Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

Effects of medium composition on the production of plasmid DNA vector potentially for human gene therapy

XU Zhi-nan (徐志南)†, SHEN Wen-he (沈文和), CHEN Hao (陈 灏), CEN Pei-lin (岑沛霖)

(*Institute of Bioengineering, School of Material Science and Chemical Engineering, Zhejiang University, Hangzhou 310027, China*)

†E-mail:znxu@zju.edu.cn

Received Feb. 10, 2004; revision accepted July 8, 2004

Abstract: Plasmid vector is increasingly applied to gene therapy or gene vaccine. The production of plasmid pCMV-AP3 for cancer gene therapy was conducted in a modified MBL medium using a recombinant *E. coli* BL21 system. The effects of different MMBL components on plasmid yield, cell mass and specific plasmid DNA productivity were evaluated on shake-flask scale. The results showed that glucose was the optimal carbon source. High plasmid yield (58.3 mg/L) was obtained when 5.0 g/L glucose was added to MMBL. Glycerol could be chosen as a complementary carbon source because of the highest specific plasmid productivity (37.9 mg DNA/g DCW). After tests of different levels of nitrogen source and inorganic phosphate, a modified MMBL medium was formulated for optimal plasmid production. Further study showed that the initial acetate addition (less than 4.0 g/L) in MMBL improved plasmid production significantly, although it inhibited cell growth. The results will be useful for large-scale plasmid production using recombinant *E. coli* system.

Key words: Plasmid DNA, Growth medium, Gene therapy
doi:10.1631/jzus.2005.B0396 **Document code:** A **CLC number:** Q782

INTRODUCTION

The difficulties associated with large-scale production of biotherapeutics provide a constant challenge to the biotechnology industry. FDA had added "therapeutic DNA plasmid vectors" to the list of well-characterized biotechnology product (DoHHs, 1996), and gene therapy has moved rapidly from laboratory scale to clinical trials. It is urgent to develop new protocols to obtain high-quality plasmids with high yields and minimal or no contamination of RNA and chromosomal DNA. Although the processes of production and purification for many gene-therapy vectors have been developed in pharmaceutical companies, the information on large-scale plasmid production is scarce and usually not available to the scientific community (Durland and Eastman, 1998).

After therapeutic DNA plasmid vectors are constructed and transferred into the host cell, the process development for producing plasmid DNA generally starts with the optimization of medium composition and culture conditions. In the past twenty years, many efficient strategies for recombinant-cell fermentation had been developed to attain high productivity of proteins in *E. coli*, *B. subtillis*, yeast, even in animal cell culture systems (SivaKesava *et al*., 1999; Ebisu *et al*., 1992; Cheng *et al*., 1997; Churgay *et al*., 1997). However, few papers were focused on the plasmid DNA production in recombinant *E. coli* fermentation process (Lahijani *et al*., 1996; O'Kennedy *et al*., 2000). It was reported that the media composition affected cell growth rate, and thus influenced plasmid copy number (Kleinman *et al*., 1986; Kim and Shuler, 1990). Therefore, the effects of medium components on cell growth and plasmid productivity should be evaluated to solve the trade-off between higher copy number and reduced growth rate. Acetate accumulation is also a major concern for medium design and high density fermentation of

recombinant *E. coli* because it inhibits cell growth and protein expression (Sun *et al*., 1993; Naka *et al*., 1997). However, according to our literature survey, the effect of acetate on plasmid DNA production has never been investigated.

A recently constructed plasmid (pCMV-AP3) for cancer gene-therapy (Xu *et al*., 2002) contains apoptin gene and operative elements in both prokaryotic and eukaryotic cells, and can duplicate automatically in *E. coli*. In this work, the medium composition and cultivation conditions were optimized for the production of plasmid pCMV-AP in a recombinant *E. coli* BL21 system.

MATERIALS AND METHODS

Strain and plasmid

Escherichia coli BL21 (DE3) was stored in our laboratory and selected as host cell. The plasmid pCMV-AP3 used in this study contained an origin of replication in *E. coli*, an ampicillin resistance gene, an apoptin gene and a eukaryotic transcription unit for the expression of apoptin to kill cancer cells selectively. The plasmids were transformed into host cells by standard procedure (Xu *et al*., 2000).

