Journal of Microbiology and Biotechnology
Vol. 4, No. 1, 13-19, 1994

Estimation and Sensitivity Analysis of Kinetic Parameters for Plasmid Stability in Continuous Culture of a Recombinant *Escherichia coli* Harboring *trp*-operon Plasmid

NAM, SOO WAN[1]*, BYUNG KWAN KIM AND JUNG HOE KIM

Department of Biotechnology, Korea Advanced Institute of Science and Technology, Taedok Science Town, Taejon 305-701
[1]Genetic Engineering Research Institute, KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea

A model equation to describe the plasmid instability in recombinant *Escherichia coli* fermentation is proposed. The equation allows one to estimate easily the two model parameters; (1) the difference in the specific growth rates between plasmid-free cells and plasmid-harboring cells (δ), and (2) the probability of plasmid loss by plasmid-harboring cells (p). The estimated values of δ and p were in the range of 0.02-0.07 and 10^{-3}-10^{-5}, respectively, and were strongly dependent on the dilution rate. As another parameter , the ratio of specific growth rates of plasmid-free cells and plasmid-harboring cells (α) was calculated and the result showed the highest value of 1.28 at the lowest dilution rate of 0.075 hr^{-1}, examined in this work. By the sensitivity analyses on the estimates of δ and p, it was found that the growth rate difference (δ) affected the plasmid instability more seriously than the probability of plasmid loss (p). Furthermore, the profound instability of plasmid at low dilution rate could be explained by the high values of α and p.

The segregational instability of the plasmid and populational dominance of plasmid-free cells in the late period of recombinant fermentation are caused mainly by two factors: the probability of a plasmid-free cell generation from a plasmid-harboring one (p) and the growth rate difference between plasmid-free and plasmid-harboring cells (δ) (1, 2, 6, 13, 22, 25). Several continuous cultures of recombinant *Escherichia coli* have been reported by many authors (3-5, 7-11, 16-18, 28), and the authors suggested that the probability of plasmid loss increased with dilution rate and thus with growth rate (5, 7, 12, 26). Furthermore, it was found that the probability of plasmid loss showed a dependency upon the specific growth rate of plasmid-harboring cell (μ^+). These kinetic parameters had been evaluated under the repressed condition of cloned-gene expression (19), and/or under the selective condition made by adding antibiotics (23). However, the repressed and/or selective conditions are not practical, since most recombinant fermentation is carried out under the derepressed or induced conditions in the production scale process.

It is very important to determine how much these factors affect the host-vector relationship under the given culture condition, especially under the derepressed or induced condition of gene expression. Although much experimental data have been reported, the measuring of these kinetic parameters for plasmid instability have not been fully appreciated most likely due to their empirical nature. It is also difficult to measure experimentally these kinetic parameters related to the genetic characteristics of recombinant microorganisms. The corresponding values of these parameters have been calculated in the past by San and Weber (21) using an integral approach, and they have been analyzed more generally by Park *et al.* (19). However, a complex form of model equation was used to represent the dynamic state of recombinant fermentation.

In our previous work, a continuous culture of a recombinant *E. coli* was conducted under the derepressed condition (42℃) and in the absence of selection pressure (antibiotic-free medium), and the plasmid stability was measured at different dilution rates (15). To analyze the experimental data on the plasmid stability kinetically, a graphical and analytical approach was developed to estimate the values of p and δ, by using a simple model equation. The estimated values of these parameters were found to be strongly dependent on the dilu-

*Corresponding author
Key words: recombinant *Escherichia coli*, continuous culture, plasmid stability, plasmid loss, sensitivity analysis

tion rate. The modelling approach proposed here also allowed us to evaluate how mach these parameters affect the plasmid stability, through a sensitivity analysis on p and δ.

KINETIC MODEL FOR PLASMID STABILITY

The growth model describing a mixed culture system with plasmid-harboring cell (X^+) and plasmid-free cells (X^-) is given by the following three reactions (6);

$$X^+ \xrightarrow{\mu^+} (2-p)X^+$$
$$X^+ \xrightarrow{\mu^+} pX^+$$
$$X^- \xrightarrow{\mu^-} 2X^-$$

where μ^+ and μ^- are the specific growth rates of the plasmid-harboring and plasmid-free cells, respectively, and p is the probability of plasmid loss per cell division. According to the above model, the dynamics of X^+ and X^- can be obtained by simple material balances and are given by a set of ordinary differential equations:

$$\frac{dX^+}{dt}=(\mu^+-D)X^+-p\,\mu^+X^+ \quad (1)$$

$$\frac{dX^-}{dt}=(\mu^--D)X^-+p\,\mu^+X^+ \quad (2)$$

where D is the dilution rate.

