YIELD PRODUCTION OF RECOMBINANT PLASMID DNA WITH ESCHERICHIA COLI IN FED-BATCH CULTURE BY PSEUDO-EXPONENTIAL FEEDING

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ABSTRACT
For preventing and treating debilitating diseases, plasmid DNA [pDNA] is emerging as the vector of choice for gene therapy and DNA vaccination. In this study, the development of a new implementation of control strategies for high-cell-density (HCD) culture fermentation of Escherichia coli was described to produce high level of cell mass for the mass production of bacterial plasmid. A yield of 2 g of pDNA/L was achieved by application of the newly developed techniques for HCD culture. We were also able to reduce the fermentation time for the highest production to only 3 h rather than 72 h. Furthermore, application of the HCD culture technique permits to keep the organism at a low growth rate to ensure high quality of the produced plasmid.

The kinetics of batch and fed-batch cultures of E. coli producing plasmid was investigated. The cell lag time, the maximum specific growth rate and (\(\mu\)) were determined as 2 h, 1.7 h\(^{-1}\) and 1.61 g·g\(^{-1}\), respectively. In the fed-batch culture, different specific growth rates were set at 2.5 h\(^{-1}\), 3.2 h\(^{-1}\), and 4.5 h\(^{-1}\) by pseudo-exponential feeding, and the expressions for the specific rate of substrate consumption, the growth kinetics and the product formation kinetics of each phase were obtained. The result shows that the concentrations of cell and plasmid can reach 93.0 g·L\(^{-1}\) and 2.0 g·L\(^{-1}\) respectively, and the quality of the DNA vaccine met the requirements for medical use.

Keywords: high-cell-density, fed-batch, DNA vaccine, E. coli, plasmid, kinetics

Introduction
The current developments in biotechnology involve production of biopharmaceutical products (BPP) like vaccines, recombinant proteins, monoclonal antibodies, etc. used for therapeutic and diagnostic purposes. Over the past several years, a significant leap forward has been made toward the development of safe and effective DNA vaccines and DNA-based therapies against human and animal diseases (1). Bacterial plasmids are the vectors of choice for DNA vaccines and short-term gene therapeutics (8).

The key component for the commercial success of any BPP is the ability to achieve large-scale manufacture. E. coli is the most commonly used host strain for heterologous protein production by recombinant DNA technology (16). Several genetically engineered E. coli strains have been developed to enhance pDNA production. Among these strains E. coli DH5α [F endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+)], λ] was engineered to give high level of supercoiled (gyrA96) and more stable pDNA (recA = recombination deficient) and to avoid plasmid degradation (end A1 = endonuclease I deficient) (22). E. coli is preferable for its relative simplicity, inexpensive and fast high-density cultivation,
well-known genetics, large number of compatible molecular tools available, and remains a valuable organism for the high-level production of recombinant proteins (13).

Since 1970, several studies have been devoted to high-cell-density cultivation techniques to achieve maximum productivity of recombinant proteins in *E. coli* (17). Research strategies were focused on improving the cultivation techniques, manipulating the bacteria’s physiology or both (25). As a result, batch, fed-batch and dialysis fermentation techniques have been developed. Fed-batch processes have most often been used to obtain high cell density (29).

The common problems in scale-up issues are plasmid instability, acetate accumulation and substrate inhibition in the high-cell-density bioreactor production system (27). Recombinant protein expression using *E. coli* as a host is frequently associated with the formation of intracellular aggregates in the form of insoluble biologically inactive inclusion bodies (20). Thus, the productivity of a recombinant protein is proportional to the final cell density and the specific yield as the amount of product formed per unit cell mass (17). It is commonly found that the higher growth rates in batch and fed-batch fermentations are associated with lower percentages of supercoiled plasmid in comparison to that obtained in simple batch fermentation (19). Overall plasmid productivity can be improved by increasing the product of two variables: (i) the copy number of plasmid per cell (specific productivity) and (ii) the amount of cell mass per unit of volume and time as cell productivity (26).

