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Automated fed-batch fermentation with feed-back controls based on dissolved oxygen (DO) and pH for production of DNA vaccines

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A fermentation process in *Escherichia coli* for production of supercoiled plasmid DNA for use as a DNA vaccine was developed using an automated feed-back control nutrient feeding strategy based on dissolved oxygen (DO) and pH. The process was further automated through a computer-aided data processing system to regulate the cell growth rate by controlling interactively both the nutrient feed rate and agitation speed based on DO. The process increased the total yield of the plasmid DNA by approximately 10-fold as compared to a manual fed-batch culture. The final cell yield from the automated process reached 60 g L^{-1} of dry cell weight (OD_{600} = 120) within 24 h. A plasmid DNA yield of 100 mg L^{-1} (1.7 mg g^{-1} cell weight) was achieved by using an alkaline cell lysis method. Plasmid yield was confirmed using High Performance Liquid Chromatography (HPLC) analysis. Because cells had been grown under carbon-limiting conditions in the automated process, acetic acid production was minimal (below 0.01 g L^{-1}) throughout the fed-batch stage. In contrast, in the manual process, an acid accumulation rate as high as 0.36 g L^{-1} was observed, presumably due to the high nutrient feed rates used to maintain a maximum growth rate. The manual fed-batch process produced a low cell density averaging 10–12 g L^{-1} (OD_{600} = 25–30) and plasmid yields of 5–8 mg L^{-1} (approximately 0.7 mg g^{-1} cells). The improved plasmid DNA yields in the DO- and pH-based feed-back controlled process were assumed to be a result of a combination of increased cell density, reduced growth rate (μ) from 0.69 h^{-1} to 0.13 h^{-1} and the carbon/nitrogen limitation in the fed-batch stage. The DO- and pH-based feed-back control, fed-batch process has proven itself to be advantageous in regulating cell growth rate to achieve both high cell density and plasmid yield without having to use pure oxygen. The process was reproducible in triplicate fermentations at both 7-L and 80-L scales.

Keywords: fed-batch fermentation; feed-back control; *E. coli* plasmid DNA; DNA vaccines

Introduction

The development of DNA vaccines and therapeutics using facilitated DNA injection technology [1,12] requires a consistent supply of clinical grade plasmid DNA. As a first step in producing large quantities of plasmid DNA, a well-controlled and efficient *Escherichia coli* fermentation process needs to be established. Requisite to a successful fermentation process are optimized cell growth and high product accumulation. Fed-batch cultures are generally preferred over batch cultures because optimal cell growth rate and nutrient consumption rates can be achieved by controlling the nutrient feeding in a desired range. A successful fed-batch culture can reach a cell density of over 100 g L^{-1} cell dry weight [8]. Several nutrient-feeding strategies have been explored to control cell growth in fed-batch cultures. They can be divided into either 'non-feed-back control' or 'feed-back control'. In the former case, the feeding rate is controlled according to a pre-determined, constant or exponential profile [14]. This type of feeding scheme requires that metabolic characteristics of the growing cells are reproducible each time in both seed and early batch culture stages, thereby matching feeding rates to actual cell growth rates. In contrast, feed-back control is directly correlated to cell activities throughout fermentation. Control parameters which have been used for feed-back control methods of fermentation include pH; pCO_2; cell density measurement or dissolved oxygen (DO) [3,6,8,10,11,13].

Most knowledge about fermentations involving recombinant *E. coli* has been obtained through studies which optimized the expression of protein products. However, the fermentation conditions for optimization of plasmid DNA production in *E. coli* could be fundamentally different. In this case, only a DNA replication step is required when plasmid DNA is the final product. RNA transcription and protein translation associated with an inserted gene are generally undesirable during plasmid production in bacterial host cells. To selectively amplify the plasmid DNA replication activity, abundant nucleotide pools and extra energy sources must be made available in a culture environment and other cellular activities should be kept to a minimum. For example, cellular protein synthesis can be inhibited with chloramphenicol or through amino acid limitation [2,5]. Also, a low cell growth rate can be maintained 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 [9,15]. A fermentation process that minimizes the amounts of non-plasmid by-products in the biomass is also desirable. In this presentation, we describe a fermentation process for production of plasmid DNA in *E. coli* that uses an automated feed-back control nutrient feeding strategy based on DO

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and pH. Cell growth and plasmid production in the process are compared to a 'manual step feed', fed-batch culture.