Considering the changes in the ratio of X^- to X^+

$$\frac{d(X^-/X^+)}{dt}=\frac{1}{X^+}\left(\frac{dX^-}{dt}-\frac{X^-}{X^+}\cdot\frac{dX^+}{dt}\right) \quad (3)$$

Substituting Eq (1) and (2) into Eq (3) gives the following differential equation:

$$\frac{d(X^-/X^+)}{dt}=p\,\mu^+\left(1+\frac{X^-}{X^+}\right)+\left(\frac{X^-}{X^+}\right)\delta \quad (4)$$

where $\delta=\mu^--\mu^+$. A positive value of δ indicates advantages of plasmid-free cells over plasmid-harboring cells in growth.

The analytical solution of Eq (4) with the initial condition, $(X^-/X^+)=0$ at $t=0$, yields

$$\frac{X^-}{X^+}=\frac{p\,\mu^+}{(p\,\mu^++\delta)}\left[\exp(p\,\mu^++\delta)\cdot t-1\right] \quad (5)$$

For a relatively stable plasmid system, the rate of plasmid loss is much smaller value than the difference in the specific growth rate. That is, $\delta \gg p\,\mu^+$. Eq (5) can be approximated to the following equations:

$$\frac{X^-}{X^+}=\frac{p\,\mu^+}{(p\,\mu^++\delta)}\cdot\exp(\delta\cdot t) \quad (6)$$

$$\ln\left(\frac{X^-}{X^+}\right)=\delta\cdot t+\ln\left(\frac{p\,\mu^+}{p\,\mu^++\delta}\right) \quad (7)$$

Consequently, the slope (δ) and intercept $[p\,\mu^+/(p\,\mu^++\delta)]$ from a plot of ln (X^-/X^+) vs time will provide the estimates for parameters δ and p, respectively.

During the fermentation period of 100% plasmid stability, μ^+ is expected to be nearly equal to the dilution rate, i.e., $\mu^+=D$. From the definition of δ, the ratio of specific growth rates (α) of plasmid-free cells and plasmid-harboring cells can be simply calculated as following;

$$\alpha=\mu^-/\mu^+=(D+\delta)/D=1+(\delta/D) \quad (8)$$

RESULTS AND DISCUSSION

Estimation of Model Parameters from Experimental Data

Continuous cultures were conducted with *E. coli* W 3110/pCRT185, a recombinant DNA strain that synthesizes *trp*-operon enzymes and thus produces tryptophan. From the experimental data of plasmid stability (15), the proposed Eq (7) was graphically applied to calculate the parameter values of p and δ. The fraction of plasmid-free cells to plasmid-harboring cells (X^-/X^+) was plotted as a function of culture time (t) for different dilution rates in Fig. 1. From this plot, two parameters of δ and p were evaluated using a linear regression method, and the results are summarized in Table 1.

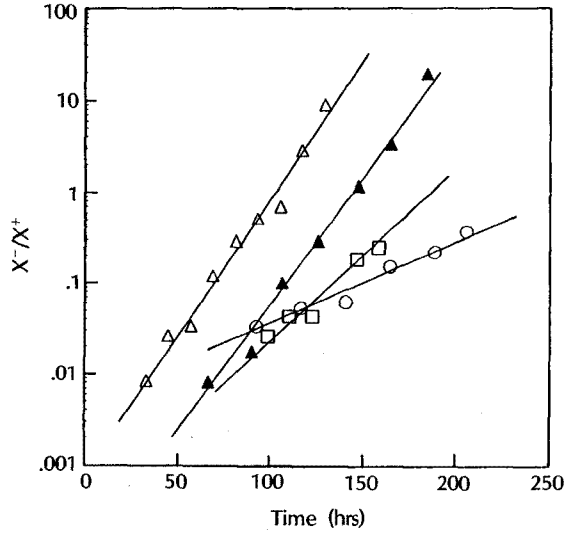

Fig. 1. Plot of $\ln(X^-/X^+)$ vs. time for the different dilution rates. (○) D=0.075 hr^{-1}; (□) D=0.15 hr^{-1}; (△) D=0.35 hr^{-1}; (■) D=0.425 hr^{-1}.