Several methods for improving the volumetric productivity of plasmid have been reported. Two major growth techniques were developed for high-density growth, fed-batch and dialysis (25). Typical plasmid fermentation media and processes result in yields of 100 mg to 250 mg of pDNA/L of culture medium containing plasmids (8). Recently, a fed-batch fermentation yield of about 1000 mg of pDNA/L was obtained after reduction of plasmid-mediated metabolic burden during growth (9). In order to improve the volumetric productivity of recombinant proteins in *E. coli* with high yield, over-expression of the recombinant protein in a fermentation process and a purification procedure allowing efficient recovery of the protein from the resultant biomass are necessary.

The aim of this study was to obtain an optimum feeding model and an appropriate strategy for induction and cultivation of *E. coli* for significantly increased specific plasmid yield with respect to cell mass, while enhancing the plasmid integrity and maintaining the supercoiled DNA content. This study focused on the process and kinetics engineering parameters of fermentation. *E. coli* and plasmid DNA production in batch and fed-batch cultivation was also developed.

**Materials and Methods**

**Plasmid and bacterial strain**
The bacterial host used for plasmid gWizHBs was *E. coli* DH5α strain, Genotype: F’ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80lacZΔM15 Δ(lacZYA-argF)U169, hsdR17( rK- mK- ), λ−, was purchased from Promega Corp. (Madison, WI, USA). The gWiz high-expression HBsAg plasmid (gWizHBsAg) 5 mg was purchased from Aldevron, USA (http://www.aldevron.com). This plasmid is 6.435 kbp long and has a pUC origin of replication.

**Chemical and other kits**
Most chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were molecular biology grade. Kanamycin monosulfate from *Streptomyces kanamyceticus* was purchased from Sigma–Aldrich, catalog number K0879 (50 mg Powder). Milli-Q grade water (18 ohm resistance) was used to make the buffers throughout the work. Good Microbiology laboratory practices were followed throughout the work.

Plasmid Mini Kit (25) with 25 QIAGEN-tip 20, Reagents, Buffers, Reference 12123 was from QIAGEN (Germantown, Maryland, USA). Restriction enzymes EcoR1, BamH1, Hind III were from New England Biolabs (Boston, MA, USA), Alul, HaeIII, Sau3A were from Promega (Madison, WI, USA).

**Bacterial culture**
Bacteria [*E. coli* DH5α] were grown under standard conditions (37 °C for 14 h to 16 h under shaking at 250 rpm) in Luria Bertoni [LB] media in the presence of kanamycin at a concentration of 50 μg·mL−1 as described before (21).

**E. coli DH5α competent cells**
*E. coli* DH5α was made competent using the CaCl2 procedure (3). Cell density was monitored by determining OD600 and was less than 10−8 cells·mL−1 (log phase of growth) as described before (21).

**Transformation with gWiz Hbs plasmid and isolation of recombinant strain**
An amount of 10 ng of the gWIZ HBs plasmid was added to 200 μL of competent cells in ice-cold Eppendorf tubes and placed at 42 °C for exactly 90 s and returned to ice for 1 min to 2 min. Aliquots of 1 mL of SOC media (2 % w/v bacto-tryptone, 0.5 % w/v bacto-yeast extract, 10 mmol·L−1 NaCl, 2.5 mmol·L−1 KCl, 10 mmol·L−1 MgCl2, 20 mmol·L−1 MgSO4, 20 mmol·L−1 glucose) were added and the bacteria were incubated for 2 h at 37 °C. The exact volume of 200 μL of transformed bacteria was plated in an LB/Kanamycin plate.

**Digestion of pDNA with restriction enzymes**
An amount of 1 μg of plasmid DNA was digested or double digested with the enzymes Hind III, BamH1 and Alul, Hae III and Sau3A in a single digestion. The reaction was performed as described before (21).

