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Influence of Growth Conditions on Plasmid DNA Production

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The obtention of high yields of purified plasmid DNA is viewed as an essential issue to be considered towards efficient production of DNA vaccines and therapeutic plasmids. In this work, *Escherichia coli* DH5α bearing the 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°C), 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 favoring 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 a small scale.

Keywords: Plasmid DNA, fermentation, filamentous bacteria, microbial growth, temperature, tryptone

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 [2, 18]. 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 [15, 27]. Despite that these vectors were firstly characterized as inefficient in transfection studies [8], the increased effectiveness recently achieved have made naked DNA gene transfer a suitable method for gene therapy [10]. 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 a highly purified plasmid DNA product is viewed as an essential issue to be considered towards efficient production of DNA vaccines and therapeutic plasmids [3].

Since pDNA accounts for less than 3% of the total contents of an *E. coli* cell (depending on plasmid size and copy number) [27], 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 [13]. 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 [4, 16], addition of chloramphenicol [5], amino acid starvation or limitation [12, 35], and reduced growth rates [25, 40].

It is well known that optimal purity can be achieved by maximizing the average supercoiled-plasmid copy number [7]. Hence, plasmid amplification can lead to higher pDNA specific yields, which is translated in a low percentage of key contaminants present in the *E. coli* lysate such as RNA, denatured genomic DNA, proteins, and lipopolysaccharides (LPS) [30] 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 [27]. 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 [17, 20]. Some new purification strategies of the supercoiled plasmid DNA isoform have been proposed [28, 29].

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All these concepts are only possible by manipulation of well-known microrganisms. In fact, *E. coli* recombinant strains are industrially important microrganisms and largely used in bioprocesses, and known for being easy to produce and because of their well-characterized genetics and cellular metabolism [24]. 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.

MATERIALS AND METHODS

Plasmid and Bacterial Strain

The bacterial host for plasmid pVAX1-*LacZ* was *E. coli* DH5α [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°C.

The bacterial strain is a relA mutant (relA⁻) that, in response to amino acid starvation or limitation, causes pDNA amplification [35]. The plasmid contains an origin of replication, which allows an increased plasmid copy number at 37–42°C, but not at 30–32°C [4].

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 was 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 30 mg/ml kanamycin solution, 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; and $MgSO_4 \cdot 7H_2O$, 240 g; and the trace mineral solution contained (per l of 1.2 M hydrochloric acid) $FeCl_3 \cdot 6H_2O$, 27 g; $ZnCl_2$, 2 g; $CoCl_2 \cdot 6H_2O$, 2 g; $Na_2MoO_4 \cdot 2H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 1 g; $CuCl_2 \cdot 2H_2O$, 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 the 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 of a mixture of amino acids and was used as the 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 5,445 ×*g* during 30 min at 4°C and the pellet was washed twice with an equal volume of 0.9% NaCl and resuspended in the semi-defined medium [38]. 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 the plasmid DNA content in the bacterial cell during fermentation is highly dynamic, leading to a strong increase of the specific pDNA yield in the middle of fermentation time (H. Huber *et al.* U.S. Patent 2005/0233421).

To ensure that tryptone was the limiting nutrient, the 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 ×*g* for 10 min in preweighed tubes and the pellet was 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 [21]. 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°C. Cell pellets were lysed using a modified alkaline lysis protocol as described previously [28]. 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°C), 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 ×*g* for 30 min in the first centrifugation and 15 min in the second one at 4°C with a Sigma 3-18 K 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 ×*g* for 30 min at 4°C. 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 ×*g* for 20 min at 4°C. 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.* [6]. 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, and 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 relative 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.