Table 1. Kinetic parameters estimated from the experimental results of continuous culture with a recombinant *E. coli*

Dilution rate D (hr^{-1})	Growth rate difference δ (hr^{-1}) ($\mu^- - \mu^+$)	Probability of plasmid loss p	Growth rate ratio α (μ^-/μ^+)
0.075	0.021	1.21×10^{-3}	1.28
0.15	0.039	1.33×10^{-4}	1.26
0.35	0.069	1.74×10^{-4}	1.20
0.425	0.060	2.93×10^{-5}	1.14

Also, the ratio of specific growth rates (α) for each dilution rate was calculated by Eq (8) and the results are summarized in Table 1.

The difference in the specific growth rate (δ) between the plasmid-free cell and plasmid-harboring cell was in the range of 0.02~0.07 and increased with the dilution rate. In contrast, the ratio of specific growth rates (α) were found to be inversely proportional to the dilution rate. The ratio of specific growth rate was found to be in the range of 1.1~1.3. This ratio was very consistent with the ratio of maximum specific growth rates, 1.23, obtained from the data on the batch culture of plasmid-free host cell ($\mu^-_{max}=0.55\ hr^{-1}$) and the recombinant one ($\mu^+_{max}=0.45\ hr^{-1}$). The observation that the ratio of growth rate, μ^-/μ^+, decreased with the increase in dilution rate, was also reported by Ryan and Parulekar in *E. coli* carrying pUC8 plasmid (20) and Wei *et al.* in *Bacillus subtilis* containing α-amylase gene (27).

The probability of plasmid loss (p) was in the range of 10^{-3}~10^{-5} and yielded the highest value of 10^{-3} at the dilution rate of 0.075 hr^{-1}. As in the similar declining manner of α, the value of parameter p was also strongly dependent on the dilution rate, and decreased with the dilution rate. However, the magnitude of decrease was more profound in the case of α, since the change of α value was 10^{-2} order, while that of parameter p was of 10^{-3}~10^{-5} order.

As seen in Fig. 1, the values of y-axis intercept are too small to evaluate the p value accurately, and thus various approaches to solve this problem have been suggested by many authors (13, 19, 21). Both of the integral form equations (19, 21) and the derivative method combined with data filtering and smoothing (13) can be used, but, in this work, those methods were not applied to estimate the p value, since those require an additional set of experiment and/or has the complexity of equation to use. Also, since the p values, calculated by above two methods, showed a little difference (13), it can be concluded that the small value of y-axis intercept was not so significant in determining the parameter p accurately.

For the high copy number of plasmids, it is generally believed that the partitioning of plasmid is achieved by a random distribution of plasmid molecules (24) and that the plasmid-free segregants appear at a frequency of less than 10^{-5} per cell generation (12, 14, 21). Most recently, San and Weber (21) reported that the probability of plasmid loss was estimated to be about 10^{-4} with *E. coli* harboring pBR322 plasmid in chemostat. In our recombinant *E. coli* system, the same order of magnitude of parameter p was observed, but the dependency of p on the dilution rate was different. In case of San and Weber (21), and Nancib and Boudrant (14), the value of p increased when the dilution rate was increased, while in our case and in the result of Park *et al.* (19) the reverse situation was observed.

Regarding the mechanism about the dependency of the probability of plasmid loss on the specific growth rate of plasmid-carrying cell, it has been known that the quantitative relationship between the plasmid replication or the plasmid content and the segregational instability should be considered carefully. If one considers the plasmid replication, the replication rate should be measured at different dilution rate and correlated to the specific growth rate. Recently, Mosrati *et al.* pointed out that it was very difficult to estimate the replication rate for the plasmid of high copy number, since the corresponding formula was not fully established due to the complex and multistep reactions of its replication (12). Another factor is the plasmid content or copy number per cell. Park *et al.* reported that the great probability of plasmid loss was estimated at the low dilution rate in which the increase of the plasmid copy number was observed (19). Therefore, they suggested that the high content of plasmid might increase the value of p, if no mechanism existed for active segregation of plasmid molecules to the daughter cells. This suggestion can be also valid in our result, since the content of pCRT185 plasmid, used in this work, reached the highest concentration at the low dilution rate (15). On the other hand, a significant effect on the plasmid stability might be also resulted from small difference in genetic information carried on the plasmid and the operating conditions such as dilution rate. Consequently, it is not clear yet whether the trend of p to increase with the dilution rate, is caused by the inherent characteristics

of the host-vector system or by factors related to the culture environmental conditions.