**Fermentation media**
Semi-defined batch medium (NTC 3018) and fed-batch media (NTC 3019) were formulated according to the methods described by Carnes et al. (8). Glycerol (50 g·L−1) and 20 g·L−1 of yeast extract, along with salts and trace metals, were used in the batch medium. The fed-batch medium consisted of a base medium similar in composition to the batch medium, but with lower concentrations of glycerol (10 g·L−1) and yeast extract (5 g·L−1), and a concentrated semi-defined nutrient feed containing 600 g·L−1 of glycerol.

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Shaking flask cultures

Two clones of strain from an LB agar plate were inoculated into two 300 mL flasks containing 50 mL LB media, and incubated on a shaker at 34 °C and 200 rpm for 10 h to 12 h. Eight 500 mL Erlenmeyer flasks, each containing 50 mL of LB media, were inoculated with 5 mL of the seed culture. These eight flasks were used to inoculate the fermentor.

Fermentation conditions

The seed cultures were started from glycerol stocks or colonies inoculated into NTC 3018 medium plus 50 µg mL⁻¹ kanamycin or 100 µg mL⁻¹ ampicillin and grown in shake flasks at 37 °C. At mid-exponential phase (D₂₀₀ = 0.3–1.0), the seed cultures were used to provide 1% inoculum for the fermentations. All fermentations were carried out in a bioreactor (BioFlo 110 and BioFlo 310, New Brunswick Scientific Edison, Edison, NJ) equipped with a built-in digital controller for pH, temperature, agitation, dissolved oxygen monitoring, and peristaltic pumps for adding acid, base, and the feeding solution. The pH and dissolved oxygen (DO) concentration were controlled at the set points by on-line monitoring. The controller was interfaced with a computer through the NBS Super Control Program. During fermentation, pH was controlled at 7.0 ± 0.1 by the automatic addition of 30% ammonium hydroxide or 10% phosphoric acid (Fig. 1). The dissolved oxygen probe was calibrated to 0% by nitrogen gas sparging and 100% with air saturation. The vessel was aerated at 1 VVM (volume of gas/volume of medium per min) and dissolved oxygen was maintained at 30% by proportional-integral control of agitation. At cell densities above approx. D₂₀₀ 20, O₂ supplementation was required and was increased automatically as needed during the rest of the fermentation to maintain 30% dissolved oxygen saturation by replacement of air by pure O₂. During fed-batch fermentations, the semi-defined nutrient feed was added either at a constant feed rate, or according to a predetermined exponential feeding strategy. Briefly, an initial amount of carbon substrate was consumed during the batch phase at a specific growth rate of µmax. Upon exhaustion of the carbon substrate, the fed-batch phase began and the nutrient feed was added continuously.

For exponential feeding, the feed rate was determined automatically by the following equation (7, 17): 

\[ F(t) = \left( \frac{\mu X}{S_f} \right) \cdot e^{\mu t}, \]

where \( f \) is the feed rate (L h⁻¹), \( \mu \) is the desired specific growth rate during the fed-batch phase (h⁻¹), \( X \) is the biomass concentration at the end of the batch phase (dry cell weight, g L⁻¹), \( S_f \) is the initial liquid volume of the culture (L), \( S \) is the limiting substrate concentration in the nutrient feed medium (g L⁻¹), \( Y_X/S \) is the yield coefficient of biomass from substrate (g g⁻¹), and \( t \) is the time elapsed from the beginning of the fed-batch phase (h).

\[ Y_X/S = 0.4 \text{ g g}^{-1}, \quad X = 4 \text{ g L}^{-1}, \quad \mu = 0.12 \text{ h}^{-1} \] were used for calculation of the feed rate for all exponential fed-batch fermentations (Fig. 1) and resulted in specific growth rates close to the desired specific growth rate during fed-batch fermentation. Typically, the final culture volume was 20% to 25% greater than the starting volume due to the addition of nutrient feed and ammonium hydroxide.

