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Short Communication

Plasmid DNA Production for Genetic Immunization in Fed-Batch Fermentation with Feed Back Controls Based on Dissolved Oxygen (DO) and pH

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Abstract

A fermentation process for production of super coiled plasmid DNA in *E. coli* for use as a DNA vaccine was developed. In the process a feed-back control nutrient feeding strategy based on pH and DO was used to regulate the cell growth rate by controlling the interactivity of the nutrient feed rate, pH and DO. The process increased the total yield of plasmid DNA by approximately 4 fold as compared to batch fermentation. The final cell yields of the process reached 56 gl^{-1} dry cell weights (OD_{550} nm 112) within 23 h. A plasmid DNA of 5.6 g l^{-1} was obtained by using an alkaline lyses method.

Keywords: Fed-batch fermentation; Feed-back control; Plasmid; DNA Vaccine

Introduction

DNA vaccines are based on the use of bacterial plasmids to deliver and express immunogenes in vaccinated hosts. Vaccine plasmids are amplified in bacteria, purified and injected into the host being vaccinated to elicit cellular and humoral immunity [1-5].

The development of DNA vaccines and therapeutics using DNA injection technology need a consistent supply of plasmid DNA [6]. For production of large quantities of plasmid DNA an efficient fermentation process needs to be established. Most of the knowledge about fermentation involving recombinant *Escherichia coli* has been obtained through studies which optimize the expression of recombinant proteins [7-11]. However the fermentation conditions for optimization of plasmid DNA production in *E. coli* could be fundamentally different and experimental data are limited. In this case only DNA replication is required when plasmid DNA is the final product [12]. RNA transcription and protein translation associated with the gene in the plasmid are generally undesirable during plasmid production in bacterial host cells [13,14]. To selectively amplify the plasmid DNA replication, abundant nucleotide pools and extra energy sources must be made available in a culture environment and other cellular activities should be kept at the minimum. For example, cellular protein synthesis can be inhibited with chloramphenicol or through amino acid limitation [15,16]. 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

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synchronize with cell division [6,17]. Because the plasmid DNA is intracellular; productivity is proportional to the final cell-density and the specific productivity (i.e. the amount of plasmid DNA produced per unit cell mass per time). High Cell-Density Fermentation (HCDF) techniques for culturing *E. coli* has been developed to improve productivity, and fed-batch processes have most often been used to obtain high cell density [19]. However, this technique has its draw backs and growth inhibitory by products, such as acetate is formed. Acetate formation can be reduced or prevented by altering the fermentation medium or optimization of feeding strategies during fed-batch fermentation. In this paper we describe a fermentation process for production of plasmid DNA coding Hepatitis B virus gene in *E. coli* that uses an automated/manually feed-back control nutrient feeding strategy based on pH and DO.

Materials and Methods

Cultures and Media

E. coli strain DH5α carrying plasmid pRC-CMVs which is about 5.6 kb long with a ColE1 origin was used. The plasmid contains the core gene of Hepatitis B virus under the control of a human cytomegalovirus (CMV) promoter, and also encodes an ampicillin resistance gene for selection [2]. The organism was cultured in the seed medium in a shake flask at 37°C for 12 h. The seed and fermentation medium which were optimized using Taguchi statistical method program (H2 media) consisted of (gL^{-1}): Na_2HPO_4, 12.8; KH_2PO_4, 3; NaCl, 0.5; NH_4CL, 1; $MgSO_4.7H_2O$, 0.24; peptone, 25; yeast extract, 7.5; glycerol, 30 and 1 ml trace elements (consisted of (g) $FeSO_4.7H_2O$, 10; $CaCl_2.2H_2O$, 2; $ZnSO_4.7H_2O$, 2.2; $MnSO_4.7H_2O$, 1; $CuSO_4.5H_2O$, 1; $(NH_4)MO_7O_{24}.4H_2O$, 0.1; $Na_2B_4O_7.10H_2O$, 0.02 dissolved in 1 l 5 M HCl). pH of the medium was adjusted to 7. $MgSO_4.7H_2O$ was sterilized separately and added into the fermentation medium following sterilization.

Batch Fermentation

Seed cultures for fermentation were prepared by inoculating an isolated colony in 100 ml of H2 medium containing (100 µg/ml) ampicillin. The flasks were incubated overnight at 37°C and 200 rpm. Lab-scale fermentation was carried out in a 3.3 l (2.5 l working volume) fermenter (BiofloIII New Brunswick Scientific). During the fermentation, pH was controlled at 7.0 with NH_4OH and HCl and temperature at 37°C. Agitation speed (500-900 rpm) was automatically feed-back controlled based on DO at a set point of 30% of air saturation. Trace element and salt solution were sterilized separately and added into the fermenter before inoculation with 5% (v/v) of the seed culture.

