7.4) without the addition of DRAQ5, as incubation time increased, bacterial viability decreased; between 15 and 30 min of incubation, a plateau of stained cells was observed (Figure 10b). When a similar *E. coli* suspension was incubated under the same conditions but with the addition of 1 µM DRAQ5, the behaviour of the cell population was similar to that observed without the addition of DRAQ5 but with a concomitantly higher decrease in bacterial viability (Figure 10b). When *E. coli* cells were incubated with increasing concentrations of DRAQ5 ranging from 1 to 10 µM, the percentage of DRAQ5 stained cells reached a plateau at a concentration of 5 µM (Figure 10c). As DRAQ5 concentration increased from 1 to 10 µM, bacterial viability decreased to almost four times less with the highest DRAQ5 concentration used (Figure 10d). As DRAQ5 concentration increased, the percentage of stained cells also increased, reaching a maximum of 93% at a concentration of 10 µM (Figure 10c).

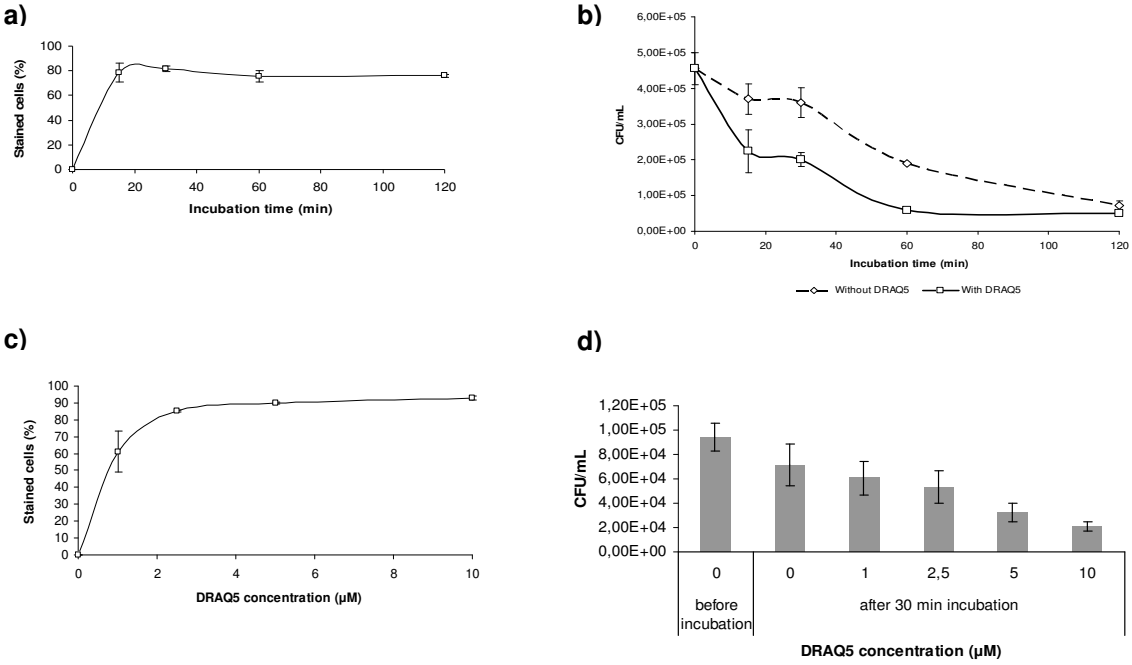

Figure 10: Effect of incubation time and DRAQ5 concentration in the percentage of stained cells and bacterial viability:

(a) percentage of DRAQ5 stained cells following different incubation times using a fixed DRAQ5 concentration (5 μM), (b) effect of incubation time in bacterial viability with and without the addition of DRAQ5 (5 μM), (c) percentage of DRAQ5 stained cells in the presence of serial DRAQ5 concentration with an incubation time of 30 min, (d) effect of DRAQ5 concentration in bacterial viability with 30 min of incubation. A total of 20 000 events were collected for this analysis.

Flow cytometric comparison of cell fixation protocols

Since DRAQ5 seems to alter the morphology of live *E. coli* cells and does not stain all the population, we investigated DRAQ5 labelling of fixed cells.

Glutaraldehyde and ethanol are two common fixatives for use in bacterial intracellular component measurements. Despite resulting in very similar populations in terms of light scatter measurements, these two fixatives exhibited very different patterns of fluorescence in the FL4 channel. Ethanol-fixed cells exhibited low fluorescence in the FL4 channel (Figure 11c), while glutaraldehyde-fixed cells exhibited a mild background fluorescence in the same channel (Figure 11d) which can cause a poor signal to noise ratio. Attending this finding, DRAQ5 and glutaraldehyde fixation should not be used concomitantly.

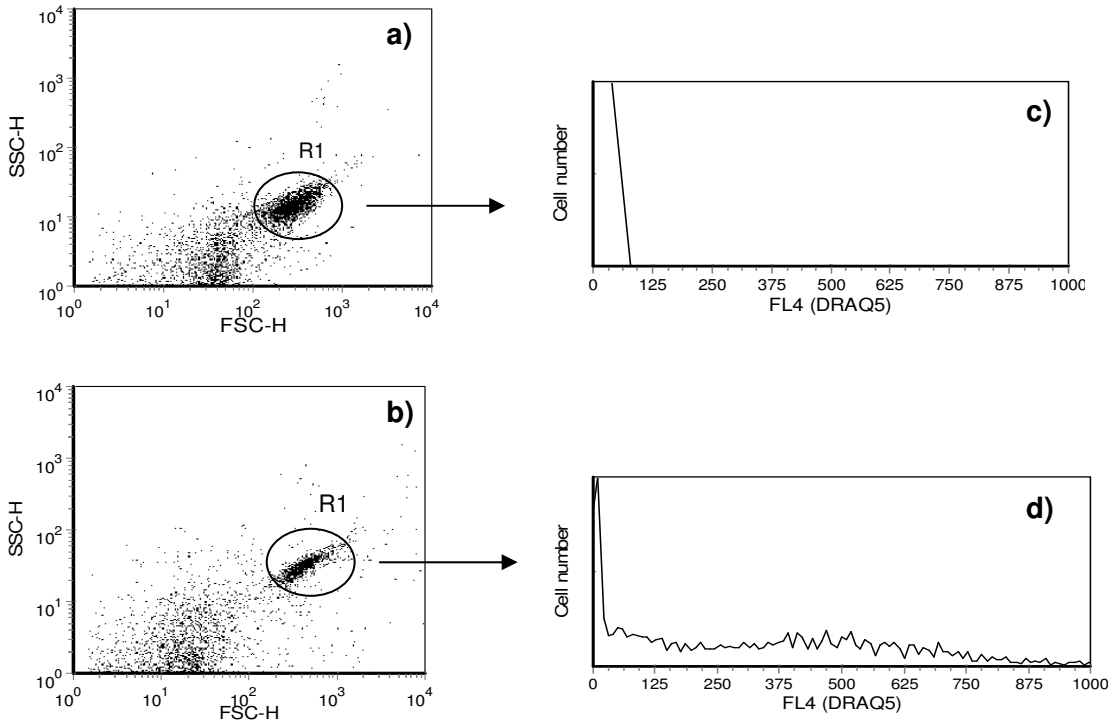

Figure 11: Effect of different fixatives on light scatter measurements and fluorescence distribution.

(a) Light scatter measurements of ethanol-fixed *E. coli* cells, (b) light scatter measurements of glutaraldehyde-fixed *E. coli* cells. Histograms showing the distribution of DRAQ5 fluorescence intensity

(FL4 channel) of fixed *E. coli* cells without staining using (c) ethanol or (d) glutaraldehyde as fixatives. R1 region corresponded to *E. coli* cells gating. A total of 20 000 events were collect for this analysis.

Labelling specificity of DRAQ5

In order to demonstrate bacterial DNA specificity of DRAQ5, cells were treated with DNase, RNase and with both these enzymes previously to DRAQ5 labelling. The analysis of light scatter parameters showed that DNase and RNase treatments were effective as indicated by the decrease in forward and side scatter parameters (data not shown). Despite the fact that DNase treatment yielded a higher FL4 channel fluorescence comparing to treatment with both enzymes, the two histograms were very similar (Figure 12b and c) to the non-stained population (Figure 12a). RNase treatment yielded a similar FL4 channel fluorescence distribution when compared to *E. coli* cells without treatment (Figure 12d and e); nevertheless, RNase-treated cells exhibited peaks with lower CV and lower mean fluorescence intensity. The fact that this stain is DNA-specific is further supported by the microscopic evaluation of DRAQ5 and FITC stained *E. coli* cells where DRAQ5-stained *E. coli* chromosomes could be observed (Figure 12).

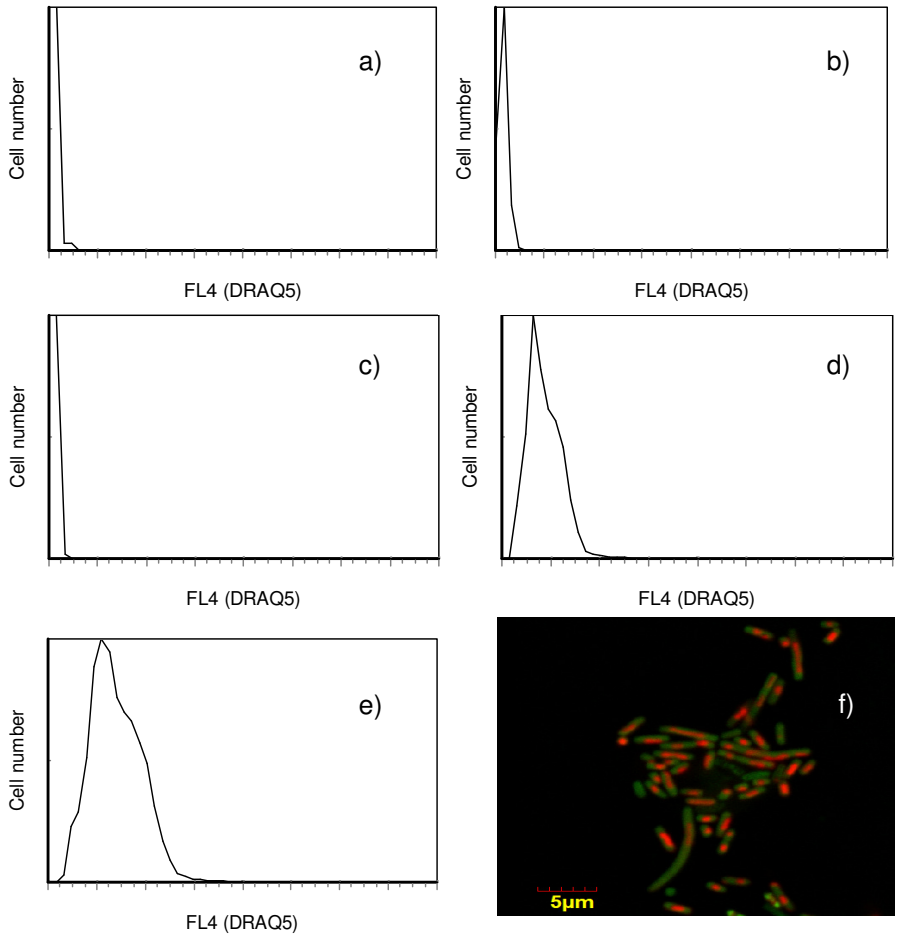

Figure 12: Effect of enzyme digestion on the DRAQ5 staining of fixed *E. coli* cells.

(a) untreated suspension without staining, (b) stained cell suspension after DNase treatment, (c) stained cell suspension after DNase and RNase treatment, (d) stained cell suspension after RNase treatment and (e) untreated and stained cell suspension, (f) confocal microscopy images of *E. coli* cells immobilized on poly(D-lysine)-coated coverslips and incubated with DRAQ5 (red) and FITC (green) as described in the Experimental section. A total of 20 000 events were collect for the flow cytometric analysis.

DRAQ5 labelling of ethanol-fixed *E. coli* cells

When ethanol-fixed *E. coli* cells were incubated with serial DRAQ5 concentrations, the fluorescence distribution histograms for DRAQ5 concentrations ranging from 5 to 10 µM were very similar (Figure 13). In order to establish an optimal stain concentration, the mean and median fluorescence intensity values were determined. The analysis of the fluorescence intensity values (62.32) and peak CV values (32%) showed that the optimal dye concentration was reached at 7.5 µM DRAQ5. At concentrations of 5 µM and 10 µM the fluorescence intensity values were lower (56.10 and 57.97, respectively) with higher peak CVs (33% and 34%).

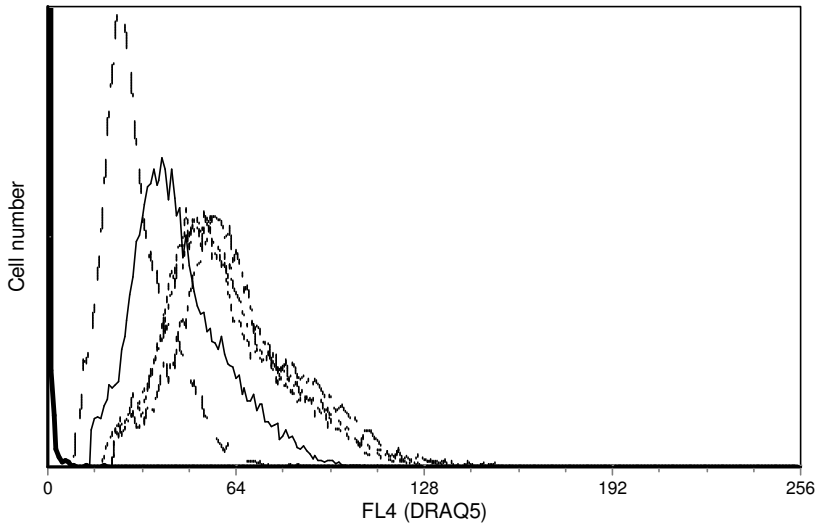

Figure 13: Typical histograms showing the distribution of DRAQ5 fluorescence intensity (FL4 channel) in a linear scale.