Comparison of Model Prediction with Experimental Data

Based on the estimated values of parameter p and δ, computaional model prediction for the plasmid stability was performed by the numerical integration of Eq (1) and Eq (2) using a fourth-order Runge-Kutta method. Comparisons of the plasmid stability between the model predictions and experimental data for dilution rates of 0.35 hr^{-1} and 0.425 hr^{-1} are presented in Fig. 2. The predicted result agreed well with the experimental data. For the other dilution rates of 0.075 hr^{-1} and 0.15 hr^{-1} the same procedure with each value of p and δ (Table 1) was performed and showed good consistency with the experimental results of plasmid stability (data not shown). This fact illustrated that the parameter values evaluated by the proposed method satisfactorily represented the characteristics of the host-plasmid system employed in this work.

From Table 1, the values of p and δ at the low dilution rate of 0.075 hr^{-1} were higher than those at higher dilution rates. In the previous paper (15), the plasmid content per g-cell was at its highest at the lowest dilution rate, and presumedly this high content of plasmid DNA provided the recombinant cell with the extra metabolic and energy burdens. Therefore, it seems most likely that the detrimental effect of metabolic burdens on the recombinant cell resulted in the highest value of p and δ at a low dilution rate. Consequently, it could be concluded that the coupled effect of the higher values of the probability of plasmid loss (p) and ratio of growth rate (μ^-/μ^+) had caused the plasmid free cells to displace the plasmid-harboring cell population

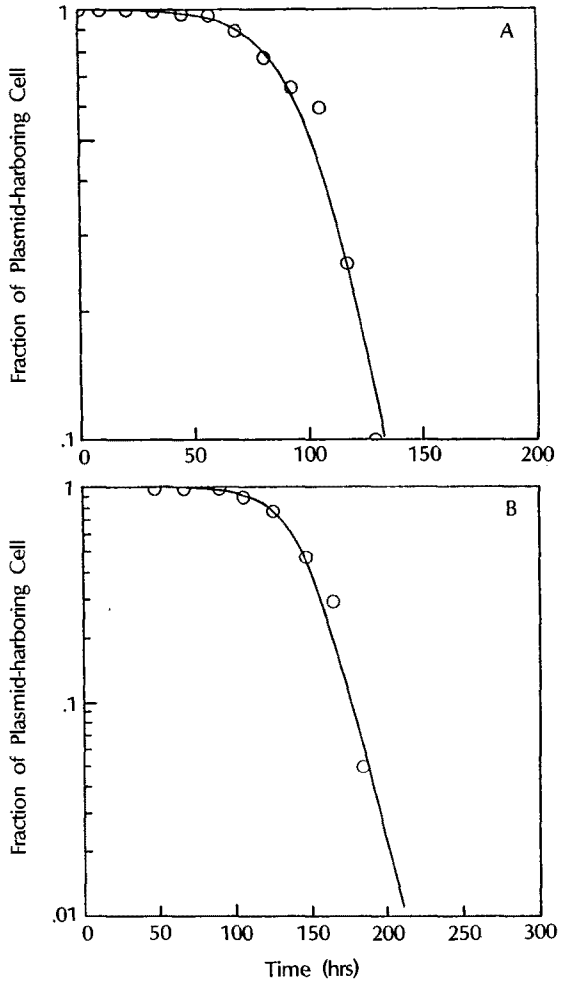

Fig. 2. Model predictions of plasmid instability at the dilution rate of 0.35 hr^{-1} (A) and 0.425 hr^{-1} (B).
The line (—) and open circle (○) represent the simulation result and experimental data on plasmid instability, respectively.