Fed-Batch Fermentation

Fed-batch fermentation was carried out in a 5 litre fermenter (Bioflo III New Brunswick Scientific). The fermentation process was initiated similar to batch fermentation. During the fermentation, pH was controlled at 7.0 by adding 20% NH_4OH and HCl. Agitation speed was automatically controlled based on DO at a set point of 30% of air saturation. A concentrated nutrient (500 g/l glycerol, 250 g/l yeast extract, 15 g/l $MgSO_4$.7H2O and 1 ml/l trace element solution), was fed into the fermenter after 10 h via an acid pump, which could be triggered by the increase of pH above respective set point (pH 7) and a nutrient pump that was controlled manually when the DO increased above 40%. Samples of 10 ml were removed from the fermenter hourly during fermentation. Cell growth was measured by determining optical density at 550 nm using a spectrophotometer (Beckman). To obtain the cell dry weight (CDW), a sample of 10 ml of fermentation broth was collected and the cells separated by filtration on a 0.45 µM filter. The filter containing the cell was dried in an oven 110°C until constant weight. The culture supernatant was used for analysis of glycerol concentration (Bohringer Mannhein kit). Diluted sample was also plated on LB agar with and without ampicillin to determine plasmid stability.

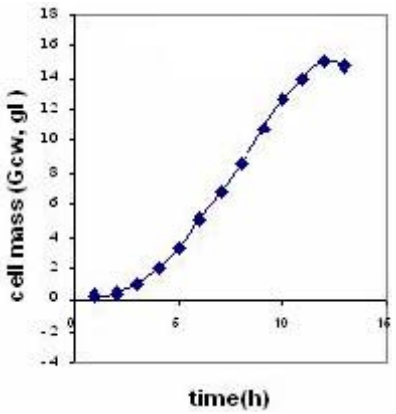

Figure 1. Growth curve of *E. coli* DH5α-pCMVs in batch method fermentation.

Plasmid DNA Analysis

For the preparation of analytical grade plasmid DNA, *E. coli* cells from 1ml broth were pelleted by centrifugation at 5000 × g for 10 min. Cells were lysed using the alkaline lysis method and the plasmid DNA was purified according to protocol (Qiagen kit). After purification, isolated plasmid DNA was electrophoresed in 1% agarose gel. The absorbance of DNA sample was measured in 260 and 280 nm. The endotoxin content of purified DNA was assessed to be free of endotoxin contamination using a Limulus Amoebocyte Lysate (LAL)-based methods (PYROGEN Gel Clot endotoxin Assay Kit) according to the manufacturer's instructions.

Result and Discussion

Batch Fermentation

Production of plasmid DNA from a recombinant *E. coli* harboring pCMVs was evaluated in batch fermentation using glycerol as carbon source. Figure 1 shows cell growth curve of the recombinant *E. coli* (pRC-CMV$_s$) in the batch process. Cell growth entered log phase 4 hrs after inoculation. A nearly constant specific growth rate (μ) of 0.5 h^{-1} was obtained under these operation conditions until t= 11h. The glycerol concentration gradually decreased and carbon source became limiting after 11 hrs and the growth rate began to fall. The cell growth at a maximum rate of 0.5 h^{-1} was apparently deleterious in the late stages of fermentation by DO and carbon source limitation. Under these conditions a cell dry weight of 15 g/l (OD = 30) was achieved with a yield of plasmid DNA of 1.3 g/l. This result indicates that cell growth was rapid, leading to a rapid stationary phase, resulting in both low cell density and yield of plasmid. In this condition growth was faster than the plasmid replication. 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} [2,12]. Therefore it has been decided to design an automated feed-back control system based on pH and DO that would meet the varied requirements associated with changing cellular activities.

Table 1. Fermentation result in using batch, fed-batch, pH-stat, fed-batch pH-DO-stat

Process	Growth rate (h^{-1})	Cell mass (gl^{-1})	Plasmid yield (gl^{-1})
Batch method	0.5	15-17	1.3
Fed-batch, pH-stat	0.5-0.7	31-32	1.25
Fed-batch, pH-DO-stat	0.1-0.5	56	5.6

Fed-Batch Fermentation

A fed-batch fermentation process with feed-back control based on pH was developed. Results showed that the cell growth entered log phase soon after inoculation (μ=μmax). Nutrient feeding was automated through feed-back control of changing the value of pH during the fermentation. In this feed-back control the pH parameter was chosen based on the fact that, when the carbon source becomes limiting in a culture, cellular activity slows down and pH rises as a result of consumption of metabolic fatty acids by cells as an alternative carbon source and the production of ammonium ions associated with protein catabolism. Acid pump which was coupled with pH controller was used for nutrient feeding. The suitable set point of pH for nutrient feed was determined as 7. The nutrient feed was automatically co-regulated with pH and the acid pump was activated each time pH rose above set point and deactivated when pH dropped below the set point. Cell growth rate initiated at μ=0.5 h^{-1} in the end of the batch mode and during the feeding was 0.5-0.7 h^{-1}. Cell density reached 32 gL^{-1} CDW (OD_{550}nm=64) within 23 h. More than 2-fold increase in cell density and yield of 1.25 gl^{-1} plasmid DNA was obtained compared to the batch mode, (Table 1). In pH-stat fermentation the yield of plasmid DNA was not higher than the batch mode due to instability of the plasmid which may be related to the high growth rate (0.5-0.7 h^{-1}).