For this purpose ethanol-fixed *E. coli* cell suspensions incubated with DRAQ5 concentrations ranging from 0 to 10 µM. A total of 20 000 events were collected for this analysis.

E. coli cell cycle analysis with DRAQ5

Ethanol-fixed *E. coli* cells stained with DRAQ5 exhibited a broad fluorescence distribution (Figure 14), possibly due to population heterogeneity, which did not allow visualizing peaks corresponding to fully replicated chromosomes. In order to evaluate the ability of DRAQ5 to resolve ploidy, cells were incubated with rifampicin and cephalexin. The analysis of fluorescence distribution showed that fluorescence peaks were sharper and with lower peak

CV values (approximately 14%) in treated cells comparing to non-treated cells (32%). When observing the fluorescence distribution histograms resultants from this incubation, two peaks were identified (Figure 14). The mean fluorescence intensity value of the first peak was approximately 24 units and the mean fluorescence intensity of the second peak was approximately 48 units (Figure 14), what indicated that these peaks probably corresponded to peaks of cells containing one or two chromosome equivalents. The analysis of treated samples taken from the fermentation at regular time periods showed that the percentage of cells with two chromosome equivalents decreases throughout fermentation time from 30% at 4 h of growth to about 15% at 9 h of growth, leading to an increase of cells with only one chromosome equivalent in the late-exponential and stationary phases of growth.

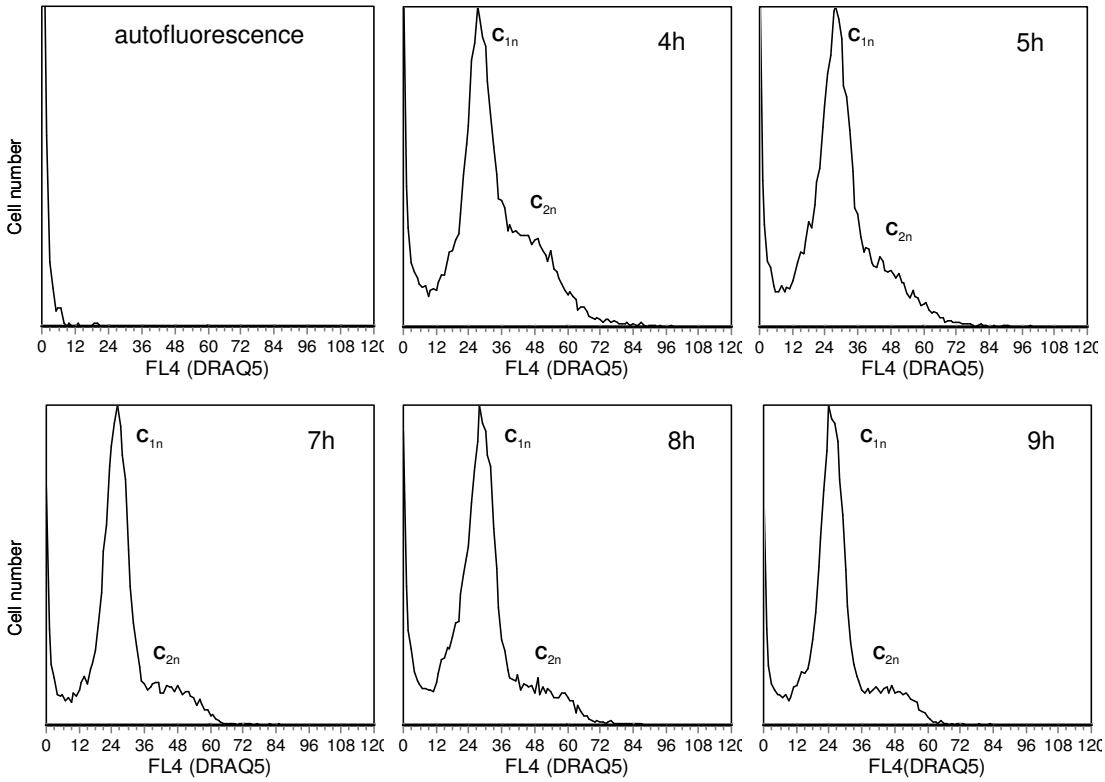

Figure 14: DNA content distributions of *E. coli* DH5 alpha.

Cells were grown in a semi-defined media and sampled for flow cytometry after incubation with rifampicin and cephalexin for 2h. A total of 20 000 events was collect for this analysis.

2.2.6. Discussion

DRAQ5 has been shown to be able to stain live and fixed eukaryotic cells [19-21] and live Gram-positive bacteria [22-23]. In this work, the ability of DRAQ5 to stain live and fixed samples of the Gram-negative bacteria *E. coli* was investigated. As DRAQ5 also proved to be useful in the analysis of the eukaryotic cell cycle [16], it was also studied in addition whether it could resolve bacterial cell cycle phases.

The incubation buffer of choice for live cell staining was PBS supplemented with EDTA in order to permeate the outer cell membrane, thus facilitating the uptake of DRAQ5. The use of this incubation buffer caused a reduction of bacterial viability that could result from the destabilizing effect of EDTA in the bacterial outer membrane [24]. The addition of DRAQ5 to *E. coli* cells resulted in a decrease in viability when compared with cells without DRAQ5 addition. This fact can be due to changes in chromatin structure and impairment of cell cycle progression [25] since, like other DNA fluorescent stains, DRAQ5 is a DNA intercalating agent. The percentage of DRAQ5-labelled live *E. coli* cells remained almost unaltered following different incubation periods, indicating that DRAQ5 is a time-independent stain and, probably, it is not pumped out from the cells. However, when analysing bacterial viability, the results showed that viability only remained unaltered between 15 and 30 minutes incubation time, which lead to the choice of an incubation period of 30 minutes.

DRAQ5-labelling of live *E. coli* cells proved to be effective at a concentration of 5 μM, yielding about 93% of stained cells. Increasing DRAQ5 concentrations caused a significant decrease in the viability of *E. coli* population. In order to obtain higher staining percentage, we tested several incubation buffers with EDTA concentrations up to 10 μM. However, higher staining percentages were not obtained, resulting in a decrease in bacterial viability (data not shown). The inability to stain 100% of the cells may be due to the fact that the *E. coli* population probably contained ghost-cells that are not stained with DRAQ5. The results obtained suggest that an optimized protocol for DRAQ5-labelling of live cells should involve the incubation of $1x10^6$ cells/mL with 5 μM DRAQ5 in PBS buffer supplemented with EDTA (pH=7.4) during 30 min at 37 ºC.

DRAQ5 proved to be specific for bacterial DNA, since DNase treatments yielded a fluorescence distribution similar to that obtained with no DRAQ5 labelling while RNase treatment yielded a fluorescence distribution similar to that of DRAQ5 labelling. Nonetheless, DRAQ5 revealed a weak fluorescence when bound to bacterial RNA.

For DRAQ5 labelling of fixed *E. coli* cells, ethanol should be preferred as fixative in detriment of glutaraldehyde, due to the absence of positive fluorescence in the FL4 channel. The titration of fixed *E. coli* cells ($1x10^6$ cells/mL) with different DRAQ5 concentrations showed that an optimal dye concentration could be achieved with 7.5 μM, based on the values of fluorescence intensity and peak CVs obtained.

Some authors have described the use of Hoechst 33342 UV-fluorescent stain to assess bacterial cell cycle phases [26]. However, UV radiation causes damages in cellular DNA [27] and benchtop flow cytometers with a UV laser are uncommon in a research laboratory. For these reasons, we further investigated the ability of DRAQ5 to resolve bacterial DNA content. The fluorescence distribution histograms obtained with rifampicin plus cephalexin treated *E. coli* cells showed two peaks, corresponding to one or two chromosome equivalents, attending

to the mean fluorescence values of those peaks. The results obtained demonstrated that this *E. coli* strain, in the conditions mentioned, has a eukaryotic-like cell cycle, since the cells only possessed one or two chromosome equivalents [28].Similar cell cycle analysis has already been described for other *E. coli* K-12 derivatives [29] in slowly-growing cultures using other fluorescent stains [17]. Furthermore, the increase in the population of cells with only one chromosome equivalent in late-exponential and stationary phases of growth is in agreement with proposed models of bacterial growth [30].

2.2.7. Conclusion

The results of this study suggest that DRAQ5 can be successfully used to stain a bacterial population in order to discriminate bacteria from other background particles. Moreover, it was demonstrated that DRAQ5 is able to stain bacterial DNA in ethanol-fixed *E. coli* cells without the need for additional laborious and expensive enzymatic treatment steps. This work showed evidence that DRAQ5 can be effectively used to monitor bacterial cell cycle since peaks of fully replicated chromosomes can be observed. In conclusion, DRAQ5 can be used alone or in combination with other commonly used fluorescent stains such as FITC or antibodies labelled with fluorescent stains, for multiparameter analysis of live and fixed gram-negative bacterial cells.

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2.3. Impact of plasmid induction strategy on overall plasmid yield and *E. coli* physiology using flow cytometry and real-time PCR

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2.3.1. Abstract

DNA-based vaccines and gene therapies have rapidly evolved since the first demonstration of *in vivo* gene expression by naked DNA injection. When optimizing their production, the major goals in the fermentation process are to maximize the specific plasmid yield, as well as plasmid quality, while minimizing plasmid-mediated metabolic burden. Due to this fact, the development of new and fast monitoring techniques able to easily assess plasmid-mediated metabolic burden and process productivity is in demand. In this work, *E. coli* DH5 alpha harboring plasmid pVAX1-*LacZ* was grown in semi-defined media using several pDNA induction strategies. The effect of the induction strategy on cell physiology and overall process productivity was monitored using multi-parameter flow cytometry and real-time qPCR. All induction strategies caused cell filamentation and decreased viability at the end of fermentation. The results also suggest that an amino acid limitation with AMP addition induction strategy resulted in the highest specific yields (20.94 mg/g) and, concomitantly, highest plasmid copy number (1070 per cell). In conclusion, amino acid limitation with AMP addition seems to be a suitable approach to be implemented at a large scale level since it does not require any additional energy and it has proved to be efficient in plasmid amplification.

Keywords: plasmid DNA; Amino acid limitation; Temperature up-shift; Flow Cytometry; Real-time qPCR

2.3.2. Introduction

Over the past years, naked/plasmid DNA gene therapy has gained more relevance, accounting, in 2009, for 17.7% of all gene therapy clinical trials worldwide (www.wiley.co.uk/genmed/clinical); being the third most used vector, right behind adenovirus and retrovirus, which account for 23.9 and 20.8% of all clinical trials, respectively. Regarding its application, naked DNA vectors are being used in clinical trials for a large variety of diseases such as cancer, non-infectious diseases like ischemia and infectious diseases like hepatitis and HIV (www.wiley.co.uk/genmed/clinical) in phase I, II and III clinical trials. Being a non-viral vector, naked DNA is particularly suitable with respect to

simplicity of use, ease of large-scale production and lack of specific immune response [1]. The major setback regarding the use of non-viral vectors is the relatively low efficiency of gene delivery, particularly for *in vivo* applications, though efforts are being made to overcome this problem [2-4].

In order to facilitate the entrance of naked/plasmid DNA into clinical trials, large amounts of clinical grade plasmids are required. To date, most bioprocesses for plasmid DNA production emphasize only downstream processing such as primary recovery [5] and purification steps [6], overlooking the fact that upstream processing steps are fundamental for the overall process performance. In fact, the fermentation process can have a tremendous impact in the plasmid DNA quality at harvest as well as plasmid DNA quality after cell lysis [7-8]. Increasing plasmid DNA yield, while decreasing process key contaminants, such as RNA and proteins are key goals for plasmid production. Several induction strategies aiming to maximize plasmid DNA copy number in *E. coli* cells have been proposed, namely temperature up-shift [9], addition of chloramphenicol [10], amino acid limitation [11] and adenosine monophosphate (AMP)-induced amplification [12].

Several studies reported that plasmid DNA amplification imposes a metabolic burden on the host strain, resulting in reduced growth rates and biomass yield [13-14]. As a result, the development of new and fast monitoring techniques capable of easily assessing plasmid-mediated metabolic burden and process productivity is in demand. Examples of such techniques are flow cytometry and quantitative real-time PCR (real-time qPCR). Flow cytometry is a powerful technique for the fast characterization of cell populations [15] and because of this, its application to microorganism-based biotechnological processes has gained more interest over the past years [16-18]. For instance, measuring cell physiological states, proliferation and viability, as well as intracellular components, is essential for making informed decisions about process control, because process performance will largely depend on the number of metabolically active cells [19]. The other major concern in plasmid DNA fermentation processes is segregational instability, which can have a tremendous impact in process productivity. In order to monitor plasmid segregational instability, the plasmid copy number (PCN) during the fermentation process must be determined. In the last years, quantitative real-time PCR techniques have evolved and its successful application to PCN determination in bacterial cells has been reported [20-22]

Although several plasmid DNA induction strategies have been described, a thorough comparison between them is necessary. In this sense, the aim of this work is to report how flow cytometry and real-time qPCR can be used to determine cell physiological states and pDNA segregational stability during pVAX1-*LacZ* plasmid induction in *E. coli* DH5 alpha cells, using AMP addition, amino acid limitation with or without AMP addition and temperature up-shift induction strategies in order to develop strategies for bioprocess optimization.

2.3.3. Materials and Methods

Plasmid, bacterial strain and growth conditions

The bacterial host for plasmid pVAX1-*LacZ* was *E. coli* DH5 alpha. The plasmid pVAX1-*LacZ* (Invitrogen, Carlsbad, USA) is 6.05 kbp long and has a pUC origin of replication. It also contains a cytomegalovirus (CMV) promoter and a kanamycin resistance gene for selection purposes.