Antifoam

Antifoam has a strong influence on the pO₂-signal. Pulsing antifoam led to strong shifts in the pO₂-signal which interfered with the detecting tool and thereby led to faulty results (15). Continuous antifoam addition in very low concentration can be a solution. Massive use of antifoam leads to a very unstable and oscillating pO₂-signal and deteriorates the oxygen transfer rate. It is a big advantage to use no chemical antifoam at all and to work with a mechanical foam breaker instead.

Analytical measurements

Culture samples were taken at key points and at regular intervals during all fermentations. Samples were analysed immediately for culture density and plasmid yield. Dry cell weight (DCW) was gravimetrically determined using the pellet fraction from 20 mL samples, after centrifugation at 12000 rpm for 10 min. The pellet was washed twice with distilled water and resuspended in 10 mL of distilled water. This was poured into preweighed aluminium cups and placed overnight in an oven at 80 °C until the mass became constant. The dried cups were weighed again to calculate the dry cell weight. Culture density was measured by optical density at 260 nm in a spectrophotometer (3). The Beer–Lambert law relates the amount of light absorbed to the concentration of the absorbing molecule. At a wavelength of 260 nm, the extinction coefficient for double-stranded DNA is 50 (µg/mL)⁻¹·cm² and for single-stranded DNA and RNA it is 38 (µg/mL)⁻¹·cm². Thus, an O.D. of 1 corresponds to 50 µg mL⁻¹ of double-stranded DNA. This method of calculation is valid for up to an O.D. of 2:

\[ \text{Concentration of double stranded DNA} = \text{Absorbance at 260 nm} \times 50 \times \text{dilution} \]

Preparation of pure pDNA

Plasmid gWiz DNA was prepared using the QIAGEN Plasmid Kits (Mini and midi size) according to the manufacturer’s instructions. These kits are based on the alkaline denaturation method for the isolation of plasmid from bacterial culture described by Birnboim and Doly (6). DNA quantification was carried out by measuring the optical density (O.D.) at 260 nm in a spectrophotometer (3).
Agarose gel electrophoresis
Agarose gels (1 %) containing 0.5 g·mL⁻¹ ethidium bromide were run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The running buffer was TAE (40 mmol·L⁻¹ Tris, 20 mmol·L⁻¹ acetic acid, 1 mmol·L⁻¹ EDTA, pH 8.0). Electrophoresis was carried out at 100 V for 1 h on an Amersham–Pharmacia Biotech (Uppsala, Sweeden) power supplier unit ECPS3000/150.

Results and Discussion
Economical considerations play a significant role when large-scale operations are considered (25). These include longevity of the process, loss of nutrients, safety and cost of working with pure oxygen and pressurized fermentors, mechanical load on agitation system, efficiency of cooling, heating and mass transfer, sensing and probing limitations, and equal distribution of ambient conditions in the whole culture. A close examination of past publications on high-density techniques for E. coli cultures reveals a wide array of final cell concentrations ranging from DCW of 20 g·L⁻¹ to 190 g·L⁻¹ (18). This significant variability can be attributed to the fact that the growth of different strains may be controlled by parameters not completely understood such as stress response and quorum sensing in E. coli (28). Better understanding of these parameters may contribute to the development of improved growth methods and recombinant protein expression.