Culture media

The basic LB medium for seed cultivation contained (g/L): tryptone, 10; yeast extract, 5.0; NaCl, 10.0. The basic composition of MMBL (modified MBL) medium was (g/L): glucose, (variable); tryptone, 10.0; yeast extract, 10.0; NaCl, 10.0; glycerol, 2.0; K_2HPO_4, 0.5; KH_2PO_4, 2.5; $(NH_4)_2HPO_4$, 2.0; $MgSO_4$, 1.0; trace metal solution, 1.0 ml/L; ampicillin, 70 mg/L (when required) (SivaKesava *et al*., 1999). The trace metal solution was prepared as follows (g/L): $FeCl_3{\cdot}6H_2O$, 0.162; $ZnCl_2{\cdot}4H_2O$, 0.0144; $CoCl_2{\cdot}6H_2O$, 0.12; $Na_2MoO_4{\cdot}2H_2O$, 0.012; $CaCl_2{\cdot}2H_2O$, 0.006; $CuSO_4{\cdot}5H_2O$, 1.9; H_3BO_3, 0.5; HCl (37%, w/v), 37 ml/L. Plackett-Burman experiments were carried out to find the key factors which influence the plasmid productivity.

Shake flask cultivation

Seed culture was prepared by inoculating a single colony from a freshly spread transformation plate into 30 ml LB media supplemented with 70 μg/ml ampicilin. The inocula were grown at 36 °C for about 5 h on a rotary shaker at 200 rpm. All the flask cultures of 30 ml MMBL medium were inoculated (10%, v/v) (in 250 ml flasks) and shaken at 36 °C at 200 rpm in an orbital shaker for 15 h unless otherwise stated.

Determination of cell density and dry cell weight

Cell density (OD_{550}) was measured spectrophotometrically. For dry cell weight assay, aliquots (10 ml) of fermentation culture were centrifuged at 10000 rpm for 10 min in pre-weighed glass tube. The supernatant was decanted and cells were resuspended in an equal volume of sterile pure H_2O and centrifuged again in the same condition. The supernatant was decanted and the cell pellets were dried to a constant weight at 105 °C.

Plasmid DNA assay

Plasmid DNA was prepared according to the standard procedure (O'Kennedy *et al*., 2000). Absorbance of DNA sample at 260 nm and 280 nm was determined with an HP spectrophotometer. Concentration of plasmid DNA was calculated from A_{260} (an A_{260} of 1 corresponds to a 50 μg/ml double stranded DNA solution). The purity of samples was checked by the ratio of absorbance at 260 nm and 280 nm.

RESULTS AND DISCUSSION

Effects of different carbon sources on plasmid production

The effects of different carbon sources in MMBL media on plasmid production are shown in Fig.1. The results showed that the highest cell mass (DCW 5.38 g/L) and plasmid content (58.3 mg/L) were obtained after 13 h cultivation when glucose was chosen as the carbon source in MMBL medium, whereas both cell mass and plasmid productivity were reduced when lactose and sucrose were used. Although use of maltose as carbon source brought about comparable plasmid content and modest cell growth, it is more expensive than glucose. Glycerol resulted in highest specific plasmid productivity (37.9 mg DNA/g DCW), but poor cell growth, and delayed the peak time for plasmid yield. So, glucose was the optimal carbon source, and its proper initial concentration should be further evaluated. Fig.2 shows that the

highest plasmid content and cell mass were obtained when initial glucose concentration was 5.0 g/L. It was obvious that 5 g/L glucose supported high growth rate and high plasmid yield, although the specific plasmid productivity was relatively low.

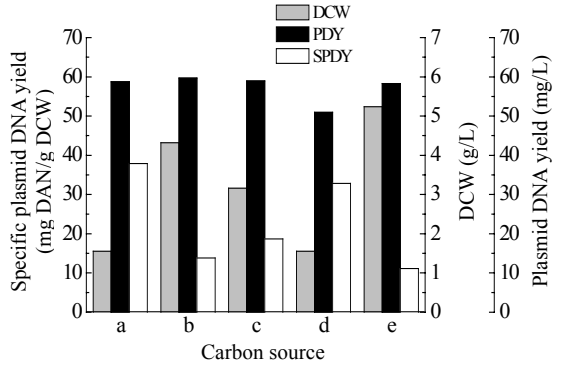

Fig.1 Effects of carbon source on plasmid production and cell growth

Different carbon source was respectively chosen in MBL media with glycerol (a), maltose (b), lactose (c), sucrose (d) and glucose (e)