The expression system was cultivated in a semi-defined medium previously developed [13, 23]. Glycerol supplementation to growth media improves specific plasmid DNA productivity and plasmid content [24]. In this sense, glycerol was used as the carbon source, at a concentration of 15 g/L, and tryptone was used as the nitrogen source. In all studies performed, cultures were started with an OD_{600} of approximately 0.2, grown in 500 mL shake flasks containing 125 mL of medium, at 250 rpm, and using the appropriate temperature in each experiment. Growth was suspended at the stationary phase. For each induction strategy used, three independent fermentation runs were performed.

Amino acid limitation and AMP addition:

For these experiments, growth was performed in the semi-defined medium with a tryptone concentration of 3 g/L, since this tryptone concentration proved to be the growth-limiting nutrient in similar conditions [13]. To ensure that tryptone was the limiting nutrient, glycerol concentration was kept to a non limiting level (15 g/L). The terminology "amino acid limitation" used in this work is in agreement with the work performed by other authors using similar conditions [11]. In order to alleviate the interference of tryptone concentration present in the pre-fermentation medium, the bacterial cells from the medium with an approximate OD of 2.6 were harvested by centrifugation of the culture at 5000 x *g* during 20 min at 4 ºC and the pellet was washed twice with an equal volume of 0.9 % NaCl and resuspended in the semi-defined medium [25].

Amino acid limitation was achieved by the cultivation of bacteria in the semi-defined medium containing 3.0 g/L tryptone at 37 ºC, until the stationary phase of growth was reached (approximately 10 h of growth).

For amino acid limitation experiments, after this initial cultivation for 10 h, cultivation was prolonged for another 8 h. For AMP addition experiments, after the initial cultivation for 10 h, Adenosine 5'-monophosphate sodium salt (AMP, Sigma-Aldrich, St. Louis, MO) at 5 mM final concentration was added to the culture. To exclude amino acid limitation in these experiments, tryptone was also added to the culture at 2 g/L final concentration and cultivation was prolonged for another 8 h. For amino acid limitation plus AMP addition

experiments, after an initial cultivation for 10 h, AMP at 5 mM final concentration was added to the culture and cultivation was prolonged for another 8 h.

Temperature up-shift

For these experiments, growth was performed in the semi-defined medium. Glycerol and tryptone concentrations were kept under non limiting levels: 15 and 20 g/L, respectively. Bacterial cells from the medium with an approximate OD of 2.6 were harvested by centrifugation of the culture at 5000 x *g* during 20 min at 4 ºC and the pellet was washed twice as mentioned above.

Temperature up-shift was achieved by the initial cultivation of bacteria for 5 h at 30 ºC, until mid-log phase was achieved. After this initial cultivation, cells were grown at 42 ºC for another 13h.

Lysis and primary isolation

Cells were recovered by centrifugation and stored at -20 ºC. Cell pellets were lysed using a modified alkaline lysis protocol as described previously [26]. After a suitable dilution, the obtained cell lysate was loaded onto the HPLC system and the agarose gel.

Analytical chromatography

HPLC was used to measure pDNA concentration and purity in *E. coli* lysates, according to the method described by Diogo *et al*. [27]. pDNA concentration was calculated as the mean of three independent samples.

Agarose-gel electrophoresis

An agarose gel electrophoresis analysis (110V, 40 min) was performed using 1 % agarose gel in TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) buffer in the presence of 0.5 µg/mL ethidium bromide.

Real-time qPCR

Sample preparation

Samples were prepared as previously described [28]. Briefly, 1 mL of cell culture containing $1.0x10^8$ cells was incubated in a 1.5 mL microtube at 95 ºC for 10 minutes followed by immediate storage at -20 ºC. The cell number in each starting sample was determined by using a correlation between the optical density of the culture and cell concentration. One unit of OD_{600} was found to correspond to a dry cell concentration of $3.97x10^8$ CFU/mL.

Real-time qPCR

Quantitative real-time PCR was used for the determination of plasmid copy number in whole *E. coli* cells. Specific primers to kanamycin resistance gene (forward: 5'-AGACAATCGGCTGCTCTGAT-3'; reverse: 5'-AGTGACAACGTCGAGCACAG-3') were used to amplify a fragment of 176 bp. Real-time PCR (IQ5 Biorad, Hercules) efficiency was determined for primer set using serial dilutions of *E. coli* cells (1:1, 1:10, 1:100, 1:1000 and 1:10000). Real-time PCR reactions were carried out using 3 µl of *E. coli* cells (sample preparation is described below) in a 20 µl reaction containing 10µl Maxima™ SYBR Green qPCR Master Mix (Fermentas, Canada) and 300nM of each primer. Reactions were incubated at 95 ºC for 10 min, followed by 30 cycles of 10 s at 95 ºC and 30 s at 60 ºC. The amplified PCR fragments were checked by melting curves: reactions were heated from 55 to 95ºC with 10 s holds at each temperature (0.05 ºC/s).

Since cell components released during the treatment at 95 ºC may inhibit PCR reaction, bacterial cell concentration was kept constant with a concentration of approximately $2.9x10^4$ cells/reaction, which is in accordance with other methods [20]. Plasmid copy number standards for the calibration curve were prepared by spiking different amounts of purified plasmid DNA with non-transformed *E. coli* DH5 alpha cells. Each reaction contained $2.9x10^4$ *E. coli* cells and different amounts of plasmid DNA. Each calibration curve was obtained as an average of three independent assays. Calculation of PCN was based on plasmid base pair number (6050 bp) and the average molecular weight of a DNA base pair (660 Daltons).

Flow cytometry

Bacterial samples were analysed on a BD Biosciences FACSCalibur. Acquisition was performed with CellQuest™ Pro Software and based on light-scatter and fluorescence signals resulting from 15 mW laser illumination at 488 nm and 635 nm. Light scatter measurements were acquired logarithmically, while fluorescence signals were acquired logarithmically with the exception of the FL-4 signal, which was acquired linearly. Signals corresponding to forward and side scatter (FSC and SSC) and fluorescence were accumulated, the fluorescence signal (pulse area measurements) was screened by FL-1 (530/30 nm), FL-3 (>670 nm) and FL-4 (661 nm) bandpass filters. Threshold levels were empirically set on SSC to further reduce electronic and small particle noise. The flow cytometer was routinely operated at low flow rate setting (12 µL sample/minute), and data acquisition for a single sample typically took 20 to 30 min. *E. coli* cells were gated according to FSC/SSC parameters. Data analysis was performed using FCS Express version 3 Research Edition (De Novo Software™, Los Angeles, USA).

DNA analysis

Flow cytometric analysis of DNA was made using ethanol-fixed cells collected at several fermentation times. The fluorochrome used for this analysis was DRAQ5 (Biostatus Limited, Leicestershire, UK), which has recently been described for DNA labelling and bacterial cell cycle analysis [29]. For DNA content analysis, $1.0x10^6$ cells were washed twice in PBS buffer (pH 7.4) and resuspended in 1 mL of PBS buffer (pH 7.4) containing 7.5 µM DRAQ5. After 30 min in the dark at 37 ºC, cells were washed twice, acquired and analysed as described above. The fluorescence signal was collected by a FL-4 (DRAQ5) bandpass filter.

Bacterial viability

For this analysis, a propidium iodide (PI) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) dual staining was used. BOX is a lipophillic and anionic fluorescent stain that accumulates intracellularly when the cytoplasmic membrane is depolarised [30]. PI binds to DNA, but cannot cross an intact cytoplasmic membrane [31].

Briefly, $1.0x10^6$ cells/mL cells taken from fermentation were washed once in PBS buffer (pH 7.4) and resuspended in 1 mL of PBS buffer supplemented with 4 mM EDTA [19] (pH 7.4) containing 2.5 µg/mL BOX (Molecular Probes®, Invitrogen, part of Life Technologies, Carlsbad, CA) and 1.0 µg/mL PI (Sigma-Aldrich, St. Louis, MO). After 15 min in the dark at 37 ºC, cells were washed twice, acquired and analysed as described above. The fluorescence signals were collected by FL-1 (BOX) and FL-3 (PI) bandpass filters.

2.3.4. Results and Discussion

E. coli DH5 alpha is a relA mutant ($relA^-$) which, in response to AMP addition or amino acid limitation with or without AMP addition, enables plasmid amplification [12]. Furthermore, in relA mutant bacteria, AMP addition increases the abundance of uncharged tRNAs which promote plasmid DNA replication [12]. Plasmid pVAX1-*LacZ* contains a pMB1-derived origin of replication, which allows an increased plasmid copy number at 37-42 ºC, but not at 30-32 ºC [9, 32]. Therefore, pDNA induction strategies used in this work were based on AMP addition, amino acid limitation with or without AMP addition as well as temperature up-shift.

Evaluation of cell morphology

Escherichia coli growth may result in an increased stress response, often leading to cell filamentation and growth cessation. This phenomenon has been described to occur in both recombinant protein [33] and plasmid DNA production processes [13]. Therefore, one of the first parameters to be assessed throughout all fermentation runs was *E. coli* morphology by forward (related to size) and side scatter (related to complexity) measurements. Since all fermentations were inoculated with the same cell suspension, all samples taken at 0 h have

very similar morphology (Figure 15). With the exception of temperature up-shift (Figure 15d), all other fermentations exhibited higher cell size in the 5 h samples, indicating cell filamentation. At 10 h of growth, cells from temperature up-shift fermentation exhibited a mild increase in cell size (Figure 15d), probably resultant from the temperature increase to 42 ºC at 5 h. At this time, cells taken from the other fermentations seemed to maintain their filamentous structure. At the end of fermentations, *E. coli* cell size seemed to remain unchanged in samples collected from amino acid limitation with or without AMP addition fermentations (Figure 15a and c). Probably due to nutrient addition (tryptone), cells from AMP addition fermentation could be arranged in two distinct populations, one of them exhibiting a filamentous structure (Figure 15b). Cells from temperature up-shift fermentation suffered a mild increase in cell size when compared with the previous sample (Figure 15d).

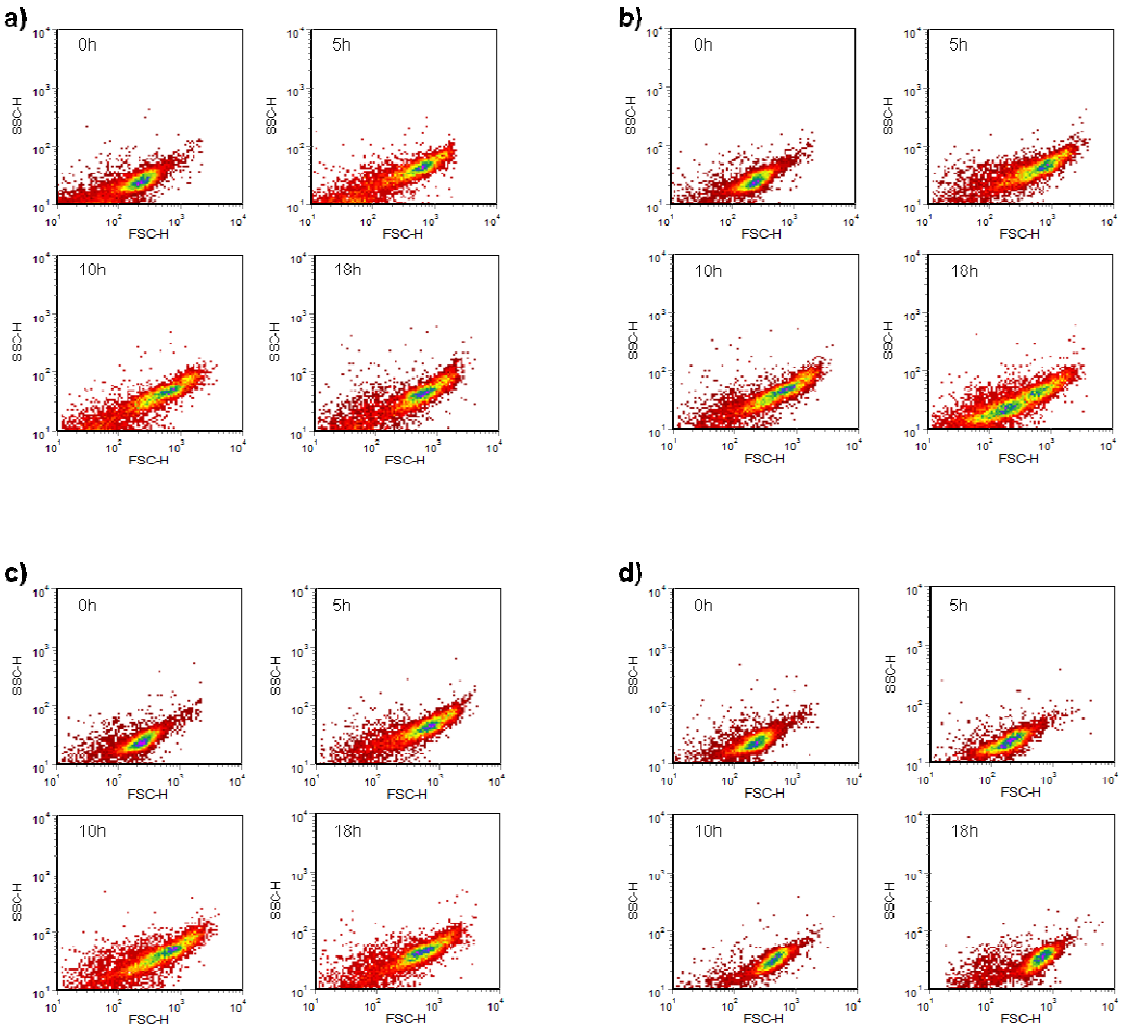

Figure 15: Light scatter measurements of cell samples taken during fermentations.