High cell density fermentation
A simple and reproducible high-cell-density fermentation method for E. coli strain is aimed at maximizing the overall plasmid production in a bioreactor. We can routinely obtain cell densities (dry cell weight) of over 93 g·l⁻¹ in a simple fed-plasmid production in a bioreactor. We can demonstrate that high levels of the plasmid can be produced in a single and efficient fermentation process, fermentation was conducted to produce biomass of approximately 100 g·L and plasmid of over 1 g·L. The fermentation was conducted as a three-stage process: batch, fed-batch, and induction. During the batch phase, the initial inoculum was allowed to grow such that the cells consumed the initial 20 g·L⁻¹. Glycerol depletion (13 h after inoculation) was indicated by a sharp increase in the concentration of dissolved oxygen. After depletion of the initial glycerol, a feed containing glycerol, phosphate, and sulfate at a constant feed rate of 10 g·h⁻¹ glycerol was initiated in a pseudo-exponential mode. The cells continued to grow exponentially until a fixed growth rate corresponding to the glycerol feed rate was achieved. The feed rate was then further increased to 10.0 g·h⁻¹ glycerol, thereby increasing the growth rate. During this phase, both phosphate and sulfate concentrations increased in the fermentation, whereas glycerol, being the rate-limiting nutrient, remained undetectable due to its immediate consumption in the reactor. During this phase, the amount of plasmid in the cells remained less than 1 % of the total biomass.

Kinetics of batch cultivation
The development of the present kinetic model was based on the following assumptions that are basically from the knowledge of fermentation biochemistry and mass balance in the bioreactor (14): 1) Glycerol is the only limiting substrate in batch fermentation; 2) There is no limitation on the nitrogen source; 3) Cultivation inhibition is brought about only by the burden of the metabolic products (aim plasmid) via temperature control. 4) The pH value is known and controlled at a constant value.

Lag phase
The initial concentrations of cells and glycerol were 1.5 g·L⁻¹ (DCW) and 30 g·L⁻¹, respectively. The concentrations of cells and glycerol were determined every hour. The initial lag phase is a period without observable growth, but chemical analysis shows much hidden metabolic turnover, indicating that the cells are adapting to the new environment and that growth will begin in due course (5). The kinetics of cell growth in the lag phase could be represented by Eq. (1).

\[ \mu = \frac{\mu_{\text{max}} \mu}{(t - t_s) + \frac{\mu}{t_{\text{eq}}}} \]

Based on the data in Fig. 2, \( t_s \) could be found easily and the maximum specific growth rate could be roughly calculated from the slope of the straight line obtained by plotting \( \ln D \) versus time \( t \) during the exponential growth phase. From Fig. 2, \( \mu_{\text{max}} \) would be 0.648 h⁻¹, close to the value from following the calculation method.
Fig. 2. Growth curve of E. coli in batch fermentation (♦), glycerol concentration, and specific growth rate (■) measured by optical density at 600 nm.

In the exponential phase, cell growth is unlimited, with nutrient in excess and inhibitors absent. The specific growth rate attained its maximum, i.e. \( \mu = \mu_{\text{max}} \).

Fig. 3. Relation between \( \mu \) (specific growth rate, h\(^{-1}\)) and D (cell density [DCW], g L\(^{-1}\)).

After lag phase

The kinetics of cell growth and substrate consumption in the exponential growth phase, the retardation phase, and the stationary phase in batch cultivation agreed with Eq. 2 and Eq. 3. The yield coefficients determined in the above-mentioned phases might be assumed as constant for a simple growth-association system free of endogeneous metabolism:

\[
\frac{dD}{dt} = \frac{U_{\text{max}} D}{G_{s} + U} \quad \text{and} \quad \frac{dU}{dt} = \frac{1}{Y_{DGs}} \frac{dD}{dt} \quad \text{(2) and (3)}
\]

For cell growth, from Fig. 2, the specific growth rate can be calculated directly according to the cell mass accumulation with time interval:

\[
\mu = \frac{1}{D^0} \frac{\Delta D}{\Delta t} \quad \text{(4)}
\]

The relationship between \( \mu \) and \( U \) could be obtained (Fig. 3) and Eadic–Hofstee illustration shows that the relationship between \( \mu \) and \( \mu/U \) is:

\[
\frac{\mu}{U} = \hat{\mu}_{\text{max}} \frac{1}{G_{s}} \frac{L_{s} + U}{G_{s}} \quad \text{(5)}
\]

The slope is the saturation constant for substrate (\( G_{s} \)). The absissa intercept represents \( \mu_{\text{max}}/G_{s} \) (Fig. 4). Based on the data shown in Fig. 2, Fig. 3 and Fig. 4, the maximum specific growth rate and the saturation constant for substrate (2) could be obtained. Most substrate was used for cell growth during this period, so the cell yield coefficient should be viewed not only as constant, but also as the “true” coefficient.