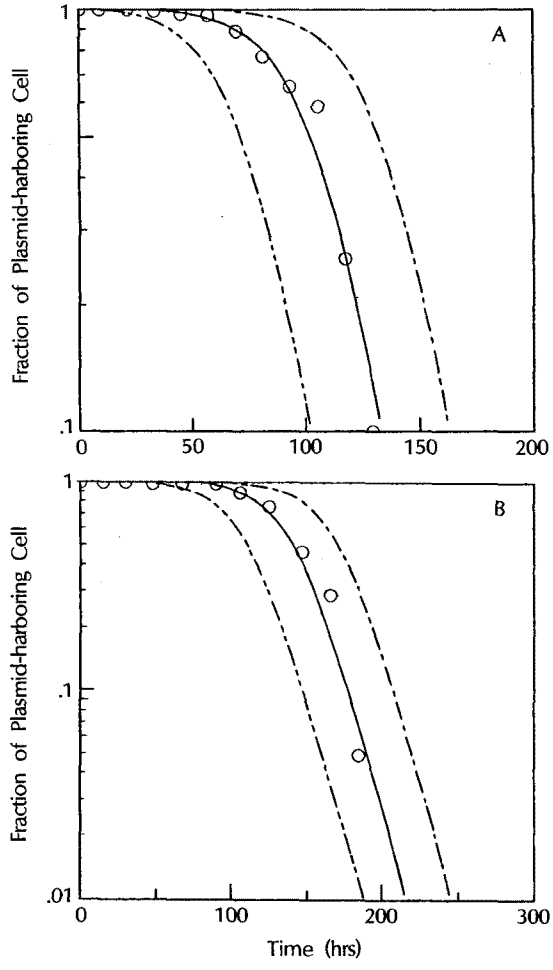

Fig. 3. Sensitivity of model prediction to the zero time, as the starting time of steady state.
The zero time deviation of ± 30 hrs was analyzed for the dilution rates of 0.35 hr^{-1} (A) and 0.425 hr^{-1} (B). The open circle (○) represents the experimental data on plasmid instability.

much faster, especially in the lower dilution rate.

In this work, the following assumptions were made: the δ and $p\,\mu^+$ remain constant throughout the experiment, and the plasmid system is relatively stable. Most recently, Mosrati *et al.* (12) suggested that the parameters of δ and p did not remain constant and increased with μ^+. Contradictory to their report, our result indicated that the model studied here is valid even with the constant condition for the parameters, and that the values of δ and p decreased with the dilution rate.

Sensitivity Analysis on Model Parameters

In Eq (1) and Eq (2), two parameters of p and δ are constants at steady state. Thus, the culture time of zero in the previous model prediction of plasmid instability was assumed at the time in which the observation of cell concentration reached a steady state. The culture time of zero, however, is subjected to the self-judgement and may be changed by the experimenter. Therefore, the prediction studies were proceeded with respect to the "zero time sensitivity".

The new zero time for the steady state of continuous culture was assumed at 30 hrs (± 30 hr) and 10 hrs (± 10 hr) before and after on the basis of the zero time determined in the previous simulation studies. The simulation results with this variation of "the zero time" for the dilution rate of 0.35 hr^{-1} and 0.425 hr^{-1} were shown in Fig. 3(A) and (B), and Fig. 4(A) and (B), respectively. From the figures, it was found that the greater the deviation of zero time, the faster or slower the appearance of plasmid-free cell. Consequently, in order to reduce the zero time effect on the plasmid instability, the zero time as the initial starting point for the steady state must be determined as carefully as possible. This can

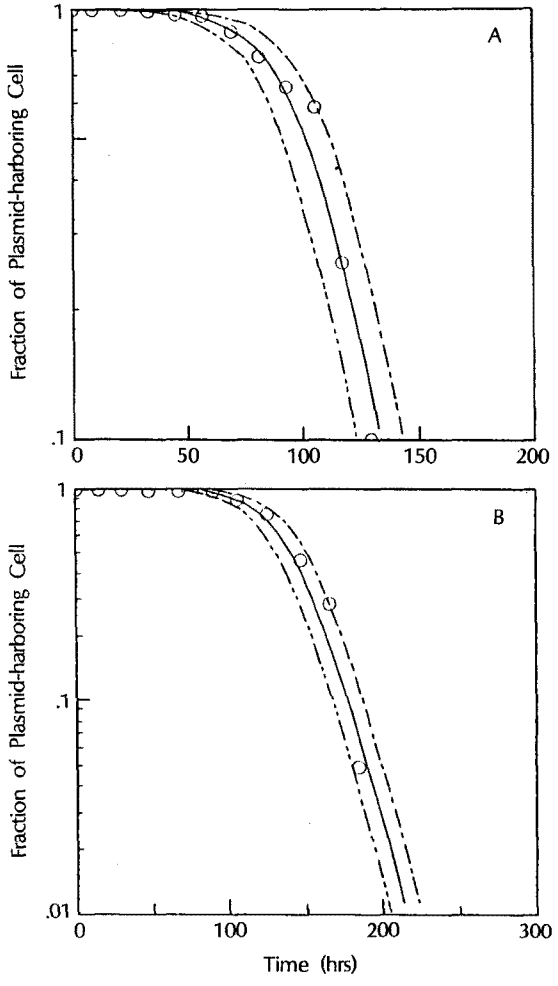

Fig. 4. Sensitivity of model prediction to the zero time, as the starting time of steady state.
The zero time deviation of ± 10 hrs was analyzed for the dilution rates of 0.35 hr^{-1} (A) and 0.425 hr^{-1} (B). The open circle (○) represents the experimental data on plasmid instability.

