Korean J. Chem. Eng., **25**(5), 1110-1114 (2008)

A fermentation strategy for production of recombinant protein subjected to plasmid instability

Shu-Jen Chen*,†, Bo-Shun Ke, and I-Chung Chiu****

*Department of Chemical and Materials Engineering, National Kaohsiung University of Applied Sciences, No. 415, Chien Kung Road, Kaohsiung 80778, Taiwan
**Department of Chemical Engineering, National Cheng Kung University, No.1, University Road, Tainan 70101, Taiwan
(*Received 25 December 2007 • accepted 27 February 2008*)

Abstract−The appearance of plasmid-losing cells in a recombinant *Escherichia coli* culture was observed when the cell mass became doubled after induction, which corresponded to the timing of cell fission. Accordingly, a two-stage fermentation strategy capable of maintaining plasmid stability without selective pressure in a recombinant *E. coli* culture was proposed. In the first stage (cell growth stage), a high cell density culture was obtained by incubating the cells in the R medium. In the second stage (producing stage), the cells were devoted to producing the recombinant protein by introducing the fresh LB medium supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG). It was necessary to prevent the doubling in the cell mass after induction; otherwise cell fission would occur and generate plasmid-losing cells. The present strategy is expected to be extensively applicable in recombinant *E. coli* cultures.

Key words: *Escherichia coli*, Induction, Plasmid Stability, Recombinant, Two-Stage Culture

INTRODUCTION

The segregational instability of plasmids is often experienced in the production of foreign proteins by a recombinant *Escherichia coli* culture. It is well known that plasmid replication and cell fission are not synchronous. The uneven distribution of plasmids during cell fission may result in the formation of plasmid-free daughter cells. Once the segregant cells are born, a competitive instability may appear owing to the growth advantage of plasmid-losing cells over plasmid-carrying cells, since the expression of plasmid-encoded genes imposes an extra metabolic burden on the host cell [1-4]. Consequently, in the absence of selective pressure, the plasmid-losing cells may quickly overwhelm the cell population.

Many methods for maintaining the stability of plasmids in a recombinant culture have been suggested. Typically, the introduction of the selective pressure provided by antibiotics is recognized as being capable of maintaining plasmid stability, although, in fact, the emergence of plasmid-losing cells is still inevitable, since the selective pressure may be relieved by antibiotics-degrading enzymes [5]. Additionally, the plasmid-carrying cells with low plasmid copy number might be damaged severely by antibiotics [6]. Cost considerations also make this strategy unfavorable for large-scale operations. Another selective pressure method is to cultivate an auxotrophic host that requires the plasmid to synthesize essential amino acids in an incomplete selective medium. However, the plasmid-losing cells can still survive, because they can ingest the particular amino acid produced by plasmid-carrying cells [7]. In addition, several engineering approaches have also been proposed for preventing plasmid instability, including cell immobilization [8], cycling the dilution rate for delaying the washout of plasmid-carrying cells in a chemostat culture [9,10], and selective recycling of plasmid-carrying cells [11]. The methods above involve complicated operations, and may not be directly applicable to industrial processes.

Since the plasmid-losing cells appear after cell division, it is suggested that the maintenance of plasmid stability in a recombinant culture may be achieved by preventing the occurrence of cell fission. Accordingly, a two-stage fermentation system without the appearance of plasmid-losing cells is proposed as follows. First, the cells are grown in a growth medium to increase cell density; then, the culture is supplemented with the fresh medium accompanied by an inducer to induce the production of recombinant protein. The occurrence of newly generated cells after induction can be decreased by avoiding the doubling in cell mass. To verify our hypothesis, the recombinant strain *Escherichia coli* JM109/pSCR05 producing creatinase was used as a model system. In this work, the timing of the appearance of plasmid instability during fermentation is first examined, and then the effectiveness of our strategy will be discussed.