Materials and methods

Cultures and media

E. coli strain DH10B (Gibco BRL, Life Technology, Inc, Gaithersburg, MD, USA) carrying plasmid pENV was cultured in a seed medium in shake flasks at 37°C for 16 h. The seed medium consisted of 6 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 3 g L^{-1} $(NH)_2SO_4$, 5 g L^{-1} dextrose (JT Baker, Phillipsburg, NJ, USA) and 5 g L^{-1} yeast extract (Difco Laboratory, Detroit, MI, USA). The plasmid product pENV of the fermentation is about 8 kb long with a ColEI origin. It contains the envelope gene of a type I HIV under the control of a human cytomegalovirus (CMV) promoter [1,12]. The plasmid also encodes a kanamycin resistance gene [1,12] for selection.

Fed-batch fermentation

Lab-scale fermentation was first carried out in a 7-L bioreactor Bioflo 3000 and later scaled up to an 80-L MP80 Mobil Pilot fermentor (both from New Brunswick Scientific Co, Edison, NJ, USA). Both fermentors were equipped with a built-in digital controller for pH, temperature, agitation, DO and peristaltic pumps for base and nutrient. The controller was also interfaced with a computer through the Advanced Fermentation Software (AFS) (New Brunswick Scientific). The AFS software provided data logging, interactive parameter processing and supervisory control to the fermentation process. The fermentation batch medium, consisting of the same composition as the seed medium, was batched in the fermentors. The initial working volumes for the 7-L and 80-L fermentors were 4 L and 50 L respectively. Glucose and $MgSO_4 \cdot 7H_2O$ were sterilized separately and added into the fermentor following sterilization. The fermentors were inoculated with 1% (v/v) of the seed, 40 ml for the 7-L and 500 ml for the 80-L fermentor. During the fermentation, pH was controlled at 7.0 with 30% NH_4OH. Agitation speed was automatically feed-back controlled based on DO at a set point of 30%. A concentrated nutrient solution (40 g L^{-1} glucose, 20 g L^{-1} yeast extract) was fed into the culture via a nutrient pump which was triggered by the increase of either DO or pH above respective set points of 50% and 7.2 for nutrient feed. The maximum nutrient pump flow rates were set to be 0.6 ml L^{-1} min^{-1} for the 7-L and 7.5 ml L^{-1} min^{-1} for the 80-L fermentor. The control parameters for the 'manual fed-batch' culture were the same as above except the nutrient feeding was done using a manually-adjusted step feed rate to maintain a glucose level of 1–3 g L^{-1}. Samples of 10 ml were removed from the broth hourly during fermentation. Cell growth was measured by optical density at 600 nm using a UV Spectrophotometer (Shimadzu OV-1201, Columbia, MD, USA) and cell dry weight (CDW). To obtain the CDW, 10–50 ml of fermentation broth (depending on the optical densities at different stages of fermentation) was taken periodically. The cells were pelleted by centrifugation for 10 min at 5000 × *g* and resuspended in deionized water before centrifuging again. This procedure was repeated for a second time. The supernatant was removed, and the residue was transferred quantitatively to a weighing dish using absolute ethanol. The dish was predried for 16 h in an oven and weighed. After the transfer, the dish with the residue was placed in the oven for another 16 h. After being cooled, the loaded dish was weighed. For the preparation of analytical grade plasmid DNA, *E. coli* cells from 1 ml broth were pelleted by centrifugation at 12 000 × *g* for 1 min and then lysed by the alkaline lysis method (see below). Diluted sample broth was also plated on LB agar plates with and without kanamycin to determine the number of plasmid-bearing and plasmid-free cells.