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 the plasmid copy number by the *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°C. Typically, individual cells of *E. coli* are rod-shaped and are approximately 800 nm wide and 2.5 μm long [32]. In fact, the cells grown at 30 and 32°C (Fig. 1A and 1B, respectively) exhibit this typical morphology. In contrast, cells grown at 37 and 40°C (Fig. 1C and 1D, respectively) show a filamentous morphology essentially found when cells elongate and replicate their DNA, but do not septate and divide [32]. With relation to *E. coli* morphology, it has been described that nutrient starved cells [11] do not apparently exhibit the filamentous structure, but this phenomenon is activated as a stress response to oxidative stress [1] 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* DH5α, resulting in downregulation of cell wall biosynthetic genes [23]. In practice, this effect is often associated with reduced growth rates and final biomass concentration [23], supporting the results obtained for the temperatures of 37 and 40°C that showed lower specific growth rate and a decrease in the final biomass concentration (Table 1), when compared with the lower temperatures under study.

Furthermore, owing to the presence of a replication origin pUC, higher specific pDNA yields (4.60 and 5.56 mg

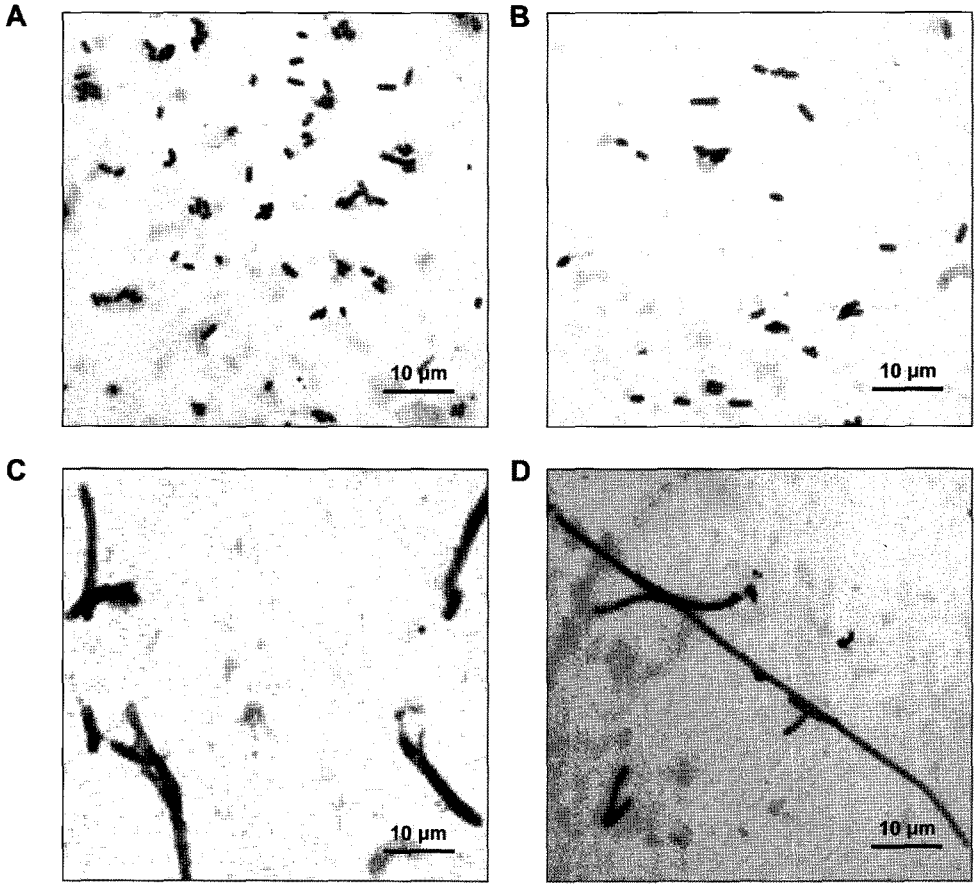

Fig. 1. Safranin staining of cellular suspension collected at late log phase of *E. coli* DH5α harboring plasmid pVAX1-LacZ grown in a semi-defined media containing 30 g/l glycerol and 20 g/l tryptone at different temperatures: **A.** 30°C; **B.** 32°C; **C.** 37°C; **D.** 40°C.