These fermentations were performed with (a) amino acid limitation without AMP addition, (b) AMP addition, (c) amino acid limitation with AMP addition and (d) temperature up-shift plasmid induction strategies. A total of 10 000 events was collected for this analysis.

Evaluation of cell physiological state and cell cycle

Since process productivity is dependent on the number of viable cells, cell viability was also assessed using PI and BOX. PI was used to assess membrane integrity, considering that healthy cells exhibit a PI-impermeable cell membrane and, if membrane integrity is compromised, PI can enter the cell where it binds to nucleic acids. BOX was used to assess membrane potential, since polarized cells are able to exclude anionic molecules such as BOX and depolarized cells allow the accumulation of BOX inside the bacterial cell. Therefore, in this work, by using a PI/BOX dual staining protocol already described [15, 34], three distinct populations can be observed: healthy cells (PI and BOX-negative cells), cells with a depolarized membrane (BOX-positive cells) and cells with a permeabilized membrane (PI and BOX-positive cells).

At the beginning of fermentations, the percentage of healthy cells was close to 100%, ranging from 94 to 96% (PI and BOX-negative cells) (Figure 16). Throughout the course of the fermentations, the percentage of healthy cells suffered a decrease to approximately 75-80%, with the exception of temperature up-shift fermentation, where healthy cells accounted for approximately 85% of the population. This decrease in bacterial viability in AMP addition and amino acid limitation with or without AMP addition fermentations was mainly due to the increase in the percentage of depolarized cells (BOX-positive cells) rather than an increase in permeabilized cells (PI and BOX positive cells) (Figure 16). This increased cell depolarization may be explained by the fact that all these three strategies are based in nutrient limitation which has proven to cause cell depolarization in *E. coli* cells [31]. Due to the limitation in nutrient and/or oxygen availability, bacterial cells often have lower cellular activity and metabolic energy that are required for the effective maintenance of cellular functions [35]. However, the majority of cells (75-80%) had intact polarized membranes, being able to withstand such fermentation conditions.

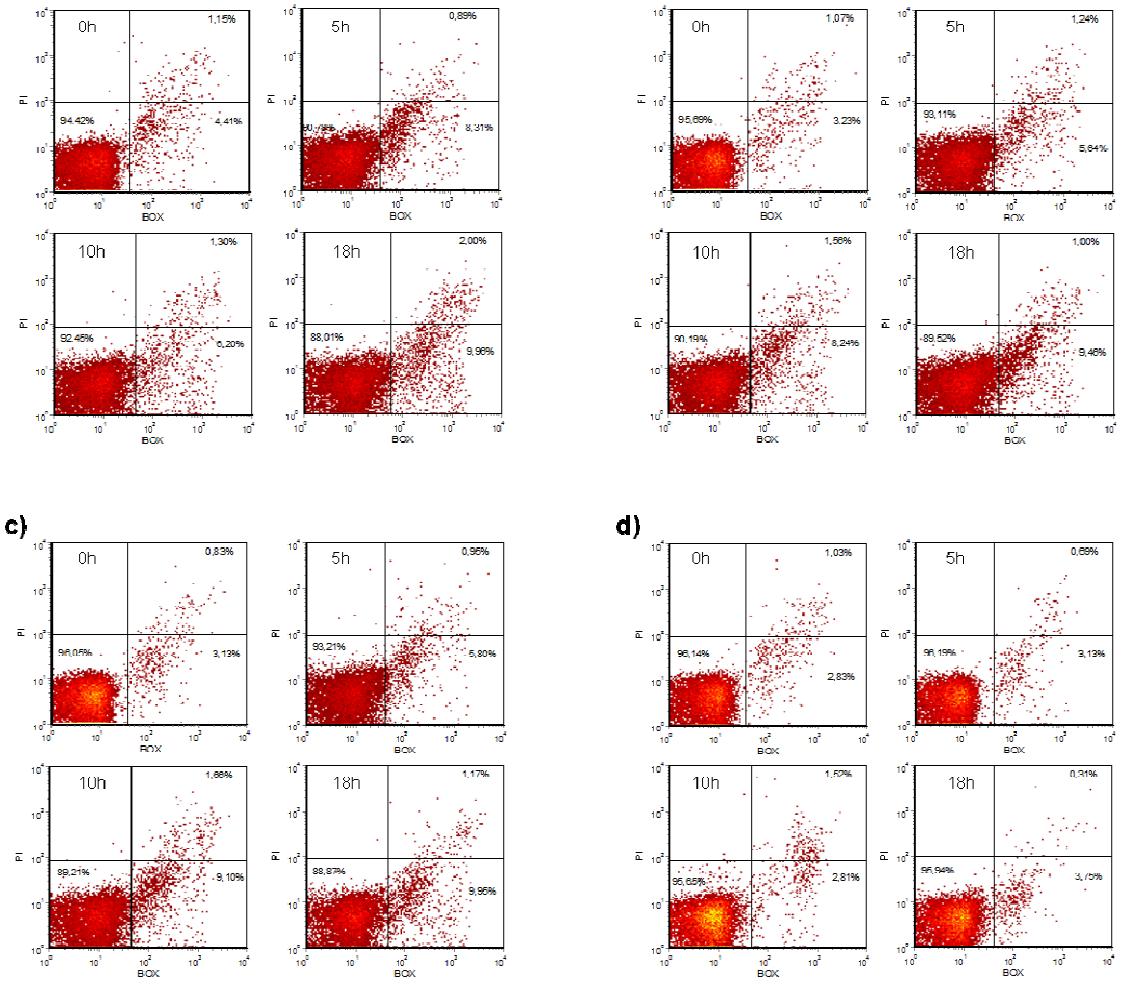

Figure 16: Dot plots of green fluorescence of cells (BOX, x axis) plotted against red fluorescence (PI, y axis) obtained with cell samples taken during fermentations.

These fermentations were performed with (a) amino acid limitation without AMP addition, (b) AMP addition, (c) amino acid limitation with AMP addition and (d) temperature up-shift plasmid induction strategies. Three main subpopulations of cells can be distinguished corresponding to: healthy cells, no staining; cells with depolarized membranes, stained with BOX; and cells with permeabilized membranes, stained with PI. A total of 10 000 events was collected for this analysis.

Cell cycle analysis provides an excellent tool to study bacterial physiology and activity states. Slowly-growing cultures of *E. coli* generally exhibit a DNA distribution with two peaks and a ridge connecting them [36]. The first peak corresponds to cells containing one chromosome equivalent, the second peak (with a double fluorescence value of the first peak) corresponds to cells containing two chromosome equivalents and the ridge corresponds to cells between one and two chromosome equivalents. For this reason, the first and second peaks correspond to cells that are not engaged in DNA replication and the ridge corresponds to replicating cells [37]. In order to assess bacterial replication, cell cycle analysis was performed using a DNA-specific dye, DRAQ5 in ethanol-fixed cells. At zero time, fluorescence distribution histograms

showed two peaks, although not very well resolved. These peaks corresponded to cells containing one or two chromosome equivalents, attending to the mean fluorescence values obtained (Figure 17). This two peak distribution, where a ridge connects the two peaks [38], corresponds to the replicating cells, showing that the inoculum contained actively-dividing cells. During fermentation, a spreading of the DNA histograms was observed (Figure 17). This could be due both to an increase in population heterogeneity and also to plasmid DNA amplification, since similar histograms have already been described in plasmid-harbouring *E. coli* fermentations [38].The most striking difference between the obtained DNA histograms obtained was a more pronounced spreading in cells taken from AMP addition and amino acid limitation with or without AMP addition fermentations (Figure 17 a, b and c). Concomitantly, these DNA histograms also exhibited higher fluorescence intensities (Figure 17 a, b and c), possibly indicating a higher chromosome content (two and four chromosome equivalents). This has been observed in cells grown under stress conditions [39], which suffer bacterial filamentation, resulting in the formation of elongated cells with multiple chromosome copies [40]. However, due to peak broadening, it was very difficult to resolve ploidy in these histograms. DNA histograms of cells taken from temperature up-shift fermentation, showed a decrease in DNA spreading at 5 h (Figure 17d), probably due to the fact that these cells were grown at 30 ºC and, consequently, pDNA amplification was lower compared with the other fermentations. After the temperature up-shift to 42 ºC, DNA histograms showed a more spread DNA distribution with increased fluorescence intensities (Figure 17d), which can corroborate the fact that this spreading is due to pDNA amplification. Even though all suspensions were at the stationary phase of growth at the end of fermentations (Figure 17a), DNA histograms did not return to the two peak distribution of a stationary culture, corresponding to characteristic cells with only one or two chromosome equivalents that are not engaged in DNA replication [38].

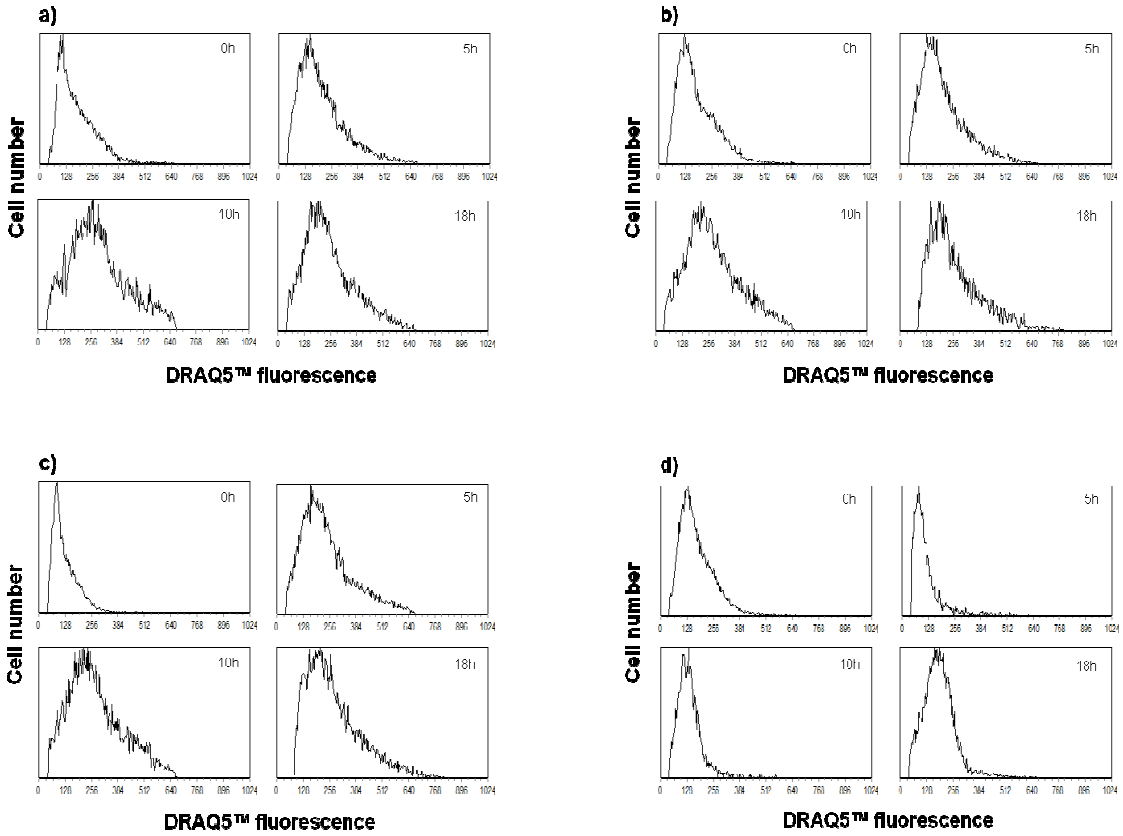

Figure 17: DNA histograms of cell samples taken during fermentations.

These fermentations were performed with (a) amino acid limitation without AMP addition, (b) AMP addition, (c) amino acid limitation with AMP addition and (d) temperature up-shift plasmid induction strategies. A total of 20 000 events was collected for this analysis.

Assessment of plasmid copy number

PCN variations in all fermentations were compared between all pDNA induction strategies used (Figure 18b). In addition, the growth curves and global PCN levels (Figure 18a and c) are presented. PCN remained practically unaltered until mid-log phase was achieved (Figure 18a and b) in all fermentations, with the exception of temperature up-shift fermentation, that suffered a PCN decrease. This decrease might be due to the fact that pVAX1-*LacZ* is not induced at a growth temperature of 30 ºC, because when the temperature increased to 42 ºC, plasmid copy number increased. Due to this fact, a temperature of 30 ºC seemed to promote segregational instability. Both amino acid limitation fermentations showed a similar PCN variation profile up to 10h of fermentation. At the end, both these fermentations revealed higher PCN when compared to fermentations using induction strategies without amino acid limitation. However, the increase was more pronounced in the amino acid limitation fermentation with AMP addition (Figure 18b). Regarding AMP addition fermentation, PCN variation throughout fermentation was not very pronounced, with only a slight PCN increase after AMP addition (10 h) when compared to PCN values obtained at 0 h. When analysing final

PCN obtained, plasmid amplification was more efficient in amino acid with AMP addition fermentations with a PCN value of 1070 per cell, whereas all the other fermentations had similar PCN values, ranging from 700-800 per cell. Almost all PCN values obtained fell within the range reported for high-copy plasmids by other authors [41] and some values were slightly higher than expected. However, it has been reported that, in certain conditions, plasmids containing a pPMB1-derived origin can reach PCN of around 2700 per cell [41]. Some authors have described that PCN values reach a maximum in the exponential phase of growth, followed by a sharp decrease when cells enter into stationary phase [20] while others refer that PCN reaches maximal values in the late exponential growth phase [28]. Our results suggest that maximum PCN values are obtained upon entry into stationary phase of growth (Figure 18a and b). There are several reasons that could explain this fact: firstly plasmid amplification occurred in amino acid limitation conditions achieved in the stationary phase of growth; secondly AMP addition also caused plasmid DNA amplification and this addition only occurred at 10 h of growth and finally a growth temperature of 42 ºC resulted in plasmid DNA amplification which did not occur at 30 ºC.