MATERIALS AND METHODS

1. Microorganism and Culture Conditions

The creatinase producing strain *Escherichia coli* JM109/pSCR05 was provided by Professor Ming-Chung Chang of the Department of Biochemistry, National Cheng Kung University, Taiwan. The plasmid pSCR05 encoding the creatinase of *Pseudomonas putida* under control of the *lac* promoter was constructed by inserting the creatinase gene into the expression vector pQE-51 (QIAGEN). In addition, the recombinant creatinase possesses a signal sequence of chitinase from *Aeromonas hydrophila* [12]. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), creatinase is secreted to the periplasm.

The recombinant *E. coli* cells were maintained at −20 °C in 50% (v/v) glycerol. Prior to fermentation, the preculture was carried out

†To whom correspondence should be addressed.
E-mail: biochen@cc.kuas.edu.tw
‡This work was presented at 13*th* YABEC symposium held at Seoul, Korea, October 20-22, 2007.

Table 1. Composition of R medium

Component	Concentration
Glucose (g/L)	27.5
KH_2PO_4 (g/L)	13.3
$(NH_4)_2HPO_4$ (g/L)	4.0
Citric acid (g/L)	1.7
$MgSO_4 \cdot 7H_2O$ (g/L)	1.2
Thiamin HCl (mg/L)	4.5
Trace metal solution (mL/L)*	10

*The trace metal solution contained (per liter): 6 g of Fe(III) citrate, 1.5 g of $MnCl_2 \cdot 4H_2O$, 0.8 g of $Zn(CH_3COO)_2 \cdot 2H_2O$, 0.3 g of H_3BO_3, 0.25 g of $Na_2MoO_4 \cdot 2H_2O$, 0.25 g of $CoCL_2 \cdot 6H_2O$, 0.5 g of $CuCl_2 \cdot 2H_2O$, and 0.84 g of ethylene-dinitrilo-tetraacetic acid-Na_2-salt$\cdot 2H_2O$.

in a 500-ml Erlenmeyer flask with a working volume of 100 mL. The preculture medium containing (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 50 mg ampicillin was adjusted to pH 7.0 with NaOH. After overnight incubation in a reciprocal shaker (120 rpm) at 37 °C, the preculture broth was inoculated to the fermenter.

The two-stage fermentations were performed in a 2.5 L fermenter (Model M-100, Tokyo Rikakikai, Japan), with a starting working volume of 1,000 ml. The medium used in the first-stage culture was R medium [13] whose composition is shown in Table 1. After the cell growth entered the stationary phase, the second stage culture was carried out by adding 400 mL of the fresh LB medium supplemented with IPTG to the fermenter for inducing the expression of the creatinase gene. All fermentations were conducted at 37 °C and pH 7.0 unless stated otherwise. The dissolved oxygen was kept above 40% by changing aeration rate and agitation speed throughout the fermentation process. If the second stage was performed with a fed-batch operation, IPTG with a desired concentration was first introduced to the broth. The fresh LB medium containing the desired concentration of IPTG was then fed into the fermenter. The feeding rate was 2 mL/min.

2. Analytical Methods

Cell concentration was measured by the absorbance of the culture broth at 600 nm with a spectrophotometer (model UV-1201, Shimadzu, Japan), correlated with dry cell mass.

The cell pellet was suspended in RO water and 5 ml of aliquots were disrupted in an ice bath by using a Vibra-Cell ultrasonic processor (Sonics, VC 130), with 1-s pulse and 1-s pause for a total period of 10 min. After sonication, the sample was centrifuged at 15,000 *g* for 15 min and the supernatant was analyzed for creatinase activity. The analysis of creatinase activity was according to the method proposed by Koyama et al. [14]. One creatinase unit was defined as the amount of enzyme required for releasing 1 μmol of urea per minute at 37 °C and pH 7.7.

The plasmid stability was determined by spreading the culture samples on the LB plates after appropriate dilution and incubated overnight at 37 °C. Then, one hundred colonies were randomly picked, and transferred aseptically onto the LB plates containing 100 mg/L of ampicillin (LB-Ap plates). After overnight incubation at 37 °C, the plasmid-carrying colonies were identified by the colonies formed on the LB-Ap plates. The proportion of plasmid-carrying cells was calculated from the number of colonies growing on LB-Ap plates divided by the number of colonies which had grown on LB plates. The plasmid stability (%) was given by multiplying the values for the proportion of plasmid-carrying cells by 100.