Table 1. Effect of growth temperature on specific growth rate, final biomass concentration, plasmid yield, and purity degree.

Growth temperature (°C)	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).

pDNA/g dcw) and purity degrees (35.68% and 45.72%) were obtained at 37 and 40°C, respectively (Table 1). This result can probably be 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 [23] leading to an accentuated filamentation of cells (Fig. 1C and 1D). As this filamentation is not observed when plasmid-free *E. coli* DH5α 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°C [4], 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°C showed lower specific pDNA yields (1.22 and 1.60 mg pDNA/g dcw) and purity degrees (12.06% and 13.91%), respectively (Table 1). This drop-off in these parameter values

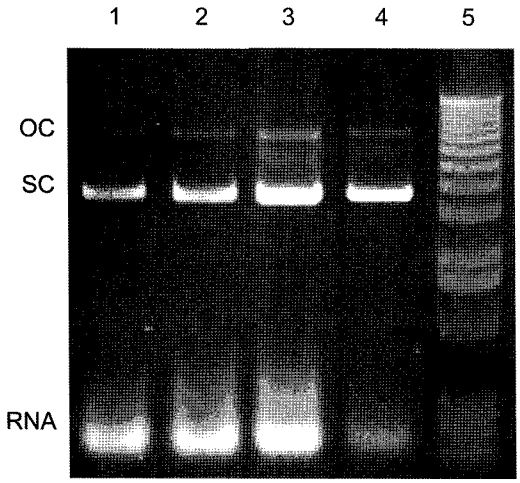

Fig. 2. Agarose gel electrophoresis of cell lysate resultant from fermentations at different temperatures: 30°C (lane 1), 32°C (lane 2), 37°C (lane 3), and 40°C (lane 4), and molecular weight marker (lane 5). Abbreviations: oc (open circular pDNA), sc (supercoiled pDNA).

and the higher percentage of RNA as a contaminant (Fig. 2, lanes 1 and 2) suggest that plasmid amplification occurred with lower extent at these temperature conditions, in accordance with the behaviour previously described [4], and could also explain the typical *E. coli* morphology observed (Fig. 1A and 1B). Despite that the percentage of RNA seems higher at 37°C (Fig. 2, 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 the open circular pDNA structure.

The fermentation performed at 40°C exhibited the highest specific yield and purity degree (Table 1), with a lower percentage of RNA (Fig. 2, 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 responses on the bacterial host, leading to a reduced host growth and viability [26]. In addition, the detrimental effect of this plasmid-imposed metabolic burden can lead to an increase of plasmid-free cells with faster growth kinetics during fermentation. Over time, these nonproductive cells become a significant fraction of the population [31], contributing to several disadvantages of industrial relevance such as plasmid loss and reduced productivity [14].

Previous works reported the influence of amino acid limitation in pDNA amplification [36, 38]. 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 [37] and bacteriophage γ [38]. 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 [36] or LB medium (with yeast extract and tryptone) [33], where limitation was achieved by cultivation of bacteria to the 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 [34]; and it also shows a 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 [19]. In this work, the possibility of studying amino acid limitation by using different tryptone concentrations for the most efficient amplification of the plasmid pVAX1-*LacZ* was evaluated.

Some authors have 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 [9]. 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 [36]. The amplification factor was calculated in the cell mass collected at the stationary phase of growth.

In order to compare the influence of tryptone concentration on bacterial growth, different cultivations were done at 37°C. The time profiles for cell density, obtained by measuring OD_{600} of samples periodically taken, are shown in Fig. 3. By comparing the growth curves, it is apparent that a 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 (Fig. 4). 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 the 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 (Fig. 4).