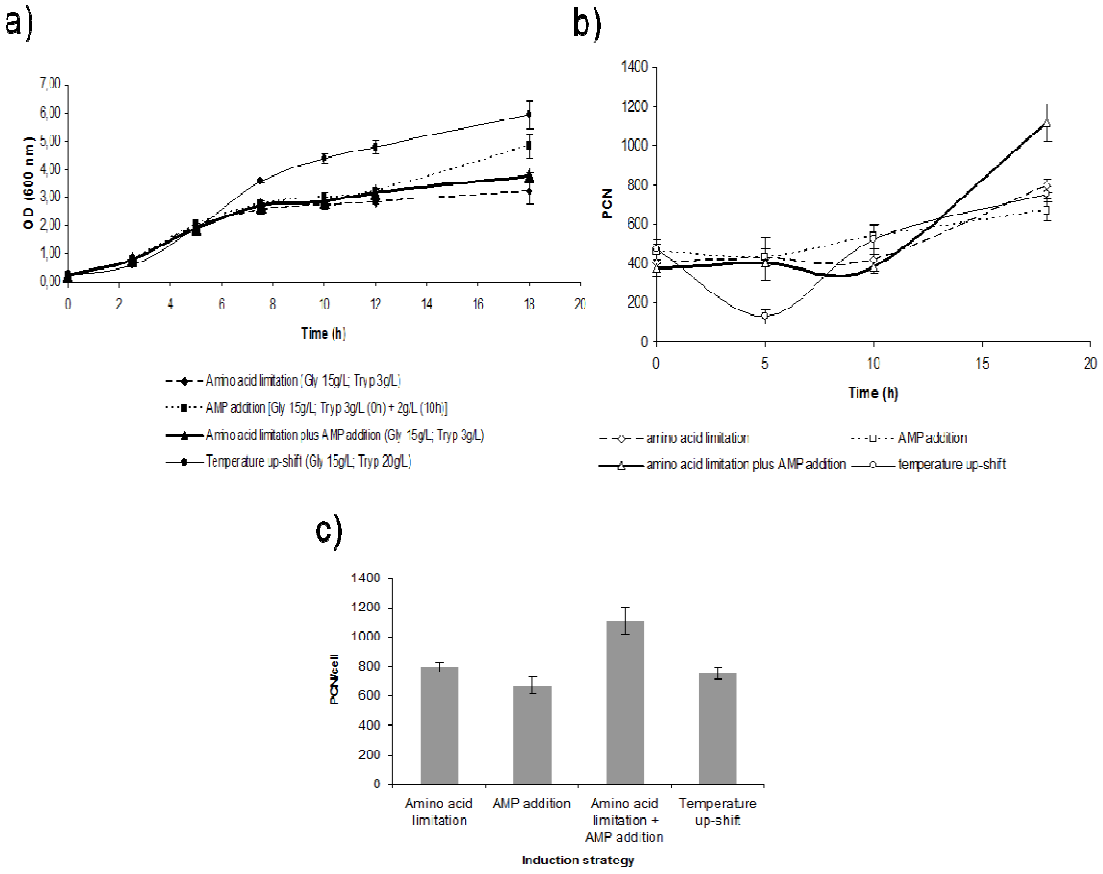

Figure 18: Effect of induction strategy on plasmid copy number per cell.

(a) growth profiles of *E. coli* DH5 alpha harboring plasmid pVAX1-*LacZ* performed in shake flasks at 250 rpm in semi-defined medium using different induction strategies, (b) plasmid copy number evolution throughout fermentation using different induction strategies and (c) comparison between the PCN

values at the end of fermentation for each induction strategy used. The mean results and standard deviations of three independent fermentation runs are presented. Abbreviations: Gly (glycerol concentration used) and Tryp (tryptone concentration used).

Plasmid DNA yield

Plasmid DNA yield, expressed as a means of specific plasmid DNA yield (Figure 19a), and plasmid DNA integrity (open circular and supercoiled isoforms) (Figure 19b) were assessed in lysates from *E. coli* cells recovered at the end of the fermentations. Amino acid limitation with AMP addition fermentation had a significantly higher specific yield (20.94 mg/g) when compared with all the other induction strategies ($p<0.001$). Amino acid limitation without AMP addition (12.33 mg/g) also had significantly higher specific yields ($p<0.001$) when compared with AMP addition (8.85 mg/g) and temperature up-shift (9.34 mg/g) induction, which showed similar specific yields. Agarose gel electrophoresis confirmed the quality of the plasmid DNA with low quantities of the open circular isoform (Figure 19b, lanes 1 to 4) and high quantities of supercoiled plasmid DNA (Figure 19b, lanes 1 to 4) with no visible bands corresponding to RNA.

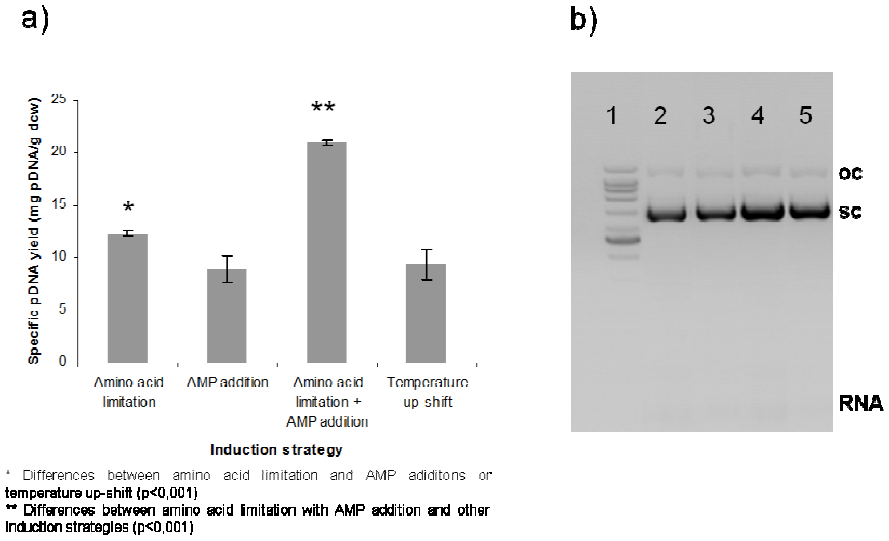

Figure 19: Effect of the induction strategy in plasmid yield and plasmid quality.

(a) plasmid DNA yield variation between all induction strategies used and (b) agarose gel electrophoresis of cell lysate resultant from fermentations with different induction strategies: amino acid limitation without AMP addition (lane 2), AMP addition (lane 3), amino acid limitation with AMP addition (lane 4) and temperature up-shift (lane 5). In Figure 19a), the mean results and standard deviation error bars of three independent fermentation runs are presented and the statistical analysis was performed using One-way ANOVA. Abbreviations: oc (open circular pDNA), sc (supercoiled pDNA) and molecular weight marker (lane 1).

2.3.5. Conclusion

The study of cell morphology and chromosome content showed that, in response to stress conditions, like plasmid-imposed metabolic burden, DNA replication occurs. Since chromosome content is higher but *E. coli* cells are not able to divide, filamentation occurs. By establishing correlations between intracellular constituents and morphology or PCN, it was possible to establish significant positive correlations between intracellular protein and DNA contents and also between protein intracellular content and cell size.

The presented work showed that, depending on the induction strategy used, different cell morphology, cell viability, plasmid copy number and plasmid specific yield were obtained. When compared with the other induction strategies, amino acid limitation with AMP addition also showed the highest plasmid segregational stability (1070 PCN per cell) and the highest plasmid specific yield (20.94 mg/g). Despite nutrient limitation, cells were able to withstand such fermentation conditions, considering that 75-80% of the cells had intact polarized membranes.

Overall, this work describes a systematic approach in order to easily assess cell physiological states and plasmid segregational stability that can be applied to the vast majority of fermentation bioprocesses using recombinant microorganisms, allowing an at-line monitoring of bioprocess performance. This approach allowed us to conclude that an amino acid limitation with AMP addition induction strategy could be the suitable strategy for plasmid DNA production platforms since it is very practical and does not require additional energy in order to rise growth temperature.

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2.4. Plasmid DNA fermentation strategies: influence on plasmid stability and cell physiology

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2.4.1. Abstract

In order to provide sufficient pharmaceutical-grade plasmid DNA material it is essential to gain a comprehensive knowledge of the bioprocesses involved; so, the development of protocols and techniques that allow a fast monitoring of process performance is a valuable tool for bioprocess design. Regarding plasmid DNA production, the metabolic stress of the host strain as well as plasmid stability have been identified as two of the key parameters that greatly influence plasmid DNA yields. The present work describes the impact of batch and fed-batch fermentations using different C/N ratios and different feeding profiles on cell physiology and plasmid stability, investigating the potential of these two monitoring techniques as valuable tools for bioprocess development and design. The results obtained in batch fermentations showed that plasmid copy number (PCN) values suffered a pronounced increase at the end of almost all fermentation conditions tested. Regarding fed-batch fermentations, the strategies with exponential feeding profiles, in contrast with those with constant feeding, showed higher biomass and plasmid yields, being the maximum values obtained for these two parameters of 95.64 OD_{600} and 344.3 mg pDNA/L, respectively, when using an exponential feed rate of 0.2 h^{-1}. Despite the results obtained, cell physiology and plasmid stability monitoring revealed that, although higher pDNA overall yields were obtained, this fermentation exhibited lower plasmid stability and percentage of viable cells. In conclusion, this study allowed clarifying the bioprocess performance based on cell physiology and plasmid stability assessment, allowing improving the overall process and not only plasmid DNA yield and cell growth.

Keywords: plasmid DNA; batch; fed-batch; cell physiology; segregational stability

2.4.2. Introduction

Over the past decades, advancements in somatic gene transfer delivery have transformed gene therapy from a futuristic fantasy to a scientific reality, envisioning gene therapy potential to significantly advance clinical medicine. Nowadays, gene therapy encompasses not only the delivery of plasmid DNA (pDNA) but also the delivery of antisense oligonucleotides (ASOs) and RNA interference (RNAi)-based systems to target cells [1]. Several vector systems have been developed to facilitate the introduction of therapeutic genetic material into cells. These include DNA- based vectors, viral vectors and, most recently, vectors that induce RNA interference [2]. Although adenoviral vectors are the most commonly used vectors in gene

therapy clinical trials, their use in clinical practice still presents some limitations [3]. These constraints can be overcome by the use of non-viral vectors which provide a more favorable risk/benefit analysis than viral vectors [4]. Regarding non-viral vectors, naked/plasmid DNA vectors are presently amongst the most widely used vectors in gene therapy, accounting for 18.7% of all clinical trials worldwide, targeting pathologies such as cancers, cardiovascular diseases, ocular diseases, infectious and monogenic diseases as well as autoimmune and neurological disorders (http://www.wiley.com/legacy/wileychi/genmed/clinical).

In order to be able to provide enough plasmid DNA material for its use in clinical trials, there is a compelling need to design better pDNA preparation processes. These processes should also meet the GMP requirements for long-term transfection of plasmid DNA suitable for application in gene therapy and DNA vaccination. Although not suitable for clinical applications, non-GMP pDNA production in a culture medium containing animal-derived products can be successfully applied for transient transfection experiments, after suitable chromatographic purification [5]. Over the last years, effort is being made in the development of production steps capable of meeting the requirements of clinical trials for large amounts of plasmid DNA [6-7]. Several recent reports have proposed pDNA production approaches using various fermentation strategies. For instance, Danquah and Forde, using a batch strategy with complex media obtained a volumetric pDNA yield of more than 60 mg/L [8]. Carnes and collaborators have proposed a continuous fermentation process for pDNA production, with two bioreactors operating at different conditions, attaining high yields of pDNA (up to 1.2 g pDNA per hour) [9]. However, fed-batch fermentation remains the most commonly used strategy for plasmid DNA production, either using feedback control [10] or predetermined feeding profiles such as exponential and constant feeding [11]. For example, Carnes *et al*. reported that in fed-batch fermentation with predetermined profiles, exponential feeding was able to produce higher pDNA amounts (1497 mg pDNA/L) when compared to the use of constant feeding rates (620 mg pDNA/L) [12]. More recently described fed-batch processes with exponential feeding using optimized strains and vectors were able to yield plasmid volumetric titres of 2220 [6] and 2600 mg pDNA/L [13]. Moreover, Listner and collaborators using a fed-batch strategy with a constant feeding profile were able to obtain plasmid DNA yields of up to 1600 mg/L [14]. Fed-batch strategies using feed-back controls based on pH-DO feed-back control [15] and DO-feed-back control [16] have yielded plasmid DNA volumetric titres of 250 mg/L and 1923 mg/L, respectively. Despite the high yields of plasmid DNA obtained in these fermentations, the impact of these strategies on key process performance parameters is not addressed.

Plasmid segregational stability is one of the keystones in recombinant fermentation processes [17] due to the existence of plasmid-free segregants even when selective pressure is exerted as a result of the leakage of the selective gene product into the media from plasmid-bearing cells [18]. Another critical parameter in recombinant fermentations is the host cell physiology

[19] since changes in cell physiological state may reflect alterations in the host metabolic burden. Although plasmid maintenance and replication *per se* can impose a metabolic burden to *E. coli* cells [20], there are other pDNA-related factors that could increase this metabolic burden. These factors comprise the use of high plasmid copy numbers [21], increased vector size [22], increased synthesis of plasmid-encoded proteins such as antibiotic resistance proteins [23] and the presence of additional elements in plasmid backbone [24]. This plasmid-related "metabolic burden" is known to result in decreased host cell growth or viability, plasmid loss, low productivity and poor product quality [20]. So far, the impact of different fermentation strategies on host cell physiology and plasmid on process performance and overall plasmid yield is scarce, with only a few reports on the effect of fermentation strategy on plasmid yield and quality [25]. Thus, the present work describes the impact of batch and fed-batch fermentations using different glycerol/tryptone ratios and different predetermined feeding profiles, such as exponential and constant feed rates, on cell physiology, plasmid stability and overall plasmid yield, investigating the potential of these two monitoring techniques as valuable tools for bioprocess development and design.