Plasmid DNA analysis

Cells were lysed by the alkaline lysis method and plasmid DNA was purified according to protocol (Promega Wizard, Madison, WI, USA). After the purification, the supercoiled DNA along with other DNAs in the sample were loaded onto and separated on a 0.8% agarose gel. After electrophoresis, the gel was analyzed and quantified using a densitometer IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA) against the standard DNA. Quantitation of DNA was also confirmed by HPLC through injecting 10 to 20 μl samples into a Nucleogen DEAE 4000-7 Anion Exchange column (Waters, Boston, MA, USA).

Results and discussion

Manual fed-batch fermentation

Initially, manual fed-batch fermentations of DH10B transformed with pENV were evaluated. High concentrations of glucose (>5 g L^{-1}) in culture caused growth suppression, inconsistent growth rates and high accumulation of acetic acid which in turn affected both cell and plasmid yields. Thus, a fed-batch culture was designed to control a low glucose residual level throughout the fermentation. The culture was fermented in batch mode with an initial glucose concentration of 5 g L^{-1}. Cell mass and glucose concentration were monitored off-line. Within 5 h, cell doubling occurred every hour before the glucose was consumed with a nearly constant specific growth rate ($\mu = 0.7\ h^{-1}$) and an average specific glucose consumption rate, $q = 1.5\ h^{-1}$. Cell growth entered log phase immediately after inoculation, $\mu = \mu_{max}$. Consequently, assuming that all the other requirements were met and glucose was the only limiting factor for growth, the relationship between cell mass and time should have been:

$$dx/dt = \mu x \quad (1)$$

$$X = X_o \exp(\mu t) \quad (2)$$

where X_o is the cell mass at the time of inoculation and X the cell mass at time t. The expected glucose depletion (or accumulation rate) rate, on the other hand, may be expressed from mass balance as:

$$ds/dt = FSi/V - qX \quad (3)$$

where F is the glucose feed rate (g h^{-1}), Si the glucose

concentration in the feed solution (g g^{-1}), q the substrate consumption rate (g g^{-1} h^{-1}) and V the fermentation working volume (L). Because the residual glucose concentration was minimized,

$$ds/dt = 0$$

$$\text{thus} \quad f = FSi/V = qX = qX_o \exp(\mu t) \tag{4}$$

where f (g L^{-1} h^{-1}) = the volumetric feed rate.

In this case, the glucose feed rate was equal to the consumption rate. From Eqn 4, the glucose consumption at any time during the log phase of fermentation could be estimated and the required feed rate determined.

Figure 1a presents a cell growth comparison between the predicted cell mass based on Eqn 1 and experimental data obtained from the off-line analyses. Actual and predicted cell growth curves were essentially superimposable with a nearly constant growth rate until t = 8 h. After 8 h, the growth rate began to fall. Figure 1b displays the predicted nutrient feed rate associated with the predicted growth rate calculated from Eqn 4 and the actual step feed rates. As expected up to 8 h, the predicted feed rates accurately reflect the manual feed rates used. Figure 1c presents the theoretical and the actual glucose residual concentrations computed from off-line analyses during the fermentation. As shown, the glucose concentration gradually decreased along with growth and then spiked at 9 h even when the addition of glucose was adjusted to a rate far lower than would have been estimated using Eqn 4. Glucose accumulation was undoubtedly associated with variable growth (μ) and glucose consumption (q) rates. The discrepancies between estimated and measured values in Figure 1 indicate that Eqns 2 and 4 are obviously too simple to meet varying cellular demands. Cell growth could be easily affected by metabolites (eg acetic acid) accumulation rates which were not taken into account. The cell growth, at a maximum rate of 0.7 h^{-1}, was apparently jeopardized in the late stages of fermentation by DO limitations and fast acetic acid accumulation (estimated at 0.36 g L^{-1} min^{-1}). Under these conditions, cell densities averaging 30 OD at 600 nm (CDW 10–12 g L^{-1}) were achieved and plasmid DNA yields were between 8–10 mg L^{-1}. These results clearly indicate that cell growth was very rapid, leading to a rapid transition to stationary phase, resulting in both low cell and plasmid DNA yields. Growth simply outpaced the rate of plasmid replication. Low copy number plasmids containing a pBR322 origin may be especially susceptible to this phenomenon [4,15]. It has been reported that a minimum acetic acid accumulation and a high plasmid copy number could be obtained when the growth rate is about 0.1 h^{-1} [4,15]. Therefore, we decided to slow down the growth rate and design an automated feed-back control system that would meet the varied requirements associated with changing cellular activities.