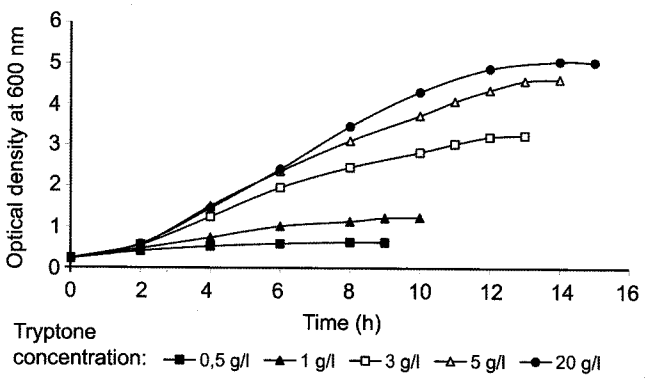

Fig. 3. Growth profiles of *E. coli* DH5α harboring plasmid pVAX1-*LacZ* performed in shake flasks at 37°C and 250 rpm in semi-defined medium containing different tryptone concentrations.

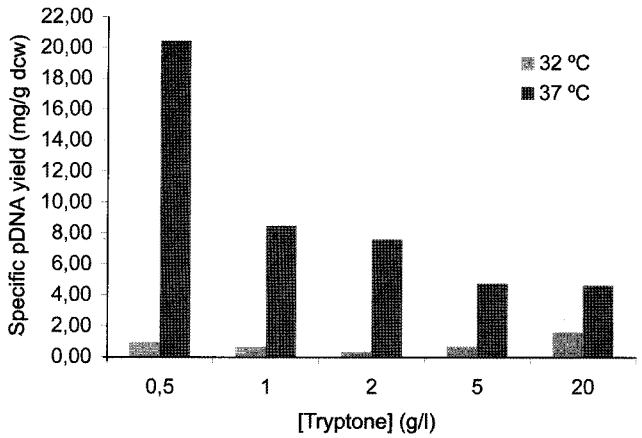

Fig. 4. pDNA specific yield variation with different tryptone concentrations at 32 and 37°C.

When the same experiments were performed at 32°C, lower specific pDNA yields were obtained, when compared with experiments performed at 37°C, and tryptone concentration seems not to influence greatly specific yields (Fig. 4). These results are in accordance with Wrobel and Wegrzyn's report [36], which described a decrease in amplification efficiency at low temperatures, when compared with 37°C, and found that the optimal temperature for amplification was 37°C, when pSC101 derived-plasmid was used.

The highest specific pDNA yield (20.43 mg pDNA/g dcw) was obtained for a tryptone concentration of 0.5 g/l at 37°C, 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 [35], 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 [22] and 16.0 mg pDNA/g dcw [39], and even when compared with similar studies using amino acid limitation in LB medium at 37°C [33] that obtained a specific plasmid yield of approximately 10.0 mg pDNA/g dcw.

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 40°C, 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°C, giving an amplification ratio of 4.3. However, when the same experiments were performed at 32°C, a lower specific

pDNA yield was obtained and tryptone concentration seems not to influence greatly specific yields. Hence, by using tryptone as the source for amino acid limitation experiments at 37°C, specific pDNA yields suffered a four-fold increase, proving that the pDNA amplification strategy analyzed here can improve pDNA yield even at a 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.