2.4.3. Materials and Methods

Plasmid and bacterial strain

The bacterial host for plasmid pVAX1-LacZ (Invitrogen, Carlsbad, USA) was *E. coli* DH5 alpha [F^- ϕ80lacZΔM15, Δ(lacZYA-argF), U169, recA1, endA1, hsdR17(r_k^-, m_k^+), phoA, supE44, thi-1, gyrA96, relA1, tonA] (Invitrogen, Carlsbad, USA). The plasmid pVAX1-LacZ is 6.05 kbp long, contains a pUC origin of replication, a cytomegalovirus (CMV) promoter and a kanamycin resistance gene for selection purposes.

Cultivation conditions

Recombinant *E. coli* DH5 alpha was cultivated in semi-defined medium [26-27] containing glycerol as carbon source, tryptone as nitrogen source. This semi-defined medium containing kanamycin sulphate 30 mg/L, with variable glycerol and tryptone concentrations, was used in all pre-cultures, batch fermentations as well as the batch phases of the fed-batch fermentations. In all fermentations performed, cultures were inoculated with a pre-culture grown in 500 mL shake flasks containing 125 mL of medium at 250 rpm at 37 ºC. To obtain a better reproducibility between assays, all cultures started with an OD_{600} of approximately 0.2. Growth was suspended at the stationary phase of growth. For each fermentation condition used, two independent fermentation runs were performed.

Bacterial fermentations were carried out in four 750 mL parallel mini-bioreactors (Infors HT, Switzerland) with 250 mL of semi-defined medium. The bioreactors were operated with strictly controlled parameters including pH, temperature, airflow, agitation, dissolved oxygen

and oxygen supplementation. The temperature was set at 37 ºC while the pH was set at 7.0 and maintained through the automatic addition of 1 M NaOH and 1 M. H2SO4. Foaming was controlled manually by the addition of antifoam agent antifoam A (Sigma-Aldrich, St. Louis, MO). Dissolved oxygen concentration was maintained at 30 % by automatic adjustment of agitation, airflow and oxygen supplementation.

For batch fermentations, different glycerol and tryptone concentrations ranging from 20 to 50 g/L were evaluated. In the case of fed-batch fermentations, the culture medium initially contained 20 g/L glycerol and 20 g/L tryptone. The feed medium had the following composition: glycerol 400 g/L; tryptone 80 g/L and magnesium sulphate heptahydrate 20 g/L for all experiments, with the exception of the fed-batch fermentations with an exponential feeding rate of 0.2 h^{-1}, where the glycerol concentration used was 600 g/L. The feed medium was supplemented with kanamycin sulphate 250 mg/L in order to prevent issues related with possible antibiotic degradation [28] and dilution in the culture medium. After a rise on the levels of dissolved oxygen and pH, the feed medium was added to the bioreactor at exponential or constant feed rates. The addition of feed medium was achieved using peristaltic pumps that were automatically controlled by IRIS software (Infors HT, Switzerland) using a feeding profile previously programmed. For exponential feeding, the feed rate was calculated automatically according to the equation described by Carnes and collaborators [12] where three different desired specific growth rates (0.06, 0.12 and 0.2 h-1) during the fed-batch phase were evaluated. In the case of constant feeding, the feed medium was added to the bioreactor in order to maintain three desired constant rates of glycerol addition: 1.6, 3.2 and 6.4 g L-1h-1. The final volumes of feeding medium added to fed-batch fermentation with exponential feeding of 0.06, 0.12 and 0.2 h-1 were, approximately, 18, 169 and 212 mL, respectively. For fed-batch fermentations with constant feeding profiles, the feeding medium volumes added to the fermentations with constant rates of 1.6, 3.2 and 6.4 g L-1h-1 were 23, 46 and 92 mL, respectively. At the end of the fermentation, cells were recovered by centrifugation at 5000× g for 20 min and the cell pellet was subjected to cell lysis as previously described [29]. Considering that the biomass obtained among fermentations was considerably different and that the amount of biomass can influence the efficacy of cell lysis procedures, a ratio between culture media volume and OD600 was maintained in order to ensure that the amount of biomass subjected to cell lysis was kept constant.

Analytical chromatography

HPLC was used to measure pDNA concentration and purity in *E. coli* lysates according to the method described by Diogo and co-workers [30]. Plasmid DNA standards were made with highly purified pVAX1-LacZ (6.05 kbp) plasmid obtained with a commercial Qiagen kit (Hilden, Germany) according to the manufacturer instructions. The concentration of pDNA in each sample was calculated using a calibration graph constructed using six pDNA standards (1-400 mg/L). These standards were prepared by measuring pDNA concentration

spectrophotometrically. pDNA concentration were calculated as the mean of three independent samples.

Real-time qPCR

Quantitative real-time PCR was used for the determination of plasmid copy number in whole *E. coli* cells according to our previously reported method [31]. PCR reactions were carried out in a Bio-Rad iQ™ 5 Real-Time PCR Detection System using the Maxima™ SYBR Green qPCR Master Mix (2x) (Fermentas, Canada).

Flow cytometry

Bacterial samples were analysed on a BD Biosciences FACSCalibur. Acquisition was performed with CellQuest™ Pro Software Light scatter measurements and fluorescence signals were acquired on a logarithmic scale, with the exception of the FL-4 signal, which was acquired on a linear scale. Data analysis was performed using FCS Express version 3 Research Edition (De Novo Software™, Los Angeles, USA).

Samples withdrawn from fermentation at regular time intervals were analysed immediately using a propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) and bis-(1,3-dibutylbarbituric acid)trimethine oxonol (BOX) (Molecular Probes®, Invitrogen, part of Life Technologies, Carlsbad, CA) dual staining protocol [31] or fixed with 70% ethanol and stored at -20ºC for DNA analysis using DRAQ5 (Biostatus Limited, UK) [32].

2.4.4. Results

Cell physiology and plasmid copy number in batch fermentations

Over the last years, several plasmid DNA production processes have been proposed. Most of these processes are based in fed-batch fermentation strategies. It has been described that batch cultivations enabled high specific growth rates and higher accumulation of metabolic by-products such as acetate, which could lead to higher plasmid instability and growth reduction [33]. Despite these disadvantages, batch cultivations are easier to perform and have generally lower cultivation times [34]. Since the carbon-nitrogen ratio (C/N) was shown to influence plasmid DNA yield and plasmid stability [35], in this work we evaluate the influence of several tryptone and glycerol concentrations on overall process performance.

In all batch conditions tested, with the exception of glycerol 20 g/L/tryptone 40 g/L, plasmid copy number achieved its highest value at the end of the fermentation (Figure 20) with an increment in PCN ranging from 222 to 87% of the initial inoculum PCN value (Table 11). Although PCN variations differ for each batch conditions tested, final PCN values were similar, ranging from 802 to 1061. In the case of glycerol 20 g/L/tryptone 40 g/L

fermentation, PCN value decreased at the end of the fermentation (Figure 20b). However, when testing different C/N ratios with a fixed tryptone concentration of 50 g/L and glycerol concentrations ranging from 30 to 50 g/L, there is no significant alteration in the PCN values obtained at the end of fermentations (ranging from 918 to 972, respectively) as well as in the PCN profiles obtained during fermentation (Figure 20d-f). In respect to cell physiology, the percentage of healthy cells suffered some fluctuations throughout the fermentation (Figure 20) that were more pronounced in the case of glycerol 30 g/L/tryptone 50 g/L fermentation (Figure 20d). The fermentation that exhibited the lowest fluctuations was the one containing the highest glycerol and tryptone concentrations, possibly because of the higher amount of nutrients available during fermentation. As opposite to plasmid stability, different C/N ratios did not seem to have a pronounced effect on the percentage of healthy cells (unstained cells) that was kept at approximately 90% at the end of fermentations (Figure 20).

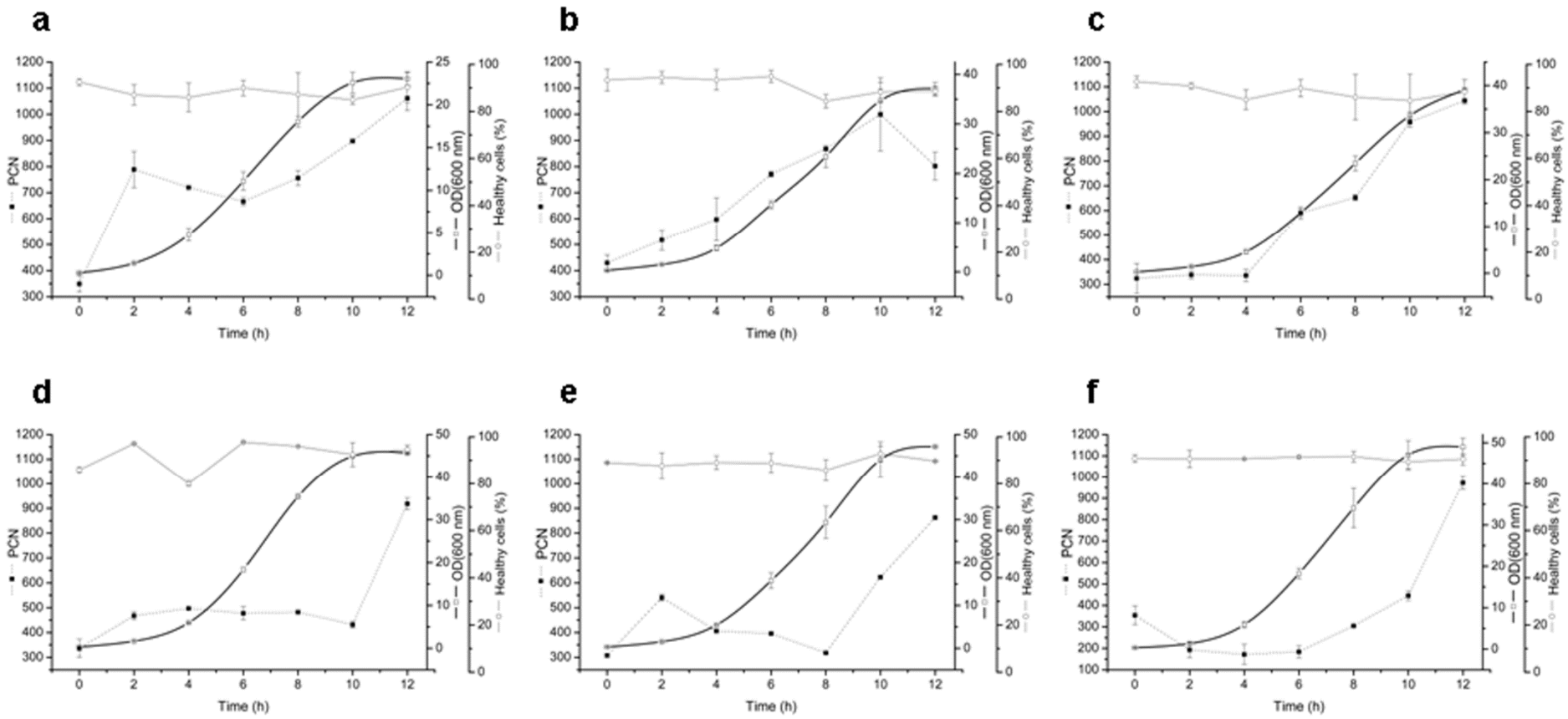

Figure 20: Variations on plasmid copy number per cell (PCN) (---■---), optical density at 600 nm (—□—) and percentage of healthy cells (—○—) during batch fermentations with (a) 30 g/L glycerol and 20 g/L tryptone; (b) 20 g/L glycerol and 40 g/L tryptone; (c) 30 g/L glycerol and 40 g/L tryptone; (d) 30 g/L glycerol and 50 g/L tryptone; (e) 40 g/L glycerol and 50 g/L tryptone; (f) 50 g/L glycerol and 50 g/L tryptone.

The mean results and standard deviations of two independent fermentation runs are presented.

When analysing DNA distributions by DRAQ5 staining of fixed-cells, all fermentations displayed similar cell cycle profiles where a shift from the two peak distribution with a ridge connecting both peaks to a more heterogeneous population with broader peaks corresponding to higher chromosome contents is obtained (Figure 21a). As expected, biomass yield and growth kinetics were dependent on glycerol and tryptone concentrations used (Figure 20); though, in some cases, the increase of 10 g/L in glycerol concentration did not have any significant effect on the final biomass, yielding only a two units increase in final biomass.