Feed-back control, fed-batch fermentation

A feed-back control, fed-batch fermentation was developed. Nutrient feeding was automated through feed-back control of changing values of DO and pH during the fermentation. These feed-back parameters were chosen based on the prin-

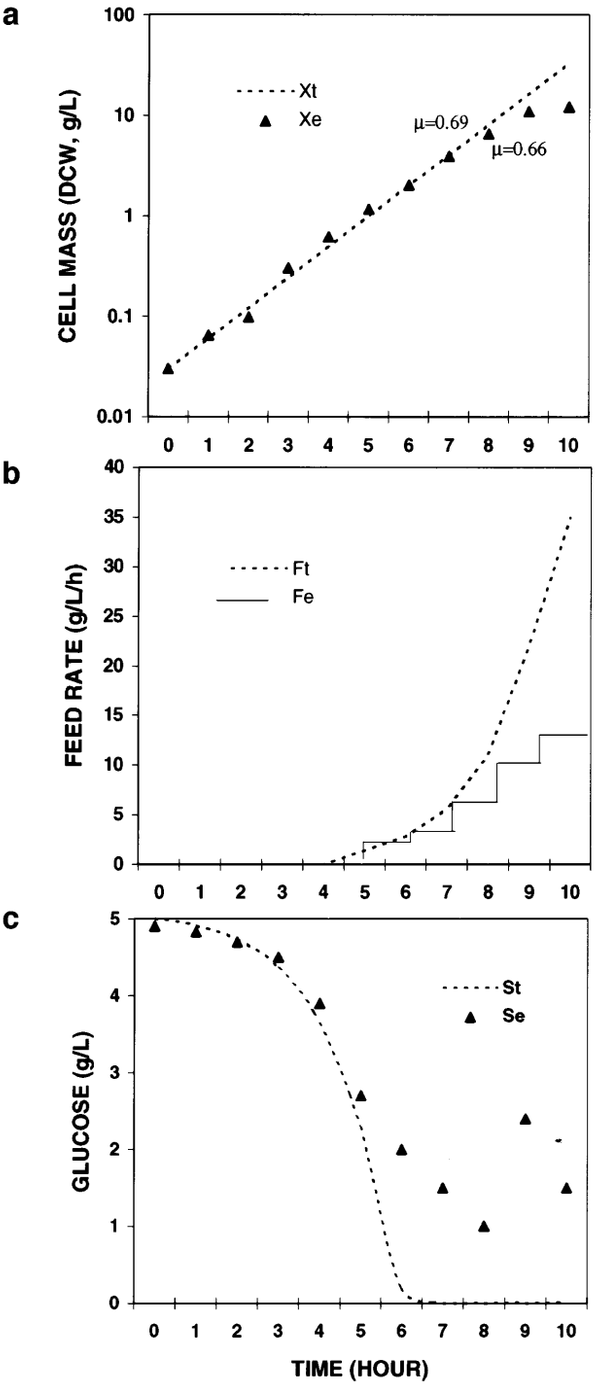

Figure 1 Manual fed-batch fermentation. Specific growth rates (μ) and specific glucose consumption rates (q) were calculated based on the data obtained from triplicate fermentations in the 7-L fermentor. The rates were then used in Eqns 2 and 4 to calculate the theoretical values of cell mass (X_1), nutrient feed rates (F_t), and glucose residual concentrations (S_t) at time of t during fermentation. The real values of each parameter were obtained by off-line measurement and plotted as X_e, F_e, and S_e. Each data point reflects a mean value from triplicate fermentations in the 7-L fermentor. (a) Profiles of the calculated growth based on a constant μ = 0.69 (---) and the actual growth (▲) from off-line measurements in the manual fed-batch culture. (b) Comparison of predicted substrate consumption rates (....) and actual substrate feed rates (—) adjusted manually. (c) Profiles of predicted glucose residual concentrations and actual glucose concentrations measured off-line during the manual fed-batch culture.

ciple that when the carbon and nitrogen sources become limiting in a culture, cellular respiratory activity slows down, resulting in a rapid increase in DO. If the carbon source is limiting, pH also rises, likely the result of consumption of metabolic fatty acids by cells as an alternative carbon source and the production of ammonium ions associated with protein catabolism. A nutrient pump was coupled with both pH and DO controllers. The set points for both pH and DO to trigger the feed were programmed through AFS software. To prevent overfeeding and to retain a slow growth rate, the set points of pH and DO for the nutrient feed were experimentally determined as 7.2 and 50%, respectively. Every time either DO or pH rose above set point, the nutrient pump was activated. The nutrient pump was deactivated when both DO and pH dropped below set points. Because early batch stage fermentation DO was high, the starting point of the nutrient pump activation was programmed based on time and decrease on DO, ie the feed-back control mechanism was not established until a certain time had passed and DO dropped below an initial set point. The maximum flow rate of the nutrient pump was set at 0.6 ml L^{-1} min^{-1} to provide sufficient nutrient supply. The control scheme for the nutrient feeding described above may be expressed as logic terms:

