J. Microbiol. Biotechnol. (2006), **16**(1), 20–24

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Production of ColE1 Type Plasmid by *Escherichia coli* DH5α Cultured Under Nonselective Conditions

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Received: December 18, 2004
Accepted: March 30, 2005

Abstract Plasmid DNA (pDNA) is a product of interest for many biopharmaceutical companies and research laboratories, because of increase in the number of gene therapy protocols that use nonviral vectors. This work was undertaken to study the effect of antibiotic and dissolved oxygen concentration (DOC) on the production of a ColE1-type plasmid (pVAX1-*LacZ*) hosted in *Escherichia coli* DH5α and cultured in a batch fermentor with 0.75 l of Terrific Broth. A decrease in the DOC from 60% to 5% was shown to increase the specific pDNA concentration approximately 1.5-fold, due to the downregulation of growth. Additionally, this increase in the pDNA concentration led to a 2.2-fold increase in the purity of cell lysates obtained after cell lysis. However, the use of higher DOC led to 2.8-fold higher volumetric productivity as a consequence of a faster growth rate, reducing the fermentation time from 24 to 8 h. Interestingly, the specific pDNA concentration, and pDNA productivity and purity were always higher (10–15%) in the absence of antibiotic. Overall, the data indicate that nonselective conditions can be used without compromising yield, productivity, and purity of pDNA.

Key words: ColE1-type plasmid, *Escherichia coli*, production, nonselective, dissolved oxygen concentration

Developments in gene therapy and DNA vaccination have increased the demand for large amounts of pharmaceutical-grade plasmid DNA [7, 8]. The majority of researchers use multicopy ColE1-type plasmids as backbones for gene therapy and vaccine constructs, and promote their replication in *Escherichia coli* host [7]. The random distribution of multicopy natural plasmids such as ColE1 at cell division usually guarantees a high segregational stability. However, innumerable observations indicate that man-made, multicopy plasmids, such as those in the pUC series, are usually lost at high rate under nonselective conditions [10]. For this reason, a selectable marker (e.g., an antibiotic marker) is usually included to create selective pressure against the predominance of plasmid-free cells, which may arise during growth. Although it may be acceptable to use antibiotics such as kanamycin under full-scale manufacturing, beta-lactams should be avoided, since the presence of residues is likely to cause allergic reactions in recipients [2]. Another concern associated with the use of antibiotic markers in gene therapy plasmid vectors is the possibility of a chance gene transfer to environmental organisms, particularly pathogens, with the consequent spread of drug resistance [13]. To avoid antibiotic resistance genes in plasmid vectors would thus have clear advantages for DNA vaccination and gene therapy.

The use of high copy number plasmids, together with an adequate selection of the culture conditions (media formulation, cultivation strategy, and antibiotic) used to grow *E. coli* cells, may lead to plasmid yields as high as 100 mg/l [3] and 220 mg/l [6]. Specific yields of ColE1-derived plasmids are known to vary inversely with the growth rate of the host because of high plasmid stability and preferential plasmid synthesis over other biochemical pathways [7]. Thus, 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 [3]. The cultivation process used to produce plasmid DNA should be selected and optimized not only on the basis of plasmid productivity, but also by taking into consideration the impact that culture conditions may have in the downstream processes used to purify plasmid [7, 8].

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In this work, we have focused on the effect of antibiotic and DOC on the plasmid productivity of a batch *E. coli* culture carried out in a lab-scale fermentor. As a model system, we have used a ColE1-type plasmid (pVAX1-*LacZ*) hosted in a DH5α strain.

MATERIALS AND METHODS

Plasmid and Host Strain

The 6,050 bp ColE1-type plasmid pVAX1-*LacZ* (Invitrogen, Carlsbad, U.S.A.), designed for the development of DNA vaccines, was used as a model plasmid. This vector contains the human cytomegalovirus (CMV) immediate-early promoter, the bovine growth hormone (BGH) polyadenylation sequence, a kanamycin resistance gene, a pMB1 origin (pUC-derived), a multiple cloning site, a T7 promoter/priming site, and a reporter (β-galactosidase) gene. *Escherichia coli* DH5α (Invitrogen, Carlsbad, U.S.A.) was used as the host strain. Cell banks were stored in 30% (v/v) glycerol at −80°C.