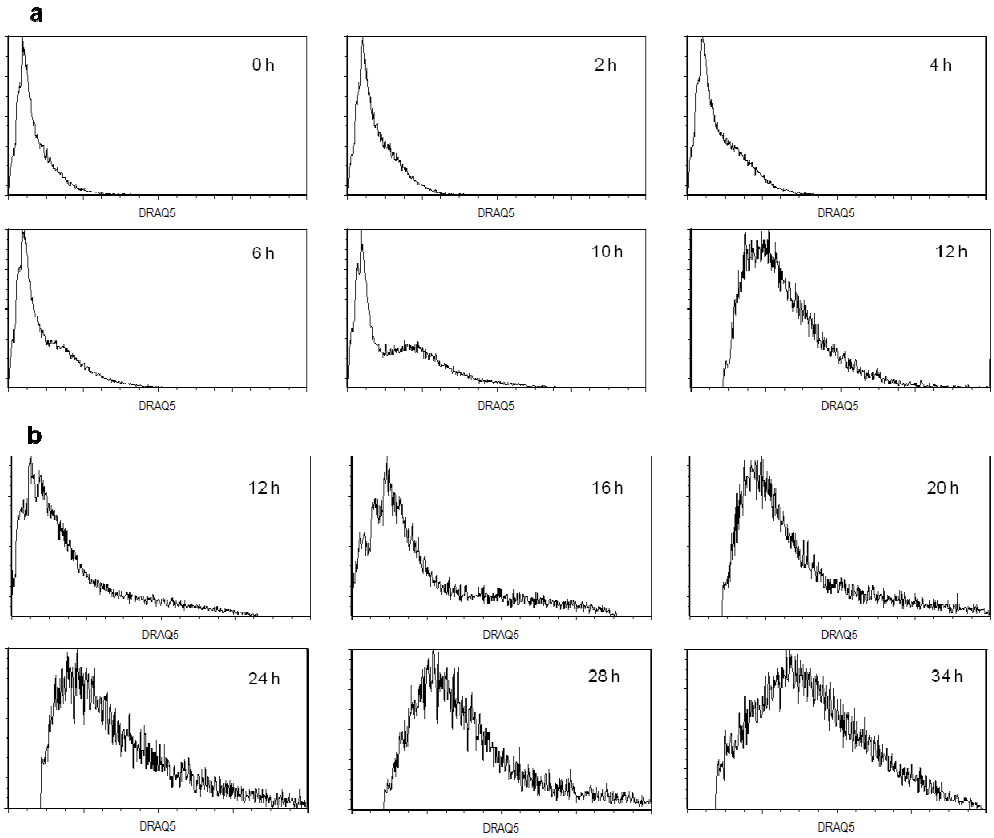

Figure 21: Typical DNA histograms of cell samples taken during (a) batch and (b) fed-batch fermentations.

A total of 30 000 events was collect for this analysis.

Cell physiology and plasmid copy number in fed-batch fermentations

It has been described that in fed-batch fermentations, higher plasmid yields were obtained when pre-determined feeding profiles were used in comparison to feeding profiles based on a feed-back control using parameters such as pH or dissolved oxygen [12, 36-37]. Since plasmid stability is thought to be closely associated with the specific growth rate [33], several desired specific growth rates were tested in exponential feed rates (0.06, 0.12 and 0.20 h^{-1}) and, in constant feed rates, different glycerol addition rates were tested in order to also obtain distinct specific growth rates. In order to obtain more reproducible results, all fed-batch

fermentations were initiated at 12 h of growth of a batch fermentation containing 20 g/L glycerol and 20 g/L tryptone.

Regarding plasmid segregational stability, PCN values suffered high fluctuations during all fed-batch fermentations, not only in the fed-batch phase but also in the batch phase (Figure 22). When comparing the PCN values obtained at the beginning of the nutrient feeding and at the end of fermentation, it can be observed that there seems to be some extent of segregational instability in fed-batch fermentations either with exponential or constant feed profiles with an overall decrease in final PCN ranging from 10.7 to 38.4 % with an maximum decrease in PCN value of 38.4 % in comparison to the initial values, the only exception being the fed-batch fermentation with a constant feed rate of 3.2 g gly L^{-1} h^{-1} (Figure 22b) where an increase of approximately 4.2% in final PCN in relation to initial PCN value is achieved. The maximum PCN decrease was observed in the fed-batch fermentation with a constant feed rate of 6.4 g gly L^{-1} h^{-1} (Figure 22c). These results showed that lower specific growth rates do not correspond necessarily to higher plasmid stability, since the lower values for plasmid instability and higher values for plasmid stability were attained both with low values of exponential (0.06 h^{-1}) and moderate values of constant (3.2 g gly L^{-1} h^{-1}) feed rates, respectively (Figure 22b and 22e).

Regarding cell physiology, the feeding strategy used did not seem to have a significant influence on cell viability (Figure 22), with the exception of the fed-batch fermentation with an exponential feed rate of 0.2 h^{-1} where, at the end of fermentation, healthy cells percentage decrease to values below 70 % (Figure 22f). This could be due to the fact that the higher dilution rate in this fermentation could negatively influence cell physiology. DNA distributions of samples collected during the fed-batch phase showed a peak broadening throughout the entire fermentation (Figure 21b), despite the fact that cell division is occurring as indicated by the increasing values of optical density. This peak broadening could indicate more pronounced population heterogeneity during the fed-batch phase that could also be related to the PCN fluctuations previously described.

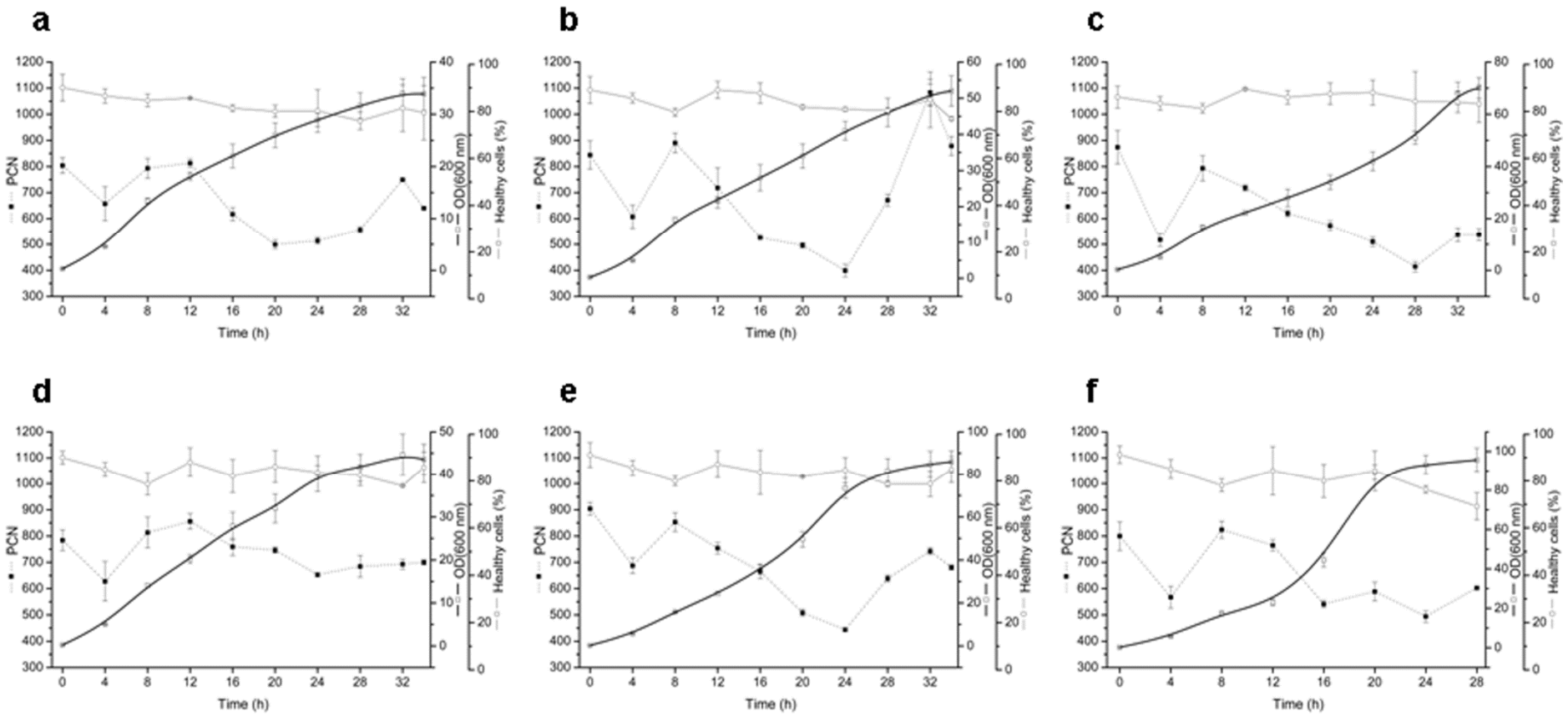

Figure 22: Variations on plasmid copy number per cell (PCN) (---■---), optical density at 600 nm (—□—) and percentage of healthy cells (—○—) during fed-batch fermentations with constant feed rates of (a) 1.6 g gly L-1h-1, (b) 3.2 g gly L-1h-1 and (c) 6.4 g gly L-1h-1 g/L and exponential feed rates of (d) 0.06 h-1, (e) 0.12 h-1 and (f) 0.20 h-1.

The mean results and standard deviations of two independent fermentation runs are presented.

Plasmid yields in batch and fed-batch fermentations

In regard to batch fermentations, the maximum specific growth rates obtained are very similar, ranging from 0.61 to 0.67 h^{-1} (Table 11). One could expect that, on account of the different glycerol and tryptone concentrations tested, different specific growth rates were attained; however, during the first hours of growth (up to 6h), where the maximum specific growth rate is achieved, the biomass growth on all fermentations is almost identical and the differences between growth are only observed close to the end of fermentation. Batch fermentations yielded moderate cell densities (up to approximately 50 OD_{600}) (Table 11) and up to 149.0 mg/L of plasmid DNA (Table 11) when using the highest tryptone and glycerol concentrations (50 g/L).

Table 11: Effect of batch fermentations with different glycerol and tryptone concentrations on final biomass concentration (expressed as optical density at 600 nm), plasmid DNA volumetric and specific yields and maximum growth rate obtained (μ_{max}).
The results are expressed as mean±standard deviation of two independent fermentation runs.

Tryptone concentration (g/L)	Glycerol concentration (g/L)	OD_{600}	pDNA volumetric yield (mg pDNA/L)	pDNA specific yield (mg pDNA/g dcw)	μ_{max} (h^{-1})	PCN variation (%)[a]
20	30	23.04±0.74	70.1±1.6	8.33±0.43	0.61±0.02	(+) 204.3±10.7
40	30	39.08±0.28	117.2±1.6	7.97±0.07	0.64±0.04	(+) 222.2±15.6
40	20	37.12±1.24	99.9±7.3	6.99±0.55	0.62±0.01	(+) 86.9±8.9
50	30	45.07±0.14	128.8±3.9	7.50±0.49	0.67±0.02	(+) 173.2±10.3
50	40	47.18±0.11	135.9±14.7	7.63±0.88	0.65±0.01	(+) 180.8±20.2
50	50	49.00±2.12	149.0±15.2	8.32±0.53	0.67±0.03	(+) 174.6±15.4

[a]This was calculated by the relation between initial PCN value and final PCN value. The (+) symbol indicates an increase in final PCN value while the (-) values indicates a decrease in final PCN value.

With respect to fed-batch fermentations (Table 12), the addition of feeding medium at a constant feed rate yielded lower specific growth rates, with a maximum value of 0.08 h^{-1} for the highest feed rate tested, with concomitant lower cell growth during the 34 h fermentation time (Table 12). Since exponential feed profiles were set to a desired specific growth rate inferior to the maximum specific growth rate (μ_{max}), the maximum specific growth rates accomplished during these fermentations were very similar to the desired growth rates defined for the feed profiles (Table 12). Although batch fermentations exhibited higher PCN values than fed-batch fermentations, the plasmid specific yields were higher in fed-batch fermentations. The differences among specific pDNA yields in fed-batch fermentations were in accordance to the previous results obtained for PCN. As expected, plasmid DNA volumetric yields were higher in fed-batch fermentations when compared to batch fermentations, due to higher biomass values obtained (Table 12). In general, constant feeding profiles yielded lower plasmid titres, probably as a consequence of the lower specific

growth rates and biomass values obtained for these fermentations at the end of the 34 h fermentation time. This lower specific growth rates can be due to the glycerol feeding rates chosen for these feeding profiles. The highest value of final biomass (95.64 OD_{600}) and concomitant highest value of pDNA volumetric yield (344.3 mg/L) was achieved using an exponential feeding profile with a desired specific growth rate of 0.2 h^{-1} (Table 12).

Table 12: Effect of different exponential and constant feed rates on final biomass concentration (expressed as optical density at 600 nm), plasmid DNA volumetric and specific yields and maximum growth rate obtained (μ_{max}).
The results are expressed as mean±standard deviation of two independent fermentation runs.

Feed profile	Feed rate	OD_{600}	pDNA volumetric yield (mg pDNA/L)	pDNA specific yield (mg pDNA/g dcw)	μ_{max} (h^{-1})	PCN variation (%)[a]
Constant	1.6 g $glyL^{-1}h^{-1}$	33.88±4.21	152.1±25.4	11.92±0.54	0.03±0.00	(-) 20.3±1.2
	3.2 g $glyL^{-1}h^{-1}$	52.00±4.21	207.3±16.5	10.18±1.98	0.05±0.01	(+) 4.2±0.5
	6.4 g $glyL^{-1}h^{-1}$	70.00±4.02	221.5±37.5	8.38±0.54	0.08±0.02	(-) 38.4±4.2
Exponential	0.06 h^{-1}	43.52±3.58	157.5±15.7	9.61±0.70	0.05±0.01	(-) 10.7±2.3
	0.12 h^{-1}	85.94±5.03	324.5±20.3	9.99±0.51	0.10±0.03	(-) 24.7±1.7
	0.20 h^{-1}	95.64±6.05	344.3±10.2	9.31±0.28	0.17±0.04	(-) 24.6±3.5

[a]This was calculated by the relation between initial PCN value and final PCN value. The (+) symbol indicates a positive variation while the (-) values indicates a negative variation.

2.4.5. Discussion

Among the described factors affecting plasmid yields, vector characteristics can have a great influence on overall plasmid yields as a result of the alterations in plasmid stability and host cell metabolic burden. For instance, the production of a plasmid containing an antibiotic resistance gene can be influenced by the use of selective pressure during fermentation [38]; although, recently, the use of antibiotic-free systems has proven to be a safer alternative, with the attainment of higher pDNA yields [39]. Furthermore, the nature and size of the gene of interest as well as the presence of CpG motifs or polyadenylation sequences can impact plasmid DNA titres obtained due to the generation of plasmid structural instability [40] and possible metabolic burden caused by the encoded gene product resultant from background translation from the plasmid promoter [41]. So, in order to alleviate these plasmid-related deleterious effects and improve plasmid stability, several alternatives have been proposed, such as the design of minimized vectors, the use of alternative promoters [42] and also the use of post-segregational killing selection [43].