Kinetics of fed-batch cultivation

When DO\(_2\) and pH began to rise, this indicated that the glycerol would be consumed up. Feeding operation started, and fed-batch cultivation began. The exponential feeding was carried out according to the method described by Henes and Sonnleitner (15).

Before induction

During this phase, it could not be assumed that \( Y_{D/U} \) is a constant, and the part of substrate consumption used for maintaining metabolism should be considered. The trend, which is contrary to the Monod chemostat model, can be explained by including the possibility of endogeneous metabolism in the model. Endogeneous metabolism means that some reactions occur in cells, which consume cell substrate. Eq. 10 can account for this effect. For the purpose of the cell maximum yield coefficient, i.e. the “true” coefficient (\( Y_{DGs} \)) and the maintain coefficient (\( c \)), a pseudo-exponential feeding strategy (10) was introduced to control the specific growth rate at 0.15 h\(^{-1}\), 0.20 h\(^{-1}\), and 0.25 h\(^{-1}\), respectively, and the feeding flow rate is expressed as:

\[
R = \frac{\mu_{\text{set}} V_{s} D_{s}}{U - U_{s}} \exp(\mu_{\text{set}} \Delta t) \quad \text{(6)}
\]

\( D^0 \) is much larger than \( D_{s} \), so \( D_{s} \) can be neglected, and \( (D^0 - D_{s}) \) becomes \( D^{0} \). Eq. 6 reduces to:

\[
R = \frac{\mu_{\text{set}} V_{s} D_{s}}{U - U_{s}} \exp(\mu_{\text{set}} \Delta t) \quad \text{(7)}
\]

At this phase, the kinetics may be represented as:

\[
\frac{dV}{dt} = R \quad \text{(8)}
\]

\[
\frac{dVD}{dt} = \mu_{\text{set}} \frac{dV}{dt} \quad \text{(9)}
\]

\[
\frac{d(UV)}{dt} = R U' - \frac{V_{\text{set}} D}{Y_{DG/UV}} - cXV \quad \text{(10)}
\]

And the initial conditions are \( V = V_{0} \), \( D = D_{0} \) and \( U = U_{0} \).

From Fig. 5, according to Eq. 11, \( Y_{D/UV} \) and \( c \) can be calculated:

\[
\Omega = \frac{1}{D} \frac{dU}{dt} = \frac{\mu_{\text{set}}}{Y_{DG/UV}} + c \quad \text{(11)}
\]

\[
\Omega = \frac{1}{D} \frac{dU}{dt} = \frac{\mu_{\text{set}}}{0.55} + 0.066 \quad \text{(12)}
\]
After induction

When the O.D. reached 90–100, the fermentation temperature was adjusted to 42 °C for induction of plasmid production. The plasmid could be accumulated by inhibition of the cell growth and metabolism by using a low metabolic active substrate and temperature change (8). The kinetics of this phase should include the formation of the product, so it may be expressed as in Eq. 8, then:

\[
\frac{d(VD)}{dt} = \frac{\mu_{\text{net}} U}{G_s + U} \times \frac{G_p}{G_p + P} \times DV \quad (13)
\]

\[
cDV = \frac{V}{Y_{r/g}} \frac{dP}{dt} \quad (14)
\]

\[
\frac{dPV}{dt} = V \frac{dD}{dt} + DV \quad (15)
\]

And the initial conditions are \( V = V_1, D = D_1 \) and \( U = U_1 \), then:

\[
\frac{dX}{dt} = \frac{\mu_{\text{max}} U}{G_s + U G_p + P} \quad (16)
\]