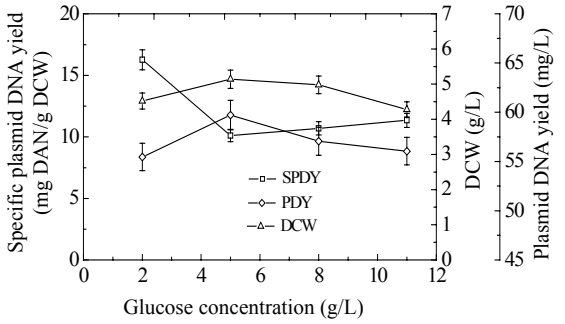

Fig.2 Effects of initial glucose concentration on plasmid production and cell growth

Values are the average of three independent determinations, and all the standard deviations are less than 5%

It was reported that glycerol is a suitable carbon source for recombinant protein production (Li and Taylor, 1994). The application of glycerol avoids repression of intermediate metabolites and accumulation of inhibitive organic acids to some extent. Therefore the effects of glycerol addition in accompany with glucose on plasmid DNA production in *E. coli* were also examined. The plasmid content and specific plasmid DNA productivity was improved by 70.6% or 67.9% respectively, when 4.0 g/L glycerol was supplemented into the medium (Table 1). Further increase of glycerol concentration would decrease plasmid yield and delay cell growth significantly.

Table 1 Effects of combined glycerol addition on plasmid production and cell growth*

Glycerol (g/L)	Relative DCW (%)	Relative PDY (%)	Relative SPDY (%)
0.0	100	100	100
2.0	126.3	96.5	76.4
4.0	101.6	170.6	167.9
6.0	113.5	100.0	88.0
8.0	125.0	38.1	30.4

*Besides 5 g/L glucose in MMBL, different levels of glycerol was respectively supplemented into the medium. All the glycerol -containing cultures were harvested at 18 h, and no further increases in plasmid yield were achieved beyond the time. Values were the average of three independent determinations. PDY: plasmid DNA yield; SPDY: specific plasmid DNA yield

Effects of nitrogen sources on plasmid production

The selection of the nitrogen source and the determination of its concentration are critical to the optimization of plasmid production in recombinant cell fermentation. In our preliminary Placett-Burman tests, two different types of nitrogen sources, yeast extract (YE) and casamino acid (CAA), were identified to be the key factors which affected plasmid productivity. The results (Fig.3) showed that the highest plasmid content and cell mass were achieved with the supplementation of 15 g/L YE to MMBL. The suitable initial CAA concentration was 20 g/L with plasmid productivity of 30.5 mg/L (Fig.4). However, compared with the peak plasmid yield (60.8 mg/L) obtained with YE supplementation, CAA was a less effective nitrogen source and abounded in the future culture experiments.

Effects of inorganic phosphate on plasmid production

Inorganic phosphates have a pH buffering capa-

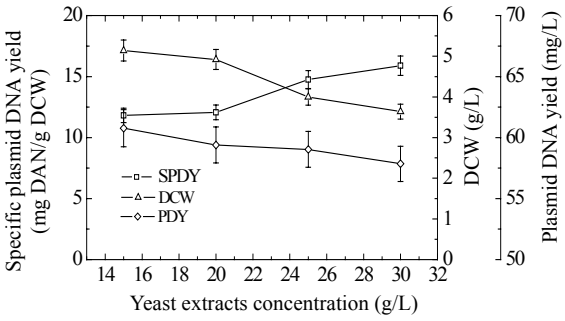

Fig.3 Effects of initial yeast extract concentration on plasmid production and cell growth

Values are the average of three independent determinations, and all the standard deviations are less than 5%

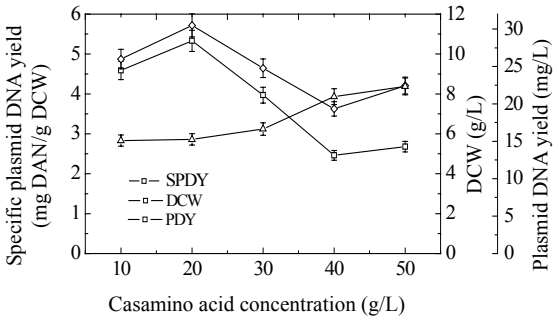

Fig.4 Effects of initial casamino acid concentration on plasmid production and cell growth

Values are the average of three independent determinations, and all the standard deviations are less than 7%

bility in the medium and provide some primary materials for DNA's biosyntheses. The effects of different levels of inorganic phosphate salts were tested when the initial pH of MMBL was adjusted to 6.8. The results (Fig.5) showed that cell mass was increased when the concentration of inorganic phosphate salts was increased from 1.0 g/L to 2.0 g/L, whereas the specific plasmid productivity was decreased steeply. With further increase of phosphate salts, only minor change in plasmid productivity and cell mass was observed. From the viewpoint of development of high density cell culture, 2.0 g/L inorganic phosphate in MMBL was essential.