Regarding batch fermentations, maximum PCN values were obtained at the end of the fermentation, at the beginning of the stationary phase of growth, which is in agreement with

our previous results [31]. The only exception to this observation was the batch fermentation with glycerol 20 g/L/tryptone 40 g/L, probably due to the fact that lower C/N are responsible for lower plasmid stability [35]. Also, in general, lower tryptone concentrations yielded higher PCN values, possibly due to some plasmid amplification that occurred in response to amino acid limitation [27]. When analysing cell viability, the percentage of healthy suffered some alterations during batch fermentations, but the percentage of healthy cells was kept to approximately 90% and DNA distribution histograms obtained were very similar for all conditions tested.

With respect to the fed-batch fermentations, several different pre-determined profiles were used, that resulted in different growth rates. The assessment of plasmid copy number revealed higher fluctuations than the ones obtained during batch fermentations and also plasmid stability was higher at intermediate pre-determined growth rates which is in line with a new model recently proposed which stated that there is an optimal specific growth rates for maximum plasmid stability at 37 ºC [44]. Since different amounts of feeding medium were added to fermentations in order to obtain the desired conditions, another factor to be taken into account is the dilution rate. Kilonzo and collaborators [45] have demonstrated that an increase in the dilution rate led to an increase in plasmid DNA stability; nonetheless, our results did not show any relation between plasmid stability and the dilution rate. Since the feed medium also contained antibiotic, another factor to be taken into account is the influence of selective pressure on plasmid segregational stability. The studies regarding antibiotic selective pressure obtained by other groups are not consensual whether the use of selective pressure can effectively improved plasmid segregational stability [28, 46]. Our results showed that this factor does not seem to influence plasmid stability, since higher antibiotic quantities added to the growth medium did not improve plasmid stability.

Similarly to PCN, cell viability also showed higher fluctuations in fed-batch fermentations that could be linked to the extended cultivation time [47]. Cell cycle analysis of fed-batch fermentations presented a pronounced peak broadening similar to the ones obtained at the end of the batch fermentations. The slight discrepancy obtained for PCN values and plasmid specific yields in fed-batch fermentations can be due to the pronounced cell filamentation obtained for these cells, which influenced OD_{600} readings [48] that in turn influenced the amount of cells analysed by real-time qPCR. Another factor to be taken into account is the fact that plasmid specific yields are calculated after cell lysis which can lead to inaccurate results due to the variability of the extraction and precipitation procedures [49].

Overall, our results for PCN variation during fermentation are similar to the ones reported for plasmid specific yields variation by other authors also using *E. coli* DH5 alpha harbouring a different plasmid [25]. However, another study using *E. coli* DH5 alpha harbouring a different plasmid has shown different trends in PCN variation during batch cultivation [50] when compared to our results and others reported [25]. Therefore, plasmid DNA characteristics can

also seem to influence plasmid copy number during fermentation. However, the trend for a reduction in cellular viability at the end of fermentations seems to be in agreement with the results reported by other authors using different *E. coli* strains [51], probably due to the physiological changes occurring in *E. coli* upon entry into the stationary phase of growth.

The optical densities as well as plasmid DNA yields (149.0 mg/L) obtained in batch fermentations are in line with those obtained for similar processes for plasmid DNA production [12, 25, 52]. As expected, the highest value of plasmid DNA yield (344.3 mg/L) was obtained using fed-batch fermentation strategy with an exponential feeding rate. Despite the fact that this pDNA yield value obtained with these bench-top mini-reactors is not comparable to some of those obtained for similar processes using large scale processes with exponential feed rates (2.0 g pDNA/L) (Carnes et al. 2009) and constant feed rates (1.2 g/L) (Listner et al. 2006), they are amongst the higher values obtained for recently developed pDNA production processes using smaller scale bioreactors (Mairhofer et al. 2010; Singer et al. 2009). Notwithstanding the fact that, so far, higher yields were described for large scale production processes, this bioreactor scale issue did not seem to influence production processes, since different scale bioreactors yielded equivalent results [53].

With the flow cytometric analysis of cell physiology and real-time qPCR measurements of plasmid stability, this study showed that the highest yield obtained for the fed-batch fermentation with an exponential feed rate of 0.2 h^{-1} did not correspond to the best fermentation performance. In fact, cell physiology and plasmid stability data obtained for this fermentation revealed lower cell viability and higher plasmid instability.

The monitoring tools described herein allow gaining new insights about process performance and providing a more comprehensive approach when the optimization of a fermentation bioprocess is needed; since, as supported by our data, final product and biomass yields cannot be seen by researchers as the only parameters to indicate the best process. The development of other monitoring tools should be seen as an important contribution to the challenging task that is the design and optimization of a robust production bioprocess.

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2.4.6. References

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Chapter III

3.1. General Conclusions

Nowadays, the challenges faced during production of plasmid DNA (pDNA) in *Escherichia coli* are similar to those dealing with the production of recombinant proteins. However, large-scale production of plasmid DNA, by itself, represents a comparatively new challenge since research on this topic is still recent and vector and strain engineering are continuously evolving, thus requiring the design and optimization of new bioprocesses. Some of the reports dealing with the optimization of plasmid DNA production processes are focused primarily in the influence of medium composition, fermentation conditions and feeding strategies on the overall pDNA mass and volumetric titre. Recently, pertinent reports addressing optimization of large-scale plasmid DNA production in *Escherichia coli* have been described with pDNA yields ranging from 600 up to 2600 mg pDNA/L.

In order to design an efficient plasmid DNA bioprocess, the first step in our research was to develop a culture medium suitable for plasmid DNA production. In this first stage, we chose a semi-defined medium containing glycerol as carbon source and tryptone as nitrogen source that was used throughout this research. After culture medium development, our first goal was to study the influence of culture conditions such as temperature and tryptone concentration on *E. coli* biomass yield and overall plasmid DNA yield primarily in shake flasks. We found that, depending on the growth temperature, different cell morphology, specific pDNA yields and plasmid purity levels on *E. coli* lysates were obtained. For instance, at higher temperatures (37 and 42 ºC), cell filamentation was observed. This cell filamentation could be related with host cell metabolic stress imposed by higher plasmid copy number, as the higher pDNA specific yields obtained for these culture temperatures suggested. Tryptone concentration clearly influenced plasmid DNA yield: as tryptone concentration increased, the pDNA content by bacterial mass, expressed as specific pDNA yield, decreased. These results demonstrated that lower tryptone concentrations and higher temperatures could significantly improve plasmid DNA yields while reducing host cell impurities.

Due to the results obtained in this first study, there was evidence that cell physiology was an important feature to monitor in plasmid DNA fermentation processes, since a possible higher host metabolic stress could be causing cell filamentation and reduced growth rates, thus affecting both biomass and volumetric product yields. Since the analysis of bacterial DNA patterns is a relevant feature in the control of bioprocesses, the next step in our research was the development and validation of a flow cytometric protocol for cell cycle monitoring during *Escherichia coli* fermentation processes. This protocol was developed using a newly developed far-red fluorescent dye, DRAQ5, which, due to its excitation and emission spectra, allows the combination with other fluorescent stain envisioning the development of multi-parametric

flow cytometry protocols. Our results demonstrated, that DRAQ5 selectively binds to bacterial DNA, and was able to resolve ploidy in ethanol-fixed *E. coli* cells, but not in live *E. coli* cells. Cell cycle analysis performed during fermentation demonstrated that this *E. coli* strain, in the conditions mentioned, has a eukaryotic-like cell cycle, since the cells only contained one or two chromosome equivalents. These findings further support the application of this flow cytometric method envisioning *E. coli* cell cycle analysis during plasmid DNA fermentation as well as in other bacterial fermentation bioprocesses.

After developing this tool for bioprocess monitoring, we further developed other flow cytometric methods for cell viability analysis and, also, real-time qPCR protocols to assess plasmid segregational stability during fermentations. After developing these new tools, we studied the impact of several plasmid DNA induction strategies on bioprocess performance. Due to the characteristics of the *E. coli* strain and plasmid used for pDNA production, pDNA induction strategies used in this work were based on AMP addition, amino acid limitation with or without AMP addition as well as temperature up-shift. In order to evaluate cell physiology alterations, the parameters studied by flow cytometry were bacterial morphology, cell viability and cell cycle. Flow cytometric analysis of bacterial morphology showed that, during fermentation, cell filamentation occurred which is in agreement with our previous results. Cell viability suffered a slight decrease during fermentations, attaining values of approximately 75-80% at the end of fermentations, meaning that the vast majority of cells still had intact polarized membranes, being able to withstand all fermentation conditions tested. Cell cycle analysis demonstrated that, after inoculation, DNA histograms exhibited a two peak distribution, with a ridge connecting them showing that the inoculum contained actively-dividing cell; however, during fermentation, a spreading of the DNA histograms was observed that could be related to an increase in population heterogeneity and also to plasmid DNA amplification.

Considering that cell physiology and pDNA segregational stability are closely related, we also evaluated plasmid stability by measuring plasmid copy number throughout fermentation. Our results showed that the initial cultivation at 30 ºC seemed to promote segregational instability, showing evidences that low culture temperatures should be avoided in plasmid production processes. Our results revealed that maximum PCN values were obtained upon entry into stationary phase of growth, and that amino acid limitation fermentations exhibited higher PCN when compared to fermentations using induction strategies without amino acid limitation. Regarding overall plasmid DNA yield, amino acid limitation with AMP addition fermentation had a significantly higher specific yield when compared with all the other induction strategies. Furthermore, agarose gel electrophoresis confirmed the quality of the final plasmid DNA obtained, showing low quantities of the open circular isoform and high quantities of supercoiled plasmid DNA with no visible bands corresponding to RNA.

When developing a bioprocess, the choice of an appropriate fermentation strategy is of crucial relevance since different productivity levels are obtained in accordance to the fermentation strategy used. In the last part of our research, we evaluated the influence of batch and fed-batch fermentations using small scale parallel bioreactors on plasmid DNA production. Since process performance is an important feature when developing a bioprocess, not only plasmid yields were evaluated but also, cell physiology and plasmid segregational stability were assessed using the methodologies already described. Several batch fermentations were performed varying glycerol and tryptone concentrations and, in the case of fed-batch fermentations, several exponential and constant pre-determined feed profiles were tested. Maximum PCN values in batch fermentations were, in general, obtained at the stationary phase of growth, which is in accordance to our previous results, and higher PCN values were obtained in the fermentations with lower tryptone concentrations. Cell viability and cell cycle analysis did not reveal significant alterations in the batch conditions tested; although the percentage of healthy cells have suffered slight fluctuations throughout the fermentation step. In fed-batch fermentations, due to the several different pre-determined profiles used, different growth rates were obtained. PCN values revealed higher variations than the ones obtained during batch fermentations and also plasmid stability was higher at intermediate pre-determined growth rates. Similarly to PCN, cell viability also showed higher fluctuations in fed-batch fermentations that could be linked to the extended cultivation time and a pronounced peak broadening was obtained for cell cycle analysis, indicating higher population heterogeneity.

With this study, we found that, although being capable of producing higher amounts of plasmid DNA, fed-batch strategies seem to impose higher stresses on the cells leading to higher plasmid instability and lower cell viability. On the contrary, batch strategies showed an improvement of plasmid segregational stability; although a slight decrease in bacterial viability was also obtained. A fed-batch fermentation strategy with exponential feeding rate enabled the attainment of the highest plasmid DNA yield (344.3 mg/L). With the flow cytometric analysis of cell physiology and real-time qPCR measurements of plasmid segregational stability, this study showed that the highest yield did not correspond to the best fermentation performance. In fact, cell physiology and plasmid stability data obtained for this fermentation revealed lower cell viability and higher plasmid instability.

In summary, in this thesis, the key goal was to improve plasmid DNA yields through the optimization of culture medium, culture conditions as well as the choice of an appropriate induction strategy that could selectively amplify plasmid DNA after the establishment of the fermentation strategy. However, during our research we encountered some evidences of metabolic stress and plasmid amplification that needed to be further explored. Therefore, although our primary task was the design of an improved bioprocess, we further developed monitoring methods that allowed us to gain a deeper knowledge about process performance

and will also enable other researchers to address similar problems that may arise when optimizing a bioprocess. The systematic approach for the easy assessment of cell physiological states and plasmid segregational stability can be applied to the vast majority of fermentation bioprocesses using recombinant microorganisms, allowing an at-line bioprocess monitoring.

3.2. Future Perspectives

Despite all the work performed during this thesis, further research is still need regarding the optimization of this plasmid DNA fermentation process, while providing further insights into bioprocess performance.

As future prospects, and following the studies already carried out, the work to be conducted may include the following aspects:

- To propose an alternative method for clone selection for construction of master cell banks, by using the techniques proposed in this thesis and others to be developed;
- To develop a model for the bioprocess, taking into account the combinatorial interactions between components of the medium, cell physiology, plasmid DNA stability and pDNA yields;
- To exploit possible production processes for other plasmids using the same strain in order to analyze how genetic characteristics, such as the origin of replication and the plasmid size can affect process performance;
- To integrate the process described in this work with all the downstream processing such as isolation, purification and application in order to develop a pilot plant for plasmid DNA manufacturing.
- To develop a process for a therapeutic plasmid, studying the influence of final cell lysates obtained in the fermentation step on its therapeutic application, namely the evaluation of transient gene expression or even the design of a therapeutic non-viral formulation for delivery into the cells.

Chapter IV

4. References

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