Batch Culture and Medium

E. coli DH5α cells were grown overnight at 37°C in 250-ml shake flasks containing 50 ml of LB medium with 30 μg/ml kanamycin at 250 rpm. Growth was suspended at late log phase (OD_{600}≈3.8). An appropriate volume (52.5 ml) of this preculture was used to start batch cultures in a CH-4103 fermentor (Infors AG, Bottmingen, Switzerland) with a working volume of 750 ml. The Terrific Broth medium used contained 1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 55 mM glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4 [9]. In some experiments, the medium was supplemented with kanamycin (30 mg/ml). Temperature was maintained at 37°C and the pH was set at 7.0±0.1 and controlled automatically by addition of 1.0 M HCl and 1.0 M NaOH. The DOC was maintained at the selected set-point value by automatic control of the agitator speed. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) of appropriately diluted samples in a Hitachi (San Jose, U.S.A.) spectrophotometer. Cells were harvested at late log phase.

Analytical Methods

Dry Cell Weight. Culture samples were periodically withdrawn from the fermentor and analyzed for biomass and plasmid concentration. Dry cell concentration (g/l) was measured by filtering 10-ml samples on a 0.22 μm membrane and subsequently oven drying the membranes at 105°C for 24 h until constant weight was achieved.

Plasmid Concentration. In order to determine the plasmid concentration, cells were first lysed under alkaline conditions [1]. The volume of culture sample to be lysed was selected so as to maintain the OD_{600}× volume relation equal to 40. This procedure was followed in order to guarantee that roughly the same mass of cells was being lysed. The selected culture volume was transferred to 30-ml centrifuge tubes and centrifuged at 14,000 ×*g* for 15 min at 4°C in a JA-10 rotor in a Beckman (Fullerton, CA, U.S.A.) centrifuge. Supernatants were discarded and cell pellets were resuspended in 1.6 ml of TE buffer [50 mM glucose, 25 mM Tris-Cl, 10 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.0]. Alkaline lysis was performed on ice by adding 1.6 ml of 200 mM NaOH and 1% (w/v) sodium dodecyl sulfate solution prechilled on ice. After complete lysis, cellular debris, genomic DNA (gDNA), and proteins were precipitated by gently adding and mixing 1.3 ml of prechilled (on ice) 3 M potassium acetate, pH 5.0. The precipitate was removed by centrifuging twice at 18,000 ×*g* (30 min, 4°C) in a JA-20 rotor using the same centrifuge. The supernatant obtained was used to determine plasmid concentration and purity by HPLC as described previously [5]. Briefly, a 4.6 cm×10 cm HIC Source 15 PHE PE column from Pharmacia Biotech (Uppsala, Sweden) was connected to a HPLC system (Merck Hitachi, Darmstadt, Germany) and equilibrated with 1.5 M ammonium sulfate in 10 mM Tris-Cl (pH 8.0). Thirty μl of a cell lysate appropriately diluted in the equilibration buffer were injected and eluted at 1 ml/min. All plasmid isoforms (supercoiled, open circular, linear) eluted at a salt concentration of 1.5 M as a single peak. After that, the salt concentration was kept to 0 M ammonium sulfate during 0.5 min in order to elute the bound species. The column was then again equilibrated with 1.5 M ammonium sulfate during 5.5 min. The absorbance was recorded at 260 nm. The plasmid pVAX1-*LacZ* was quantified through a calibration curve constructed with standards of the model plasmid (1.25 to 20 μg/ml). HPLC analysis was also used to assess the purity of lysates of samples collected throughout cell cultivation. This HPLC purity was defined as the percentage of the plasmid peak area, when compared with the total area of the peaks on the chromatogram, as described previously [5]. Specific plasmid DNA concentration is defined as milligram of plasmid per gram of dry weight cells.

Plasmid Stability. Plasmid stability was assessed by plating cells as follows. Culture samples were appropriately diluted in saline buffer (0.9% w/v NaCl) and plated onto LB agar plates with and without kanamycin. Following incubation at 37°C for 24 h, the ratio of plasmid-containing cells to plasmid-free cells was determined by counting the number of colonies grown on kanamycin-free and kanamycin-containing plates.

Gel Electrophoresis. Cell lysates obtained as described above were analyzed by horizontal electrophoresis using 15-cm 1% agarose gels (Hoefer HE99X unit, Uppsala, Sweden), run at 25 V overnight. TAE (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) was used as the running buffer, and the gels were stained with ethidium bromide (0.5 μg/ml).

Table 1. The effect of antibiotic and DOC on the plasmid concentration and productivity of a laboratory-scale batch *E. coli* culture. The data presented refer to the endpoint of the fermentation.