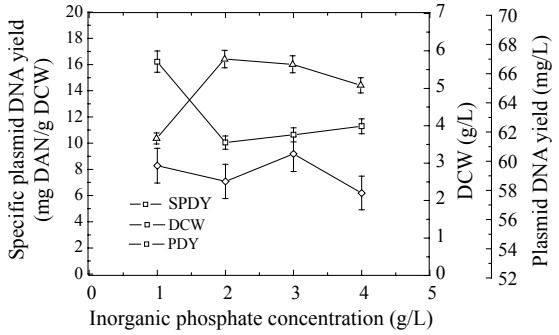

Fig.5 Effects of inorganic phosphate concentration on plasmid production and cell growth

Values are the average of three independent determinations, and all the standard deviations are less than 5%

Effects of acetate on plasmid production

It is well known that acetate inhibits cell growth and represses recombinant protein expression. Many culture strategies were developed to delineate acetate accumulation in high-density recombinant cell fermentation (Konstantinov *et al*., 1990; Robbins and Taylor, 1989). In order to efficiently produce plasmid DNA in large-scale, an optimal strategy of high density cultivation may be employed with a variety of fed-batch protocols. Therefore, it is imperative to investigate the effects of acetate on cell growth and plasmid production in recombinant *E. coli* system. The effects of acetate were investigated in our recombinant *E. coli* system by supplementing acetate to the MMBL containing 3.0 g/L glucose. The acetate produced by 3.0 g/L glucose in the MMBL was low and negligible in the experiment. The result (Fig.6) showed that cell growth was heavily inhibited with acetate addition, but the plasmid production was enhanced if less than 3.0 g/L acetate was added, and up to 15.1% improvement of plasmid yield was achieved with 2.0 g/L acetate supplementation in the MMBL. It was also clear that specific plasmid DNA yield (SPDY) was increased significantly with acetate addition in the medium, and that the highest SPDY (9.0 mg DNA/g DCW) was achieved when 3.0 g/L acetate was supplemented. It suggested that, targeting at plasmid DNA production, without induction of plasmid transcription, the inhibition of cell growth by acetate might increase plasmid copy number. Therefore, contrary to the inhibition of acetate on the expression of recombinant proteins, acetate acid might not be a crucial bottleneck for high density cell cultivation targeting at plasmid DNA production with a recombinant *E. coli* system.

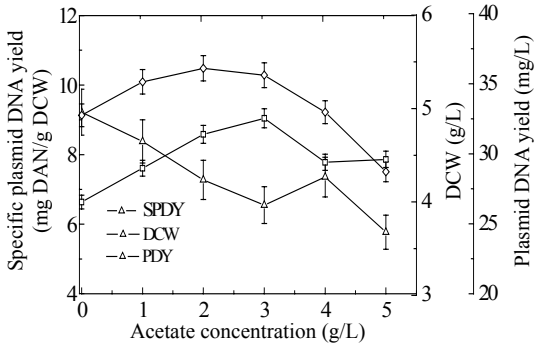

Fig.6 Effects of initial acetate addition on plasmid production and cell growth

Besides 3 g/L glucose in MMBL, different levels of acetate were respectively supplemented into the medium. All the acetate-containing cultures were harvested at 18 h, and no further increases in plasmid yield were achieved beyond the time. Values are the average of three independent determinations, and all the standard deviations are less than 7%

CONCLUSION

Among five different carbon sources, glucose was preferred for the production of plasmid pCMV-AP in *E. coli*. High plasmid yield (58.3 mg/L) was achieved on shake flask scale when 5.0 g/L glucose was added to MMBL. Glycerol could be supplemented to increase plasmid yield up to 70.6% as a complementary carbon source because of its high specific plasmid DNA productivity. Compared with casamino acid, yeast extract was a more effective nitrogen source to support high plasmid yield, and 15.0 g/L yeast extract was optimal supplement in MMBL. The plasmid DNA yield was not influenced significantly by inorganic phosphate, but the addition of 2.0 g/L inorganic phosphate salts was essential for maintaining cell growth. It is well known that acetate accumulation inhibits cell growth and protein expression in recombinant *E. coli* system. On the contrary, acetate addition of less than 3.0 g/L improved plasmid DNA yield obviously in our recombinant *E. coli* BL21 system. It suggested that low growth rate led to high specific plasmid DNA yield, then improved plasmid DNA yield if the cell growth was not inhibited seriously with acetate.