REFERENCES

1. Ackerley, D. F., Y. Barak, S. V. Lynch, J. Curtin, and A. Matin. 2006. Effect of chromate stress on *Escherichia coli* K-12. *J. Bacteriol.* **188:** 3371–3381.
2. Anderson, W. F. 1998. Human gene therapy. *Nature* **392:** 25–30.
3. Carnes, A., C. Hodgson, and J. Williams. 2004. Optimization of *E. coli* fermentation for plasmid DNA production. *Mol. Ther.* **9:** S310
4. Carnes, A. E., C. P. Hodgson, and J. A. Williams. 2006. Inducible *Escherichia coli* fermentation for increased plasmid DNA production. *Biotechnol. Appl. Biochem.* **45:** 155–166.
5. Clewell, D. B. 1972. Nature of ColE1 plasmid replication in *Escherichia coli* in presence of chloramphenicol. *J. Bacteriol.* **110:** 667–676.
6. Diogo, M. M., J. A. Queiroz, and D. M. Prazeres. 2003. Assessment of purity and quantification of plasmid DNA in process solutions using high-performance hydrophobic interaction chromatography. *J. Chromatogr. A* **998:** 109–117.
7. Diogo, M. M., J. A. Queiroz, and D. M. Prazeres. 2005. Chromatography of plasmid DNA. *J. Chromatogr. A* **1069:** 3–22.
8. Diogo, M. M., J. A. Queiroz, G. A. Monteiro, S. A. Martins, G. N. Ferreira, and D. M. Prazeres. 2000. Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnol. Bioeng.* **68:** 576–583.
9. Herman, A., A. Wegrzyn, and G. Wegrzyn. 1994. Combined effect of stringent or relaxed response, temperature and *rom* function on the replication of pUC plasmids in *Escherichia coli*. *Acta Biochim. Pol.* **41:** 122–124.
10. Herweijer, H. and J. A. Wolff. 2003. Progress and prospects: Naked DNA gene transfer and therapy. *Gene Ther.* **10:** 453–458.
11. Imlay, J. A. and S. Linn. 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. *J. Bacteriol.* **169:** 2967–2976.
12. Kay, A., R. O'Kennedy, J. Ward, and E. Keshavarz-Moore. 2003. Impact of plasmid size on cellular oxygen demand in *Escherichia coli*. *Biotechnol. Appl. Biochem.* **38:** 1–7.
13. Kelly, W. J. 2003. Perspectives on plasmid-based gene therapy: Challenges for the product and the process. *Biotechnol. Appl. Biochem.* **37:** 219–223.
14. Kumar, P. K. R., H. E. Maschke, K. Friehs, and K. Schugerl. 1991. Strategies for improving plasmid stability in genetically modified bacteria in bioreactors. *Trends Biotechnol.* **9:** 279–284.
15. Li, Y., X. Y. Dong, and Y. Sun. 2005. High-speed chromatographic purification of plasmid DNA with a customized biporous hydrophobic adsorbent. *Biochem. Eng. J.* **27:** 33–39.
16. Lahijani, R., G. Hulley, G. Soriano, N. A. Horn, and M. Marquet. 1996. High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperature-controllable point mutation. *Hum. Gene Ther.* **7:** 1971–1980.
17. Levy, M. S., R. D. O'Kennedy, P. Ayazi-Shamlou, and P. Dunnill. 2000. Biochemical engineering approaches to the challenges of producing pure plasmid DNA. *Trends Biotechnol.* **18:** 296–305.
18. Luo, D. and W. M. Saltzman. 2000. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat. Biotechnol.* **18:** 893–895.
19. Matsui, T., H. Sato, S. Sato, S. Mukataka, and J. Takahashi. 1990. Effects of nutritional conditions on plasmid stability and production of tryptophan synthase by a recombinant *Escherichia coli*. *Agric. Biol. Chem.* **54:** 619–624.
20. Middaugh, C. R., R. K. Evans, D. L. Montgomery, and D. R. Casimiro. 1998. Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* **87:** 130–146.
21. Neubauer, A., J. Soini, M. Bollok, M. Zenker, J. Sandqvist, J. Myllyharju, and P. Neubauer. 2007. Fermentation process for tetrameric human collagen prolyl 4-hydroxylase in *Escherichia coli*: Improvement by gene optimisation of the PDI/beta subunit and repeated addition of the inducer anhydrotetracycline. *J. Biotechnol.* **128:** 308–321.
22. O'Kennedy, R. D., C. Baldwin, and E. Keshavarz-Moore. 2000. Effects of growth medium selection on plasmid DNA production and initial processing steps. *J. Biotechnol.* **76:** 175–183.
23. Ow, D. S., P. M. Nissom, R. Philp, S. K. W. Oh, and M. G. S. Yap. 2006. Global transcriptional analysis of metabolic burden due to plasmid maintenance in *Escherichia coli* DH5α during batch fermentation. *Enzyme Microb. Technol.* **39:** 391–398.
24. Ozkan, P., B. Sariyar, F. O. Utkur, U. Akman, and A. Hortacsu. 2005. Metabolic flux analysis of recombinant protein overproduction in *Escherichia coli*. *Biochem. Eng. J.* **22:** 167–195.
25. Reinikainen, P., K. Korpela, V. Nissinen, J. Olkku, H. Soderlund, and P. Markkanen. 1989. *Escherichia coli* plasmid production in fermenter. *Biotechnol. Bioeng.* **33:** 386–393.
26. Ricci, J. C. D. and M. E. Hernandez. 2000. Plasmid effects on *Escherichia coli* metabolism. *Crit. Rev. Biotechnol.* **20:** 79–108.
27. Shamlou, P. A. 2003. Scaleable processes for the manufacture of therapeutic quantities of plasmid DNA. *Biotechnol. Appl. Biochem.* **37:** 207–218.
28. Sousa, F., S. Freitas, A. R. Azzoni, D. M. Prazeres, and J. Queiroz. 2006. Selective purification of supercoiled plasmid DNA from clarified cell lysates with a single histidine-agarose chromatography step. *Biotechnol. Appl. Biochem.* **45:** 131–140.
29. Sousa, F., T. Matos, D. M. Prazeres, and J. A. Queiroz. 2008. Specific recognition of supercoiled plasmid DNA in arginine affinity chromatography. *Anal. Biochem.* **374:** 432–434.
30. Stadler, J., R. Lemmens, and T. Nyhammar. 2004. Plasmid DNA purification. *J. Gene Med.* **6:** S54–S66.
31. Summers, D. K. 1991. The kinetics of plasmid loss. *Trends Biotechnol.* **9:** 273–278.
32. Takeuchi, S., W. R. DiLuzio, D. B. Weibel, and G. M. Whitesides. 2005. Controlling the shape of filamentous cells of *Escherichia coli*. *Nano Lett.* **5:** 1819–1823.
33. Wang, Z., L. Xiang, J. Shao, and G. Wegrzyn. 2007. Adenosine monophosphate-induced amplification of ColE1 plasmid DNA in *Escherichia coli*. *Plasmid* **57:** 265–274.