$$F = fm\ E_t\ (E_{pH} + E_{o_2})$$

where $(E_{pH} + E_{o_2}) \leq 1$
F: the actual feed rate, ml L^{-1} min^{-1}; fm: the maximum pump flow rate, ml L^{-1} min^{-1}; E_t: a time factor to establish the feed-back control scheme,

$E_t = 0$, when $t < 5$h

$E_t = 1$, when $t > 5$h

E_{pH}: a pH factor related to the pH upper set point,

$E_{pH} = 1$, when pH > 7.2

$E_{pH} = 0$, when pH < 7.2

E_{o_2}: a DO factor related to the DO upper set point,

$E_{o_2} = 1$, when DO $>50\%$

$E_{o_2} = 0$, when DO $< 50\%$

When the above scheme was translated into the AFS equation it became:

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NutA1.SP = CMP(TIME(EFT),5)*100*OR(CMP
           (DO.CV,50),CMP(PH.CV,7.2))
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Because the nutrient feed rate was regulated by DO and pH both of which affect ultimate growth rate, two effects could be manifested. First, cells could go through repeated nutrient depletion as reflected by drastic changes in DO. Second, DO rises above the set point might only drive a minimum nutrient supply, barely enough for cell maintenance but not enough to achieve a substantial growth to high cell density. To move this 'demand and supply' balance forward, the agitation speed was coupled with the same DO controller and regulated by a lower set point of 30% in a way that every time DO dropped below 30% due to excess nutrients and vigorous cell growth, the agitation speed was increased by 2% min^{-1} of the previous rpm. The agitation rate no longer increased when DO was above 30%. This type of control was also achieved through the AFS equation function shown as:

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AGIT.SP = LIMIT (1 + 0.01*CMP(DO.CV,30),
          150, 1000)
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Figure 2 presents a typical fermentation time course of pENV-transformed *E. coli* DH10B using the automated feed-back control strategy. Cell growth was supported initially (first 5 h) by the batched-in nutrients. Subsequently, DO levels went below the upper set point (50%) for the nutrient pump. At this point the control for the nutrient pump was activated. During this first 5 h of the fermentation, the agitation speed was identical to the starting speed. After this time, increased growth caused a depletion