Antibiotic	% O_2 (v/v)	$t_{harvest}$ (h)	Cells (g/l)	μ (h^{-1})	pDNA concentration		pDNA productivity		Purity (%)	Reference
					(mg/l)	(mg/g_{cell})	(mg/h.l)	(mg/h.g_{cell})		
Yes	5	24	4.7	0.23	56.4	12.0	2.35	0.50	9.9	This work
No	5	25	5.4	0.49	66.9	12.4	2.68	0.50	11.3	This work
Yes	60	8	7.0	0.70	52.3	7.47	6.54	0.93	4.7	This work
No	60	8	6.9	0.70	60.2	8.72	7.53	1.09	5.1	This work
Yes	10–50	24	60.0	0.4	100.0	1.67	4.17	0.07	NA	[3, 4]
Yes	20	21.5	50*	0.25	220.0	4.4**	10.2	0.20**	NA	[6]
Yes	NA	6	4.8	NA	49.3	10.3	8.2	1.72	NA	[6]
No	NA	20	3.5	0.33	60.0	17.1	3.0	0.86	NA	[12]

NA, not available; *, estimated based on 1 OD_{600}=0.5 g/l dry cell weight and from data in Fig. 5 of [6]; **, estimated based on a 50 g/l dry cell weight.

RESULTS AND DISCUSSION

The results on the effect of antibiotic and DOC on the plasmid concentration and productivity of a batch *E. coli* culture carried out in a laboratory-scale fermentor are shown in Table 1. Data published in the literature are also included for comparison. Overall, the plasmid production data presented here fall within the typical ranges reported for other plasmid/host systems replicated under other fermentation conditions.

Under oxygen limitation (5% DOC), the specific growth rate was half the value obtained when antibiotic was present in the medium (0.23/h vs 0.49/h). These differences were not detected when cultivation was carried out at 60% DOC, since a specific growth rate of 0.7/h was independent of the presence or absence of antibiotic (Fig. 1A). These results can be due to an aerobic respiratory metabolism of *E. coli* at 60% DOC, which is not limited by oxygen, thus leading to a high-energy content (ATP and NADH). Consequently, the amount of energy and metabolites available are sufficient to allow for a high rate of cell growth.

The volumetric (Fig. 1B) and specific plasmid (Table 1) concentrations indicate a high segregational stability of the model plasmid even at 60% DOC, a behavior that is typical of natural multicopy plasmids [11]. These findings were confirmed by plating cells, harvested from cultures grown at 5% and 60% DOC with no antibiotic, onto media with and without antibiotic. These experiments indicated that almost 100% of the cells harvested contained plasmid (data not sown). This was also evident from agarose gel electrophoresis analysis, which showed no plasmid loss during growth (Fig. 2). Furthermore, the increase in plasmid copy number under nonselective conditions, as shown by the specific and volumetric plasmid concentration data (Table 1, Fig. 1B), indicates that the presence of kanamycin somehow reduces plasmid copy number even at high growth rates (60% DOC). For example, this could result from the inhibition of the synthesis of enzymes involved in plasmid replication.

The higher specific plasmid concentrations obtained at the end of cultivation under low DOC when compared with those obtained at higher DOC (Table 1) suggest that plasmid copy number increases with a decrease of growth rate. This inverse relationship between growth rate and plasmid copy number is a well-known characteristic of ColE1-type plasmids, usually attributed to high plasmid stability and privileged plasmid synthesis over other biochemical pathways [7].

The results in Fig. 1B show that maximum volumetric plasmid concentrations are obtained at maximum cell concentration. However, from a production point of view, this is not the best time to harvest cells. This is clear from the plots of plasmid productivity as a function of time (Fig. 1C), especially in the case when cells were cultivated at low DOC without antibiotic. In fact, harvesting cells at 7 h instead of 24 h would result in a 52% increase in plasmid productivity (4.10 mg/h.l vs 2.68 mg/h.l). For 60% DOC, maximum productivity almost coincides with maximum volumetric concentration.

Cultivation processes used to produce plasmid DNA should be selected and optimized not only on the basis of plasmid productivity, but also by taking into consideration the impact that culture conditions may have during subsequent purification steps [7, 8]. Thus, the amount of plasmid obtained after performing alkaline lysis at different times throughout cultivation, relative to the other host impurities (purity), was determined from the HPLC chromatograms as described previously [5] (Fig. 1D). The main variable affecting the purity was time. Nevertheless, a 2-fold increase in purity was observed by changing DOC from high to low. The longer the cells remained in the fermentor, the higher was the purity obtained. This increase in the purity partially results from the degradation of RNA as cells approach the stationary phase, as shown clearly in Fig. 2 for the case of the culture with 60% DOC and no antibiotic.