RESULTS AND DISCUSSION

1. Examination of the Timing of Plasmid Segregation

Traditionally, introducing antibiotics to a fermentation broth is the most common strategy for maintaining the plasmid stability in a recombinant culture. However, our preliminary experiments (data not shown) showed that the addition of selective pressure (ampicillin) was not sufficient to ensure the stability of plasmids throughout the fermentation since ampicillin was degraded by the secretion of β-lactamase. In addition, the creatinase yield obtained from the culture with antibiotics was comparable to that obtained from the culture without selective pressure. Furthermore, when considering the cost of antibiotics, the effectiveness of maintaining plasmid stability by the addition of selective pressure is limited. It is thus worth deducing a plasmid-maintaining strategy that operates without selective pressure.

The timing of the decrease in plasmid stability was examined in a 1,000-mL batch culture containing two-fold concentrated LB medium (pH 6.0). The inducer IPTG was introduced at the fermentation time of 2 h, corresponding to a cell concentration of 0.6 g/L. As shown in Fig. 1, after IPTG induction, the stability of plasmid dropped abruptly from 100% to around 50% when the cell mass doubled from 0.6 g to around 1.2 g. A further decrease in plasmid stability from 50% to 25% was found at the second doubling time of cell growth (from ca. 1.2 to 2.4 g). The period for cell mass doubling can be viewed as the time length required for completing a cell life cycle. The stepwise drop in plasmid stability with a percentage of ca. 50% at each doubling time of cell mass implied that the newly generated cells after cell fission were plasmid-free cells. Accordingly, it was thought that the expression of recombinant creatinase posed a metabolic burden on the cells, which resulted in the generation of plasmid-free cells during cell division. Additionally, most of the plasmid-free cells were generated at the doubling time of the cell mass.

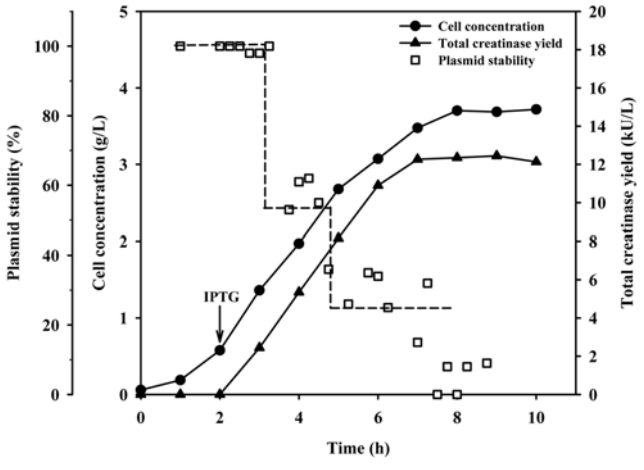

Fig. 1. Time course analysis of the change of plasmid stability. The cells were cultivated in the two-fold concentrated LB medium (pH 6.0). IPTG with a final concentration of 0.5 mM was added at the fermentation time of 2 h.