References

Cheng, C., Huang, Y.L., Yang, S.T., 1997. A novel feeding strategy for enhanced plasmid stability and protein production in recombinant yeast fed-batch fermentation. *Biotechnol. Bioeng.*, **56**:23-31.

Churgay, L.M., Kovacevic, S., Tinsley, F.C., Kussow, C.M., Millican, R.L., Miller, J.R., 1997. Purification and characterization of secrected human leptin in baculovirus-infected insect cells. *Gene*, **190**:131-137.

DoHHs, S., 1996. Elimination of establishment license application for specified biotechnology and specified synthetic biological products. *Notice Fed. Reg.*, **61**:24227-24233.

Durland, R.H., Eastman, E.M., 1998. Manufacturing and quality control of plasmid-based gene expression systems. *Advanced Drug Delivery Reviews*, **30**:33-48.

Ebisu, S., Takagi, H., Kadowaki, K., Yamagata, H., Udka, S., 1992. Production of human epidermal growth factor by *Bacillus brevis* increased with use of a stable plasmid from *B. Brevis* 481. *Biosci. Biotechnol. Biochem.*, **56**: 812-813.

Kim, B.G., Shuler, M.L., 1990. Analysis of pBR322 replication kinetics and its dependency on growth rate. *Biotechnol. Bioeng.*, **36**:233-242.

Kleinman, M.J., Gingold, E.B., Stanbury, P.E., 1986. The stability of the yeast plasmid pJDB248 depends on the growth rate of the culture. *Biotechnol. Lett.*, **8**:225-230.

Konstantinov, K., Kishimoto, M., Seki, T., Yoshida, T., 1990. A balanced Do-stat and application to the control of acetic acid excrection by recombinant *E. coli*. *Biotechnol. Bioeng.*, **36**:750-758.

Lahijani, R., Hulley, G., Soriano, G., Horn, N.A., Marquet, M., 1996. High-yield production of pBR322-derived plasmids intended for human gene therapy by employing a temperure-controllable point mutation. *Hum. Gene Ther.*, **7**:1971-1980.

Li, X., Taylor, K.B., 1994. Effect of glucose on expression parameters of recombinant protein in *E. coli* during batch growth in complex medium. *Biotechnol. Prog.*, **10**:160-164.

Naka, K., Risxhke, M., Sato, S., Markl, H., 1997. Influence of acetic acid on the growth of *Escherichia coli* K12 during high-cell-density cultivation in a dialysis reactor. *Appl. Microbiol. Biotechnol.*, **48**:597-601.

O'Kennedy, R.D., Baldwin, C., Keshavarz-Mmoore, E., 2000. Effects of growth medium selection on plasmid DNA production and initial processing steps. *J Biotechnol.*, **76**:175-183.

Robbins, J.W., Taylor, K.B., 1989. Optimization of *Escherichia-coli* growth by controlled addition of glucose. *Biotechnol. Bioeng.*, **34**:1289-1294.

SivaKesava, S., Xu, Z.N., Chen, Y., Hackett, J., Huang, R., Lam, E., Lam, T., Siu, K., Wong, R., Wong, W., 1999. Production of excreted human epidermal growth factor (hEGF) by an efficient recombinant *Escherichia coli* system. *Proc. Biochem.*, **34**(9):893-900.

Sun, W., Lee, C., George, H.A., 1993. Acetate inhibition on growth of recombinant *E. coli* and expression of fusion protein. *Biotechnol. Lett.*, **15**:809-814.

Xu, Z., Liu, G., Cen, P., Wong, W.K.R., 2000. Factors influencing secretive production of human epidermal growth factor (hEGF) with recombinant *E. coli* K12. *Bioproc. Eng.*, **23**:669-674.

Xu, Z.N., Chen, H., Tian, J., Cen, P.L., 2002. Studies on Cultivation Conditions for Efficient Production of Gene-therapy Vectors in *E. coli*. Proceeding of the Tenth NCBE, Hangzhou, China, p.43-48.