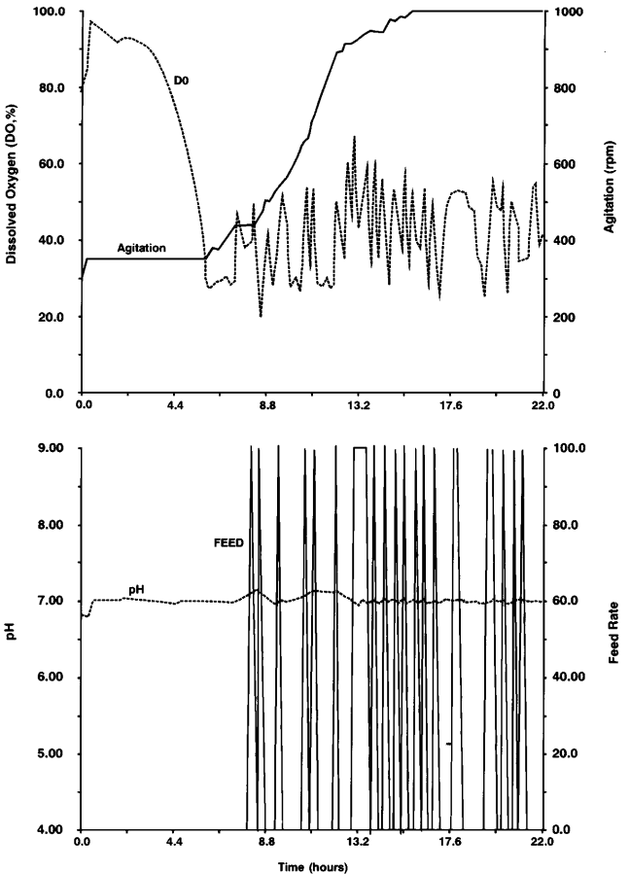

Figure 2 A typical profile of the controlling parameters: pH, DO, agitation and nutrient pump output during the DO- and pH-based feed-back control fermentation of plasmid pENV in *E.coli* in the 7-L fermentor. DO was controlled between 30–50% by automatically feeding a nutrient solution (40% glucose, 20% yeast extract) and increasing agitation speed when DO was below the 30% of the lower set point. The profiles of those parameters have been very similar in triplicate fermentations with the same plasmid (data not shown).

in DO below the lower set point (30%) for the agitation control. At this point, agitation speed began to rise at the pre-set increment rate until the DO exceeded the lower set point (30%), then stabilized. Between hours 7 and 9 of the fermentation the carbon source was depleted, followed by depletion of the nitrogen source as indicated by the DO and pH spikes. Further limitations of carbon and nitrogen sources caused the DO and pH to rise above the respective upper set points (50% and 7.2) thereby activating the nutrient pump. In this experiment, the nutrient pump was controlled in 'On-off' rather than PID modes, hence 100% output was reached immediately. The nutrient pump remained at the maximum flow rate until DO and pH fell below each upper set point. Then the pump rate was automatically reset to zero flow rate until the next cycle was initiated. When a 1-h portion was enlarged on the data-logging screen, the pump activity was seen to follow exactly the DO and pH excursions (data not shown). pH and DO spikes took place at different stages of fermentation, indicating varying cell demands for the carbon or nitrogen sources. Hence, the simultaneous use of both pH and DO for feed-back control provided a control advantage that would not have been afforded by using either parameter alone for feed-back control.