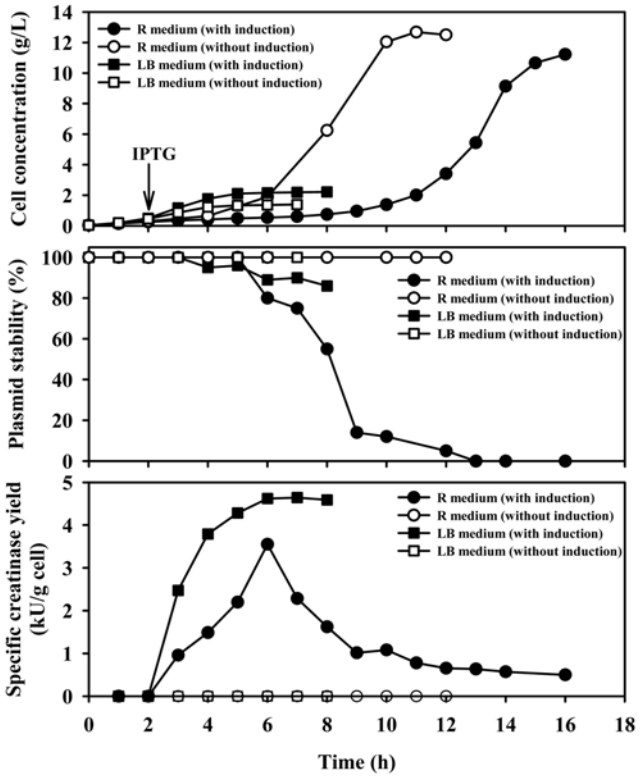

Fig. 2. Cell growth and creatinase production in LB and R medium with or without IPTG. IPTG with a final concentration of 0.5 mM was added at the fermentation time of 2 h.

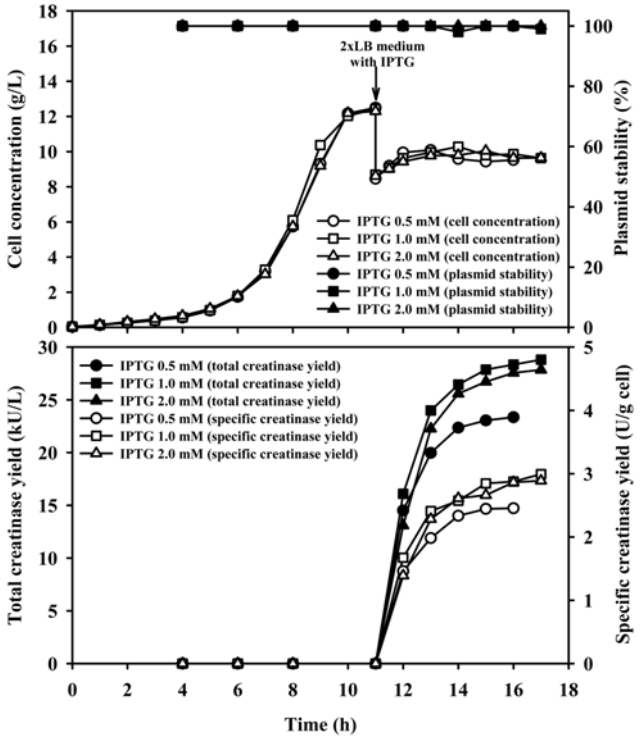

Fig. 3. Plasmid stability and creatinase production in a two-stage culture. In the first stage, the cells were cultivated in the R medium with 27.5 g/L of glucose. In the second stage, two-fold concentrated LB medium with various concentration of IPTG was supplemented.

2. Media Design for the Two-stage Culture

Based on the timing of plasmid loss, one would consider that the maintenance of plasmid stability may be achieved by decreasing the occurrence of cell fission after IPTG induction. Accordingly, a two-stage fermentation strategy was proposed. In the first stage, the cells were cultivated in a growth medium to a desirable cell concentration; then, in the second stage, creatinase was produced in a producing medium supplemented with IPTG. For fulfilling the requirements of the strategy, different media should be prepared individually for cell grow and creatinase production. Fig. 2 shows the growth of cells, the stability of plasmid and the specific yield of creatinase obtained from R medium and LB medium. In the absence of IPTG, a final cell concentration of 1.2 and 12.5 g/L could be obtained from LB and R medium, respectively; thus, R medium is obviously more suitable for being a growth medium. In addition, there were no plasmid-losing cells observed in the cultures without the inducer. For investigating the creatinase production, 0.5 mM IPTG was added to the fermentation broth at a cell concentration of 0.6 g/L. It can be seen that the introduction of IPTG to R medium inhibited the growth of cells; however, the addition of IPTG to LB medium was beneficial to cell growth. The stability of plasmid in the R medium culture was significantly lower than that in the LB medium culture, which might be due to multiple occurrences of cell fission in the R medium culture. In addition, the specific creatinase yield obtained from LB medium was higher than that obtained from R medium, indicating that LB medium is more appropriate for enzyme production. The decrease in the creatinase yield obtained from the R medium culture might be attributed to the instability of plasmids. Thus, based on the characteristics of the media, R medium was used in the first stage as the growth medium and LB medium was used in the second stage as the producing medium.