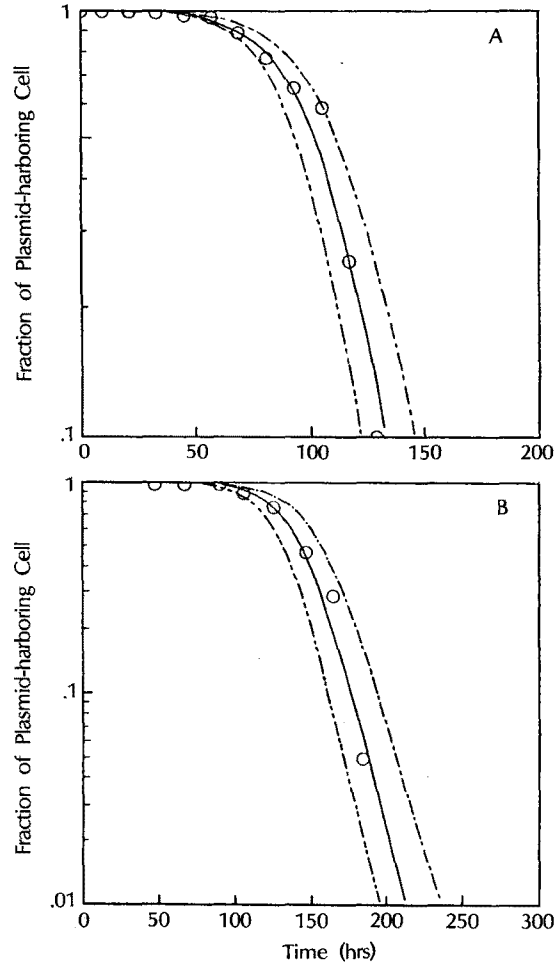

Fig. 5. Sensitivity of model prediction to the parameter δ.
The ± 10% deviation in δ was analyzed for the dilution rates of 0.35 hr^{-1} (A) and 0.425 hr^{-1} (B). The open circle (○) represents the experimental data on plasmid instability.

be achieved through the on-line monitoring or more frequent sampling of culture after the feeding of nutrients. In addition, to study which parameter has the significant effect on the plasmid instability, the sensitivity of model parameters was investigated. In this study, the simulated predictions in which the parameters deviated from the experimentally estimated parameters were compared to the experimental results.

The predicted results at the dilution rates of 0.35 and 0.425 hr^{-1} with 10% deviation of the estimated values of p and δ (Table 1), are shown in Fig. 5 and Fig. 6. It was elucidated that a small error in the parameter led to quite a large disagreement between the simulation results and the experimental observations. A 10% error in the growth rate difference (δ) resulted in a curve that suggests either a much faster or a much slower disappearance rate of the plasmid-harboring cell (Fig. 5). On the other hand, from the simulation results or the 10% deviation of the probability of plasmid loss (p) (Fig. 6), the model was quite insensitive to the small error in the value p. This insensitivity can be explained by the magnitude of the order in two parameters, i.e., growth rate difference (δ) had an order of 10^{-2}, but the probability of plasmid loss (p) had 10^{-4}. The 10^{-2} order difference between these two parameters demonstrates that the major factor for plasmid instability is the growth rate difference. As shown in the Eq (8), the growth rate ratio α is proportional to the parameter δ. It is also expected that the higher value of α at the low dilution rate will result in the faster growth of plasmid-free cells, and thus result in the rapid disappearing rate of plasmid-harboring cells, as observed with δ.