34. Wang, Z. L., G. W. Le, Y. H. Shi, and G. Wegrzyn. 2001. Medium design for plasmid DNA production based on stoichiometric model. *Process Biochem.* **36:** 1085–1093.
35. Wegrzyn, G. 1999. Replication of plasmids during bacterial response to amino acid starvation. *Plasmid* **41:** 1–16.
36. Wrobel, B. and G. Wegrzyn. 1997. Amplification of pSC101 replicons in *Escherichia coli* during amino acid limitation. *J. Biotechnol.* **58:** 205–208.
37. Wrobel, B. and G. Wegrzyn. 1997. Differential amplification efficiency of pMB1 and p15A (ColE1-type) replicons in *Escherichia coli* stringent and relaxed strains starved for particular amino acids. *Microbiol. Res.* **152:** 251–255.
38. Wrobel, B. and G. Wegrzyn. 1997. Replication and amplification of lambda plasmids in *Escherichia coli* during amino acid starvation and limitation. *FEMS Microbiol. Lett.* **153:** 151–157.
39. Xu, Z. N., W. H. Shen, H. Chen, and P. L. Cen. 2005. Effects of medium composition on the production of plasmid DNA vector potentially for human gene therapy. *J. Zhejiang Univ. Sci. B* **6:** 396–400.
40. Zabriskie, D. W. and E. J. Arcuri. 1986. Factors influencing productivity of fermentations employing recombinant microorganisms. *Enzyme Microb. Technol.* **8:** 706–717.