At each \( \Delta t \) interval in this period, the kinetics is expressed as:

\[
\frac{dU}{dt} = \frac{R U^\prime}{V} - \frac{1}{Y_{r/g}} \frac{\mu_{\text{max}} U}{G_s + U G_p + P} D - cVD - \frac{1}{Y} \frac{dP}{dt} \quad (17)
\]

\[
\frac{dP}{dt} = \gamma \frac{dX}{dt} + \delta D \quad (18)
\]

Based on the experimental data of fed-batch cultivation, the parameters were evaluated by software MATLAB (4, 12, 24). The kinetic parameters of fed-batch cultivation were calculated from the data shown in Fig. 4. The \( R^2 \) of cell growth models, glycerol consumption model and plasmid production model were at 0.95, which indicated that the experimental data agreed well to the kinetic models (Eqs. 19–21) as shown in Fig. 6.

![Fig. 4. The Eadic–Hofstee relationship between \( \mu \) (specific growth rate, h\(^{-1}\)) and \( \alpha/U \) (glycerol concentration, g L\(^{-1}\)).](image)

These models would be a very useful reference to design expert control systems. The fed-batch experimental results were close to the model predictions, which proved that the developed model could be used to design fed-batch fermentation. It can also be used as a reference for other feeding strategies of fed-batch fermentation such as the exponential feed.

![Fig. 5. The relation between \( \Omega \) (specific rate of substrate disappearance, h\(^{-1}\)) and \( \mu \) (specific growth rate, h\(^{-1}\)).](image)

![Fig. 6. Time courses of batch and fed-batch culture of *E. coli* for plasmid production.](image)

Before the induction, the specific growth rate was respectively controlled at 0.15 h\(^{-1}\), 0.20 h\(^{-1}\) and 0.25 h\(^{-1}\) by pseudo-exponential feeding during the fed-batch cultivation. After the induction, the specific growth rate was maintained around 0.05 h\(^{-1}\). The final cell density reached 78.5 g L\(^{-1}\), 73.4 g L\(^{-1}\) and 68.8 g L\(^{-1}\) (DCW), and the volumetric concentrations of plasmid were 10.2 g h\(^{-1}\), 10.15 g h\(^{-1}\) and 9.6 g L\(^{-1}\), respectively.

**Quality of pDNA**

The gWizHBs plasmid was prepared from a fresh single colony both at a small analytical scale, from a 3 mL *E. coli* gWizHBs culture, as well as at medium preparative scale in 50 mL culture in LB media supplemented with Kanamycin.
as shown in “Materials and Methods”. The plasmid DNA quality was tested by agarose gel electrophoresis. As shown in Fig. 7, the supercoiled plasmid has a certain shape in the gel as it interacts with more molecules of stain such as ethidium bromide, tending to make it more fluorescent than other DNA forms. Fig. 7 also shows that the majority (over 90%) of the DNA is in the supercoiled form, revealing high quality plasmid DNA preparation.

Fig. 7. Quality control of plasmid preparations in agarose gel; evaluation of the ratio of the gWiz plasmid ccc form versus the other form was done through visual estimation of the fluorescence due to ethidium bromide bound to the plasmid DNA. Here >90% of plasmid gWiz HBs is in the ccc form.

Conclusions
The technique of pseudo-exponential feeding in high-cell-density culture of E. coli for plasmid production was proved to be robust, dynamic, and reliable. The production of wanted plasmid is significantly increased compared to other strategies. Furthermore, the mean experimental data of batch and fed-batch cultivation were used to develop a mathematical model. Model parameters were determined by the Eadic–Hofstee illustration, common induction methods and the non-linear regression technique. The model simulations were compared with the experimental results of batch and fed-batch to evaluate the accuracy of the model developed. It can also be used as a reference for other feeding strategies of fed-batch fermentation.

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REFERENCES