Overall, the data presented here indicate that, if a ColE1-type plasmid (pVAX1-*LacZ*) is hosted in a DH5α strain, a high segregational stability can be obtained under

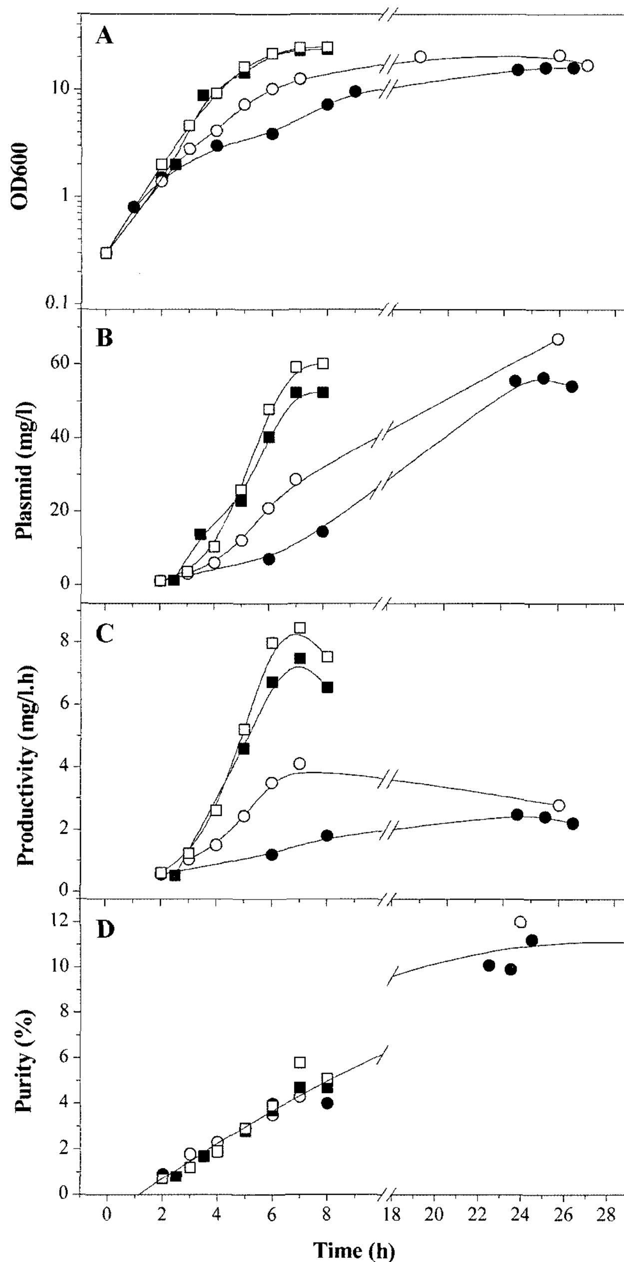

Fig. 1. The effect of antibiotic and DOC on the plasmid productivity of a batch *E. coli* culture carried out in a laboratory-scale fermentor. A. cell growth; B. volumetric plasmid concentration; C. plasmid productivity; and D. HPLC purity of cell lysates.
(●) 5% DOC, 30 μg/ml kanamycin; (○) 5% DOC, no kanamycin; (■) 60% DOC, 30 μg/ml kanamycin; (□) 60% DOC, no kanamycin.

nonselective conditions. This eliminates the need to exert selective pressure by adding antibiotics to the cultivation medium. Furthermore, it is possible to envisage a highly stable, multicopy plasmid vector, devoid of a selection marker, which can be propagated in a nonmanipulated host. The use of low DOC clearly increased the number of plasmid copies per cell, consequently leading to higher volumetric and specific plasmid concentrations and further to a higher

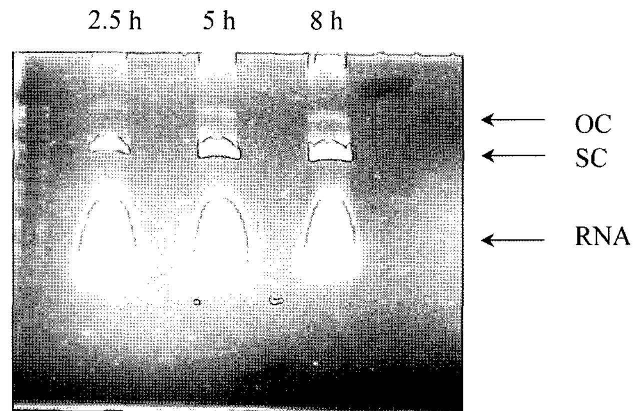

Fig. 2. Agarose gel electrophoresis analysis of samples collected throughout a culture carried out in a laboratory-scale fermentor with 60% DOC and without antibiotic.
Cells were lysed as described and 45 μl were loaded in each well. Plasmid isoforms (OC-open circular, SC-supercoiled) and RNA are shown.

purity of lysates after cell disruption. Nevertheless, plasmid productivity was higher at higher DOC, owing a faster cell growth. Clearly, the choice of the best DOC for a given system should be made by compromise between plasmid copy number, productivity, impact in the downstream processing, and costs.

Acknowledgment

This work was supported by the Portuguese Ministry of Science and Technology (POCTI/BIO/43620/2000 and Ph. D. grant BD/21241/99 to M.M. Diogo).

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