DO was maintained well between the upper and lower set points during a typical 24-h fermentation without the need for oxygenation with pure O_2 or high mixing power. Cell growth rate initiated at $\mu = 0.69$ during the batch mode and later stabilized at $\mu = 0.13$ (Figure 3). Glucose residuals were virtually undetectable (below 0.1 g L^{-1}) after the culture went into the fed-batch mode. Acid production became undetectable after 9 h (data not shown). Cell density finally reached 60 g L^{-1} DCW (OD_{600} = 121), nearly a 4-fold increase over the cell density obtained in the manual fed-batch culture. A very similar growth and nutrient consumption profile was also observed in four scale-up runs in the 80-L fermentor (data not shown). This may be reasonable because the same ranges of pH and DO control set points

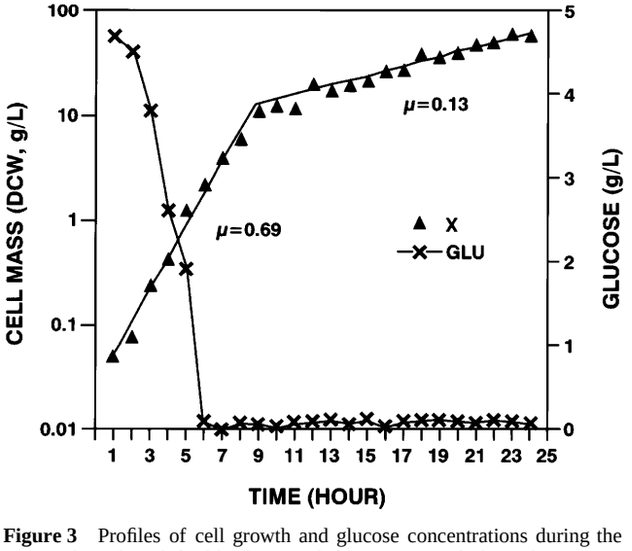

Figure 3 Profiles of cell growth and glucose concentrations during the DO- and pH-based feed-back control fermentation of plasmid pENV in *E.coli* DH10B in the 7-L fermentor. The data shown are mean values obtained from three fermentation runs.

Table 1 Fermentation results in 7-L and 80-L fermentors using the manual and the DO-stat processes

Fermentor (L)	Process	Growth rate (h^{-1})	Cell mass (g L^{-1})	Plasmid yield (mg L^{-1})
7	manual	0.7–1	35–45	8–10
7	DO-stat	0.1–0.3	80–105	82–98
80	manual	0.5–0.7	35–45	6–8
80	DO-stat	0.08–0.15	72–90	70–80

were used at both 7-L and 80-L scales. The same DO tension achieved via high back pressure and lower agitation speed in the 80-L stainless steel vessel did not seem to affect the culture's performance. A very straight-forward scale-up was obtained (Table 1). Plasmid DNA yields in feed-back control, fed-batch fermentations were amplified nearly 10-fold over manual fed-batch cultures, reaching 100 μg ml^{-1} (*vs* 5–8 mg L^{-1}) (Figures 4, 5). Although these data were from the 7-L fermentor, a similar yield had been obtained consistently from the 80-L scale as well.

Plasmid yields per gram of cells were also increased from 0.7 mg g^{-1} cells in the manual process to 1.7 mg g^{-1} cells using the pH/DO feed-back control. The observed improvement in the plasmid yields was probably in part due to the increased cell density. However, additional reasons for this increased yield might also be postulated. It has been observed [15] that plasmid content increases as specific growth rate decreases. When the rate of plasmid replication is insufficient to keep pace with host division (growth) rate, plasmid copies are partitioned unequally between daughter cells, resulting in plasmid-free segregants (or low plasmid content), the so-called segregational instability of plasmid DNA [7,15]. Therefore, slower growth should be more favorable for plasmid to partition properly and to replicate more efficiently, because cellular metabolic