3. Plasmid Maintenance in the Two-stage Culture

The verification of our strategy is shown in Fig. 3. In the first stage, the cells grew to a cell concentration of 12.5 g/L in the R medium with 27.5 g/L of glucose. In the second stage, 400 ml of the 2-fold concentrated LB medium supplemented with IPTG was introduced to the fermenter. During the enzyme producing stage, the cell concentration increased from 8.9 to 10.5 g/L (equivalent to the cell mass increasing from 12.5 to 14.7 g), implying that most of the cells had not experienced cell division. It can be seen that the stability of the plasmid was always kept at 100% during the enzyme producing phase, irrespective of the IPTG concentration in the culture. It is evident that the decrease in the occurrence of cell fission by avoiding the doubling in cell mass is favorable for the maintenance of plasmid stability. Fig. 3 also demonstrates that the production of creatinase increased from 23 to 28 kU/L when the IPTG concentration increased from 0.5 to 1.0 mM. This increase in the creatinase yield could be attributed to the enhancement in the expression of the recombinant gene. The expression of a foreign gene may pose a metabolic burden on the host cell, which subsequently resulted in the rapid increase of plasmid free segregants [15,16]. However, Fig. 3 shows that once the division of cells was prevented, the host cells could harbor the plasmids stably, even though the metabolic burden was present.

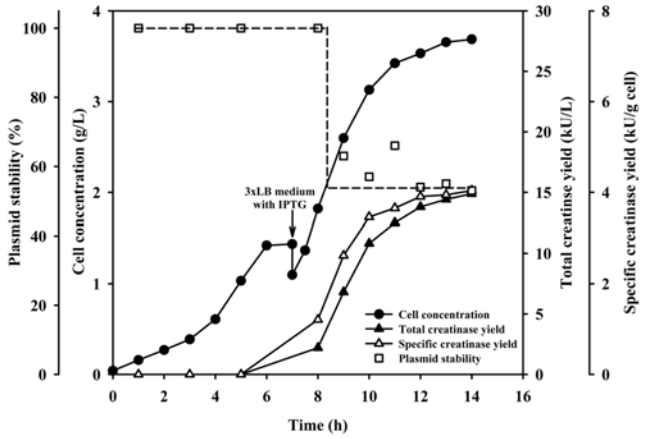

Fig. 4. Plasmid loss during the enzyme producing stage after the doubling of the cell mass. In the first stage, the cells were cultivated in the R medium with 3 g/L of glucose. In the second stage, three-fold concentrated LB medium with 0.5 mM of IPTG was supplemented to the fermenter.

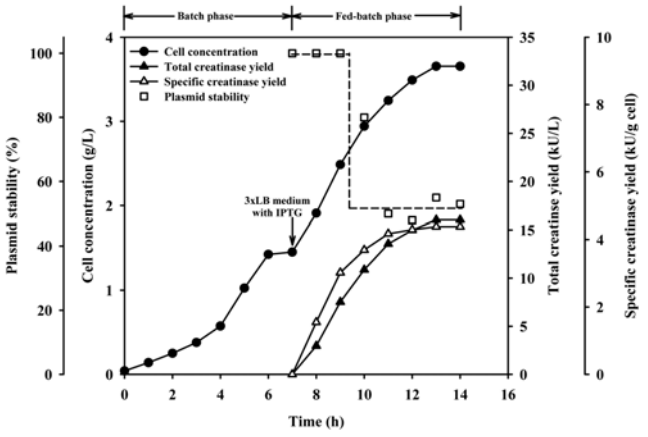

Fig. 6. Plasmid loss in a fed-batch culture. In the batch stage, the cells were cultivated in the R medium with 3 g/L of glucose. In the fed-batch stage, three-fold concentrated LB medium was fed with a flow rate of 2 mL/min. IPTG concentration was kept at 0.5 mM.