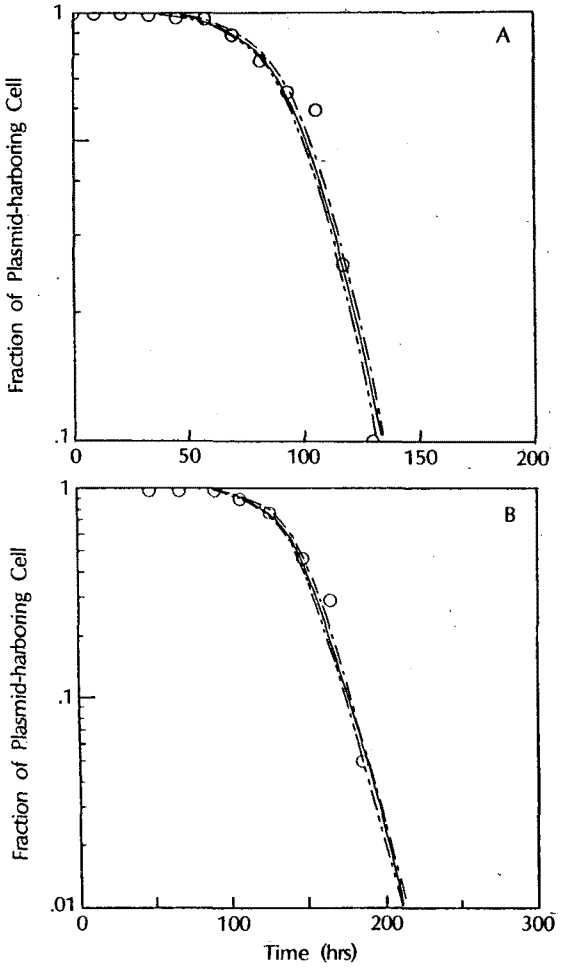

Fig. 6. Sensitivity of model prediction to the parameter p. The $\pm$10% deviation in p was analyzed for the dilution rates of 0.35 hr^{-1} (A) and 0.425 hr^{-1} (B). The open circle (○) represents the experimental data on plasmid instability.

As a result, the simulation performed in this work fits well the the experimental data, and constitutes evidence that the model equation proposed here is valid. The sensitivity studies indicate that an accurate estimation of the parameter δ and zero time is more important than that of the parameter p, for a good prediction of the experimental result. Furthermore, it was found that the significant instability of plasmid at the low growth rates can be explained by the high values of growth rate ratio α and probability of plasmid loss p, which are caused by the additional metabolic burdens due to the higher content of plasmid DNA or plasmid copy number.

REFERENCES

1. Caulcott, C.A., A. Dunn, H.A. Robertson, N. Cooper, M.E. Brown and P.M. Rhodes. 1987. Investigation of the effect of growth environment on the stability of low-copy-number plasmids in *Escherichia coli*. *J. Gen. Microbiol.* **133**: 1881-1889.
2. Cooper, N.S., M.E. Brown and C.A. Caulcott. 1987. A mathematical method for plasmid stability in microorganisms. *J. Gen. Microbiol.* **133**: 1871-1880.
3. DiBiasio, D. and C. Sardononi. 1986. Stability of continuous culture with recombinant organisms. *Ann. N.Y. Acad. Sci.* **469**: 111-117.
4. Dwivedi, C.P., T. Imanaka and S. Aiba. 1981. Instability of plasmid-harboring strain of *E. coli* in continuous culture. *Biotechnol. Bioeng.* **24**: 1465-1468.
5. Helling, R.B., T. Kinney and J. Adams. 1981. The maintenance of plasmid-containing organisms in populations of *Escherichia coli*. *J. Gen. Microbiol.* **123**: 129-141.
6. Imanaka, T. and S. Aiba. 1981. A perspective on the application of genetic engineering: Stability of recombinant plasmid. *Ann. N.Y. Acad. Sci.* **369**: 1-14.
7. Impulsup, A., P. Caunt and P.F. Greenfield. 1989. Effect of growth rate on stability of a recombinant plasmid during continuous culture of *Saccharomyces cerevisiae* in non-selective medium. *J. Biotechnol.* **10**: 171-180.
8. Jones, S.A. and J. Melling. 1984. Persistence of pBR322-related plasmids in *Escherichia coli* grown in chemostat