Chen *et al.* 1997 indicated that the yield of plasmid DNA is related to the growth rate of bacteria during the fermentation. The lower growth rate was resulted to the higher plasmid yield, in addition Reininkainen *et al.* 1989 found that plasmid yields obtained from bacterial culture were highest when growth went into stationary phase. During stationary phase cellular metabolic activities including RNA transcription and protein translation are usually minimum whereas, plasmid replication rate might increase. So we developed a feed-back control, fed-batch fermentation process based on pH and DO to improve production of supercoiled plasmid DNA, which should increase the cell density and productivity of the process. In this method cell density reached 56 gl^{-1} CDW (OD_{550}nm=112) within 22 h (Fig. 2).

The yield of plasmid increased as specific growth rate decreased (0.1 h^{-1}). When the rate of plasmid replication is insufficient to keep race with host division rate, plasmid copies are partitioned unequally between daughter cells, resulting in plasmid-free segregates, the so-called segregation instability of plasmid [18]. Therefore, slower growth rate should be more favorable for plasmid to partition properly and to replicate more

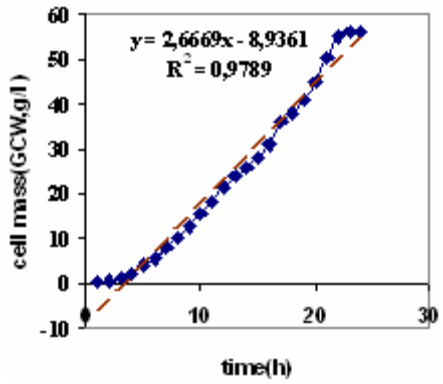

Figure 2. Growth curve of *E. coli*-DH5-α carrying DNA Vaccine plasmid pCMVs in fed-batch cultivation by pH- and DO-stat control.

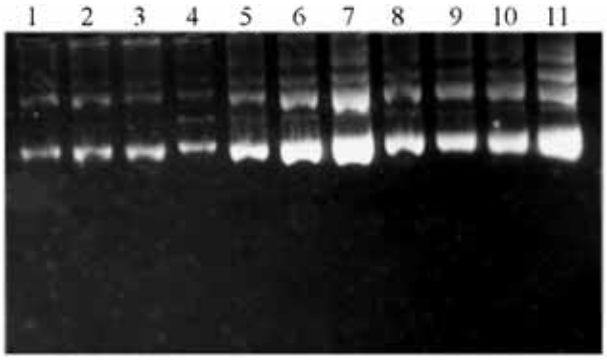

Figure 3. Agarose gel analysis of the plasmid DNA. Lanes 1-11 sample from different hours of fermentation. Plasmid DNA was purified from 1 ml fermentation broth using an alkaline lysis method and 2µl of DNA was loaded on the 1% agarose gel.

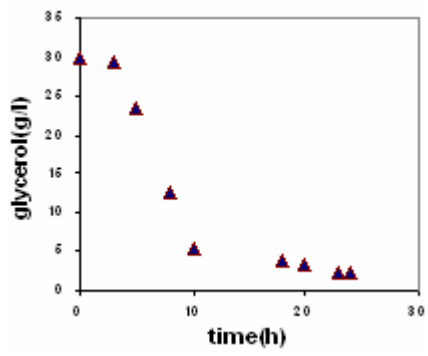

Figure 4. Profiles of glycerol concentration during the pH and DO-based feed-back control fermentation of in *E. coli* DH5α harboring DNA vaccine plasmid pCMVs.

efficiently because cellular metabolic activity is generally low at slower growth rate, leaving more nutrients and energy available for plasmid replication. The growth rate of 0.1 h^{-1} was favorite for maximize yields of pCMVs. DNA plasmid was extracted and analyzed by agarose gel electrophoresis. The concentration of DNA was 5.6 gl^{-1} (100 mg g^{-1} cell weight as measured by spectrophotometer with high ratio of the supercoiled DNA). The DNA was endotoxin free as analyzed by LAL test which could be used in DNA vaccine development programs (Fig. 3).

We have developed a feed-back control, fed-batch fermentation process based on pH and DO to improve production of pure and endotoxin free supercoiled plasmid DNA up to 5-fold (Table 1) when compared with the batch mode. The improved plasmid DNA yield in this process might be a result of the increased cell density, decreased growth rate (µ) from 0.5 h^{-1} to 0.1 h^{-1} and density glycerol as carbon source (Fig. 4). The productivity of this process is much higher than the previous works [19,20] which may be suitable for large scale production of DNA vaccine. Automatic feed-back control, fed-batch fermentation based on DO and pH should increase the cell density and productivity of the process further that will be carried out in the future work.

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