The decrease in plasmid stability at the time of cell concentration doubled during the enzyme producing phase is observed in Fig. 4. During the creatinase-producing stage, the cell concentration increased from 1.1 to 3.7 g/L. Notably, the plasmid stability decreased from 100% to around 50% when cell concentration increased from 1.1 to around 2.2 g/L, which corresponded to the timing of the cell mass doubling. The result indicates that the plasmid-losing cells originated during the division of host cells which experienced a metabolic burden imposed by the expression of creatinase gene.

4. Plasmid Maintenance in a Fed-batch Culture

Recombinant protein is often produced in a fed-batch culture; therefore, our strategy was further investigated by using a fed-batch operation. Fig. 5 shows that the cells were grown in the R medium with 27.5 g/L of glucose in the first stage. The fed-batch culture began at a fermentation time of 11 h. During the fed-batch stage, 400 mL of 2-fold concentrated LB was fed with a flow rate of 2 mL/min and the concentration of IPTG in the fermentation broth was kept at 1.0 mM throughout. During the creatinase-producing phase, the cell mass in the fermenter increased from 12.9 to 14.3 g. Since a doubling in cell mass was not attainable, it was expected that no plasmid-losing segregant would appear in the culture. The results, shown in Fig. 5, agreed with our prediction.

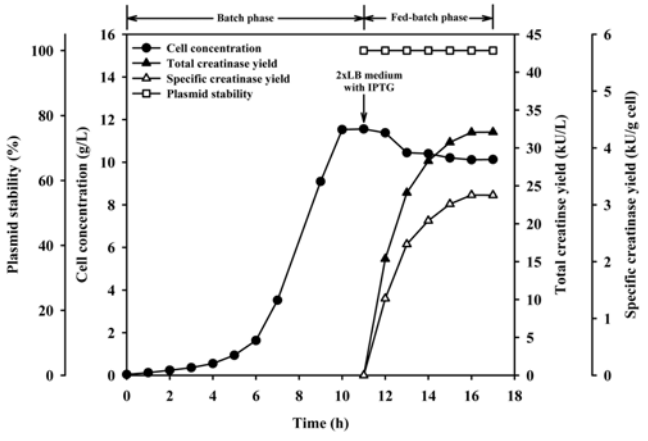

Fig. 5. Maintenance of plasmid stability in a fed-batch culture. In the batch stage, the cells were cultivated in the R medium with 27.5 g/L of glucose. In the fed-batch stage, two-fold concentrated LB medium was fed with a flow rate of 2 mL/min. IPTG concentration was kept at 1.0 mM.

Based on our hypothesis, the plasmid-losing cells were expected to appear after the cell mass was doubled in a fed-batch production culture. Therefore, the change of plasmid stability during creatinase production in a fed-batch culture was further examined. As shown in Fig. 6, the cells in the growth phase were cultivated in the R medium with 3 g/L of glucose. The fed-batch culture began at the fermentation time of 6 h. Hereafter, 400 mL of 3-fold concentrated LB was fed with a flow rate of 2 mL/min and the concentration of IPTG in the fermentation broth was kept at 0.5 mM throughout the fed-batch stage. In the second stage, the cell mass increased from 1.5 to 5.1 g. As expected, the decrease of plasmid stability started when the cell mass doubled from ca. 1.5 to 3.0 g; the timing was in accord with the occurrence of cell fission. The results give strong evidence that plasmid-losing cells occur when the increased cell mass is larger than the cell mass at IPTG induction, irrespective of whether in batch or fed-batch cultures.

CONCLUSIONS

The principle for protein production in a recombinant *E. coli* culture without plasmid instability was illustrated. The production of recombinant protein should be performed in a two-stage culture without selective pressure. The first stage is designed as the cell growth stage from which a high cell density culture is obtained. The production of recombinant protein is arranged in the second stage, when the producing medium with an inducer is added. In order to maintain plasmid stability, the doubling in the cell mass must be avoided during the second stage. This strategy does not require expensive antibiotics and is convenient to operate, and thus can be expected to be adopted in performing recombinant cultures.

ACKNOWLEDGMENTS

This research was supported by National Kaohsiung University of Applied Sciences, Taiwan (project No. KUAS-94-SB-001).

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