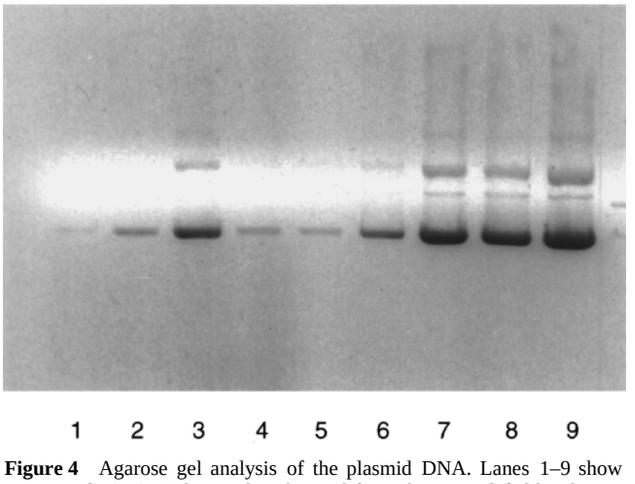

Figure 4 Agarose gel analysis of the plasmid DNA. Lanes 1–9 show respectively 3, 6, 10-h samples obtained from the manual fed-batch process and 3-, 6-, 10-, 14-, 18-, and 24-h samples from the DO- and pH-based feed-back control, fed-batch process in the 7-L fermentor. Plasmid DNA was purified from 1 ml fermentation broth using an alkaline lysis method and 10 μl of DNA was loaded on the 0.8% agarose gel. Bands from top: genomic DNA, open circular plasmid DNA and supercoiled plasmid DNA.

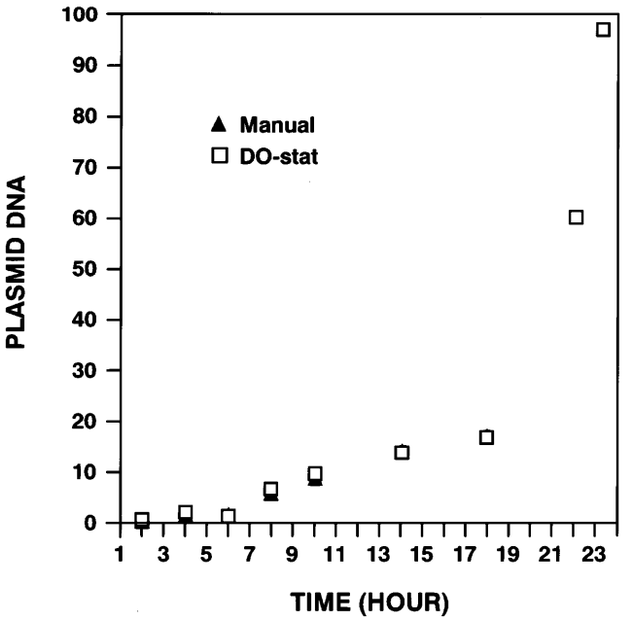

Figure 5 Comparison of plasmid DNA yields during the DO- and pH-based feed-back control fermentation (□) and the manual fed-batch culture (▲) in the 7-L fermentor. Plasmid DNA concentrations were determined by using the densitometer and confirmed by HPLC (Materials and methods). The data shown are the mean values obtained from three fermentation runs with the same plasmid. The scale for plasmid DNA is up to 100 μg ml^{-1}.

activity is generally low at slower growth rate, leaving more nutrients and energy available for plasmid replication. The optimal growth rate needed to maximize yields of pENV in these experiments was $\mu = 0.13$. Reinikainen *et al* [9] found that plasmid yields obtained from cultured bacteria were highest when growth went into stationary phase. During the stationary phase, cellular metabolic activities including RNA transcription and protein translation are usually at a minimum. Hence, plasmid replication rates might have been increased. It is possible that the late stage fed-batch process might create a similar environment. Hecker *et al* [5] discovered that in *E. coli* relA$^-$ strains which can not synthesize guanosine tetraphosphate (ppGpp) when amino acids limit cell growth, pBR322 yields have amplified following arginine starvation. The DH10B strain is relA$^+$. Nonetheless overall nitrogen limitation might suppress protein synthesis in DH10B cells. As a result, the plasmid DNA yields could be amplified. A better understanding of plasmid DNA metabolism in bacterial cells will require additional research.

We have developed an automated feed-back control, fed-batch fermentation process based on DO and pH. The process has proven to be reproducible and scalable. This feed-back control, fed-batch process provided a means to improve plasmid yields up to 10-fold when compared with manual fed fermentation. Additional experiments to test this process with different bacterial strains and plasmids are under way.

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