cultures. *FEMS Microbiol. Lett.* **22**: 239-243.
9. Kim, S.H. and D.D.Y. Ryu. 1984. Instability kinetics of *trp* operon plasmid ColE1-*trp* in recombinant *Escherichia coli* MV12 [pVH5] and MV12*trp*R [pVH5]. *Biotechnol. Bioeng.* **26**: 497-502.
10. Lauffenburger, D.A. 1985. Stability of colicin plasmids in continuous culture: Mathematical model and analysis. *Biotechnol. Prog.* **1**: 53-59.
11. Lin-Chao, S. and H. Bremer. 1986. Effect of the bacterial growth rate on replication control of plasmid pBR322 in *Escherichia coli*. *Mol. Gen. Genet.* **203**: 143-149.
12. Mosrati, R., N. Nancib and J. Boudrant. 1993. Variation and modelling of the probability of plasmid loss as a function of growth rate of plasmid-harboring cells of *Escherichia coli* during continuous cultures. *Biotechnol. Bioeng.* **41**: 395-404.
13. Nancib, N., R. Mosrati and J. Boudrant. 1992. Study of population dynamics for a recombinant bacterium during continuous cultures: Application of data filtering and smoothing. *Biotechnol. Bioeng.* **39**: 398-407.
14. Nancib, N. and J. Boudrant. 1992. Effect of growth rate on stability and gene expression of a recombinant plasmid during continuous culture of *Escherichia coli* in a non-selective medium. *Biotechnol. Lett.* **14**: 643-648.
15. Nam, S.W., B.K. Kim and J.H. Kim. 1994. Plasmid stability and cloned-gene expression in continuous culture of recombinant *Escherichia coli* under derepressed condition. *J. Microbiol. Biotechnol.* **4**: (in press).
16. Nilsson, J. and S.G. Skogman. 1986. Stabilization of Escherichia coli tryptophan-production vectors in continuous cultures: A comparison of three different systems. *Bio/Technol.* **4**: 901-903.
17. Noack, D., M. Roth, R. Geuther, G. Muller and K. Undisz. 1981. Maintenance and genetic stability of vector plasmid pBR322 and pBR325 in *Escherichia coli* K12 strains grown in a chemostat. *Mol. Gen. Genet.* **184**: 121-124.
18. Nugent, M.E., S.B. Primrose and W.C.A. Tacon. 1983. The stability of recombinant DNA. *Dev. Ind. Microbiol.* **24**: 271-285.
19. Park, S.H., D.D.Y. Ryu and S.B. Lee. 1991. Determination of kinetic parameters related to the plasmid instability for the recombinant fermentation under repressed condition. *Biotechnol. Bioeng.* **37**: 404-414.
20. Ryan, W. and S.J. Parulekar. 1991. Recombinant protein synthesis and plasmid instability in continuous cultures of *Escherichia coli* JM103 harboring a high copy number plasmid. *Biotechnol. Bioeng.* **37**: 415-429.
21. San, K.Y. and A.E. Webber. 1989. Data analysis of plasmid maintenance in a CSTR. *Biotechnol. Bioeng.* **33**: 451-459.
22. Siegel, R. and D.D.Y. Ryu. 1985. Kinetic study of instability of recombinant plasmid pPLc23*trpAI* in *E. coli* using two-stage continuous culture system. *Biotechnol. Bioeng* **27**: 28-33.
23. Stephens, M.L. and G. Lyberatos. 1988. Effect of cycling on the stability of plasmid-bearing microorganisms in continuous culture. *Biotechnol. Bioeng* **31**: 464-469.
24. Summers, D.K. and D.J. Sherratt. 1984. Multimerization of high copy number plasmid causes instability: ColE1 encodes a determinant essential plasmid monomerization and stability. *Cell.* **36**: 1097-1103.
25. Walmsley, R.M., D.C.J. Gardner and S.G. Oliver. 1983. Stability of a cloned gene in yeast grown in chemostat culture. *Mol. Gen. Genet.* **192**: 361-365.
26. Weber, A.E. and K.Y. San. 1987. Persistence and expression of the plasmid pBR322 in *Escherichia coli* K12 cultured in complex medium. *Biotechnol. Lett.* **9**: 757-760.
27. Wei, D., S.J. Parulekar, B.C. Stark and W.A. Weigand. 1989. Plasmid stability and α-amylase production in batch and continuous cultures of *Bacillus subtilis* TN106[pAT5]. *Biotechnol. Bioeng.* **33**: 1010-1020.
28. Wouters, J.T.M., F.L. Driehuis, P.J. Polaczek, Marie-Lousie H.A. van Oppenraay and J.G. van Andel. 1980. Persistence of the pBR322 plasmid in *Escherichia coli* K12 grown in chemostat cultures. *Ant. Leeuwenh.* **46**: 353-362.

(Received November 4, 1993)